UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

VANESSA ABREU SANCHES MARQUES COSTA

Effect of different irrigation protocols on the removal of mono and multispecies biofilms, depth of penetration and changes in the dentin surface

Efeito de diferentes protocolos de irrigação na remoção de biofilmes mono e multiespécies, profundidade de penetração e alterações da superfície dentinária

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Tese constituída por artigos apresentada à Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Doutor em Ciências no Programa de Ciências Odontológicas Aplicadas, na área de concentração Endodontia.

Orientador: Prof. Dr. Rodrigo Ricci Vivan

Versão Corrigida

BAURU
2021

Abreu Sanches Marques Costa, Vanessa
Effect of different irrigation protocols on the removal of mono and multispecies biofilms, depth of penetration and changes in the dentin surface / Vanessa Abreu Sanches Marques. -- Bauru, 2021.
104p. : il. ; 31 cm.

Tese (doutorado) -- Faculdade de Odontologia de Bauru, Universidade de São Paulo, 2021.

Orientador: Prof. Dr. Rodrigo Ricci Vivan

Nota: A versão original desta dissertação/tese encontra-se disponível no Serviço de Biblioteca e Documentação da Faculdade de Odontologia de Bauru – FOB/USP.

Comitê de Ética no Uso de Animais da da FOB-USP

Protocolo nº: 003/2018

Data: 12/03/2018

FOLHA DE APROVAÇÃO

DEDICATÓRIA

Primeiramente à Deus, pelo dom da vida e por ter me concedido aqueles que são minha base: minha família.

Dedico este trabalho, também, aos meus pais José Sanches Marques (*in memoriam*), Jeovanete França Abreu Marques, minha irmã Erika Abreu Sanches Marques e meu marido, Beethoven Estevão Costa, que são meus exemplos de dedicação e amor!

AGRADECIMENTOS

À **Deus**, pela minha família e amigos, por guiar meus passos, acalmar meu coração nos momentos de dificuldade e me mostrar os caminhos a seguir.

À minha mãe, **Jeovanete França Abreu Marques**, a pessoa que mais admiro e desejo proteger no mundo, meu primeiro amor. Exemplo de dedicação, coragem, amor, força e fé. Agradeço por sempre estar ao meu lado, me apoiar e me incentivar em toda e qualquer situação, com suas palavras que acalentam meu coração. Eu te amo. Obrigada por ser tão genuína, pura e doce. Deus me presenteou nesta vida, me dando o maior exemplo de pessoa, como minha mãe!

Ao meu pai, **José Sanches Marques** (*in memoriam*), tenho certeza que está feliz por mim aí no céu! És meu anjo da guarda e com toda certeza, meu maior incentivador nos estudos. Que legado o senhor deixou, pai. É uma honra ser sua filha. Obrigada por tudo!

A minha irmã, **Erika Abreu Sanches Marques**, minha grande apoiadora, amiga, incentivadora e minha companheira de vida. Obrigada por sempre ter uma palavra que me acalma, que cuida, que ama. Minha metade morena, como te admiro. Eu te amo muito!

Ao meu marido, **Beethoven Estevão Costa**. Meu amor, você é o melhor presente que esta jornada me proporcionou. Agora meu companheiro de vida! Obrigada por ter sido tão compreensível nos dias mais difíceis, por segurar minha mão quando precisei, por cuidar tão bem de nós e por estarmos construindo uma história linda. Eu te amo, meu amor!

Aos meus sogros, **Magda da Costa** e **Luiz Eduardo da Costa** e meu cunhado, **Filipe Costa,** por me receberem tão bem em minha nova família. Obrigada pelo carinho. Amo vocês!

Ao meu orientador **Prof. Dr. Rodrigo Ricci Vivan**. Lembro-me do primeiro dia que o senhor me recebeu na Faculdade de Odontologia de Bauru, nossa primeira conversa em que me abriu as portas ao doutorado. Jamais imaginei tudo que estava por vir. Como sou grata a ti, por todo crescimento profissional que me foi proporcionado neste período. Não posso deixar de dizer o quanto sou admiradora do seu trabalho e da sua didática. O entusiasmo que o senhor transmite seus conhecimentos me inspira. O senhor é um endodontista extremamente competente e um grande mestre! Agradeço todas as conversas, cafés na cantina (o senhor me ensinou que os momentos de descontração são os mais valiosos), incentivos, por tudo me ensinou e por todas as oportunidades de crescimento. Foi uma honra trabalhar e aprender contigo! Obrigada, professor.

Ao **Prof. Dr. Marco Antonio Hungaro Duarte**, agradeço por nos ajudar na concretização deste trabalho e nos demais que, juntamente com o Prof. Rodrigo, me confiaram e permitiram realizar durante este período. Admiro muito o senhor como pessoa e profissional. O senhor é um exemplo para nós, sempre com ótimas ideias na pesquisa, visão crítica na ciência e nos transmite seus conhecimentos com extrema competência e humildade. Agradeço também pelas imensas oportunidades de crescimento profissional e pessoal que o senhor me proporcionou. É uma honra conviver e aprender com quem sempre foi referência para mim. Muito obrigada, professor.

Aos professores do departamento de endodontia da FOB/USP, **Prof. Dr. Clóvis Monteiro Bramante, Profa. Dra. Flaviana Bombarda de Andrade, Prof. Dr. Roberto Brandão Garcia e Prof. Dr. Norberti Bernardineli**, por toda atenção, paciência, convivência agradável e pelos valiosos ensinamentos transmitidos, principalmente durante a minha especialização. Tenho um imenso carinho, admiração e respeito pela trajetória de cada um.

Às professoras da banca examinadora, **Clarissa Teles Rodrigues** e **Francine Benetti**. Agradeço por, prontamente, aceitarem nosso convite. Cada uma foi escolhida com muito carinho, afinal, tenho muita admiração pela trajetória e história de vocês. Obrigada por contribuírem com a minha formação e participarem desse momento especial em minha vida.

Agradeço aos demais professores, de graduação (em nome da **Profa. Dra. Mirian Marubayashi Hidalgo**, minha grande incentivadora na endodontia) ou na pós-graduação (em nome do **Prof. Dr. Eloi Dezan-Junior**, que me abriu as portas ao mestrado) que participaram da minha formação, como profissionais, ou como pessoas.

Ao **Prof. Dr. Paulo Henrique Weckwerth**, obrigada por nos ajudar na construção dessa pesquisa, por dispor as cepas bacterianas e pela ajuda com as dúvidas na parte microbiológica do trabalho.

Ao **Prof. Dr. Guilherme Ferreira da Silva** e **Prof. Dr. Leonardo Rigoldi Bonjardim**, os quais tive a oportunidade de conhecer, trabalhar em parceria e aprender. Sou admiradora do modo como lecionam! Vocês, com toda certeza, fazem parte da minha formação. Obrigada por me receberem tão bem!

Aos demais **Professores dos Programa de Pós-Graduação da FOB/USP** por todos os conhecimentos transmitidos através das disciplinas cursadas durante o doutorado. Muito obrigada.

À **Angélica Cristina Fonseca, Claudia Cristina Biguetti, Rafaela Fernandes Zancan** e **Talita Tartari** que ajudaram em etapas cruciais desta pesquisa. Vocês me estenderam a mão e me ensinaram quando eu ainda não dominava o assunto. Me ajudaram a dar os primeiros passos no desenvolvimento do trabalho. Aprendi muito com cada uma de vocês. Muito obrigada e desejo sucesso a todas.

Aos alunos de iniciação científica, **Gabriela de Santi Sodré, Matheus Rinaldi Lucio Martins**, **Paulo Roberto Jara de Souza.** Agradeço pelas conversas, pelas risadas em dias de experimento, por confiarem a mim este momento importante da vida de vocês e por tanta troca de conhecimento. Saibam que aprendi muito com cada um e com os trabalhos que desenvolvemos ao longo desta jornada. Desejo muito sucesso a vocês.

À minha grande amiga, **Letícia Citelli Conti**. Como agradecer a minha maior companheira durante toda esta trajetória. Sempre digo que você é um presente que a pós-graduação me deu lá em Araçatuba. Sou eternamente grata por tudo que vivemos e aprendemos juntas nesta jornada. Minha parceira nos laboratórios durante o dia ou até tarde da noite, nos cafés na cantina, almoços no bandeco, finais de semana, idas para casa, nas boas conversas, nos momentos foi preciso incentivo, na conversa amiga ou no abraço de irmã. Serei eternamente grata a Deus por esta amizade tão madura e tão sólida que temos. E sei que vou poder contar contigo para tudo que precisar. Conte também, comigo. Minha eterna amiga-irmã-cupida-madrinha! Amo você, Lê!

Aos colegas de Pós-Graduação do departamento de Endodontia. Obrigada por todos os momentos compartilhados, os cafés, as conversas, os períodos de descontração. Desejo sucesso à todos: **Arthur Costa Lemos, Bruno Piazza, Clarissa Teles Rodrigues, Denise Ferracioli Oda, Ericson Janolio de Camargo, Ericke Mucke Silva, Flavia Darius Vivacqua, Francine Cesário, Gabriela Piai, Gabryella do Nascimento Camilo, Glaucia Beatriz Golçalves, German Diaz, Gislene Cristina Vertuan, India Olinta de Azevedo Queiroz, Jessica Coelho, Jussaro Alves Duque, Larissa Borges, Leticia Citelli Conti, Letycia Acyoli, Lyz Cristina Furquim Canali, Lorena de Mello Alcantara Garrido, Marcelo Pomini, Mariana Maciel Batista Borges, Maricel Rosário Cárdenas Cuellar, Melissa Esther Rivera Peña, Michel Espinosa Klymus, Milena Perraro Martins, Mirela Barros, Murilo Priori Alcalde, Paulo Roberto Jara de Souza, Pedro Cesar Gomes Titato, Pedro Henrique Calefi, Rafaela Fernandes Zancan, Renan Diego Furlan, Renato Piai, Roberto Osaki, Samilla Monteiro, Shirley Evelin Jaen, Talita Tartari, Victor de Moraes Cruz, Victor Feliz**, **Yahir Muñoz Valencia.**

Aos funcionários, **Suely Regina Bettio, Edimauro de Andrade, Zuleica Valderes Roberto** (Departamento de Endodontia), **Audria Veronez** (Departamento de Dentística), **Elias** (Setor da Esterilização), **Cássia Regina Pereira Schucheman**, **Celina Maria de Souza, Érika Uliam Kuriki, Regina Célia de Oliveira** (Setor da Urgência),**Thelma Lopes Silva** (Laboratório de Bioquímica), **Maira Cristina Rondina Couto** (Laboratório de Histologia da Unisagrado), **Ana Letícia Palombo Momesso, Fátima Cassador Carvalho** (*in memoriam*), **Leila Regina da Silva, Vera Lygia Santos Butignoli Caetano** (Secretaria da Pós-Graduação) por estarem sempre dispostos a ajudar quando precisei e pela boa convivência em todos esses anos. Nada acontece sem vocês. Muito obrigada.

Ao centro integrado em pesquisa (CIP) da FOB-USP, em nome da **Márcia Sirlene Zardin Graeff** e **Marcelo Miranda Ribeiro Lopes**, pela disponibilidade e inestimável colaboração. Vocês foram fundamentais para a realização desta pesquisa.

Assim também agradeço a todos que direta ou indiretamente ajudaram para realização desse trabalho, muito obrigado!

AGRADECIMENTOS INSTITUCIONAIS

À **Faculdade de Odontologia de Bauru – USP** na pessoa do diretor Prof. Dr. Carlos Ferreira dos Santos e do vice-diretor Prof. Dr. Guilherme dos Reis Pereira Janson.

À **Comissão de Pós-Graduação da Faculdade de Odontologia de Bauru**, na pessoa da Presidente Prof. Dra. Izabel Regina Fischer Rubira de Bullen.

Ao curso de **Pós-Graduação em Ciências Odontológicas Aplicadas**, na **área de Concentração em Endodontia da Faculdade de Odontologia de Bauru – USP** na pessoa de Prof.Dr. Marco Antonio Hungaro Duarte.

Ao **Centro Integrado de Pesquisa (CIP)**, na pessoa da coordenadora Profa. Dra. Ana Flávia Sanches Borges, por permitir o uso das dependências do laboratório para o desenvolvimento do projeto de pesquisa.

À **Disciplina de Endodontia**, do Departamento de Dentística, Endodontia e Materiais odontológicos, na pessoa do Prof. Dr. Marco Antonio Hungaro Duarte por permitir o uso das dependências e laboratório para o desenvolvimento do projeto de pesquisa.

Ao **Departamento de Física, da Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto (FFCLRP - USP)**, na pessoa do Prof. Dr. Luciano Bachmann, por permitir o uso das dependências e laboratório para o desenvolvimento de parte do projeto de pesquisa.

À **Fundação Oswaldo Cruz (FioCruz, Rio de Janeiro – RJ),** pelo envio da Coleção de Microrganismos de Referência em Vigilância Sanitária (CMRVS) que contribuiu para o desenvolvimento de parte desta pesquisa.

À **Coordenação de Aperfeiçoamento Pessoal de Nível Superior (CAPES)**, pelo apoio financeiro e concessão da bolsa de doutorado para execução deste trabalho.

“Espalhe amor, seja amor. No caminho, uma flor tem valor, mesmo se tiver espinhos. Traga paz, viva em paz. Tá tudo bem, seja capaz de provocar algum sorriso no rosto de alguém.
Se a vida te convida pra dançar num ritmo descontrolado... injusto. Respire fundo. Deixe o seu coração de janela aberta, deixa ser desperta. Sinta o gosto do muito ou do pouco, se entrega, pode ir sem pressa. Viver é mesmo assim, com o tempo tudo se acerta”

Respire fundo – Gabi Luthai

RESUMO

Efeito de diferentes protocolos de irrigação na remoção de biofilmes mono e multiespécies, profundidade de penetração e alterações da superfície dentinária

O objetivo do presente estudo foi avaliar a ação antimicrobiana contra biofilmes mono e multiespécies, as alterações químicas na superfície da dentina, por reflexão totalmente atenuada em espectroscopia por transformada de Fourier no infravermelho (ATR-FTIR), microdureza, coloração com *Picrosirius Red* e a influência do tempo de contato e ativação de irrigantes na penetração dentinária por tomografia de coerência óptica (OCT), após diferentes protocolos de irrigação. Amostras de dentina bovina (N=20) foram submetidas aos protocolos de irrigação após indução *in vitro* de biofilmes. Corante Live/Dead e um microscópio confocal de varredura a laser foram usados para medir a porcentagem de células vivas. As amostras para ATR-FTIR (N=10) foram analisadas antes e após os tratamentos e as proporções de amida III/fosfato e carbonato/fosfato foram determinadas. Para a OCT, foram obtidas imagens transversais das amostras (N=5), antes e após os protocolos de irrigação, e obtida a profundidade média de penetração (μ). A microdureza (N=12) foi medida com indentador Knoop sob carga de 25 g por 15 segundos, antes e após os tratamentos. As amostras passaram por um processamento histológico, para coloração *Picrosirius Red* (N=12), após os regimes de irrigação. Os dados foram comparados estatisticamente (P <.05). Para o biofilme de *E. faecalis*, os grupos PUI tiveram a melhor efeito antimicrobiano independente da ação química (P <.05). Easy Clean atuou melhor com clorexidina, independente do tempo ou NaOCl em tempo de contato maior (P <0,05). Contra *E. faecalis* e *S. oralis*, apenas Easy Clean com solução salina, independente do tempo, não teve ação antimicrobiana (P> .05), assim como PUI ou Easy Clean em menor tempo de agitação com solução salina (P> .05), para o biofilme de *E. faecalis* com *C. albicans*. O tempo de contato do NaOCl (30 minutos) intensificou a dissolução do colágeno dentinário reduzindo a relação amida III/fosfato (P< .05). Na relação carbonato/fosfato, não houve diferenças intergrupos (P> .05), apenas alterações intragrupo na irrigação convencional, PUI e EasyClean com solução salina (P <.05). Os valores de difusão dos irrigantes na dentina revelam na análise intragrupo que, exceto para a irrigação convencional com soro fisiológico, houve uma difusão

significativa dos irrigantes, através dos protocolos testados (*P* <.05). Na análise intergrupo, não foram observadas diferenças significativas (*P* > .05). Exceto para o grupo solução salina, imerso em 30 minutos, não houve diferenças estatisticamente significativas em relação aos seus valores iniciais de microdureza (*P*> .05) e não apresentaram diferenças intergrupos (*P*> .05). Todos os grupos apresentaram birrefringência para fibras esverdeadas,amarelas e vermelhas, demonstrando a dinâmica de maturação da matriz. A agitação dos irrigantes pela técnica PUI e rotação contínua, assim como a associação com os fatores tempo e ação química, favorecem a redução microbiana na superfície dentinária entre os biofilmes estudados. O uso de NaOCl por tempo prolongado ou com agitação contínua causa maior desproteinação da matriz orgânica da dentina em relação ao soro fisiológico. Alguns íons carbonato são removidos na fase inorgânica da dentina pela solução salina. A agitação e tempo de contato das soluções irrigantes de hipoclorito de sódio, clorexidina ou salina e o maior tempo de contato favorecem a penetração desses irrigantes na dentina radicular. Os regimes de irrigação aplicados neste estudo não são capazes de alterar a microdureza dentinária. As soluções de clorexidina e NaOCl, quando em contato com a dentina por um período prolongado, podem causar uma desorganização na rede fibrilar ou modificar a estrutura morfológica do substrato dentinário.

Palavras-chave: Biofilmes. Espectroscopia de infravermelho com transformada de Fourier. Tomografia de coerência óptica.

ABSTRACT

Effect of different irrigation protocols on the removal of mono and multispecies biofilms, depth of penetration and changes in the dentin surface

The aim of the present study was to evaluate the antimicrobial action against mono and multispecies biofilms, the chemical changes in dentine surface, by Attenuated Total Reflectance in Fourier Transform Infrared Spectroscopy (ATR-FTIR), microhardness, *Picrosirius Red* staining and the influence of contact time and activation of irrigants on dentinal penetration by Optical Coherence Tomography (OCT), after submission to different irrigation protocols. Dentine samples from bovine teeth (N=20) were submitted to the irrigation protocols after in vitro induction of monospecies and dual-species biofilms. Live/dead dye and a confocal laser scanning microscope were used to measure the percentage of living cells. The samples for ATR-FTIR (N=10) were analyzed before and after the treatments and the proportions of amide III / phosphate and carbonate / phosphate were determined. For the OCT, cross-sectional images were obtained from the samples (N=5), before and after the irrigation protocols, and the mean depth of penetration (μ) was obtained. Microhardness (N=12) was measured with Knoop indenter under a 25 g load for 15 seconds, before and after treatments. Samples passed through a histological processing to Sirius Red staining (n=12) after irrigation schemes. Data were statistically compared (P <.05). For the *E. faecalis* biofilm, the PUI groups had the best antimicrobial action regardless of chemical action (P <.05). Easy Clean worked best with chlorhexidine, regardless of time or NaOCl in longer contact time (P <.05). Against *E. faecalis* and *S. oralis*, only Easy Clean with saline solution, regardless of time, did not have an antimicrobial action (P >.05), as well as PUI saline solution, for the biofilm of *E. faecalis* with *C.albicans*, and Easy Clean in less agitation time with saline solution (P >.05).The irrigant contact time intensified the dissolution of the dentin collagen reducing the amide III/phosphate ratio when in contact for 30 minutes with NaOCl (P <.05); In the carbonate/phosphate ratio, there were no intergroup differences (P >.05), only intra-group changes in conventional irrigation, PUI and EasyClean treatments with saline solution (P <.05). The diffusion values of irrigants in dentin reveal in the intragroup analysis that, except for conventional irrigation with saline, there was a significant diffusion of irrigants, through the tested protocols (P <.05). In the intergroup analysis, no significant

differences were observed (*P* >.05). Except for the saline group, immersed in 30 minutes, there were no statistically significant differences in relation to their initial microhardness values (*P* >.05) and there were no intergroup differences (*P* >.05). All groups showed birefringence for greenish, yellow and red fibers, demonstrating the maturation dynamics of the matrix. The agitation of irrigants by the PUI technique and continuous rotation with Easy clean, as well as the association with the factors of time and chemical action, favors the microbial reduction on the dentin surface among the studied biofilms. The use of NaOCl for prolonged time or with continuous agitation causes greater deproteination of the organic matrix of dentin compared to saline solution. Some carbonate ions are removed in the inorganic phase of the dentine by the saline solution. The agitation and contact time of the sodium hypochlorite, chlorhexidine or saline irrigating solutions and the longer contact time favor the penetration of these irrigants in the root dentin. The irrigation regimes applied in this study are not capable of altering dentinal microhardness. The solutions of chlorhexidine and NaOCl, when in contact with the dentin for a prolonged period, can cause a disorganization in the fibrillar network or modify the morphological structure of the dentin substrate.

Keywords: Biofilms. Spectroscopy fourier transform infrared. Tomography optical coherence.

SUMÁRIO

1 Introduction

1 INTRODUCTION

Biomechanical preparation is directly related to the cleaning and modeling of root canals, being an extremely important stage in endodontic treatment in view of the anatomical complexity that canals systems present (VERTUCCI, 1984; VILLAS-BOAS et al., 2011). Currently, studies with computerized microtomography allow us to evaluate the quality of the root canal instrumentation, showing that even with the technological variety of the instruments developed, more than 40% of the root surfaces are not prepared mechanically (VERSIANI et al., 2011; VERSIANI et al., 2013). Studies show that the irrigant is not able to spread throughout the root canal (VERSIANI et al., 2015) and may lead to treatment failure, since the intracanal biofilm will remain untouched and disinfection will be compromised.

The endodontic biofilm consists of multiple microorganisms, from bacteria involved in persistent infections, predominantly in endodontic failures, such as *Enterococcus faecalis* (PINHEIRO et al., 2003; SIQUEIRA & RÔÇAS, 2004) and *Streptococus oralis* (SIQUEIRA & RÔÇAS, 2009) as well such as the participation of yeasts, such as *Candida albicans* (DIOGO et al., 2017). The selection of microbial species occurs not only during the chemical-mechanical treatment of the canal, but certain species that colonize root canals may also have the advantage of surviving as a result of adaptation to the modified environment caused by the endodontic therapy itself (BYSTROM & SUNDQVIST, 1983; 1985; DE PAZ et al., 2003). This allows a microbial persistence inside the canal systems in face of endodontic treatment, as is the case of lesions in apical periodontitis due to the presence of refractory microorganisms (SIQUEIRA & RÔÇAS, 2008) that adhere to surfaces that are difficult to access, such as lateral canals, apical ramifications and isthmus areas (RICUCCI et al., 2009; RICUCCI et al., 2013). Thus, the importance of using auxiliary chemical solutions and irrigation protocols that promote greater intracanal disinfection and, consequently, the removal of biofilm, stands out (VAN DER SLUIS et al., 2006; ORDINOLA-ZAPATA et al., 2014).

Among the irrigation solutions most used in endodontic treatment, there is sodium hypochlorite (NaOCl) and chlorhexidine gluconate. Sodium hypochlorite is highly recommended in endodontic therapy due to its broad antimicrobial spectrum

and the ability to solubilize organic tissue (MOHAMMADI, 2008), despite having cytotoxic potential (HULSMANN & HAHN, 2000), while chlorhexidine has much lower toxicity to that of sodium hypochlorite (SPANGBERG et al., 1973). However, this is indicated as a final irrigating solution due to its good antibacterial property when the necrotic and tissue remains have already been removed (ZEHNDER, 2006).

However, if, on the one hand, these substances have the ability to cause desired effects, they can also change the structure of organic and inorganic compounds, such as collagen, affecting the mechanical properties of dentin (SANTOS et al., 2006; WACHLAROWICZ et al., 2007; PASCON et al., 2009; ZHANG et al., 2010). Tartari et al. (2016), evaluating changes in dentin composition by different concentrations of sodium hypochlorite, using the Fourier transform spectroscopy technique in the infrared, concluded that the increase in exposure time and in the concentration of NaOCl solution leads to an increase in dentin collagen deprotection. As for chlorhexidine, Moreira et al. (2009), evaluated the structure of bovine dentin after irrigation with 5.25% NaOCl and 2% chlorhexidine gel, and concluded that the second group did not promote changes in the morphological structure of the organic matrix of dentin, since it showed uniformity in its fibrillar network similar to that of the control group. However, results on possible changes in root dentin after the use of these substances are still scarce in the literature, as well, it is not known exactly what the ideal action time of irrigating solutions in contact with dentin to obtain desirable properties and which cause damage to it.

In summary, understanding the behavior in relation to the contact time and chemical action of irrigators is essential to optimize biofilm removal strategies during root canal irrigation. However, there is a need for more studies to be carried out in order to elucidate the effects of the chemical-mechanical action and the contact time of the irrigant on dentin collagen and in mono and multispecies biofilms when these chemicals are subjected to different irrigation protocols. Therefore, the objective of our work was to evaluate the ability to remove mono and multispecies biofilms (*Enterococcus faecalis*, *Streptococcus oralis* and *Candida Albicans*), the diffusion of irrigating solutions and changes in the dentin surface using sodium hypochlorite 2, 5%, 2% chlorhexidine and 0.9% saline at different times and irrigation protocols, using bovine tooth blocks.

2 Articles

2 ARTICLES

The articles presented in this thesis were written according to instructions and guidelines for article submission presented in (1) Journal of Endodontics, (2) International Endodontic Journal, (3) International Endodontic Journal, (4) Brazilian Oral Research.

2.1 ARTICLE 1 - The influence of chemical action, time of contact and activation of irrigant, in the removal of mono and dual-species biofilms

Abstract

Introduction: The aim of the present study was to evaluate the antimicrobial action against mono and multispecies biofilms using sodium hypochlorite (NaOCl) 2.5%, chlorhexidine 2%, and saline 0.9% at different times of agitation protocols. **Methods:** Dentine samples from bovine teeth (*N* = 20) were submitted to the following irrigation protocols after *in vitro* induction of monospecies and dual-species biofilms: G1: 3 cycles of 20 seconds of ultrasonic agitation with 2.5% NaOCl (PUI); G2: 6 cycles of 20 seconds of ultrasonic agitation with 2.5% NaOCl (PUI); G3: 3 cycles of 20 seconds of agitation with EasyClean, with 2.5% NaOCl; G4: 6 cycles of 20 seconds of agitation with EasyClean, with 2.5% NaOCl; G5 to G8 received the same protocols described above, but the 2.5% NaOCl was replaced by 2% chlorhexidine (CHX); and from G9 to G12 the 2.5% NaOCl was replaced by 0,9% saline. Live/dead dye and a confocal microscope were used to measure the percentage of living cells. Data were statistically compared (P <.05). **Results:** For the E. faecalis biofilm, G1-G2, G4 to G10 showed better antimicrobial effectiveness (P <.05). Against E. faecalis and S. oralis, the lowest percentage of live bacteria was in G1 to G10 (P >.05) and for the biofilm of E. faecalis with C.albicans, the greatest reduction was for groups G1-G8 and G12 (P <.05). **Conclusion:** The agitation of irrigants by the PUI technique and continuous rotation with Easy clean, as well as the association with the factors of time and chemical action, favors the microbial reduction on the dentin surface among the studied biofilms.

Key Words: Antimicrobial action, biofilms, chlorhexidine, sodium hypochlorite, ultrasonic.

Introduction

The presence of microorganisms in the pulp tissue is a prerequisite for the development of apical periodontitis (1). The mechanical chemical preparation effectively reduces the microbiota in infected teeth, but not enough to obtain a complete antisepsis, a consequence of the limitations of access to instruments and irrigators due to the various anatomical complexities (2). Therefore, the persistence of these pathogens even after instrumentation of root canals has been a challenge for endodontic treatment (3), since the elimination of microorganisms may not be uniform due to the vulnerability of the species involved (4).

The initial adhesion of microorganisms to surfaces is the first stage of colonization and formation of biofilms, such as Enterococcus faecalis, which has the ability to survive due to the ease in adhering to dentin, occupying dentinal tubules, forming communities organized in biofilms and presenting resistance even after intracanal procedures, resulting in persistent or chronic infections (5,6). E. faecalis is the species commonly found in up to 90% of retreatment cases, followed by less frequent bacteria such as Streptococcus (7). A percentage of yeasts, such as Candida albicans, have been associated with persistent root canal infections that do not respond favorably to endodontic therapy (8).

Accordingly, the irrigating solutions must show high antimicrobial activity, dissolved organic tissues, and inorganic debris from necrotic tissue, remove debris and to inactivate endotoxin (9). New strategies, such as irrigation devices and agitation regimes, have been used in order to increase the flow and diffusion of irrigants, promoting a greater reach of substances in areas of anatomical complexity, in order to obtain an adequate disinfection of the canal system. root and repair of periapical tissues (10,11,12). However, the agitation time, chemical action and solution renewal are important (13,14).

To date, no studies have been found in the literature regarding the removal of mono and dual species biofilms, composed of both bacteria and yeast, correlating longer agitation time and chemical action of irrigating solutions. Thus, the aim of the present study was to evaluate the ability to remove mono and multispecies biofilms (*Enterococcus faecalis, Streptococcus oralis and Candida albicans*) using 2.5% sodium hypochlorite, 2% chlorhexidine, and 0.9% saline at different times. agitation

protocols, using blocks of bovine teeth. The null hypothesis was that the irrigating solutions were not able to remove the biofilm according to the proposed protocols.

Methods

Irrigation solutions

Concentrated (5.25%) NaOCl solution (Fórmula e Ação, São Paulo – SP - Brazil) was diluted in distilled water to produce solution with 2.5% concentration that were confirmed by iodometric titration. The solutions obtained were stored, protected from the light in airtight plastic bottles in a refrigerator at 4°C, and removed one hour before the experiments to reach room temperature. A 2% chlorhexidine digluconate (Biodinâmica, Ibiporã – Paraná – Brazil) solution was also used for the experiments and a 0.9% physiological saline solution was used as a control.

Antimicrobial Test and Microscopic Analysis

Biofilm Growth. Microbiological procedures were conducted under aseptic conditions in a laminar flow chamber (VecoFlow Ltda, Campinas, SP, Brazil). For E. faecalis, 15 µL standard strain (American Type Culture Collection [ATCC] 29212) were put into 3 mL sterile brain-heart infusion (BHI) (Oxoid, Basingstoke, UK) at 37°C in air for growth overnight. For S.oralis, 15 µL standard strain (Fundação Oswaldo Cruz, Rio de Janeiro, Brazil - ATCC 10557) were put into 3 mL sterile brain-heart infusion (BHI) (Oxoid, Basingstoke, UK) at 37°C in air for growth overnight. For C. albicans, 15 µL standard strain were seeded on Petri dishes containing Sabouraud Dextrose Agar (SDA). The SDA dishes were incubated in a bacteriological oven at 37±1°C for 24 hours (15).

For dual-species of bacteria biofilm, the same procedure described was used with *E. faecalis* (ATCC 20219) and *S. oralis*, each one separately and at different times to ensure that no contamination occurred. After growth overnight, bacterial density was adjusted at $0{,}25 \times 10^8$ cells/mL for *E. faecalis* (ATCC 29212) and 10^8 cells/mL for *S. oralis* with a spectrophotometer (UV-VISIBLI, Shimadzu, Japan) at an optical density of 1 at 580 nm according to the 0.5 MacFarland standard.

For dual-species of bacteria and yeast biofilm, the same procedure described was used with *E. faecalis* (ATCC 20219) and *Candida albicans* (ATCC 10231) each one separately and at different times to ensure that no contamination occurred. After growth overnight, bacterial density was adjusted at 10^8 cells/mL for *E.*

faecalis and 10^7 cells/mL for *Candida albicans* with a spectrophotometer (UV-VISIBLI, Shimadzu, Japan) at an optical density of 1 at 530 nm for yeast according to the 0.5 MacFarland standard.

Dentin Surface Infection. Dentin blocks, obtained from bovine central incisors with fully developed roots using trephine drills 4.0 mm in diameter under abundant irrigation, were subsequently sterilized in an autoclave. After density adjustment, the dentin surfaces were infected. For *E. faecalis* biofilm, 1 dentin block + 100 µL *E. faecalis* + 900 µL BHI were inserted into each well of a 24-well multiwell plate.

For dual-species of bacteria biofilm, 1 dentin block + 120 µL *S. oralis* initially + 830 µL BHI were put into each well according to Hoedke *et al.* 2018 methodology (16). Four hours later, 50µL of *E. faecalis* were added.

For dual-species of bacteria and yeast biofilm, 1 dentin block + 900 µL *C. albicans* initially + 1000 µL BHI. Two days after the initial contamination, 100 µL of *E. faecalis* will be added.

For growth of both types of biofilms, all plates were incubated aerobically at 37°C for 21 days (monospecies biofilm and dual dual-species of bacteria biofilm) and 19 days (dual-species of bacteria and yeast biofilm). The BHI was refreshed every 2 days.

Root canal irrigation model. A root canal irrigation model was developed based on the Ordinola-Zapata *et al.* 2014 methodology (17), using bovine incisor roots. The root canals were standardized with 12 mm in length and apically enlarged with the 40.06 instrument (MK life, Porto Alegre, RS, Brazil). Subsequently, a drilling was performed (3.5 × 3.5 mm) at 3 mm from the apical foramen to adjust the dentin blocks.

The specimens were fixed and sealed at the drilling site with the treated side facing the root canal (17), allows the adaptation of the treated area of the dentin block at the same level as the apical area of the root canal of a bovine incisor tooth. Each root was used a maximum of five times. For the irrigation protocols, the groups were divided as follows: G1: 3 cycles of 20 seconds of ultrasonic agitation (E1, Helse Dental Technology, Santa Rosa de Viterbo, São Paulo, Brazil), with 2.5% NaOCl (PUI); G2: 6 cycles of 20 seconds of ultrasonic agitation with 2.5% NaOCl (PUI); G3: 3 cycles of 20 seconds of agitation with EasyClean 25/04 (Easy Equipamentos Odontológicos, Belo Honrizonte, MG, Brazil), with 2.5% NaOCl; G4: 6 cycles of 20 seconds of agitation

with EasyClean, with 2.5% NaOCl; G5 to G8 received the same protocols described above, but the 2.5% NaOCl was replaced by 2% chlorhexidine (CHX); and from G9 to G12 the 2.5% NaOCl was replaced by 0,9% saline.

The same volume of irrigant was used for all treatments (6mL). The irrigating solutions were applied with a Navitip 30G needle (Ultradent Products, Inc, South Jordan, Utah, USA) inserted 2 mm from the apex, in all groups. For groups with ultrasonic agitation the model PM100 EMS (E.M.S. Electro Medical Systems S.A., Nyon, Switzerland) was used.

Microbiological Analysis. Biofilm viability was analyzed using the SYTO 9/propidium iodide technique (Live/Dead BacLight Viability Kit; Molecular Probes, Eugene, OR). After protocols irrigation, the blocks were washed with phosphate-buffered saline and stained in a dark environment with 15 µL of the dyes for 20 minutes. Then, they were washed again and directly observed by inverted confocal laser scanning microscopy (Leica TCS-SPE; Leica Biosystems CMS, Mannheim, Germany). Four confocal "stacks" of random areas were obtained for each sample with a 40× oil lens. In total, there were 5 samples per group, thus 20 stacks for each protocol. For quantification, LAS X software (Leica Application Suite X) software was used to calculate percentage of live and dead cells in the biomass.

Statistical analysis

The Shapiro-Wilk test was used to verify the normality of data from all analyses, and absence of normality was observed. Therefore, statistical comparisons between the groups were made by the Kruskal-Wallis and Dunn tests. The significance level was established at 5%.

Results

Table 1 presents median and minimum and maximum values of the percentage of live cells of the different biofilms after the irrigation protocols. For the E. faecalis biofilm (Fig. 1), the weakest antimicrobial activity occurred in groups G3, G11 and G12, with no statistical differences in relation to the control group ($P > .05$). Groups G1, G2, G4, G5, G6, G7, G8, G9 and G10 showed better effectiveness in antimicrobial action ($P < .05$). For the biofilm of two bacterial species (Fig. 2), the highest percentage of

live bacteria was found in groups G11 and G12 ($P > .05$) and the lowest, in groups G1 to G10 ($P < .05$). For the E. faecalis biofilm with C. albicans (Fig. 3), the best antimicrobial action was for groups G1, G2, G3, G4, G5, G6, G7, G8 and G12 ($P < .05$). Groups G9, G10 and G11 did not show statistical differences from the control group ($P < .05$).

Table 1. Median (med) and minimum and maximum (min–max) values of the percentage of live cells of *E. faecalis, E. faecalis* and *S. oralis, E. faecalis* and *C. albicans* biofilms after the irrigation protocols

		E. faecalis	*E. faecalis and S. oralis*	*E. faecalis and C. albicans*
Groups		**Med (Min – Max)**	**Med (Min – Max)**	**Med (Min – Max)**
	Control	98.47^{A} (72.32 - 99.94)	92.96^{A} (76.06 - 99.63)	94.46^{A} (36.77 - 99.85)
Sodium hypochlorite	G1 - PUI (3 cycles)	50.0^{CD} (1.39 - 95.37)	63.47^{BCD} (0.17 - 97.84)	13.56^{BCE} (0.09 - 67.81)
	G2 - PUI (6 cycles)	20.57^{BD} (0.0 - 73.95)	53.37^{BCE} (0.0 - 97.65)	0.67^{BC} (0.0 - 99.09)
	G3 - Continuous Rotation (3 cycles)	59.62^{AC} (0.02 - 99.89)	36.415^{BDE} (0.12 - 81.39)	5.82^{BC} (0.0 - 87.48)
	G4 - Continuous Rotation (6 cycles)	2.69^{B} (0.0 - 92.65)	33.768^{BCE} (3.45 - 87.01)	4.65^{C} (0.05 - 67.59)
Chlorhexidine	G5 - PUI (3 cycles)	25.78^{BC} (0.03 - 84.99)	50.499^{BC} (2.50 - 98.76)	30.31^{BCD} (0.39 - 98.84)
	G6 - PUI (6 cycles)	26.47^{BC} (2.42 - 75.47)	30.713^{BDE} (0.76 - 95.58)	47.20^{BD} (4.30 - 99.62)
	G7 - Continuous Rotation (3 cycles)	49.93^{BC} (1.00 - 81.02)	9.354^{E} (0.90 - 53.27)	20.59^{BCD} (7.93 - 80.53)
	G8 - Continuous Rotation (6 cycles)	14.51_{BD} (2.48 - 71.13)	21.769^{BDE} (0.39 - 97.06)	31.43^{BCD} (0.91 - 97.39)
Saline solution	G9 - PUI (3 cycles)	40.09^{BC} (3.48 - 94.23)	53.696^{BCE} (2.74 - 82.11)	75.56^{AD} (2.08 - 99.39)
	G10 - PUI (6 cycles)	33.40^{BC} (11.09 - 67.87)	58.554^{BC} (2.77 - 99.98)	60.25^{ADE} (8.13 - 98.35)
	G11 - Continuous Rotation (3 cycles)	68.05^{AC} (0.77 - 99.36)	86.574^{AC}(21.27 - 97.88)	59.72^{ADE} (3.96 - 93.79)
	G12 - Continuous Rotation (6 cycles)	80.23^{AC} (3.30 - 99.76)	70.705^{ACD}(12.36 - 97.54)	39.43^{BCD} (1.40 - 92.10)

Different capital letters in the column indicate statistically significant intergroup differences (*P-value <0.05*).

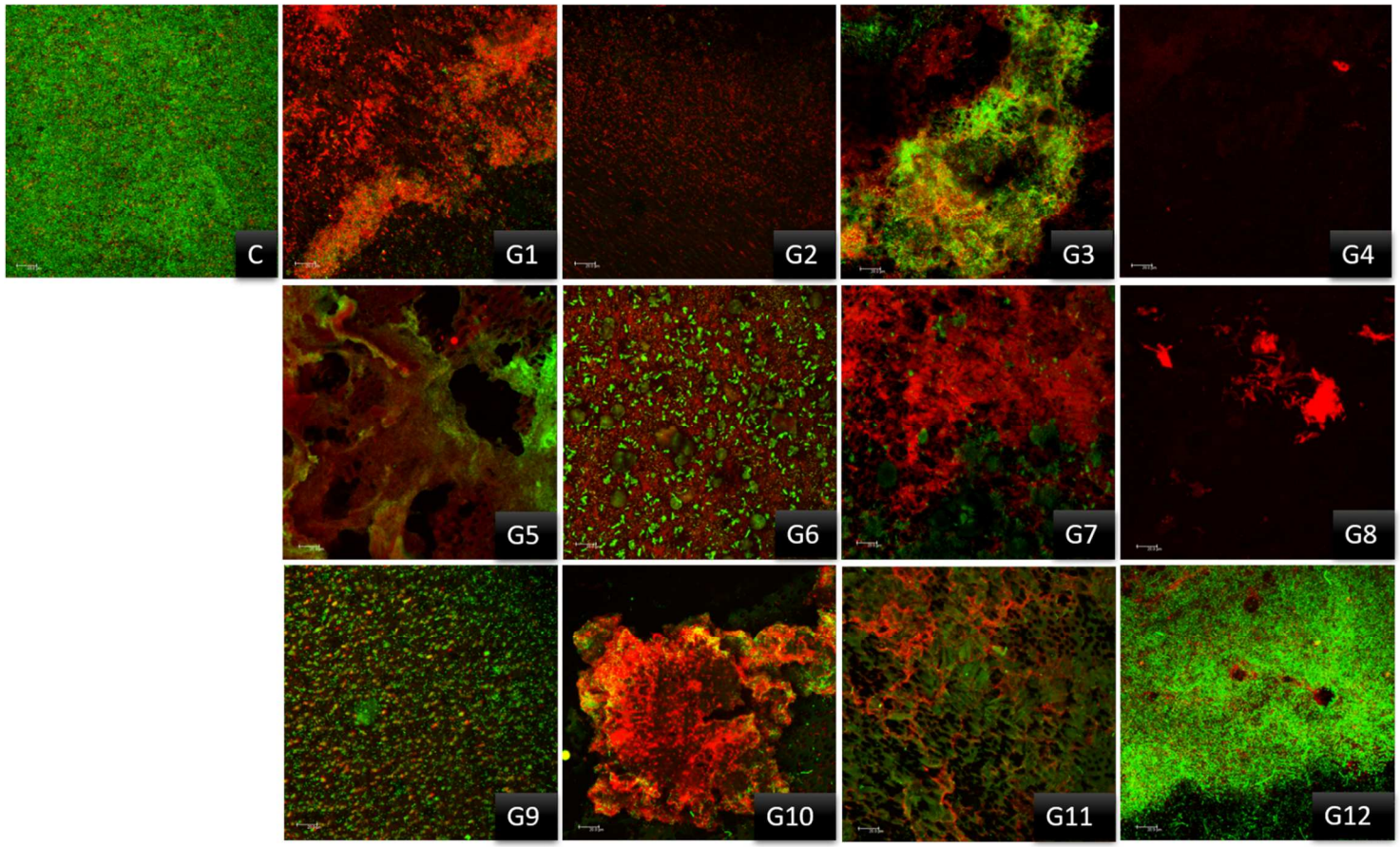

Figure 1. Confocal laser scanning microscopy of monospecies biofilm of *Enterococcus faecalis*, 21 days. (C) Control Group; (G1) 3 cycles of 20 seconds of agitation with 2.5% NaOCl (PUI); (G2) 6 cycles of 20 seconds of agitation with 2.5% NaOCl (PUI); (G3) 3 cycles of 20 seconds of agitation with EasyClean, with 2.5% NaOCl; (G4) 6 cycles of 20 seconds of agitation with EasyClean, with 2.5% NaOCl; (G5-G8) same protocols described above, but the 2.5% NaOCl was replaced by 2% chlorhexidine (CHX); (G9-G12) the 2.5% NaOCl was replaced by 0,9% saline. Live cells are indicated in green, and dead cells are indicated in red. Each picture represents an area of 275 × 275 µm.

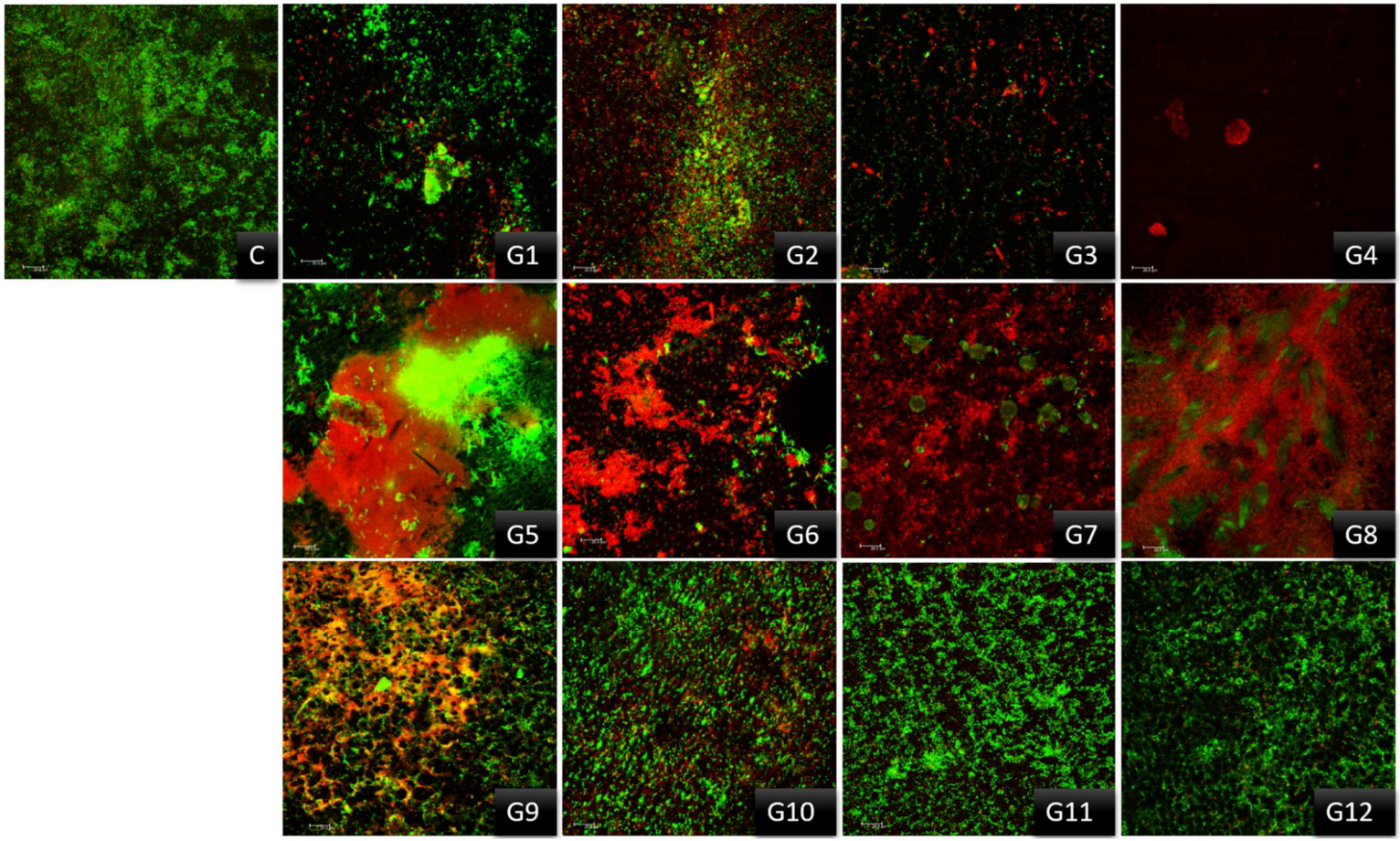

Figure 2. Confocal laser scanning microscopy of dual-species biofilm of *Enterococcus faecalis* and *Streptococcus oralis*, 21 days. (C) Control Group; (G1) 3 cycles of 20 seconds of agitation with 2.5% NaOCl (PUI); (G2) 6 cycles of 20 seconds of agitation with 2.5% NaOCl (PUI); (G3) 3 cycles of 20 seconds of agitation with EasyClean, with 2.5% NaOCl; (G4) 6 cycles of 20 seconds of agitation with EasyClean, with 2.5% NaOCl; (G5-G8) same protocols described above, but the 2.5% NaOCl was replaced by 2% chlorhexidine (CHX); (G9-G12) the 2.5% NaOCl was replaced by 0,9% saline. Live cells are indicated in green, and dead cells are indicated in red. Each picture represents an area of 275 × 275 µm.

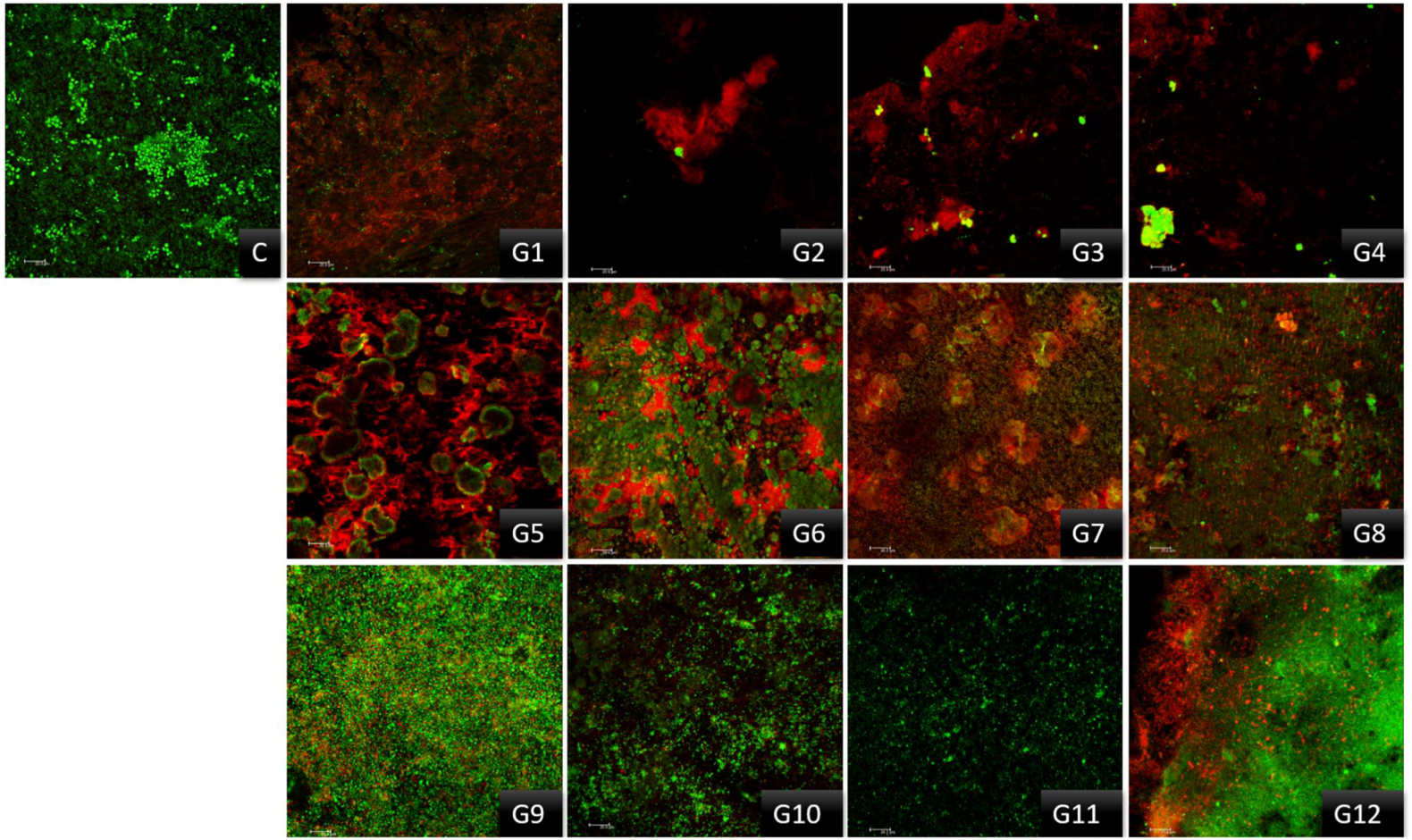

Figure3. Confocal laser scanning microscopy of dual-species biofilm of *Enterococcus faecalis* and *Candida albicans*, 19 days. (C) Control Group; (G1) 3 cycles of 20 seconds of agitation with 2.5% NaOCl (PUI); (G2) 6 cycles of 20 seconds of agitation with 2.5% NaOCl (PUI); (G3) 3 cycles of 20 seconds of agitation with EasyClean, with 2.5% NaOCl; (G4) 6 cycles of 20 seconds of agitation with EasyClean, with 2.5% NaOCl; (G5-G8) same protocols described above, but the 2.5% NaOCl was replaced by 2% chlorhexidine (CHX); (G9-G12) the 2.5% NaOCl was replaced by 0,9% saline. Live cells are indicated in green, and dead cells are indicated in red. Each picture represents an area of 275 × 275 µm.

Discussion

In this study, a root canal model was used in an attempt to reproduce the conditions in vivo, with dentin plus biofilm and irrigation in the root canals following protocols in a clinically representative manner. The outcome was the percentage of remaining biofilm assessed by laser scanning confocal microscopy. Studies have examined the efficacy of cleaning against the activation of irrigants in different devices, as part of the final irrigation procedure, reporting favorable disinfection (18). However, in our study we sought to activate the solutions in longer times than usually proposed on surfaces with one or two bacterial species and the association with yeasts. The results demonstrate that the association of the factors of time, chemical action and agitation of irrigants is necessary for microbial reduction. As there were differences between the protocols tested in the different biofilms, compared to the control group, the null hypothesis was rejected.

Passive ultrasonic irrigation (PUI) has been used to agitate irrigating solutions, through the use of an insert coupled to ultrasound. This method induces the formation of cavitation and acoustic waves, improving the cleaning capacity in root canal systems (19,20). In our results, the activation of the solutions by the PUI technique had the high capacity to remove biofilm in monospecies and dual bacterial species, regardless of the solution and agitation time. For yeast biofilms, the chemical effect of the associated solutions potentiated the action of this device. These results agree with studies that have shown that a final irrigation combining PUI removes more debris (21) and bacteria (22) when compared to conventional irrigation. The root canals of the present study were inoculated with C. albicans and E. faecalis for 19 days. However, this greater difficulty in removing dual species biofilm can be explained by the ability of C. albicans to adapt to severe conditions of survival, to have good surface adhesion and to form hyphae (23) that can penetrate deeply into the dentinal tubules and increase their pathogenesis (24). Similarly, E. faecalis tolerates disturbances in ecological conditions (such as lack of nutrients, oxygen tension and bacterial relationships) entering a viable state, capable of surviving, adapting and maintaining its pathogenicity and other biological functions (25). Thus, chemical action was also necessary to obtain an ideal disinfection. This result was already expected, since NaOCl has the ability to dissolve tissues and a disinfectant effect (26), while chlorhexidine digluconate (CHX), despite not dissolving organic matter, has a high antibacterial activity (27).

The other device used in our study, Easy Clean, a plastic instrument, size 25/04, is recommended for mechanical use in reciprocal (28) and rotational movement which has shown good results in cleaning lower molars and removing debris (29). In our results, Easy Clean had no effect with saline solution, on monofilm and dual bacterial species. Only against bacteria and yeasts, this device showed its mechanical effectiveness, when activated in 6 cycles in the group with saline solution. Laboratory studies that investigate the antimicrobial effects of different disinfection methods must use models that resemble in vivo conditions (30). A double dense bacterial species in vitro biofilm (E. faecalis and S. oralis) was used due to its strong collateral bonds that improve the cohesion and adhesion of the biofilm (16). In addition, an E. faecalis biofilm; E. faecalis and S. oralis of 21 days was formed based on a previous study that observed the formation of mature biofilms after this period of time (31), whereas the biofilm of E. faecalis and C. albicans was formed in a time of 19 days. In this case, the time of formation may have been a factor that influenced these results, since in these results an inert solution (sodium chloride) was used, being free of the chemical action by the irrigation and acting only the physical-mechanical action of the Easy Clean device, which possibly promoted a turbulence and agitation of the irrigating solution throughout the instrument (28). On the other hand, Easy Clean enhanced the action of NaOCl, when activated for 6 cycles, against E. faecalis and was more effective when agitation with chlorhexidine occurred, even in a shorter time (G7), compared to PUI NaOCl (G1) and PUI chlorhexidine (G5) in the biofilm of S. oralis and E. faecalis.

Studies report that inside the root canals because it is a highly complex system, the surface contact between irrigant-biofilm is limited, especially due to the morphological characteristics, such as isthmus and lateral canals and apical delta (32). In addition, it is not known whether the volume of irrigant present in the root canal contains, in the case of sodium hypochlorite, sufficient free chlorine to allow diffusion in the present biofilm. The effectiveness of chlorhexidine has already been reported in previous studies (33). Its antimicrobial activity is based on a positively charged molecule that interacts with negatively charged phosphate groups present in the bacterial cell wall, allowing the chlorhexidine molecule to penetrate the bacteria and leading to intracellular effects (34). Thus, these results are in line with other studies that confirm that in addition to the chemical action, the time, renewal and agitation of the solutions (14,22,32,35) are essential in cleaning mechanically inaccessible areas, reaching clinically acceptable levels.

Under the conditions of the present study, the protocols used were effective in the microbial reduction of mono and dual species biofilms. However, the association of chemical substances and agitation time play important roles in cleaning root canals.

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2.2 ARTICLE 2 - Fourier Transform Infrared Spectroscopy analysis of the dentin composition after treatment with different irrigation solutions associated or not with agitation methods

Abstract

Aim To evaluated the chemical changes on dentine surface, by Attenuated Total Reflectance in Fourier Transform Infrared Spectroscopy (ATR-FTIR), after submission to different irrigation protocols.

Methodology Dentine samples from bovine teeth were submitted to the following irrigation protocols (n=10): G1- 30 minutes with 2.5% sodium hypochlorite (NaOCl) without agitation; G2- 5 minutes of conventional irrigation and continuous aspiration with 2.5% NaOCl; G3- 3 cycles of 20 seconds of ultrasonic agitation with 2.5% NaOCl (PUI); G4- 3 cycles of 20 seconds of agitation with EasyClean, with 2.5% NaOCl; G5 to G8 received the same protocols described above, but the 2.5% NaOCl was replaced by 0.9% saline. The samples were analyzed by ATR-FTIR before and after treatments and the proportions of amide III/phosphate and carbonate/phosphate were determined. For intragroup comparison data were subjected to the Wilcoxon test ($\alpha < 0.05$) and for intergroup comparison to Kruskal-Wallis and Dunn tests ($\alpha < 0.05$).

Results The long contact time of the irrigant (30 min) intensified the dissolution of dentine collagen reducing the amide III/phosphate ratio in G1, showing significant differences to G5 and G6 ($P < 0.05$); also, G3 e G4 were different from G5 ($P < 0.05$). In the intragroup analysis, there were statistical differences in G1, G4 and G5 ($P < 0.05$). In the carbonate/phosphate ratio, no intergroup differences were observed ($P > 0.05$), only intragroup changes in the treatments of G6, G7, G8 ($P < 0.05$).

Conclusion The use of NaOCl for prolonged time or with agitation by continuous rotation causes greater deproteination of the organic matrix of dentin compared to other agitation methods and to saline solution. Some carbonate ions were removed from the inorganic phase of the dentine by the saline solution.

Introduction

The physical and chemical effects of irrigating solutions during biomechanical preparation are essential for cleaning and disinfection in areas of difficult access since the root canal systems have great anatomical complexity and microbial diversity (Peters *et al.* 2001, Villas-Boas *et al.* 2011).

Sodium hypochlorite (NaOCl) has become the most used irrigant in endodontic therapy due to its broad spectrum of antimicrobial activity and the ability to dissolve organic tissues (Siqueira *et al.* 2000, Stojicic *et al.* 2010). However, if it is accidentally injected into the periapical tissues, causes damage mainly by oxidation of proteins (Pashley *et al.* 1985, Hülsmann & Hahn 2000). Sodium chloride (saline) is an inert substance (Harrison, 1984).

Studies show that some solutions can modify the components of the organic matrix of dentin, especially collagen (Cohen *et al.* 1970), and decrease the amount of phosphate present in the root dentin (Tsuda *et al.* 1996), changing the capacity of adhesion and sealing of the endodontic sealers, which contributes considerably to the mechanical properties of the structure (Marending *et al.* 2007, Lisboa *et al.* 2013), or affect the interaction between some adhesive systems and dentin at the time of rehabilitation (Tulunoglu *et al.* 1998).

The literature highlights the importance of activation protocols to promote greater intracanal cleaning and disinfection (van der Sluis *et al.* 2006, Duque *et al.* 2017) in areas of difficult access. However, its necessary to understand if the undesirable modifications in the organic and inorganic components of the dentin are enhanced by the agitation of the solutions used to optimize the disinfection strategies during root canal irrigation.

The transformation of hard tissues samples in powder to perform some analysis leads to dehydration of the substrate. The infrared spectroscopy of biological samples of sliced hard tissues is of great importance because it allows to evaluate the chemical composition of these tissues in their natural form, without losing water (Bachmann *et al.* 2003). This technique showed to have also other advantages for the dentine analysis, such as, the simplicity and sensitivity, requirement of minimal sample preparation, and non-destructivity that allows the characterization of alterations after some treatments (Di Renzo *et al.* 2001a, 2001b, Zhang *et al.* 2010a, 2010b, Tartari *et al.* 2018).

However, the effect of the activation of different solutions in the organic and inorganic components of the dentine was not studied. This way, the objective of the present study was to evaluate, by Attenuated Total Reflectance of Fourier Transform Infrared Spectroscopy (ATR-FTIR), the chemical changes in the composition of the dentin surface promoted by 2.5% NaOCl and 0.9% saline at different times and irrigation protocols. The null hypothesis was that the tested irrigation protocols were not able to cause changes in the dentinal components.

Materials and Methods

Irrigation solutions

Concentrated (5.25%) NaOCl solution (Fórmula e Ação, São Paulo – SP - Brazil) was diluted in distilled water to produce a solution with 2.5% concentration that were confirmed by iodometric titration. The solutions obtained were stored, protected from the light in airtight plastic bottles in a refrigerator at 4°C, and removed one hour before the experiments to reach the room temperature. A 0.9% physiological saline solution was used as a control.

Samples preparation and initial analysis by ATR-FTIR

Dentin slices with approximately 0.8 mm thicknesses were cut from bovine crowns on the Isomet 1000 cutting machine (Buehler Ltd.; Lake Bluff, IL, USA), totaling one hundred and twenty samples (n = 10) with approximately 3 mm x 3 mm x 3 mm (length x width x thickness) according to the Tartari *et al.* 2016 methodology.

One surface of the dentin specimens was wet polished with 4000 grain silicon carbide abrasive papers (Buehler; Lake Bluff, IL, USA) and alpha alumina suspensions with 1, 0.3 and 0.05 microns (Struers; Ballerup, Denmark) until a flat and smooth surface was obtained. The samples were immersed in distilled water between the suspensions to remove any polishing residue. They were then dried with absorbent paper and the polished surface positioned on the diamond crystal that was the internal reflection element from the Fourier Transform Infrared (FTIR) Spectrometer Nicolet 380 (Thermo Fisher Scientific Inc.; Waltham, MA, USA) and the absorbance spectra were collected by the technique of Attenuated Total Reflection (ATR), between wavenumbers of 4000 and 400 cm^{-1} at resolution of 1 cm^{-1} using 32 scans and was recorded using the OMNIC Spectra Software (Tartari *et al.* 2018).

Root canal irrigation model

A root canal irrigation model was developed based on the Ordinola-Zapata *et al.* 2014 methodology, using bovine incisor roots. The crowns were removed, the root canals were standardized with 12 mm in length and apically enlarged with the 40.06 instrument (MK life, Porto Alegre, RS, Brazil). Subsequently, a drilling with 3.5 × 3.5 mm was performed 3 mm from the apical foramen.

The dentine specimens previously prepared and analysed were fixed and sealed at the drilling site with the polished side facing the root canal. As in the Ordinola-Zapata *et al.* 2014 methodology, this model allowed the adaptation of the dentin blocks at the apical area of the root canal of a bovine incisor tooth. Each root was used a maximum of five times. The samples were then distributed in the following groups, according to the irrigation protocols (n=10): G1, 30 minutes with 2.5% sodium hypochlorite without agitation; G2, 5 minutes of conventional irrigation and continuous aspiration with 2.5% sodium hypochlorite; G3, three passive ultrasonic agitation (PUI) of 20 seconds each with 2.5% sodium hypochlorite; G4, three agitations with EasyClean for 20 seconds each, with 2.5% sodium hypochlorite; the groups from G5 to G8 received the same protocols as above replacing NaOCl 2.5% by 0,9% saline.

In all groups, the blocks were fixed and 6 mL of the irrigants were injected into the roots with a Navitip 30G needle (Ultradent Products, Inc, South Jordan, Utah, USA) inserted 2 mm from the apex. For groups G1 and G5, the total volume was deposited within the roots in 30 minutes (1 mL every 5 minutes). For groups G2 and G6, 1.2ml of irrigant was deposited per minute. For groups G3, G4, G7 and G8, 2ml of irrigant was deposited previously to each 20 second cycle. For groups with ultrasonic agitation the Irrissonic (E1) 20/01 insert (Helse Dental Technology, Santa Rosa de Viterbo, São Paulo, Brazil) was used in an ultrasound unit PM100 EMS (E.M.S. Electro Medical Systems S.A., Nyon, Switzerland) at power 1 according to the manufacturer's recommendations. The agitation was conducted at 2 mm of the working length (WL) with vertical movements in the buccal-lingual directions. For groups in continuous rotation, the procedure was performed using the Easy Clean instrument size 25/04, passively inserted 2 mm from the WL coupled to the counter-angle and operated with a micromotor at approximately 20.000 rotations per minute (KaVo Kerr Group, Charlotte, NC).

After the irrigation, the samples were transferred to a microtube containing 1.5 mL of distilled water and rinsed for 1 min with 15 s of agitation in an ultrasonic tube.

They were then dried with absorbent paper and the ATR-FTIR spectra recorded again. A typical absorbance spectrum obtained from a disc of untreated dentin is shown in Figure 1.

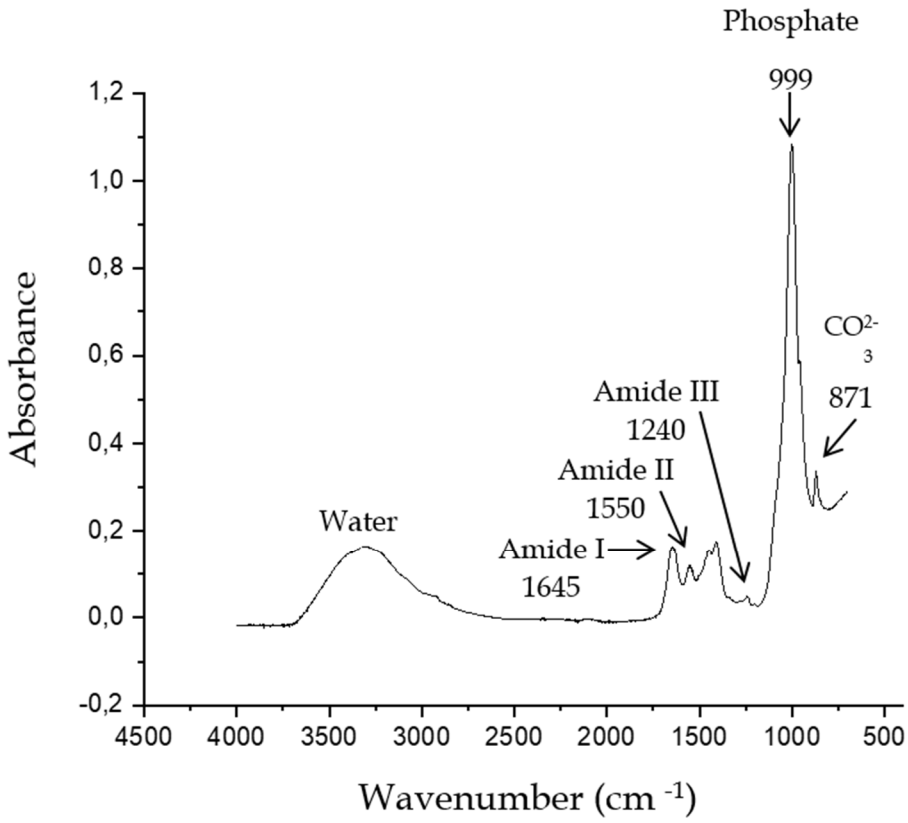

Figure 1 Absorbance spectrum of untreated dentin with the absorption peaks of the main dentin components

Statistical analysis

The previous analysis of the data did not show normal distribution, according to Shapiro-Wilk test. For intragroup comparison, data were submitted to the Wilcoxon test. The Kruskal-Wallis test with Dunn's test were used to detect intergroup differences. The value of $P < 0.05$ was considered significant.

Results

Table 1 presents the median and interquartile range values of amide III/phosphate ratio before and after the irrigation protocols. The irrigant contact time caused a greater collagen deproteination that resulted in a reduction in this ratio in G1, showing significant differences to G5 and G6 ($P < 0.05$); also G3 e G4 were different from G5 ($P < 0.05$). The intragroup comparison revealed that G1 and G4 caused the greatest

reductions in the proportion of amide III/phosphate. Otherwise, G5 caused the greatest increase in this ratio ($P < 0.05$).

Table 2 shows the results of dentin treatment in the proportion of carbonate/phosphate. When compared to the initial dentin composition, significant differences were observed only in G6, G7 and G8 ($P < 0.05$). No intergroup differences were observed ($P > 0.05$).

Table 1 Median (Med) and interquartile range (IQR) values for the ratio of amide/phosphate on dentin surface before and after the irrigation protocols and their reductions in percentages (%). The ratio values are multiplied by 10^{-3}.

Groups		**Before Med (IQR)**	**Post-treatment Med (IQR)**	**Alteration in amide/phosphate ratio (%) Med (IQR)**
Sodium hypochlorite	G1 – 30 min	9.46 (7.38 / 13.7)[a]	6.05 (5.01 / 9.90)[b]	-30.36[A] (-42.33 / -12.36)
	G2 – Conventional irrigation	11.33 (9.13 / 13.1)[a]	11.13 (10.1 / 13.1)[a]	0.86[ABCD] (-15.85 / 9.28)
	G3 – PUI	12.65 (10.1 / 14.0)[a]	10.05 (8.71 / 12.5)[a]	-11.12[ABC] (-14.77 / -8.42)
	G4 – Continuous Rotation	10.96 (9.86 / 13.4)[a]	8.96 (8.53 / 11.1)[b]	-14.42[AB] (-28.09 / -2.72)
Saline solution	G5 – 30 min	10.18 (7.56 / 12.4)[a]	12.06 (8.24 / 14.9)[b]	11.07[D] (1.08 / - 26.51)
	G6 – Conventional irrigation	13.21 (11.8 / 14.5)[a]	13.15 (11.5 / 16.0)[a]	2.88[BCD] (-9.32 / 11.68)
	G7 – PUI	12.56 (10.0 /15.3)[a]	11.01 (8.49 / 15.6)[a]	-0.07[ABCD] (-17.27 / 6.25)
	G8 – Continuous Rotation	10.47 (9.06 / 14.6)[a]	10.03 (9.54 / 13.4)[a]	-1.08[ABCD] (-8.10 / 3.21)

Different capital letters in the column indicate statistically significant intergroup differences (Kruskal-Wallis test and Dunn's post hoc, *P-value* < *0.05*). Different lower case letters in rows indicate statistically significant intragroup differences in the two periods evaluated (Wilcoxon text, *P-value* < *0.05*). Negative values in percentage means reduction in the amide III/phosphate ratio.

Table 2 Median (Med) and interquartile range (IQR) values for the ratio of carbonate/phosphate on dentin surface before and after the irrigation protocols and their reductions in percentages (%). The ratio values are multiplied by 10^{-3}.

Groups		Before Med (IQR)	Post-treatment Med (IQR)	Alteration in carbonate/phosphate ratio (%) Med (IQR)
Sodium hypochlorite	G1 – 30 min	23.5 (19.7 / 24.5)[a]	22.49 (19.7 / 23.6)[a]	0.75 (-5.73 / 3.51)[A]
	G2 – Conventional irrigation	22.49 (20.4 / 23.9)[a]	19.94 (18.8 / 23.2)[a]	-8.15 (-17.86 / 10.62)[A]
	G3 – PUI	20.16 (18.7 / 22.6)[a]	21.24 (18.3 / 22.9)[a]	-1.48 (-15.74 / 10.75)[A]
	G4 – Continuous Rotation	19.64 (18.0 / 22.3)[a]	18.3 (17.3 / 22.7)[a]	-7.03 (-17.25 / 4.42)[A]
Saline solution	G5 – 30 min	23.84 (19.9 / 25.1)[a]	21.73 (20.3 / 23.2)[a]	-2.45 (-9.33 / 3.18)[A]
	G6 – Conventional irrigation	21.09 (19.7 / 23.1)[a]	19.42 (17.2 / 21.6)[b]	-6.45 (-15.52 / -4.44)[A]
	G7 – PUI	22.12 (20.1 / 25.3)[a]	19.76 (18.7 / 24.5)[b]	-6.62 (-12.74 / -1.53)[A]
	G8 – Continuous Rotation	22.67 (21.2 / 23.6)[a]	19.89 (19.7 / 22.6)[b]	-7.76 (-15.73 / -1.81)[A]

Different capital letters letters in the column indicate statistically significant intergroup differences (Kruskal-Wallis test and Dunn's post hoc, *P-value< 0.05*). Different lower case letters in rows indicate statistically significant intragroup differences in the two periods evaluated (Wilcoxon text, *P-value < 0.05*). Negative values in percentage means reduction in the carbonate/phosphate ratio

Discussion

In the present study, changes in the chemical composition of dentin by different irrigation protocols with NaOCl and saline solutions were evaluated. This study demonstrates that NaOCl has the ability to deproteinate collagen from dentin in large quantities and, on the other hand, saline can cause a small reduction in the carbonate component in the inorganic portion of this structure when applied to an irrigation method. The null hypothesis tested must be rejected, since there were differences in the effects that irrigants have on the dentin composition throughout the treatments.

A wide range of methodologies has been used previously to determine changes in the chemical composition of dentin promoted by different concentrations of NaOCl over time. Although this experimental model has been used in previous investigations, significant efforts have been made in this study, in order to faithfully reproduce the conditions *in vivo*. The bovine incisor dentin has a structure similar to that of human molar dentin (Schilke *et al.* 2000) and reduces variables such as mineralization, age, caries influence, periodontal disease, donor ethnicity, etc. (Marshall *et al.* 1997), allowing a more standardized substrate for analysis. Furthermore, no differences were observed between the mineral matrix of the bovine and human tissues, only a change in intensity of the absorption bands (Bachmann *et al.* 2003).

Instead of being submerged or undergoing grinding process as recommended in other methodologies, in this study the dentin blocks were coupled to the bovine roots and the irrigants were deposited in the root canals following protocols in a clinically representative manner (Ordinola-Zapata *et al.* 2014, Ghorbanzadeh *et al.* 2016, Generali *et al.* 2018).

The organic matrix of dentin is basically formed by collagen in addition to other components in small quantities (Provenza 1964). According to the FTIR analysis, dentin treatment showed that NaOCl leads to collagen decreases time and agitation-dependent (Table 1). Although there were no statistical differences in G2 and G3, when the surface was exposed for 30 minutes (G1) or subjected to a protocol of continuous rotation (G5) with this irrigant, the removal of the organic phase of the mineralized dentin subsurface was considerably more severe. This lower amide III/phosphate ratio has also been observed in other studies (Zhang *et al.* 2010a, Zhang *et al.* 2010b), in which NaOCl concentrations caused a time-dependent linear increase in the removal of the organic phase from mineralized dentin. Collagen degeneration was also evident when NaOCl was used alone in studies with scanning electron microscopy (SEM) and

transmission electron microscopy (TEM), in which the authors observed displacement of the collagen surface and thinning of the fibrils (Wagner *et al*. 2017). The destruction of the collagen matrix of dentin should be investigated from a clinical point of view, as it can affect the adhesion of filling cements that chemically bind to dentin collagen (Alamoudi *et al.* 2019) and can interfere both in its mechanical strength and in the bonding strength in the adhesion of materials restorers (Wagner *et al.* 2017) and reduce fracture resistance of the tooth surface (Marending *et al*. 2007).

The prolonged treatment with NaOCl (G1) showed statistical differences from the G5 and G6 (saline solution), which was also reported by Atabek *et al.* 2014, applying the substances used in this study. On the other hand, a decrease in collagen by NaOCl in concentrations greater than 2.5% was also observed (Hu *et al.* 2010, Tartati *et al.* 2016) through the ATR-FTIR analysis. Ultrastructural changes in dentin collagen caused by NaOCl and, potentiated by PUI, promoting peritubular and intertubular erosion were observed (Wagner *et al.* 2017). It is believed that the ultrasonic activation of NaOCl increases its reaction rate by a synergistic effect of increasing the temperature, flow and cavitation of the irrigant (Macedo *et al.* 2010). However, although some aspects have been clarified, the exact mechanism of operation is still not fully understood (Macedo *et al.* 2010). In our results, there was no decrease in the amide III/phosphate ratio when the solutions were agitated by passive ultrasonic irrigation in the intragroup analysis. Collagen denaturation was greater when NaOCl was agitated with continuous rotation. The instrument easy clean has been proven to be an effective device for the cleaning of root canals (Duque *et al.* 2017). Kato *et al*. 2016 observed that the activation of irrigation with the Easy Clean system promoted a more efficient removal of dentin debris in the apical region of the root canals when compared to PUI. Probably the results obtained in this study are due to the mechanical action and the speed of rotation which produces turbulence of the irrigating solution throughout the instrument. No study was found in the literature that evaluated the effects of agitation of solutions by Easy Clean on the chemical composition of dentin that would allow comparison with the results of this study.

In the organic phase of dentin, had an increase in the amide/phosphate ratio when the saline solution was applied for 30 minutes. Although in the studies by Tartati *et al.* 2018, the saline solution also caused changes in the dentin surface, these changes were not enough to significantly change the carbonate/phosphate ratio as in our results. The apparent inconsistencies between these findings can be attributed to

variations in dentin substrates, as well as the time of irrigation and methods of application of irrigants. As for the dentin mineral matrices, these are mainly composed of carbonated hydroxyapatite crystals, being identified in the infrared absorption of hydroxyl ions, carbonate and phosphate radical (Bachmann *et al.* 2003). In the present study, a reduction in the carbonate/phosphate ratio occurred in the saline groups after application of irrigation methods (conventional, PUI and continuous rotation). The changes in NaOCl groups and saline solution used for 30 min were not significant. Hu *et al.* 2010 report that NaOCl with different concentrations and exposure time did not change the carbonate/phosphate ratios on dentin surfaces, indicating that NaOCl may not change the inorganic components in dentin. The reduction of the carbonate/phosphate ratio in the substrate immersed in saline solution promotes the formation of calcium phosphate crystals that contribute to an excellent cohesive bonding force on the substrate surface and can subsequently receive the filling material or dentin adhesive (Alamoudi *et al.* 2019).

Further studies are needed to understand the action of the irrigation solution agitation protocols on the dentin surface, however, the choice of solution, concentrations and application time is important to obtain the correct sanitization without compromising the quality of the dentin structure.

Conclusions

Based on the experimental methods and results, it can be concluded that the use of NaOCl for prolonged time or with continuous agitation causes a greater deproteination of the organic matrix of dentin, when compared to other agitation methods and saline solution agitated or not. Some carbonate ions are removed in the inorganic phase of the dentin by the saline solution. Thus, the choice of solution, concentrations and application time is important to obtain the correct sanitization without compromising the dentinal structure.

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2.3 ARTICLE 3 - Influence of contact time and activation of irrigation solutions on dentinal penetration: Optical Coherence Tomography analysis.

Abstract

Aim To evaluated the influence of contact time and activation of irrigants on dentinal penetration by Optical Coherence Tomography (OCT).

Methodology Dentine samples from bovine teeth were submitted to the following irrigation protocols (n=5): G1: 30 minutes in 2.5% sodium hypochlorite (NaOCl) without agitation; G2: 5 minutes of conventional irrigation and continuous aspiration with 2.5% NaOCl; G3: 3 cycles of 20 seconds of ultrasonic agitation with 2.5% NaOCl (PUI); G4: 3 cycles of 20 seconds of agitation with EasyClean, with 2.5% NaOCl; G5 to G8 received the same protocols described above, but the 2.5% NaOCl was replaced by 2% chlorhexidine (CHX); and from G9 to G12 the 2.5% NaOCl was replaced by 0,9% saline. Transverse images were obtained from the samples, before and after the irrigation protocols and the average depth of penetration (μ) was obtained. For intra-group comparisons, data were submitted to the t-paired test ($\alpha<0.05$) and for intergroup comparison to the one-way analysis of variance (ANOVA) with Tukey's multiple-comparison test ($\alpha<0.05$)

Results The diffusion values of irrigants in dentin reveal in the intragroup analysis that, except for conventional irrigation with saline, there was a significant diffusion of irrigants, through the tested protocols ($P < 0.05$). In the intergroup analysis, no significant differences were observed ($P > 0.05$).

Conclusion The agitation and contact time of the sodium hypochlorite, chlorhexidine or saline irrigating solutions and the longer contact time favor the penetration of these irrigants in the root dentin. OCT proved to be an efficient and non-invasive method to detect the diffusion of irrigants.

Introduction

The microorganisms that invade the pulp tissue, trigger the development of apical periodontitis, and are disseminated throughout the root canal system, including the dentinal tubules (Ricucci & Siqueira 2010) and other areas of difficult access to instrumentation. Current treatment strategies, therefore, focus mainly on reducing bacterial load to levels that achieve periradicular repair (Siqueira & Rôças 2008). However, 35-40% of the root canal walls can remain without mechanical preparation (Peters *et al.* 2001, Versiani *et al.* 2011, Versiani *et al.* 2013) and the greatest emphasis on treatment is on irrigating solutions. The depth of penetration of irrigants in areas of anatomical complexity is a desirable factor, as it can optimize the disinfection of the root canal system and the treatment prognosis (Zou *et al.* 2010, Solana *et al.* 2017)

Although the solutions considerably reduce the number of microorganisms on the surface of root dentin via conventional irrigation (CI), bacteria located deeper in the dentinal tubules often remain unchanged (Wong & Cheung 2013, Azim *et al.* 2016, Vatkar *et al.* 2016). A possible way to increase root canal disinfection is to activate the solutions, to increase the penetration of irrigants in areas of anatomical complexities, such as isthmus, apical deltas and dentinal tubules (Gulabivala *et al.* 2010).

Passive ultrasonic irrigation (PUI) has been applied to agitate irrigating solutions, through the use of an insert coupled to ultrasound. This method induces the formation of cavitation and acoustic waves, improving the cleaning capacity in root canal systems (van der Sluis *et al.* 2010, Guerreiro-Tanomaru *et al.* 2015). Studies have shown that a final irrigation combining PUI removes more debris (Urban *et al.* 2017) and bacteria (Gründling *et al.* 2011) when compared to conventional irrigation.

Another device recently launched and studied is Easy Clean, a plastic instrument, size 25/04, recommended for mechanical use in reciprocal movement which has shown good results in cleaning the walls of the mesial canal of lower molars (Kato *et al.* 2016). Its effectiveness in rotational movement, among devices that promote agitation of the irrigating solution, for cleaning and removing debris has also been reported (Duque *et al.* 2017, Cesario *et al.* 2018, Marques *et al.* 2018).

The penetration of irrigants by means of dentin blocks has already been evaluated by several methods, however, for the first time, we used optical coherence tomography to assess the dentin diffusion of irrigants. Optical coherence tomography (OCT) was first reported by Fujimoto *et al.* in 1991 (Huang *et al.* 1991). Clinically, OCT

systems have been widely used for ophthalmology (Hangai *et al.* 2007), dermatology (Pagnoni *et al.* 1999), gastroenterology (Poneros *et al.* 2001) and dentistry (Busanello *et al.* 2019). Ultrasonography, computed tomography and magnetic resonance are technologies with a significant impact on medical research and clinical practice, but new tools are needed for scientific research (Le *et al.* 2010). The advantages of OCT stand out because it is a non-invasive, non-contact technique, using close infrared light (ie, without ionizing radiation) and reaching a high axial resonance, in the order of 10 µm with real-time images (Azevedo *et al.* 2011).

Currently, studies compare the concentration and time of action of irrigants in relation to agitation techniques and few report directly the diffusion of these substances in dentin. Thus, the main objective of this study was to evaluate the influence of contact time and the activation of 2.5% sodium hypochlorite, 2% chlorhexidine and 0.9% saline in dentin diffusion, in a root canal irrigation model, using optical coherence tomography (OCT). The null hypothesis tested was that the irrigation protocols tested with the different irrigators did not show differences in the capacity of dental penetration.

Materials and Methods

Irrigation solutions

Concentrated (5.25%) NaOCl solution (Fórmula e Ação, São Paulo – SP - Brazil) was diluted in distilled water to produce a solution with 2.5% concentration that were confirmed by iodometric titration. The solutions obtained were stored, protected from the light in airtight plastic bottles in a refrigerator at 4°C, and removed one hour before the experiments to reach the room temperature. A 2% chlorhexidine digluconate (Biodinâmica, Ibiporã – PR – Brazil) solution was also used for the experiments and a 0.9% physiological saline solution was used as a control.

Samples preparation and initial analysis by optical coherence tomography

Dentin slices with approximately 0.8 mm thicknesses were cut from bovine crowns on the Isomet 1000 cutting machine (Buehler Ltd.; Lake Bluff, IL, USA), totaling one hundred and twenty samples (n = 10) with approximately 3 mm x 3 mm x 3 mm (length x width x thickness) according to the Tartari *et al.* 2016 methodology.

One surface of the dentin specimens was wet polished with 4000 grain silicon carbide abrasive papers (Buehler; Lake Bluff, IL, USA) and alpha alumina suspensions with 1, 0.3 and 0.05 microns (Struers; Ballerup, Denmark) until a flat and smooth

surface was obtained. The samples were immersed in distilled water between the suspensions to remove any polishing residue. They were then dried with absorbent paper and the polished surface positioned inside a petri dish, fixed on an XY translator and covered with distilled water.

The structural characterization of the samples was performed using a OCT-OCS1300SS (Thorlabs, New Jersey, USA), which uses a superluminescent light emitting diode (SLED) operating at 930 nm, power of 2 mW and resolution of 6,2 μm, capable of presenting up to 8 images per second. The maximum depth of the image is 1.6 mm, the maximum lateral scan is 6.0 mm (Monteiro *et al.* 2011). In the most common OCT systems, a Michelson interferometer is used to provide a cross-sectional image of the dispersed samples, with non-invasive evaluation and without contact with their surfaces. In this system, the light is guided by an optical fiber to a 2 × 2 coupler, where the light is divided into 2 beams, one of which is sent to a reference mirror and the other to the sample. The scattering of light is measured due to the proportional relationship between the reduction in light penetration and the density and thickness of the specimen (Lambert-Beer Law), and results in layers of different gray scale densities within the sample according to the protocol of treatment used.

Root canal irrigation model

A root canal irrigation model was developed based on the Ordinola-Zapata *et al.* 2014 methodology, using bovine incisor roots. The crowns were removed, the root canals were standardized with 12 mm in length and apically enlarged with the 40.06 instrument (MK life, Porto Alegre, RS, Brazil). Subsequently, a drilling with 3.5 × 3.5 mm was performed 3 mm from the apical foramen.

The dentine specimens previously prepared and analysed were fixed and sealed at the drilling site with the polished side facing the root canal. As in the Ordinola-Zapata *et al.* 2014 methodology, this model allowed the adaptation of the dentin blocks at the apical area of the root canal of a bovine incisor tooth. Each root was used a maximum of five times. The samples were distributed in the following groups, according to the irrigation protocols (n=5): G1, 30 minutes in 2.5% sodium hypochlorite without agitation; G2, 5 minutes of conventional irrigation and continuous aspiration with 2.5% sodium hypochlorite; G3, three ultrasonic agitation (PUI) 20 seconds each, with 2.5% sodium hypochlorite; G4, 3 agitations with EasyClean 25/04 20 seconds

each, with 2.5% sodium hypochlorite; G5 a G8 received the same protocol as above replacing NaOCl 2,5% by CHX 2%; e G9 a G12 by 0,9% saline.

In all groups, the blocks were fixed and the irrigants injected into the roots. The same volume of irrigant was used for all treatments (6mL). The irrigating solutions were applied with a Navitip 30G needle (Ultradent Products, Inc, South Jordan, Utah, USA) inserted 2 mm from the apex, in all groups. For groups with ultrasonic agitation the Irrissonic (E1) 20/01 insert (Helse Dental Technology, Santa Rosa de Viterbo, São Paulo, Brazil) was used in an ultrasound unit PM100 EMS (E.M.S. Electro Medical Systems S.A., Nyon, Switzerland) at power 1 according to the manufacturer's recommendations. The agitation was conducted at 2 mm of the working length (WL) with vertical movements in the buccal-lingual directions. For groups in continuous rotation, the procedure was performed using the Easy Clean instrument size 25/04, passively inserted 2 mm from the WL coupled to the counter-angle and operated with a micromotor at approximately 20.000 rotations per minute (KaVo Kerr Group, Charlotte, NC).

After the irrigation, the samples were transferred to a microtube containing 1.5 mL of distilled water and rinsed for 1 min with 15 s of ultrasonic agitation.

Transverse images were obtained in OCT, before and after the irrigation protocols, from the beginning of one of the sample margins to the other, totaling 800 frames in each reading. This procedure provided a complete internal mapping. Three fields, starting from the center of the sample, were initially selected by the MatLab R219a software. In all images the interface of the polished surface was not measured and the same region was defined for all samples (22μ depth), to analyze the displacement that occurs in the gray scale level after the application of the irrigant. In this way, the depth of penetration into the dentin structure can be visualized and measured. The value of the average intensity of diffusion was measured using the software Zen 2.3 lite, to define the corresponding area (μ) of reflection of these irrigators, in the different protocols.

Statistical analysis

The collected data showed normal distribution and were submitted to the one-way analysis of variance (ANOVA) with Tukey's multiple-comparison test ($\alpha<0.05$) to detect differences between the groups. For intra-group comparisons, data were submitted to the t-paired test ($\alpha<0.05$). The value of $P < 0.05$ was considered significant.

Results

Table 1 shows the mean and standard deviation of the diffusion values of irrigants by optical coherence tomography before and after the irrigation protocols. Except for the G10 group, there was a significant diffusion of irrigants through the dentin sample, through the tested protocols ($P<0.05$). In the intergroup analysis, no significant differences were observed ($P> 0.05$). The representative images can be seen in Figure 1.

Table 1 Mean and standard deviation and P values (paired t-test) for optical coherence tomography analysis on dentin surface before and after the irrigation protocols.

Groups		**Before** **X ± SD**	**Post-treatment** **X ± SD**	***P value*** **(paired t-test)**
Sodium hypochlorite	G1 – 30 min	62,0± 16,1^{Aa}	105,4±25,5^{Ba}	= 0,006
	G2 – Conventional irrigation	57,3±11,6^{Aa}	89,5±8,8^{Ba}	= 0,007
	G3 - PUI	72,1±23,1^{Aa}	88,3±14,2^{Ba}	= 0,029
	G4 – Continuous Rotation	61,6±22,8^{Aa}	81,3±11,1^{Ba}	= 0,042
Chlorhexidine	G5 – 30 min	60,8±22,7^{Aa}	76,7±15,7^{Ba}	= 0,028
	G6 – Conventional irrigation	67,7±15,9^{Aa}	80,3±22,6^{Ba}	= 0,031
	G7 - PUI	68,4±17,5^{Aa}	98,0±26,5^{Ba}	= 0,034
	G8 – Continuous Rotation	53,5±10,9^{Aa}	86,9±26,5^{Ba}	= 0,032
Saline solution	G9 – 30 min	57,7±16,7^{Aa}	89,5±7,1^{Ba}	= 0,008
	G10 – Conventional irrigation	62,1±16,5^{Aa}	78,5±24,5^{Aa}	= 0,233
	G11 - PUI	89,1±17,8^{Aa}	111,8±23,2^{Ba}	= 0,043
	G12 – Continuous Rotation	57,6±19,9^{Aa}	84,0±5,0^{Ba}	= 0,028

Same lower case letters in the column indicate the absence of statistical difference intergroup (*P-value* >0.05). Different capital letters in rows indicate statistically significant intragroup differences in the two periods evaluated (*P-value* <0.05).

Figure 1 Structural characterization by optical coherence tomography of untreated and treated dental surfaces treated according to the protocols: G1: 30 minutes in 2.5% sodium hypochlorite (NaOCl) without agitation; G2: 5 minutes of conventional irrigation and continuous aspiration with 2.5% NaOCl; G3: 3 cycles of 20 seconds of ultrasonic agitation with 2.5% NaOCl (PUI); G4: 3 cycles of 20 seconds of agitation with EasyClean, with 2.5% NaOCl; G5 to G8 received the same protocols described above, but the 2.5% NaOCl was replaced by 2% chlorhexidine (CHX); and from G9 to G12 the 2.5% NaOCl was replaced by 0,9% saline.

***Legend:* NT - Untreated sample; T: Sample treated

Discussion

In this study, many precautions were taken to reproduce the conditions in vivo: the bovine dentin used has a structure similar to that of human dentin (Schilke *et al.* 2000) allowing a more standardized substrate for the analysis, the irrigants were deposited in the root canals in order to reproduce the dynamics and impact in the apical thirds, the apical foramina were sealed to create a closed environment and the root canals were instrumented up to a diameter of 40.06, since smaller dimensions can reduce the efficiency of irrigations (van der sluis *et al.* 2005).

Since the substrate obtained artificially will be used in future laboratory tests, the diffusion of irrigants must be measured by a non-destructive method. The present study is the first report in which the penetration of substances into dentin was assessed using optical coherence tomography. The OCT is a non-invasive technique and spatial resolution can be as high as 10 times that obtained by the ultrasound technique (Fujimoto *et al.* 1995). Data acquisition in OCT is based on lowcoherence laser interference principles (Otis *et al.* 2000), and the scanning of the light beam generates a backscatter signal according to the sample depth. Through this method, this study demonstrates that the applied protocols substantially improve the diffusion of irrigants on the dentin surface, with the exception of conventional irrigation with 0.9% saline solution. However, there were no significant differences between the systems involved for each protocol, therefore, the null hypothesis was accepted.

Irrigating solutions are necessary to assist disinfection in areas that are out of reach of the instruments, since the mechanical action does not have the ability to remove bacteria in their entirety in the root canal. Long-lasting microorganisms have been found at depths of up to 420 μm in human dentin (Kakoli *et al.* 2009), such as *Enterococcus faecalis* which is capable of extending up to 500 μm (Haapasalo & Ørstavik 1987) and may contribute to the persistence of periradicular disease (Siqueira & Rôças 2008). Therefore, clinically representative models can better inform effective strategies for endodontic disinfection and currently different activation techniques are recommended to promote the efficiency of irrigating solutions.

Although no statistical differences were observed between groups in our results, the use of agitation protocols is recommended in relation to conventional irrigation in clinical practice, as it improves the penetrability of irrigants in dentinal tubules and other areas of anatomical complexity, reducing toxic products and substrates necessary for bacterial growth on inaccessible and un-instrumented surfaces (Mohmmed *et al.*

2018). This can be explained by the shear stresses and the hydrodynamic pressures generated in the irrigators during the PUI that are significantly higher and more evenly distributed (Chen *et al.* 2014). Duque et al. 2017 observed that the same methods of activating the irrigating solution used in our study, provided better cleaning of the canal and isthmus when compared to conventional irrigation.

As for the low diffusion of saline solution through conventional irrigation, it can be explained by the high surface tension (54.0 dyne / cm) of this substance compared to the other substances. On the other hand, Munoz & Camacho - Cuadra (2012) demonstrated that irrigants administered through conventional syringes passively reflect towards the pulp chamber, thus limiting their potential for penetration. The use of agitation techniques, therefore, promotes disinfection of the entire root canal system during treatment.

In our study, we investigated the most commonly used irrigation regimens and inserted a prolonged time of contact with the dentin to evaluate this diffusion among the different solutions. Despite not showing differences between themselves, in 30 minutes of contact without intervention, all substances show considerable diffusion rates when penetrating bovine dentin. Even in the face of methodological variations, these trends are consistent with the findings of other studies that observed that, shaking irrigants, as well as promoting longer contact times, enhances penetration into root dentin (Virdee *et al.* 2020). Zou *et al.* 2010, demonstrated that temperature, time and viscosity are variables that affect the depth of penetration in the dentinal tubules. On the other hand, in this methodological protocol the sample was evaluated without the presence of interference, unlike the complex processes that occur inside the root canals, since in the presence of the structure of a biofilm there is an influence on the chemical efficacy and physical action of endodontic irrigators (Busanello *et al.* 2018).

Thus, the inability of instruments driven by anatomical difficulties in debridling the entire root canal system (Peters *et al.* 2001) justifies the need to design effective irrigation regimes.

Conclusion

In conclusion, an OCT proved to be an efficient and non-invasive method to detect the diffusion of irrigants in view of the irrigation protocols tested in bovine dentin. No significant differences were observed in the penetration of sodium hypochlorite 2.5%, chlorhexidine 2% and saline 0.9% when in contact with dentin for a prolonged time, or

by conventional irrigation, PUI or combined with Easy Clean. However, communicators improve data on the diffusion of irrigants on the dentin surface, with the exception of conventional irrigation with 0.9% saline solution.

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2.4 ARTICLE 4 - Effects of reducing agents on birefringence dentin collagen and microhardness after use of different irrigation regimens

Abstract

This study evaluate whether different irrigation procotols alter the dentin ultrastructure, by analyzing microhardness (n=12) and microscopy of polarized light and *Picrosirius Red* staining (n=12). Dentine samples from bovine teeth were submitted to the following irrigation protocols: G1: 30 minutes in 2.5% sodium hypochlorite (NaOCl) without agitation; G2: 5 minutes of conventional irrigation and continuous aspiration with 2.5% NaOCl; G3: 3 cycles of 20 seconds of ultrasonic agitation with 2.5% NaOCl (PUI); G4: 3 cycles of 20 seconds of agitation with EasyClean, with 2.5% NaOCl; G5 to G8 received the same protocols described above, but the 2.5% NaOCl was replaced by 2% chlorhexidine (CHX); and from G9 to G12 the 2.5% NaOCl was replaced by 0,9% saline. Paired t test and Kruskal-Wallis tests were used to analyze the results with a significance level set at 5%. It was observed that, except in group G9, the irrigation regimes tested did not reveal statistically significant differences from their initial values of microhardness ($p > .05$) and no intergroup differences were observed ($p > .05$). All groups showed birefringence for greenish, yellow and red fibers, demonstrating the maturation dynamics of the matrix Considering the total fibers, no statistically significant difference was detected between the groups. It can be concluded that the irrigation regimes applied in this study are not capable of altering dentinal hardness. The solutions when in contact with the dentin for a long time, can cause a disorganization in the fibrillar network or modifies the morphological structure of the dentin substrate.

Introduction

Irrigation protocols play an important role in modeling and sanitizing root canal systems, being essential for the success of endodontic treatment.[1] The use of irrigation solutions agitation devices, such as ultrasound[2,3] or continuous rotation devices,[4] has been widely used, as they spread these substances inside the root canals, reaching areas previously untouched during biomechanical preparation.[5]

Different auxiliary chemical agents have been proposed, however, sodium hypochlorite (NaOCl) is still the most used irrigating solution in endodontics, due to its property of dissolving organic tissues,[6] while chlorhexidine diguclonate (CHX) has been used due to its broad antimicrobial capacity and low toxicity7, despite not having solvent action of necrotic tissues.[7]

However, if on the one hand these substances have the ability to act causing desired effects, they can modify the structure of dentin, changing the organic matrices, such as collagen.[8] This effect can influence the bond strength of endodontic cements or restorative materials, putting at risk the quality and durability of direct restorations and the cementation of fiber posts[9] or result in a less resistant and more brittle substrate, affecting fracture resistance,[10] a since reaching the inorganic structure of dentin, they can cause changes in dentin microhardness.[11]

Tartari et al.[12] observed that the increase in the exposure time and in the concentration of NaOCl solution leads to an increase in the deprotection of the dentin collagen. Moreira et al.[13] evaluated the structure of bovine dentin after irrigation with 5.25% NaOCl and 2% chlorhexidine gel, by polarized light microscopy and staining with Picrosirius Red, and concluded that the second group did not promote changes in the morphological structure of the organic matrix of dentin, for having uniformity in its fibrillar network similar to that of the control group. However, results on possible changes in root dentin after the use of these substances are not fully elucidated. In addition, it is not known exactly which is the ideal protocol in which the chemical and disinfectant action of the irrigating solutions in contact with the dentin has to obtain desirable properties without causing damage to it.

Thus, understanding the relationship of contact time, chemical action and agitation of solutions is essential to optimize disinfection strategies during root canal irrigation and to clarify whether there are major undesirable changes in the chemical composition of dentin. Thus, the objective of the present study was to evaluate whether different irrigation procotols with 2.5% sodium hypochlorite, 2% chlorhexidine and

0.9% saline alter the dentin ultrastructure, by analyzing microhardness and microscopy of polarized light and *Picrosirius Red* staining. The null hypothesis tested was that the tested irrigation regimes were not able to cause changes in the dentinal matrix.

Materials and Methods

Irrigation solutions

Concentrated (5.25%) NaOCl solution (Fórmula e Ação, São Paulo – SP - Brazil) was diluted in distilled water to produce solution with 2.5% concentration that were confirmed by iodometric titration. The solutions obtained were stored, protected from the light in airtight plastic bottles in a refrigerator at 4°C, and removed one hour before the experiments to reach room temperature. A 2% chlorhexidine digluconate (Biodinâmica, Ibiporã – PR – Brazil) solution was also used for the experiments and a 0.9% physiological saline solution was used as a control.

Samples preparation and initial analysis by Microhardness and Picrosirius red

Dentin blocks from bovine central incisors with fully developed roots were obtained using trephine drills 4.0 mm in diameter under abundant irrigation. For *Picrosirius red* polarization method, no polishing was performed on the samples. For microhardness, surface was ground flat using 600-grit, 800 and 1,200-grit SiC (Extec Corp., Enfield, CT, USA) paper under running water (Politriz APL-4, Arotec, Cotia, SP, Brazil). Between each polishing cycle, blocks were submitted to ultrasound cleaning (Ultrasonic Cleaner Mod. USC 750; Unique Ind. e Com. de Produtos Eletrônicos Ltda., São Paulo, SP, Brazil), for 2 min. In the end, the samples were polished with felt paper (Extec Corp., CT, USA) and diamond suspension 1µ (ERIOS Equipamentos Eireli, São Paulo, SP, Brazil).

The microhardness values of untreated samples were recorded using Knoop indentations (Shimadzu Corporation, Tokyo, Japan) with either 25g loads, for 15s. Starting from the center of the sample, three indentations were made along the lumen of the root canal, following a straight line towards the other. The average of these values was used to compare changes in microhardness before and after irrigation protocols. For this, the specimens were randomly distributed in twelve groups mentioned above (N=12).

Root canal irrigation model

A root canal irrigation model was developed based on the Ordinola-Zapata et al.[14] methodology, using bovine incisor roots. The crowns were removed, the root canals were standardized with 12 mm in length and apically enlarged with the 40.06 instrument (MK life, Porto Alegre, RS, Brazil). Subsequently, a drilling was performed with a 3.0 mm drill trephine at 3 mm from the apical foramen to adjust the dentin blocks.

The specimens were fixed and sealed at the drilling site with the treated side facing the root canal, as in the Ordinola-Zapata et al.[14] methodology, allows the adaptation of the treated area of the dentin block at the same level as the apical area of the root canal of a bovine incisor tooth. Each root was used a maximum of five times. For the irrigation protocols, the groups were divided as follows: G3, three ultrasonic agitation (PUI) 20 seconds each, with 2.5% sodium hypochlorite; G4, three agitations with EasyClean 25/04 20 seconds each, with 2.5% sodium hypochlorite; G5 a G8 received the same protocol as above replacing NaOCl 2,5% by CHX 2%; e G9 a G12 by 0,9% saline.

In all groups, the blocks were fixed and the irrigants injected into the roots. The same volume of irrigant was used for all treatments (6mL). The irrigating solutions were applied with a Navitip 30G needle (Ultradent Products, Inc, South Jordan, Utah, USA) inserted 2 mm from the apex, in all groups. For groups with ultrasonic agitation the Irrissonic (E1) 20/01 insert (Helse Dental Technology, Santa Rosa de Viterbo, São Paulo, Brazil) was used in an ultrasound unit PM100 EMS (E.M.S. Electro Medical Systems S.A., Nyon, Switzerland) at power 1 according to the manufacturer's recommendations. The agitation was conducted at 2 mm of the working length (WL) with vertical movements in the buccal-lingual directions. For groups in continuous rotation, the procedure was performed using the Easy Clean instrument size 25/04, passively inserted 2 mm from the WL coupled to the counter-angle and operated with a micromotor at approximately 20.000 rotations per minute (KaVo Kerr Group, Charlotte, NC).

To avoid the prolonged effect of the solutions, the samples received 40mL for 1 min of distilled water in an ultrasound vat. The post-treatment indentations were performed again and in the same way, in each sample. The final microhardness values were then recorded.

Picrosirius red polarization method

After treatment, circular samples (N=12) were immediately fixed in 10% buffered formalin. Then, were washed in tap water for 24h and immersed in 10% ethylenediaminetetraacetic acid (EDTA) for processing in paraffin and 4 µm thick cuts. To make possible the identification and analysis of collagen amount and quality by the birefringence of its fiber bundles organization, histological slices were stained with *Picrosirius-red.* According to the organization of collagen fiber orientation, the color of the birefringence spectrum can vary from yellow-green (immature and thin fibers) to yellow-red (mature and thick fibers), thus reflecting the thickness and packaging of the fibers.[15] Specifically, when the Picrosirius red tissue is under polarized light, only collagen fibers appear in the birefringence field, while other components of the tissue remain on the black background and are excluded from this analysis. Four central fields of the samples were analyzed under a polarized light microscope with ×20 magnification. The intensity of birefringence from greenish to yellow, green, and red color collagen fibers was measured using ImageJ (version 1.36) software to define the corresponding area (pixel^2) of these fibers, as well as total birefringent fibers.[16,17]

Statistical analysis

The previous analysis of the data did not show normal distributions (Shapiro - Wilk). For intra-group comparisons, data were submitted to the t-paired test for microhardness ($p < .05$). To detect differences between the groups was performed the Kruskal - Wallis and Dunn multiple comparison test ($p < .05$) for microhardness and *Picrosirius Red*. The significance level was set at 5%.

Results

The mean and standard deviation in microhardness in the different experimental groups, before and after treatments, are shown in table 1. It was observed that, except in group G9, the irrigation regimes tested did not reveal statistically significant differences from their initial values of microhardness ($p > .05$). The G9 group showed an increase in the microhardness value after treatment, compared to the initial value ($p < .05$). No intergroup differences were observed ($p > .05$).

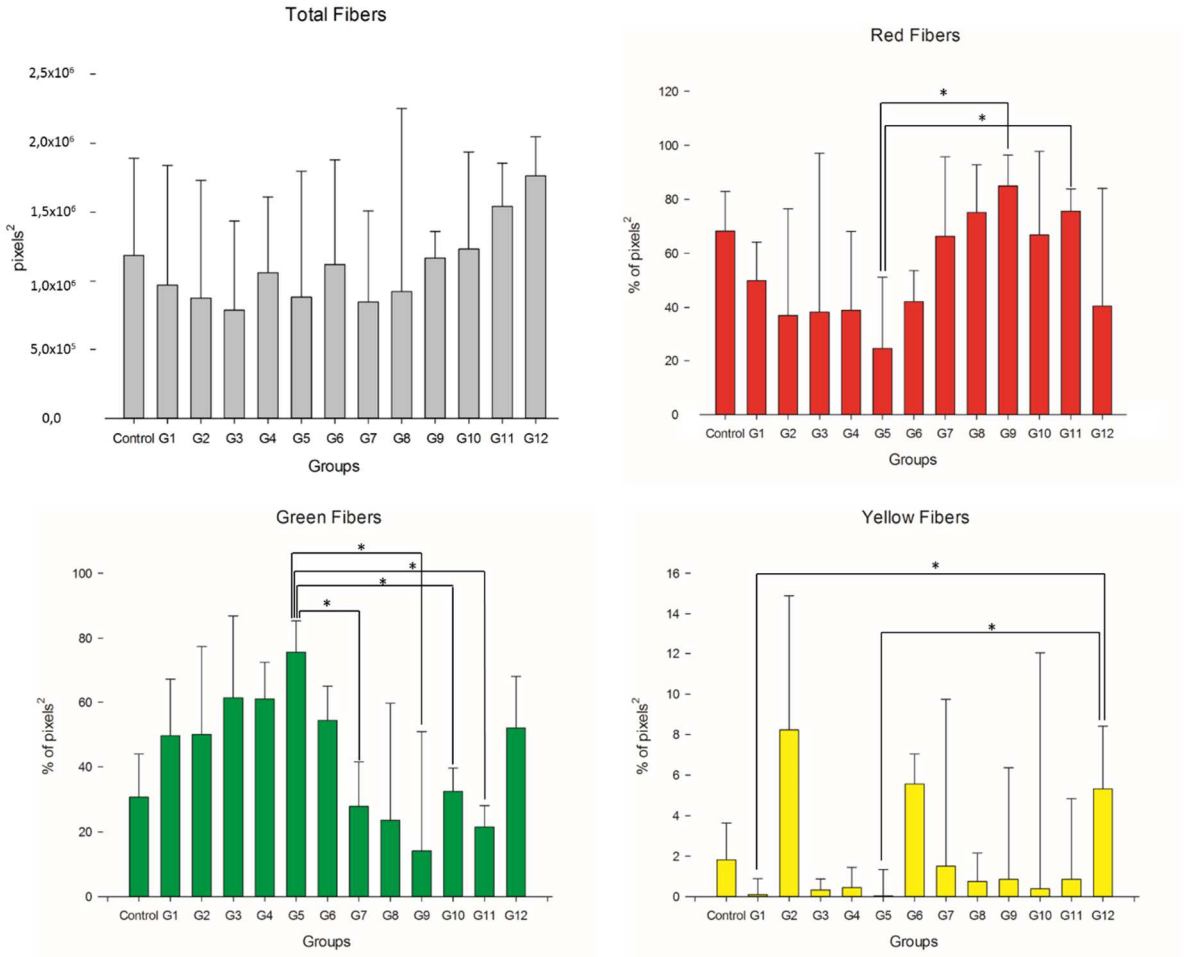
The results of *Picrosirius Red* analysis are presented in Graphic 1. All groups showed birefringence for greenish, yellow and red fibers, demonstrating the maturation dynamics of the matrix (Figure 1). Considering the total fibers, no statistically significant

difference was detected between the groups. In the yellow fibers, although the groups showed a reduced amount of these fibers, there was a statistical difference between G1 and G5 with G12 ($p < .05$). It was also detected in the red fibers, a statistical difference between G5 with G9 and G11 ($p < .05$). In the green fibers, only the G5 group showed a statistical difference with the G7, G9, G10 and G11 groups ($p < .05$).

Table 1 Mean and standard deviation and P values (paired t-test) for microhardness analysis on dentin surface before and after the irrigation protocols.

Groups		**Before** **X ± SD**	**Post-treatment** **X ± SD**	$P\ value$ (paired t-test)
Sodium hypochlorite	G1 – 30 min	40.3 ± 7.8^{Aa}	35.0 ± 9.5^{Aa}	= 0,176
	G2 – Conventional irrigation	42.2 ± 6.6^{Aa}	47,7 ± 13.7^{Aa}	= 0,175
	G3 - PUI	41.1 ± 7.1^{Aa}	41.7 ± 8.9^{Aa}	= 0,873
	G4 – Continuous Rotation	41.1 ± 6.3^{Aa}	45.9 ± 11.9^{Aa}	= 0,237
Chlorhexidine	G5 – 30 min	41.5 ± 7.3^{Aa}	37.8 ± 12.0^{Aa}	= 0,505
	G6 – Conventional irrigation	41.5 ± 6.6^{Aa}	40.4 ± 11.3^{Aa}	= 0,722
	G7 - PUI	40.0 ± 4.8^{Aa}	43.3 ± 9.3^{Aa}	= 0,215
	G8 – Continuous Rotation	44.5 ± 12.4^{Aa}	39.4 ± 6.0^{Aa}	= 0,193
Saline solution	G9 – 30 min	40.9 ± 6.5^{Aa}	45.4 ± 9.0^{Ba}	= 0,012
	G10 – Conventional irrigation	41.1 ± 5.7^{Aa}	36.6 ± 5.3^{Aa}	= 0,053
	G11 - PUI	41.8 ± 6.3^{Aa}	46.2 ± 11.5^{Aa}	= 0,251
	G12 – Continuous Rotation	41.7 ± 5.7^{Aa}	38.5 ± 16.4^{Aa}	= 0,532

Same lower case letters in the column indicate the absence of statistical difference intergroup (*P-value >0.05*). Different capital letters in rows indicate statistically significant intragroup differences in the two periods evaluated (*P-value <0.05*).



Graphic 1. Quantification of collagen fiber bundles by *Picrosirius*-polarization method for protocols irrigation. Intensity of birefringence measured from total area of collagen fibers, as well as greenish, yellow and red collagen fibers. Results are presented as median of pixels^2. Symbols (asterisk) indicate significant statistical difference ($P < 0.05$) between groups.

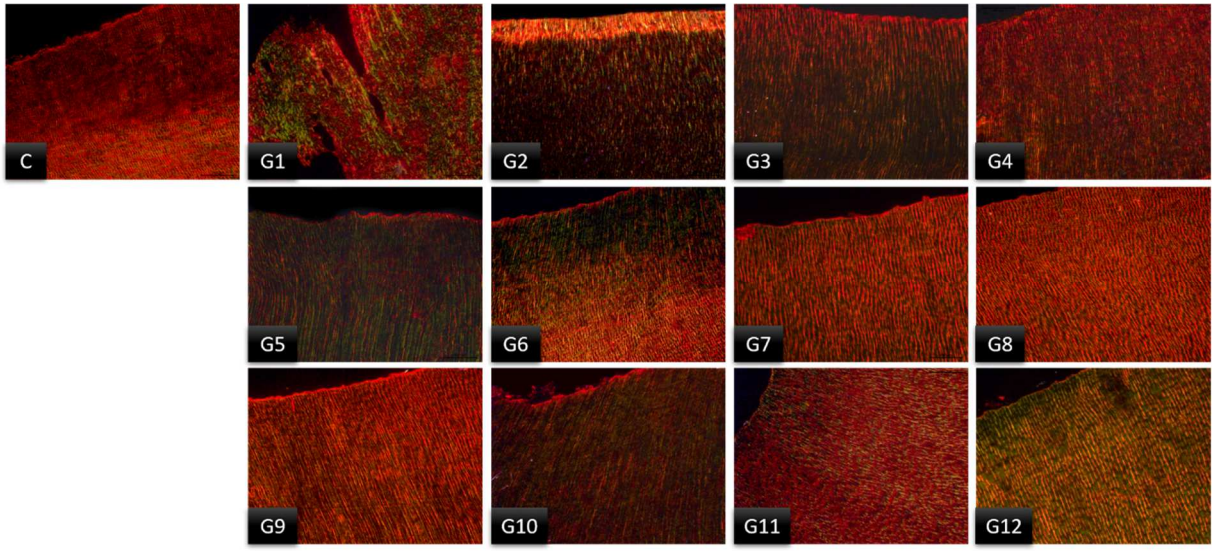


Figure 1. *Picrosirius* stain using polarized light microscopy of dentin surfaces treated according to the protocols: G1: 30 minutes in 2.5% sodium hypochlorite (NaOCl) without agitation; G2: 5 minutes of conventional irrigation and continuous aspiration with 2.5% NaOCl; G3: 3 cycles of 20 seconds of ultrasonic agitation with 2.5% NaOCl (PUI); G4: 3 cycles of 20 seconds of agitation with EasyClean, with 2.5% NaOCl; G5 to G8 received the same protocols described above, but the 2.5% NaOCl was replaced by 2% chlorhexidine (CHX); and from G9 to G12 the 2.5% NaOCl was replaced by 0,9% saline. The organic matrix showed uniformity of the fibrillar network in all groups, except for G1. G5, has a predominance of greenish fibers. All figures have the same magnification.

Discussion

Information on dentin structure and collagen integrity is important due to the influence on the bond strength of endodontic cements or the durability of restorative materials. To be closer to the clinical scenario, a root canal irrigation model was used, with bovine teeth blocks attached. The null hypothesis of the present study was partially accepted, since the irrigation regimes tested were not able to cause changes in dentinal microhardness, only group G9 showed an increase in the microhardness value after treatment. For *Picrosirius Red* analysis, the groups demonstrated the maturation dynamics of the collagen matrix.

In the present study, we used the Knoop indenter to assess changes in dentinal microhardness, the most suitable method for analyzing superficial dentin, the region closest to the canal lumen.[18,19] In our results, there were no changes in microhardness between the tested irrigation protocols. Although the substances used in our study are the most applied in endodontic clinical treatment, they do not dissolve inorganic dentin particles.[20] This property falls on chelating substances, such as EDTA,[21] which promote demineralization and, consequently, soften the surface, reducing dentin hardness.[22] This was expected since the irrigating solutions used do not promote the loss of calcium in the dentin. That is, they are not demineralizing, capable of removing the smear layer and, thus, preventing the formation of a smear layer during instrumentation.[20]

For this same purpose, only the group who was in prolonged contact (30 minutes) with saline, which is an inert substance,[23] showed an increase of the value of hardness. Another study, however, did not observe any change in the same analysis period with this.[19] However, Alamoudi et al.[24] explains that, a substrate immersed in saline solution promotes the formation of calcium phosphate crystals that contribute to an excellent cohesive bonding force on the sample surface and can subsequently receive the filling material or dentin adhesive.

The modification of dentin collagen after treatment with chemical substances can be observed by polarized light microscopy (PLM). Collagen is the main structural protein (90%) of the organic matrix of dentin[25] and contributes to the ultrastructural stability and tensile strength of dentin.[26] Because collagen molecules are rich in basic amino acids, they react intensely with acid dyes, as is the case with *Picrosirius Red*, promoting an evidence of the natural birefringence of collagen fibers when a polarized light passes through its long axis.[27] The shade of birefringence emitted by collagen

fibers of different thicknesses can vary from green, indicating thinner and more dispersed fibers, to yellow to red, indicating gradually thicker and more organized fibers with a higher degree of compaction.[27]

In our results, the three shades of birefringence were seen in all groups, with different amounts of the types of fibers present in the samples, according to the treatments (Figure 1). In addition, a less organized substrate with immature fibers is observed when in prolonged contact with chlorhexidine. This amount of green fibers is reduced when chlorhexidine is agitated by the passive ultrasonic irrigation technique. It is known that exposure of the extracellular matrix in collagen tissues by acidic substances can trigger the metalloproteinase activity of the extracellular matrix (MMP), capable of degrading collagen at the interface between the restorative material and dentin.[28] Some studies have reported that CHX-based materials, such as cavity disinfectants or endodontic irrigators, can negatively interfere with some adhesive systems.[29,30] This can lead to a reduction in the mechanical resistance of collagen fibrils, by favoring proteolytic degradation of dentinal tissue,[31] which can lead to premature loss of restorations.[32]

On the other hand, substances such as 2% chlorhexidine are known to be used and used as protease inhibitors, indicating that inhibition of (MMP) may be beneficial in the preservation of hybrid layers,[28] and with evidence of increased durability of restorations.[33,34]

For the groups treated with saline solution, there was a reduction in immature fibers, when the samples were in prolonged contact with this substance, or by conventional irrigation and also by passive ultrasonic agitation. Concomitantly, there is a predominance of mature and thick fibers in these groups. In addition, these groups did not show statistical differences from the control group, being similar to the pattern of fiber organization, which was already expected, as it is an inert substance.[23]

Regarding the irrigation regimes with NaOCl, it can be noted that there is a disorganization in the fibrillar network, and in the morphological pattern of the collagen surface only when exposed to this substance for 30 minutes, however, in general, they did not cause changes in the quantities collagen fibers, showing similar results to the control group. This was probably because NaOCl was used in relatively low concentration. In previous studies, damage to collagen was reported when irrigated only with NaOCl ,[13,35] also for a period longer than 30 minutes, however, the solution was used in greater concentration (5%), throughout the irrigation process. Thus, the

attribution to NaOCl for collagen degradation and the effect on the mechanical properties of dentin is reported by some authors as concentration dependent[10,36] and time-dependent.[10] It is important to highlight that changes in the structure, as occurred with NaOCl, and in the composition of dentin, such as for chlorhexidine, both in prolonged application times, can lead to a reduction in the fracture resistance of the teeth and can alter the adhesion of endodontic cements. and rehabilitation materials.[37,38] Thus, the choice of the appropriate chelating solution to be used in the final irrigation protocol, assumes an important role in order to preserve the organic and inorganic components of dentin, without compromising the ages following endodontic therapy.[37,38]

Conclusions

Thus, based on the experimental methods and results, it can be concluded that the irrigation regimes applied in this study are not capable of altering dentinal hardness. On the other hand, 2% chlorhexidine when in contact with the dentin for a long time, can cause a disorganization in the fibrillar network. NaOCl, despite not causing a change in the amount of mature and thick fibers, modifies the morphological structure of the dentin substrate. However, the choice of solution, concentrations and application time is necessary to obtain a correct preparation of the root canal, without compromising the quality of the dentin collagen network.

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3 Discussion

3 DISCUSSION

New strategies, such as irrigating devices and agitation regimes, have been employed in order to increase the flow and diffusion, promoting a greater reach of substances in order to obtain an adequate disinfection of the root canal system and, consequently, the repair of periapical tissues (VAN DER SLUIS et al., 2006; GU et al., 2009; GULABIVALA et al., 2010). This is because the physical and chemical effects of irrigating solutions during biomechanical preparation are essential for cleaning in areas that are difficult to access, since root canal systems have great anatomical complexity and microbial diversity (PETERS et al., 2001; VILLAS-BOAS et al., 2011).

When endodontic treatment fails, bacteria resistant to biomechanical preparation remain in the root canal system, perpetuating endodontic infection and apical periodontitis is formed. *Enterococcus faecalis* is a facultative, gram-positive anaerobic bacterium, common in up to 90% of retreatment cases. This microorganism is able to invade dentinal tubules and remain viable, but not cultivable even in ecological disturbances (lack of nutrients, oxygen tension and bacterial relationships), in addition to having virulence factors that allow it to form biofilms and the ability to if they adhere to the dentin surface (LLEO et al., 1998; LOVE et al., 2001; PINHEIRO et al., 2003). Less frequent bacteria such as *Streptococcus oralis* (ROÇAS et al., 2004) and a percentage of yeasts, such as *Candida albicans*, have been associated with persistent root canal infections that do not respond favorably to endodontic therapy (VALERA et al., 2001; MERGONI et al., 2018).

For this, irrigating solutions must have antimicrobial activity, the ability to dissolve organic, inorganic tissues and inactivate endotoxins (ZEHNDER, 2006). Thus, a root canal model was used in this study, in order to reproduce the conditions in vivo, with dentin blocks initially added with biofilm, and root canal irrigations following representative clinical protocols, were performed in an attempt to remove mono and dual species biofilms, composed of both bacteria and yeast, correlating a longer stirring time and chemical action of irrigating solutions. Through laser scanning confocal microscopy it was possible to determine the viability profile of multispecies biofilms and the isolated biofilm of *E. faecalis*, visualizing live and dead cells and quantifying them after treatments in infected bovine dentin. The results demonstrate that the association

of the factors of time, chemical action and agitation of irrigants is necessary for microbial reduction.

The activation of the solutions by the passive ultrasonic irrigation (PUI) technique had the high capacity to remove biofilm in monospecies and dual bacterial species, regardless of the solution and agitation time. For yeast biofilms, the chemical effect of the solutions associated with improved the action of this device. It has been shown that this technique increases the cleaning capacity in root canal systems (VAN DER SLUIS et al., 2010, GUERREIRO-TANOMARU et al., 2015) due to the cavitation action and acoustic wave formation inside the root canals. These results agree with other studies that confirm that in addition to the chemical action, the time, renewal and agitation of the solutions (GRÜNDLING et al., 2011, PLOTINO et al., 2019, PEREIRA et al., 2020, VIRDEE et al., 2020) are essential in cleaning mechanically inaccessible areas, reaching clinically acceptable levels.

As part of the methodology of our study, we used optical coherence tomography (OCT) in order to assess the influence of contact time and the activation of irrigants on dentin diffusion, in the same root canal irrigation model. OCT is a non-invasive, spatial resolution can be as high as 10 times that obtained by the ultrasound technique (FUJIMOTO et al., 1995). The acquisition of data in the OCT is based on lowcoherence laser interference principles (OTIS et al., 2000), and the scanning of the light beam generates a backscatter signal according to the depth of the sample. Through this method, this study demonstrates that the applied protocols substantially improve the diffusion of irrigants on the dentin surface, with the exception of conventional irrigation with 0.9% saline solution, which is in line with the microbiological results, explained above. Munoz & Camacho-Cuadra (2012), demonstrated that irrigants administered using conventional syringes passively reflect towards the pulp chamber, thus limiting their potential for penetration. The use of agitation techniques, therefore, are recommended in clinical practice, compared to conventional irrigation, as they promote disinfection throughout the root canal system during treatment, due to the high penetrability of irrigants in dentinal tubules and other areas of complexity. anatomical, reducing toxic products and substrates necessary for bacterial growth on inaccessible and non-instrumented surfaces (MOHMMED et al., 2018).

On the other hand, changes in the collagen matrix and in the amount of dentin phosphate are reported in the literature, as they can undergo changes on the

part of the irrigants used, implying changes in adhesion and sealing with endodontic cements, or the bonding of adhesive systems (COHEN et al., 1970; TSUDA et al., 1996; TULUNOGLU et al., 1998; LISBOA et al., 2013). For this, the ATR-FTIR methodology was used, as it aims at the chemical characterization by infrared Fourier transform spectroscopy (FTIR) of solid samples such as dental tissues, employing a spectroscopy technique known as ATR (Atenuated Total Reflectance). This technique is important because it restricts the interaction of infrared radiation to just a few microns of tissue, which guarantees a chemical analysis of a layer of superficial tissue, ideal for analyzing samples that have had their surface treated, such as irrigation of the root canal. In addition, the infrared spectroscopy of biological samples of sliced hard tissues is of great importance for some research areas, as it is possible to evaluate the chemical composition of the tissues, without losing water, as is the case with methodologies that use powder, making the substrate dehydrated, different from what would be human dentin (BACHMANN et al., 2003).

According to the FTIR analysis, the treatment of dentin according to the protocols applied in our study showed that NaOCl leads to a collagen reduction dependent on time (30 minutes) and agitation in continuous rotation. We believe that our results are due to the mechanical action and the speed of rotation that produces turbulence of the irrigating solution throughout the Easy Clean instrument (Easy Dental Equipment, Belo Horizonte, MG, Brazil), since the effectiveness of this device has already been observed in the removal of dentin debris in the apical region when compared to the passive ultrasonic irrigation (KATO et al., 2016) and in the cleaning of the root canals (DUQUE et al., 2017).

As for the dentin mineral matrices, these are composed mainly of crystals of carbonated hydroxyapatite, being identified in the infrared absorption of hydroxyl ions, carbonate and phosphate radical (BACHMANN et al., 2003). In the present study, a reduction in the carbonate/phosphate ratio occurred in the saline groups after application of irrigation methods (conventional, PUI and continuous rotation) and the changes in NaOCl were not significant. HU et al. (2010), report that NaOCl with different concentrations and exposure time did not change the carbonate/phosphate ratios on dentin surfaces, indicating that NaOCl may not change the inorganic components in dentin. The reduction of the carbonate/phosphate ratio in the substrate immersed in saline solution promotes the formation of calcium phosphate crystals that

contribute to an excellent cohesive bonding force on the substrate surface and can subsequently receive the filling material or dentin adhesive (ALAMOUDI et al., 2019).

Regarding changes in dentinal structure, in our study, we used the Knoop indenter to assess changes in microhardness, the most suitable method for analyzing superficial dentin, the region closest to the lumen of the canal (CRUZ-FILHO et al., 2011; TARTARI et al., 2018). In our results, there were no changes in microhardness between the tested irrigation protocols. Although the substances used in our study are the most applied in endodontic clinical treatment, they do not dissolve inorganic dentin particles (LESTER et al., 1977). This property falls on chelating substances, such as EDTA (ZHANG et al., 2010), which promote demineralization and, consequently, softening of the surface, reducing dentinal hardness (TANEJA et al., 2014). This was expected since the irrigating solutions used do not promote the loss of calcium in the dentin. That is, they are not demineralizers, capable of removing the smear layer (LESTER et al., 1977).

For an analysis of collagen fibers, we observed a less organized substrate, with immature fibers, when in prolonged contact with chlorhexidine. This amount of green fibers is reduced when chlorhexidine is agitated by the passive ultrasonic irrigation technique. It is known that exposure of the extracellular matrix in collagen tissues by acidic substances can trigger the metalloproteinase (MMP) activity of the extracellular matrix, capable of degrading collagen at the interface between the restorative material and dentin (PASHLEY et al., 2004). This can lead to a reduction in the mechanical resistance of collagen fibrils, by favoring proteolytic degradation of dentinal tissue (CAUSTON & JOHNSON 1979), which can lead to premature loss of restorations (AGEE et al., 2000).

For NaOCl, it can be noted that there was a disorganization in the fibrillar network, and in the morphological pattern of the collagen surface only when exposed to this substance for 30 minutes, however, in general, they did not cause changes in the amounts of collagen fibers, presenting similar results to the control group. This was probably because NaOCl was used in relatively low concentration. The attribution to NaOCl to collagen degradation and the effect on the mechanical properties of dentin is reported by some authors as concentration dependent (MARENDING et al., 2007; ZHANG et al., 2010) and time-dependent (ZHANG et al., 2010).

4 Conclusions

4 CONCLUSIONS

Based on the applied methods, it can be concluded that the protocols used were effective in the microbial reduction of mono and dual species biofilms and, with the exception of conventional irrigation with 0.9% saline solution, substantially improve the diffusion of irrigants on the dentin surface. In addition, optical coherence tomography proved to be an efficient and non-invasive method to evaluate the distribution of irrigation protocols tested on bovine dentin.

The irrigation regimes applied in this study are not capable of altering dentinal hardness. On the other hand, the use of NaOCl for a prolonged time or in continuous agitation causes a greater deproteination of the organic dentin matrix, in relation to the saline solution. Some carbonate ions are removed in the inorganic phase of the dentin by the saline solution. The solutions when in contact with the dentin for a long time, can cause a disorganization morphological structure of the dentin substrate. Thus, the choice of solution, concentrations and application time is important to obtain the correct sanitization without compromising the quality of the dentinal structure.

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