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.

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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.

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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