UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

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Enterococcus faecalis: development of a final irrigant to destroy it, analysis of its survival after endodontic treatment and its ability to stimulate pro-inflammatory cytokines after stressed

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Orientador: Prof. Dr. Marco Antonio Hungaro Duarte

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FOLHA DE APROVAÇÃO

DEDICATÓRIA

Dedico este trabalho à minha mãe ***Vera*** *e minha irmã* ***Maria Helena****, responsáveis por construir minhas memórias e me fazer compreender que o importante não é a casa onde moramos, mas onde, em nós, a casa mora.*

[...Quem quer passar além do Bojador
Tem que passar além da dor.
Deus ao mar o perigo e o abismo deu,
Mas nele que espelhou o céu...]

Fernando Pessoa

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Nascemos com um fio invisível. Ao longo do tempo vamos colocando pérolas neste fio e – lentamente – vamos formando nossa história.

No término do meu doutoramento, posso dizer que entendi – graças às pérolas colecionadas até aqui - que por mais longa que seja a estrada nunca ficarei longe deste lugar. Nestes seis anos de USP meu fio invisível e pouco habitado ganhou tantas pérolas que hoje, o colar dá voltas no meu pescoço me ensinando que a vida não foi feita para ser pouca e o mundo não foi feito para ter medida.

Em 2014 minha lembrança me leva aos corredores desta universidade. Depois de uma prova longa e difícil – a imagem de um homem simpático e brincalhão, de uma simplicidade única. Passou por nós (alguns candidatos à vaga de mestrado), nos cumprimentou, fazendo graça da nossa expressão deslumbrada ao nos depararmos com a bibliografia viva dos cimentos. Convivi de perto com a majestade do professor Ivaldo Gomes de Moraes que me acolheu – naquele momento de iniciação ao novo em uma Universidade com o peso do nome e da qualificação que sempre teve em pesquisa – entendendo que eu sentia o mesmo desamparo de uma criança no primeiro dia de escola, dando-me ânimo para continuar.

Lembrar-se do professor Ivaldo é ouvir eco no silêncio. Ele nos deixou como legado a possibilidade de praticarmos o amor e a gentileza, mesmo num lugar tão imperfeito como o mundo.

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Mia Couto, autor moçambicano, num prefácio escreveu: “Não é segurando nas asas que se ajuda um pássaro a voar. O pássaro voa simplesmente porque o deixam ser pássaro”. Voei mais alto que meus sonhos. Hoje entendo que isso só foi possível graças à sua paciência, dedicação e humildade - que aos poucos - me fizeram acreditar na minha capacidade de ingressar no mundo da pesquisa.

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Vivemos – infelizmente no Brasil - uma tentativa de desmonte da educação e ciência, tendo como consequência o aumento de “verdades relacionadas a senso comum ” que sem qualquer embasamento toma corpo. É o que podemos chamar de uma Ode ao Obscurantismo que nós, como discentes e docentes de Universidades Públicas comprometidas com pesquisas não podemos e não iremos aceitar de braços cruzados, por isso, celebro o término do meu doutorado com agradecimento especial à FAPESP (Processo 2016/25133-1 e 2018/03554-0) pelo incentivo moral e financeiro a todos os pesquisadores.

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ABSTRACT

Aim: Ethylene diamine tetracetic acid (EDTA) is used in root canal therapy prior to obturation as a calcium chelator to remove the *smear layer*. Enhancing the antibacterial properties of EDTA can reduce the irrigation steps and thus reduce treatment time. Furthermore, the use of sodium hypochlorite (NaOCl) can be minimized. There is no clear consensus regarding the ideal irrigation protocol, based on the physical-chemical properties of dentin. As Hydraulic calcium silicate cements (HCSC) are interactive with dentin the effects of previous irrigation might influence sealer adhesion and antimicrobial action. **Methodology:** Article 1. 17% EDTA without additives and with 1% benzalkonium chloride, 1% N-acetylcysteine or 2% chlorhexidine together with saline control were tested through: antibacterial properties towards monospecies biofilm of *Enterococcus faecalis* which was inoculated in human dentin either before exposure to irrigating solution or after (confocal laser scanning microscope), ability of removal of smear layer (scanning electron microscope), dentin wettability by the sessile drop method (goniometer), surface tension of solutions (pendent drop test) and viability of fibroblast cells (reducing MTT assay); Article 2. The irreversible damage to dentin produced by NaOCl (2 or 5%), chelating agents (17% EDTA or 17% EDTA + 1% benzalkonium chloride) isolated or in a irrigant protocol and saline were assessed considering: dentin microhardness, morphology and mineral content (scanning electron microscopy and energy dispersive spectroscopy), organic/inorganic matter (Fourier transform infrared spectroscopy), surface roughness and Young`s modulus (atomic force microscope). Characterization of 4 sealers (AH Plus, BioRoot, MTA Fillapex and Total Fill BC Sealer) and their interaction with the dentin interface were analyzed by two techniques (scanning electron microscopy/energy dispersive sectroscopy and confocal laser scanning microscope); Article 3. the effect of 2% NaOCl and 17% EDTA coated dentin on the antimicrobial activity of HCSC (BioRoot, MTA Fillapex and Total Fill BC Sealer) and AH Plus towards *E. faecalis* inside dentinal tubules was assed by confocal laser scanning microscope. **Results/ Conclusion:** Article 1. The additives did not alter the EDTA cytotoxicity or smear layer removal capacity, but increased its antibacterial action. Lower surface tension and better wettability of the irrigants was obtained by the addition benzalkonium chloride or N-acetylcysteine to EDTA. The EDTA benzalkonium chloride solution was the best for prevention of bacteria adherence and dentin

wettability; Article 2. Highly concentrated NaOCl followed by EDTA significantly changed inorganic matter dissolution, microhardness and induced a rougher topography on the dentin surface with eroded dentinal tubules. As smear layer was still present on surfaces in NaOCl+chelator groups, the use of EDTA Benzalkonium Chloride (EDTA-BC) or NaOCl final flush was required for further disinfection. Further irrigation with NaOCl resulted in erosion of the dentin around the tubules and *smear-layer*-free surfaces. Based on CLSM analysis of dentin, the protocols NaOCl/EDTA/NaOCl, or NaOCl/EDTA-BC matched with AH Plus, BioRoot BCS and Total Fill sealers. Microhardness progressively decreased in protocols containing NaOCl 5%, and was recovered after MTA Fillapex or Total Fill application; Article 3. The sealer hydration influenced HCSC antimicrobial activity, as BioRoot BCS a water-based sealer provided the best antimicrobial activity against *E. faecalis.* EDTA coated dentin do not harm HCSC antimicrobial action. A smear-free surface enhanced killed bacteria inside dentinal tubules for all sealers.

Keywords: sodium hypochlorite; EDTA; benzalkonium chloride; chlorexidine; N-acetylcysteine; Bio Root BCS; MTA Fillapex; Total Fill; AH Plus; *Enterococcus faecalis*.

RESUMO

Objetivo. O ácido etilenodiamino tetra-acético (EDTA) é usado na terapia do canal radicular antes da obturação como um quelante de cálcio para remover a *smear layer*. O aumento das propriedades antibacterianas do EDTA pode reduzir as etapas da irrigação e, assim, reduzir o tempo de tratamento. Além disso, o uso de hipoclorito de sódio pode ser minimizado. Não há consenso claro quanto ao protocolo de irrigação ideal, baseado nas propriedades físico-químicas da dentina. Como os cimentos hidráulicos de silicato de cálcio (HCSC) são interativos com a dentina os efeitos da irrigação prévia podem influenciar a adesão do cimento e sua ação antimicrobiana. **Metodologia:** Artigo 1. EDTA 17% sem aditivos ou com cloreto de benzalcônio 1%, N-acetilcisteína 1% ou clorexidina 2% foram testados tendo solução salina como controle através de: propriedades antibacterianas para biofilme monoespécie de *Enterococcus faecalis* inoculado em dentina humana antes da exposição à solução irrigante ou depois (microscópio de varredura a laser confocal), capacidade de remoção da *smear layer* (microscópio eletrônico de varredura), molhabilidade da dentina pelo método da gota séssil (goniômetro), tensão superficial das soluções (teste da gota pendente) e viabilidade de células fibroblastos (redução do ensaio MTT); Artigo 2. Os danos irreversíveis à dentina produzidos por NaOCl (2 ou 5%), agentes quelantes (EDTA 17% ou EDTA 17% + cloreto de benzalcônio 1%) e solução salina isolados ou em uso sequencial foram avaliados considerando: microdureza da dentina, morfologia e conteúdo mineral (microscopia eletrônica de varredura e espectroscopia de energia dispersiva), matéria orgânica/inorgânica (espectroscopia infravermelha transformada Fourier), rugosidade superficial e módulo de Young (microscópio de força atômica). A caracterização de 4 cimentos (AH Plus, BioRoot, MTA Fillapex e Total Fill BC Sealer) e sua interação com a interface dentinária foram analisadas por duas técnicas (microscopia eletrônica de varredura/espectroscopia dispersiva de energia e microscópio de varredura a laser confocal); Artigo 3. HCSC (BioRoot, MTA Fillapex e Total Fill BC Sealer) e AH Plus foram expostos a dentina tratada com 2% NaOCl e 17% EDTA e sua ação antimicrobiana em relação ao *E. faecalis* dentro dos túbulos dentinários foi acessada através de microscópio de varredura a laser confocal. **Resultados/Conclusão:** Artigo 1. Os aditivos não alteraram a citotoxicidade do EDTA ou a capacidade de remoção da *smear layer*, mas

aumentaram sua ação antibacteriana. A tensão superficial mais baixa e a melhor molhabilidade dos irrigantes foram obtidas pela adição do cloreto de benzalcônio ou N-acetilcisteína ao EDTA. A solução de EDTA-cloreto de benzalcônio (EDTA BC) foi melhor na prevenção da adesão de bactérias a dentina e no aumento de sua molhabilidade; Artigo 2. O NaoCl altamente concentrado seguido por EDTA mudou significativamente a dissolução de matéria inorgânica, microdureza e induziu uma topografia mais áspera na superfície da dentina com túbulos dentinários erodidos. Como a *smear layer* ainda estava presente nas superfícies dos grupos NaOCl+quelantes, o uso do EDTA-BC ou NaOCl na irrigação final é necessário para uma maior desinfecção. A irrigação adicional com NaOCl resultou na erosão da dentina ao redor dos túbulos e superfícies de *smear layer*. Com base nas análise do CLSM na dentina, os protocolos NaOCl/EDTA/NaOCl, ou NaOCl/EDTA-BC combinam com os cimentos AH Plus, Bio Root e Total Fill. A microdureza da dentina diminuiu progressivamente nos protocolos que continham NaOCl 5%, e foi recuperada após a aplicação do MTA Fillapex ou Total Fill BC Sealer; Artigo 3. A hidratação do cimento influenciou a atividade antimicrobiana dos HCSC uma vez que o BioRoot BCS, um cimento à base de água, forneceu a melhor atividade antimicrobiana sobre *E. faecalis*. A dentina tratada com EDTA não prejudica a ação antimicrobiana dos HCSC. Uma superfície sem *smear-layer* resultou em um maior número de bactérias mortas dentro de túbulos dentinários para todos os cimentos.

Palavras-Chave: hipoclorito de sódio; EDTA; cloreto de benzalcônio; clorexidina; N-acetilcisteína; Bio Root BCS; MTA Fillapex; Total Fill; AH Plus; *Enterococcus faecalis*.

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1 INTRODUCTION

1 INTRODUCTION

The irrigation protocol is an important step which effects the success of root canal therapy (1, 2). Used throughout the biomechanical preparation, sodium hypochlorite is recommended as the main irrigant due to it BioRoot BCSoad antimicrobial spectrum and capacity for dissolving organic matter, such as pulp remenants, bacterial components and their by-products (1). The chelating agent disodium or trisodium ethylenediaminetetracetic acid (EDTA) is used as adjunct irrigant to remove the inorganic portion of the *smear layer* (3). The low antibacterial ability of EDTA, when compared with sodium hypochlorite or chlorhexidine necessitates a third stage of final irrigation with these solutions, with the purpose of increasing disinfection of root canal system (4-6). Although sodium hypochlorite is the gold standard for root canal irrigation, the third stage of irrigation might cause further degradation of organic tissues with deleterious effects on the collagen matrix and integrity of the dentin structure (7). Improvement in the antibacterial properties of EDTA would result in a reduction in use of the sodium hypochlorite and the use of one final irrigating solution achieving both the chelating and antimicrobial effect.

Persistent aggression of bacteria present in the root canal space induces and perpetuates diseases on pulp and surrounding periodontal tissues. Enhancing the antibacterial properties of EDTA can reduces the amount to harmful bacteria, but might not reach levels compatible with healing and regeneration of the periapical tissues (8). Viable bacteria often remain on untouched areas of the canal system by instruments and irrigants (9) such as isthmus, dentinal tubules, accessory canals and apical ramifications (8, 10). Root canal filling would compensated this faulty by trap remained bacteria inside these anatomical complexities. As dentin-sealer hermetic seal is not easy to achieve (11) the release of antimicrobial compounds by cements would prevent bacteria to reach pathways to periapical tissues.

The wide range of chemical compositions of different sealer types require distinct dentine pretreatments for optimal adhesion (12). AH Plus (AH) is an epoxy resin-based sealer that presents two mechanisms of adhesion to dentin: mechanical and chemical. The *smear layer* removal performed by EDTA improves sealer tags penetration into dentinal tubules and thus AH mechanical bond (13). The collagen

exposed by EDTA chemically bond to the epoxy rings of AH to initiate it setting reaction (14). These sealing ability can be impaired when sodium hypochlorite is applied due it proteolytic action on the organic phase of dentin. (15)

Hydraulic Calcium Silicate Cements (HCSC) do not depend on EDTA for a good dentin seal, but rather on chemical interaction. A ion exchange occurs after cement hydration whereas the release of Ca^{2+} ions from the calcium silicate particles of cement reacts with OH^- ions derived from water on tissue fluids resulting in calcium hydroxide (16). The highly alkaline environment stimulates the precipitation of hydroxyl apatite on the material surface that creates a chemical bond with dentin. It also provides antimicrobial action for the sealer, as the release of calcium hydroxide unable the survive of the majority of endodontopathogens (17-20). Literature theorizes that the moist on *smear layer* BioRoot BCS positive effects on the biological properties of a HCSC and decreased leakage on HCSC/smeared than smear-free dentin(21, 22) In addition, EDTA`s ability to chelate calcium ions is shown to disrupt HCSC hydration (23) compromising their release of calcium hydroxide, sealing ability, hardness, flexural and bond strength (21, 24, 25). Although HCSCs had a similar composition, the HCSC hydration might also influence calcium hydroxide release. As a water-based sealer BioRoot BCS do not rely on OH^- ions derived from tissue fluids to form calcium hydroxide like MTA Fillapex presented in a 2-paste system (26) or Total Fill, a premixed ready-to-use injectable sealer (27).

The aim of this study was to investigate: Article 1. the antibacterial action, *smear layer* removal, wettability, surface tension and cytotoxicity of EDTA and modified EDTA chelating agents. Furthermore, the wettability of dentin and adhesion of bacteria were analyzed after treatment with the irrigant solutions; Article 2: the irreversible damage to dentin produced by eleven irrigant protocols containing NaOCl, chelating agents and saline considering: dentin microhardness, morphology, mineral content, organic/inorganic matter, surface roughness and Young`s modulus of dentin and how these protocols would affect the dentin/sealer interface of four different sealers; Article 3: the influence of coated dentin with two different protocols (1. NaOCl and 2. NaOCl-EDTA) on the antimicrobial properties of HCSCs towards *Enterococcus faecalis* intratubular infection, considering the presence of *smear layer*for the non-chelator protocol.

2 ARTICLES

2 ARTICLES

2.1 ARTICLE 1 - Improvement of antimicrobial and physical properties of EDTA for effective removal of smear layer

2.2 ARTICLE 2 - Influence of different endodontic protocols on the dentin structure and adhesive dentin-sealer interface

2.3 ARTICLE 3 - The presence of *smear-layer* affects the antimicrobial action of root canal sealers

2.1 ARTICLE 1 - **Improvement of antimicrobial and physical properties of EDTA for effective removal of smear layer**

ABSTRACT

Aim. Ethylene diamine tetracetic acid (EDTA) is used in root canal therapy prior to obturation as a calcium chelator to remove the smear layer. EDTA presents low antibacterial properties. Enhancing the antibacterial properties of EDTA can reduce the irrigation steps and thus reduce treatment time. Furthermore, the use of sodium hypochlorite can be minimized. The aim of this study was to assess the physical, chelating, antibacterial properties and citotoxicity of EDTA, used with and without antibacterial additives.

Methodology. 17% EDTA without additives and with 1% benzalkonium chloride, 1% N-acetylcysteine or 2% chlorhexidine together with saline control were tested. The antibacterial properties of the solutions were assessed using a monospecies biofilm of *Enterococcus faecalis* which was inoculated in human dentine either before exposure to irrigating solution or after. Live/dead dye and a confocal microscope were used to measure the percentage of living cells and biovolume of both. The chelating effect was tested by assessing the ability of removal of smear layer using a scanning electron microscope (SEM). The irrigants were also evaluated for dentin wettability by the sessile drop method using a goniometer and the surface tension of solutions was verified by the pendent drop test. The effect of irrigating solutions on the viability of fibroblast cells was assessed by reducing MTT and crystal violet assay.

Results. The additives did not alter the EDTA cytotoxicity or smear layer removal capacity, but increased its antibacterial action. Lower surface tension and better wettability of the irrigants was obtained by the addition benzalkonium chloride or N-acetylcysteine to EDTA. The EDTA-benzalkonium chloride solution was the best for prevention of bacteria adherence and dentin wettability.

Conclusions. The addition of antibacterial substances to EDTA resulted in improvement in antibacterial action against *E. faecalis* biofilm, without compromising the EDTA chelating action and cytotoxicity. EDTA + benzalkonium chloride also decreased the adherence of *E. faecalis* to dentin coated with it, lowered EDTA surface tension and allowed better wettability of dentin, standing out from the other solutions. Therefore, the use of this association as a final rinse in endodontic treatment should be considered.

INTRODUCTION

The irrigation protocol is an important step which effects the success of root canal therapy. (1, 2) One of it goals is the removal of smear layer represented by debris on the dentin wall arising from organic and inorganic matter formed during root canal instrumentation.The smear layer promotes dentin tubule obliteration, making it difficult for the intracanal medication and sealers to penetrate (28), and may increase the adhesion of microorganisms initiating the process of biofilm formation. (29) Used throughout the biomechanical preparation, sodium hypochlorite is recommended as the main irrigant due to it broad antimicrobial spectrum and capacity for dissolving organic matter, such as pulp remenants, bacterial components and their by-products.(1) The chelating agent disodium or trisodium ethylenediaminetetracetic acid (EDTA) is used as adjunct irrigant to remove the inorganic portion of the smear layer.(3). The low antibacterial ability of EDTA, when compared with sodium hypochlorite or chlorhexidine necessitates a third stage of final irrigation with these solutions, with the purpose of increasing disinfection of root canal system. (4-6)

Although sodium hypochlorite is the gold standard for root canal irrigation, it is a dangerous chemical if ingested and causes severe bruising when it comes in contact with the soft tissues mainly at high concentrations.(30) Furthermore, sodium hypochlorite causes degradation of the dentin organic matrix. (31) Improvement in the antibacterial properties of EDTA would result in a reduction in use of the sodium hypochlorite and the use of one final irrigating solution achieving both the chelating and antimicrobial effect. The addition of antibacterial agents such as benzalkonium chloride, chlorhexidine and N-acetylcysteine would potentially achieve this.(32-35) The low surface tension of these mixtures, could increase EDTA wettability, leading it to reach deeper areas into the dentinal tubules (36) that could contain bacteria. The removal of smear layer in these areas could also improve the adhesion of filling materials to the dentin.

The aim of this study was to investigate the antibacterial action, smear layer removal, wettability, surface tension and cytotoxicity of EDTA and modified EDTA chelating agents. Furthermore, the wettability of dentin and adhesion of bacteria were analyzed after treatment with the irrigant solutions. The null hypothesis was that the modified EDTA solutions would show similarity to one another and to pure EDTA.

MATERIAL AND METHODS

Five irrigating solutions were investigated. These included G1. 17% EDTA , (Biodinâmica Química e Farmacêutica LTDA, Paraná, Brazil); G2. 17% EDTA + 1% benzalkonium chloride (Bauru Fórmulas, São Paulo, Brazil); G3. 17% EDTA + 1% N-acetylcysteine (Bauru Fórmulas, São Paulo, Brazil); G4. 17% EDTA + 2% chlorhexidine (Bauru Fórmulas, São Paulo, Brazil); G5. saline solution.

2.1 Antibacterial Testing

The microbiological procedures were conducted under aseptic conditions in a laminar flow chamber (VecoFlow Ltda, Campinas, SP, Brazil). Dentin blocks were obtained from the cervical area of bovine central incisors with fully developed roots, by using trephine drills in 4.0-mm diameter (Neodent, Curitiba, PR, Brazil), under copious irrigation. The incisors were positioned laterally to provide access to the most flattened portion of the root. The root was perforated with a trephine attached to a contra-angle handpiece positioned perpendicular to the teeth passing through the mesial and distal dentin walls, thus obtaining two dentin blocks measuring 4 mm x 1.2 mm (diameter x thickness). The dentin surface of the discs were wet polished in a circular grinding machine (Arotec, São Paulo, SP, Brazil) using 500 and 800 grit SiC papers (Buehler, Lake Bluff, IL, USA) on the pulp surface to smoothen it. The smear layer formed during dentin specimen preparation, was removed by submerging the specimens in 17% EDTA for 5 min for the biofilm assay and after this, they were sterilized by autoclaving at 121° for 20 minutes. For the adhesion test the samples were autoclaved after being polished, with no treatment.

2.1.1 Antibacterial Test for E. faecalis Biofilm

For *Enterococcus faecalis (E. faecalis)* biofilm, 15 µL standard strain (ATCC 29212) was put into 3 mL sterile brain heat infusion (BHI) broth (Oxoid, Basingstoke, UK) for growth overnight at 37° in air. After this, bacterial density was adjusted at 10^8cells/mL for *E. faecalis* (ATCC 29212) in a spectrophotometer (UV-VISIBLI, Shimadzu, Japan) at an optical density (OD) of 1 at 600 nm according to the 0.5 MacFarland standard. A dentin block + 100 µL of E. faecalis + 1900 µL of BHI was put into each well of a 24-well multiwell plate. For biofilm growth, all plates were incubated aerobically at 37°C for 21 days. The BHI was refreshed every 2 days. After the incubation period, the infected samples were washed with 1 mL of distilled water to remove loosely adherent planktonic bacteria. Then they were randomly divided into

five groups (n = 20) according to the experimental solutions. The contact test was performed by immersing dentin samples for 5 minutes in 5 mL ofthe experimental solutions. After the established time of contact with the solutions, the blocks were washed with phosphate-buffered saline (PBS).

2.1.2 Adhesion of E. faecalis to the dentin surface

After sterilization the dentin blocks were treated with 5 mL of the irrigant solutions for 5 minutes, and then neutralized with saline solution. For the sample contamination, *Enterococcus faecalis* standard strain (ATCC 29212) was grown overnight at 37° in air, having the bacterial density adjusted at 10^7cells/mL (as described in Section 2.4.1). Following this, a dentin block + 25 µL of *E. faecalis* + 975 µL of BHI was put into each well of a 24-well multiwell plate for a period of one hour. The specimens were washed extensively with 2 mL of phosphate buffered saline (PBS) to remove the microorganisms not adhered to the surface of the block,

2.1.3 Microbiological Analysis

After washing the dentin blocks prepared in Sections 2.4.1 and 2.4.2 with phosphate-buffered saline (PBS), they were stained with 15 µL of the SYTO 9/propidium iodide dye (Live/Dead, Baclight, Invitrogen, Eugene, OR) for 15 minutes in a dark environment. Then they were washed again, and directly observed using an inverted confocal laser scanning microscope (Leica TCS-SPE; Leica Biosystems CMS, Mannheim, Germany). Two confocal “stacks” for random areas were obtained for each sample using a 40x oil lens. In total, there were 10 samples per group, therefore, 20 stacks for each medication. For quantification purposes, bioImage_L software (www.bioImageL.com) was used to calculate the total biovolume and the percentage of red (dead cells) found after the antibacterial treatment.

2.2 Smear Layer Removal

Dentin blocks were obtained from bovine maxillary incisors, by using a 4 mm trephine drill (Neodent, Curitiba, PR, Brazil) under copious irrigation. The dentin surface of the discs were wet polished in a circular grinding machine (Arotec, São Paulo, SP, Brazil) using 300 and 600 grit SiC papers (Buehler, Lake Bluff, IL, USA) for 30 s each to produce a standardized smear layer. To prove the presence of smear layer on dentin walls, low vacuum scanning electron microscopy (LVSEM) (Apex

Express (FEI, Eidoven, Holland) images were captured at 550x magnification (Figure 1 Initial Images). Subsequently the blocks were randomly divided into 5 Groups (*n = 10*), treated for 5 minutes with the respective irrigating and chelating agents. New images were captured (final images) by SEM, according to the previously described parameters.

The presence of smear layer, was scored by three previously calibrated and blinded evaluators assessing the images obtained: Score 1 - no smear layer, all dentinal tubules open; Score 2 - small amount of smear layer, more than half of the dentinal tubules open; Score 3 - homogenous smear layer covering the root canal wall, less than half of the dentinal tubules open; Score 4 - complete root canal wall covered by a homogeneous smear layer, no open dentinal tubules. The difference in the quantity of smear layer before and after use of the irrigant solutions was measured, and corresponded to the value of cleaning the dentin block.

2.3 Determining the angle of contact by the sessile drop method (Wettability)

Dentin slices with dimensions of 4 × 4 × 0.8 mm (length × width × thickness) were obtained from crowns of bovine incisor teeth. The dentin surface of the discs were wet polished in a circular grinding machine (Arotec, São Paulo, SP, Brazil) using 1200 grit SiC papers (Buehler, Lake Bluff, IL, USA) for 30 s to obtain a flat surface. Then, the specimens were immersed in distilled water and submitted to ultrasonic agitation for 1 min to remove the residues from polishing.

The contact angle measurements were taken with a Goniometer (Drop Shape Analyzer DSA25B) coupled to a Progressive 1/3""CCD camera with filter, high performance acquisition plate, optical length and fixed focus system with a field of view of 11 X 8.2 mm, manual dosage system, syringe, needles and measurement software.

This occurred in two ways:

Test 1: a sessile drop of 1.0 µL of test solution at ambient temperature was dispensed on the dentin specimen surface with the aid of a micropipette;

Test 2: a sessile drop of 1.0 µL of physiological solution at ambient temperature was dispensed from a micropipette into the dentin specimen surface (treated for 5 minutes with the solutions in question) and then dried in an oven. The software measured the angle of contact formed. Ten measurements per group were made.

2.4 Surface tension determination by the pendant drop method

The contact angle measurements were taken with a Goniometer (Drop Shape Analyzer DSA25B) coupled to a Progressive 1/3""CCD camera with filter, high performance acquisition plate, optical length and fixed focus system with a field of view of 11 X 8.2 mm, manual dosage system, syringe, needles and measurement software. One 1.0 µL drop of the irrigant agents in question was dispensed from the needle. Before the drop fell, the computer program calculated the surface tension or interfacial tension by studying the profile of this pendant drop, in a process that could be divided into three parts: acquiring the image and extracting the contour, smoothing the contour of the drop, and finally calculating the surface or interfacial tension.

2.5 Cytotoxicity Analysis

Initially, the following stock solutions were prepared by dilution in Dulbecco's modified eagle medium (DMEM) obtaining the following concentrations: Solution 1: EDTA (17%, 8.5%, 4.25%, 2.125%); Solution 2: EDTA (17%, 8,5%, 4,25%, 2,125%) + benzalkonium chloride (1%, 0.5%, 0.25%, 0.125%); Solution 3: EDTA (17%, 8,5%, 4,25%, 2,125%) + N-acetylcysteine and Solution (1%, 0.5%, 0.25%, 0.125%) 4: EDTA (17%, 8.5%, 4.25%, 2.125%) + chlorhexidine (2%, 1%, 0.5%, 0.25%).

2.5.1 Cell Culture

For the assessment of cell expression, murine NIH/3T3 fibroblasts (ATCC) were grown in DMEM, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. For subculture, they were incubated with trypsin (0.25%) for 3 minutes at 37°C, followed by trypsin inactivation with medium containing 10% FBS. After centrifugation at 1,200 rpm for 5 minutes, the pellet was resuspended in the respective media and the cells were cultured in bottles for further experiments. Cells were incubated at 37ºC in a humid atmosphere containing 5% CO_2. For cell viability assays, MTT and crystal violet, $1x10^4$ cells / well were plated in 96-well plates. After 24h of incubation, the culture medium was replaced with respective irrigators concentrations for period of 3h. Positive and negative control groups were also carried out, the cells being treated, respectively, with 10% FBS medium and filtered ultrapure water. Control groups were not exposed to the irrigating solutions.

2.5.2 MTT Assay

After treatment time (3h), a solution containing 0.5 mg MTT per mL of medium was added to the plate and they were incubated in a humid atmosphere at 37°C for 4h. After removal of the MTT, dimethylsulfoxide (DMSO) was added for 30 minutes at room temperature. Absorbance was determined by optical density spectrophotometer with 562nm filter (FluoStar OPTIMA, microplate fluorescence reader). (37)

2.6 Statistical analysis

The data of all testes were submitted the Kolmorov-Smirnov test to evaluated the normality. Only the wettability test values presented normal distribution. To the wettability, the Anova e tukey were used to compare the groups. In the other tests the groups were compared using the Kruskal-Wlallis and Dunn tests. The significant level was 5%.

RESULTS

The percentage of viable cells of *E. faecalis* biofilm after 5 minutes treatment with the EDTA-based solutions are presented in Table 1 and represented in Figure 1 by confocal laser scanning microscopy images. The EDTA without additives and saline solution had the lowest antibacterial action against *E. faecalis* biofilm. ($P < 0.05$) There was no statistical differences among the other groups ($P>0.05$).

Table 2 shows the median, minimum and maximum values of bacteria adhesion after 1 hour of contamination on irrigant coated dentin blocks, and also it viability. The bacteria adhesion was avaliated by biovolume of bacteria after extensively irrigation with PBS to detache the non-adherent cells using confocal laser scaning microscopy and live and dead dye, represented by Figure 2. EDTA + benzalkonium chloride shows the repelling effects of surfaces coated with EDTA add to a surfactant, having the lowest amount of *E. faecalis* biovolume ($P < 0.0001$) and viability of cells ($P < 0.0001$) when compared to the other groups.

The Kappa test showed great concordance (0.91) between the examiner at the smear layer evaluation. Table 3 shows the median, minimum and maximum values of smear layer scores before and after the experimental treatments. In the initial analysis (before treatment) there was no difference among the groups ($P > 0.05$). After treatment, all the EDTA-based solutions removed the smear layer significantly when compared with the saline solution ($P < 0.05$).

Table 4 shows the mean values and standard deviation of surface tension and wettability of the tested solutions by the contact angle measurement (WI). It also shows the wettability values of saline solution after treated dentin with the tested solutions by the contact angle measurement (WD). Lower surface tension and wettability values (WI) were observed to occur in the Groups EDTA + benzalkonium chloride and EDTA + N-acetylcysteine, which differed statistically from the other groups ($P < 0.05$). Significant difference also occurred in the surface tension comparison of Group saline solution with Groups EDTA and EDTA + N-acetylcysteine ($P < 0.05$).The group that favors the wettability of saline solution (WD) in coated dentin was EDTA/benzalkonium, which differed statistically from all groups ($P < 0.005$).

Figure 3 shows the effect of the irrigating solutions at different concentrations on NIH⁄3T3 fibroblasts viability by MTT assay at 3h of exposure. All the groups exhibited high cytotoxicity at all dilutions tested when compared to the control ($P < 0.05$).

DISCUSSION

The null hypothesis was rejected because differences were observed in antimicrobial action, adhesion of bacteria to dentin, wettability properties and surface tension of the irrigants when compared to unmodified EDTA.

EDTA was introduced in Endodontics by Østby in 1957 in the form of a 15.5% aqueous solution and pH 7.3 (Nygaard-Østby 1957) and is one of the most frequently used chelating agents for root canal irrigation.(1, 38) However, the commercial form of EDTA (17%), has low antibacterial action, and non-existent antibiofilm action (39, 40), which leads to a final rinse with an antimicrobial agent, such as sodium hypochlorite to optimize disinfection. (Yamada et al. 1983, Zehnder 2006).

Concerns related to the extrusion of a concentrated sodium hypochlorite solution exist due to it acute injuring effects when in contact with vital tissues.(30) Furthermore, it proteolytic action on the collagen matrix of dentin can reduces the elastic modulus and flexural strength of the latter. (41) Using a calcium chelator as a final irrigant with antimicrobial properties will reduce the need to use excessive amounts of sodium hypochlorite solution to disinfect the root canal space. This will reduce the harmful effects of the the hypochlorite solution and also reduce treatment time. For this purpose

active substances benzalkonium chloride, n-acetylcisteine and chlorhexidine were added to the EDTA.

Benzalkonium chloride is a cationic agent belonging to the group of quaternary ammonium composites. Its antimicrobial action is related to the change in ionic resistance of the bacterial cell membrane. (42) N-acetylcysteine (NAC) is a potent antioxidant containing thiol and widely used mucolytic agent. It is effective in reducing extracellular polysaccharide production, disrupting mature biofilms.(43-45) Both of them are supposed to enhance the wettability of liquids, and also reduce the adhesion of bacteria to dentine, harming the formation of biofilm, which is a desirable effect for a final irrigant. (34, 35) But their use with EDTA has not been explored.

Chlorhexidine digluconate seens to have desirable caractheristics as well because of its adsorption to dentine (46), that leads to antimicrobial residual activity, a phenomenon known as substantivity. (47) It is a cationic compound that binds to bacteria cells that are charged negatively, causing an osmotic dequilibrium. At low concentration can cause leak out ions as potassium and phosphorous, causing a bacteriostatic effect.(48) At high concentration is a bactericidal agent with a broad spectrum of action due to precipitation and/or coagulation of the cytoplasm of bacterial cells. (33)

The antimicrobial activity of these substances added to EDTA were tested on *Enterococcus faecalis* biofilm. The bacterial communities in the root canal are found as biofilms (49) which are wrapped by an extracellular matrix protected them and increasing their resistance to antimicrobials by about 1000 times when compared to planktonic bacteria.(50) EDTA affected the membrane integrity in *E. faecalis* after 5 minutes of exposure (51), however, the EDTA concentration used was 50 mmol/L, that corresponds to 38.7% aqueous solution. This high amount of EDTA reduced microhardness, increasing calcium loss, resulting in not only smear layer removal, but erosion of dentin.(52) In this study, 17% EDTA alone did not affect the biofilm having the same behavior as saline solution. As expected, the mixtures had superior effect against biofilm than EDTA. (Table 1)

The use of a surfactant is important in biofilm disruption since proteins and polysaccharides on the bacterial cell surface attach irreversibly to specific receptors on the substrate (dentin) in a hydrophobic interaction.(53) Surfactants can change this high-affinity binding acting on the surfaces, by either changing bacteria hydrophobicity and bacterial surface charge.(54) This was verified in the current study where the

addition of benzalkonium chloride resulted in the lowest amount of *E. faecalis* biovolume. (Figure 2) This results are in accordance with literature that shows that adding benzalkonium chloride to sodium hypochlorite on dentin promoted inhibition of adhesion, and consequently formation of biofilm.(34) Therefore, EDTA/benzalkonium chloride showed effectiveness not only preventing the formation of biofilm and root canal re-infection, but also against *E. faecalis* biofilm itself when compared to pure EDTA. Chlorhexidine mixture with EDTA forms a white-colored precipitate, formed by neutralization of the cationic chlorhexidine by the anionic EDTA. (55) In this study, the mixture did not have a reduced chelating action when compared with that of pure EDTA, increasing it antibacterial action, but it might had affect the effect of the adsorption of chlorexidine to dentin, (56) no residual antimicrobial effect against *E. faecalis* in it adhesion. Adding a detergent (surface active agent) in this mixture may avoid this, based on a commercial irrigating solution called QMiX. (Dentsply Tulsa, Maillefer, Ballaigues, Switzerland) (57) EDTA is known to expose the dentin collagen. Although studies showed the ability of *E. faecalis* to adhere to collagen, (58) this study is in agreement with literature (29) that the adhesion of *E. faecalis* is not increased by the use of EDTA.

The use of a chelating agent is necessary for effective smear layer removal.(3) The chelation of calcium ions by EDTA was not negatively influenced by any of the additives tested. (Table 3) The surfactant effect further enhances this action possibly by increasing the penetration of the mixtures into uninstumented areas, (59) thereby increasing the disinfection of root canal systems. This study, for the first time showed that the addition of the surfactant benzalkonium chloride or the mucolytic agent N-acetylcysteine to EDTA led to a reduction in surface tension of the latter, increasing its wettability on dentin. (Table 4) When EDTA is applied to dentin, the hydroxyapatite is removed (inorganic component) leading to exposure of the collagen fibers, and diminishing the free surface energy.(60) This chemical change in the composition of dentin results in a more hydrophobic surface.(61) This may explain the better measurements of the dentin surface wettability when it was treated with physiological solution in comparison with EDTA. Whereas, a surfactant compound can change the energy of the surface, explaining why the mixture of EDTA with benzalkonium chloride favored the wettability of saline solution in the treated dentin.

Few studies have evaluated the cytotoxicity of EDTA.(62-65) In this study, the irrigating solutions, in serial dilutions, were applied to murine NIH/3T3 fibroblasts cells,

and the cytotoxicity was conducted using a colorimetric assay, MTT, measuring the number of viable cells present assessing cells mitochondrial metabolic activity.(66) Ideally, an irrigation solution is expected to have antimicrobial properties with as low of a toxicity as possible. For sodium hypochlorite and other solutions, as the concentration of irrigant is increased, unwanted cytotoxic effects are produced. (64) Nevertheless, as shown in the literature (64) and in Figure 3, the cytotoxicity of EDTA was not dose-dependent in the dilutions used in both works, having negative effects in the cells even in lower concentrations. (2,25%) EDTA at 1%, and 0.1% [46] or lower concentrations (65) have displayed reduced cytotoxic effects. If future studies shows that these low concentrations does not affect the EDTA smear layer removal, they could be a clinical potentially reality.

CONCLUSION

The addition of benzalkonium chloride, N-acetylcysteine and chlorhexidine to EDTA resulted in improvement in antibacterial action against *E. faecalis* biofilm, without compromising the EDTA chelating action and cytotoxicity. EDTA + benzalkonium chloride also decreased the adherence of *E. faecalis* to dentin coated with it, lowered EDTA surface tension and allowed better wettability of dentin, standing out from the other solutions. Therefore, the use of this association as a final rinse in endodontic treatment should be considered.

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Figure 1. Confocal laser scanning microscopy of *Enterococcus faecalis* biofilm treated with (G1)17% EDTA, (G2) 17% EDTA + 1% Benzalkonium Chloride, (G3) 17% EDTA + N-acetylcysteine, (G4) 17% EDTA + Chlorhexidine and (G5) Saline Solution. Live cells are indicated in green, and dead cells are indicated in red. Each piture represents an area of 275 x 275 mm.

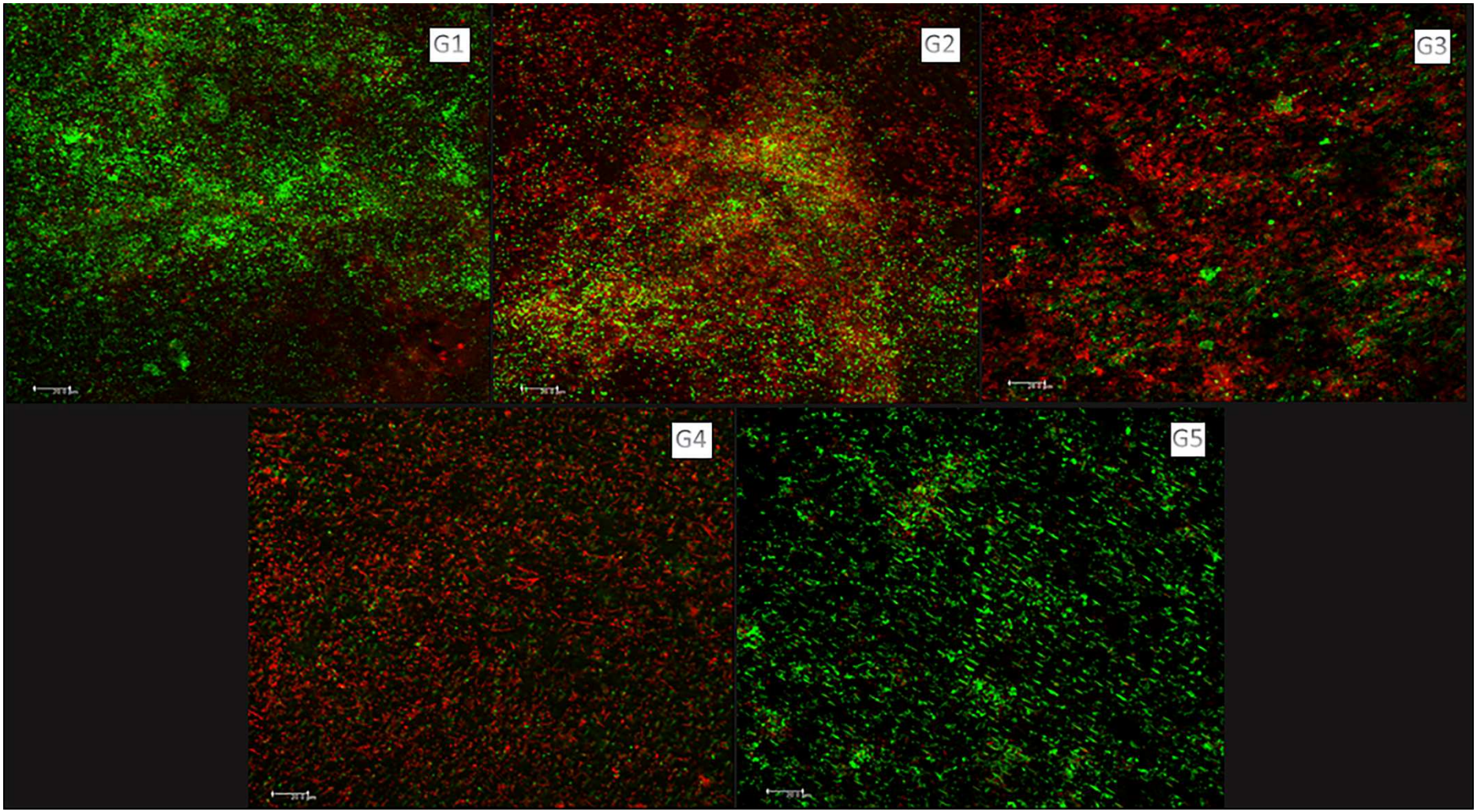

Figure 2. Confocal laser scanning microscopy of *Enterococcus faecalis* cells adhesion after one hour of contamination on dentin surfaces treated with (G1)17% EDTA, (G2) 17% EDTA + 1% Benzalkonium Chloride, (G3) 17% EDTA + N-acetylcysteine, (G4) 17% EDTA + Chlorhexidine and (G5) Saline Solution. Live cells are indicated in green, and dead cells are indicated in red. Each picture represents an area of 275 x 275 mm.

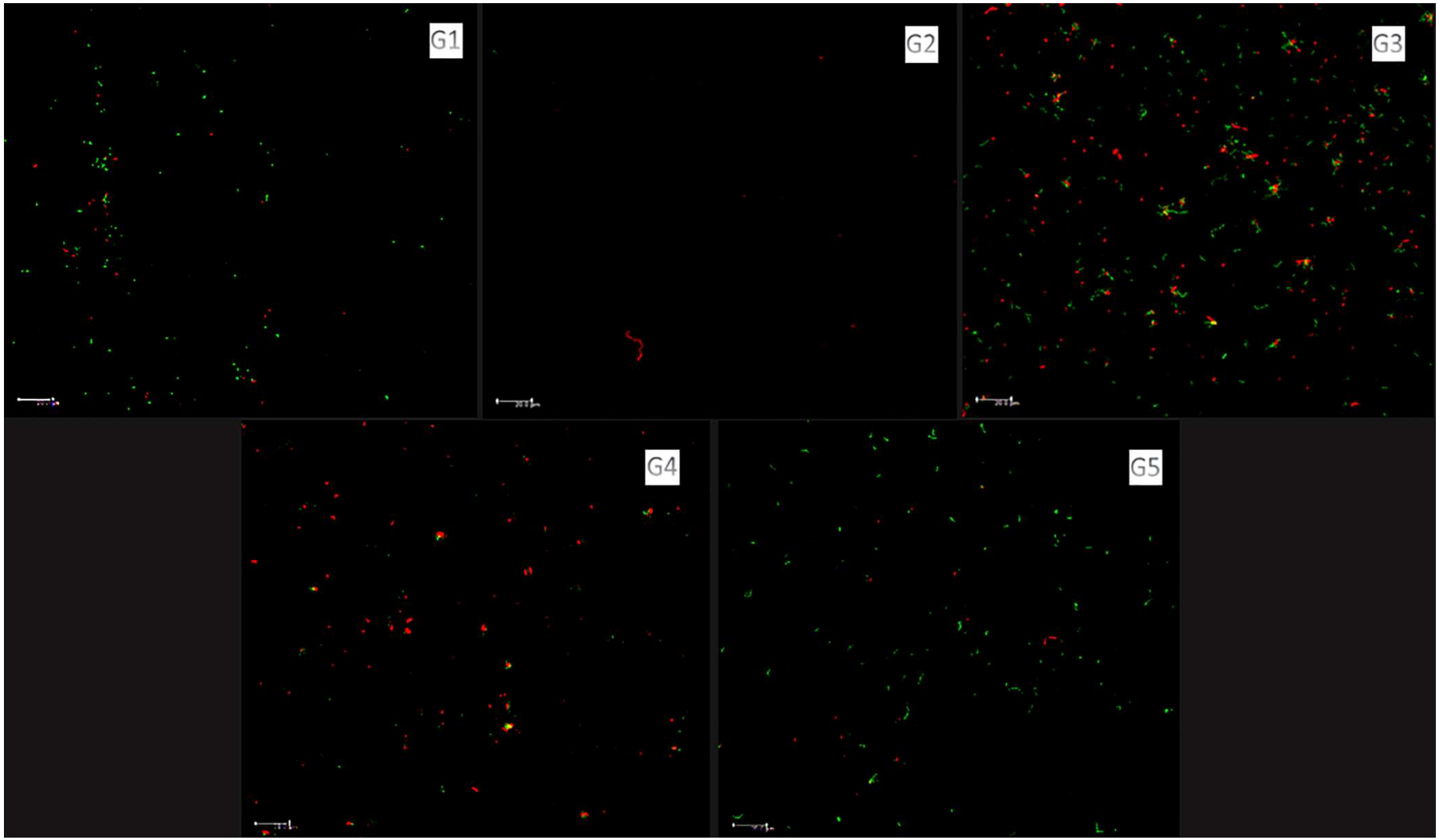

Figure 3. Effect of the irrigators at different concentrations on NIH⁄3T3 fibroblasts viability by MTT assay at 3h of exposure. Different letters indicate a statistically significant difference ($p<0.05$).

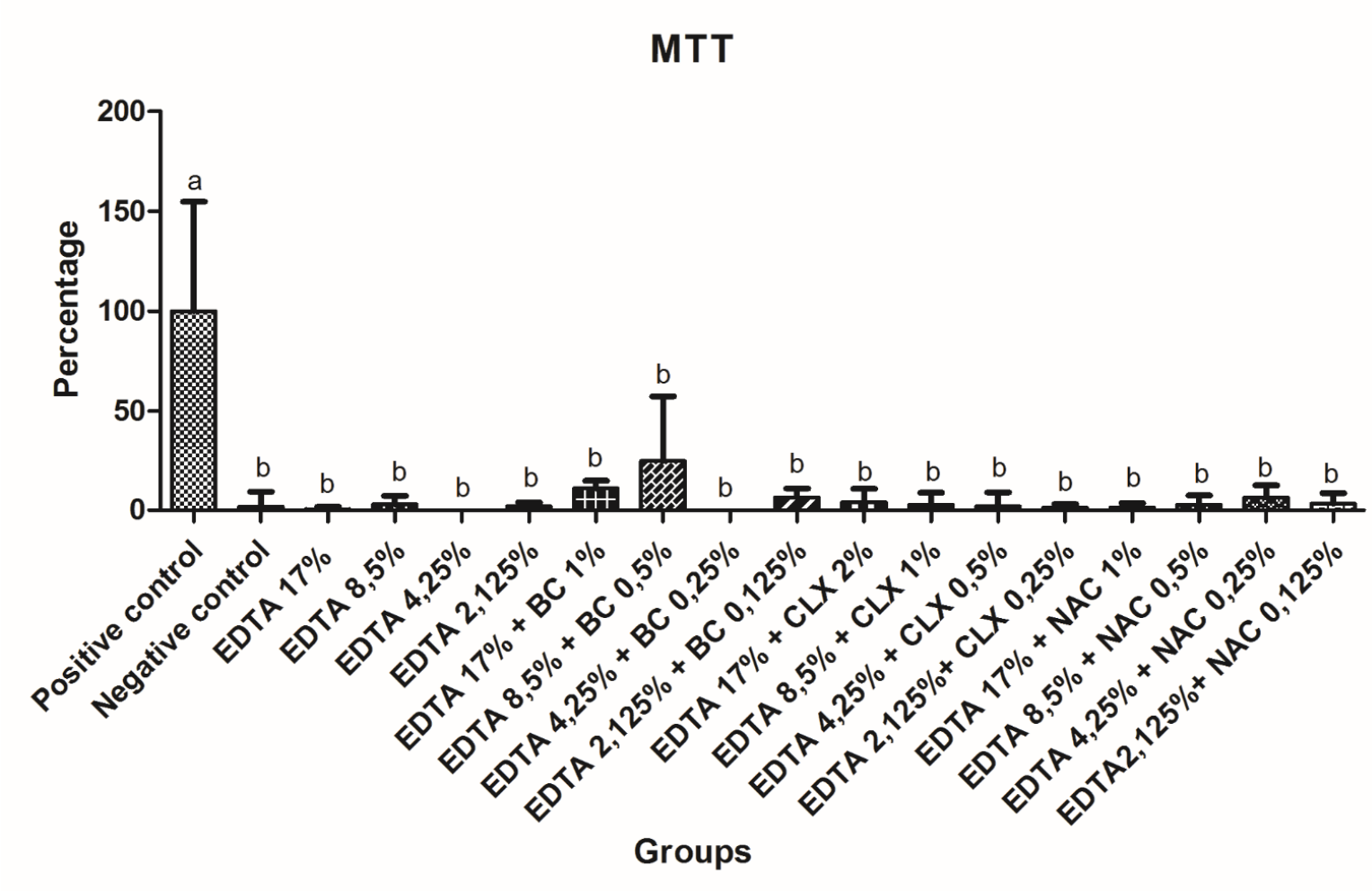

Table 1 - Median (Med), minimum and maximum (Min–Max) values of the percentage of live cells (% LC) of different biofilms after contact with the experimental solutions for 5 minutes.

	EDTA	**EDTA/BC**	**EDTA/CLX**	**EDTA/NAC**	**SS**
% LC	74,86 ^{A}	53,67 ^{B}	39,27 ^{B}	62,97 ^{B}	84,50^{A}
	(38,95 - 95,43)	(20,75 – 77,11)	(24,44 – 90,41)	(29,05 – 76,96)	(42,9 – 96,75)

Kruskal-Wallis with a Dunn post hoc P value <.05. Different capital letters in rows indicate statistically significant intergroup differences.

Table 2- Median (Med), minimum and maximum (Min–Max) values of the percentage of live cells (% LC) and biovolume of bacteria adherence after 1 hour of contamination on the dentin coated with the experimental solutions for 5 minutes.

	EDTA	**EDTA/BC**	**EDTA/CLX**	**EDTA/NAC**	**SS**
%LC	87,57^{A}	0,59^{B}	90,4^{A}	72,82^{A}	91,36^{A}
	(4,40 – 99,94)	(0,0 – 32,33)	(49,79 – 100)	(10,67 – 98,19)	(19,5 – 100)
Biovolume	670,5^{A}	134,5^{B}	994,5^{A}	854,5^{A}	804^{A}
	(58 – 4678)	(1 – 6346)	(78 – 7880)	(151 – 5162)	(80 – 14626)

Kruskal-Wallis with a Dunn post hoc P value <.05. Different capital letters in rows indicate statistically significant intergroup differences.

Table 3- Median (Med), minimum and maximum (Min - Max) scores for the presence of smear layer in each group before and after 5 min of immersion in the irrigation solutions.

	EDTA		**EDTA/BC**		**EDTA/CLX**		**EDTA/NAC**		**SS**	
	T-Inicial	*T-5min*	*T-inicial*	*T-5min*	*T-inicial*	*T-5min*	*T-inicial*	*T-5min*	*T-inicial*	*T-5min*
Aqueous	4^{Aa}	2^{Ba}	4^{Aa}	2^{Ba}	4^{Aa}	2^{Ba}	4^{Aa}	2^{Ba}	4^{Aa}	4^{Ab}
Vehicle	(3-4)	(1-4)	(3-4)	(1-4)	(3-4)	(1-3)	(3-4)	(1-3)	(4-4)	(4-4)

Score 1 - no smear layer, all dentinal tubules open;

Score 2 - small amount of smear layer, more than half of the dentinal tubules open;

Score 3 - homogenous smear layer covering the root canal wall, less than half of the dentinal tubules open;

Score 4 - complete root canal wall covered by a homogeneous smear layer, no open dentinal tubules.

Friedman P-value <0.05; different capital letters in rows indicate statistically significant intragroup differences, different lowercase letters in columns indicate statistically significant intergroup differences.

Table 4- Line 1: Mean and standard deviation values of surface tension for all the irrigants. Line 2: The mean and standard deviation values of the wettability of the irrigants on dentin measured by contact angle measurement. (WI) Line 3: Median (Med), Mininum and Maximun (Min-Max) values of surface wettabilivty of dentin after been coated with the irrigant solutions by contact angle measurement. (WD)

	EDTA	**EDTA/BC**	**EDTA/CLX**	**EDTA/NAC**	**SS**
Surface Tension	65,52±1,87^{A}	34,38±0,46 ^{C}	68,45±0,98 ^{A}	35,8±1,53 ^{C}	54,17±1,32 ^{B}
WI	50,80±7,74^{A}	18,23±5,76^{B}	58,57±13,5^{A}	30,92±11,29^{B}	54,57±18,45^{A}
WD	67,95^{AB} (44,1 – 75,3)	20,30^{C} (7,3 – 32,1)	74,30^{A} (33,8 – 89,7)	39,95^{BC} (10,9 – 66,6)	39,10^{BC} (21,7- 49,0)

One-way Anova with Tukey post-hoc P value <0,05. Different capital letters in rows indicate statistically significant intergroup differences.

2.2 ARTICLE 2 - Influence of different endodontic protocols on the dentin structure and adhesive dentin-sealer interface

ABSTRACT

Objective. The use of sodium hypochlorite (NaOCl) and ethylene diamine tetracetic acid (EDTA) to chemo-mechanically prepare the root canal may cause irreversible damage to dentin. Furthermore the dentin substrate may not be favourable for the sealer interaction. The aim of this research was to assess a number of chemicals employed on their own or in series and investigate their effect on tooth structure and also to investigate which irrigation protocol is the best for the use of hydraulic calcium silicate cement (HCSC) sealers thus guidign the clinicians on matched irrigation-obturation strategies for optimized root canal therapy.

Methods. Different irrigating solutions including NaOCl (2 and 5%), 17% EDTA, 1% Benzalkonium Chloride (BC) and saline used on their own or in a sequence were employed and the change in dentin microhardness, ultrastructure and mineral content (Scanning Electron Microscopy and Energy Dispersive Spectroscopy; SEM/EDS), organic/inorganic matter (Fourier transform infrared spectroscopy; FT-IR), surface roughness and Young`s modulus (Atomic Force Microscope; AFM) were investigated. Furthermore, four root canal sealers were characterized by SEM/EDS and their interaction with the dentin was analyzed by assessing the changes in microhardness of the dentin after sealer placement and also the sealer to dentin interface by SEM/EDS and Confocal Laser Scanning Microscopy (CLSM).

Results/Conclusion. Protocols E/F significantly altered the mineral content of root dentin causing it to crack. Previous degradation of the organic portion of smear layer on the root canal surface by NaOCl favored removal of Ca^{2+} by chelating agents with widening of the dentinal tubules. Highly concentrated NaOCl followed by EDTA significantly changed inorganic matter dissolution, microhardness and induced a rougher topography on the dentin surface with eroded dentinal tubules. As smear layer was still present on surfaces in NaOCl+chelator Groups, the use of EDTA Benzalkonium Chloride (EDTA-BC) or NaOCl final flush was required for further disinfection. In protocol D, no significant alteration was noted for the mixture EDTA-surfactant indicating a weaker chelating solution. Further irrigation with NaOCl resulted in erosion of the dentin around the tubules and *smear-layer*-free surfaces. Dehydration

of Hydraulic Calcium Silicate Cements in high vacuum SEM *caused* material shrinkage *and conflicting data with CLSM, in* which specimens were kept hydrated. Based on CLSM analysis of dentin, Protocols A/B (NaOCl/EDTA/NaOCl), or C/D (NaOCl/EDTA-BC) matched with AH Plus, Bio Root and Total Fill sealers. Microhardness progressively decreased in protocol B, and was recovered after MTA Fillapex or Total Fill application. As MTA Fillapex presented gaps on dentin/sealer interface, Total Fill appeared to be the best match for protocol B. All sealers presented a rich dye-infiltrated layer raising doubt about the existence of a “Mineral Infiltrated Zone”.

1. Introduction

The technological advancement in endodontic instruments has enabled clinicians to speed up the biomechanical preparation stage (67). As time of action of irrigant solutions in root canals has been reduced, the use of more concentrated antimicrobial agents during and after instrumentation are probably necessary to prevent the tooth from being a source of infection. NaOCl is the irrigant mainly used in contemporary endodontic practice, followed by EDTA (68). The antimicrobial activity and efficacy of NaOCl for dissolving organic tissue, such as remnants from the canal space, is positively corelated to it concentration (69, 70). Further degradation may lead to deleterious effects on the collagen matrix and integrity of the dentin structure (7). EDTA is an inorganic debris-dissolving irrigant, although may act on the mineral content of root canals, causing peritubular and intertubular dentinal erosion (71).The latter may be worsened by the low surface tension of the surfactant Benzalkonium chloride (BC), used in a mixture with EDTA to enhance it poor antimicrobial action (In review: Zancan RF, 2020). As none of these irrigants can be regarded as optimal with alternating the use of these chemicals in clinical practice, their action enables a better outcome of the treatment to be achieved, in terms of cleaning and disinfection of root canal (68, 72). However, from a mechanical point of view, progressive dissolution of the organic and inorganic compounds of dentin may predispose to post-treatment root fracture.

Hydraulic Calcium Silicate Cements (HCSCs) have been promoted for their bioactivity with calcium-hydroxide-releasing properties when in the presence of biological fluids. The high pH induces a “mineral infiltrated zone”, consequently leading to mineralized tissue deposition, creating a self-adhesive bond with root canal walls (73-75). These chemical bonds seem to have a positive effect on reinforcing the remaining tooth structure (76). In this sense, sealers may cause recovery of the mechanical properties of dentin. Although the sealers tested had a similar composition, they varied in their presentation and dependence on humidity in the root canal to set. BioRoot RCS (Septodont, France) is a water-based sealer (77), MTA Fillapex (Angelus, Londrina, Brazil) - the first MTA-based sealer that was developed - is presented in a 2-paste system (26), and TotalFill BCS (FKG Dentaire SA, La Chaux-de-Fonds, Switzerland) also known as Endosequence BC sealer (Brasseler, Savannah, GA, USA) is a premixed bioceramic endodontic sealer(27).

Sealers are also required to create a bacteria-resistant seal that has good interaction with the dentin-sealer interface, because microorganisms present in persistent or secondary intraradicular infection may reach the periapical tissues and be responsible for endodontic failure (78, 79). However, the choice of sealer needs to match with the previous irrigation protocol, since they both interact with dentin. For example, the sealer bond strength of the gold standard AH Plus - an epoxy resin-based sealer - appears to be benefit from EDTA (80), while Hydraulic Calcium Silicate Cements (HCSCs) may be harmed by it (81).

Health care professionals must be aware of their duty relative to “primum non nocere”, with reference to avoiding unnecessary harm or injury to patients and potential medico-legal complications. Clinicians in the field of endodontic treatment need a reliable source of reference to guide them to finding a safe irrigant protocol that matches with the sealer, to provide a suitable obturation that will prevent bacteria from percolating into the periapical tissues, weakening the tooth or even fracturing it. The assessment of the effects of irrigation protocols on dentin characteristics were directly analyzed by means of microhardness testing, Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS), Fourier transform infrared spectroscopy (FT-IR) and Atomic Force Microscopy. The characterization of sealers and sealer/dentin interfacial characteristics were assessed using Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS), Confocal Laser Scanning Microscopy (CLSM), and a fluorescent marker. This study hypothesis was that changes in environmental water content could have an effect on the data for HCSCs.

2. Materials and Methods

The following irrigating solutions were used:

- Sodium Hypochlorite 2% and 5% (NaOCl-2, NaOCl-5; Cerkamed, Stalowa Wola, Poland)
- 17% ethylene diamine tetracetic acid (EDTA; Sigma, Stoinheim, Germany)
- 17% EDTA and Benzalkonium Chloride 1% (EDTA-BC; Sigma, Stoinheim, Germany)
- Saline Solution (Fisher Scientific, UK)

EDTA was prepared by mixing 17 g of the powder (99,9% of purity) (Sigma, Stoinheim, Germany) in 50 mL of deionized water. Sodium hydroxide (Sigma, Stoinheim, Germany) was added to aid the solubility of the powder and pH was stabilized with hydrochloric acid, to 7.4. The solution was made up to 100 mL and pH buffered by adding 0.02M of Phosphate (Sigma, Stoinheim, Germany). For the preparation of EDTA 17% Benzalkonium Chloride 1%, 1.05 mL of BC (95% of purity) (Sigma, Stoinheim, Germany) was added in 99 mL of EDTA before the stabilization of pH and the addition of 0.02 M of Phosphate.

For the saline solution 0.904 g of NaCl powder (99,53% of purity) (Fisher Scientific, UK) was mixted in 100 mL of deionized water, obtaining a 0.9% solution.

Irrigation Protocols

The following irrigating sequences shown in Table 1 were employed in the irrigation protocols.

Table 1: Irrigants tested alone (NaOCl, EDTA, EDTA-BC and saline) or combined on different sequences.

Protocols	Groups	Irrigants
A	1.	NaOCl 2
	2.	NaOCl 2 – EDTA
	3.	NaOCl 2 – EDTA - NaOCl 2
B	4.	NaOCl 5
	5.	NaOCl 5– EDTA
	6.	NaOCl 5 – EDTA - NaOCl 5
C	1.	NaOCl 2
	7.	NaOCl 2 – EDTA-BC
D	4.	NaOCl 5
	8.	NaOCl 5 – EDTA-BC
E	9.	EDTA
F	10.	EDTA-BC
G	11.	Saline Solution

The details of irrigation protocols undertaken are shown in Table 2.

Table 2: The amount and velocity of the antimicrobials and chelators undertaken during the irrigation protocols.

Antimicrobial (NaOCl)	5 mL/each instrument (S1, SX, S2, F1, F2, F3) 1mL/10 sec flow rate
Chelator or Chelator/Antimicrobial (EDTA or EDTA-BC)	5 mL/3 minutes
Antimicrobial (NaOCl final irrigation)	5 mL/ 3 minutes

Assessment of effect of irrigation protocol on dentin characteristics

I- Dentin preparation

Extracted human maxillary incisors were obtained from the Dental School Tissue Bank (ethical approval number: 14/EM/2811- BCHCDent397) and were embedded in auto polymerizing epoxy resin (Epoxyfix; Struers GmbH, Ballerup, Denmark) to enable sectioning of the root along its long axis using a hard tissue microtome (Isomet, Buhler, Lake Buff, USA). The resulting halves were then ground progressively using finer diamond discs (Stuers ApS, Ballerup, Denmark) and pastes (Stuers ApS, Ballerup, Denmark) on an automatic polishing machine (Buehler Phoenix Beta Grinder/Polisherm, **Dusseldorf**, Germany), finishing with a silicon suspension of 1 μm.

II- Microhardness testing

The measurement of the dentin microhardness was obtained by a Durascan 20 Vickers microhardness tester (Emco Test, Kuchl, Austria) at a magnification of x40 employing 9.807N load and a 15-second dwell time. The results were given by Knoop hardness number (KHN). The samples (*n=3*) were submitted to the protocol treatments shown in Table 1 and 2. Afterward each irrigation regime five indentations were made along the medium third of dentin and a mean value was obtained for each sample. The differences between treated and untreated (initial) KHN measurements of dentin were used to assess alterations on its microhardness.

III- Dentin morphology and composition

a. Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS)

In order to prepare treated samples for SEM they were dried in a vacuum desiccator, attached to aluminum stubs and sputter coated with a conductive layer of gold using a TK8842 Gold Target (Emitech Limited, Ashford, United Kington). The specimens were analyzed by scanning electron microscopy (SEM; Zeiss MERLIN Field Emission SEM, Carl Zeiss NTS GmbH, Oberkochen, Germany) at 2000× magnification. For Energy Dispersive Spectroscopy (EDS) analyses, the follow parameters were used, EHT= 20 kV, Iprobe = 1000 pA and WD= 8.5 mm for a 35° take off (elevation angle). The alterations of dentin surface by different irrigation protocols were measured by the monitoring changes in the elements calcium, phosphate and chlorine. Each exposed dentin sample (*n=3*) was analyzed in triplicate.

b. Fourier transform infrared spectroscopy (FT-IR)

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

IV- Atomic Force Microscopy (AFM) Analysis

NanoWizard® 3 AFM (JPK Instruments, Germany) and a cantilever with a TAP 150GD-G silicon tip gold reflex coating, resonant frequency of 150 kHz, and a spring constant of 5 N/m (Budget Sensors, Bulgaria) were used for imaging and measure Young`s modulus of dentin. After the cantilever calibration, the atomic termination of AFM probe

sweep across the dentin surface making a constant force of 125 nanonewtons. (*n=3*) The measurements were 100 nm apart in a 1 μm^2 for Young's Modulus, considering the values of cantilever deflection and contact region of the force curve using the Hertzian model. The images of 5 μm^2 were processed and analyzed using the commercial software NanoScope Analysis (Bruker Corporation, version 1.5, Leeds, UK).

Sealer characterization and assessment of the dentin to sealer interface

The sealers undertaken to fill the root canal after irrigant protocols are shown in Table 3.

Table 3: Sealers undertaken on the filling of root canal

1	AH Plus (AH; DENTSPLY DeTrey GmbH, Konstanz, Germany);
2	Bio Root (BR; Septodont, Saint Maur-des-Fosses, France);
3	MTA Fillapex (MF; Angelus Dental Solutions, Londrina, SP, Brazil);
4	Total Fill BC Sealer (TF; FKG Dentaire, La-Chaux-de-Fonds, Switzerland).

V- Sample preparation

The Incisors root canals (*n=3*) were instrumented up to the real working length (1mm short of the apical foramen) with the ProTaper System (Dentsply Maillefer, Ballaigues, Switzerland) up to a master apical file size F3 using an in-and-out pecking motion of about 3 mm in amplitude with a light apical pressure. With the aid of a plastic syringe and capillary tip cannula that coincided with the apical foramen (Ultradent, South Lake City, USA) abundant irrigation was performed according to the irrigation protocol in tables 1 and 2. The sealers (table 3) were prepared following the manufacturer's recommendations, and them dispensed in the root canal using a syringe. Following obturation, the samples were stored in an oven at 37 °C for 7 days. After that, they were embed in auto polymerizing epoxy resin (Epoxyfix; Struers GmbH, Ballerup, Denmark), then sectioned longitudinally for SEM/EDS analysis and cross sectioned for Confocal Laser Scanning Microscope (CLSM) using a hard tissue microtome and polished using an automatic polishing machine as described previously. (section I)

VI- Microhardness testing

The microhardness of dentin coated with different irrigant protocols followed by the obturation of the root canal was assessed as described in section II.

VII- Sealer characterization and assessment of the dentin to sealer interface

a. Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS)

The methodologies were performed as described in section III (a). SEM employed in the secondary electron imaging (SE1) mode was used to obtain: 2000x images of the sealer morphology; 100x images of the sealers morphology and their interaction with dentin and 500x images of dentin-sealer interface in the mid-root portion of the root canal. The EDS detector was performed for sealer chemically map on randomly selected areas and chemical characterization of the dentin after root canal obturation 50µm distant about to dentin-sealer interface.

b. Confocal Laser Scanning Microscope (CLSM)

To allow analysis under the CLSM, each sealer was mixed with Orange G dye (16230 Sigma-Aldrich, Dorset, UK) to an approximate concentration of 0.1%. (*n*=*3*). After specimens preparation (section V), they were analyzed a CLSM (Leica Microsystems GmbH, Mannheim, BadenWürttemberg, Germany) with x60 water immersion objective with a excitation/emission wavelength of 494/521 nm. Four randomly selected images were made *per* sample.

3. Results

Asessment of effect of irrigation protocol on dentin characteristics

I. Microhardness

The mean of Knoop hardness number (KHN) measurements of root dentin according to the treatment group in different protocols are shown in Figure 1. For protocols A and C that used the 2% concentration of NaOCl, no difference between groups was observed ($p = 0.45$ and $p = 0.73$). However, there was a significant reduction in dentin microhardness in protocols B and D when NaOCl is used in it higher concentration (5%) ($p =0.02$; $p= 0.002$). The irrigants EDTA (Protocol E), EDTA-BC (Protocol F) and Saline Solution (Protocol G) alone did not affect the microhardness of dentin ($p = 0.70$;

p = 0.10; p =0.86). Nevertheless, when EDTA was applied in sequence with NaOCl 5% in protocol B the dentin microhardness was significantly reduced. (p = 0.02) The use of EDTA-BC after NaOCl-5 in protocol D did not affect the dentin microhardness. (p>0.05)

II. Morphology and composition of dentin

a. Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS)

The scanning electron micrographs of the dentin treated with different irrigation protocols are shown in Figure 2. In both Protocols A and B, when the NaOCl is used as the first irrigant, it is shown clearly that the smear layer is not removed. Small deposits were observed on the dentin surface (Figure 2a). High magnification of these deposits (Figure 3a) reveals the cuboid shape of the crystals which is typical of sodium chloride. For both protocols the use of EDTA led to the widening of the dentinal tubules which was more effective in the 2% sodium hypochlorite group. After EDTA irrigation some crystals of sodium chloride were visible inside the tubules in certain areas shown in high magnification in Figure 3b. Further irrigation with sodium hypochlorite resulted in the erosion of the dentin around the tubules (Figure 2c). This is shown at higher magnification in Figure 3c. The use of EDTA BC after the NaOCl as in Protocols C and D shows widening of the dentinal tubules with some erosion shown in high power in Figure 3c. The dentinal tubules were less evident than when using EDTA after the hypochlorite as in Protocols A and B. After the use of chelators small amounts of smear layer were present inside dentinal tubules for Protocols A, B, C and D (Figure 2b), which disappear after the final flush of sodium hypochlorite. The use of EDTA and EDTA-BC as sole irrigating solutions in protocols E and F showed widening of the dentinal tubules also with some sclerosis (Figure 2d) at the periphery and presence of smear layer. Saline used on its own as in Protocol G did not have any effect on the smear layer removal.

EDS analysis are shown in table 6 and described in section V (a).

b. Fourier transform infrared spectroscopy (FT-IR)

Figure 4 represents the infrared spectral region between 1600 and 750 cm^{-1} of untreated dentin showing the absorption peaks amide III (1298–1216 cm^{-1}), phosphate (1170–780 cm^{-1}) and carbonate (888–816 cm^{-1}).

Figures 5 and 6 show values for the ratios of amide III/phosphate and phosphate/carbonate respectively, on the dentin surface before and after immersion in the irrigants contained in the protocols: A, B, C, D, E, F and G.

NaOCl first and final flush causes a decrease on organic matter by the deproteinization of collagen on dentin that is recovered by the follow use of chelators. There is no significant difference between groups except for Protocol D ($p=0.004$) whereas the higher concentrated NaOCl is followed by EDTA-BC. Even when EDTA and EDTA-BC (Protocols E/F) were used alone the collagen ratio increased (Figure 6; $p = 0.089$ and $p = 0.06$) due to the dissolution of inorganic matter (Figure 7; $p = 0.05$ and $p = 0.128$) that creates a partially demineralized collagen matrix. The results show a relevant tendency towards significant values, though. When comparing the protocols using 2% (Protocol A) and 5% (Protocol B) NaOCl, the latter seems to stimulate the demineralization of dentin ($p = 0.015$). This does not happen in protocol D that used EDTA-BC as a final irrigator. Carbonate/phosphate ratio was left intact by saline solution. The small drop on amide III/phosphate was not sufficient to alter significantly dentin.

III. Atomic Force Microscopy (AFM) Analysis

Figure 7 and 8 indicates the Young's modulus and representative AFM scanning phototopographs of dentin coated with the irrigation protocols, respectively. NaOCl first flush, chelators used alone and saline alters significantly the Young's Modulus of dentin. A progressive decrease on the latter occurs on the irrigation sequence NaOCl + chelator, except for NaOCl-2 + EDTA-BC. The values after NaOCl-2 and 5 final flush have no difference with NaOCl-2 first flush and initial, respectively. AFM phototopographs revealed a totally covered non treated dentin (initial) by the *smear layer*. The attenuation on image colors after NaOCl first flush on dentin is related to a decrease of *smear layer* height on it topography. When the chelators are used alone (Protocols E and F) just a few tubes were found on dentin. The protocols NaOCl+chelator result in a wider range of open dentinal tubules, whereas NaOCl-5 promotes a rougher surface of dentin than NaOCl-2. A smoother surface and widening of the dentinal tubules were found after NaOCl final flush (protocols A/B).

Sealer characterization and assessment of the dentin to sealer interface

VI. Microhardness

The microhardness values of the dentin having as variable the sealer or the irrigation protocols are presented in tables 4 and 5, respectively. Table 4 compared each sealer data with the values of non obturated dentin (section II). Indicating a recover of dentin, the sealers MF and TF enhanced KHN dentin values when compared with the other groups. No statistic difference was shown for AH, BR and non obturated dentin. On table 5 no statistic difference was seen among the irrigation protocols regardless the sealer.

IV. Sealer characterization and assessment of the dentin to sealer interface

a. Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS)

The 4 sealers were characterized by scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS), as shown in Figure 9. EDS revealed the presence of calcium (Ca), carbon (C), oxygen (O), silicon (Si) and aluminum (Al) for all sealers. AH shiny particles (letter a, spectrum 2) had high amounts of calcium (Ca) and tungsten (Tg), indicating calcium tungstate (ICDD: 01-085-0443) as being one of the radiopacifiers. The second was zirconium oxide (ICDD: 00-037-1484) indicated by the presence of zirconium (Zr) and oxygen (O) (spectrum 1 and 2). BR and TF also contained zirconium oxide as radiopacifier, as may be seen in spectrum 1 (letter b,d). The presence of chlorine was found in BR, because it contained calcium chloride that accelerated its setting. No bismuth (Bi) was indicated in the EDS analyses of the area selected for MF (letter c) although its manufacturer indicated the presence of bismuth oxide as a radiopacifier. The same occurred for TF, since calcium phosphate was stated by the manufacturers, but no traces of phosphate (P) were shown.

At lower magnifications (100x), MF and TF displayed porosity on their surfaces, when compared with AH and BR that showed homogenous filling of the root canal (Figure 10).

The sealing ability of the sealers at the interface of dentin coated with different irrigation protocols, was shown by SEM as illustrated in Figures 11, 12, 13 and 14. At 500x magnification, all HCSCs appeared to have a dryer and amorphous structure

when compared with AH. The use of chelating agents (EDTA or EDTA-BC) in Protocols E and F promoted good marginal adaptation of all sealers to dentin. The epoxy resin sealer AH had thinner spaces at the dentin-sealer interface when compared with the HCSCs. AH-dentin interface showed a good seal except for the NaOCl First Flush Groups. A outstanding seal was noted for Groups NaOCl/chelator, NaOCl-5/EDTA/NaOCl-5 and EDTA. None of the sealers showed completely gap-free regions along the samples.

EDS analysis for calcium, phosphate, chlorine and silicon ions are represented in Tables 6 and 7. In Table 6 the data of sealers were considered without regard to the irrigation protocol. HCSCs had calcium values than those of AH, although no statistical difference was found (Table 6). All sealers enhanced the calcium values of non-obturated dentin. Chlorine remained on dentin after applying the irrigation protocols. BR had the highest chlorine values among the sealers, due to the presence of this ion within its composition. Larger amounts of silicon (Si) were found on dentin for AH than that found for HCSCs.

In Table 7 the irrigation protocol was consider irrespective of the sealer. The lowest calcium and phosphate values were found for the chelator Groups (Protocols E/F). Higher amounts of chlorine where found Group NaOCl-5+EDTA + NaOCl-5. The sequence NaOCl+EDTA+NaOCl (Protocols A/B) allowed the best sealer penetration, confirmed by highest amounts of silicon on dentin.

b. Confocal Laser Scanning Microscope (CLSM)

The sealing ability of AH, BR, MF and TF were shown in Figures 15, 16, 17 and 18 respectively, at the dentin interface coated with different irrigation protocols (Table 1/2). Irrespective of the irrigation protocol, all sealers penetrated into the dentinal tubules. At magnification by CLSM, AH showed a good seal on dentin for all groups. A rich dye infiltrated zone appeared at the AH-dentin interface of non-chelator groups (NaOCl-2/5 and Saline) and at the AH-gutta percha interface for Group EDTA-BC. For HCSCs, good marginal adaptation was achieved for BR and TF with the exception of Protocol A (NaOCl/EDTA) used for the former, and Protocols A and F for the latter. AH displayed more porosity than BR and TF. MF displayed voids and cracks on its surface, with better interaction with dentin for Protocols B/D (NaOCl/chelator), E/F (chelators) and G (saline).

A band of rich dye infiltrated dentin was also seen in Protocol A (NaOCl-2) for HCSCs; Protocol B/D (NaOCl-5, NaOCl-5/chelator) for BR; Protocol A (NaOCl-2/EDTA/NaOCl-2), D (NaOCl-5/EDTA-BC) and G (saline) for MF and Protocol A/C (NaOCl/chelator), E (EDTA-BC) and A/B (NaOCl/EDTA/NaOCl) for TF. For chelator groups (Protocols E/F), lines on dentin were shown for AH and BR. For MF they were shown for Protocol B/D (NaOCl/chelator) and E (EDTA). Although it was not a point of interest of this study, the sealer-gutta percha interface had larger spaces for HCSCs than for AH.

Illustrative images of the sealer-dentin interface with possible situations that may change the optical properties of fluorescence-mode CLSM are present in Figure 19. Letters A and B1 show that gaps on sealer-dentin interface may expose the entrance of dentinal tubules and lead to a bright line beneath the cement. The sealer with great flowability (HF) more easily masked this situation with superimposition of stained dentinal tubules, while the lower penetration pattern might enhance the optical illusion of “MIZ”. Variations in the angular orientation of dentin cuts and illustrative images of S-shaped dentinal tubules based on Shirazi, 2017 (82) are shown in B2. Figure C shows dense clusters of stained dentin debris that may have been created after the teeth were sectioned;

4. Discussion

A combination of several irrigant solutions has been proposed to achieve the objectives of root canal treatment (68, 83). The sequence and irrigant solutions used defined the characteristics of the dentin surface and its interaction with the sealers used in the root filling. Sodium hypochlorite is a strong base used throughout the biomechanical preparation procedure, due its unique capacity to dissolve organic and necrotic tissues as pulp, bacterial and biofilm remnants(69, 70). It is capable of precipitating sodium chloride crystals inside the dentinal tubules and in the main root canal (Figure 2; 3a,b), which may cause a bactericidal effect resulting from the loss of water content from the bacterial cells (84). The concentration of sodium hypochlorite depends on the chlorine content it releases, which is positively correlated to its tissue-dissolving ability (85, 86). Chlorine is a strong oxidant that degrades long peptide chains of collagen (87), resulting in N-chloroamines (88) that could be indicated by traces of chlorine on the surface of dentin after flushing with NaOCl (Table 7). NaOCl deproteination is not uniform. The smear layer created by the combined use of hand

and rotary instruments has different amounts of organic mass (89) which creates an irregular degradation pattern of superficial and sub-superficial encapsulated collagen. In topographic analysis by AFM the intensity of image color is proportional to surface height. The thicker smear layer on untreated dentin is represented by the strongest colors, while the organic matter dissolution by NaOCl on the surface of dentin is shown by the decrease in its intensity (Figure 8). In the structural architecture of dentin, the soft tissue collagen is encapsulated and protected by nanocrystalline carbonated apatite. Since NaOCl has a low molecular weight (74.4 Da) it spreads on the intrafibrillar water volume of apatite-encapsulated collagen matrix (7). The contact surface of collagen with sodium hypochlorite leads to oxidative chemical degradation of collagen from the “superficial subsurface” of mineralized dentin, creating a collagen-depleted mineral ghost layer (Figure 5 and 6) with a brittle structure (7, 90). The mechanical properties of dentin are changed in 5 minutes of collagen degradation by the higher concentration of NaOCl (5%) (Figure 1)

Advocated as an adjunct irrigant to NaOCl, the Ethylenediaminetetraacetic acid (EDTA) disodium salt removes the inorganic matter of the *smear layer*. Its reaction with the calcium ions in hydroxyapatite crystals can alter the mineral content of root dentin (Table 7) (91). Benzalkonium Chloride is a surfactant that enhances the antimicrobial action of ETDA and its wettability on dentin (International Zancan 2020). Despite the reduced surface tension of the mixture, there was no improvement in the ability of EDTA to remove the smear layer (92), as shown in SEM images (Figure 2C,D,F); or lead to a decrease in the microhardness of dentin, caused by the addition of a surfactant, as suggested in the literature (93). When these chelators were used alone, the dentinal tubules were less evident than they were with the treatment of sodium hypochlorite + chelator (Figure 2,8) (94). The spectra obtained from FTIR analysis showed that previous degradation of the organic portion of smear layers on the root canal surface by sodium hypochlorite (Figure 5) led to further exposure of the inorganic matter (Figure 6), data that were shown as decreases in the amide III/phosphate ratio and increases in the carbonate/phosphate ratio respectively, favoring the removal of Ca^{2+} by the chelators (95, 96). Although the irrigant sequence NaOCl + chelator led to the widening of the dentinal tubules, small amounts of smear layer remained on the dentin surface even after 3 minutes of flushing with the chelator (Figure). As smear layer removal is time dependent, the optimum working time of 1

minute recommended in literature for EDTA (71, 97) was not enough time for effectively cleaning the surface when it was used as a final flush.

The higher concentrated NaOCl followed by EDTA significantly changed the dissolution of inorganic matter and microhardness of dentin (Figure 1,7). These results strengthened the idea that subsurface damage caused by NaOCl-5 was higher, resulting in a collagen-depleted mineral ghost layer with unbound crystallites and non-uniform deproteinization channels (7). The absence of statistical differences in the decrease in organic matter could be explained by the sub superficial measurements of FTIR that penetrated to a depth of 1–2 μm (98), masking the damages caused by NaOCl at a deeper level. EDTA exposed the underlying destruction caused by NaOCl, explaining the rougher topography on dentin surface (Figure 8) with eroded dentinal tubules than those caused by NaOCl-2 + EDTA or EDTA itself (Figure 2). A three-dimensional structure with decalcified areas could be generated by the deep penetration of EDTA into the non uniform channels and empty spaces caused by NaOCl-5, creating a brittle structure. There was no significant reduction in microhardness and carbonate/phosphate ratios for dentin coated with NaOCl-5 followed by EDTA-BC, indicating a weaker chelating solution. All data suggested that the erosion of dentin was not exclusively induced by NaOCl or a chelator, but by the way the two were applied in an irrigation sequence.

Therefore, the idea that EDTA would leave an unprotected demineralized collagen-rich dentin appeared to be wrong, since the conditions of the organic matrix were dependent on the previous NaOCl irrigation (concentration, renewal and time of action). The increase in dentin organic matter produced by EDTA (Figure 5) could be composed of either exposed collagen rich fibrils or peptide chains cleaved along their lengths by the deproteination effect of NaOCl. Acids produced by acidogenic bacteria that remain in the root canal after obturation may slowly dissolve the collagen-depleted apatite crystallites within the mineral ghost layer creating clinical pathways for bacteria to enter (99). A final rinse with NaOCl appeared to be beneficial for enhancing disinfection in the root canal and removing rests of organic debris (Figure 2,5) (68, 83). The exposed and compromised fibrils were flushed away from the dentin surface, creating a flatter, smear layer-free surface (Figure 2,8). Presumably, at a subsurface level, the irregular dentin structure compromised by previous degradation of its organic and inorganic matter created faster pathways for spreading the NaOCl. As this occurred at a deeper level, peptide fragments and unbound crystallites, created by

deproteinization caused by the final flush with NaOCl, may have had more difficulty with reaching the surface, which was morphologically perceived as canal wall erosion in the intertubular dentin. Whereas peptide fragments and unbound crystallites on surface were easily dissolved resulting in enlarged tubular orifice diameters that were larger than those of the tubules (Figure 2, 3c) (71, 100, 101).

The Collagen component plays a significant role in the toughness of mineralized tissues (102). The unusual stability of collagen due the presence of strong intermolecular cross-links in the fibrils (103, 104), make dentin microhardness more susceptible to the more highly concentrated organic solvent because of its concentration-dependent effect (7, 105). Despite the continuous decrease in microhardness shown by the Knoop Hardness test (KHT) after sequential irrigant treatment, nanomechanical analysis and FTIR data showed no statistical differences in stiffness between untreated dentin and that coated with a final flush of NaOCl (Young`s modulus), and in the band area of mineral content, respectively. Knoop tests measure the ability of the dentin to resist the penetration of a diamond point applied with a load of a 9.8 N. (98) Whereas, by AFM a force curve was performed of a probe with 1.25×10^{-7} N load in contact with the dentin, by means of a laser beam projected onto the upper surface of the cantilever coupled to the tip (106). Although both tests used a force per unit area, the latter acted on a nano scale, while the former acted on a micro scale area (a difference of 3 decimal places). As the force applied in the AFM test was 7 decimal places lower than that of the KHT, the contact pressure applied was lower for AFM. We presumed that this force was not enough to deform the superficial, apatite-rich layer of collagen-sparse dentin, but was enough to deform the collagen exposed by EDTA, which showed significant differences in all protocols. As the cantilever sensed the nano indentation forces that were generated between the probe and the superficial sample surface, this method seemed to be more sensitive to the superficial topography than to microhardness, in which micro indentations had a limited depth of <19 μm (98).

The current study investigated 1 epoxy resin-based sealer, 1 containing salicylate resin and MTA and 2 hydraulic calcium silicate cements (HCSCs), assessing their morphology, composition and interfacial characteristics with modified dentin, by SEM, EDS and CLSM analysis. Complete and adequate filling of root canals prevents leakage of bacteria from the oral environment or those that persist in root canals from migrating into the apical periodontium (78). Gaps between the gutta-percha and the

internal walls of the root canal were filled by sealers. Used as a control, AH Plus (AH) - an epoxy resin-based sealer - was presented as a 2-component paste that contained zirconium oxide and calcium tungstate as radiopacifiers (Figure 9). Although there was evident migration of silicon from sealer to tooth for HCSCs (74), AHPlus had the highest values of silicon on dentin. The presence of silicon oil in its composition and its extensive penetrability into dentinal tubules might explain the results (Table 6). AHPlus interacted with dentin through the sealer tags that penetrated into the dentinal tubules forming a mechanical (107) and chemical bond. The latter occurs when the amino groups of the dentinal collagen bond to epoxy rings of AH Plus(14, 80). The proteolytic action of NaOCl on collagen (Figure 5) made this interaction poor (80), enhancing the gaps at dentin-sealer interface (Figure 11). As there were organic and inorganic parts of smear layer present in the saline solution coated dentin, a lower level of dentinal tubule penetration was shown. Chelators removed the inorganic smear layer, exposing dentin collagen fibrils, which positively affected the sealing ability of AHPlus (Figure 11; Protocols F,G). NaOCl + chelator groups enabled the formation of a thinner AHPlus-dentin interface (108, 109) with better results for EDTA than EDTA-BC. The widening of dentinal tubules in protocols A/B, when compared with C/D (Figure 2) might have allowed a deeper mechanical bond between AHPlus and dentin. Despite the the AHPlus dentin seal featured in Group NaOCl-5+EDTA+NaOCl-5, there were concerns about it use relative to the decrease in the mechanical properties of dentin (Figure 1).

Hydraulic calcium silicate cements (HCSCs) are mainly composed of calcium, silica, carbon and oxygen, and contain different radiopacifiers and additives (Figure 9). Aluminum was indicated in the EDS analyses for the 4 sealers. Aluminum release into the bloodstream, in the presence of HCSCs, has been shown to increase oxidative stress levels in erythrocytes, liver samples and the brain of tested animals. (110, 111). HCSCs are interactive rather than inert, and are affected by the environment, such as water content (74). Their fine hydrophilic particles absorb water during hydration of the powder, and expand during solidification. The water content in the pore spaces between the cement grains is replaced with hydration products, while the setting of material proceeds, thereby decreasing it porosity (112, 113). Although hydration of the majority of HCSCs occurs within the first few days, complete hydration may even take one or two years (114). High vacuum SEM could cause dehydration of the water content (115), which dried off leaving empty spaces, giving the HCSC surfaces a sandy

and dry aspect (Figures 9,10,12,13,14). A dense and homogeneous surface that did not absorb water was noted for AH Plus (Figure 9,10,11). When the analyses by CLSM were made under hydration conditions, a denser structure was found for TF and BR, with higher porosity for AH (Figures 15,16,18). The conflicting results for AH could also be explained by differences in methodologies. SEM provided overlapped images of all layers of the teeth analyzed in one image, thereby hiding the gaps and porosity that were displayed by CLSM, which dismembered these layers.

Although the sealers tested had a similar chemistry, they behaved in different ways relative to bonding and morphology. BR, composed of a powder and a water based liquid, had zirconium oxide as a radiopacifier (Figure 9). Calcium Chloride accelerated its setting, which was not dependent on the wetness of the root canal (77), thus explaining the higher amounts of chlorine on dentin for this sealer (Table 6). As the pore spaces were already filled with hydration products resulting from the faster setting process, BR was less vulnerable to SEM dehydration, and had a better surface morphology than MF and TF (Figure 9). However, SEM dehydration *appeared to cause* material shrinkage. Poor adaptation of the BR with dentin was noted in SEM images (74) (Figure 12), while the opposite was shown for the majority of irrigant protocols by CLSM, in which the specimens were kept hydrated (Figure 16).

MTA Fillapex, presented in a 2-paste system was the first Portland-based sealer developed in salicylate resin matrix. The bismuth oxide radiopacifier made it more prone to staining tooth structures (26) and interfered with MTA hydration (16). In its new version no traces of bismuth were found, as this had been replaced by calcium tungstate radiopacifier (Figure 9) (74). The presence of titanium is related to titanium dioxide that enhances its physical properties (116). As MTA Fillapex contains a resin matrix and 40% of MTA in its composition (117) the polymerization of resin may happen at an intermediate point between Stage 1 (hydrolysis and ion exchange) when portlandite is released, and Stage 2 cement matrix setting with formation of an amorphous water containing layer (114), explaining the porosity and gaps inside the obturation mass even in CLSM images. Sealers containing salicylate resin in their composition, such as MF, undergo shrinkage-related stress during the setting reaction (118). This contraction enhanced the unfilled spaces inside the obturation mass and led to detachment of the MTA Fillapex from dentinal walls, forming gaps at the sealer-dentin interface (108, 119). The presence of sealer inside dentinal tubules followed by gaps at the sealer-dentin interface in Protocol A (NaOCl) and B (NaOCl,

NaOCl/EDTA/NaOCl) confirmed the contraction of MF (Figure 17). If the dentin/sealer adhesion force were greater than the contraction force of MF, the sealer would remain attached to the dentin, with cracks in it. This indicated a strong bond between sealer and coated dentin with protocol B/D (NaOCl/Chelator) or F (EDTA), despite studies having shown that the ability of EDTA to chelate calcium ions inhibited calcium hydroxide formation, thereby affecting the hydration mechanism of MTA. (23) .

Total Fill, a premixed ready-to-use injectable calcium phosphate silicate cement paste, had zirconium oxide and tantalum oxide as a radiopacifier. The presence of tantalum and phosphorus were not indicated in the selected areas, by EDS analysis (Figure 9). Its setting and hydration depended on the wetness of the root canal. The literature has suggested that at least 168 hours in high humidity would be necessary for propagating TF setting from the cement surface downwards (27). The failure of complete TF setting was noted during this period. This could have been caused by non-reaction of the entire water content or the lack of it. Hydrated cutting and polishing of the incompletely set sample could cause a loss of TF mass as a result of material dissolution (Figure 10). As TF enhanced the microhardness of dentin, the humidity appears to have reached the sealer inside dentinal tubules, resulting in its setting (Table 4). It is unclear whether the *smear layer* would block fluids that migrate via dentinal tubules, or whether the moist environment of root canals (120) would be sufficient to allow the setting of TF to occur *in vivo*. In the CLSM analysis, the Groups consisting of Protocols B (NaOCl-5), A/B (NaOCl/EDTA/NaOCl), C/D (NaOCl/EDTA-BC), E and G *had the best dentin-sealer interface.*

Root fillings made by a single-cone technique are popular amongst many dentists because of its simplicity. It was selected in this study because it is less technique and operator sensitive than the other root filling techniques (121). Although the focus of the study was the sealer-dentin interface, considerable gaps at the sealer-gutta percha interface were noted by CLSM, for HCSCs when compared with AH. The manufacturer's recommendations stated that modified gutta-percha coated with bioceramic nanoparticles, called Total fill BC points, obtained higher bond strength values with Total fill than with the conventional type. The bond between the former and HCSCs should be tested for use as an adequate filling.

HCSCs release $Ca(OH)_2$ in the presence of biological fluids, thereby increasing the pH of the environment. The alkaline caustic etching derived from $Ca(OH)_2$ forms a ion exchange layer within the structure of dentin, just beneath the cement where

mineral diffusion occurs, called the “mineral infiltration zone” (MIZ). The alkaline environment also stimulates the expression of alkaline phosphatase responsible for hard tissue formation, creating a chemical bond between the sealer and dentin. The MIZ is identified by CLSM and fluorescent markers. (73-75).

There is a lack of scientific evidence to substantiate the bioactivity of HCSCs (114). Previous studies have shown no ultrastructural or chemical changes in the transition zone at the cement-dentin interfaces (122) and a lower push-out bond strength for HCSCs than for AH (123). This study showed a richly dye-infiltrated layer for both AH and HCSCs. Changes in the optical properties of sealer-dentin interface may be associated with the angular orientation of dentinal tubules and dentin cut (Figure 19,B), dentin debris, quality of flow, and adhesion of sealer-dentin interface. Dentinal tubules were arranged in an S-shaped curve that led inwards towards the pulp chamber (Figure 19,B) (82). As the dentinal tubules originated from the root canal walls in an angled direction, the entrance of the tubules was oblique to the specimen long axis. This resulted in brighter points or small lines under CLSM analysis, which differentiated them from the mostly longitudinal cross-sectional view of the dentinal tubules, equally achieved for longitudinal or transversal cutting directions (124). The uniform decrease in the diameter and number of tubules per square millimeter in the proximity of the root canal to cementum (125) may lead to a bright line around the root canal walls. The exposure of this line in CLSM images might increase with gaps at the sealer-dentin interface (Figure 19,A), explaining the brighter band at the AH-dentin interface for NaOCl Groups. Under pressure, the sealer drains into dentinal tubules from one point to another, with a certain loss of energy. A high level of flowability leads to further sealer/dye entering into the dentinal tubules and thus producing brighter lines. As dentinal tubules have dimensions to the order of 1 μm, several tubules at different depths may be superimposed on the same image, thus masking anatomical differences; while a low flowability with tag-like sealer structures (73) near the root canal walls followed by its lower penetration pattern enhanced the “MIZ” optical illusion (Figure 19,A). This may explain the favoritism of BR at the expense of MF or AH in forming the rich dye layer (74, 75) .

Considering Figure 19 (c) and the non uniform pattern of lines on dentin for Protocol F (BR) and Protocol B (NaOCl/EDTA; MF) brighter areas could also be interpreted as an accumulation of dye within dentin debris after sectioning the teeth, which, at this magnification, appeared as “dense” clusters of stained material. The debris deposits

might have occurred more frequently in the transition zone due the space between dentin and cement. As debris is capable of reaching different depths, polishing the sample would not be enough to remove the surface debris.

Interestingly, a richly dye-infiltrated layer was formed high above the interface for the majority of the chelators groups. The great loss of dentin mineral content in these groups (Table 7) may have weakened the teeth causing long and straight cracks.

The use of sodium hypochlorite in its “full strength” in protocol B, practiced for by the majority of American endodontists, progressively decreases the dentin microhardness, which may enhance fatigue crack propagation (126) changing the vertical fracture resistance of teeth (127). Interestingly, the irrigation protocol had no affect on dentin microhardness after obturation (Table 5), suggesting a recovery of dentin by the root canal sealers. Although this article theorized about the non-existence of “MIZ”, the biomineralization ability of HCSCs may exist. Despite the fact that BR is also a HCSC, only MF and TF had increased dentin microhardness (Table 4). TF and MF had higher flow values of than those of the gold standard AH Plus, (128, 129), while BioRoot RCS had lower values than those specified in ISO 6876 (2012) (77). High flowability allows cements to fill spaces that are difficult to access, and to penetrate into the damaged structure left by the irrigation protocol and around collagen fibrils, entrapping them into dentin structure, which may have a positive effect on reinforcing the remaining tooth structure.

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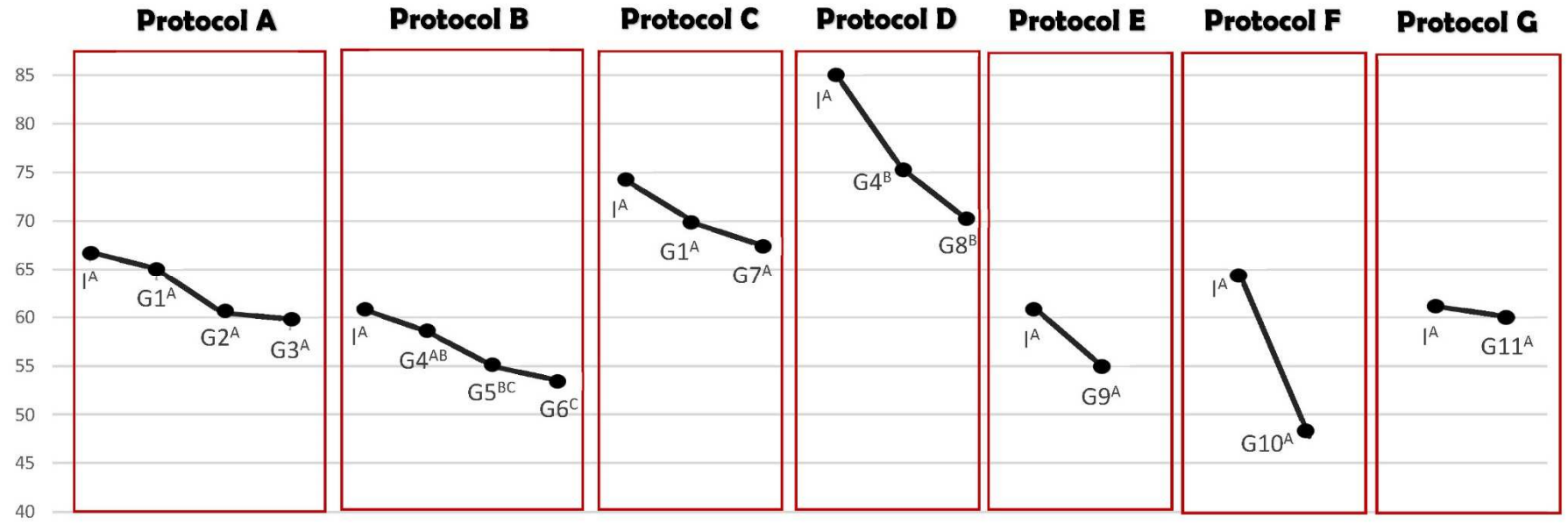
Figure 1. Knoop hardness number (vertical axis) of root canal dentin before and after irrigation treatments (G1-G11; horizontal axis) presented in different treatment protocols indicating the differences in microhardness of dentin.



Different capital letters indicate significant intragroup differences in the protocols.

Figure 2. Representative images at 2000x magnification after the protocol treatments on the surface of dentin.

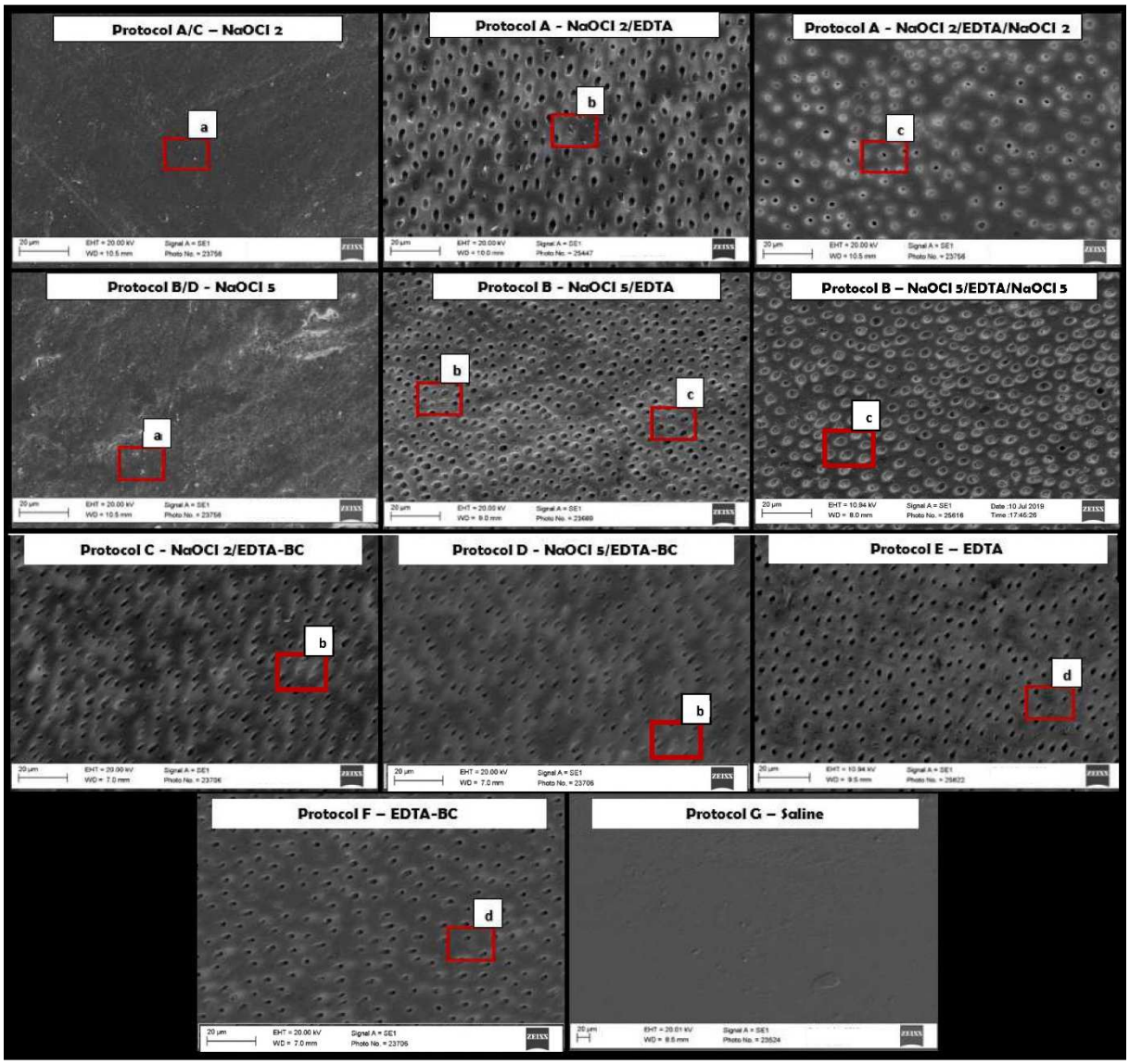


Figure 3. High magnification images of the areas marked by the letters A, B and C in Figure 2. These indicate the presence of sodium chloride crystals on dentin. (letter A -2000x and letter B- 3000x; yellow arrows) and the presence of smear layer (letter C - 4000x; pink arrows) and erosion (letter D - 3000x; red arrows) inside the dentinal tubules.

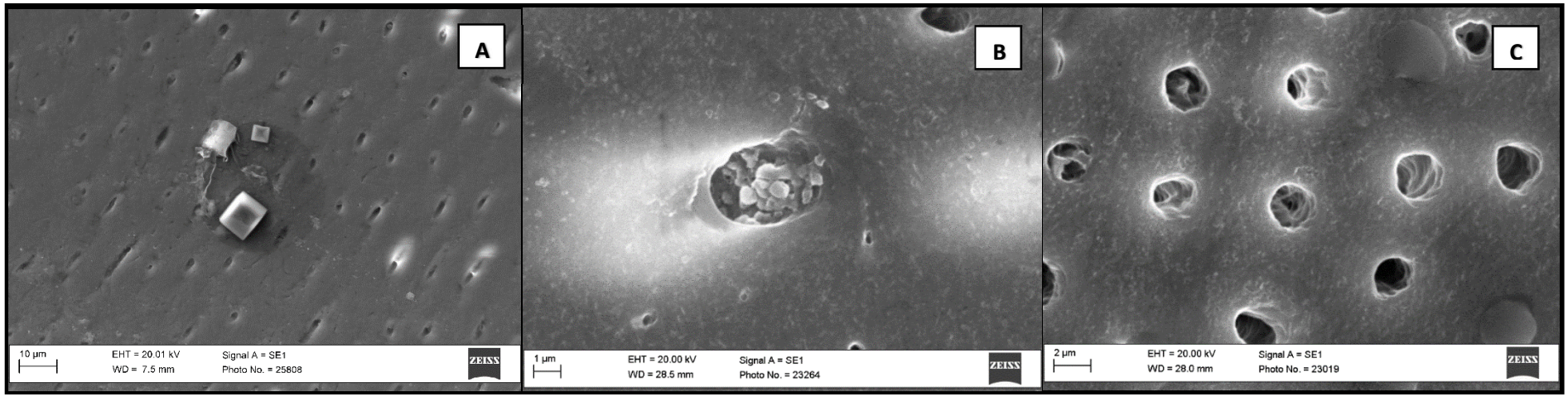


Figure 4. Representative infrared spectral region between 1600 and 750 cm^{-1} of untreated dentin showing the absorption peaks of the main dentin components.

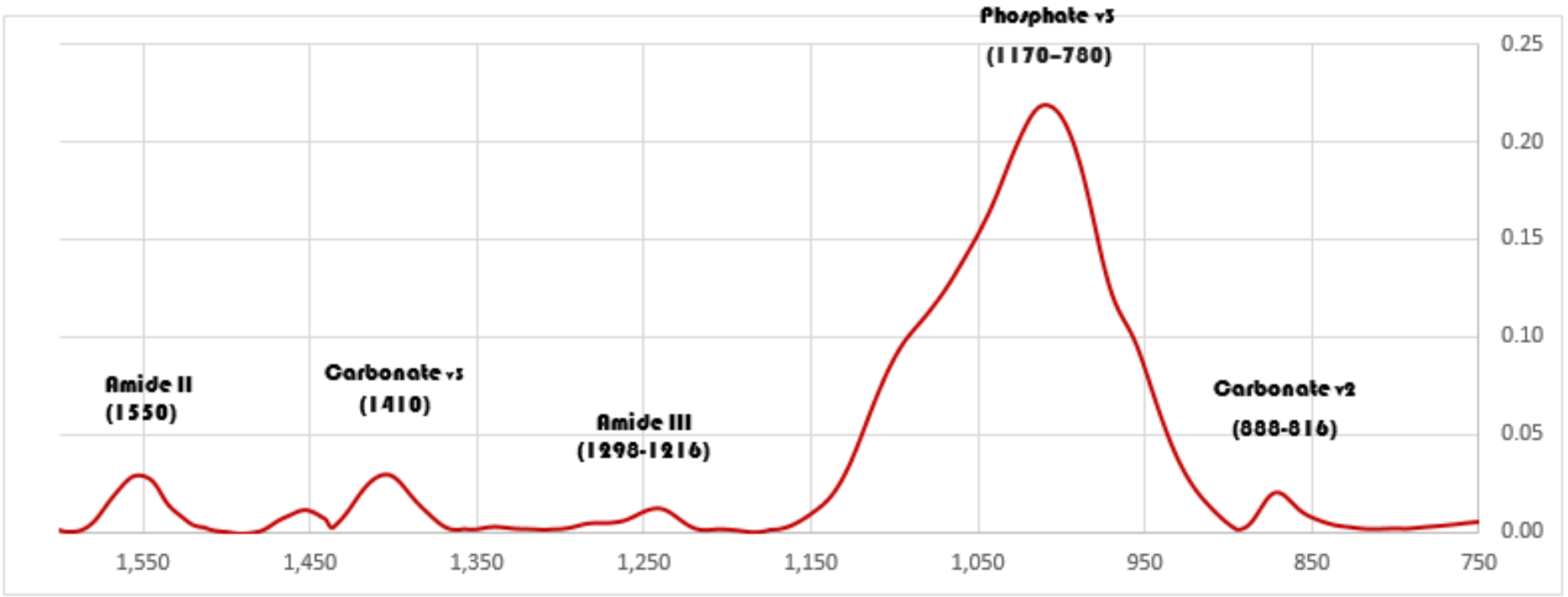
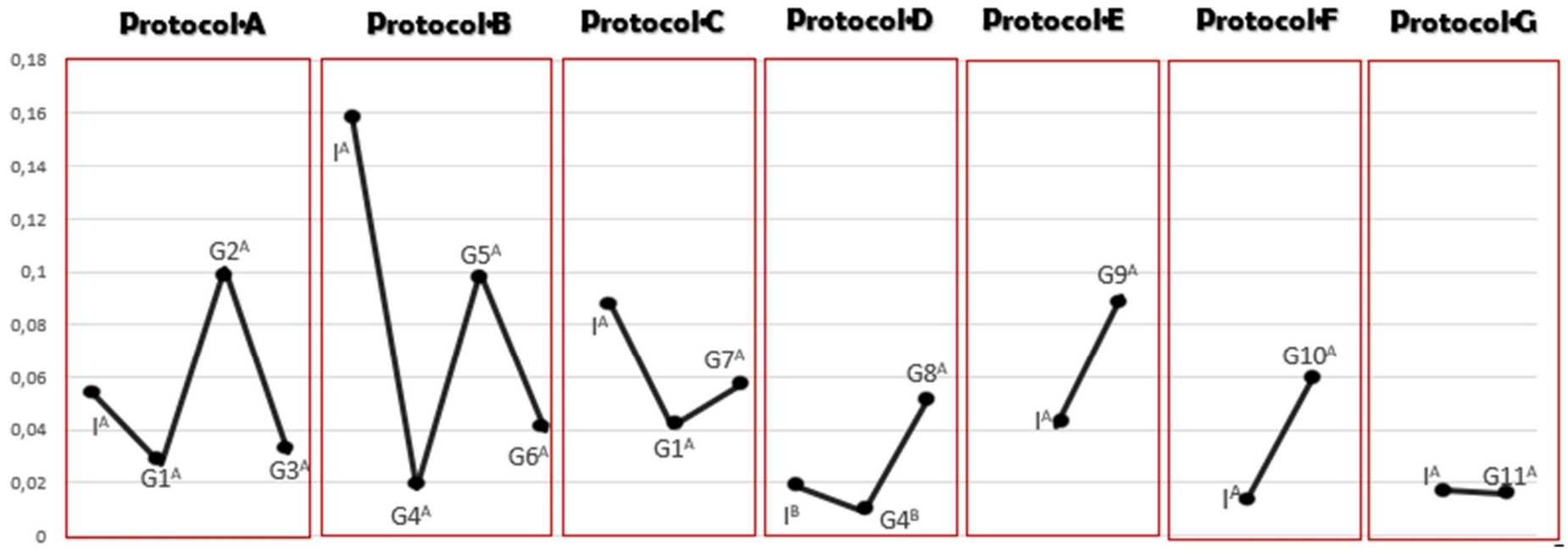
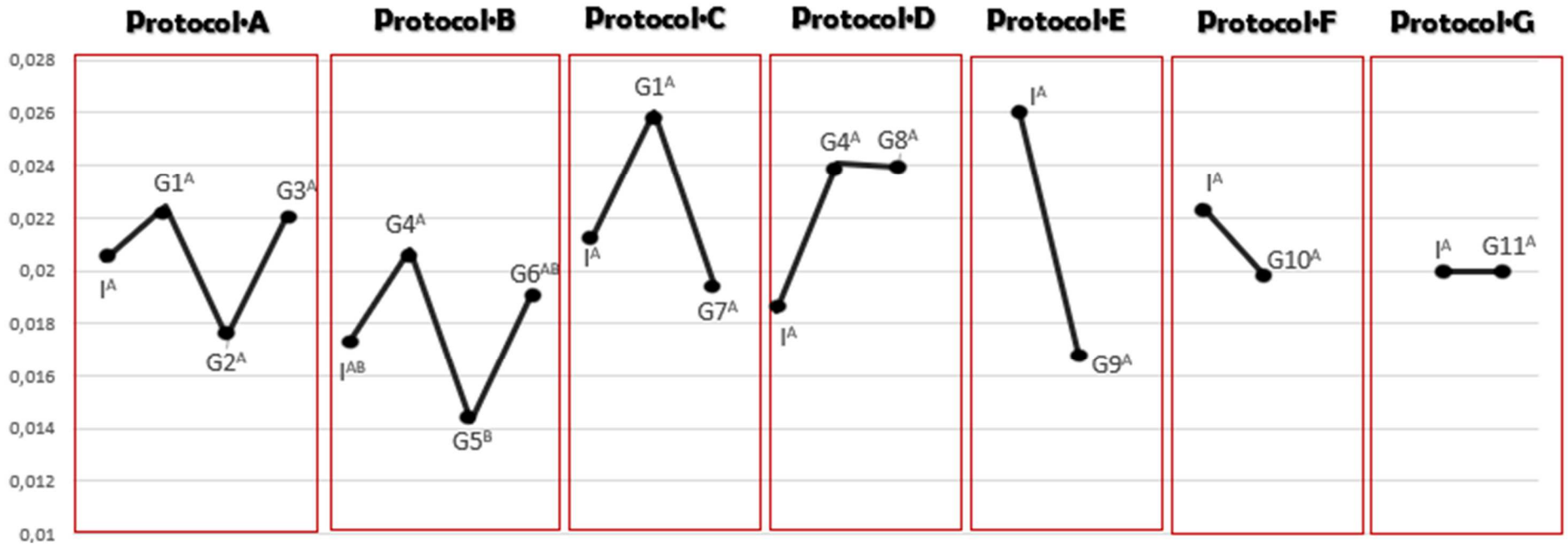


Figure 5. Ratio values of amide III/phosphate bands (vertical axis) on dentin surface before (initial -I) and after immersion in the irrigation solutions (G1-G11; horizontal axis) as indicated in protocols A – G which are detailed in Table 1.



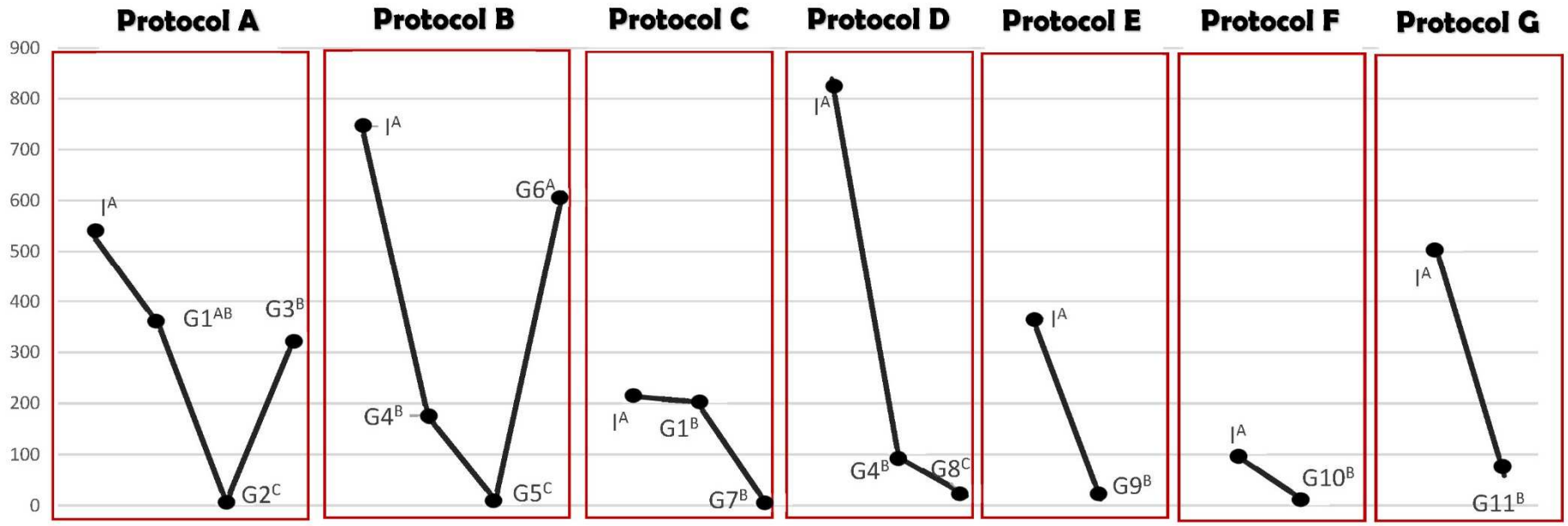
Different capital letters indicate significant intragroup differences in the protocols.

Figure 6. Ratio values of carbonate/phosphate bands (vertical axis) on dentin surface before (initial -I) and after immersion in the irrigation solutions (G1-G11; horizontal axis) as indicated in protocols A – G which are detailed in Table 1.



Different capital letters indicate significant intragroup differences in the protocols.

Figure 7. Young`s modulus values of dentin (vertical axis) before (initial -I) and after immersion in the irrigation solutions (G1-G11; horizontal axis) as indicated in protocols A – G which are detailed in Table 1.



Different capital letters indicate significant intragroup differences in the protocols.

Figure 8. Atomic Force Microscope topography of the dentin surface before (initial -I) and after immersion in the irrigation solutions G1: NaOCl 2; G2: NaOCl 2 - EDTA; G3: NaOCl 2 - EDTA – NaOCl 2; G4: NaOCl 5; G5: NaOCl 5 - EDTA; G6: NaOCl 5 - EDTA – NaOCl 5; G7: NaOCl 2 – EDTA-BC; G8: NaOCl 5 – EDTA-BC; G9: EDTA; G10: EDTA-BC and G11: Saline Solution as indicated in Protocols A – G which are detailed in Table 1.

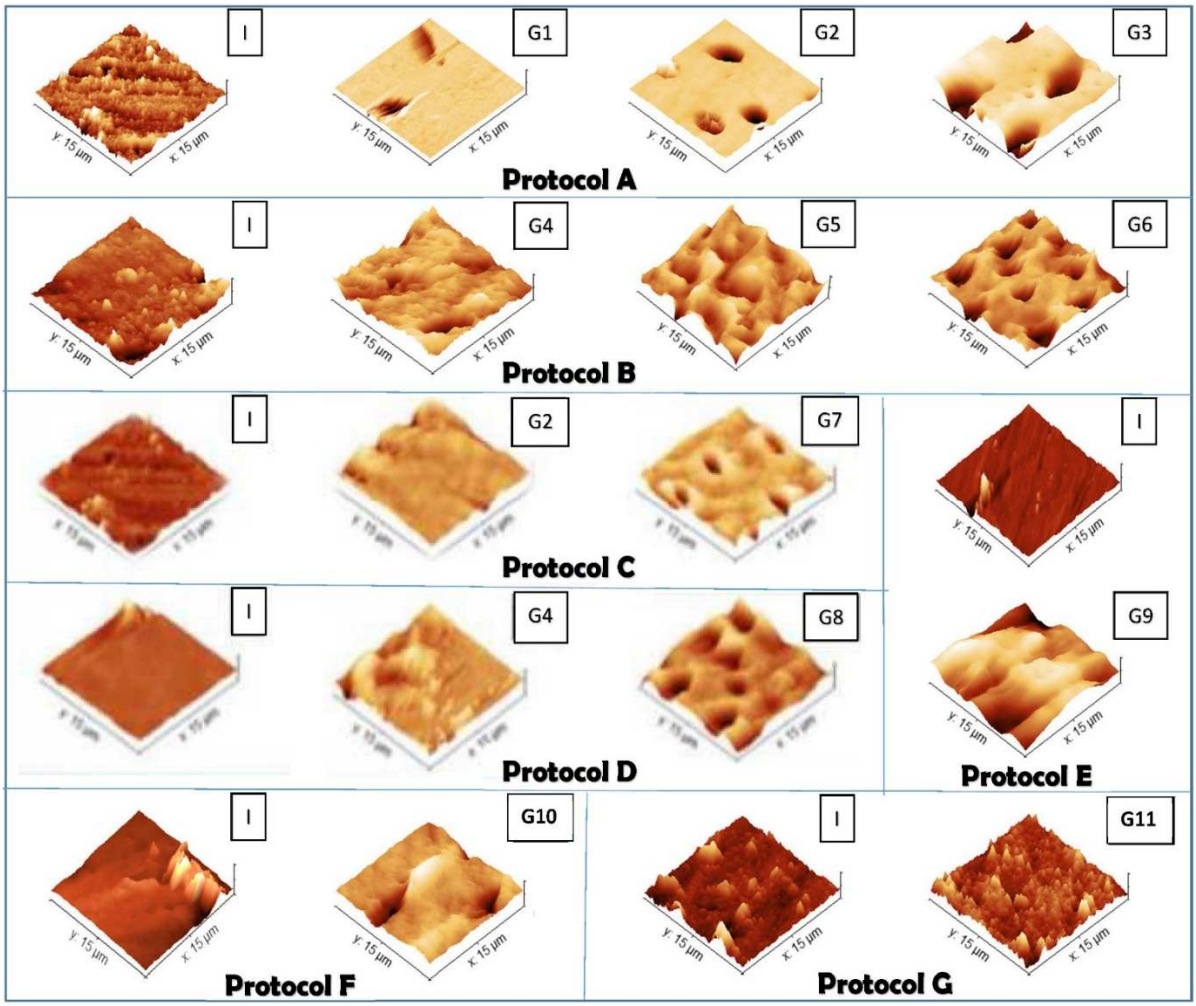


Figure 9. Representative Scanning Electron Microscope images and corresponding Energy Dispersive Spectroscopy (EDS) spectrum of AH Plus, Bio Root, MTA Fillapex and Total Fill sealers at 2000x magnification.

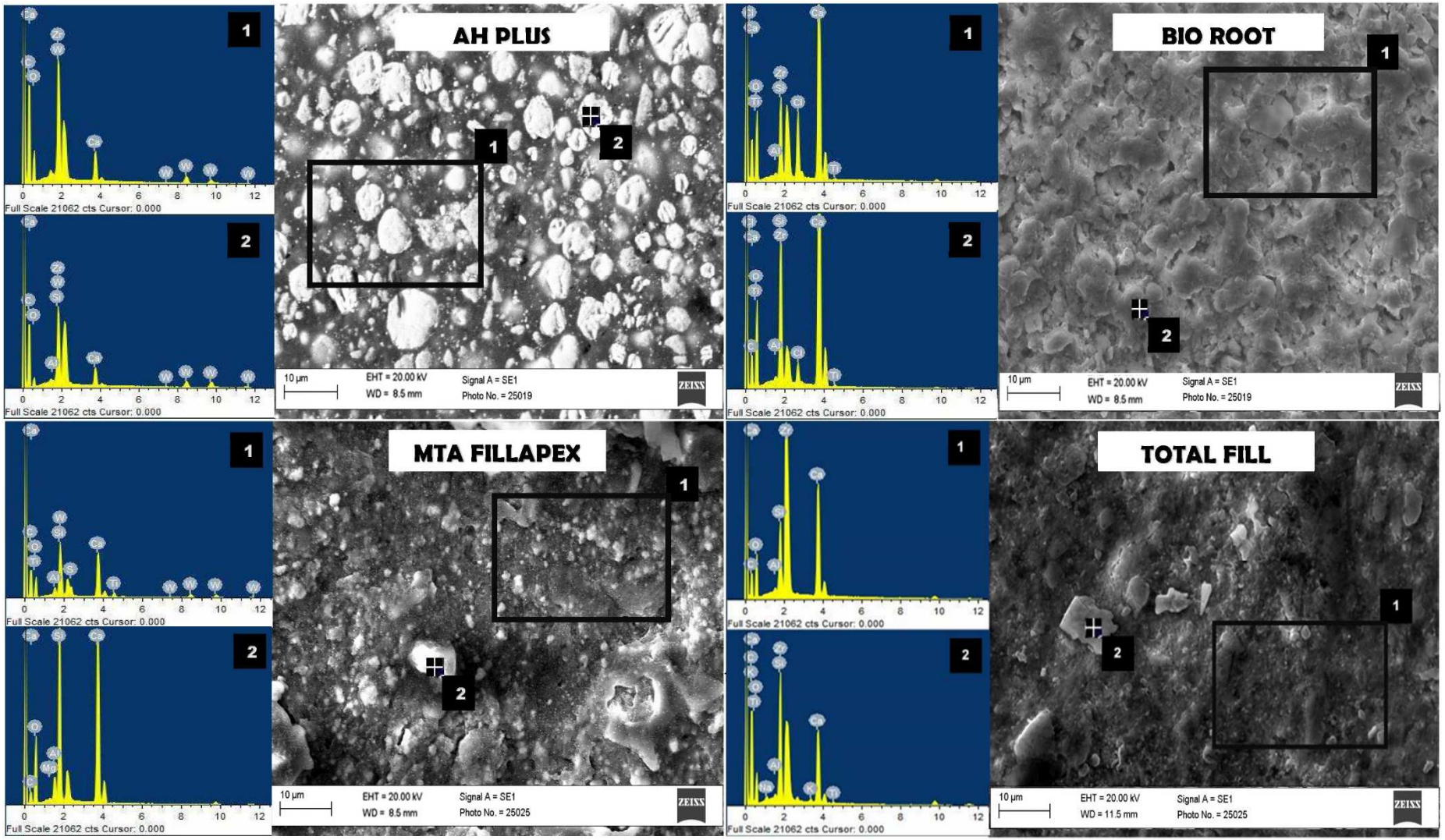


Figure 10. Representative Scanning Electron Microscope images of the sealers AH Plus, Bio Root, MTA Fillapex and Total Fill and it interaction with dentin at a lower (100x) magnification

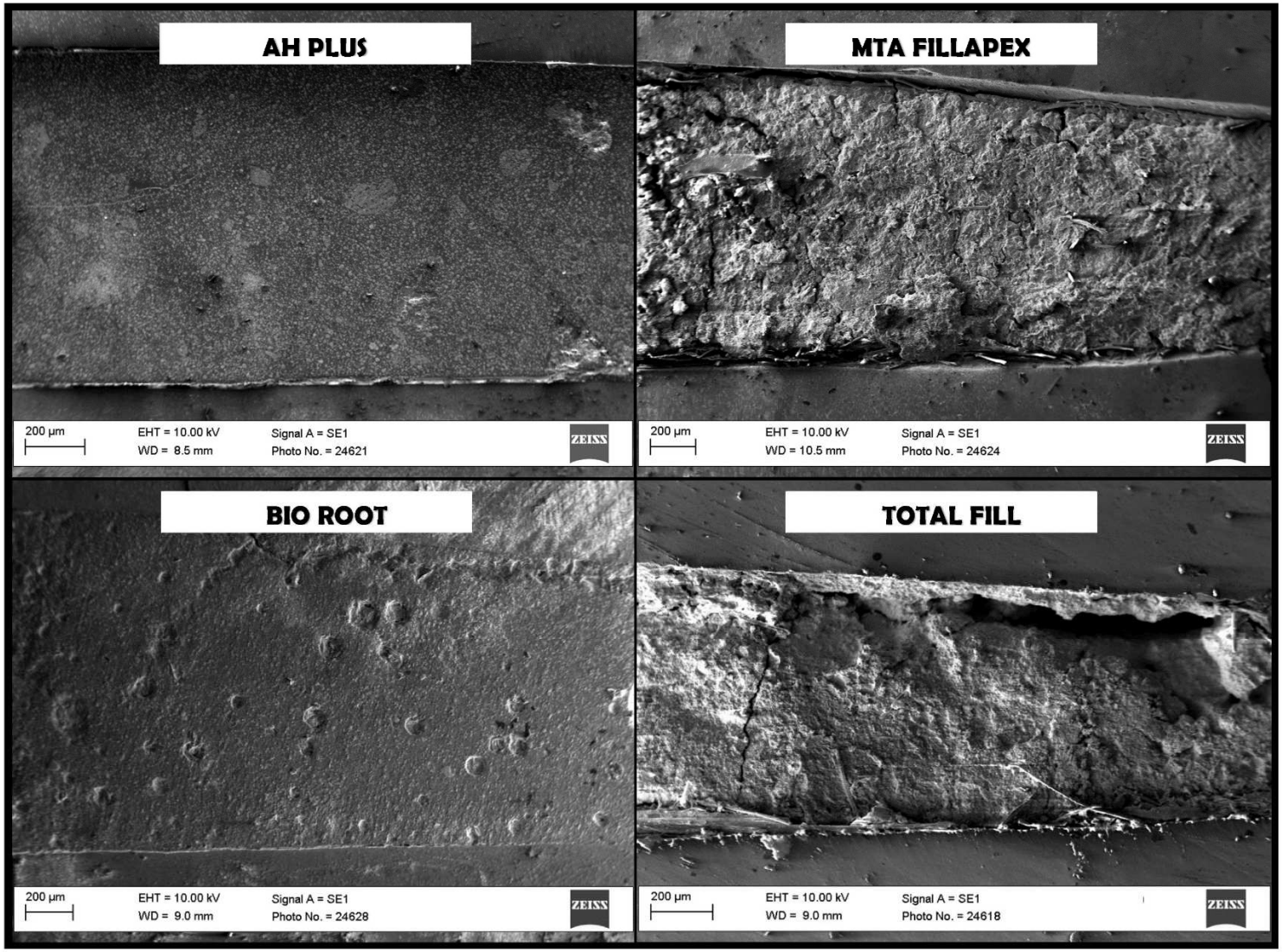


Figure 11. Representative Scanning Electron Microscope images of interface between AH Plus (AH) sealer and dentin coated with different irrigant protocols at 500x magnification.

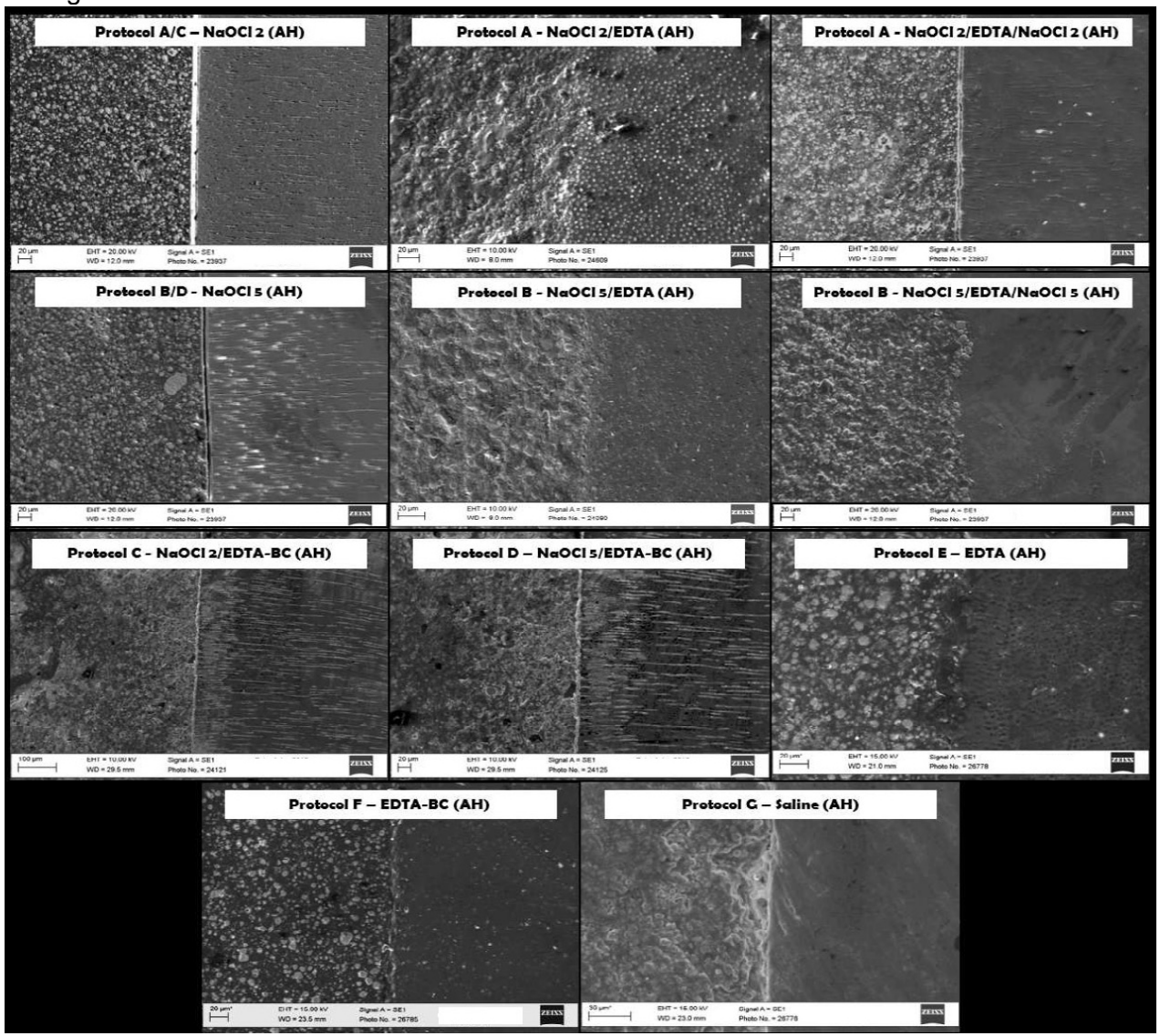


Figure 12. Representative Scanning Electron Microscope images of interface between Bio Root (BR) sealer and dentin coated with different irrigant protocols at 500x magnification.

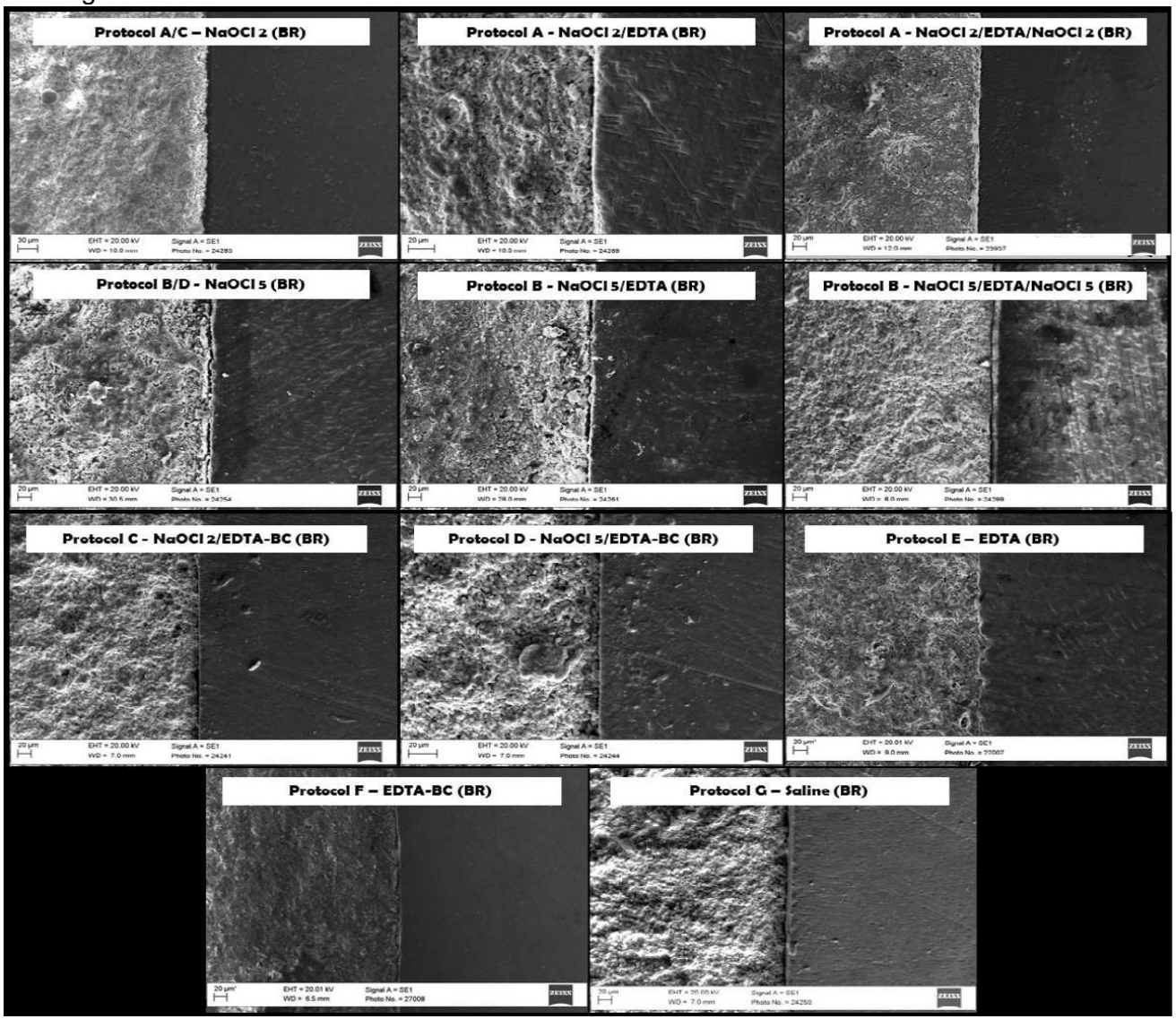


Figure 13. Representative Scanning Electron Microscope images of interface between MTA Fillapex (MF) sealer and dentin coated with different irrigant protocols at 500x magnification.

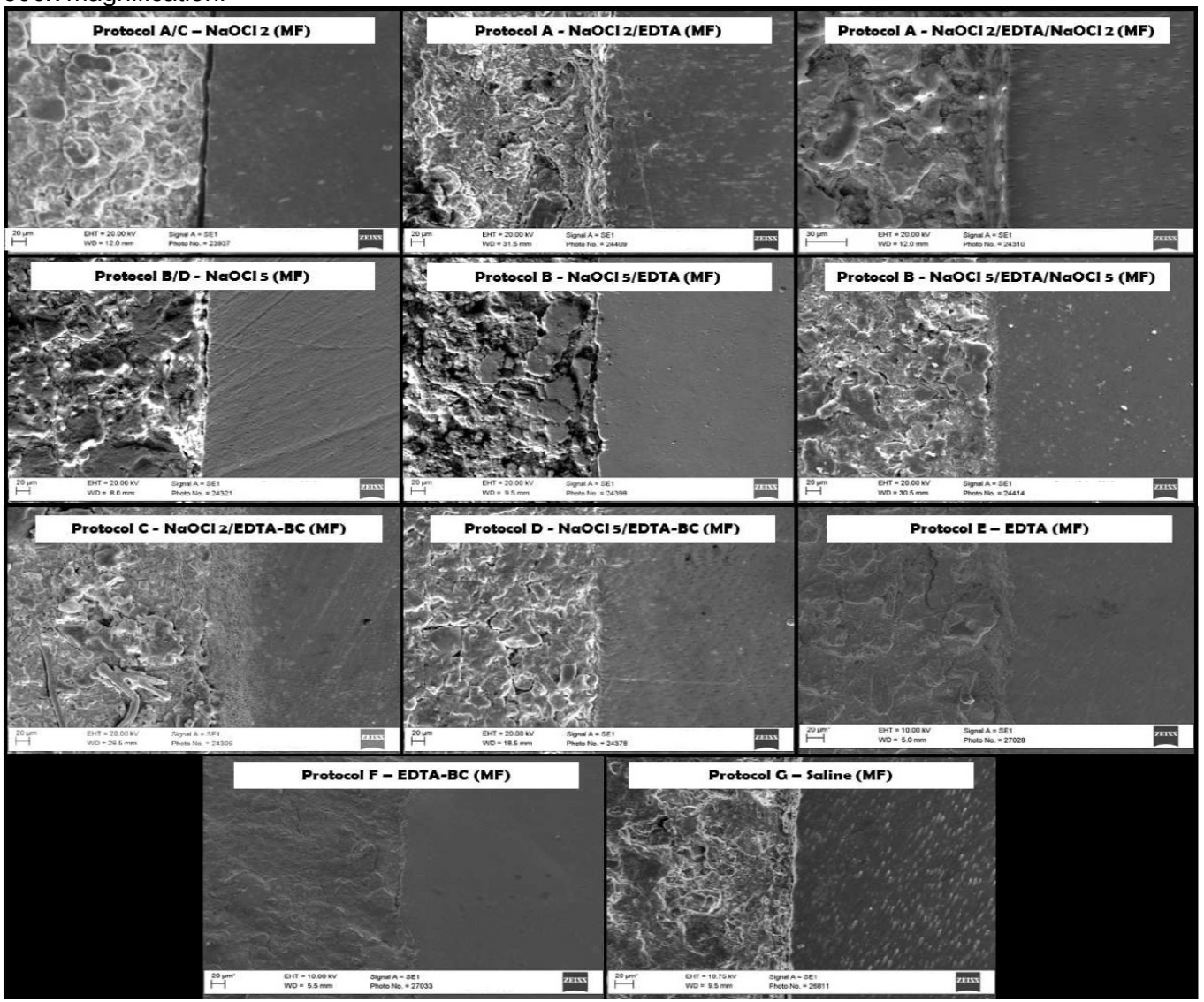


Figure 14. Representative Scanning Electron Microscope images of interface between Total Fill (TF) sealer and dentin coated with different irrigant protocols, at 500x magnification.

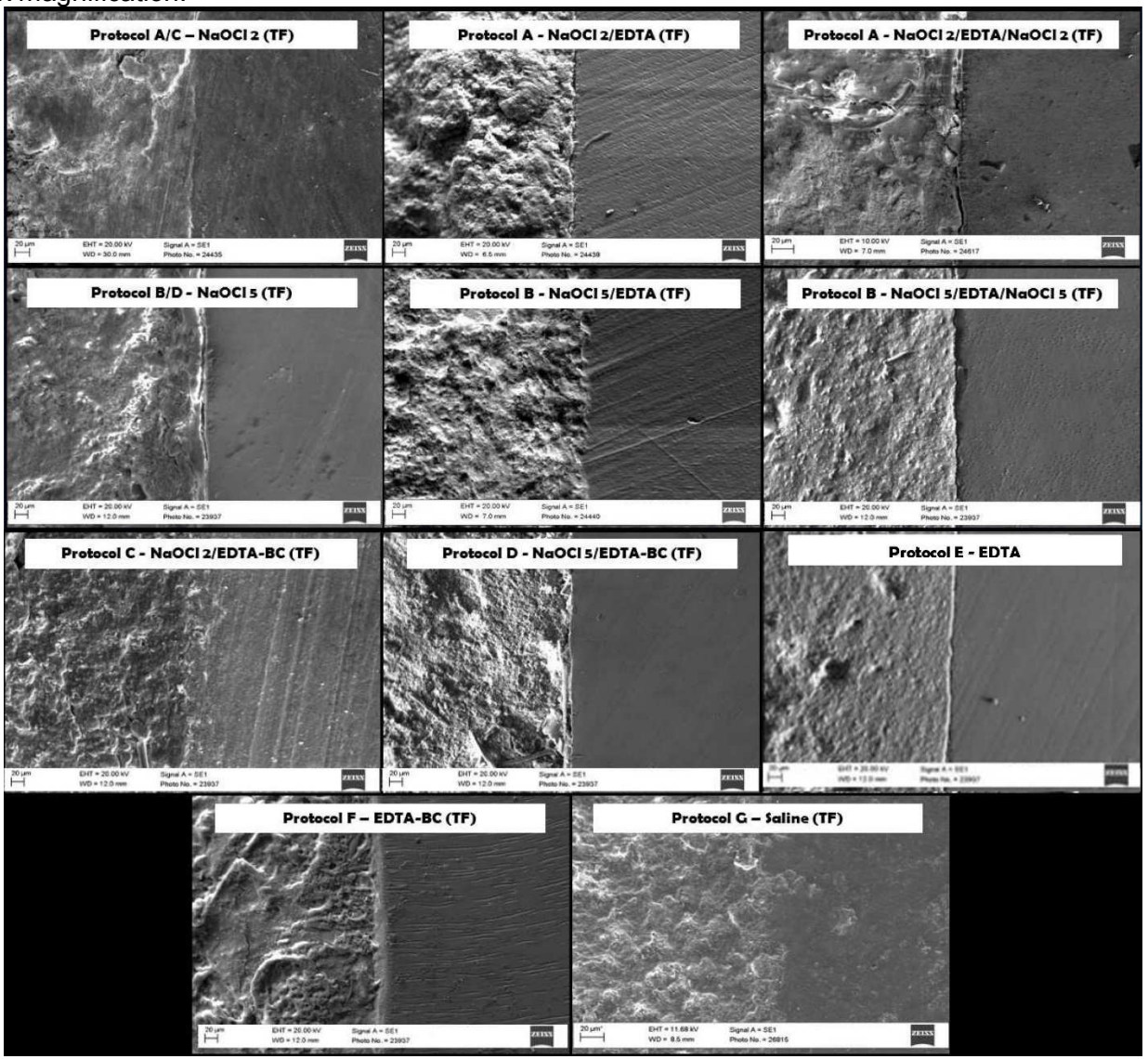


Figure 15. Representative fluorescence-mode CLSM images of sealer-dentin interface demonstrated by Orange G added to AH Plus sealer.

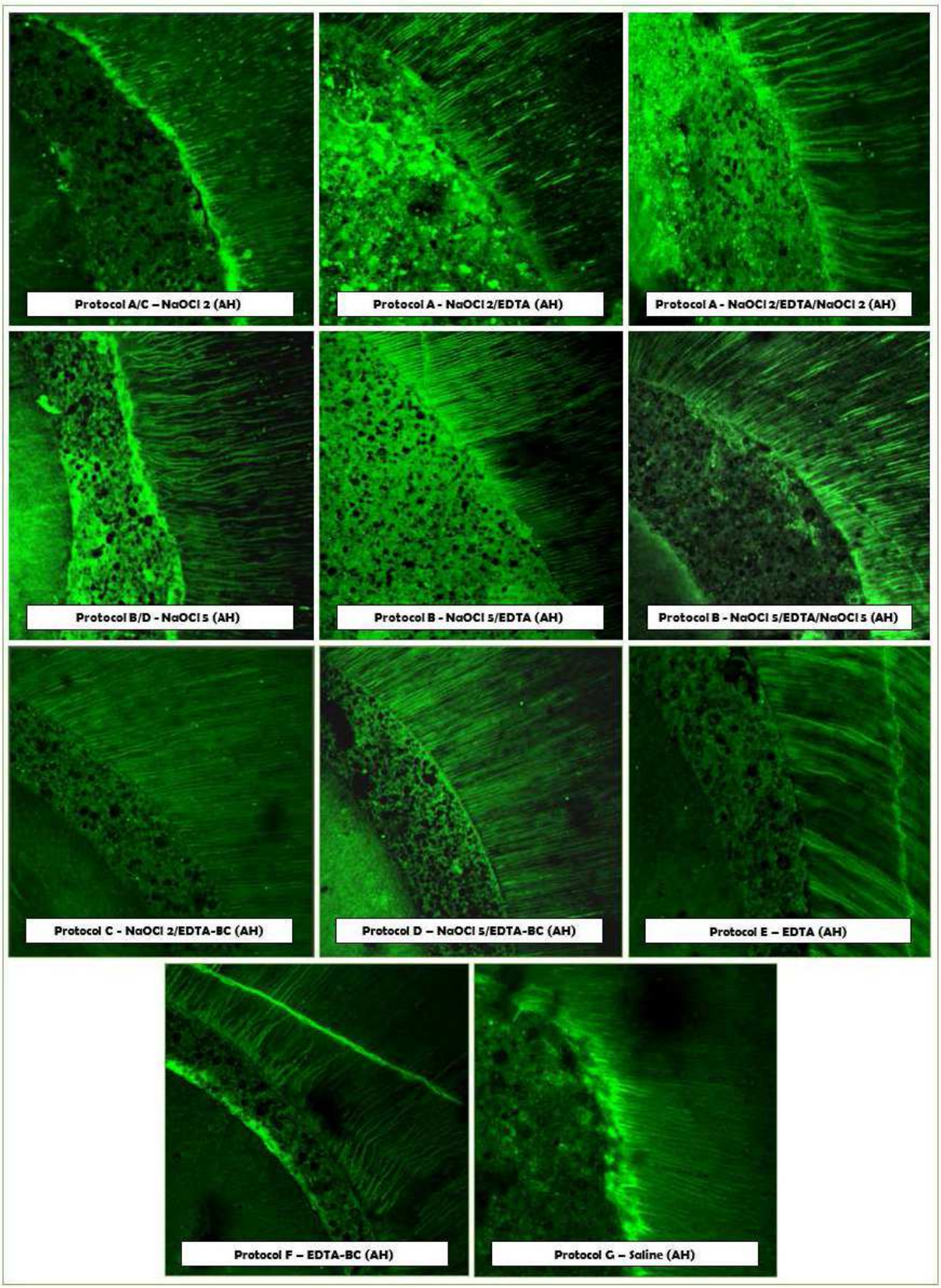


Figure 16. Representative fluorescence-mode CLSM images of sealer-dentin interface demonstrated by Orange G added to Bio Root sealer.

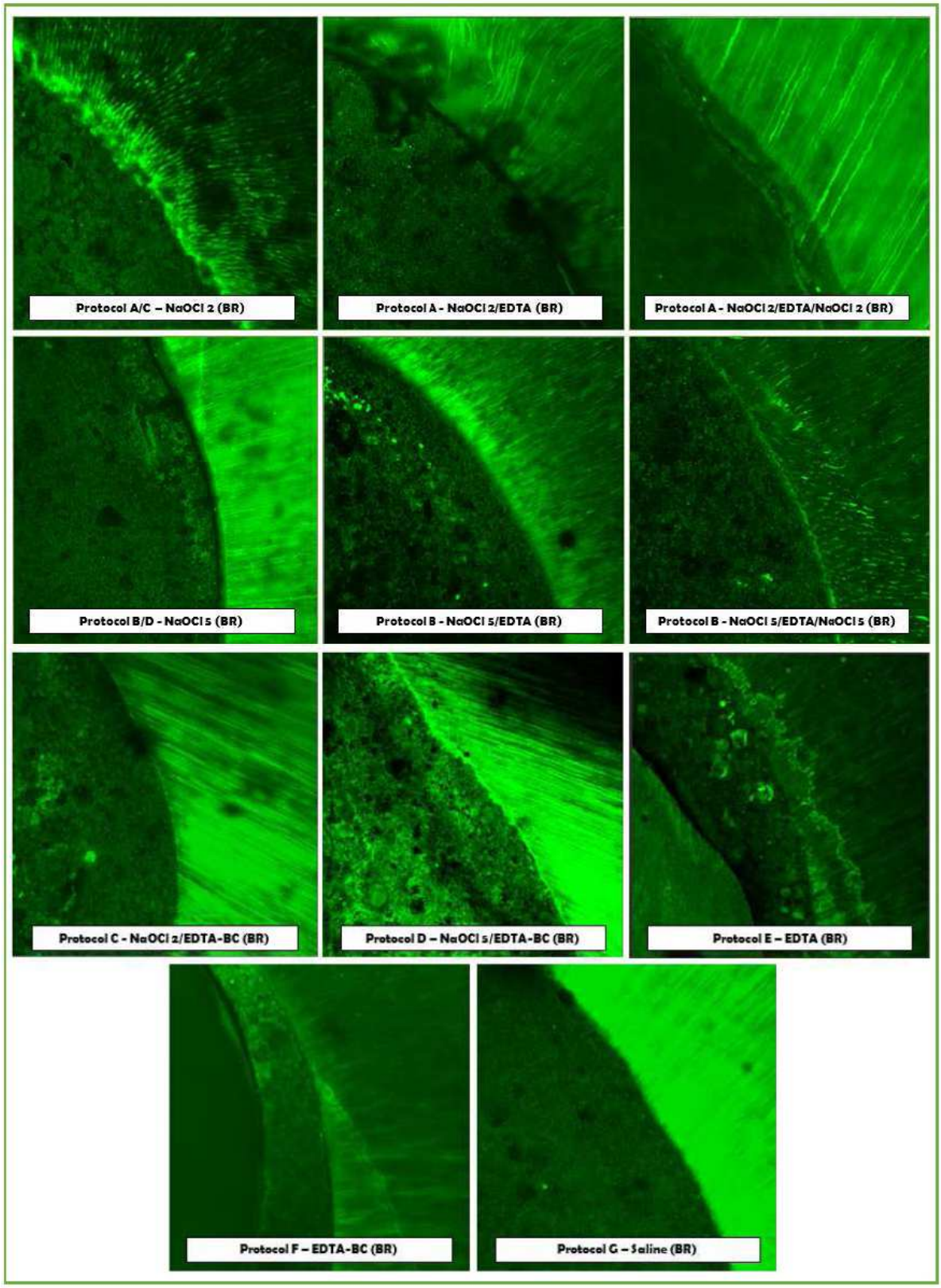


Figure 17. Representative fluorescence-mode CLSM images of sealer-dentin interface demonstrated by Orange G added to MTA Fillapex sealer.

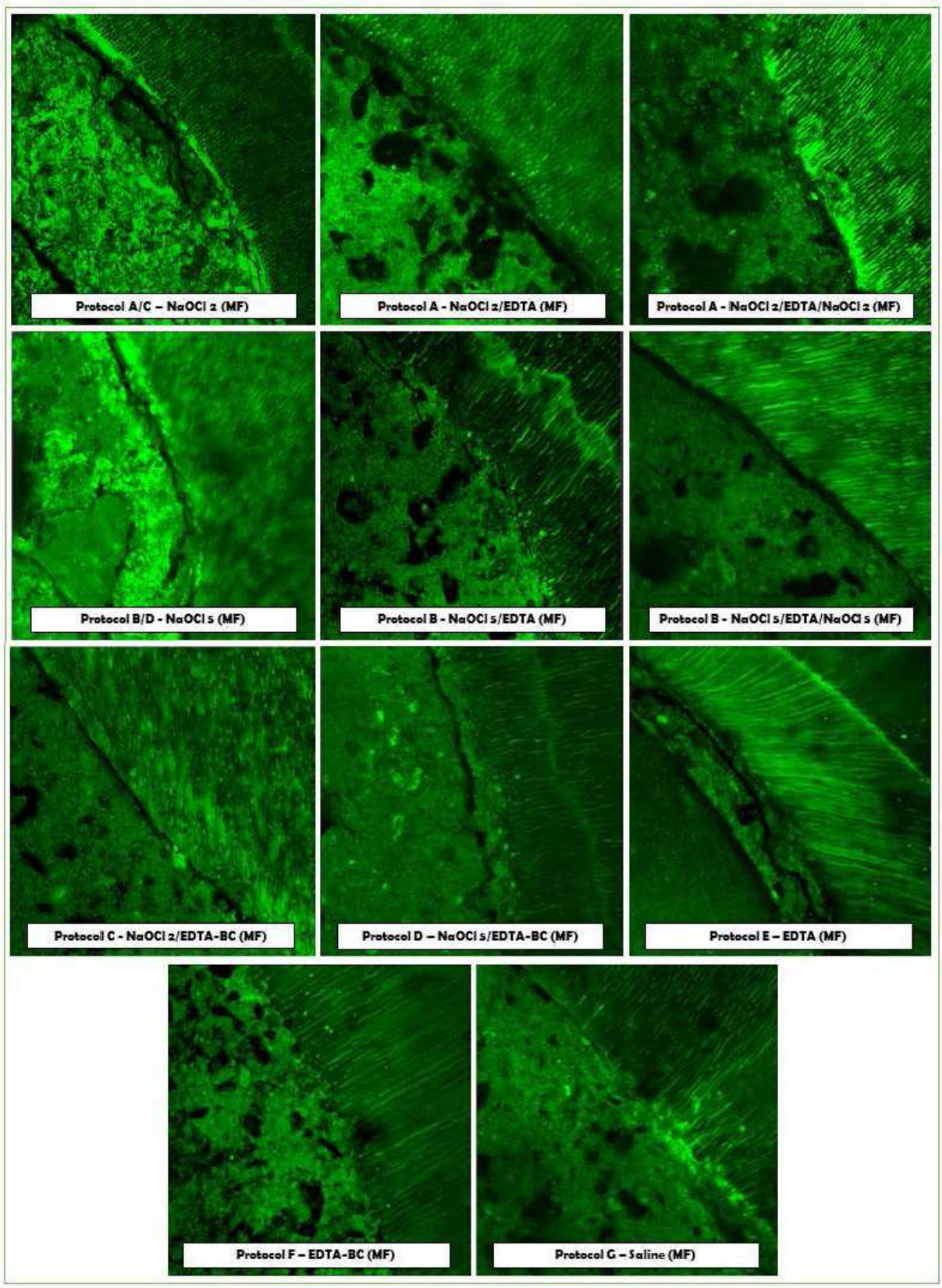


Figure 18. Representative fluorescence-mode CLSM images of sealer-dentin interface demonstrated by Orange G added to Total Fill sealer.

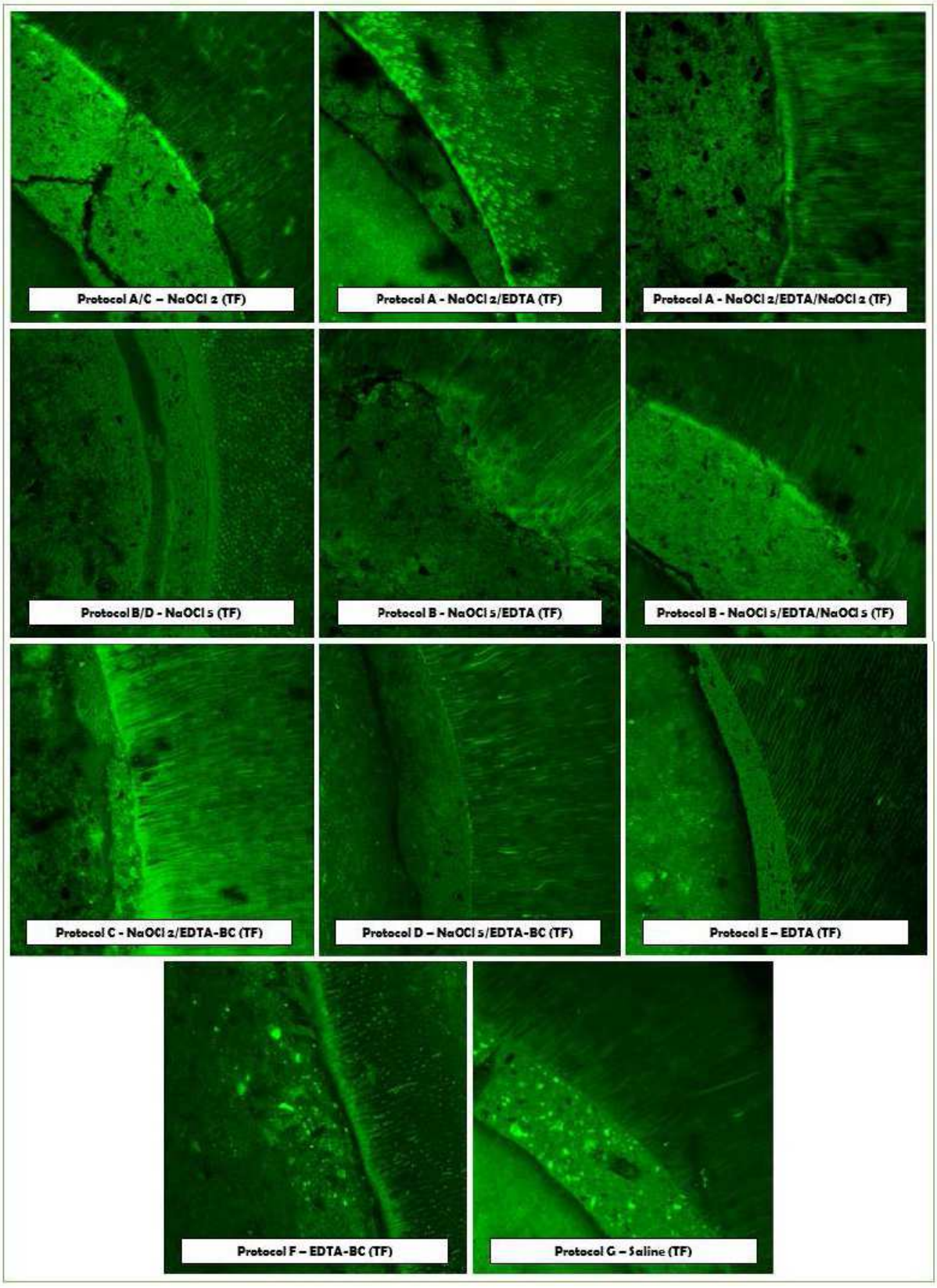


Figure 19. Representative images of A: oblique entrance of dentinal tubules exposed by gaps in dentin, and penetrability into dentinal tubules of sealers with low (LF) or high flow (HF); B: S shape of dentinal tubules and different angles at which dentin may be cut and C: dense clusters of stained dentin debris that may have been created after sectioning teeth.

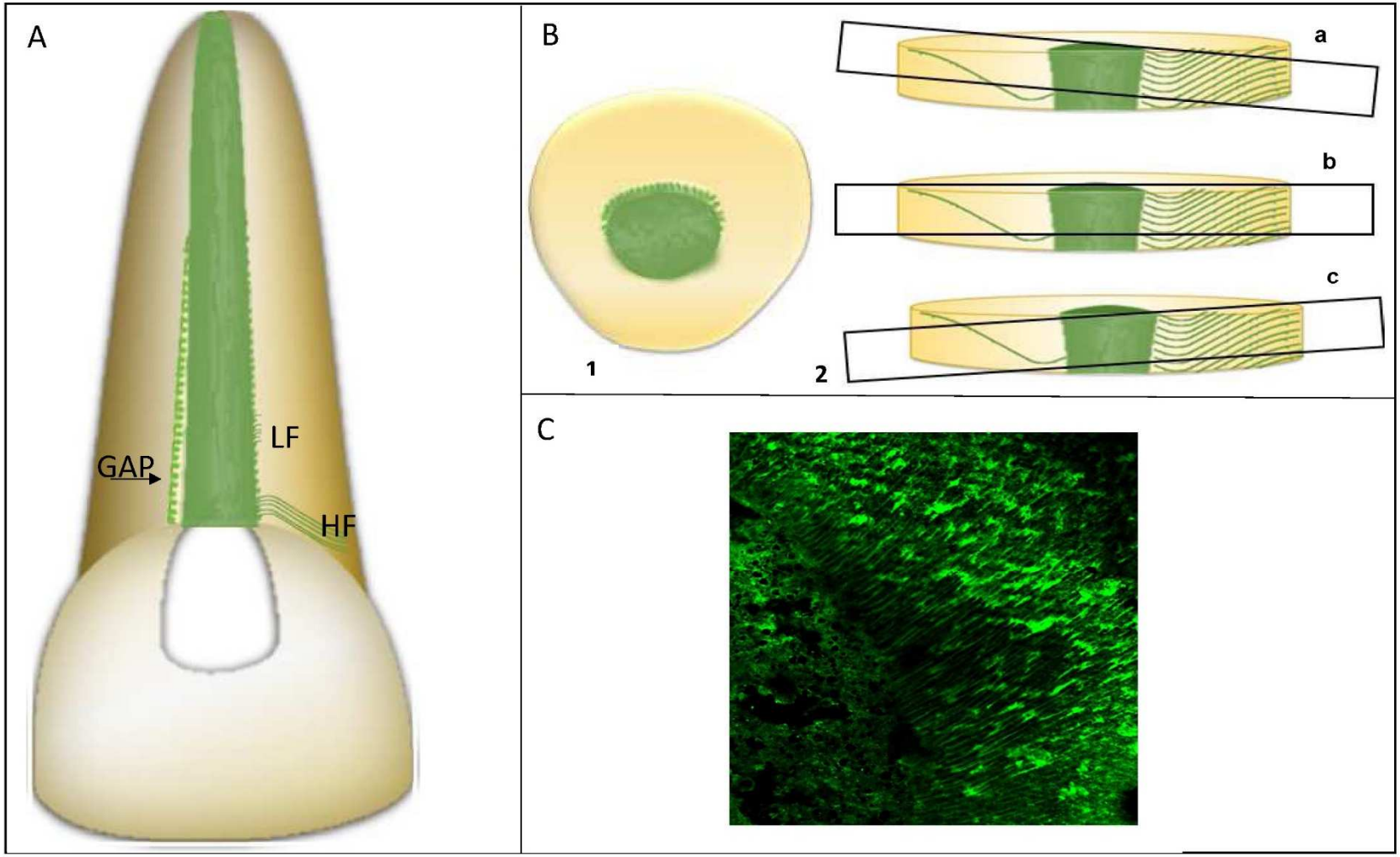


Table 1. Median (med) and minimum and maximum (min–max) values of the percentage of live cells of *Enterococcus faecalis* inside dentinal tubules after contact with the sealers for a week.

	AH Plus	Bio Root	MTA Fillapex	Total Fill	Control
Live cells	76,18^{AB} (50,02 – 98,26)	53,06^{C} (15,82 – 73,00)	67,00^{BC} (50,93 – 92,21)	76,92^{AB} (61,54 – 94,89)	94,49^{A} (60,57 – 99,65)

Kruskal-Wallis with a Dunn post hoc P value <.05. Different capital letters in rows indicate statistically significant intergroup differences in the same biofilm.

2.3 ARTICLE 3 - The presence of *smear-layer* affects the antimicrobial action of root canal sealers

ABSTRACT

Introduction. Approaches using sealers are based on leaching of antimicrobial agents to prevent bacteria remaining after chemomechanical preparation from maintaining persistent periapical diseases. Hydraulic calcium silicate cements (HCSC) interact with dentin, therefore, irrigation protocols used prior to their application might affect HSCS properties. In this study, the influence of dentin coated with NaOCl and EDTA on the antimicrobial properties of HCSCs and an epoxy resin sealer was assessed, considering the presence of *smear layer* for the protocol without use of chelating agent. **Material and Methods.** Standard semi-cylindrical root segments (obtained from human teeth) were irrigated with 2% NaOCl (Protocol A) or 2% NaOCl + 17% EDTA (Protocol B). Afterwards, they were placed in filter tubes and centrifuged with *Enterococcus faecalis* suspension at 1400 *g*, 2000 *g*, 3600 *g*, and 5000 *g,* twice each in sequence, for 5 minutes. Contamination lasted for 5 days and during this time, the procedure was repeated after each 48 hours. The samples were then filled with: AH Plus (G1), BioRoot BCS (G2), MTA Fillapex (G3) and Total Fill (G4). After 7 days live/dead dye and confocal laser scanning microscopy were used to measure the percentage of living cells. **Results.** For both protocols BioRoot BCS presented the greatest antimicrobial action, followed by MTA Fillapex. AH Plus and Total Fill showed no statistically significant differences when compared with Control, however, the mean number of bacteria killed were slightly higher in Protocol 2. The presence of *smear layer* reduced the antimicrobial action of all sealers. **Conclusion.** Sealer hydration influenced the antimicrobial activity of hydraulic calcium silicate cements (HCSC). EDTA coated dentin did not harm HCSC antimicrobial action. A *smear-layer*-free surface increased killing of bacteria inside dentinal tubules for all sealers. Although BioRoot BCS provided the best antimicrobial activity against *E. faecalis,* it failed to achieve effective root canal disinfection, and the previous steps for root canal cleaning should be implemented.

1. Introduction

Spreading and flushing the irrigant NaOCl throughout the root canal space provides broad spectrum antimicrobial action and dissolution of necrotic tissues but fails to attain bacteria in inaccessible, remote areas of the canal system (130). Bacteria can spread to a depth of approximately >500 micrometers into the dentinal tubules, while penetration of NaOCl might be limited to the first 100-μm layer (131). EDTA is a weak chelator used in conjunction with NaOCl for complete *smear layer* removal. Despite its low antimicrobial action, bacteria might be trapped within dentinal tubules, limited by the enhanced sealer penetration after its use. This is beneficial for inert sealers such as AH Plus, because its mechanical bond to dentin prevails (132). However, the wide range of chemical compositions of different types of sealers require distinct dentin pretreatments for optimal performance (12). Hydraulic Calcium Silicate Cements (HCSC) do not depend on EDTA to produce a good dentin seal, but rather on chemical interaction. HCSCs set through a hydration reaction forming calcium hydroxide - the byproduct responsible for their antimicrobial activity and chemical bond to dentin (133). In the Literature, there are theories that the moisture in *smear layer* has positive effects on the biological properties of HCSC and decreases leakage of HCSC through dentin with smear layer when compared with smear-layer-free dentin (21, 22). In addition, the EDTA ability to chelate calcium ions has been shown to disrupt HCSC hydration (23) compromising their sealing ability, hardness, flexural and bond strength (21, 24, 25).

As it is not easy to achieve a hermetic seal with a dentin-sealer (11) the antimicrobial compounds, such as calcium hydroxide, released by HCSC would prevent bacteria from reaching pathways to the periapical tissues. BioRoot RCS is a tricalcium silicate–based sealer prepared by hand-mixing its powder with water. This might benefit its action against bacteria, since some HCSCs depend on the humidity of root canal to hydrate them, among them Total Fill BCS - a calcium phosphate silicate–based sealer; and MTA Fillapex - a salicylate resin MTA cement–based sealer. Although it has been proved that HCSCs interact with their environment and were affected by variations in pH, temperature and humidity (77, 133), studies with antimicrobial agents have been conducted in the absence of dentin by mans of the agar diffusion (ADT) and direct contact (DCT) tests (134-136) .

No studies have evaluated the effect of the presence of smear layer on the disinfection of dentinal tubules. In this research, a modified noninvasive confocal laser scanning

(CLSM) method was used to assess the influence of dentin coated with two different protocols: A. NaOCl and B. NaOCl-EDTA on the antimicrobial properties of HCSCs and Epoxy resin sealers against intratubular infection *by Enterococcus faecalis*, considering the presence of *smear layer* for the protocol without use of a chelating agent.

2. Material and Methods

2.1 Tooth preparation

Extracted caries-free maxillary human incisors (ethical approval number: 14/EM/2811-BCHCDent397) were prepared following previously described protocols (137) with modifications. The teeth were horizontally sectioned by using a hard tissue microtome (Isomet, Buhler, Lake Buff, USA) at 1 and 5 mm below the cementoenamel junction, thereby obtaining cylindrical root segments standardized to a length of 4 mm. Subsequently, the root canal was enlarged with a Gates-Glidden bur #6 (1.5 in diameter) (Tulsa Dentsply, Tulsa, OK) under water cooling. Each root segment was sectioned into two semi cylindrical halves using a hard tissue microtome (Isomet, Buhler, Lake Buff, USA). The outer cementum was ground with 600-grit silicon paper (Carbine; Buehler Ltd) and the specimen size was adjusted to 4 × 4 × 2 mm to fit the inner wall of a 2 mL filter tube (Pall Corporation, Ann Arbor, MI). The samples were then sterilized by autoclave at 121 °C for 20 min. After this the specimens were immersed in Protocol A: NaOCl (5 minutes) or Protocol B: NaOCl (5 minutes) – EDTA (3 minutes).

2.2 Dentin infection

The antimicrobial activity tests were conducted under aseptic conditions in a laminar flow chamber (Monmouth Guardian MSC1200, UK). For *Enterococcus faecalis* infection, a 45 µl standard strain (American Type Culture Collection [ATCC 29212]) was placed in 9 mL sterile brain-heart-infusion (BHI; Oxoid, Basingstoke, UK) at 37ºC in air for growth overnight. Subsequently, a spectrophotometer (Jenway 7315, Staffordshire, UK) was used to adjust the bacterial density to 10^7 cells/mL in BHI broth, at an optical density of 1 at 600 nm according to the 0.5 MacFarland standard.

Then, the dentin specimens were placed inside the filter tubes with the root canal side facing upwards, followed by addition of 500 µL of this suspension. The tubes were centrifuged at 1400*g*, 2000*g*, 3600*g*, and 5000*g* in a sequence, twice for each sample, for 5 minutes. After each centrifugation, the suspension was replaced with a new one. The samples were then incubated at 37°C in BHI broth and in air; after every 48 hours the procedure was repeated. The contamination lasted for 5 days.

2.3 Antimicrobial test

The dentin specimens were removed from each tube, followed by rinsing in sterile water for 1 minute and air drying. The specimens were randomly divided into four experimental groups according to the sealer applied: AH Plus (DENTSPLY DeTrey GmbH, Konstanz, Germany), Bio Root (Septodont, Saint Maur-des-Fosses, France), MTA Fillapex (Angelus Dental Solutions, Londrina, SP, Brazil), Total Fill (FKG Dentaire, La-Chaux-de-Fonds, Switzerland) and a Control Group that received no treatment. The root canal was dried with sterile absorbent papers and the sealers were applied using a cavity liner applicator achieving an approximate thickness of 0.5 mm. The samples were kept in an oven at 37 °C in 100% relative humidity for 7 days. For Confocal Laser Scanning Microscopy (CLSM) analysis, the sealer was removed from the root canal surface and the semi cylindrical samples were vertically sectioned by using a hard tissue microtome (Isomet, Buhler, Lake Buff, USA), resulting in a fresh surface that provided a longitudinal view of the dentin canals.

2.4 Confocal Laser Scanning Microscopy analysis

Bacterial viability was analyzed using the SYTO 9/propidium iodide technique (Live/Dead BacLight Viability Kit; Molecular Probes, Eugene, OR). This involved washing the dentin discs with 100 µl phosphate-buffered saline (PBS) to eliminate intracanal medication residues and the samples were subsequently stained with 30 µl of the dye, in a light free environment, for 10 min. The samples were then examined with an inverted Leica TCS-SPE confocal microscope (Leica Microsystems GmbH, Mannheim, Baden-Württemberg, Germany) using a 40X magnification water immersion lens. Four confocal “stacks” of random areas were obtained for each sample. In total, there were 5 samples per group, thus a total of 20 operative fields per group. For quantification bioImage_L software (www.bioImageL.com) was used to

calculate the percentage of live (green) and dead (red) cells found after the sealer application.

3. Results

Table 1 presents the percentage of live cells in the dentinal tubules after contact with different sealers influenced by the previous irrigation protocol applied on dentin. In the dentin with a smear layer (Protocol A) BioRoot BCS showed the highest level of antimicrobial action ($p < 0.05$) followed by MTA Fillapex. AH Plus and Total Fill showed no statistically significant differences ($p > 0.05$) when compared with the Control Group that showed no action against *Enterococcus faecalis*. Dentin coated with NaOCl-EDTA (Protocol B), enhanced intratubular antimicrobial action for all sealers, however, intragroup differences were shown only for BioRoot BCS and AH Plus ($p<0.05$). The order of *E.faecalis* susceptibility to the sealers remained the same as it was for Protocol A. Figure 1 shown representative confocal laser scanning microscope images of *Enterococcus faecalis* infected dentinal tubules obturated with (*G1*) AH Plus, (*G2*) Bio Root, (*G3)* MTA Fillapex, (G4) Total Fill and (G5) control with previous irrigation with Protocols A. NaOCl and B. NaOCl+EDTA after viability staining.

4. Discussion

Intratubular infection models are essential to enable tests of the efficacy of chemicals to be performed for laboratory assessment of their capacity to disinfect remote areas of root canals. HCSC are interactive with dentin, therefore, experimental models should be chosen with care. Since 1987, when Hapassalo and Orstavik described the first *in vitro* model for dentin, the use of NaOCl and a chelating agent were recommended for opening dentinal tubules (138) and this was followed in literature (17, 69, 139) to test bacterial penetration (140). However, it would be incoherent to evaluate how previous irrigation would affect the antimicrobial activity of sealers, since the real protocol performed would be NaOCl+Chelator+Tested Solution. As these are hydrophilic materials, dentin wettability changed by the previous application of irrigants might also modify the pattern of HCSC penetration into dentinal tubules. With the purpose of finding a reliable dentin-sealer interaction, pilot studies were conducted according to the methodology described by Ma J in 2011 (69); by changing the previous irrigation protocol to A. NaOCl or B. NaOCl-EDTA. The power of

centrifugation forced the bacteria into dentinal tubules (69) even against the smear layer barrier and allowed the distribution of a high level of dentin infection in both irrigation protocols, as shown in Figure 1.

The antimicrobial action of HCSCs occurs after cement hydration, whereas the release of Ca^{2+} ions from the calcium silicate particles of cement reacts with the water molecules dissociated from tissue fluids, resulting in rhombohedral portlandite crystals (calcium hydroxide) (16, 133). EDTA has been proved to harm these mechanisms due its chelating ability in the absence of dentin (23), whereas the only source of Ca^{2+} ions would be those released by HCSC. Based on the clinical scenario, in this study, we analyzed the residual effect of NaOCl (Protocol A) and NaOCl-EDTA (Protocol B) on dentin, relative to the antimicrobial action of HCSCs. We theorized that the better results achieved by HCSC when followed by Protocol B were because: 1. the peaks of Ca^{2+} extraction from dentin might have been active before placement of the sealer, as EDTAC reached its peak within 15 minutes of coming into contact with dentin (141); 2. there was a preference for Ca^{2+} extraction from dentin because of the greater contact of EDTA with this substrate (142) than the sealer; 3. Ca^{2+} released through the sealer surpassed the levels of Ca^{2+} extracted by EDTA; and/or 4. the *smear*-layer-free dentin and its greater wettability (56) favored the diffusion of hydraulic cements and fluids through dentinal tubules, increasing the ion exchange and hydration of HCSC (56).The highly alkaline environment resulting from the ionic dissociation of calcium hydroxide, disrupt bacterial cell metabolism due to protein denaturation (143). The inorganic debris covering the root canal walls act as a mechanical barrier to these ions (144-146) protecting bacteria from antibacterial effect of sealers. The decrease in pH values in circumjacent dentin (147), as dentinal tubules, might have retarded bacterial growth (pH 10.5-11), but might not have reached levels that would be sufficient to kill *E. faecalis,* considering that some species can survive at pH 11.5 (148). The low availability of hydroxyl ions also restricted damage to the bacterial cytoplasmic membrane and DNA caused by coming into direct contact with them (149, 150).

The hydration of HCSC may significantly influence calcium hydroxide release. In a non-randomized clinical trial a single cone technique with BioRoot RCS can achieve a predictable success rate in the range of 84% to 90% similar to the gold standard AH Plus (151). As a water-based sealer, BioRoot BCS does not rely on OH^- ions derived from tissue fluids to form calcium hydroxide, as does MTA Fillapex presented in a 2-paste system (26) or Total Fill, a premixed ready-to-use injectable sealer (27). When

these sealers were compared BioRoot BCS showed higher portlandite peaks and Ca^{2+} ion leaching (152) which agreed with it greater effect on *E. faecalis* viability for both dentin protocols (Table 1). This is in agreement with literature that reported the superiority of BioRoot BCS against intratubular infection and in the direct contact test (17, 153). The viscosity of dentinal fluid flow should be also investigated, as vehicles have an influence on rates of ionic dissociation and diffusion of calcium hydroxide (154, 155).

The low antimicrobial effect of Total Fill against *E. faecalis* might have been influenced by insufficient levels of calcium hydroxide released. The hydration of Total Fill cannot be assumed *in vivo* and conditions of tissue fluids can not be monitored or reproduced *in vitro*. The Total Fill specimen discs completely exposed to 100% relative humidity required 10 days to achieve their final setting (27), raising doubt about their complete hydration in a period of 7 days enclosed within the root canal, as occurred in this study. Total Fill hydration might be harmed by: its lower calcium silicate content, as it is a biphasic sealer (27), directly related to Ca^{2+} ion leaching; its entire dependence on an aqueous environment to hydrate and set, and its presentation as a water-free paste (152). The higher susceptibility of *E. faecalis* to Total Fill achieved by Wang Z (20) might be explained by the wider dentinal tubule openings obtained by means of previous irrigation with a stronger chelating agent than the one used in the present study. This allowed better sealer penetration and enhanced antimicrobial action, while maintaining the order of susceptibility, since no differences were found between TF and AH in both studies. The direct contact test disregarded the dentin-buffering effect and might have benefitted Total Fill hydration by the water content of the bacterial suspension, thereby explaining the better results found for the antimicrobial action of Total Fill (134, 135).

MTA Fillapex is chemically based on mineral trioxide aggregate and salicylate resin. Irrespective of being a pioneer on the HCSC market, it does not release calcium hydroxide (156). The mildly alkaline pH (156, 157) correlated to its ability to release Ca^{2+} ions (156) 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 (158, 159).

Epoxy resin AH exhibited antibacterial activity through formaldehyde and bisphenol A diglycidyl ether (BDE) released during the curing process (160, 161) Although formaldehyde may act as an irritant inducing long-term periapical inflammation, (162)

relatively minute amounts were detected from amines reacting with epoxy for speeding up AH polymerization. This warranted the use of AH as an endodontic sealing cement (163) but also implied a pattern of low antimicrobial activity that was proved in this and other studies (Table 1) (164-166). This was not helped by the small amounts of residual BDE released from incomplete polymerization of dental materials (167). When limited by the presence of a *smear layer*, the antimicrobial agents released by AH did not reach lethal levels against *E. faecalis* within the dentinal tubules. The use of EDTA improved the penetration of sealer tags into dentinal tubules and therefore, improved the antimicrobial action and mechanical bond of AH.

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Figure 1. Representative confocal laser scanning microscope images of *Enterococcus faecalis* infected dentinal tubules obturated with (*G1*) AH Plus, (*G2*) Bio Root, (*G3)* MTA Fillapex, (G4) Total Fill and (G5) control with previous irrigation with Protocols A. NaOCl and B. NaOCl+EDTA after viability staining.

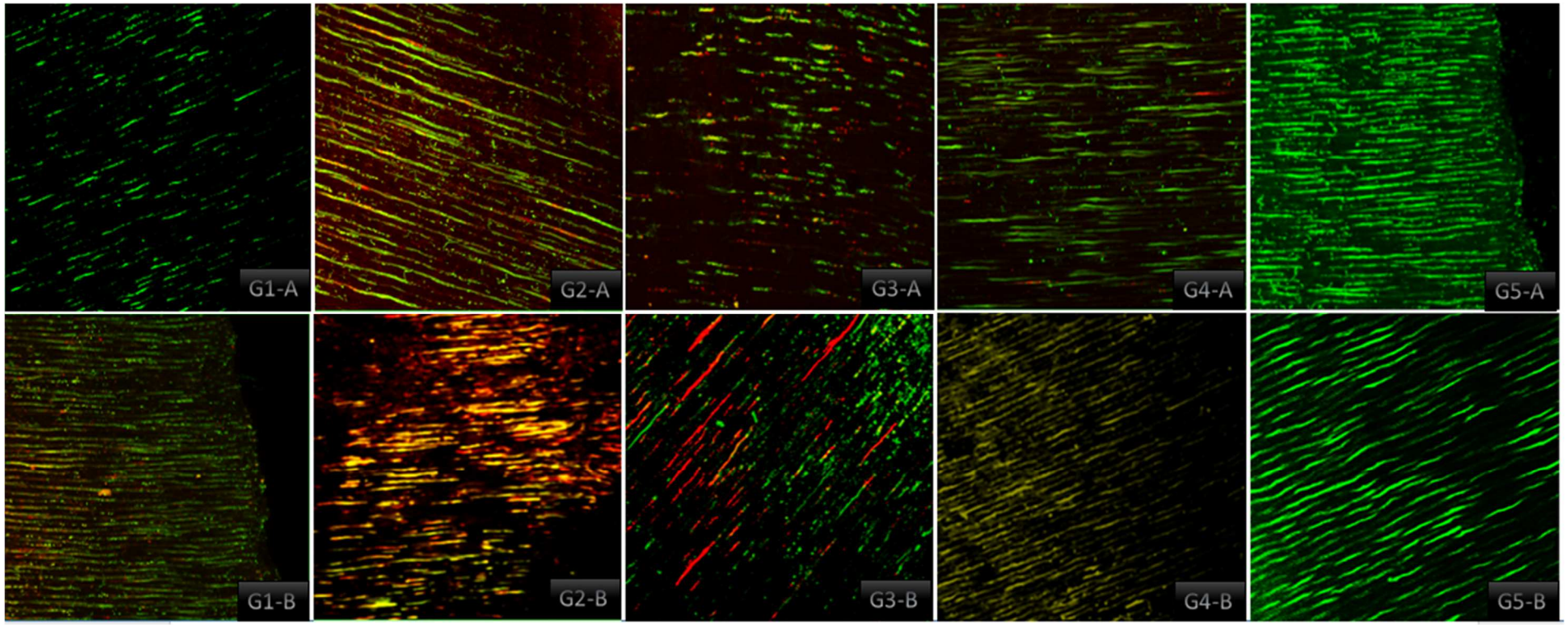

Table 1. Median (med) and minimum and maximum (min–max) values of the *Enterococcus faecalis* percentage of live cells inside dentinal tubules after 7 days of exposure to the sealers followed by to different irrigant protocols: A. NaOCl and B. NaOCl-EDTA.

	AH Plus	BioRoot BCS	MTA Fillapex	Total Fill	Control
Protocol A	81,63^{Aa} (63,99 – 96,42)	70,09^{Ba} (59,23 – 82,53)	77,43^{ABa} (50,74 – 98,46)	88,06^{Aa} (54,23 – 96,96)	83,46^{Aa} (68,01 – 99,10)
Protocol B	76,18^{ABb} (50,02 – 98,26)	53,06^{Cb} (15,82 – 73,00)	67,00^{BCa} (50,93 – 92,21)	76,92^{ABa} (61,54 – 94,89)	94,49^{Aa} (60,57 – 99,65)

Protocol 1: Bartlett's test for equal variances P value <05. Protocol 2: 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.

3 DISCUSSION

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A combination of several irrigant solutions has been proposed to achieve the objectives of root canal treatment (68, 83). The sequence and irrigant solutions used defined the characteristics of the dentin surface and its interaction with the sealers used in the root filling. Sodium hypochlorite is a strong base used throughout the biomechanical preparation procedure, due its unique capacity to dissolve organic and necrotic tissues as pulp, bacterial and biofilm remnants (69, 70). The concentration of sodium hypochlorite depends on the chlorine content it releases, which is positively correlated to its tissue-dissolving ability (85, 86). Chlorine is a strong oxidant that degrades long peptide chains of collagen (87), resulting in N-chloroamines (88) that could be indicated by traces of chlorine on the surface of dentin after flushing with NaOCl (Article 2;Table 7). NaOCl deproteination is not uniform. The *smear layer* created by the use of hand or rotary instruments has different amounts of organic mass (89) which creates an irregular degradation pattern of superficial and sub-superficial encapsulated collagen. In topographic analysis by AFM the intensity of image color is proportional to surface height. The thicker *smear layer* on untreated dentin is represented by the strongest colors, while the organic matter dissolution by NaOCl on the surface of dentin is shown by the decrease in its intensity (Article 2;Figure 8). In the structural architecture of dentin, the soft tissue collagen is encapsulated and protected by nanocrystalline carbonated apatite. Since NaOCl has a low molecular weight (74.4 Da) it spreads on the intrafibrillar water volume of apatite-encapsulated collagen matrix (7). The contact surface of collagen with sodium hypochlorite leads to oxidative chemical degradation of collagen from the “superficial subsurface” of mineralized dentin, creating a collagen-depleted mineral ghost layer (Article 2; Figure 5 and 6) with a brittle structure (7, 90). The mechanical properties of dentin are changed in 5 minutes of collagen degradation by the higher concentration of NaOCl (5%) (Article 2; Figure 1)

Ethylenediaminetetraacetic acid (EDTA) was introduced in Endodontics by Østby in 1957 in the form of a 15.5% aqueous solution and pH 7.3 and is one of the most frequently used chelating agents for root canal irrigation.(1, 38). Advocated as an adjunct irrigant to NaOCl, the EDTA removes the inorganic matter of the *smear layer*.

Its reaction with the calcium ions in hydroxyapatite crystals can alter the mineral content of root dentin (Article 2; Table 7) (91). When used alone, the dentinal tubules were less evident than they were with the treatment of sodium hypochlorite + chelator (Article 2;Figure 2,8) (94). The spectra obtained from FTIR analysis showed that previous degradation of the organic portion of *smear layer* on the root canal surface by sodium hypochlorite (Article 2;Figure 5) led to further exposure of the inorganic matter (Article 2;Figure 6), data that were shown as decreases in the amide III/phosphate ratio and increases in the carbonate/phosphate ratio respectively, favoring the removal of Ca^{2+} by the chelators (95, 96). Although the irrigant sequence NaOCl + chelator led to the widening of the dentinal tubules, small amounts of *smear layer* remained on the dentin surface even after 3 minutes of flushing with the chelator (Article 2; Figure 2). As *smear layer* removal is time dependent, the optimum working time of 1 minute recommended in literature for EDTA (71, 97) was not enough time for effectively cleaning the surface when it was used as a final flush. A final rinse with NaOCl appeared to be beneficial for enhancing disinfection in the root canal and removing rests of organic debris (Article 2; Figure 2,5) (68, 83). Although, further irrigation with NaOCl resulted in smear-layer-free surfaces, it leads to erosion of the dentin around the tubules and a progressively decrease in dentin microhardness when in higher concentration. Concerns related to the extrusion of a concentrated sodium hypochlorite solution exist due to it acute injuring effects when in contact with vital tissues (30).

The commercial form of EDTA (17%), has low antibacterial action, and non-existent antibiofilm action (39, 40), in agreement with this study. Using a calcium chelator as a final irrigant with antimicrobial properties will reduce the need to use excessive amounts of sodium hypochlorite solution to disinfect the root canal space. This will reduce the harmful effects of the hypochlorite solution and also reduce treatment time. For this purpose, active substances such as benzalkonium chloride, n-acetylcisteine and chlorhexidine were added to the EDTA and tested on *Enterococcus faecalis* biofilm. The bacterial communities in the root canal are found as biofilms (49) which are wrapped by an extracellular matrix protected them and increasing their resistance to antimicrobials by about 1000 times when compared to planktonic bacteria.(50) As expected, the mixtures had superior effect against biofilm than EDTA (Article 1; Table 1).

The use of a surfactant is important in biofilm disruption since proteins and polysaccharides on the bacterial cell surface attach irreversibly to specific receptors on the substrate (dentin) in a hydrophobic interaction (53). Surfactants can change this high-affinity binding acting on the surfaces, by either changing bacteria hydrophobicity and bacterial surface charge.(54) This was verified in the current study where the addition of benzalkonium chloride resulted in the lowest amount of *E. faecalis* biovolume (Article 1; Figure 2). This results are in accordance with literature that shows that adding benzalkonium chloride to NaOCl on dentin promoted inhibition of adhesion, and consequently formation of biofilm (34). Therefore, EDTA/benzalkonium chloride (EDTA-BC) showed effectiveness not only preventing the formation of biofilm and root canal re-infection, but also against *E. faecalis* biofilm itself when compared to pure EDTA.

The addition of the surfactant benzalkonium chloride or the mucolytic agent N-acetylcysteine to EDTA led to a reduction in surface tension of the latter, increasing its wettability on dentin. (Article 1; Table 4) When EDTA is applied to dentin, the hydroxyapatite is removed (inorganic component) leading to exposure of the collagen fibers, and diminishing the free surface energy (60). This chemical change in the composition of dentin results in a more hydrophobic surface (61). This may explain the better measurements of the dentin surface wettability when it was treated with physiological solution in comparison with EDTA. Whereas, a surfactant compound can change the energy of the surface, explaining why the mixture of EDTA with benzalkonium chloride favored the wettability of saline solution in the treated dentin. Despite the reduced surface tension of the EDTA-BC, there was no improvement in the ability of EDTA to remove the *smear layer*(92), as shown in SEM images (Article 2; Figure 2C,D,F).

The current study investigated 1 epoxy resin-based sealer, 1 containing salicylate resin and MTA and 2 hydraulic calcium silicate cements (HCSCs), assessing their morphology, composition and interfacial characteristics with modified dentin, by SEM, EDS and CLSM analysis. It also analyzed the residual effect of NaOCl and NaOCl-EDTA on dentin, towards these sealers antimicrobial action.

Complete and adequate filling of root canals prevents leakage of bacteria from the oral environment or those that persist in root canals from migrating into the apical periodontium (78). Gaps between the gutta-percha and the internal walls of the root

canal were filled by sealers. Used as a control, AH Plus - an epoxy resin-based sealer - was presented as a 2-component paste that contained zirconium oxide and calcium tungstate as radiopacifiers (Article 2; Figure 9). AH Plus exhibited antibacterial activity through formaldehyde and bisphenol A diglycidyl ether (BPA) released during the curing process (160, 161) Although formaldehyde may act as an irritant inducing long-term periapical inflammation (162) relatively minute amounts were detected from amines reacting with epoxy for speeding up AH Plus polymerization. This warrant the use as an endodontic sealing cement (163) but also implied a pattern of low antimicrobial activity that was proved in this and other studies (Article 3; Table 1) (164-166). This is not helped by the small amounts of residual BPA released from incomplete polymerization of dental materials (167). When limited by the presence of a *smear layer*, the antimicrobial agents released by AH did not reach lethal levels against *E. faecalis* within the dentinal tubules. The use of EDTA improved the penetration of sealer tags into dentinal tubules and therefore, improved the antimicrobial action and mechanical bond of AH. NaOCl + chelator groups enabled the formation of a thinner AH Plus-dentin interface (108, 109) with better results for EDTA than EDTA-BC. The widening of dentinal tubules in protocols A/B, when compared with C/D (Article 2; Figure 2) might have allowed a deeper mechanical bond between AH Plus and dentin. Despite the AH Plus dentin seal featured in Group NaOCl-5+EDTA+NaOCl-5, there were concerns about it use relative to the decrease in the mechanical properties of dentin (Article 2; Figure 1).

Hydraulic calcium silicate cements (HCSCs) are mainly composed of calcium, silica, carbon and oxygen, and contain different radiopacifiers and additives (Article 2; Figure 9). HCSCs are interactive rather than inert, and are affected by the environment, such as water content (74). Their fine hydrophilic particles absorb water during hydration of the powder, and expand during solidification. The water content in the pore spaces between the cement grains is replaced with hydration products, while the setting of material proceeds, thereby decreasing it porosity (112, 113). Although hydration of the majority of HCSCs occurs within the first few days, complete hydration may even take one or two years (114). High vacuum SEM could cause dehydration of the water content (115), which dried off leaving empty spaces, giving the HCSC surfaces a sandy and dry aspect (Article 2; Figures 9,10,12,13,14). A dense and homogeneous surface that did not absorb water was noted for AH Plus (Article 2;

Figure 9,10,11). When the analyses by CLSM were made under hydration conditions, a denser structure was found for Total Fill and BioRoot BCS, with higher porosity for AH Plus (Article 3; Figures 15,16,18). The conflicting results for AH Plus could also be explained by differences in methodologies. SEM provided overlapped images of all layers of the teeth analyzed in one image, thereby hiding the gaps and porosity that were displayed by CLSM, which dismembered these layers.

The antimicrobial action of HCSCs occurs after cement hydration, whereas the release of Ca^{2+} ions from the calcium silicate particles of cement reacts with the water molecules dissociated from tissue fluids, resulting in rhombohedral portlandite crystals (calcium hydroxide) (16, 133). EDTA has been proved to harm these mechanisms due its chelating ability in the absence of dentin (23), whereas the only source of Ca^{2+} ions would be those released by HCSC. Based on the clinical scenario, in this study, we analyzed the residual effect of NaOCl and NaOCl-EDTA on dentin, relative to the antimicrobial action of HCSCs. We theorized that the better results achieved by HCSC when followed by NaOCl-EDTA were because: 1. the peaks of Ca^{2+} extraction from dentin might have been active before placement of the sealer, as EDTAC reached its peak within 15 minutes of coming into contact with dentin (141); 2. there was a preference for Ca^{2+} extraction from dentin because of the greater contact of EDTA with this substrate (142) than the sealer; 3. Ca^{2+} released through the sealer surpassed the levels of Ca^{2+} extracted by EDTA;; and/or 4. the *smear*-free dentin and its greater wettability (56) favors the diffusibility of hydraulic cements and fluids through dentinal tubules, increasing ion exchange and hydration of HCSC (56).The highly alkaline environment coming from calcium hydroxide ionic dissociation, disrupt bacteria cell metabolism due protein denaturation (143). The inorganic debris covering the root canal walls act as a mechanical barrier for these ions (144-146) protecting bacteria from antibacterial effect of sealers. The decreased on pH values in circumjacent dentine (147), as dentinal tubules, might have retarded bacterial grown (pH 10.5-11), but might not have reached levels that would be sufficient to kill *E. faecalis,* that can survive at pH 11.5 (148). The low availability of hydroxyl ions also restricted damage to the bacterial cytoplasmic membrane and DNA caused by coming into direct contact with them (149, 150).

Although the sealers tested had a similar chemistry, they behaved in different ways relative to bonding and morphology. As a water-based sealer, BioRoot BCS does

not rely on OH^- ions derived from tissue fluids to form calcium hydroxide, as does MTA Fillapex presented in a 2-paste system (26) or Total Fill, a premixed ready-to-use injectable sealer (27). When these sealers were compared BioRoot BCS showed higher portlandite peaks and Ca^{2+} ion leaching (152) which agreed with it greater effect on *E. faecalis* viability for both dentin protocols (Article 3; Table 1). This is in agreement with literature that reported the superiority of BioRoot BCS against intratubular infection and in the direct contact test (17, 153). BioRoot BCS was less vulnerable to SEM dehydration, and had a better surface morphology than MTA Fillapex and Total Fill (Article 2; Figure 9). However, SEM dehydration appeared to cause material shrinkage. Poor adaptation of the BioRoot BCS with dentin was noted in SEM images (74) (Article 2; Figure 12), while the opposite was shown for the majority of irrigant protocols by CLSM, in which the specimens were kept hydrated (Article 2; Figure 16).

MTA Fillapex contains a resin matrix and 40% of MTA in its composition (117). Regardless of being the first Portland-based sealer developed in salicylate resin matrix., it does not release calcium hydroxide (156). The mildly alkaline pH (156, 157) correlated to its ability to release Ca^{2+} ions (156) 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 (158, 159). Sealers containing salicylate resin in their composition, such as MTA Fillapex, undergo shrinkage-related stress during the setting reaction (118). This contraction enhanced the unfilled spaces inside the obturation mass and led to detachment of the MTA Fillapex from dentinal walls, forming gaps at the sealer-dentin interface (108, 119). The presence of sealer inside dentinal tubules followed by gaps at the sealer-dentin interface in Protocol A (NaOCl) and B (NaOCl, NaOCl/EDTA/NaOCl) confirmed the contraction of MTA Fillapex (Article 2; Figure 17). If the dentin/sealer adhesion force were greater than the contraction force of MTA Fillapex, the sealer would remain attached to the dentin, with cracks in it. This indicated a strong bond between sealer and coated dentin with protocol B/D (NaOCl/Chelator) or F (EDTA), despite studies having shown that the ability of EDTA to chelate calcium ions inhibited calcium hydroxide formation, thereby affecting the hydration mechanism of MTA. (23) .

Total Fill showed no statistically significant differences with control on it antimicrobial effect of Total Fill towards *E. faecalis*, although the average amount of

killed bacteria was slightly higher in *a smear-layer*-free dentin. Total Fill hydration might be harmed by: its lower calcium silicate content, as it is a biphasic sealer (27), directly related to Ca^{2+} ion leaching; its entire dependence on an aqueous environment to hydrate and set, and its presentation as a water-free paste (152). The hydration of Total Fill cannot be assumed *in vivo* and conditions of tissue fluids can not be monitored or reproduced *in vitro*. Total Fill specimen discs complete exposed in 100% relative humidity required 10 days to achieve the final set (27), raising doubt about it complete hydration on 7 days enclosed into the root canal, as performed in this study. Hydrated cutting and polishing of the incompletely set sample could cause a loss of Total Fill mass as a result of material dissolution (Article 2; Figure 10). As Total Fill enhanced the microhardness of dentin, the humidity appears to have reached the sealer inside dentinal tubules, resulting in its setting (Article 2; Table 4). It is unclear whether the *smear layer* would block fluids that migrate via dentinal tubules, or whether the moist environment of root canals (120) would be sufficient to allow the setting of Total Fill to occur *in vivo*. In the CLSM analysis, the Groups consisting of Protocols B (NaOCl-5), A/B (NaOCl/EDTA/NaOCl), C/D (NaOCl/EDTA-BC), E and G had the best dentin-sealer interface.

There is a lack of scientific evidence to substantiate the bioactivity of HCSCs (114). Previous studies have shown no ultrastructural or chemical changes in the transition zone at the cement-dentin interfaces (122) and a lower push-out bond strength for HCSCs than for AH Plus (123). This study showed a richly dye-infiltrated layer for both AH Plus and HCSCs. Changes in the optical properties of sealer-dentin interface may be associated with the angular orientation of dentinal tubules and dentin cut (Article 2; Figure 19,B), dentin debris, quality of flow, and adhesion of sealer-dentin interface. Dentinal tubules were arranged in an S-shaped curve that led inwards towards the pulp chamber (Article 2; Figure 19,B) (82). As the dentinal tubules originated from the root canal walls in an angled direction, the entrance of the tubules was oblique to the specimen long axis. This resulted in brighter points or small lines under CLSM analysis, which differentiated them from the mostly longitudinal cross-sectional view of the dentinal tubules, equally achieved for longitudinal or transversal cutting directions (124). The uniform decrease in the diameter and number of tubules per square millimeter in the proximity of the root canal to cementum (125) may lead to a bright line around the root canal walls. The exposure of this line in CLSM images

might increase with gaps at the sealer-dentin interface (Article 2; Figure 19,A), explaining the brighter band at the AH Plus-dentin interface for NaOCl Groups. Under pressure, the sealer drains into dentinal tubules from one point to another, with a certain loss of energy. A high level of flowability leads to further sealer/dye entering into the dentinal tubules and thus producing brighter lines. As dentinal tubules have dimensions to the order of 1 µm, several tubules at different depths may be superimposed on the same image, thus masking anatomical differences; while a low flowability with tag-like sealer structures (73) near the root canal walls followed by its lower penetration pattern enhanced the "MIZ" optical illusion (Article 2; Figure 19,A). This may explain the favoritism of BR at the expense of MTA Fillapex or AH Plus in forming the rich dye layer (74, 75).

Considering Figure 19 (c) and the non uniform pattern of lines on dentin for Protocol F (BioRoot BCS) and Protocol B (NaOCl/EDTA; MTA Fillapex) brighter areas could also be interpreted as an accumulation of dye within dentin debris after sectioning the teeth, which, at this magnification, appeared as "dense" clusters of stained material. The debris deposits might have occurred more frequently in the transition zone due the space between dentin and cement. As debris is capable of reaching different depths, polishing the sample would not be enough to remove the surface debris.

Interestingly, a richly dye-infiltrated layer was formed high above the interface for the majority of the chelators groups. The great loss of dentin mineral content in these groups (Article 2; Table 7) may have weakened the teeth causing long and straight cracks.

The use of sodium hypochlorite in its "full strength" in protocol B, practiced for by the majority of American endodontists, progressively decreases the dentin microhardness, which may enhance fatigue crack propagation (126) changing the vertical fracture resistance of teeth (127). Interestingly, the irrigation protocol had no affect on dentin microhardness after obturation (Table 5), suggesting a recovery of dentin by the root canal sealers. Although this article theorized about the non-existence of "MIZ", the biomineralization ability of HCSCs may exist. Despite the fact that BioRoot BCS is also a HCSC, only MTA Fillapex and Total Fill had increased dentin microhardness (Table 4). Total Fill and MTA Fillapex had higher flow values of than those of the gold standard AH Plus, (128, 129), while BioRoot RCS had lower values

than those specified in ISO 6876 (2012) (77). High flowability allows cements to fill spaces that are difficult to access, and to penetrate into the damaged structure left by the irrigation protocol and around collagen fibrils, entrapping them into dentin structure, which may have a positive effect on reinforcing the remaining tooth structure.

4 CONCLUSION

4 CONCLUSION

Considering these experimental conditions and methodologies, this study found that Protocols E/F (EDTA/EDTA-BC) significantly altered the mineral content of root dentin causing it to crack. Previous degradation of the organic portion of *smear layer* on the root canal surface by NaOCl favored removal of Ca^{2+} by chelating agents with widening of the dentinal tubules. Highly concentrated NaOCl followed by EDTA significantly changed inorganic matter dissolution, microhardness and induced a rougher topography on the dentin surface with eroded dentinal tubules. As *smear layer* was still present on surfaces in NaOCl+chelator groups, the use of EDTA+antimicrobial or NaOCl final flush was required for further disinfection. Although the addition of benzalkonium chloride, N-acetylcysteine and chlorhexidine to EDTA resulted in improvement in antibacterial action against *E. faecalis* biofilm, without compromising the EDTA chelating action and cytotoxicity, EDTA + benzalkonium chloride (EDTA-BC) also decreased the adherence of *E. faecalis* to dentin coated with it, lowered EDTA surface tension and allowed better wettability of dentin, standing out from the other solutions. Therefore, the use of this association as a final rinse in endodontic treatment should be considered. In protocol D (5NaOCl/EDTA-BC), no significant alteration was noted for the mixture EDTA-surfactant indicating a weaker chelating solution. Further irrigation with NaOCl resulted in erosion of the dentin around the tubules and smear-layer-free surfaces. Dehydration of HCSC in high vacuum SEM *caused* material shrinkage *and conflicting data with CLSM, in* which specimens were kept hydrated. Based on CLSM analysis of dentin, Protocols A/B (NaOCl/EDTA/NaOCl), or C/D (NaOCl/EDTA-BC) matched with AH Plus, Bio Root and Total Fill sealers. Microhardness progressively decreased in protocol B (5NaOCl/EDTA/5NaOCl), and was recovered after MTA Fillapex or Total Fill application. As MTA Fillapex presented gaps on dentin-sealer interface, Total Fill appeared to be the best match for protocol B. All sealers presented a rich dye-infiltrated layer raising doubt about the existence of a “Mineral Infiltrated Zone”.

Regarding the residual effect of NaOCl and NaOCl-EDTA on dentin, towards HCSC`s antimicrobial action, for both protocols BioRoot BCS presented the greatest antimicrobial action, followed by MTA Fillapex. AH Plus and Total Fill showed no

statistically significant differences with control, although the average amount of killed bacteria was slightly higher in a *smear-free* dentin. The sealer hydration influenced hydraulic calcium silicate cements (HCSC) antimicrobial activity. EDTA coated dentin do not harm HCSC antimicrobial action. A *smear-free* dentin enhanced killed bacteria inside dentinal tubules for all sealers. Although BioRoot BCS provided the best antimicrobial activity against *E. faecalis,* it failed in achieve an effective disinfection on root canal, and the previous steps for root canal cleaning should not be ignored.

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