UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

TALITA TARTARI

Effects of different irrigation regimes in physico-chemical properties of dentin and consequences of changes in the adhesion of microorganisms and AH Plus sealer

Efeitos de diferentes regimes de irrigação nas propriedades físico-químicas da dentina e consequências das alterações na adesão de microrganismos e do cimento endodôntico AH Plus

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Tese constituída por artigos apresentada a 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. Clovis Monteiro Bramante

Versão Corrigida

BAURU
2017

Tartari, Talita

T178e Effects of different irrigation regimes in physico-chemical properties of dentin and consequences of changes in the adhesion of microorganisms and AH Plus sealer / Talita Tartari. – Bauru, 2017.

159 p. : il. ; 31cm.

Tese (Doutorado) – Faculdade de Odontologia de Bauru. Universidade de São Paulo

Orientador: Prof. Dr. Clovis Monteiro Bramante

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

Comitê de Ética da FOB-USP
Protocolo nº: 016/2017
Data: 18 de agosto de 2017

FOLHA DE APROVAÇÃO

DEDICATÓRIA

Dedico essa tese...

...com muito carinho e amor à minha **família**, pelo apoio e incentivo incondicional em todos os momentos dessa jornada.

Aos meus pais, **Arnildo e Ivani**, que se sacrificaram para que eu pudesse estudar e ter uma boa formação profissional. Vocês não tiveram essa mesma oportunidade, porém são “Doutores” na vida e como verdadeiros “Mestres” educaram seus filhos. Devo tudo o que sou a vocês, e se sinto orgulho de mim e do lugar onde cheguei é porque vocês seguraram a minha mão.

Aos meus irmãos, **Tania e Tailor,** por serem meu porto seguro. Mesmo distantes nunca me sinto sozinha, pois sei que posso contar com vocês sempre que precisar. Vocês são os melhores irmãos que a vida poderia ter me dado.

Aos meus sobrinhos, **Alan e Gabriel**, por preencherem minha vida com muita alegria e amor e fazerem da nossa família o lugar para onde sempre se quer voltar. Amor incondicional.

Amo muito vocês!

AGRADECIMENTO ESPECIAL

Ao meu orientador,

Prof. Dr. Clovis Monteiro Bramante,

“Papi soberano”, seu amor pela profissão é inspirador. O senhor é um exemplo de profissional a ser seguido. Agradeço a Deus por ter me dado a oportunidade de trabalharmos juntos. Obrigada por compartilhar a sua sabedoria, pela orientação e pelo trato respeitoso e aberto que sempre teve comigo. Obrigada por me ouvir sempre que eu chegava empolgada com uma nova ideia ou descoberta. Na sua condição de orientador, o senhor poderia ter imposto suas vontades, mas preferiu acreditar em mim e deixar que eu alçasse voo em busca de saciar a minha sede por conhecimento na área em que eu desejava. Não tenho palavras para agradecer ao senhor por isso e por ter estado ao meu lado indicando o melhor caminho a seguir.

A ti, minha eterna admiração e gratidão.

AGRADECIMENTOS

À energia que ilumina o meu caminho e me mantém firme na busca da realização de todos os meus projetos de vida, que eu chamo de **Deus**.

Ao **Prof. Dr. Marco Antonio Hungaro Duarte**, a tua disponibilidade para ajudar, o teu amor pelo ensino e pela pesquisa, o teu desprendimento e apreço quando solicitado e acima de tudo a tua humildade, são alguns dos teus exemplos que levarei comigo para sempre. Obrigada pela significante contribuição no desenvolvimento de toda esta tese e por ter acreditado no potencial do meu trabalho sem nem me conhecer direito. Nunca vou esquecer do senhor largando tudo para ir comprar uma cuba ultrassônica a fim de que eu pudesse realizar um experimento. Obrigada por sempre ter aquela referência que faz toda a diferença para indicar e por mostrar que são as perguntas difíceis nos tiram da zona de conforto e nos fazem crescer quando vamos em busca da resposta.

Ao **Prof. Dr. Renato Menezes da Silva**, por ter aceitado me orientar e receber para fazer doutorado sanduíche na The University of Texas School of Dentistry at Houston. Você foi como um pai quando estive em Houston, sempre se preocupando com meu bem-estar e dando palavras de incentivo quando eu quis fraquejar. Você mostrou com seu próprio exemplo que quando definimos um objetivo o caminho para chegar até ele pode ser muito difícil, pois muitas vezes teremos que abdicar do convívio com a família e dos momentos de lazer. Porém, se mantivermos o foco e aceitarmos que nossas escolhas tem um preço, quanto atingirmos a nossa meta e olharmos para trás iremos perceber que os momentos de dificuldade serviram para nos tornarem mais fortes. Obrigada por me ensinar que quando temos certeza do que queremos para nossas vidas, nada é impossível.

Ao **Prof. Dr. Luciano Bachmann**, por ter me acolhido no seu laboratório e pela confiança em mim depositada durante o período em que fui para Ribeirão Preto fazer as análises em FTIR. Acredito que uma das habilidades mais importantes que um professor pode ter é a capacidade de tornar simples os assuntos mais difíceis e você com certeza tem esse dom. Obrigada por ser uma das primeiras pessoas a me incentivar a ir atrás do meu sonho de estudar no exterior. Obrigada por todo o

aprendizado que foi muito além do meio acadêmico. São poucas as pessoas com o seu nível de formação que possuem a humildade de sair da sua sala para sentar com um aluno no laboratório a fim de ensinar a maneira correta de realizar um experimento. Desejo poder chegar ao seu nível profissional e ter essa mesma humildade e simplicidade. Obrigada por sempre deixar uma porta aberta para novas parcerias e acreditar no potencial.

À **Profa. Dra. Ariadne Letra**, por ter me acolhido em Houston e pelo exemplo de competência e dedicação à profissão. Obrigada por mostrar que devemos ser fortes nos momentos de dificuldade, pois a maneira como enfrentamos os desafios é que define quem somos e nos faz conseguir o respeito e a admiração das pessoas. Sabemos que ser mulher no meio acadêmico não é fácil, pois nossas conquistas tendem a ser menos reconhecidas, porém você ensinou que alcançar esse reconhecimento é possível e que para isso não precisamos deixar nossa feminilidade e delicadeza de lado.

Ao **Prof. Dr. Ivaldo Gomes de Moraes,** um ser humano com o coração gigante, que foi capaz de abrir as portas de sua casa para acolher a mim e aos meus amigos no seio de sua família quando não pudemos passar o dia das mães com nossas mães. Uma pessoa com uma alma tão pura e uma sensibilidade tão grandes que não tenho palavras para descrever. Sempre mais preocupado com o bem-estar dos outros que com o seu próprio bem-estar. Obrigada por mostrar que prestar atenção aos detalhes faz toda a diferença na hora de realizar um experimento e interpretar os resultados. Obrigada por mostrar que é possível unirmos serenidade a uma mente brilhante e inquieta. “Profinho querido”, o senhor é como um pai para mim e com certeza para muitos outros alunos do departamento.

Ao **Prof. Dr. Rodrigo Ricci Vivan**, um profissional tão novo e ao mesmo tempo com muito conhecimento clínico e científico para transmitir. Você é um exemplo de que com dedicação e esforço somos capazes de chegar aonde quisermos. Sua disposição, energia e vontade de trabalhar servem de inspiração para os alunos e as pessoas que convivem com você. Obrigada por acreditar em minha pesquisa, acreditar em mim e pelas muitas vezes em que me deu palavras de apoio nessa minha

trajetória na FOB-USP. Obrigada por aceitar participar da minha banca de doutorado e assim ajudar a engrandecer esse trabalho.

À **Profa. Dra. Flaviana Bombarda**, pelo incentivo, amizade e atenção que me foram dados durante todos esses anos de doutorado. Obrigada por sua contribuição e carinho sempre.

Ao **Prof. Dr. Norberti Bernardineli**, pelos ensinamos transmitidos, pela sua imensa boa vontade e disposição em ajudar. Lembro sempre do senhor entrando na sala de convivência para dar conselhos que serviam tanto para nossa vida acadêmica quanto pessoal.

Ao **Prof. Dr. Roberto Brandão Garcia,** pelo conhecimento compartilhado e por ter feito parte da minha caminhada na pós-graduação em Bauru. O senhor é um exemplo de que gentileza e educação no trato com as pessoas nunca são demais.

To **Dr. Ransome van der Hoeven**, for opening the doors of your laboratory for me which allowed me to perform microbiology experiments freely in Houston. Your sense of humor always ensured hearty laughter for me and Caroline Wichnieski especially when we were stressed out at the challenges of experiments. Thank you for the numerous times you made yourself available to talk with me when I had doubts about my essays.

À **Profa. Dra. Patricia de Almeida Rodrigues**, minha eterna orientadora. Obrigada por ter me incentivado a ingressar na pós-graduação e posteriormente a fazer o doutorado na FOB-USP. Mesmo distante sei que posso contar com você! Obrigada por ter acreditado de mim desde quando eu estava na especialização em endodontia.

Aos professores **Dr. Antônio David Normando** e **Dr. José Roberto Pereira Lauris,** pelo suporte dado quando tive dúvidas quanto ao tipo de análise estatística que eu deveria fazer.

Ao **Prof. Dr. Miguel Jafelicci Junior**, por ter me recebido no Instituto de Química da Universidade Estadual Paulista Júlio de Mesquita Filho de Araraquara para a realização das análises de molhabilidade.

À **Profa. Dra. Linda Wang**, por ter disponibilizado a máquina de ensaios universal para a realização dos experimentos de push-out e por ter providenciado o treinamento para uso do equipamento.

Aos funcionários do departamento de Endodontia da FOB-USP, **Andressa Barraviera Laureano, Edimauro de Andrade e Suely Regina Bettio**. Obrigada pela convivência e amizade. Vocês são pessoas com as quais sempre pude contar para dar boas gargalhadas e para ajudar naqueles momentos de imprevistos.

Aos meus amigos e colegas **Clarissa Teles Rodrigues, Denise Ferracioli Oda, Jussaro Alves Duque, Lyz Cristina Furquim Canali e Rafaela Fernandes Zancan**, obrigada por terem me presenteado com as suas amizades. Vocês me deram suporte e apoio, me incentivaram a lutar por meus sonhos, mesmo sabendo que a realização desses sonhos iria nos deixar distantes fisicamente. A vida nos uniu e não foi por acaso, vocês são verdadeiros irmãos para mim. Palavras não serão capazes de descrever a gratidão que tenho por vocês, pela nossa amizade, pela atenção e carinho dados nas horas difíceis e alegres. Obrigada por aceitarem compartilhar das minhas angustias, aflições, alegrias e conquistas da vida pessoal e acadêmica.

À minha amiga e colega **Caroline Wichnieski**, um presente de Houston para minha vida. Uma parceira para todas as horas, tanto na vida pessoal quanto profissional. Uma colega que está sempre disposta a ouvir e dar conselhos e que se precisar passa a noite junto no laboratório para que os experimentos sejam concluídos dentro do prazo. Durante vários meses dividimos muitas gargalhadas, dúvidas e inseguranças e também a certeza de que crescemos juntas e seremos amigas para sempre. Por ser canhota posso dizer que você foi meu “segundo braço esquerdo” em Houston e vai morar para sempre no meu coração.

Aos queridos amigos e colegas **Amanda Maliza, Bruno Martini Guimarães, Marcela Milanezi, Pablo Amoroso,** apesar de termos entrado no doutorado em datas diferentes considero que somos da mesma turma. Obrigada pela imensa amizade de vocês, pelo companheirismo, incentivo e presença tanto nos momentos alegres quanto nos mais difíceis.

Aos amigos e colegas da pós-graduação **Bruno Piazza, Ericson Janolio de Camargo, Fernanda Fernandes, Francine Cesário, Layla Vasconcelos, Lincoln Fruch, Mariana Borges, Murilo Priori Alcalde, Samuel Lucas Fernandez, Paloma Minotti, Sávio Brands, Thais Pereira, Raquel Midena Mesquita, Bruno Cavalini Cavenago**. Obrigada pela amizade de vocês. Nosso convívio durante o curso trouxe incontáveis momentos de alegria e também grande aprendizado para todos.

Aos demais amigos e colegas de pós-graduação **Arthur Lemos, Ericke Mucke, Gislene Cristina, Glaucia Gonçalves, Marcelo Pomini, Maricel Cuellar, Melissa Rivera, Michel Klymus, Milena Perraro, Renan Furlan, Pedro Titato, Vanessa Marques, Victor Cruz e Yahir Munoz** obrigada por todo o carinho, apesar de não termos tido a oportunidade de convivermos por muito tempo.

Às minhas amigas **Daliane Soraia Maia Palha** e **Darla Luzia Ropellato Fernandez** pelo incentivo, companheirismo e apoio irrestritos desde o momento em que decidi ingressar na pós-graduação. Que nossa amizade se perpetue por toda a vida! Vocês preenchem meu coração de alegrias, cumplicidade e tranquilidade para enfrentar o dia-a-dia. Obrigada por vibrarem com minhas conquistas como se elas fossem de vocês e por comemorarem junto comigo cada etapa superada nesse caminho.

To **Imran Nadir,** for your companionship, constant encouragement and unwavering confidence in me in the past year. At the most challenging times when I was close to giving up, your support and feedback reinforced my self-confidence, convinced me of my abilities and renewed my self-belief. Thank you for teaching me that to achieve our goals, we must choose one battle at a time so we can fully focus on and execute the task at hand. Thank you for believing in me even when I did not believe in myself. Most importantly, thank you for showing me that our search for knowledge must be constant throughout our lives and we should be proud of this search.

Ao meu amigo e colega **Ronald Ordinola Zapata**, uma das primeiras pessoas a me oferecer suporte quando entrei na pós-graduação em Bauru, me dando ideias e conselhos para a realização de experimentos. Você também foi a primeira pessoa a me mostrar a possibilidade de um novo horizonte em minha carreira e uma das pessoas que mais me incentivou e deu conselhos para conseguir atingir esse novo objetivo.

À minha amiga **Leticia Chavez**, que mesmo me conhecendo a pouco tempo já me apoiou em várias batalhas e conquistas. Você é uma pessoa maravilhosa e com um coração enorme.

Às técnicas **Thelma Lopes e Larissa,** do Laboratório de Bioquímica pelo treinamento e suporte no preparo dos irrigantes, realização dos experimentos de titulometria iodométrica e rugosidade.

À **Marcia Graeff** e ao **Marcelo Milanda Ribeiro**, que colaboram para que este trabalho se concretizasse compartilhando seus conhecimentos e dando treinamentos em diversos tipos de análises.

Ao **Rodolfo Debone Piazza,** aluno de Doutorado em Química da Universidade Estadual Paulista Júlio de Mesquita Filho de Araraquara, pelo treinamento para a realização das análises de molhabilidade.

Ao **Kaique Mutter Haleplian,** pela ajuda quando tive dúvidas nos experimentos em FTIR.

À **Elaine Cristina Consolmagno**, pelo treinamento e orientações para a realização dos experimentos de push-out.

To **Ali Naji**, for the individual attention and support along with your willingness to train and help me with my experiments in Houston.

A **todos os amigos e familiares** que entenderam minha ausência e torceram pelo meu sucesso e felicidade sempre. Muito obrigada!

AGRADECIMENTOS INSTITUCIONAIS

À Faculdade de Odontologia de Bauru da Universidade de São Paulo (FOB-USP), na pessoa Diretora Profa. Dra. Maria Aparecida de Andrade Moreira Machado.

À Comissão de Pós-graduação da FOB-USP, na pessoa do Prof. Dr. Guilherme dos Reis Pereira Janson.

Ao Coordenador do programa de Pós-graduação em Endodontia da FOB-USP, Prof. Dr. Marco Antonio Hungaro Duarte.

To the School of Dentistry of the University of Texas Health Science Center at Houston.

Às funcionárias da Pós-Graduação da FOB-USP, Ana Letícia Palombo Momesso, Fátima Cassador Carvalho e Leila Regina da Silva Yerga Sanches por sempre estarem dispostas a atender e ajudar.

À funcionária do Comitê de Ética em Pesquisa Animal da FOB-USP, Maristela Petenuci Ferrari.

Ao Centro Integrado de Pesquisas da FOB-USP, na pessoa do Prof. Dr. Rodrigo Cardoso de Oliveira.

Ao Laboratório de Bioquímica da FOB-USP, na pessoa da Profa. Dr. Marilia Buzalaf.

À Faculdade de Filosofia Ciências e Letras de Ribeirão Preto da Universidade de São Paulo, na pessoa do Prof. Dr. Luciano Bachmann.

Ao Instituto de Química da Universidade Estadual Paulista Júlio de Mesquita Filho de Araraquara, na pessoa do Prof. Dr. Miguel Jafelicci Jr.

Ao Laboratório de Nanotecnologia do Centro Nacional de Pesquisa em Energia e Materiais, nas pessoas do Prof. Dr. Christoph Friedrich Deneke e do Prof. Dr. Carlos Alberto Rodrigues Costa.

A todos os professores e funcionários da FOB-USP, que me receberam com sorrisos e disposição.

À Fundação de Amparo à Pesquisa do Estado de São Paulo que tornou a elaboração dessa tese possível, graças aos auxílios na forma de bolsas de estudo (Processos FAPESP 2013/19789-3 e 2015/09958-8).

“They did not know it was impossible,

so they did it.”

Mark Twain

RESUMO

Efeitos de diferentes regimes de irrigação nas propriedades físico-químicas da dentina e consequências das alterações na adesão de microrganismos e do cimento endodôntico AH Plus

Além dos efeitos desejados, as soluções químicas utilizadas para auxiliar os instrumentos endodônticos na limpeza e desinfecção do sistema radiculares podem causar alterações nas propriedades físico-químicas da dentina e consequentemente afetar a adesão de cimentos endodônticos e microrganismos às paredes do canal radicular. Contudo, os efeitos de novos irrigantes e protocolos de irrigação ainda são desconhecidos. Os objetivos desta tese foram verificar as alterações nas propriedades de alguns irrigantes quando utilizados combinados em misturas, definir o tempo necessário para a remoção da camada de *smear layer* por um novo irrigante, determinar a capacidade de dissolução de matéria orgânica e os efeitos de algumas soluções e protocolos de irrigação nas propriedades físico-químicas de dentina e avaliar a adesão de microrganismos e cimento AH Plus à dentina após a submissão desta a diferentes sequências de irrigação. Em todos os experimentos com dentina as amostras utilizadas foram obtidas a partir de dentes bovinos. Nas análise realizadas nesta tese as seguintes soluções foram testadas isoladas e combinadas em diferentes protocolos de irrigação: solução salina (controle), hipoclorito de sódio (NaOCl), ácido etilenodiaminotetraacético trisódico ($EDTAHNa_3$), ácido etilenodiaminotetracético tetrassódico alcalino ($EDTANa_4$), clorexidina (CHX), ácido peracético (PAA) e ácido etidrônico (HEDP). O $EDTAHNa_3$ e o $EDTANa_4$ foram testados em relação aos seus efeitos sobre o teor de cloro livre do NaOCl. As soluções foram misturadas em uma proporção de 1:1 e a titulação iodométrica das misturas realizada em diferentes intervalos de tempo. O tempo necessário para a remoção da *smear layer* de amostras de dentina pela solução de $EDTAHNa_3$ a 17% e diferentes concentrações de $EDTANa_4$ isoladas e misturadas com NaOCl foi determinado com o auxílio do microscópio eletrônico de varredura (SEM). A capacidade de dissolução de matéria orgânica pelo NaOCl foi determinada pesando fragmentos de músculo bovino antes e depois da imersão em soluções de 1%, 2,5% e 5% de NaOCl em diferentes períodos de tempo. Além disso, os efeitos do $EDTAHNa_3$, $EDTANa_4$ e HEDP na dissolução de

matéria orgânica pelo NaOCl foram avaliados. As alterações produzidas por todas as soluções isoladas e alguns protocolos de irrigação nos componentes orgânicos e inorgânicos da superfície da dentina foram analisadas pela técnica de reflexão total atenuada em espectroscopia no infravermelho por transformação de Fourier (ATR-FTIR). Espectros de absorbância foram coletados da superfície da dentina antes e após a imersão das amostras nos irrigantes, e foram calculadas as razões das bandas de amida III/fosfato e carbonato/fosfato. Para quantificar a adesão da CHX à dentina mineralizada e à dentina desmineralizada por diferentes protocolos de irrigação, foram determinadas as áreas da banda associada a CHX com pico em 1492 cm^{-1} em espectros obtidos por ATR-FTIR. Os efeitos de diferentes protocolos de irrigação na rugosidade e molhabilidade da superfície da dentina foram medidos com um rugosímetro de bancada e a técnica de gota séssil, respectivamente. Para os ensaios de adesão de microrganismos, amostras foram preparadas e tratadas da mesma maneira e com os mesmos protocolos de irrigação utilizados nos testes de rugosidade e molhabilidade. Em seguida, *Candida albicans* e *Enterococcus faecalis* foram mantidos em contato com a dentina por 2 horas e as amostras foram analisadas no microscópio confocal de varredura laser (CLSM). Testes de push-out foram realizados para determinar o impacto de diferentes protocolos de irrigação na resistência de união à dentina do cimento AH Plus ao longo do tempo. Canais de dentes incisivos de bovinos foram instrumentados, irrigados e em seguida obturados utilizando apenas o cimento AH Plus. Metade das amostras foi submetida a avaliação de push-out 7 dias após a obturação e a outra metade após 20 meses. Os resultados dos experimentos mostraram que o $EDTAHNa_3$ causou uma perda quase completa e imediata do cloro livre do NaOCl, enquanto o $EDTANa_4$ promoveu um declínio lento e concentração dependente. A *smear layer* foi removida apenas por soluções descalcificantes e em cerca de 1 min pelo $EDTAHNa_3$ a 17% e em 5 min pelo $EDTANa_4$, tanto isolados ou misturados com o NaOCl. O aumento da concentração de NaOCl e do tempo de contato com os fragmentos de músculo bovino intensificou a dissolução da matéria orgânica. As misturas de NaOCl com $EDTANa_4$ e HEDP foram capazes de dissolver as amostras de músculo ao longo do tempo, no entanto, o $EDTAHNa_3$ afetou fortemente a capacidade de dissolução do NaOCl quando eles foram misturados. Os resultados dos experimentos com ATR-FTIR mostraram que o aumento da concentração do NaOCl intensificou a desproteinização do colágeno da dentina com

redução da relação amida III/fosfato. Para o mesmo agente de descalcificação, quanto maior a concentração e o tempo de imersão, maior a remoção de fosfato, exposição da matriz de colágeno e consequentemente o aumento da proporção amida III/fosfato. O PAA causou os maiores aumentos na relação amida III/fosfato, seguido de $EDTAHNa_3$, $EDTANa_4$ e HEDP e esta ordem foi mantida nos protocolos em que o NaOCl foi usado antes dos agentes descalcificantes. O NaOCl necessitou aproximadamente 0,5 min para desproteinizar a matriz de colágeno exposta após a remoção de fosfato pelo $EDTAHNa_3$ e o PAA. A relação carbonato/fosfato diminuiu após 30 s de imersão das amostras em soluções de NaOCl a 1%, 2,5% e 5%, sem mais alterações ao longo do tempo. O carbonato da dentina foi removido mais rápido do que o fosfato por todos os agentes descalcificantes empregados sozinhos e nos protocolos de irrigação em que o uso do NaOCl foi seguido pelo uso do $EDTAHNa_3$, PAA e HEDP. Para os protocolos de irrigação que associam o NaOCl com soluções quelantes, o último irrigante utilizado definiu as proporções finais de amida II/fosfato e carbonato/fosfato da dentina. Para as análises da adesão da CHX em ATR-FTIR, os resultados mostraram que a adsorção deste irrigante à dentina foi potencializada quando agentes quelantes foram utilizados antes da CHX. Em relação aos experimentos de rugosidade da superfície, a solução salina, o NaOCl, o HEDP e a CHX não alteraram a rugosidade da dentina, mas o $EDTAHNa_3$ e o PAA a aumentaram. A molhabilidade da superfície aumentou após o uso de todos os irrigantes, sendo que o HEDP causou os maiores aumentos. Nos ensaios de adesão dos microrganismos, a *smear layer* e o colágeno exposto pelos agentes quelantes favoreceram a adesão de *E. faecalis*. A adesão da *C. albicans* foi maior em superfícies com *smear layer* ou mais mineral. O uso de CHX como irrigante final reduziu a adesão de ambos os microrganismos. A molhabilidade não influenciou a adesão dos microrganismos, enquanto o aumento da rugosidade parece potencializar a adesão do *E. faecalis*. Os experimentos de resistência de união do AH Plus à dentina mostraram que a irrigação com NaOCl e a mistura de NaOCl + $EDTANa_4$ produziram valores de resistência de união em 7 dias mais baixos em comparação com NaOCl + $EDTAHNa_3$, NaOCl + $EDTAHNa_3$ + NaOCl, NaOCl + $EDTAHNa_3$ + CHX e a mistura de NaOCl + HEDP. Após 20 meses, os valores mais baixos foram obtidos nos grupos irrigados com NaOCl e NaOCl + $EDTAHNa_3$. Os grupos de NaOCl + $EDTAHNa_3$ + NaOCl, mistura de NaOCl + HEDP e mistura de NaOCl + $EDTANa_4$ apresentaram

valores de força de união por push-out em 20 meses semelhante aos valores em 7 dias. Foi possível concluir que as soluções de irrigação testadas neste estudo têm diferentes efeitos na matéria orgânica e inorgânica e elas podem afetar as ações umas das outras quando misturadas. Independentemente de serem utilizadas isoladas ou combinadas em protocolos de irrigação, os irrigantes causam modificações nas propriedades físico-químicas dentinárias que influenciam na adesão do cimento AH Plus a curto e longo prazo e na adesão de microrganismos à superfície em casos de recontaminação.

Palavras-chave: *Candida albicans*; Dentina; *Enterococcus faecalis*; Irrigantes do canal radicular; Cimentos de resina; Propriedades de superfície.

ABSTRACT

Effects of different irrigation regimes in physicochemical properties of dentin and consequences of changes in the adhesion of microorganisms and AH Plus sealer

Besides of the desired effects, the chemical solutions used to assist the endodontic instruments in the cleanliness and disinfection of the root canal system can also cause changes in the physicochemical properties of dentin, and consequently affect the adhesion of endodontic sealers and microorganisms to the root canal walls. However, the effects of new irrigators and irrigation protocols remain unknown. The objectives of this thesis were to verify the alterations in the properties of some irrigants when used combined in mixtures, to define the time necessary for the smear layer removal by a new irrigant, to determine the organic matter dissolution capacity and the effects in the physicochemical properties of dentin of some irrigation solutions and protocols, and to evaluate the adhesion of microorganisms and AH Plus sealer to dentin after its submission to different irrigation sequences. In all experiments with dentin, the samples used were obtained from bovine teeth. In the analysis performed in this thesis, the following solutions were tested isolated and combined in different irrigation protocols: saline solution (control), sodium hypochlorite (NaOCl), trisodium ($EDTAHNa_3$), alkaline ethylenediaminetetraacetic acid tetrasodium ($EDTANa_4$), chlorhexidine (CHX), peracetic acid (PAA), and etidronic acid (HEDP). The $EDTAHNa_3$ and $EDTANa_4$ were tested in relation to their effects on the free chlorine content of NaOCl. The solutions were mixed in a 1:1 ratio and the iodometric titration of the mixtures performed in different time intervals. The time necessary for smear layer removal from dentin samples by solutions of $EDTAHNa_3$ and different concentrations of $EDTANa_4$ isolated and mixed with NaOCl was determined with the aid of the scanning electron microscope (SEM). The capacity of NaOCl to dissolve organic matter was determined by weighting fragments of bovine muscle before and after immersion in solutions of 1%, 2.5%, and 5% of NaOCl in different periods of time. Also, the effects of $EDTAHNa_3$, $EDTANa_4$ and HEDP on the organic matter dissolution by NaOCl were evaluated. The alterations produced by all solutions isolated and some irrigation protocols in the organic and inorganic components of the dentin surface were analysed by the attenuated total reflectance of Fourier transform infrared spectroscopy

(ATR-FTIR) technique. Absorbance spectra were collected from the dentin surface before and after immersion of samples in the irrigants and the ratios of the amide III/phosphate and carbonate/phosphate bands were calculated. To quantify the adhesion of CHX to mineralized dentin and to dentin demineralized by different irrigation protocols, the areas of the band associated with CHX with the peak in 1492 cm^{-1} were determined in spectra obtained by ATR-FTIR. The effects of different irrigation protocols in the roughness and wettability of dentin surface were measured with a benchtop roughness tester and the sessile drop technique, respectively. For the assays of microorganisms' adhesion, samples were prepared and treated the same way and with the same irrigation protocols used in the roughness and wettability tests. Following, *Candida albicans* and *Enterococcus faecalis* were maintained in contact with the dentin for 2 hours and the samples were analyzed on the confocal laser scanning microscope (CLSM). Tests of push-out were performed to determine the impact of different irrigation protocols on the dentin bonding strength of AH Plus sealer over time. Canals of bovine incisors teeth were instrumented, irrigated and following obturated using only the sealer AH Plus. Half of the samples were submitted to push-out assessment 7 days after the obturation and the other half 20 months later. The results of the experiments showed that the $EDTAHNa_3$ caused an almost complete and immediate loss of free available chlorine from NaOCl, whilst $EDTANa_4$ promoted a slow and concentration-dependent decline. The smear layer was removed only by decalcifying solutions and in about 1 min by the 17% $EDTAHNa_3$ and 5 min by the $EDTANa_4$, both isolated or mixed with NaOCl. The increase in NaOCl concentration and contact time with the samples intensified the dissolution of organic matter. The mixtures of NaOCl with $EDTANa_4$ and HEDP were able to dissolve the fragments of bovine muscle over-time, however, the $EDTAHNa_3$ strongly affected the NaOCl dissolution capacity when they were mixed. The results of ATR-FTIR experiments showed that the increase in the NaOCl concentration intensified the deproteination of the dentin collagen with a reduction in the amide III/phosphate ratio. For the same decalcifying agent, the higher the concentration and immersion time the greater the removal of phosphate, exposure of the collagen matrix and consequently the increases in amide III/phosphate ratio. The PAA caused greater increases in amide III/phosphate ratio, followed by $EDTAHNa_3$, $EDTANa_4$ and HEDP and this order was maintained in the protocols in which NaOCl was used before the decalcifying agents. NaOCl required

approximately 0.5 min to deproteinate the collagen matrix exposed after phosphate removal by $EDTAHNa_3$ and PAA. The carbonate/phosphate ratio decreased after 30 s of samples immersion in solutions of NaOCl at 1%, 2.5% and 5% with no more alterations over time. The carbonate of the dentine was removed faster than phosphate by all decalcifying agents employed alone and in the irrigation protocols in which the use of the NaOCl was followed by the use of the $EDTAHNa_3$, PAA and HEDP. For irrigation protocols that associate NaOCl with chelating solutions, the last irrigant used defined the final dentine amide III/phosphate and carbonate/phosphate ratios. For the ATR-FTIR analysis of CHX adhesion, the results showed that the adsorption of this irrigant to the dentin was potentiated when chelating agents were used prior to the CHX. In relation to the experiments of surface roughness, the saline solution, NaOCl, HEDP and CHX did not alter the roughness of the dentin, but $EDTAHNa_3$ and PAA increased it. The wettability of the surface increased after the use of all irrigants, being the HEDP to cause the greater increases. In the assays of microorganisms' adhesion, the smear layer and collagen exposed by the chelating agents favored the adhesion of *E. faecalis*. The *C. albicans* adhesion was major in surfaces with smear layer and more mineral. The use of CHX as the final irrigant reduced the adhesion of both microorganisms. The wettability did not influence the microorganisms' adhesion, while increases in roughness seems to potentiate the adherence of *E. faecalis*. The experiments of bond strength of AH Plus to the dentin showed that the irrigation with NaOCl and mixture of NaOCl + $EDTANa_4$ produced the lowest push-out bond strength values in 7 days compared to NaOCl + $EDTAHNa_3$, NaOCl + $EDTAHNa_3$ + NaOCl, NaOCl + $EDTAHNa_3$ + CHX and the mixture of NaOCl + HEDP. After 20 months the lowest values were obtained in the groups irrigated with NaOCl and NaOCl + $EDTAHNa_3$. The groups of NaOCl + $EDTAHNa_3$ + NaOCl, mixture NaOCl + HEDP, and mixture NaOCl + $EDTANa_4$ presented values of push-out bond strength in 20 months similar to the values in 7 days. It was possible to conclude that the irrigation solutions tested in this study have different effects in the organic and inorganic matter and some of them can affect the action of each other when mixed. Independent of being used isolated or combined in irrigation protocols, these irrigants cause modifications in the dentin physicochemical properties that influence the adhesion of AH Plus sealer in short and long term and the microorganisms' adherence to the

surface in cases of recontaminations.

Keywords: *Candida albicans*; Dentin; *Enterococcus faecalis*; Root canal irrigants; Resin cements; Surface properties.

SUMMARY

1 INTRODUCTION

1 INTRODUCTION

During the endodontic therapy, chemical solutions are used to assist the instruments in the process of shaping and cleaning (ZEHNDER, 2006). The physical and chemical effects of the irrigants are crucial in the biomechanical preparation phase to help reaching the surfaces untouched by the endodontic files, which correspond to at least 35% of the canal walls (PETERS, O. A.; SCHONENBERGER; LAIB, 2001). The physical action of the solutions is generated by the flow of the liquids throughout the root canal system and is responsible for the mechanical removal of the content of the canal, such as tissue remnants, dentin chips and microorganisms (HAAPASALO et al., 2010; SIQUEIRA et al., 1999). Among the chemical actions are the antimicrobial activity, tissue dissolution and removal of the smear layer (BYSTROM; SUNDQVIST, 1983; ZEHNDER, 2006).

However, besides of the desired effects, the irrigation solutions showed to act on the structure of dentin altering the ratio of the surface components (ARI; ERDEMIR, 2005; DOĞAN; ÇALT, 2001). The changes in the original proportion of dentin components can influence physicochemical properties of this mineralized tissue like microhardness, permeability, solubility, roughness and wettability (ARI; ERDEMIR, 2005; BALLAL; MALA; BHAT, 2010; COBANKARA; ERDOGAN; HAMURCU, 2011; HU; LING; GAO, 2010; PEREZ-HEREDIA et al., 2008; TARTARI; DE ALMEIDA RODRIGUES SILVA; et al., 2013; TARTARI; DUARTE JUNIOR; et al., 2013). The alterations in these properties might affect the adhesion of dental materials, including resin based sealers and endodontic sealers to the root canal walls (DE-DEUS; NAMEN; et al., 2008; NEELAKANTAN et al., 2011; NEELAKANTAN et al., 2012; NUNES et al., 2008), and influence the adhesion of microorganisms to dentin (JARAMILLO et al., 2012; KISHEN et al., 2008; MEI et al., 2011; SEN et al., 2003).

To promote the disinfection of the root canal system it has been recommended the use of irrigants with antimicrobial capability during the biomechanical preparation (BRITO et al., 2009). Based on this, the sodium hypochlorite (NaOCl) remain the main irrigant used in endodontics, because besides of the broad antimicrobial action it also presents the unique organic matter dissolution capability (ZEHNDER, 2006). However, as the NaOCl has nonspecific oxidizing and

proteolytic effects it denatures the collagen component of dentin (DI RENZO et al., 2001; HU; PENG; et al., 2010; ZHANG; KIM; et al., 2010) and altering the ratio of its components (ARI; ERDEMIR, 2005; DOĞAN; ÇALT, 2001; PEREZ-HEREDIA et al., 2008). These modifications result in reduction of the microhardness (PATIL; UPPIN, 2011; TARTARI; DE ALMEIDA RODRIGUES SILVA; et al., 2013) and increase in the wettability of the surface (HU; LING; et al., 2010). In relation to the roughness, the studies are controversial, because some of them showed increases in this surface property (HU; LING; et al., 2010; PATIL; UPPIN, 2011) while others no (PASCON et al., 2014; TARTARI; DUARTE JUNIOR; et al., 2013). However, the formers presented a methodological bias that is the no standardization of the initial roughness (EL FENINAT et al., 2001).

However, during the biomechanical preparation with NaOCl, there is the formation of the smear layer that is composed of organic residues from the pulp, dentin and microorganisms, and inorganic residues from dentin and instruments (SEN; WESSELINK; TURKUN, 1995). This layer adheres to the walls of the root canal, blocking the opening of dentin tubules (SEN et al., 1995) and making difficult the bacterial invasion (LOVE; CHANDLER; JENKINSON, 1996; PETERS, L. B.; WESSELINK; MOORER, 2000). On the other hand, studies showed that the smear layer removal reduces the adhesion of some types of microorganisms (SEN et al., 2003; SEN; SAFAVI; SPANGBERG, 1997; TURK; ATES; SEN, 2008; YANG; BAE, 2002; YANG et al., 2006) and improves the sealing of the root canal obturation (KOKKAS et al., 2004; SALEH et al., 2002; SHAHRAVAN et al., 2007).

An irrigation with NaOCl after the canal instrumentation is able to remove the organic portion of the smear layer, but to remove its inorganic components and completely clean dentin walls is necessary to use chelating solutions or demineralizing agents, such as the ethylenediaminetetraacetic acid (EDTA) (HAAPASALO et al., 2014; ZEHNDER, 2006). However, new demineralizing solutions have been proposed to aid in the process of sanification (LOTTANTI et al., 2009; ZEHNDER et al., 2005). Recently the peracetic acid (PAA) was suggested as a strong candidate to substitute the EDTA (DE-DEUS et al., 2011; LOTTANTI et al., 2009) because this substance, besides to remove the inorganic components of smear layer, is sporicidal, bactericidal, fungicidal and virucidal in concentrations below 0.5%, even in the presence of proteins (MCDONNELL; RUSSELL, 1999). When PAA is used after the instrumentation it might

be able to increase the disinfection of the root canal system previously prepared with NaOCl (GIRARD et al., 2005).

The etidronic acid (HEDP), a solution that prevents the bone resorption and is used systemically in patients that have osteoporosis and Paget diseases (RUSSELL, 2011), was also suggested as a substitute to the chelators commonly applied (ZEHNDER et al., 2005). This irrigant can be used mixed with NaOCl solutions, without short term loss of the desired properties of both compounds (BIEL et al., 2017; ZEHNDER et al., 2005). It has been hypothesized that when this mixture is used during the biomechanical preparation the smear layer is never formed (GIRARD et al., 2005; LOTTANTI et al., 2009). However, the HEDP is a weak chelating agent that needs 300 s to completely remove the smear layer at concentrations of 9 and 18% (DE-DEUS; ZEHNDER; et al., 2008), contrarily to 17% EDTA and 0.5% and 2.25% PAA that need 60 s to completely clean the dentin surface (DE-DEUS et al., 2011).

Studies showed that the decalcifying agents also remove dentin minerals exposing the collagen matrix, reducing the microhardness, increasing the permeability, roughness and wettability (ARI; ERDEMIR, 2005; ARI; ERDEMIR; BELLI, 2004; BALLAL et al., 2010; DOĞAN; ÇALT, 2001; HU; LING; et al., 2010; PATIL; UPPIN, 2011; PEREZ-HEREDIA et al., 2008; TARTARI; DE ALMEIDA RODRIGUES SILVA; et al., 2013). The exposition of the collagen matrix can contribute to microorganisms adherence and biofilm formation because the collagen has a fundamental role in the adhesion capability of certain types of microorganisms, such as *Enterococcus faecalis* (KISHEN et al., 2008; NALLAPAREDDY et al., 2000). The collagen also showed to benefit the adhesion of the resin based sealers (NEELAKANTAN et al., 2015; NEELAKANTAN et al., 2012; PRADO; SIMAO; GOMES, 2013), but if the filling material does not completely infiltrate the exposed collagen matrix the adhesion can be compromised (DE-DEUS; NAMEN; et al., 2008; DE MUNCK et al., 2005; PASHLEY et al., 2004; SCHWARTZ, 2006; TAY et al., 2006).

Therefore, it has been suggested the use of the NaOCl solutions after the chelating agents with the intent to remove the collagen fibers from the surface, in a process called deproteination (DI RENZO et al., 2001). This process could result in a reduction of *E. faecalis* capability to adhere to dentin (KISHEN et al., 2008; LOVE, 2001). On the contraty, the removal of this collagen matrix will expose the mineralized

subsurface that can favor the adhesion of microorganisms like *Candida albicans,* which showed to have the adherence and growth improved in the presence of magnesium and calcium ions (HOLMES; CANNON; SHEPHERD, 1991; KLOTZ et al., 1993), and prejudice the adhesion of the sealers that need the collagen matrix to adhere (NEELAKANTAN et al., 2015; NEELAKANTAN et al., 2011; NUNES et al., 2008).

An alternative for the final flushes with NaOCl is the use of chlorhexidine (CHX) solutions. The CHX has been proposed as potential substitute for NaOCl during the biomechanical preparation, especially in the cases of resistant microorganisms, allergies and incomplete root formation, due to the excellent antimicrobial effect and biocompatibility (GOMES et al., 2013). However, CHX does not act on the rests of organic matter (ZEHNDER, 2006), seeming to be more promisor as a final irrigant after the use of decalcifying agents, with the advantage that it adsorbs to the dentin surface (RÖLLA; LOE; SCHIOTT, 1970; VIANNA; GOMES, 2009). Once binded to tissues, it is slowly released in a phenomenon called substantivity (PARSONS et al., 1980) that extends its antimicrobial activity and reduces significantly the adhesion of microorganisms (BACA et al., 2011; KISHEN et al., 2008; KOMOROWSKI et al., 2000; YANG et al., 2006; ZAMANY; SAFAVI; SPANGBERG, 2003). This solution showed to cause only minors alterations in dentin structure (ARI et al., 2004; PATIL; UPPIN, 2011) and does not affect the adhesion of resin based sealers to the root canal (NEELAKANTAN et al., 2011; ROCHA et al., 2012). It has been also been suggested that this substance has an inhibitory effect on the matrix metaloproteinases increasing the long-term stability of the interface between the dental materials and dentin (CARRILHO et al., 2007; HEBLING et al., 2005).

Besides of the surface composition, characteristics of the substratum, such as roughness, surface free energy, and wettability, are believed to also influence the initial microorganisms adherence and the adhesion of dental materials (MARSHALL et al., 2010; QUIRYNEN; BOLLEN, 1995; TANG et al., 2011; TEUGHELS et al., 2006). Studies showed that high roughness and free energy provide are more favorable surfaces for colonization and biofilm formation (AN; FRIEDMAN, 1998; QUIRYNEN; BOLLEN, 1995; SUBRAMANI et al., 2009; TEUGHELS et al., 2006) and also increase the micromechanical interlocking of the dental materials (BAIER, 1992; MARSHALL et al., 2010; TAGAMI; TAO; PASHLEY, 1990; TAO; PASHLEY, 1988). However, recent papers reported that the surface free energy appears to be more important than the

roughness for the formation of fungal biofilm (BURGERS et al., 2010) and that the increase on roughness did not stimulate the initial microbial adhesion (DO NASCIMENTO et al., 2013; FERREIRA RIBEIRO et al., 2016) or improve the bond strength of dental materials to the surfaces (GONZAGA et al., 2015; SEVGICAN et al., 2004).

Based on the literature, it was possible to observe that it is necessary to use more than one irrigation solution to better clean and disinfect of the root canal system and that these solutions can alter the dentin physicochemical properties. However, in the case of new irrigants and irrigation protocols some of the modifications produced remain unknown, and it is not possible to suggest the best irrigation protocol to reduce the adhesion of the microorganisms in cases of recontaminations, and at the same time improve the long-term stability of the adhesion of endodontic sealers to dentin.

Therefore, the objectives of this research, according to the sequence of the articles inserted in the thesis, were:

- 1st article: To determine, by iodometric titration, the effects of 10% and 20% alkaline tetrasodium ethylenediaminetetraacetic acid ($EDTANa_4$) and 17% trisodium ethylenediaminetetraacetic acid ($EDTAHNa_3$) in the free available chlorine of NaOCl; To evaluate the capability of NaOCl to dissolve organic matter when mixed with 10% and 20% $EDTANa_4$ and 17% $EDTAHNa_3$; To define, by scanning electron microscopy (SEM), the time necessary for the smear layer removal by the chelators alone and mixed with NaOCl.

- 2nd article: To evaluate the effect of individual and combined use of NaOCl, HEDP, and $EDTAHNa_3$ on the dissolution of organic matter at several time periods, by using samples of bovine muscle tissue.

- 3rd article: To determine the capability of 1%, 2.5% and 5% NaOCl to dissolve organic matter; To evaluate, by the attenuated total reflectance of Fourier transform infrared spectroscopy (ATR-FTIR) tech-

nique, the chemical alterations on the composition of the dentin surface produced by the same concentrations of NaOCl, at different exposure times.

- 4th article: To determinate of the effects of $EDTAHNa_3$, HEDP, $EDTANa_4$ and PAA at several concentrations and exposure times on the organic and inorganic components of dentine surfaces and to evaluate the effects of several combinations of these decalcifying agents with NaOCl on dentin, by the ATR-FTIR technique.

- 5th article:
  5.1 To quantify, by ATR-FTIR, the CHX adhesion to mineralized dentin and to dentin submitted to irrigation protocols employing NaOCl and different chelating agents previously to the final flush with CHX.

The analysis performed until the step 5.1 were decisive to define the concentrations and the time of use of the chemical solutions in the irrigation protocols tested in the experiments of the steps 5.2 and 6.

5.2 To measure the roughness and wettability of dentin surface after the use of different irrigation protocols with a benchtop roughness tester and sessile drop technique, respectively; and to quantify, by means of confocal laser scanning microscopy (CLSM), the adhesion of *E. faecalis* and *C. albicans* to the dentin surface submitted to the same protocols applied in the experiments of roughness and wettability.

- 6th article: To determine, by push-out assessment, the impact of different irrigation protocols in dentin adhesion of the AH Plus sealer in 7 days and 20 months after the root canal filling.

2 ARTICLES

2 ARTICLES

2.1 Article 1 - **Mixture of alkaline tetrasodium EDTA with sodium hypochlorite promotes in vitro smear layer removal and organic matter dissolution during biomechanical preparation.**

INTERNATIONAL ENDODONTIC JOURNAL

doi:10.1111/iej.12595

Mixture of alkaline tetrasodium EDTA with sodium hypochlorite promotes *in vitro* smear layer removal and organic matter dissolution during biomechanical preparation

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Abstract

Tartari T, Oda DF, Zancan RF, da Silva TL, de Moraes IG, Duarte MAH, Bramante CM. Mixture of alkaline tetrasodium EDTA with sodium hypochlorite promotes *in vitro* smear layer removal and organic matter dissolution during biomechanical preparation. *International Endodontic Journal.*

Aim The aim of this study was to determine the following: (i) the quantity of free chlorine in mixtures of equal proportions of sodium hypochlorite (NaOCl) with trisodium ethylenediaminetetraacetic acid ($EDTAHNa_3$) and alkaline tetrasodium ethylenediaminetetraacetic acid ($EDTANa_4$); (ii) organic matter dissolution; and (iii) the time necessary to remove the smear layer by these irrigants alone and when mixed.

Methodology The solutions were mixed in a 1 : 1 ratio and then iodometrically titrated over time to determine the quantity of free available chlorine. The capability of organic matter dissolution by the solutions alone and the mixtures of irrigants was analysed by weighing bovine muscle tissue specimens before and after submission to the following groups ($n = 10$): G1 0.9% saline solution (control), G2 2.5% NaOCl, G3 17% $EDTAHNa_3$, G4 10% $EDTANa_4$, G5 20% $EDTANa_4$, G6 5% NaOCl + 17% $EDTAHNa_3$, G7 5% NaOCl + 10% $EDTANa_4$ and G8 5% NaOCl + 20% $EDTANa_4$. The times necessary for smear layer removal were determinated on discs of bovine dentine with a standardized smear layer produced with SiC papers using a scanning electron microscope that did not require the samples to be sputter coated. The dentine discs were submitted to the same experimental groups previously described ($n = 10$) over several time periods, and the photomicrographs acquired were scored for the presence of smear layer. The parametric data of tissue dissolution were analysed using two-way ANOVA and one-way ANOVA with Tukey's *post hoc* tests ($\alpha < 0.05$), whilst nonparametric data of smear layer removal were analysed by Friedman test ($\alpha < 0.05$) and the Kruskal Wallis test with Dunn's *post hoc* ($\alpha < 0.05$).

Results $EDTAHNa_3$ caused an almost complete and immediate loss of free available chlorine from NaOCl, whilst $EDTANa_4$ promoted a slow and concentration-dependent decline. The organic matter was not dissolved in the control group, EDTA groups or the mixture of NaOCl + 17% $EDTAHNa_3$ group ($P > 0.05$). NaOCl alone and the associations of NaOCl + $EDTANa_4$ dissolved tissue at all periods analysed ($P < 0.05$). The smear layer was not removed in the control and NaOCl groups ($P > 0.05$). The smear layer was removed at 1 min in the NaOCl + 17% $EDTAHNa_3$ group ($P < 0.05$); 2 min in 17% $EDTAHNa_3$ group ($P < 0.05$); and 5 min in 10% $EDTANa_4$, 20% $EDTANa_4$, 5% NaOCl + 10% $EDTANa_4$ and 5% NaOCl + 20% $EDTANa_4$ groups ($P < 0.05$).

Conclusions Alkaline $EDTANa_4$ was slower in removing the smear layer than $EDTAHNa_3$, but when mixed with NaOCl during biomechanical canal preparation promoted organic matter dissolution and smear

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layer removal simultaneously. However, the mixing of NaOCl and $EDTANa_4$ should be performed immediately before use to prevent the reduction of free available chlorine.

Keywords: dissolution, EDTA, free available chlorine, organic matter, smear layer, sodium hypochlorite.

Received 1 September 2015; accepted 6 December 2015

Introduction

A protocol has been recommended that includes use of NaOCl during biomechanical canal preparation to dissolve organic matter and kill microorganisms, followed by calcium complexing agents such as ethylenediaminetetraacetic acid (EDTA) to remove the inorganic components of the smear layer and finally a rinse with an antimicrobial agent, such as NaOCl, to optimize disinfection (Yamada *et al.* 1983, Zehnder 2006). However, the EDTA that remains inside the root canal, when in the form of disodium and trisodium salt, can rapidly interact with the sodium of the NaOCl used for final irrigation in a stoichiometric reaction forming chlorine gas (Fig. 1) (Baumgartner & Ibay 1987), and reducing the free available chlorine of the NaOCl (Zehnder *et al.* 2005). On the other hand, the degradation and deactivation of EDTA by NaOCl is very slow and does not compromise its clinical performance (Grawehr *et al.* 2003, Rossi-Fedele *et al.* 2012). One way to solve this problem and to simplify this protocol is to use a weak chelator such as etidronic acid (HEBP) that has alkaline pH, and can be mixed with NaOCl during canal preparations without causing significant short-term loss in the desired properties of both compounds (Lottanti *et al.* 2009).

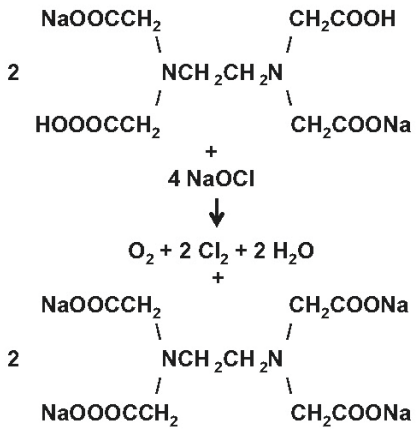

Figure 1 Stoichiometric reaction between NaOCl and disodium EDTA.

Based on the stoichiometric reaction between disodium EDTA and NaOCl, another form of EDTA that should be tested in a potential mixture with NaOCl is its tetrasodium salt $EDTANa_4$ at alkaline pH. $EDTANa_4$ is weaker in the removal of the smear layer than disodium EDTA, because although the pH of the solution is higher, with a greater dissociation of the molecules and an increase in the attraction for calcium ions, the availability of calcium ions from hydroxyapatite for chelation is lower (O'Connell *et al.* 2000). Due to its low chelating capability, the use of $EDTANa_4$ in endodontics has been forgotten. But this characteristic can be advantageous for the use of this irrigant when associated with NaOCl throughout biomechanical preparation, because as with HEBP, only subtle changes will affect the dentine structure (De-Deus *et al.* 2008). In addition, studies have revealed that the alternating irrigation regimen of NaOCl and EDTA promotes greater disinfection (Byström & Sundqvist 1985, Soares *et al.* 2010), probably because EDTA removes the smear layer, destabilizes (Cavaliere *et al.* 2014) and disrupts biofilms (Bryce *et al.* 2009), improving the effectiveness of antimicrobial drugs (Zehnder *et al.* 2005, Cavaliere *et al.* 2014).

Based on the literature, it is possible to affirm that the use of a single irrigant with antimicrobial action and the ability to dissolve organic and inorganic matter during the biomechanical preparation could reduce the treatment time (Lottanti *et al.* 2009) and favour canal disinfection (Soares *et al.* 2010). The objective of this study was to test a potential chelating solution when mixed with NaOCl by determining the effects of 10% and 20% $EDTANa_4$ and 17% $EDTAHNa_3$ on: (i) the free available chlorine of NaOCl; (ii) comparing their capability to dissolve organic matter; and (iii) the time necessary for smear layer removal of these irrigants alone and when mixed. The null hypothesis tested was that 10% and 20% $EDTANa_4$ and 17% $EDTAHNa_3$ have similar

effects on the free available chlorine of NaOCl, tissue dissolution capability and smear layer removal time.

Materials and methods

Solutions

The solutions tested were 2.5% (wt/vol, pH 12.1) and 5% NaOCl (pH 12.2), 17% ethylenediaminetetraacetic acid as trisodium salt ($EDTAHNa_3$, pH 7.5) and 10% (pH 12.0) and 20% (pH 12.2) ethylenediaminetetraacetic acid as tetrasodium salt ($EDTANa_4$). The 17% $EDTAHNa_3$ solution used in the experiments was a commercially available product (Biodinâmica Química e Farmacêutica Ltda., Ibiporã, PR, Brazil). The 2.5% and 5% NaOCl (Sigma-Aldrich, St. Louis, MO, USA) and the 10% and 20% $EDTANa_4$ (Labsynth, Diadema, SP, Brazil) solutions were prepared by mixing pure chemicals with distilled water.

Available chlorine in mixtures of chelators with NaOCl

The NaOCl solutions were iodometrically titrated using potassium iodide, soluble starch, acetic acid and sodium thiosulphate (British Pharmacopoeia 1973) to confirm their available chlorine (HOCl/OCl) content before the experiments. Aqueous mixtures were prepared by combination of distilled water, 17% $EDTAHNa_3$ and 10% and 20% $EDTANa_4$ with 5% NaOCl in a 1 : 1 ratio. The mixtures were iodometrically titrated in duplicate to determine their available chlorine content immediately after mixing the solutions and then 10 min, 30 min, 1 h and 1 day later. The pH of the mixtures was measured using a calibrated pH meter (Corning 430, Corning Incorporated, Corning, NY, USA) at the same time periods. Between measurements, the solutions were stored in dark containers at 25 °C.

Tissue dissolution

The groups tested ($n = 10$) in the tissue dissolution assay are presented in Table 1. The mixtures employed in G6, G7 and G8 were prepared immediately before the experiments.

Samples of bovine muscle tissue with defined surfaces (2 mm width × 2 mm thickness × 6 mm length) and similar weights (55.2 ± 1.98 mg) were obtained. After initial weighing on an electronic balance (FX-300; A&D Company, Tokyo, Japan) with a accuracy of 0.1 mg, each sample was assigned to the groups previously described and immersed for 5 min in individual tubes containing 10 mL of the test solutions and agitated in an ultrasonic tub (Cristófoli, Campo Mourão, PR, Brazil) for 15 s per min of immersion. Next, specimens were rinsed with distilled water for 30 s to remove the irrigants, blotted dry and weighed again. This procedure was repeated to obtain data after 5, 10 and 15 min of immersion. The solutions were renewed immediately before each time interval of immersion.

Table 1 Groups tested in tissue dissolution and smear layer removal assays

Groups	
G1 –	0.9% physiological saline solution (control);
G2 –	2.5% NaOCl;
G3 –	17% $EDTAHNa_3$;
G4 –	10% $EDTANa_4$;
G5 –	20% $EDTANa_4$;
G6 –	5% NaOCl and 17% $EDTAHNa_3$ mixed in equal parts, resulting in a solution with 2.5% NaOCl + 8.5% EDTA;
G7 –	5% NaOCl and 10% $EDTANa_4$ mixed in equal parts, resulting in a solution with 2.5% NaOCl + 5% $EDTANa_4$;
G8 –	5% NaOCl and 20% $EDTANa_4$ mixed in equal parts, resulting in a solution with 2.5% NaOCl + 10% $EDTANa_4$.

The collected data was normally distributed and presented homogeneity of variance. Two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to detect differences between the specimen weights before and after immersion. One-way analysis of variance (ANOVA) and Tukey's multiple comparison tests were used to detect differences between the groups at the same immersion periods. All hypotheses were tested at a 95% confidence level.

Smear layer removal

For the smear layer removal assay, coronal dentine slices with approximately 1.8-mm thickness were obtained from bovine teeth. Discs of 5.0 mm diameter were cut from the dentine slices using trephine drills (Neodent, Curitiba, PR, Brazil). The surface of the discs that were directed towards the pulp cavity were wet polished in a circular grinding machine (Arotec, São Paulo, SP, Brazil) using 300 and 600 grit SiC papers (Buehler, Lake Bluff, IL, USA) for 30 s each to produce a standardized smear layer. The polished surface was

marked using a diamond tip to facilitate disc positioning in the scanning electron microscope (SEM) and the analysis of the same area was carried out at different time periods. Images of the dentine samples with magnification of 500 × were obtained with an Aspex Express SEM (FEI Europe, Eidhoven, The Netherlands) with a 20 keV beam. This device does not require the samples to be sputter coated, allowing the same specimen to be evaluated before and after submission to different kinds of treatments.

The samples were then distributed into eight groups as described in Table 1 ($n = 10$). Each dentine disc was exposed to 1.5 mL of the test solutions in a microtube for four cumulative experimental time periods (1, 2, 3 and 5 min), with ultrasonic agitation performed for 15 s per min of immersion. After each immersion period, the dentine discs were rinsed for 30 s with 10 mL of distilled water to remove the irrigants and were dried in an oven at 37 °C for 15 min. Next, the samples were positioned in the SEM in the same manner as in the initial analysis, and new images were obtained. In this way, each sample served as its own control. The solutions were renewed before each experimental immersion interval because the time required for SEM analysis may have led to a reduction in the free available chlorine in the mixtures.

The photomicrographs acquired were scored independently by two previously trained, calibrated (Kappa score = 0.92) and blinded evaluators. The following scores for the presence of smear layer in dentine discs were assigned:

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

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

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

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

The scores attributed by the examiners were compared, and when a difference was found, the two evaluators jointly examined the sample to reach an agreement on the score of the disc.

Since scores were used to determine smear layer removal, the nonparametric Friedman test ($\alpha < 0.05$) was used to compare the same group over time, and the Kruskal Wallis test with Dunn's *post hoc* ($\alpha < 0.05$) was used to compare the different groups at the same time periods.

Results

Available chlorine in mixtures of chelators with NaOCl

Table 2 presents the percentage of free available chlorine relative to the theoretical maximum and the pH of the mixtures over time. The NaOCl aqueous solution when mixed with distilled water remained stable during the experimental period, presenting 100% of the theoretical maximum expected 1 day after mixing. The $EDTANa_4$ solutions caused minor reductions in the free available chlorine content of NaOCl, and the 17% $EDTAHNa_3$ solution caused an almost complete loss of free chlorine immediately upon mixing. The mixtures of 5% NaOCl with 10% $EDTANa_4$, 20% $EDTANa_4$ and 17% $EDTAHNa_3$ immediately after mixing resulted in 93.5%, 89% and 16%, respectively,

Table 2 Amounts of free available chlorine in percentage of the maximum content possible to be obtained after dilution and the measured pH in 1 : 1 mixtures of distilled water and the various chelators with 5% sodium hypochlorite over time

	Available chlorine in mixtures of chelators with NaOCl									
	Immediately after mixing		Mixture after 10 min		Mixture after 30 min		Mixture after 1 h		Mixture after 1 day	
Mixtures	Available chlorine	pH	Available chlorine	pH	Available chlorine	pH	Available chlorine	pH	Available chlorine	pH
5% NaOCl + distilled water	100%	12.1	100%	12.1	100%	12.1	100%	12.1	100%	12.1
5% NaOCl + 17% $EDTAHNa_3$	16%	8.0	11.5%	7.9	10%	7.9	8.5%	7.9	0.0%	8.8
5% NaOCl + 10% $EDTAHNa_4$	93.5%	12.1	90%	11.9	83%	9.2	62%	9.0	0.0%	9.5
5% NaOCl + 20% $EDTAHNa_4$	89%	12.0	83.5%	9.2	65%	9.1	49%	9.0	0.0%	9.8

of the maximum content of free available chlorine possible to be obtained in the mixtures that had the value of 2.5% observed in the mixture of 5% NaOCl and distilled water. After 10 min, these values became 90%, 83.5% and 11.5%, respectively, and in 30 min, the available chlorine content was 83%, 65% and 10%, respectively, of the theoretical maximum expected. The reductions in quantities of available chlorine occurred over time, and after 1 h, the mixtures of 5% NaOCl with 10% and 20% $EDTANa_4$ had 62% and 49% of free available chlorine, respectively. In contrast, the mixture of NaOCl with 17% $EDTAHNa_3$ had only 8.5%. There was no free available chlorine in all mixtures on the 1-day analysis.

Tissue dissolution

As shown in Table 3, there was a significant reduction in the weights of samples after treatment in G2, G7 and G8 between all periods analysed ($P < 0.05$). In G1, G3 and G6, there was no tissue dissolution ($P > 0.05$). Significant increases in tissue weights over time were observed in G4 and G5 ($P < 0.05$).

The intergroup comparison revealed that after 5 min, G2, G7 and G8 caused similar reductions ($P > 0.05$) in the weights of the samples that were significantly greater ($P < 0.05$) than the reductions detected in the other groups. In addition, at 5 min G1 was similar to G3 ($P > 0.05$), and G6 was similar to G1, G3 and G4 ($P > 0.05$). After 10 min, G7 was associated with significantly more dissolution than G2 and G8 ($P < 0.05$) that were similar to each other ($P > 0.05$), and promoted significantly more dissolution than G1, G3 and G6 ($P < 0.05$). After 15 min, G7 had the same dissolution capability as G2 ($P > 0.05$) but significantly higher than that of G8 ($P < 0.05$), whilst G2 was similar to G8 ($P > 0.05$). These groups in turn dissolved more muscle tissue than G1, G3 and G6 ($P < 0.05$) which were similar ($P > 0.05$). On the other hand, in all periods analysed, G5 promoted significantly more increase in the weight of muscle tissue fragments than G4 ($P < 0.05$).

Smear layer removal

The distribution of scores for smear layer removal is presented in Table 4. With the exception of saline solution (G1, $P > 0.05$) and NaOCl alone (G2, $P > 0.05$), all other solutions and mixtures completely removed the smear layer during the periods analysed. In G6, all specimen surfaces were cleaned in 1 min ($P < 0.05$). In G3, significant differences were detected from before immersion to 1 min ($P < 0.05$)

Table 3 Mean (X) and standard deviation (SD) of the weights in milligrams of tissue fragments, and differences from the initial weight after 5, 10 and 15 min of immersion in the individual solutions and mixtures

Tissue dissolution assay							
	Initial weight	After 5 min of immersion		After 10 min of immersion		After 15 min of immersion	
Groups	X ± SD	X ± SD	Difference from initial weight (%)	X ± SD	Difference from initial weight (%)	X ± SD	Difference from initial weight (%)
G1 – Saline	$56.2 \pm 1.3^{A,a}$	$53.9 \pm 2.8^{C,a}$	−4.0	$54.6 \pm 1.7^{C,a}$	−2.8	$54.8 \pm 1.8^{C,a}$	−2.4
G2 – 2.5% NaOCl	$55.7 \pm 1.9^{A,a}$	$48.8 \pm 2.9^{D,b}$	−12.3	$39.4 \pm 2.3^{D,c}$	−29.0	$29.6 \pm 3.7^{DE,d}$	−46.8
G3 – 17% $EDTAHNa_3$	$55.2 \pm 1.4^{A,a}$	$53.8 \pm 1.7^{C,a}$	−3.2	$53.4 \pm 2.0^{C,a}$	−3.9	$54.7 \pm 2.5^{C,a}$	−1.6
G4 – 10% $EDTAHNa_4$	$54.1 \pm 1.5^{A,c}$	$58.4 \pm 2.0^{B,b}$	+7.9	$60.8 \pm 2.3^{B,a}$	+12.3	$61.5 \pm 1.8^{B,a}$	+13.6
G5 – 20% $EDTAHNa_4$	$56.6 \pm 1.8^{A,b}$	$67.9 \pm 2.91^{A,a}$	+19.8	$67.0 \pm 3.7^{A,a}$	+18.3	$68.3 \pm 3.3^{A,a}$	+20.6
G6 – Mixture of 5% NaOCl + 17% $EDTAHNa_3$	$54.6 \pm 2.4^{A,a}$	$54.9 \pm 1.5^{BC,a}$	+0.5	$55.1 \pm 2.0^{C,a}$	+0.9	$54.5 \pm 2.1^{C,a}$	−0.1
G7 – Mixture of 5% NaOCl + 10% $EDTAHNa_4$	$55.1 \pm 2.2^{A,a}$	$45.5 \pm 3.8^{D,b}$	−17.4	$35.3 \pm 4.0^{E,c}$	−35.9	$27.3 \pm 5.0^{E,d}$	−50.4
G8 – Mixture of 5% NaOCl + 20% $EDTAHNa_4$	$55.4 \pm 2.0^{A,a}$	$48.6 \pm 3.3^{D,b}$	−12.2	$40.4 \pm 3.5^{D,c}$	−27.0	$33.2 \pm 3.8^{D,d}$	−40.0

Two-way ANOVA $P < 0.05$; different lowercase letters in rows indicate statistically significant intragroup differences. One-way ANOVA $P < 0.05$; different capital letters in columns indicate statistically significant intergroup differences in the same time period.

with complete smear layer removal out of 8 specimens. All G3 specimens were cleaned within 2 min. Significant differences from before immersion were detected in G4, G5, G7 and G8 at 2 min ($P < 0.05$). In G4, G5, G7 and G8, complete smear layer removal was observed at 2 min out of 2, 6, 5 and 2 specimens, respectively, and at 3 min out of 7, 9, 8 and 8 specimens, respectively. All specimens in these groups were free of the smear layer within 5 min.

The intergroup comparisons revealed that smear layer removal in G3, G4, G5, G6, G7 and G8 was similar after 3 min of disc immersion in the solutions ($P > 0.05$). However, these groups were significantly different from G1 and G2 ($P < 0.05$), which were similar to each other ($P > 0.05$).

Discussion

The null hypothesis was rejected in part, as 10% and 20% $EDTANa_4$ associated with NaOCl favoured greater free available chlorine and tissue dissolution capability than $EDTAHNa_3$, and required a longer time to remove the smear layer both when used alone and mixed with NaOCl. However, the null hypothesis was partially accepted as 5% and 10% $EDTANa_4$ and 17% $EDTAHNa_3$ did not dissolve organic matter.

In the iodometric titration of the mixtures, the $EDTANa_4$ at alkaline pH caused a slow and concentration-dependent reduction in free available chlorine of NaOCl (Table 2), showing that this mixture can be used without short-term loss of the desired properties of NaOCl. However, $EDTAHNa_3$ caused an almost complete loss of the free chlorine of NaOCl immediately after mixing, suggesting this association is not suitable. Similar results of NaOCl neutralization by several kinds of EDTA have been demonstrated (Grawehr *et al.* 2003, Girard *et al.* 2005, Zehnder *et al.* 2005). The 1-day analysis period was performed to verify the stability of the mixtures. However, free available chlorine was not observed at this time period, suggesting that the mixtures should be prepared immediately before use.

The dissolution tests were performed with bovine muscle because this kind of tissue is obtained easily, enable the tests of all groups to be performed on the same day and allow a better standardization of the surface area of each sample (Stojicic *et al.* 2010, Tartari *et al.* 2015). The results of this assay revealed that only NaOCl was able to dissolve organic matter

Table 4 Median (Med), minimum and maximum (Min - Max) scores for the presence of smear layer in each group of 10 dentine discs and number of discs free of smear layer (N) before and after 1, 2, 3 and 5 min of immersion in the irrigation solutions and mixtures

	Smear layer removal assay									
	Before immersion		After 1 min of immersion		After 2 min of immersion		After 3 min of immersion		After 5 min of immersion	
Groups	Med (Min – Max)	*N*	Med (Min – Max)	*N*	Med (Min – Max)	*N*	Med (Min – Max)	*N*	Med (Min – Max)	*N*
G1 – Saline	4 (4–4)^{A,a}	0	4 (4–4)^{A,a}	0	4 (4–4)^{A,a}	0	4 (4–4)^{A,a}	0	4 (4–4)^{A,a}	0
G2 – 2.5% NaOCl	4 (4–4)^{A,a}	0	4 (4–4)^{A,a}	0	4 (4–4)^{A,a}	0	4 (4–4)^{A,a}	0	4 (4–4)^{A,a}	0
G3 – 17% $EDTAHNa_3$	4 (4–4)^{A,a}	0	1 (1–2)^{BC,b}	8	1 (1–1)^{B,b}	10	1 (1–1)^{B,b}	10	1 (1–1)^{B,b}	10
G4 – 10% $EDTAHNa_4$	4 (4–4)^{A,a}	0	3 (2–4)^{ABC,ab}	0	2 (1–3)^{AB,bc}	2	1 (1–2)^{B,c}	7	1 (1–1)^{B,c}	10
G5 – 20% $EDTAHNa_4$	4 (4–4)^{A,a}	0	2.5 (2–4)^{ABC,ab}	1	1 (1–3)^{B,bc}	6	1 (1–2)^{B,c}	9	1 (1–1)^{B,c}	10
G6 – Mixture of 5% NaOCl + 17% $EDTAHNa_3$	4 (4–4)^{A,a}	0	1 (1–1)^{C,b}	10	1 (1–1)^{B,b}	10	1 (1–1)^{B,b}	10	1 (1–1)^{B,b}	10
G7 – Mixture of 5% NaOCl + 10% $EDTAHNa_4$	4 (4–4)^{A,a}	0	3 (1–4)^{ABC,ab}	1	1.5 (1–3)^{B,bc}	5	1 (1–2)^{B,bc}	8	1 (1–1)^{B,c}	10
G8 – Mixture of 5% NaOCl + 20% $EDTAHNa_4$	4 (4–4)^{A,a}	0	4 (2–4)^{AB,ab}	0	2 (1–3)^{AB,bc}	2	1 (1–2)^{B,c}	8	1 (1–1)^{B,c}	10

Friedman *P*-value <0.05; different lowercase letters in rows indicate statistically significant intragroup differences. Kruskal–Wallis with Dunn's *post hoc* *P*-value <0.05; different capital letters in columns indicate statistically significant intergroup differences in the same time period.

(Table 3) which is in agreement with previous studies (Naenni *et al.* 2004, Irala *et al.* 2010, Stojicic *et al.* 2010, Tartari *et al.* 2015). With relation to the mixtures, only when associated with $EDTANa_4$ was the dissolution capability of NaOCl not altered (Table 3). Another solution that can be mixed with NaOCl is etidronic acid; however, this substance reduces the organic matter dissolution capability of NaOCl (Tartari *et al.* 2015). In a pilot study, the mixtures of NaOCl and $EDTAHNa_3$ at alkaline pH and $EDTANa_4$ at neutral pH (data not shown) were also tested and found to have a significant decrease in the capability of NaOCl to dissolve organic matter. This suggests that the interaction between EDTA and NaOCl is influenced by the pH of the solution and by the quantities of sodium ions present in the chelating agent. In the $EDTANa_4$ and NaOCl mixtures, the initial pH values were alkaline and decreased over time (Table 2). As these mixtures had an alkaline pH, the balance of hypochlorous acid/hypochlorite ion ($HOCl/OCl^-$) is maintained in the hypochlorite ion side that has greater tissue dissolving capability than hypochlorous acid (Christensen *et al.* 2008, Macedo *et al.* 2010, Ballal *et al.* 2011, Jungbluth *et al.* 2011, Rossi-Fedele *et al.* 2011, Tartari *et al.* 2015).

In the tissue specimens immersed in 10% and 20% $EDTANa_4$, significant increases in fragment weights occurred at all periods analysed (Table 3). New solutions were prepared and the analyses of the groups were repeated, producing the same results. Although the specimens were blotted dry, this procedure was performed similarly for all groups to remove the excess of the solutions present on the surface without manipulating the samples. Based on this fact, the composition of some bovine muscle fragments was analysed before and after treatment with 17% $EDTAHNa_3$, 10% $EDTANa_4$ and 20% $EDTANa_4$ by energy-dispersive spectroscopy (EDS), and the results revealed increases in the quantities of sodium ions of 1.7, 2.5 and 5.1 times for 17% $EDTAHNa_3$, 10% $EDTANa_4$ and 20% $EDTANa_4$, respectively. These major increases of sodium ions in the specimen surfaces treated by $EDTANa_4$ may have induced greater tissue hydration by the water present in the solutions than that caused by $EDTAHNa_3$ observed in previous studies (Naenni *et al.* 2004, Stojicic *et al.* 2010, Tartari *et al.* 2015). The greatest hydration may have also resulted in the greater dissolution observed in G7. This did not occur in G8, because in this group, the $EDTANa_4$ also led to greater loss in the free chlorine of the mixture.

The smear layer removal experiment does not mimic the clinical condition; however, the time necessary to remove the smear layer in some groups is unknown and should be determined. Using dentine discs of bovine teeth, which have a similar structure (Schilke *et al.* 2000) and chemical composition to human dentine (Bachmann *et al.* 2003, Botta *et al.* 2012) and allow a better standardization, the smear layer removal was analysed in a SEM after different periods of immersion in the solutions. Although at higher pH, the chelation efficiency of EDTA is greater due to the higher ratio of ionized molecules in the solution (Sreebny & Nikiforuk 1951, O'Connell *et al.* 2000), the $EDTANa_4$ at alkaline pH was less effective in removing the smear layer than $EDTAHNa_3$ at pH 7.5, both alone and in the mixtures (Table 4), corroborating with a previous study (O'Connell *et al.* 2000). This may be due to the constant product solubility of dentine, which leads to a lack of dentine demineralization at alkaline pH and a lack of available calcium ions for chelation (Sreebny & Nikiforuk 1951, O'Connell *et al.* 2000). The more rapid smear layer removal observed in G6 can be explained by the presence of free available chlorine in the mixture (Table 2), which assisted $EDTAHNa_3$ by dissolving the organic portion of the smear layer. Although the polishing was standardized, variations in the time required to remove the smear layer within the same group may be a result of differences in the thickness of the smear layer produced.

$EDTANa_4$ at alkaline pH is a promising calcium complexing agent when mixed with NaOCl during biomechanical canal preparation, with no loss of the desired properties of the individual components. This will reduce the complexity of the ideal irrigation protocol, and consequently, the treatment time. As $EDTANa_4$ is a weaker chelator than $EDTANa_3$, minor physicochemical alterations in root canal dentine can be expected. However, future studies are needed to evaluate the association of $EDTANa_4$ and NaOCl on dentine properties and root canal disinfection.

Conclusion

Alkaline $EDTANa_4$ was slower in removing smear layer than $EDTAHNa_3$, but when mixed with NaOCl during biomechanical canal preparation promoted organic matter dissolution and smear layer removal simultaneously. The mixing of

NaOCl and $EDTANa_4$ should be performed immediately before use.

Acknowledgements

This study was supported by State of São Paulo Research Foundation, FAPESP (2013/19789-3 and 2012/06738-9).

Conflict of interest

The authors have stated explicitly that there are no conflict of interests in connection with this article.

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2.2 Article 2 - **Etidronate causes minimal changes in the ability of sodium hypochlorite to dissolve organic matter**

INTERNATIONAL ENDODONTIC JOURNAL

doi:10.1111/iej.12329

Etidronate causes minimal changes in the ability of sodium hypochlorite to dissolve organic matter

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Abstract

Tartari T, Guimarães BM, Amoras LS, Duarte MAH, Silva e Souza PAR, Bramante CM. Etidronate causes minimal changes in the ability of sodium hypochlorite to dissolve organic matter. *International Endodontic Journal*.

Aim To evaluate the effect of individual and combined use of sodium hypochlorite (NaOCl), etidronate (HEDP) and ethylenediaminetetraacetic acid (EDTA) in tissue dissolution.

Methodology Sixty fragments of bovine muscle tissue were prepared and their weights determined on a precision scale. The samples were then distributed in the following groups ($n = 10$): G1 saline solution (control); G2 17% EDTA; G3 18% HEDP; G4 2.5% NaOCl; G5 mixture of 5% NaOCl + 17% EDTA; and G6 mixture of 5% NaOCl + 18% HEDP. The specimens in each group were immersed in the solutions for 5, 10 and 15 min and reweighted at each time period. Analysis of variance (ANOVA) and Tukey's multiple-comparison tests ($\alpha<0.05$) were applied to identify the intragroup and intergroup differences.

Results G1, G2, G3 and G5 did not dissolve the organic matter. G4 and G6 significantly reduced the weights of specimens at all periods. Amongst the groups, the difference in ability to dissolve organic matter was greater and significant in the following order G4 = G6 > G5 = G3 = G2 = G1 after 5 min of immersion and G4 > G6 > G5 = G3 = G2 = G1 after 10 and 15 min of immersion.

Conclusion The only solution capable of dissolving organic matter was NaOCl. In the mixtures analysed, this ability was arrested by EDTA; however, it was minimally affected by the HEDP, proving that this combination, if used during the biomechanical preparation, is able to dissolve of organic matter.

Keywords: dissolution, edetic acid, etidronic acid, organic matter, sodium hypochlorite.

Received 25 March 2014; accepted 31 May 2014

Introduction

Chemical solutions are required in conjunction with mechanical preparation to assist in the cleaning and shaping of canals (Zehnder 2006). Tissue-dissolving capability of irrigating solutions is important to enhance cleaning (Turkun & Cengiz 1997), but this characteristic depends on several factors, such as its temperature and concentration, the contact area with tissues, time of action, mechanical agitation, renewal frequency and the ratio of solution volume to the mass of organic tissue (Cunningham & Balekjian 1980).

Several irrigants have been proposed for use, such as sodium hypochlorite (NaOCl), chlorhexidine (CHX), ethylenediaminetetraacetic acid (EDTA), citric acid and MTAD solutions (Zehnder 2006). Sodium hypochlorite remains the most recommended and popular solution for root canal treatment, mainly for its good antimicrobial and tissue-dissolving properties (Zehnder

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et al. 2002). However, these solutions do not act on the inorganic components of the smear layer (Zehnder 2006). Therefore, it is necessary to use a Ca^{+2} chelating agent, such as EDTA to remove the inorganic components of smear layer. The EDTA may be used to perform a final flush (Villegas *et al.* 2002) or alternately with NaOCl during the preparation of the root canal (Soares *et al.* 2010).

Irrigant solutions are not completely removed from the root canal unless aspired or dried, before applying the next irrigant. As a result, endodontic irrigants can interact with each other (Zehnder *et al.* 2005, Prado *et al.* 2013). Recently, etidronate solution (HEDP) emerged as a substitute for the commonly used chelates, because it can be mixed with NaOCl, without interfering with its antimicrobial properties in the short term (Zehnder *et al.* 2005). It was also suggested that when the mixture is used during biomechanical preparation, the smear layer is not formed (Girard *et al.* 2005, Lottanti *et al.* 2009). In addition, hard-tissue debris accumulation in the isthmus area is reduced (Paqué *et al.* 2012). The HEDP is a weak chelator, therefore, is less aggressive than EDTA on dentine (Lottanti *et al.* 2009); however, if used for a final flush, these solutions need 300 s to completely remove the smear layer (De-Deus *et al.* 2008).

Tissues from different sources have been used in studies investigating the tissue-dissolving ability of irrigants including muscle tissue and palatal mucosa from pigs (Hasselgren *et al.* 1988, Zehnder *et al.* 2002, Naenni *et al.* 2004, Christensen *et al.* 2008), muscle tissue and dental pulps from cattle (Turkun & Cengiz 1997, Spano *et al.* 2001, Okino *et al.* 2004, Stojicic *et al.* 2010, Slutzky-Goldberg *et al.* 2013), connective tissue from rats (Hand *et al.* 1978) and liver from rabbits (Moorer & Wesselink 1982).

Little is known about the consequences of chemical interactions between HEDP and NaOCl. It seems likely that, if NaOCl and HEDP remain active when mixed, and this mixture is used during biomechanical preparation, the NaOCl with HEDP will dissolve pulp debris and eliminate micro-organisms, whilst preventing smear layer formation. This may make the final irrigation unnecessary, thus reducing the time of treatment. The objective of this study is to evaluate the effect of individual and combined use of NaOCl, HEDP and EDTA on tissue dissolution at several time periods. The null hypothesis is that the HEDP and EDTA mixtures do not interfere in the tissue dissolution ability of NaOCl.

Materials and methods

Solutions

The substances evaluated in the present study were saline solution, NaOCl, HEDP and EDTA. Physiological saline (0.9% sodium chloride) was acquired in a pharmacy and used as a control. A stock solution of NaOCl (Sigma-Aldrich, St. Louis, MO, USA) was iodometrically titrated to determine its content of available chlorine. Then, the solution was diluted to 2.5% and 5% NaOCl using distilled water. To obtain solutions of 18% HEDP (Zschimmer & Schwarz Mohsdorf GmbH & Co KG, Burgstädt, Germany), the pure chemical was mixed with distilled water. The 17% EDTA (Sigma-Aldrich) was prepared by dissolving disodium EDTA (Sigma-Aldrich) in distilled water with the aid of sodium hydroxide (NaOH) (Sigma-Aldrich); the pH was adjusted to 7.0 by adding hydrochloric acid (HCl) (Sigma-Aldrich).

All chemical substances were prepared and used just after the mixing.

Tissue dissolution

Bovine muscle tissue was cut into pieces of 2 × 2 × 6 mm using a stainless steel blade immediately after slaughtering the animals.

The samples were randomly assigned to six groups ($n = 10$), blotted dry and weighed on an electronic balance (FX-300; A&D Company, Tokyo, Japan) to determinate the initial weight of each sample in mg.

The irrigating solution groups tested were:

G1 0.9% physiological saline solution (control);
G2 18% HEDP;
G3 17% EDTA;
G4 2.5% NaOCl;
G5 5% NaOCl and 17% EDTA mixed in equal parts, resulting in a solution with 2.5% NaOCl + 8.5% EDTA;
G6 5% NaOCl and 18% HEDP mixed in equal parts, resulting in a solution with 2.5% NaOCl + 9% HEDP.

The solutions employed in groups G5 and G6 were mixed with a plastic spatula (Golgran, São Paulo, SP, Brazil) immediately before immersion of the specimens. The pH of the solutions and the mixtures were measured just before starting each test, using a pH meter (Hanna instruments, São Paulo, SP, Brazil).

All experiments were performed at a room temperature of 25 °C and with the water in an ultrasonic tub at 32 °C to mimic the average intracanal temperature (Cunningham & Balekjian 1980). Containers were placed into an ultrasonic tub and then filled with 15 mL of irrigation solutions. Next, the tissue fragments were submerged individually in the solutions. Ultrasonic agitation was performed for 15 s per each minute during the 5-min incubation period.

After 5 min in the solutions, the samples were immediately submerged in distilled water for 30 s to remove the irrigation solutions. They were next blotted dry and reweighed for comparison with the initial values. This procedure was repeated three times to obtain data after 5, 10 and 15 min of immersion in the solutions.

Statistical analysis

The collected data showed normal distribution and homoscedasticity. Analysis of variance (ANOVA), followed by the Tukey's multiple-comparison test, was used to detect any differences between the specimen weights before and after the various submersion periods and used to detect any differences between the groups at the same time periods. All hypotheses testing were performed at a 95% confidence level.

Results

The pH of the solutions, mean value, standard deviation and percentage difference of the initial mean for the weight of fragments of bovine muscle tissue before and after 5, 10 and 15 min of submersion in the irrigation solutions are presented in Table 1. The intragroup comparisons showed a significant decrease ($P < 0.01$) in weight of the fragments after all time periods of immersion in the NaOCl solution (G2) and in the mixture of NaOCl + HEDP (G6). The other groups were not associated in loss of weight at any time period.

The intergroup comparison showed that after 5 min, the reduction in weights was significant ($P < 0.01$) and occurred in the followings order G4 = G6 > G5 = G3 = G2 = G1, with no difference between G4 and G6. In the 10- and 15-min time periods, a significant ($P < 0.01$) difference between G4 and G6 was identified and occurred in this order G4 > G6 > G5 = G3 = G2 = G1.

Discussion

The null hypothesis was rejected because the association of HEDP or EDTA with NaOCl negatively interfered with the dissolution ability of the NaOCl, with the greater interference promoted by the EDTA.

This knowledge is important as the remnants of organic matter present inside the root canal system can serve as a substrate for the growth of microorganisms that survive biomechanical preparation or that contaminate the root canal after treatment (Love 2001, Gomes-Filho *et al.* 2008) and compromise the root filling (De-Deus *et al.* 2012).

Bovine muscle tissue was used instead of dental pulp due to the availability and easier standardization of the surface area of each sample, which can

Table 1 Mean (X), standard deviation (SD), and difference to T0 mean (%) in weight (mg) before (T0) and after 5 (T5), 10 (T10), and 15 (T15) min of submersion to the irrigation solutions

Groups	pH	T0 X ± SD	T5 X ± SD	Difference to T0 mean (%)	T10 X ± SD	Difference to T0 mean (%)	T15 X ± SD	Difference to T0 mean (%)
G1- Saline	6.8	$54.6 \pm 1.9^{A,a}$	$54.8 \pm 2.6^{A,a}$	+0.3	$54.1 \pm 2.4^{A,a}$	−0.9	$52.6 \pm 2.5^{A,a}$	−3.6
G2- EDTA 17%	7.0	$54.7 \pm 1.7^{A,a}$	$53.7 \pm 1.6^{A,a}$	−1.8	$53.7 \pm 1.6^{A,a}$	−1.8	$54.6 \pm 2.2^{A,a}$	−0.1
G3- HEDP 18%	10.8	$54.3 \pm 1.8^{A,a}$	$55.6 \pm 2.0^{A,a}$	+2.3	$54.8 \pm 2.4^{A,a}$	+0.9	$54.5 \pm 2.1^{A,a}$	+0.3
G4- NaOCl 2.25%	11.8	$54.9 \pm 1.8^{A,a}$	$49.9 \pm 2.12^{B,b}$	−9.1	$41.0 \pm 3.2^{C,c}$	−25.3	$32.5 \pm 3.58^{C,d}$	−40.8
G5- Mixture of NaOCl 5% + EDTA 17%	7.4	$54.3 \pm 2.2^{A,a}$	$54.8 \pm 2.4^{A,a}$	+0.9	$55.1 \pm 2.5^{A,a}$	+1.4	$54.6 \pm 2.6^{A,a}$	+0.5
G6- Mixture of NaOCl 5% + HEDP 18%	11.2	$55.4 \pm 1.3^{A,a}$	$52.0 \pm 2.1^{B,b}$	−6.1	$46.8 \pm 2.1^{B,c}$	−15.5	$40.6 \pm 2.5^{B,d}$	−26.7

Different lowercase letters in rows indicate statistically significant intragroup differences (*P*-value <0.01); different capital letters in columns indicate statistically significant intergroup differences in the same time period (*P*-value < 0.01).

influence the results (Turkun & Cengiz 1997, Stojicic *et al.* 2010). The tissue samples had an equal surface area with dimensions of 2 × 2 × 6 (H × W × L) and with similar initial weights in all groups (Table 1).

In the present study, the organic matter was dissolved only in G4 group where NaOCl was used alone, and G6, where it was mixed with HEDP in equal parts. However, this ability was greater for G4 after 10 and 15 min (Table 1). Concentrated solutions of NaOCl have higher antimicrobial activity and faster tissue dissolution rates (Spångberg *et al.* 1973). However, the biocompatibility of NaOCl is inversely proportional to its concentration (Spångberg *et al.* 1973, Estrela *et al.* 2002). There was no tissue dissolution when the fragments were immersed in saline solution, chelating agents and the mixture of NaOCl with EDTA (G1, G2, G3 and G5 Table 1). These results agree with previous studies (Hand *et al.* 1978, Hasselgren *et al.* 1988, Grawehr *et al.* 2003, Naenni *et al.* 2004, Okino *et al.* 2004, Irala *et al.* 2010, Stojicic *et al.* 2010). An increase in specimen weight was observed after 5-min exposure in G1, G3 and G5 and after 10- and 15-min exposure in G3 and G5, which can probably be explained by tissue hydration that probably occured as a result of absorption of water present in the solutions, as observed in previous studies (Hand *et al.* 1978, Naenni *et al.* 2004, Stojicic *et al.* 2010).

The tissue dissolution capacity of NaOCl solutions is a direct function of their free available chlorine, (Hand *et al.* 1978, Estrela *et al.* 2002, Zehnder *et al.* 2002) which consists of hypochlorous acid (HOCl) and the hypochlorite ion (OCl) (Macedo *et al.* 2010). Hypochlorous acid has a powerful bactericidal effect and prevails in more acidic solutions. On the other hand, a greater tissue dissolution capacity is related to the OCl , which prevails in alkaline solutions (Macedo *et al.* 2010). In this study, the mixture of NaOCl with EDTA had a pH of 7.4 and no tissue dissolution, whereas the mixture of NaOCl with HEDP had a pH of 11.2 with a greater tissue dissolution capability (Table 1). An explanation for the nondissolution of organic matter by the mixture of NaOCl with EDTA is that there is a reduction on the production of the OCl ion, which is responsible for its dissolution ability (Macedo *et al.* 2010). Another explanation is that there is an interaction of NaOCl with the chelators, leading to chlorine gas evaporation and a reduction of free available chlorine in solution (Baumgartner & Ibay 1987, Prado *et al.* 2013).

Nguy & Sedgley (2006) graphically demonstrated, using bioluminescent bacteria, that the second solution does not displace the first in the apical third of root canals, even after 6 mL of irrigation with a needle 1 mm from the apex, which may lead to an interaction between irrigants. To minimize interactions between irrigants that employs chelating solutions alternately with NaOCl, their aspiration is recommended, using such devices as the Endovac, followed by drying with paper points before the use of the other chemical substances.

A minimal change in organic matter dissolution observed when analysing the mixture of NaOCl with HEDP was probably due to the fact that this substance is a weak chelator (De-Deus *et al.* 2008, Tartari *et al.* 2013), able to cause some reduction in the free available chlorine content 1 h after mixing (Zehnder *et al.* 2005). However, adverse effects may occur during root canal treatment because NaOCl and HEDP remain active during use. Studies have shown that this may result in an increase in the surface roughness of dentine and in apical root canal deviation (Tartari *et al.* 2013, Silva e Souza *et al.* 2014).

It is difficult to make inferences from the results of laboratory studies, because of the variables that may be present in clinical practice. The results of this type of study are of value, because it is possible to standardize the solutions and the tissue fragments in terms of their origin, surface size and storage conditions (Irala *et al.* 2010). Due to this, the results allowed an accurate determination of the capacity of different solutions and their mixtures for organic matter dissolution. However, future studies are needed to evaluate the association of these substances simulating clinical use.

Conclusion

The only solution tested capable of dissolving organic matter was NaOCl. In the mixtures analysed, this ability was arrested by EDTA; however, it was only minimally affected by HEDP, proving that this combination, if used during biomechanical preparation, is likely to dissolve organic matter.

Acknowledgements

This study was supported by State of São Paulo Research Foundation, FAPESP (2013/19789-3 and 2012/06738-9).

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2.3 Article 3 - **Tissue dissolution and modifications in dentin composition by different sodium hypochlorite concentrations**

www.scielo.br/jaos
http://dx.doi.org/10.1590/1678-775720150524

Tissue dissolution and modifications in dentin composition by different sodium hypochlorite concentrations

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Submitted: November 4, 2016 - Modification: January 20, 2016 - Accepted: February 15, 2016

ABSTRACT

Sodium hypochlorite (NaOCl) remains the most used irrigation solution during root canal preparation because of characteristics such as wide-spectrum antimicrobial activity and organic tissue dissolution capacity. However, these solutions can alter dentin composition and there is no consensus on the optimal concentration of NaOCl to be used. Objectives: To determine the organic matter dissolution and changes in dentin chemical composition promoted by different concentrations of NaOCl over time. Material and Methods: Fragments of bovine muscle tissue were weighed before and after 5, 10, and 15 min of immersion in the groups (n=10): G1- 0.9% saline solution; G2- 1% NaOCl; G3- 2.5% NaOCl; and G4- 5% NaOCl. Bovine dentin fragments were subjected to the same irrigants and absorption spectra were collected by Attenuated Total Reflectance of Fourier Transform Infrared Spectroscopy (ATR-FTIR) before and after 0,5, 1, 2, 3, 5, 8, and 10 min of immersion in the solutions. The ratios of the amide III/phosphate and carbonate/phosphate absorption bands were determined. The tissue dissolution and carbonate/phosphate ratios were submitted to the two-way analysis of variance (ANOVA) with Tukey's multiple-comparison test ($\alpha<0.05$) and to the one-way analysis of variance with Tukey's ($\alpha<0.05$). The amide III/phosphate ratio was analyzed by Friedman test ($\alpha<0.05$) and the Kruskal-Wallis test with Dunn's post-hoc ($\alpha<0.05$). Results: The increase in NaOCl concentration and contact time intensified the dissolution of organic matter and dentin collagen with reduction in the amide III/phosphate ratio. Significant differences between all groups ($p<0.05$) were observed in the dissolution of organic matter at 10 min and in the amide III/phosphate ratio between the saline solution and 5% NaOCl at 5 min. The carbonate/phosphate ratio decreased significantly in G2, G3, and G4 after 0,5 min of immersion ($p<0.05$), but more alterations did not occur in the subsequent periods ($p>0.05$). Intergroup differences were not observed in this ratio ($p>0.05$). Conclusions: The increase in the exposure time and in the concentration of NaOCl solution lead to an increase in the tissue dissolution and dentin collagen deproteination. Furthermore, some carbonate ions are removed from the dentin inorganic phase by the NaOCl.

Keywords: Dentin. Dissolution. Fourier transform infrared spectroscopy. Organic matter. Sodium hypochlorite.

INTRODUCTION

The physical and chemical effects of the irrigation solutions used in endodontics are crucial for cleaning and disinfection, since studies have shown that a large number of root dentin walls remain untouched after biomechanical preparation[25]. Different auxiliary chemical agents have been proposed, however, sodium hypochlorite (NaOCl) solutions are the most widely used for endodontic procedures because of their characteristics such as wide-spectrum antimicrobial activity and organic

tissue dissolution capacity[29,33]. However, there is no consensus regarding the ideal concentration of NaOCl to be used.

An increase in the number of microorganisms was observed when intracanal medicament was not used between the treatment sessions and this fact was assigned to the organic tissue that remained in the root canal and provided ideal conditions for bacterial growth[9]. Possible ways to improve the tissue dissolution by NaOCl are the increase in the pH[7], the concentration and temperature of the solutions, ultrasonic agitation, and prolonged working time[29,32]. However, the increase in concentration of NaOCl solutions can lead to undesirable effects such as an increase in toxicity to the periapical tissues[13].

NaOCl solutions can also act in the dentin changing its chemical composition[19]. In mineralized dentin, the collagen fibrils are encapsulated by apatite crystals, thus the dimensions of molecules that can penetrate in the dentin structure should be smaller[35]. NaOCl molecules can penetrate in the apatite-encapsulated collagen matrix because of their low molecular weight (74.4 Da)[35], and as a nonspecific oxidizing and proteolytic agent, can oxidize the organic matrix, denature the collagen, and adversely affects the mechanical properties of dentin[24,34]. The effects of NaOCl solutions on the collagen of the dentin organic matrix may also affect the sealing ability and the adhesion of resin-based cements and root canal sealers that chemically bond to the dentinal collagen[17,22].

In addition, with the technological advancement in endodontics, the biomechanical preparation phase is becoming faster, and the use of more concentrated irrigants for adequate sanitization is probably necessary. Therefore, it is important to know how much the increase in NaOCl concentration, with the objective to enhance sanitization, improves the organic matter dissolution without causing much undesirable alterations of the chemical composition of the dentin. The aim of the present study was to determine the dissolution capacity of organic matter and the chemical alterations on the composition of the dentin surface produced by different concentrations of NaOCl at different exposure times. The null hypothesis tested was that the different concentrations of NaOCl solutions have similar capacity of tissue dissolution and effects on dentin composition and act similarly over time.

MATERIAL AND METHODS

Irrigation solutions

Concentrated (10-15%) NaOCl solution (Sigma-Aldrich; St. Louis, MO, USA) was diluted in distilled water to produce solutions with 1%, 2.5%, and 5% concentrations 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 0.9% physiological saline solution was used as a control. The pHs of the solutions were determined before the experiments using a calibrated pH meter.

Tissue dissolution

Bovine muscle tissue was acquired on the day of the experiment and kept refrigerated in 100% humidity. The muscle was cut with scalpel blades in pieces with 2x2x6 mm (width x thickness x length) and the specimens obtained were weighed on the FX-300 electronic balance (A&D Company; Tokyo, Japan). To do the sample calibration, the data obtained were submitted to statistical analysis to verify and ensure that all groups were statistically similar before the beginning of the experiment. Next, the samples were submitted to one of the following solutions (n=10): G1– 0.9% physiological saline solution (control); G2– 1% NaOCl; G3– 2.5% NaOCl; and G4– 5% NaOCl.

Specimens from each group were immersed for 5 min in individual containers filled with 10 mL of the test solution. All the containers were placed in an ultrasonic tub to agitate the irrigants for 15 s per each minute. Next, the specimens were submerged in distilled water for 0,5 min to remove the irrigation solutions. They were then blotted with filter paper and re-weighed. This procedure was repeated 3 times to obtain data of 5, 10, and 15 min of immersion. The solutions were renewed before each immersion period to simulate clinical conditions and to prevent saturation. All test procedures were done at room temperature (25°C).

ATR-FTIR

Crowns of bovine teeth were removed at the cementoenamel junction using a diamond disc at low-speed under water cooling. Then, the incisal of the crowns were removed in the same way. Each crown was then longitudinally sectioned in the mesiodistal direction in the Isomet 1000 cutting machine (Buehler Ltd.; Lake Bluff, IL, USA) to obtain the buccal and lingual portions. Slices with approximately 0.8 mm thicknesses were obtained from these crown halves. The slices were then cut again with a diamond disc at low-speed to remove the surrounding enamel and to obtain twenty specimens with approximately 4 mm x 4 mm x 0.8 mm (length x width x thickness) (Figure 1).

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 and 0.3 microns (Struers; Ballerup, Denmark) until a flat and smooth surface was obtained. Finally, the specimens were

immersed in distilled water and ultrasonicated for 1 min to remove any residue from the polishing. 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.

The specimens were randomly assigned to the previously described four groups (n=5). Each specimen was placed inside a microtube containing 1.5 mL of the solutions for 0,5 min and ultrasonicated for 15 s. Next, they were transferred to a microtube containing 1.5 mL of distilled water and rinsed for 1 min with 15 s of ultrasonic agitation. They were then dried with absorbent paper and the ATR-FTIR spectra recorded again. Specimens were replaced in the solutions for additional 0,5 min following the same protocol described to collect the new spectra. This process was sequentially repeated to obtain the spectra at time intervals of 0, 0.5, 1, 2, 3, 5, 8 and 10 min. However, after obtaining the 1 min spectrum, the ultrasonic agitation was performed for 15 s per each minute of immersion in the irrigants. To ensure the effectiveness of the solutions, they were renewed after the time intervals of 2, 5, and 8 min.

A typical absorbance spectrum obtained from a disc of untreated dentin is shown in Figure 2. In this spectrum the peaks between 3.750–750 cm^{-1} were identified. The areas of the absorption bands of phosphate (PO_4^{3-}), carbonate (CO_4^{3-}) , and amide III of each spectrum were determined. The wavenumber values employed for the area integrations for the amide III were between 1298–1216 cm^{-1} spectral range, between 888–816 cm^{-1} for the carbonate, and between 1170–780 cm^{-1} for the phosphate. Inside the phosphate spectral range there is the carbonate band at 888–816 cm^{-1}, whose value was subtracted to obtain the real value of the area of phosphate band.

To evaluate the effects of NaOCl solutions on the chemical composition of dentin, two parameters were calculated. The first was the amide III/phosphate ratio that was used to determine the collagen deproteination by NaOCl. The amide III band was chosen, for in this region of 1298–1216 cm^{-1} there is no overlapping with bands of other dentin components. Instead, in bands of amides A and B at 3115 and 2860 cm^{-1} and amide I at 1645 cm^{-1}, overlapping occurs with water bands, and in bands assigned to amide II, present at around 1550 cm^{-1}, the overlapping occurs with carbonate bands[3]. The effect of NaOCl in the inorganic phase of the dentin was evaluated using the carbonate/phosphate (PO_4^{3-})/(CO_4^{3-})ratio, which was the second parameter. The ratios were obtained by taking the quotient between the areas of the bands.

The amide III/phosphate ratio was measured to evaluate how the amide III or the phosphate changed when immersed in NaOCl solution. For example, when this ratio decreases, it means that the amount of amide III (organic matter) decreased

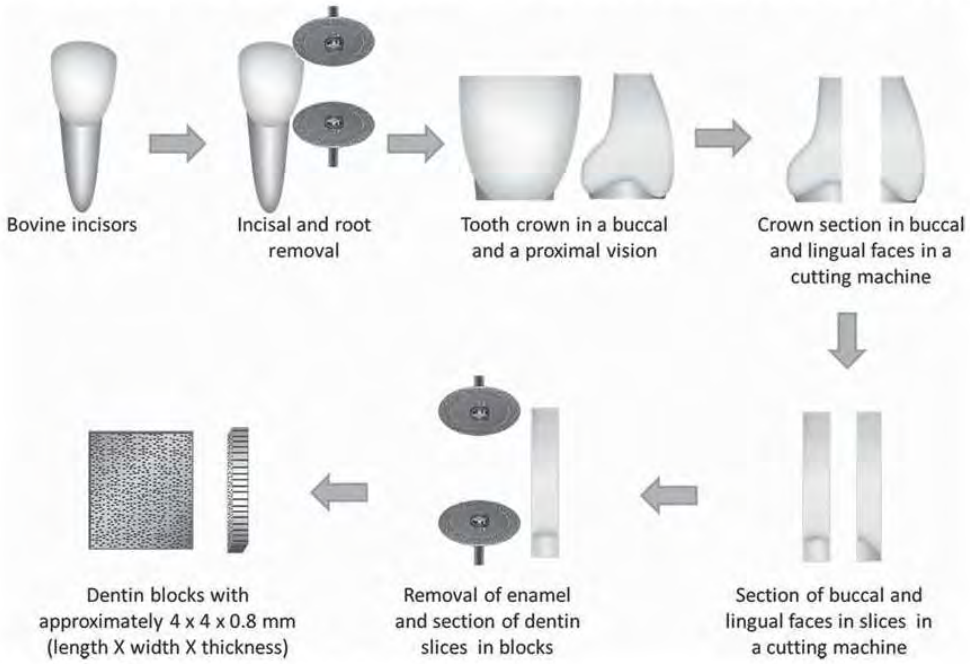

Figure 1- Sample preparation for the ATR-FTIR analysis

compared with the phosphate (inorganic matrix). However, when employing a chemical agent that removes organic matter and inorganic matter simultaneously, this ratio could stay unaltered. The carbonate/phosphate ratio is employed to evaluate the dissolution of the inorganic matrix; this ratio measures the carbonate dissolution in relation to the phosphate radical.

Statistical analysis

The collected data of tissue dissolution and carbonate/phosphate ratios showed normal distribution, and were submitted to the two-way analysis of variance (ANOVA) with Tukey's multiple-comparison test (α<0.05) to detect intragroup differences over time and the one-way analysis of variance with Tukey's (α<0.05) to detect any differences between the groups at the same time period.

The amide III/phosphate ratio exhibited abnormal distribution. The nonparametric Friedman test (α<0.05) was used to detect intragroup differences among different periods of immersion and the Kruskal-Wallis test with Dunn's *post-hoc* (α<0.05) test was used to detect intergroup differences in the same period.

RESULTS

Tissue dissolution

Table 1 presents the pHs of the solutions, mean value and standard deviation of the weight of fragments of bovine muscle tissue and percentage difference between the initial weight of the fragments and the weight after immersion in different solutions over time. The saline solution did not alter the weight of fragments between the periods analyzed (p>0.05). Tissue dissolution was directly dependent on the concentration of NaOCl solutions as well as the immersion time. The intragroup comparisons showed significant decrease in weight of the fragments for all immersion time periods in 1, 2.5, and 5% NaOCl (p<0.01). The intergroup comparison showed that the reduction in weights was higher with the increase in the concentration of NaOCl. Statistical differences between the groups were significant (p<0.01) in 5 min between G4 and all other groups, the G3 was equal to G2 but different from G1, and G2 was equal to G1. In 10 and 15 min of immersion, the intergroup differences were identified in the following order for tissue dissolution: G4>G3>G2>G1.

ATR-FTIR

Table 2 presents the results of the amide III/phosphate ratio for dentin treated with irrigants. The saline solution did not alter this ratio between the periods analyzed (p>0.05). In G2, G3, and G4, the collagen was deproteinated by NaOCl solutions from the first period of immersion, resulting in decreases in the amide III/phosphate ratio. Intragroup significant differences (p<0.05) for the initial dentin composition were identified after 5 min of immersion in all NaOCl concentrations. There were no intergroup significant differences between all NaOCl concentrations in all periods analyzed

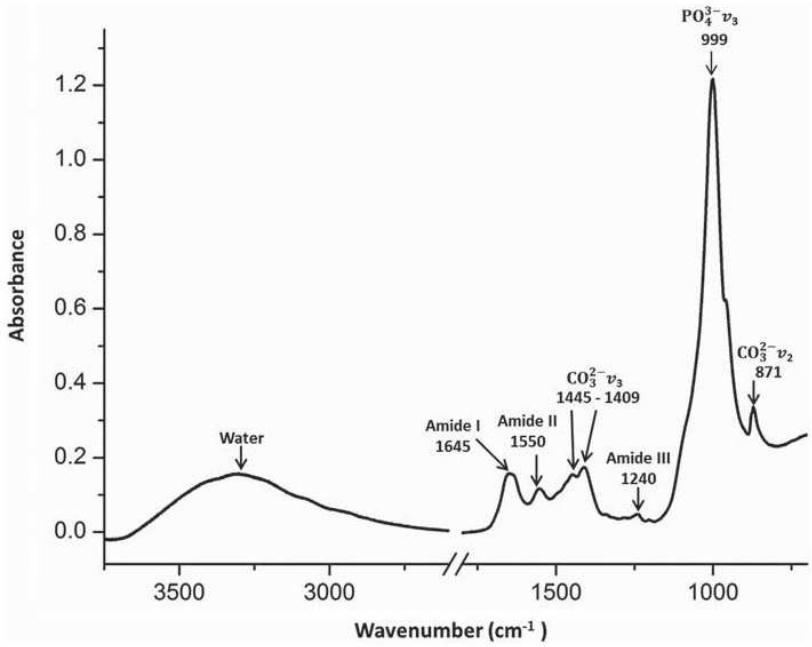

Figure 2- Absorbance spectrum of untreated dentin with the absorption peaks of the main dentin components

(p>0.05), however, statistical differences were identified between the 5% NaOCl and the saline solution after 5 min of immersion. The effects of 1% and 2.5% NaOCl in the amide III/phosphate ratio were lower than the effects of 5% NaOCl, with no statistical differences (p>0.05) for the saline solution.

Regarding the carbonate/phosphate ratio, all irrigants caused a decrease in its initial proportion (Table 3). However, only the NaOCl solutions produced significant intragroup changes (p<0.05) that were identified immediately after 0,5 min of immersion. Significant changes in this ratio were not observed between this time interval and the subsequent periods (p>0.05). Although the NaOCl solutions caused higher changes in the carbonate/phosphate ratio than saline solution, in the intergroup comparisons, no significant differences were identified between all groups in the periods analyzed (p>0.05).

Table 1 - pH of the different irrigation solutions and the mean (X) and standard deviation (SD) in mg of the weights of bovine muscle tissue fragments before and after different periods of immersion in the irrigators and the reduction in weight of the fragments in percentage.

GROUPS	pH	Initial weight	Weight after 5 min of immersion		Weight after 10 min of immersion		Weight after 15 min of immersion	
		X ± SD	X ± SD	Reduction in weight of the fragments (%)	X ± SD	Reduction in weight of the fragments (%)	X ± SD	Reduction in weight of the fragments (%)
G1- Saline	6.4	55.8 ± 1.8^{A,a}	53.5 ± 3.1^{A,a}	-4.12	54.5 ± 2.6^{A,a}	-2.3	54.0 ± 2.7^{A,a}	-3.2
G2- 1% NaOCl	11.7	55.9 ± 1.5^{A,a}	50.9 ± 1.8A^{B,b}	-8.9	44.1 ± 1.6^{B,c}	-21.1	36.2 ± 2.1^{B,d}	-35.2
G3- 2.5% NaOCl	12.05	54.7 ± 2.0^{A,a}	48.8 ± 3.0^{B,b}	-10.7	38.9 ± 2.1^{C,c}	-28.8	29.6 ± 3.5^{C,d}	-45.8
G4- 5% NaOCl	12.3	55.9 ± 2.2^{A,a}	36.9 ± 3.0^{C,b}	-33.9	21.8 ± 2.5^{D,c}	-61	12.2 ± 1.5^{D,d}	-78.1

Different lowercase letters in rows indicate statistically significant intragroup differences (Two-way Anova P < 0.01); Different capital letters in columns indicate statistically significant intergroup differences in the same time period (One-way Anova P<0.01)

Table 2- Median (Med), minimum and maximum (Min – Max) values for the ratio of amide III/phosphate in dentin surface before and after immersion in the irrigation solutions in different periods of time. The ratio values are multiplied by 10-3.

GROUPS	Initial ratio Med (Min - Max)	0.5 min Med (Min - Max)	1 min Med (Min - Max)	2 min Med (Min - Max)	3 min Med (Min - Max)	5 min Med (Min - Max)	8 min Med (Min - Max)	10 min Med (Min - Max)
G1 - Saline solution	7.8 (5.3-9.9)^{A,a}	8.2 (5.1-10.7)^{A,a}	7.7 (5.5-11.1)^{A,a}	7.8 (5.6-11.4)^{A,a}	8.7 (5.0-12.0)^{A,a}	8.6 (5.3-11.6)^{A,a}	7.9 (6.0-11.7)^{A,a}	8 (5.7-12.1)^{A,a}
G2 - 1% NaOCl	6.3 (4.9-7.0)^{A,a}	6.2 (4.6-7.0)^{A,ab}	5.1 (3.7-6.9)^{A,ab}	5.1 (3.5-6.5)^{A,abc}	4.6 (3.7-6.0)^{A,abc}	4.3 (3.5-6.1)^{AB,bc}	4.1 (2.6-5.3)^{AB,bc}	3.7 (2.0-5.0) ^{AB,c}
G3 - 2.5% NaOCl	4.9 (4.0-9.1) ^{A,a}	4.6 (3.7-8.8)^{Aa}	3.9 (3.7-7.5) ^{A,ab}	3.5 (3.4-7.8)^{A,abc}	3.7 (3.2-6.9)^{A,abc}	3.5 (2.9-6.3)^{AB,bc}	3 (2.4-6.7)^{AB,bc}	2.4 (2.0-5.9)^{AB,c}
G4 - 5% NaOCl	6.7 (4.4-7.7)^{A,a}	5.9 (3.6-7.0)^{Aa}	5.1 (3.4-6.4) ^{A,ab}	4.8 (3.1-5.9)^{A,abc}	4.4 (3.0-5.6)^{A,abc}	3.9 (2.2-4.5)^{B,bc}	2.9 (1.5-3.3)^{B,bc}	1.9 (1.2-2.9)^{B,c}

Different lowercase letters in rows indicate statistically significant intragroup differences (Friedman p<0.05); Different capital letters in columns indicate statistically significant intergroup differences in the same time period (Kruskal-Wallis and Dunn *post-hoc* p<0.05)

Table 3- Mean (X) and standard deviation (SD) values for the ratio of carbonate/phosphate in dentin surface before and after immersion in the irrigation solutions in different periods of time.

GROUPS	Initial ratio (X ± SD)	0.5 min (X ± SD)	1 min (X ± SD)	2 min (X ± SD)	3 min (X ± SD)	5 min (X ± SD)	8 min (X ± SD)	10 min (X ± SD)
G1 - Saline solution	19.3 ± 1.5^{A,a}	19.3 ± 1.4^{A,a}	19.3 ± 1.6^{A,a}	19.0 ± 1.7^{A,a}	18.8 ± 1.2^{A,a}	19.0 ± 1.3^{A,a}	18.9 ± 1.4^{A,a}	19.1 ± 1.2^{A,a}
G2 - 1% NaOCl	18.9 ± 1.7^{A,a}	18.2 ± 1.5^{A,b}	17.7 ± 1.7^{A,b}	17.7 ± 1.6^{A,b}	17.7 ± 2.0^{A,b}	17.8 ± 1.7^{A,b}	17.8 ± 1.6^{A,b}	17.7 ± 1.6^{A,b}
G3 - 2.5% NaOCl	19.1 ± 1.8^{A,a}	18.1 ± 2.0^{A,b}	17.4 ± 2.1^{A,b}	17.4 ± 1.6^{A,b}	17.4 ± 1.5^{A,b}	17.4 ± 1.9^{A,b}	17.5 ± 1.5^{A,b}	17.9 ± 2.1^{A,b}
G4 - 5% NaOCl	19.2 ± 0.9^{A,a}	17.7 ± 1.0^{A,b}	17.5 ± 1.1^{A,b}	17.2 ± 1.0^{A,b}	17.1 ± 0.8^{A,b}	17.2 ± 1.3^{A,b}	17.9 ± 1.0^{A,b}	17.8 ± 1.4^{A,b}

Different lowercase letters in rows indicate statistically significant intragroup differences (Two-way Anova and Tukey *post-hoc* P<0.05); Different capital letters in columns indicate statistically significant intergroup differences in the same time period (One-way Anova and Tukey *post-hoc* P<0.05)

DISCUSSION

In the present study, the tissue dissolution capability and the changes in the dentin chemical composition by different concentrations of NaOCl solutions were assessed. The results demonstrated that NaOCl can dissolve the organic matter and deproteinate the collagen of dentin in high quantities; and otherwise, it can cause a small reduction in the carbonate component of the inorganic phase of the dentin.

The null hypothesis tested has to be rejected, since there were differences between the concentrations of NaOCl solutions in the ability of tissue dissolution and in the effects on dentin composition over time.

A concentration and time-dependent organic tissue dissolution capacity was observed for the NaOCl solutions (Table 1), as previously found in other studies[1,7,12,14,29]. NaOCl exerts a nonspecific, non-coagulating digestive effect on vital and necrotic tissues[14,26] by direct contact between free available chlorine molecules and organic matter[20]. The pH of the solution influences the biological effects of NaOCl by determining the equilibrium of the freely available chlorine[4], i.e., the sum of concentrations of hypochlorous acid and hypochlorite anion ($HOCl/OCl^-$)[4,5]. Acid solutions have a powerful bactericidal effect because of the prevalence of HOCl. The OCl^- has a powerful oxidative effect that promote higher tissue dissolution and is more abundant in alkaline solutions[4]. Previous studies did not find differences in the tissue-dissolving properties of NaOCl at the same concentrations and different alkaline pHs of 9 and 12[7,33]. Although there were differences between the pHs of the solutions tested (Table 1) they were small and may not influence the tissue dissolution capability of the irrigants.

Tissues from different sources were used in studies about the tissue dissolving ability of irrigation solutions[1,7,8,12,14,29]. Bovine muscle was chosen because of the availability and easier standardization of the specimens[29,30]. To prevent the confounding factors in the dissolution analysis, the specimens were prepared with similar mass and surface areas. The same temperature and volume of the solutions were used for all groups, and to simulate the solution flow in the root canal during the root canal preparation, the solutions were agitated in an ultrasonic tub.

The bovine incisor dentin has a similar structure and number of tubuli of human molar dentin[28] and permits the achievement of a more standardized substrate for analyses. There are no differences between the mineral matrices of human and bovine dentin, and from the bovine collagen and demineralized human dentin, only differences in intensities of absorption bands are observed[3,6]. The samples were prepared as slices to maintain the natural structure and to prevent changes in tissue composition, because the grinding processes can cause water loss to the ambient, shift the wavenumber, and alter the intensity of the absorption bands[3].

In the dentin vibrational spectrum, it is possible to observe bands related to water, to hydroxyapatite, that originate from the carbonate and phosphate groups and to the organic matrix from the groups present in the collagen such as amides I, II, and III[3]. The treatment of dentin showed that the NaOCl leads to concentration-dependent collagen depletion (Table 2). Although there are no statistical differences between the G2, G3, and G4 groups, the removal of the organic phase from the superficial subsurface of mineralized dentin was considerably more severe for the 5% NaOCl, with significant differences for the saline solution from the 5 min of immersion. This lower amide III/phosphate

ratio was also observed for higher concentrations of NaOCl in other studies[2,15,35]. The NaOCl acts on the dentin creating deproteination channels that leads to a non-uniform effect[10], leaving unbound hydroxyapatite and an apatite-rich and collagen sparse dentin subsurface[10,11]. The destruction of the dentin collagen matrix results in a less tough and more brittle substrate[10,18] that might facilitate the fatigue crack propagation during cyclic stresses[16,34] and increase the susceptibility of crown or root fracture[34]. The destructive effect of NaOCl on the dentin is irreversible and if the chelating agent is subsequently employed, it removes the collagen-depleted apatite phase and exposes the underlying destruction caused by NaOCl, which is morphologically perceived as canal wall erosion[35].

In the present study, a time-dependent effect in the reduction of the amide III/phosphate ratio was identified in all NaOCl concentrations (Table 2). The results indicate that there was a slow and continuous degradation of collagen from the dentin surface and they are in accordance with previous studies that also observed that the removal of the organic phase from the dentin is time-dependent[34,35]. Other studies reported an initial reduction in the collagen with a plateau in dentin deproteination reached over time for the same NaOCl concentration[2,10,15,21]. The plateau was not observed in this research; however, there was a reduction in the rate of deproteination over time. This reduction may be related to the fact that the collagen present on the dentin surfaces is quickly hydrolyzed and removed, and after the process it reverts to the deeper and unexposed collagen that is encapsulated by hydroxyapatite, being less vulnerable to the destructive effects of NaOCl and showing little changes over time[10,15].

Carbonate groups may occupy phosphate and hydroxyl ions sites in bone and teeth apatite. These substitutions affect the crystallinity of the apatites and can accelerate the dissolution process of the tooth structure[23,31]. In the present study, a significant reduction in the carbonate/phosphate ratio occurred in all NaOCl concentrations tested after 0,5 min of immersion, but a plateau was observed after this immersion period (Table 3). Since the solutions were renewed to ensure their effectiveness, this plateau probably occurs after the removal of carbonate from the surface, because the accessibility to the groups that are in subsurfaces layers of dentin makes them less susceptible to the action of the NaOCl solutions. These results confirmed that carbonate groups are more soluble than phosphate groups[23,31] and are in accordance with a previous study that also observed that the NaOCl treatment removes some carbonate ions from the inorganic dentin structure, while at the same time it deproteinates the organic matter[27]. This is consistent with the very low solubility expected for apatite mineral in alkaline solution[11] and may be potentiated by the ultrasonic agitation.

The design of the present study does not directly reflect the clinical conditions, but allows quantitative evaluations regarding the different concentrations and time exposure of NaOCl solutions. This study confirmed the advantage of using a longer contact time and higher concentrations of NaOCl to promote tissue dissolution. However, it increases the alterations in dentin composition and the risks of periapical tissue damage from inadvertent extrusion. Based on these results, the use of NaOCl at lower concentrations, such as 1 and 2.5%, demonstrates to be effective in promoting a suitable dissolution of organic tissue present in the root canal system and preventing a pronounced damage to the dentin structure.

CONCLUSIONS

The findings of this study indicated that the increase in the exposure time and in the concentration of NaOCl solution lead to an increase in the tissue dissolution and dentin collagen deproteination. Moreover, some carbonate ions are removed from the dentin inorganic phase by the NaOCl.

ACKNOWLEDGEMENTS

This study was supported by the State of São Paulo Research Foundation, FAPESP (2013/19789-3 and 2012/02460-6).

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2.4 Article 4 - **Analysis of the effects of several decalcifying agents alone and in combination with sodium hypochlorite on the chemical composition of dentine**

INTERNATIONAL ENDODONTIC JOURNAL

doi:10.1111/iej.12764

Analysis of the effects of several decalcifying agents alone and in combination with sodium hypochlorite on the chemical composition of dentine

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Abstract

Tartari T, Bachmann L, Zancan RF, Vivan RR, Duarte MAH, Bramante CM. Analysis of the effects of several decalcifying agents alone and in combination with sodium hypochlorite on the chemical composition of dentine. *International Endodontic Journal*.

Aim To investigate the effects of several decalcifying agents alone and in combination with sodium hypochlorite (NaOCl) on the organic and inorganic components of dentine using attenuated total reflectance in Fourier transform infrared spectroscopy (ATR-FTIR).

Methodology Dentine slices from bovine teeth were submitted to ($n = 5$) the following: 0.9% saline, 9% and 18% etidronic acid (HEDP), 5% and 10% tetrasodium EDTA ($EDTANa_4$), 17% trisodium EDTA ($EDTAHNa_3$), and 0.5% and 2.0% peracetic acid (PAA) for 0.5 10 min; and to the combinations: G1 mixture 5% NaOCl + 18% HEDP (5 and 10 min); G2 mixture 5% NaOCl + 10% $EDTANa_4$ (5 and 10 min); G2 2.5% NaOCl (5 min) + 17% $EDTAHNa_3$ (1 min); G3 2.5% NaOCl (5 min) + 0.5% PAA (1 min); G4 2.5% NaOCl (5 min) + 9% HEDP (5 min). Specimens of G2, G3 and G4 received final flushes with 2.5% NaOCl for 0.5 10 min. Amide III/phosphate and carbonate/phosphate ratios of the spectra collected from the dentine specimens before and after immersion in the solutions were determined. Data were submitted to one-way repeated measures and one-way ANOVA.

Results For the same decalcifying agent, the higher the concentration and immersion time the greater the removal of phosphate, exposure of collagen matrix and consequently the increases in amide III/phosphate ratio. However, significant differences were found only between the two concentrations of PAA ($P < 0.05$). PAA caused greater increases in this ratio, followed by $EDTAHNa_3$, $EDTANa_4$ and HEDP, and this order was retained in the combinations with NaOCl. This ratio was significantly reduced in G1 ($P < 0.05$) and not altered in G2 ($P > 0.05$). Due to collagen degradation, the amide III/phosphate ratio reduced significantly after the use of NaOCl in G3, G4 and G5 ($P < 0.05$). NaOCl required approximately 0.5 s to deproteinate the collagen matrix exposed after phosphate removal by $EDTAHNa_3$ and PAA. The carbonate of dentine was removed more rapidly than phosphate by all decalcifying agents alone and in G3, G4 and G5. In the combinations with NaOCl, the last irrigant used defined the dentine amide III/phosphate and carbonate/phosphate ratios.

Conclusions HEDP and $EDTANa_4$ caused minor whilst $EDTAHNa_3$ and PAA caused greater demineralization of dentine; both effects were time and concentration dependent. NaOCl degraded the dentine organic matrix more rapidly when it was exposed. Combinations of NaOCl and decalcifying agents can

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be used to create dentine surfaces with varying compositions for interaction with endodontic sealers.

Keywords: decalcifying agents, dentine, Fourier transform infrared spectroscopy, sodium hypochlorite, therapeutic irrigation.

Received 11 September 2016; accepted 13 March 2017

Introduction

An irrigation protocol based on a combination of several irrigants has been proposed to achieve the objectives of root canal treatment (Yamada *et al.* 1983, Zehnder 2006), as no single irrigant has all the properties of an ideal irrigant (Zehnder 2006, Haapasalo *et al.* 2010). The use of sodium hypochlorite (NaOCl) has been proposed during biomechanical preparation to promote tissue dissolution and kill microorganisms. The use of decalcifying agents such as disodium or trisodium ethylenediaminetetraacetic acid (EDTA) to remove debris and the inorganic phase of the smear layer has also been avocated. Lastly, a flush with an antiseptic such as NaOCl or chlorhexidine is indicated to enhance disinfection further (Yamada *et al.* 1983, Zehnder 2006).

Several solutions have been proposed as substitutes for EDTA with the aim of simplifying and reducing the time of irrigation. Amongst the possible substitutes for EDTA are etidronic acid (HEDP), tetrasodium ethylenediaminetetraacetic acid with alkaline pH ($EDTANa_4$) and peracetic acid (PAA) (Lottanti *et al.* 2009, De-Deus *et al.* 2011, Tartari *et al.* 2017). HEDP and $EDTANa_4$ are weak chelating agents that need 5 min to completely remove the smear layer (De-Deus *et al.* 2008b, Tartari *et al.* 2017). During and after biomechanical preparation, these solutions can be mixed with NaOCl solutions without short-term loss of the desired properties of both compounds (Girard *et al.* 2005, Zehnder *et al.* 2005, Tartari *et al.* 2015, 2017). Besides its ability to remove the inorganic components of the smear layer in 1 min (De-Deus *et al.* 2011), PAA has an antimicrobial action even in the presence of proteins (Lensing & Oei 1985), thereby eliminating the need for a final irrigant (Lottanti *et al.* 2009).

However, in addition to the desired effects, the irrigants also act on dentine causing changes to its organic and inorganic components (Hennequin *et al.* 1994, Doğan & Çalt 2001, Ari & Erdemir 2005, Cobankara *et al.* 2011). These alterations can affect the sealing ability and adhesion of dental materials including resin-based cements and endodontic sealers (De-Deus *et al.* 2008a, Neelakantan *et al.* 2011, 2012), and the nature and force of adhesion of microorganisms to dentine (Kishen *et al.* 2008, Tang *et al.* 2011).

The changes promoted by the irrigants on the composition of dentine can be analysed directly and indirectly by several techniques such as energy-dispersive X-ray spectroscopy (EDS) (Zelic *et al.* 2014, Wang *et al.* 2016), Raman spectroscopy, atomic force microscopic imaging (AFM) (Zelic *et al.* 2014), Sirius red dye under polarized light microscopy (Moreira *et al.* 2011), microhardness and roughness tests (Ballal *et al.* 2010) and attenuated total reflectance in Fourier transform infrared spectroscopy (ATR-FTIR) (Tartari *et al.* 2016). As the chemical components of dentine such as phosphate, carbonate, organic matter and water strongly absorb the infrared radiation, the ATR-FTIR technique is indicated to determinate the quantities of these compounds in dentine (Bachmann *et al.* 2003, Bachmann & Zezell 2010, Zhang *et al.* 2010a, Botta *et al.* 2012). It requires samples with a flat and polished surface that makes it difficult to simulate clinical conditions such as the effects of the instruments associated with the irrigants. However, the ATR-FTIR method is advantageous for its simplicity and sensitivity, requiring minimal sample preparation and allowing the analysis of the surfaces of thick samples in their natural state, and nondestructivity (Di Renzo *et al.* 2001a,b, Zhang *et al.* 2010a,b). The ATR-FTIR method allows the characterization of alterations on the chemical composition of dentine surfaces and subsurface before and after it being submitted to physical or chemical treatments (Di Renzo *et al.* 2001a,b, Bachmann *et al.* 2005, Tartari *et al.* 2016).

This study used the ATR-FTIR technique and had two objectives: (i) to determinate the effects on the organic and inorganic components of dentine surfaces when submitted to trisodium EDTA ($EDTAHNa_3$), HEDP, $EDTANa_4$ and PAA at several concentrations and exposure times, and (ii) to evaluate the effects of several combinations of these decalcifying agents with

NaOCl. The null hypotheses tested were that all decalcifying agents and all combinations of irrigants altered the composition of dentine over time in a similar way.

Materials and methods

Solutions

The 0.9% saline solution, 9% and 18% HEDP, 5% and 10% $EDTANa_4$, 17% $EDTAHNa_3$, 0.5% and 2% PAA were tested alone and combined with NaOCl at 2.5% and 5%. The 0.9% saline solution and 17% $EDTAHNa_3$ (Biodinâmica Química e Farmacêutica Ltda., Ibiporã, PR, Brazil) used in the experiments were commercially available products. All other solutions were prepared in the laboratory. To obtain solutions of 9% and 18% HEDP (Zschimmer & Schwarz, Burgstädt, Germany), 0.5% and 2% PAA (Sigma-Aldrich, St. Louis, MO, USA) and 5% and 10% $EDTANa_4$ (Labsynth, Diadema, SP, Brazil), the chemicals were mixed with distilled water. The stock solution of NaOCl (Sigma-Aldrich) had its free chlorine concentration determined by iodometric titration and was then diluted in distilled water to obtain the 2.5% and 5% NaOCl solutions. All these irrigants were prepared immediately before the experiments and stored in airtight, dark containers at 5 °C.

Sample preparation

Dentine slices with dimensions of 4 × 4 × 0.8 mm (length × width × thickness) were obtained from crowns of bovine incisor teeth. The outer surface of each specimen was submitted to sequential polishing with silicon carbide abrasive paper with 4000 grit (Buehler, Lake Bluff, IL, USA) and alpha alumina suspensions with 1 and 0.3 μm (Struers, Ballerup, Denmark). This procedure achieves a flat and smooth surface that favoured the absorbance of infrared radiation. Then, the specimens were immersed in distilled water and submitted to ultrasonic agitation for 1 min to remove the residues from polishing.

FTIR analysis – decalcifying agents

The compositional analysis of all specimens was performed using the Nicolet 380 FTIR spectrophotometer (Thermo Scientific Inc., Waltham, MA, USA) with a diamond ATR set-up (Smart OMNI-Sampler, Thermo Scientific Inc.). The specimens were dried with absorbent paper to avoid excessive dehydration so as to reproduce tissue characteristics found in the clinical environment (Botta *et al.* 2012). For the measurement, the specimens were positioned with the polished surface in contact with the diamond crystal of the ATR set-up and the initial spectra were recorded. The ATR-FTIR spectra of each sample were obtained with 1 cm^{-1} resolution, with 32 scans in the range of 4000 and 400 cm^{-1}, and were recorded using the OMNIC Spectra Software (Thermo Electron Scientific Instruments LLC, Madison, WI, USA).

Then, the specimens were assigned to the following eight groups ($n = 5$): G1 0.9% physiological saline solution (control); G2 9% HEDP; G3 18% HEDP; G4 5% $EDTANa_4$; G5 10% $EDTANa_4$;G6 17% $EDTAHNa_3$; G7 0.5% PAA; G8 2% PAA. These specimens were placed inside microtubes containing 1.5 mL of the solutions for 0.5 min and ultrasonicated for 15 s. Subsequently, to remove the irrigants, the specimens were transferred to microtubes containing 1.5 mL of distilled water and immersed for 1 min, of which 15 s were under ultrasonic agitation. Then, the specimens were dried with absorbent paper, and a new infrared spectrum was recorded. These steps were repeated resulting in a sequential treatment with spectra measurement of samples at time intervals of 0, 0.5, 1, 2, 3, 5, 8 and 10 min. The solutions were changed at time intervals of 2, 5 and 8 min to prevent the depletion of the decalcifying effect. After achieving the spectrum of specimen treated for 1 min, the ultrasonic agitation time was changed to only 15 s per each minute of immersion in the solutions.

The infrared bands considered for this study were amide III (1298 1216 cm^{-1}), phosphate ($PO_4^{3-}\nu_3$, 1170 780 cm^{-1}) and carbonate ($CO_3^{2-}\nu_2$, 888 816 cm^{-1}). The areas under the considered bands were determined after the baseline tracing, and the values obtained were used to calculate the amide III/phosphate and carbonate/phosphate ratios and the relative alterations in the contents of inorganic and organic components of dentine (Tartari *et al.* 2016).

FTIR analysis – combinations of irrigants

After the analysis of the effects of the decalcifying agents, several concentrations of these substances were chosen and employed associated with NaOCl solutions. New samples were prepared and distributed in to five groups ($n = 5$) according to the following combinations: G1 mixture of 5% NaOCl and 18% HEDP in a proportion of 1 : 1 (5 and 10 min); G2 mixture of 5% NaOCl and 10% $EDTANa_4$ in a proportion of 1 : 1

(5 and 10 min); G3 2.5% NaOCl (5 min) + 17% $EDTAHNa_3$ (1 min) + 2.5% NaOCl (0.5, 1, 3, 5 and 10 min); G4 2.5% NaOCl (5 min) + 0.5% PAA (1 min) + 2.5% NaOCl (0.5, 1, 3, 5 and 10 min); G5 2.5% NaOCl (5 min) + 9% HEDP (5 min) + 2.5% NaOCl (0.5, 1, 3, 5 and 10 min);

These samples were submitted to irrigants in the same way as described above, and the spectra were collected at the beginning and after immersion in each solution. For the final flush, the NaOCl was renewed after 5 min. The alterations in dentine composition produced by the combinations of irrigants were also determined by the amide III/phosphate and carbonate/phosphate ratios.

Statistical analysis

The values of amide III/phosphate and carbonate/phosphate ratios were normally distributed according to the Shapiro Wilk test, allowing the use of parametric statistical tests. The data of the same experimental group were submitted to one-way repeated measures analysis of variance (ANOVA) and Tukey's multiple-comparison test to detect any differences in the composition of the specimens before and after immersion in the irrigants. One-way analysis of variance (ANOVA) and the *post hoc* Tukey tests were used to detect differences amongst groups at the same period of immersion. All hypotheses tests were performed at a 99% confidence level.

Results

Decalcifying solutions

Table 1 presents the mean and standard deviation values of amide III/phosphate ratio for all decalcifying agents at different time intervals. Except for 9% and 18% HEDP, the use of all other decalcifying agents resulted in significant increases in this ratio ($P < 0.05$) due to dissolution of apatite and the creation of a partially demineralized collagen matrix. For all irrigation solutions, as the concentrations and their contact time with dentine increased, greater changes were observed on the surface, although there were no significant differences between 9% and 18% HEDP and between 5% and 10% $EDTANa_4$ at all periods analysed ($P > 0.05$). Between 0.5% and 2.0% PAA, the differences were identified immediately after 0.5 min of immersion ($P < 0.05$). Significant differences to the initial amide III/phosphate ratios in the intragroup comparison occurred after 3, 10, 10, 8 and 10 min of immersion in the solutions of 5% and 10% $EDTANa_4$, 17% EDTA, 0.5% and 2.0% PAA, respectively. The intergroup comparisons revealed that 2% PAA caused the greatest increases in the amide III/phosphate ratio, followed by 0.5% PAA and 17% EDTA, whilst minor changes were caused by 5% and 10% $EDTANa_4$ and 9% and 18% HEDP. After 10 min of immersion, the alterations caused were in the following order 2.0% PAA > 0.5% PAA = 17% EDTA > 10% $EDTANa_4$ = 5% $EDTANa_4$ = 18% HEDP = 9% HEDP = saline ($P < 0.05$).

In Table 2, the results of dentine treatment with the decalcifying agents in the carbonate/phosphate ratio are presented. When compared to the initial dentine composition, significant differences in this ratio ($P < 0.05$) were observed after 5, 5, 2, 10, 5, 8 and 1 min of sample immersion in 9% HEDP, 18% HEDP, 5% $EDTANa_4$, 10% $EDTANa_4$, 17% EDTA, 0.5% PAA and 2% PAA solutions, respectively. As with the amide III/phosphate ratio, the intergroup comparisons confirmed that PAA caused the greatest alterations in the carbonate/phosphate ratio, followed by 17% $EDTAHNa_3$, whilst minor changes were caused by 5% and 10% $EDTANa_4$ and 9% and 18% HEDP. At 10 min, the order of intergroup alterations for this ratio was 2% PAA = 0.5% PAA ($P > 0.05$) and major than all other groups ($P < 0.05$), whereas 0.5% PAA had similar effects than 17% $EDTAHNa_3$ ($P > 0.05$) and major than all other groups ($P < 0.05$). The 17% $EDTAHNa_3$ had similar effects to 5 and 10% $EDTANa_4$ ($P > 0.05$), and greater effects than the other groups ($P < 0.05$), whereas 5% and 10% $EDTANa_4$, 9% and 18% HEDP and saline were similar to each other ($P > 0.05$).

Although the saline solution caused changes in the dentine surface, these changes were not sufficient to alter significantly the amide III/phosphate and carbonate/phosphate ratios ($P > 0.05$).

Combinations of irrigants

The results of dentine treatment with the different combinations on amide III/phosphate ratios are shown in Table 3. Collagen dissolution by NaOCl was higher than the demineralization induced by HEDP in G1, with significant decreases in amide III/phosphate ratio over time ($P < 0.05$). In G2, the effects of NaOCl and $EDTANa_4$ were similar, preserving the natural proportion of organic/inorganic matter of dentine ($P > 0.05$). The first 5 min of immersion in NaOCl

Table 1 Mean (X) and standard deviation (SD) values for the ratio of amide III/phosphate on dentine surface before and after immersion in the irrigation solutions at different periods of time

Groups	Initial ratio X ± SD	0.5 min X ± SD	1 min X ± SD	2 min X ± SD	3 min X ± SD	5 min X ± SD	8 min X ± SD	10 min X ± SD
Saline solution	$7.5 \pm 1.9^{A,a}$	$7.9 \pm 2.2^{B,a}$	$8.1 \pm 2.1^{BC,a}$	$8.0 \pm 2.2^{BC,a}$	$8.2 \pm 2.7^{B,a}$	$8.2 \pm 2.6^{C,a}$	$8.3 \pm 2.3^{C,a}$	$8.3 \pm 2.5^{C,a}$
9% HEDP	$5.5 \pm 0.8^{A,a}$	$5.7 \pm 0.8^{B,a}$	$5.6 \pm 0.8^{C,a}$	$5.7 \pm 0.7^{C,a}$	$5.8 \pm 1.0^{B,a}$	$5.9 \pm 0.6^{C,a}$	5.9 ± 0.8^{Ca}	$6.2 \pm 0.8^{C,a}$
18% HEDP	$5.3 \pm 1.6^{A,a}$	$5.5 \pm 1.6^{B,a}$	$5.5 \pm 1.6^{C,a}$	$5.6 \pm 1.5^{C,a}$	$5.8 \pm 1.7^{B,a}$	$5.8 \pm 1.7^{C,a}$	$6.3 \pm 1.9^{C,a}$	$6.5 \pm 2.1^{C,a}$
5% $EDTANa_4$	$7.4 \pm 1.7^{A,b}$	$7.8 \pm 2.0^{B,b}$	$8.9 \pm 2.6^{BC,ab}$	$8.9 \pm 2.5^{BC,ab}$	$9.5 \pm 2.7^{B,a}$	$10.3 \pm 2.4^{C,a}$	$11.4 \pm 3.0^{C,a}$	$12.8 \pm 3.3^{C,a}$
10% $EDTANa_4$	$7.8 \pm 1.5^{A,b}$	$8.9 \pm 1.2^{B,ab}$	$9.5 \pm 1.1^{BC,ab}$	$10.1 \pm 2.3^{BC,ab}$	$11.3 \pm 2.3^{B,ab}$	$12.6 \pm 2.5^{C,ab}$	$14.4 \pm 3.4^{C,ab}$	$16.8 \pm 3.6^{C,a}$
17% $EDTAHNa_3$	$7.0 \pm 2.0^{A,b}$	$11.3 \pm 2.8^{B,b}$	$14.3 \pm 3.7^{BC,b}$	$19.0 \pm 5.1^{BC,b}$	$26.1 \pm 7.8^{B,ab}$	$44.3 \pm 10.0^{BC,ab}$	$64.6 \pm 17.5^{B,ab}$	$90.6 \pm 21.2^{B,a}$
0.5% PAA	$6.8 \pm 2.1^{A,c}$	$11.3 \pm 1.8^{B,c}$	$20.2 \pm 6.2^{B,bc}$	$31.0 \pm 11.9^{B,bc}$	$49.4 \pm 11.7^{B,bc}$	$79.8 \pm 22.5^{AB,abc}$	$97.1 \pm 21.6^{B,ab}$	$112.7 \pm 33.8^{B,a}$
2% PAA	$6.4 \pm 1.6^{A,b}$	$23.4 \pm 5.1^{A,b}$	$44.8 \pm 10.2^{A,b}$	$77.0 \pm 25.4^{A,b}$	$102.9 \pm 49.8^{A,b}$	$121.2 \pm 46.9^{A,b}$	$134.7 \pm 39.6^{A,b}$	$167.9 \pm 60.7^{A,a}$

The mean and standard deviation values are multiplied by 10^{-3}; different lowercase letters in rows indicate statistically significant intragroup differences (one-way repeated measures ANOVA and Tukey *post hoc* *P*-value <0.01); different capital letters in columns indicate statistically significant intergroup differences in the same time period (one-way ANOVA and Tukey *post hoc* *P*-value <0.01).

Table 2 Mean (X) and standard deviation (SD) values for the ratio of carbonate/phosphate on dentine surface before and after immersion in the irrigation solutions at different periods of time

Groups	Initial ratio X ± SD	0.5 min X ± SD	1 min X ± SD	2 min X ± SD	3 min X ± SD	5 min X ± SD	8 min X ± SD	10 min X ± SD
Saline solution	$19.3 \pm 1.5^{A,a}$	$19.3 \pm 1.4^{A,a}$	$19.3 \pm 1.6^{A,a}$	$19.0 \pm 1.7^{A,a}$	$18.8 \pm 1.2^{A,a}$	$19.0 \pm 1.3^{A,a}$	$18.9 \pm 1.4^{AB,a}$	$19.1 \pm 1.2^{A,a}$
9% HEDP	$20.1 \pm 0.8^{A,a}$	$19.7 \pm 0.7^{A,a}$	$19.5 \pm 0.5^{A,ab}$	$19.5 \pm 0.5^{A,ab}$	$19.3 \pm 0.6^{A,ab}$	$19.1 \pm 0.7^{A,b}$	$19.0 \pm 0.7^{A,b}$	$18.9 \pm 0.6^{A,b}$
18% HEDP	$19.8 \pm 0.6^{A,a}$	$18.8 \pm 1.0^{A,ab}$	$18.5 \pm 1.1^{A,ab}$	$18.3 \pm 1.1^{A,ab}$	$18.4 \pm 1.2^{A,ab}$	$18.1 \pm 0.8^{AB,b}$	$18.1 \pm 0.5^{AB,b}$	$18.1 \pm 1.4^{A,b}$
5% $EDTANa_4$	$18.1 \pm 1.1^{A,a}$	$17.6 \pm 1.2^{A,ab}$	$17.7 \pm 1.1^{A,abc}$	$17.0 \pm 1.1^{A,bc}$	$17.0 \pm 1.0^{A,bc}$	$16.4 \pm 1.0^{AB,bc}$	$16.4 \pm 1.2^{ABC,bc}$	$16.4 \pm 1.4^{AB,c}$
10% $EDTANa_4$	$19.0 \pm 0.4^{A,a}$	$18.2 \pm 0.6^{A,ab}$	$17.9 \pm 0.9^{A,ab}$	$17.7 \pm 0.6^{A,ab}$	$17.9 \pm 0.7^{A,ab}$	$17.8 \pm 0.9^{AB,ab}$	$17.7 \pm 0.5^{AB,ab}$	$17.6 \pm 0.7^{AB,b}$
17% $EDTAHNa_3$	$18.3 \pm 1.3^{A,a}$	$18.3 \pm 1.4^{A,a}$	$18.1 \pm 2.0^{A,a}$	$17.7 \pm 1.9^{A,ab}$	$17.1 \pm 1.3^{A,ab}$	$16.6 \pm 1.6^{AB,b}$	$15.6 \pm 2.2^{BC,b}$	$14.0 \pm 2.0^{BC,c}$
0.5% PAA	$19.6 \pm 1.3^{A,a}$	$19.7 \pm 1.6^{A,a}$	$19.4 \pm 1.6^{A,a}$	$18.9 \pm 2.3^{A,a}$	$17.9 \pm 1.8^{A,a}$	$15.5 \pm 2.9^{B,ab}$	$13.1 \pm 1.8^{C,b}$	$12.5 \pm 1.5^{CD,b}$
2% PAA	$19.0 \pm 0.6^{A,a}$	$18.9 \pm 1.1^{A,a}$	$16.2 \pm 1.5^{A,b}$	$13.4 \pm 0.9^{B,bc}$	$12.1 \pm 1.5^{B,cd}$	$10.3 \pm 1.1^{C,cd}$	$9.5 \pm 2.4^{D,cd}$	$9.1 \pm 1.1^{D,d}$

The mean and standard deviation values are multiplied by 10^{-3}; different lowercase letters in rows indicate statistically significant intragroup differences (one-way repeated measures ANOVA and Tukey *post hoc* *P*-value <0.01); different capital letters in columns indicate statistically significant intergroup differences in the same time period (one-way ANOVA and Tukey *post hoc* *P*-value <0.01).

caused a slow, but significant reduction in amide III/phosphate ratios in G3, G4 and G5, compared to the initial value ($P < 0.05$), without intergroup differences ($P > 0.05$). When used in the combinations, the decalcifying agents had different rates of actions on dentine. The $EDTAHNa_3$ and PAA acid significantly increased this ratio compared to the initial value ($P < 0.05$), whereas in G5, HEDP kept this value similar to the initial level ($P > 0.05$). The alterations in amide III/phosphate ratios after the use of decalcifying agents were identified in the following intergroup order G4 > G3 > G5 = G1 = G2 ($P < 0.05$). The NaOCl effects on the collagen exposed by PAA and $EDTAHNa_3$ were faster than on the collagen of mineralized dentine; it required approximately 0.5 min to deproteinate all fibres and restore the dentine surface to its natural proportion of amide III/phosphate in G3 and G4. After the 0.5 min of final flush with NaOCl, the speed of deproteination decreased and became similar to that observed in the first 5 min of immersion in NaOCl. There were no differences amongst G3, G4 and G5 after 3 min of final flush with NaOCl ($P > 0.05$).

The one-way repeated measures analysis of variance of the carbonate/phosphate ratio revealed that it was not altered in G1 and G2 over time ($P > 0.05$; Table 4). In G3, G4 and G5 significant reductions in this ratio were identified after the initial immersion in NaOCl ($P < 0.05$). After the use of decalcifying agents, this ratio was similar to the initial value in G3 and G4 ($P > 0.05$); however, it continued to decrease in G5 ($P < 0.05$). The final flush with NaOCl decreased this ratio again in G3 and G4, and differences from the baseline value were found after 3 and 0.5 min, respectively ($P < 0.05$). However, no more changes in carbonate/phosphate ratio occurred after these time intervals. Intergroup comparisons revealed that all groups had similar effects in this ratio in the periods analysed ($P > 0.05$).

Discussion

In this study, the null hypotheses tested whether all decalcifying agents and all combinations of solutions altered the chemical composition of dentine in a similar way. Both of these hypotheses were rejected. The results showed that (i) the various decalcifying agents decreased the bands associated with phosphate and carbonate apatite at different rates, (ii) the combinations of solutions had both different types and rates of actions on dentine that depended on the solutions employed. Furthermore, the last irrigation solution used defined the characteristic of the dentine surface available for interaction with the material used in the root filling or the microorganisms in case of recontamination.

The spectra obtained from samples submitted to PAA and $EDTAHNa_3$ revealed a shift in the position of the band assigned to amide I after immersion in the solutions (data not shown). This fact is related to the overlapping between the amide I band and a band of water and shows that this band is not reliable for use in the analysis of dentine composition. Based on this, the changes in dentine components were determined by the ratios of amide III/phosphate ($PO_4^{3-}\nu_3$) and carbonate ($CO_3^{2-}\nu_2$)/phosphate ($PO_4^{3-}\nu_3$) bands (Tartari *et al.* 2016).

The evaluated decalcifying agents demineralized dentine and created surfaces rich in collagen but apatite-sparse, which were expressed as increases in the amide III/phosphate ratio (Table 1). However, the results showed that the alterations caused on dentine by these solutions occurred at different rates. The HEDP at concentrations of 9% and 18% did not alter the amide III/phosphate ratio of the dentine, and the $EDTANa_4$ at 5% and 10% caused minor reductions in the phosphate groups. These results are in accordance with previous studies that reported that HEDP and $EDTANa_4$ were weak chelating solutions (O'Connell *et al.* 2000, Zehnder *et al.* 2005, Lottanti *et al.* 2009, Tartari *et al.* 2017). The 2.0% PAA was the strongest demineralizing agent tested, as it caused substantial phosphate removal and exposure of the collagen matrix. Consequently, it produced major increases in the amide III/phosphate ratio when compared with all other groups. However, the increases in this ratio caused by 0.5% PAA were similar to that promoted by 17% $EDTAHNa_3$. When comparing the changes produced by irrigants on the amide III/phosphate ratio during the time required for each solution to remove the smear layer, it was observed that 17% $EDTAHNa_3$ and 0.5% and 2.0% PAA used for 1 min removed an amount of dentine mineral much higher than 5% and 10% $EDTANa_4$, and 9% and 18% HEDP used for 5 min (Table 1 and Fig. 1). These results showed that HEDP and alkaline $EDTANa_4$ at the concentrations tested were weaker chelating solutions that cause little alteration or demineralization on dentine structures agreeing with previous studies (O'Connell *et al.* 2000, De-Deus *et al.* 2008b, Tartari *et al.* 2017). PAA in concentrations above 0.5% should be

Table 3 Mean (X) and standard deviation (SD) values for the ratio of amide III/phosphate on dentine surface before and after immersion in each irrigation solution of the different irrigation combinations

Groups	Initial ratio X ± SD	Mixture (5′) X ± SD	Mixture (5′) X ± SD						
G1 – Mixture of 5% NaOCl + 18% HEDP (5 and 10′)	$7.1 \pm 1.7^{A,a}$	$5.6 \pm 1.4^{A,ab}$	$4.5 \pm 1.8^{C,b}$	–	–	–	–	–	–
G2 – Mixture of 5% NaOCl + 10% $EDTANa_4$ (5 and 10′)	$6.5 \pm 2.1^{A,a}$	$6.6 \pm 1.9^{A,a}$	$6.5 \pm 2.0^{C,a}$	–	–	–	–	–	–
			Decalcifying agent (1′ or 5′) X ± SD	NaOCl (0.5′) X ± SD	NaOCl (1′) X ± SD	NaOCl (3′) X ± SD	NaOCl (5′) X ± SD	NaOCl (10′) X ± SD	
G3 – 2.5% NaOCl (5′) + 17% EDTA (1′) + 2.5% NaOCl (0.5, 1, 3, 5 and 10′)	$6.6 \pm 1.6^{A,bc}$	$5.0 \pm 1.5^{A,d}$	$10.8 \pm 1.9^{B,a}$	$6.6 \pm 1.6^{A,b}$	$6.3 \pm 1.6^{A,bc}$	$5.4 \pm 1.4^{A,cd}$	$5.2 \pm 1.7^{A,d}$	$4.0 \pm 1.3^{A,d}$	
G4 – 2.5% NaOCl (5′) + 0.5% PAA (1′) + 2.5% NaOCl (0.5, 1, 3, 5 and 10′)	$6.8 \pm 0.9^{A,bc}$	$5.5 \pm 0.6^{A,de}$	$15.5 \pm 1.6^{A,a}$	$7.4 \pm 1.0^{A,b}$	$6.2 \pm 0.9^{A,cd}$	$5.2 \pm 1.0^{A,e}$	$4.7 \pm 1.2^{A,ef}$	$3.8 \pm 0.9^{A,f}$	
G5 – 2.5% NaOCl (5′) + 9% HEDP (5′) + 2.5% NaOCl (0.5, 1, 3, 5 and 10′)	$5.0 \pm 1.3^{A,a}$	$4.0 \pm 0.9^{A,bcd}$	$4.5 \pm 1.2^{C,ab}$	$4.2 \pm 1.3^{B,b}$	$4.0 \pm 1.1^{B,bc}$	$3.7 \pm 1.2^{A,c}$	$3.4 \pm 1.1^{A,d}$	$2.7 \pm 1.3^{A,d}$	

The mean and standard deviation values are multiplied by 10^{-3}; different lowercase letters in rows indicate statistically significant intragroup differences (one-way repeated measures ANOVA and Tukey *post hoc P*-value <0.01); different capital letters in columns indicate statistically significant intergroup differences in the same time period (one-way ANOVA and Tukey *post hoc P*-value <0.01).

Table 4 Mean (X) and standard deviation (SD) values for the ratio for the ratio of carbonate/phosphate on dentine surface before and after immersion each irrigation solution in the different irrigation combinations

GROUPS	Initial ratio X ± SD	Mixture (5′) X ± SD	Mixture (5′) X ± SD							
G1 – Mixture of 5% NaOCl + 18% HEDP (5 and 10′)	$19.4 \pm 0.5^{A,a}$	$18.8 \pm 1.1^{A,a}$	$18.2 \pm 0.9^{A,a}$	–	–	–	–	–	–	–
G2 – Mixture of 5% NaOCl + 10% EDTANa4 (5 and 10′)	$19.2 \pm 1.1^{A,a}$	$18.2 \pm 1.8^{A,a}$	$18.5 \pm 2.3^{A,a}$	–	–	–	–	–	–	–
			Decalcifying agent (1′ or 5′)	NaOCl (0.5′)	NaOCl (1′)	NaOCl (3′)	NaOCl (5′)	NaOCl (5′)	NaOCl (5′)	NaOCl (10′)
		NaOCl (5′) X ± SD	X ± SD	X ± SD	X ± SD	X ± SD	X ± SD	X ± SD	X ± SD	X ± SD
G3 – 2.5% NaOCl (5′) + 17% EDTA (1′) + 2.5% NaOCl (0.5, 1, 3, 5 and 10′)	$19.1 \pm 1.8^{A,a}$	$17.8 \pm 2.1^{A,ab}$	$17.9 \pm 1.8^{A,ab}$	$17.8 \pm 1.2^{A,ab}$	$17.9 \pm 1.2^{A,ab}$	$17.5 \pm 1.8^{A,b}$	$17.6 \pm 1.8^{A,b}$	$17.6 \pm 1.8^{A,b}$	$17.4 \pm 1.0^{A,b}$	$17.9 \pm 1.6^{A,b}$
G4 – 2.5% NaOCl (5′) + 0.5% PAA (1′) + 2.5% NaOCl (0.5, 1, 3, 5 and 10′)	$18.6 \pm 0.5^{A,a}$	$17.3 \pm 0.9^{A,b}$	$18.0 \pm 1.2^{A,ab}$	$17.5 \pm 1.3^{A,b}$	$17.5 \pm 0.8^{A,b}$	$17.3 \pm 0.8^{A,b}$	$17.4 \pm 1.0^{A,b}$	$17.4 \pm 1.0^{A,b}$	$17.4 \pm 1.0^{A,b}$	$17.7 \pm 0.9^{A,b}$
G5 – 2.5% NaOCl (5′) + 9% HEDP (5′) + 2.5% NaOCl (0.5, 1, 3, 5 and 10′)	$19.1 \pm 2.0^{A,a}$	$17.3 \pm 2.0^{A,b}$	$16.9 \pm 2.3^{A,b}$	$17.1 \pm 2.2^{A,b}$	$17.3 \pm 2.2^{A,b}$	$16.8 \pm 2.1^{A,b}$	$16.7 \pm 2.3^{A,b}$	$16.7 \pm 2.3^{A,b}$	$16.7 \pm 2.3^{A,b}$	$16.9 \pm 2.0^{A,b}$

The mean and standard deviation values are multiplied by 10^{-3}; different lowercase letters in rows indicate statistically significant intragroup differences (one-way repeated measures ANOVA and Tukey *post hoc P*-value <0.01); different capital letters in columns indicate statistically significant intergroup differences in the same time period (one-way ANOVA and Tukey *post hoc P*-value <0.01).

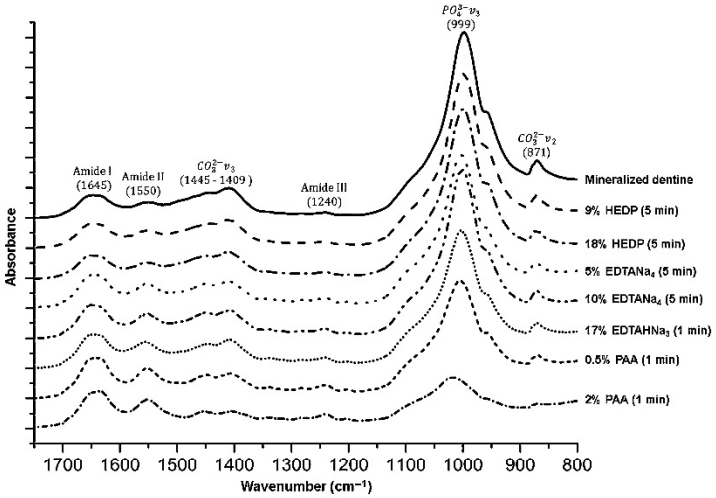

Figure 1 Superimposition of the representative infrared spectral region between 1750 and 800 cm^{-1} showing the different intensities of the bands on mineralized dentine and on dentine submitted to the decalcifying agents for the time required for each solution to remove the smear layer.

used carefully due its higher demineralizing ability (Cobankara *et al.* 2011).

Another important fact is that despite the 0.5% PAA solution having a quarter of the concentration of the 2.0% solution, the increases in the amide III/phosphate ratio caused by the former were about half of the increases produced by the latter (Table 1). These results in relation to phosphate removal may be caused by two factors. One factor is that mineral depletion close to the surface may lead to a slower diffusion rate through the exposed collagen layer, resulting in a diffusion-controlled process that decreased the etching rate (Marshall *et al.* 1993, Di Renzo *et al.* 2001a). The second factor is that the penetration of the infrared evanescent wave in dentine is limited; thus, the data collected only represent the superficial subsurface characteristics. If the demineralizing front was beyond the limit of device detection, any spectral changes would have been undetectable (Di Renzo *et al.* 2001a, Zhang *et al.* 2010b).

The analysis of the alterations in the amide III/phosphate ratio produced by the combinations of solutions showed that NaOCl promoted the deproteination of dentine collagen matrix, confirming results of previous studies (Di Renzo *et al.* 2001b, Borges *et al.* 2008, Hu *et al.* 2010, Zhang *et al.* 2010b, Atabek *et al.* 2014, Tartari *et al.* 2016). In the present study, the NaOCl was used for 5 min in the first step of the combinations applied in G3, G4 and G5, because although NaOCl is used clinically for longer periods, each time that a file is used against the root canal walls, the dentine on the surface is removed and a new layer of dentine is exposed. During this first step, the NaOCl slowly denatured the collagen fibrils encapsulated by apatites on mineralized dentine. This is because they are less vulnerable to the effects of NaOCl, which has to penetrate between crystals to act (Zhang *et al.* 2010a,b). However, the collagen exposed after the use of decalcifying agents was quickly attacked by the solution (Table 3 and Fig. 2). The demineralization promoted by 0.5% PAA and 17% EDTAHNa$_3$ after 1 min of immersion had different rates. However, the final flush with 2.5% NaOCl needed about 0.5 min to completely remove the collagen matrix exposed by these two decalcifying agents and restore the dentine surface to its natural amide III/phosphate ratio in G3 and G4. As HEDP did not cause significant dentine demineralization, the final irrigation with NaOCl for 0.5 min resulted in an amide III/phosphate ratio different from the initial value presented by G5. Another important observation made was that after the removal of the exposed collagen, the deproteination process reverted to that observed for encapsulated collagen on mineralized dentine, being much slower and showing little changes over time (Di Renzo *et al.* 2001b, Tartari *et al.* 2016).

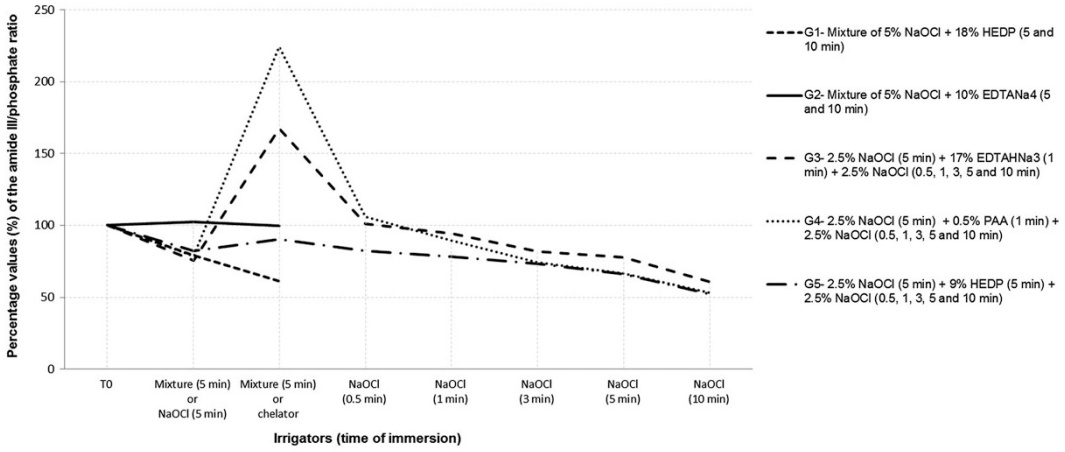

Figure 2 Differences in percentage (%) to the initial value (T0) of the amide III/phosphate ratio on dentine surface and subsurface after the immersion in each irrigation solution of the different combinations.

In the combinations of solutions, the required immersion time in the decalcifying agents to completely remove the smear layer was based on the literature (De-Deus *et al.* 2008b, 2011, Tartari *et al.* 2017). As in the analysis of the effects of each decalcifying agent alone, the results obtained with the combinations showed different degrees of dentine demineralization and consequently in the exposure of collagen matrix, depending on the decalcifying agent used (Tables 1 and 3). The use of decalcifying agents after NaOCl increased the amide III/phosphate ratio, PAA being the solution that caused the major increase, followed by $EDTAHNa_3$ and HEDP, respectively (Table 3). In G5, the amide III/phosphate ratio remained similar to the initial value after the immersion in HEDP. In G2, the different effects of NaOCl and $EDTANa_4$ had similar rates, whilst $EDTANa_4$ was demineralizing the dentine, the NaOCl was deproteinating the exposed collagen. This way the amide III/phosphate ratio remained similar to the original value. In G1, the action of NaOCl on organic matter prevailed on the removal of mineral phase by HEDP and this was expressed as a reduction in the amide III/phosphate ratio over time (Table 3).

This study revealed that strong decalcifying agents such as $EDTAHNa_3$ and PAA produce a major demineralization, exposing amino groups of the dentinal collagen. The amino groups of collagen can bond chemically with endodontic sealers such as AH Plus, improving the bond strength between the material and dentine (Neelakantan *et al.* 2015). However, a major decalcification of the root canal wall can result in a suboptimal infiltration of the material into the demineralized dentine, forming a weak bond and accelerating interfacial degradation (Perdigao *et al.* 2001, Pashley *et al.* 2004, De Munck *et al.* 2005, Schwartz 2006, De-Deus *et al.* 2008a). Alternatively, restoring dentine surfaces to their natural composition can also provide a more favourable and stable adhesion of dental materials over time (Gwinnett 1994, Wakabayashi *et al.* 1994, Di Renzo *et al.* 2001b). The same principle might be applicable in case of root canal reinfections. Some microorganisms, such as Enterococcus faecalis, have an affinity for collagen and can express factors that increase their adhesion in collagen-rich surfaces (Nallapareddy *et al.* 2000a,b, Hubble *et al.* 2003). In such cases, combinations of solutions that expose major quantities of collagen fibres on dentine surface create a better substrate for the adherence of these microorganisms (Nallapareddy *et al.* 2000a, Kishen *et al.* 2008). On the other hand, the removal of exposed collagen matrix by a final rinse with NaOCl could reduce the number of adhering bacteria and biofilm formation (Kishen *et al.* 2008).

On the inorganic phase of dentine, the substitution of phosphate and hydroxyl ions in apatites by carbonate groups decreases crystallinity and increases the apatite solubility (LeGeros *et al.* 1967, Sakae *et al.* 2003, Yao *et al.* 2009). This increase in solubility of carbonate compared to phosphate was observed through significant decreases in the initial carbonate/

phosphate ratio. These results were produced by all the decalcifying agents alone and by the NaOCl applied in combination with the solutions over time (Table 2 and 4), corroborating with the findings of previous studies (Sakae *et al.* 1988, Borges *et al.* 2008, Tartari *et al.* 2016). The use of 2.5% NaOCl for 5 min was sufficient to affect the inorganic content of dentine, irrespective of treatment with the demineralizing solutions. In the combinations, the 0.5% PAA and 17% $EDTAHNa_3$ used for 1 min increased the carbonate/phosphate ratio restoring to the surface a value similar to that presented by dentine before treatments. This reflects their high demineralizing action that should have promoted similar effects on the different inorganic components of dentine (Table 4). On the other hand, in G5 the HEDP decreased this ratio, which may be due to its weak chelating ability that should have dissolved the carbonate faster than phosphate groups, because of the higher solubility of the former. In G1 and G2, this ratio was not altered, possibly because it was easier for HEDP and $EDTANa_4$ to remove the phosphate groups from the surface by removing the unbound hydroxyapatite crystals resulting from the action of the NaOCl.

Within the limitations of this study, it is suggested that the decalcifying agents tested have different degrees of action on the mineral phase of dentine and the effects are directly related to the decalcifying capability of the solution, its concentration and immersion time. The combinations of solutions produced surfaces with different chemical functional groups suitable for different kinds of adhesion with dental materials, and these groups are directly related to the last solution applied. Based on these facts, studies should be undertaken to analyse the influence of these different characteristics of dentine surfaces on sealing ability and adhesion of different endodontic sealers and sealers used for cementing fibre posts over time and on the adhesion of microorganisms in case of reinfections.

Conclusions

HEDP and $EDTANa_4$ solutions cause minor demineralization whilst $EDTAHNa_3$ and PAA caused a greater demineralization of dentine, both being time and concentration dependent. NaOCl degraded the collagen of the organic matrix of dentine more rapidly when it was exposed than when it was covered by the hydroxyapatite. The combined and sequential treatment of dentine with NaOCl and different decalcifying agents can be used to obtain a dentine surface rich or poor in collagen or to restore its organic/inorganic natural composition for interaction with endodontic sealers, according to the last irrigation solution used.

Acknowledgments

This study was supported by State of São Paulo Research Foundation, FAPESP (2013/19789-3 and 2012/02460-6).

Conflict of Interest

The authors have stated explicitly that there are no conflict of interests in connection with this article.

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2.5 Article 5 - **Effects of Different Irrigation Protocols on Dentin Surface Properties, Adsorption of Chlorhexidine and Microorganisms' Adhesion to Dentin**

The article presented in this thesis was written according to the International Endodontic Journal instructions and guidelines for article submission

ABSTRACT

Aim To investigate the effects of combinations of different irrigants on the roughness and wettability of dentin and on the adhesion of *Enterococcus faecalis* and *Candida albicans* and the adsorption of chlorhexidine (CHX) to the dentin surface.

Methodology Dentin samples were prepared, and after standardisation of the surface roughness, they were subjected to the following: G1 - saline solution; G2 - sodium hypochlorite (NaOCl); G3 - NaOCl + ethylenediaminetetraacetic acid (EDTA); G4 - NaOCl + peracetic acid (PAA); G5 - NaOCl + etidronate (HEDP); G6 - NaOCl + EDTA + CHX; G7 - NaOCl + PAA + CHX; and G8 - NaOCl + HEDP + CHX. After the different treatments, the roughness and wettability were measured. In the assays for the assessment of adhesion of microorganisms, after 2 h of contact with the microorganisms, the samples were analysed using a confocal laser scanning microscope. Absorption spectra were collected by attenuated total reflectance of Fourier transform infrared spectroscopy before and after immersion of the dentin samples in each solution of G6, G7 and G8 and in a solution of 2% CHX at different time intervals. The areas of the band associated with CHX with the peak at 1492 cm^{-1} were determined.

Results Saline solution, NaOCl, HEDP and CHX did not alter the roughness of the dentin, but EDTA and PAA did. Dentin surface wettability increased after the use of all irrigants, with HEDP causing the highest increase. The presence of smear layer or exposed collagen favoured the adhesion of *E. faecalis*. The adhesion of *C. albicans* was highest on surfaces with smear layer or mineral rich. The use of CHX as a final irrigant reduced the adhesion of both microorganisms. The wettability did not influence the adhesion of microorganisms, while increases in roughness seemed to enhance the adherence of *E. faecalis.* The adsorption of CHX to the dentin was significant after 1 min of immersion of the mineralised samples in the irrigant, and the use of chelating agents prior to CHX potentiated this adsorption.

Conclusions The irrigation solutions differently affect the properties of dentin, and these modifications influence the adhesion of *E. faecalis* and *C. albicans* and the adsorption of CHX to the dentin surface.

Keywords: Bacterial adhesion, *Candida albicans*, Chlorhexidine, *Enterococcus faecalis*, Irrigation solutions, Surface properties.

INTRODUCTION

The presence of microorganisms in the root canal system or in the periapical area is the most important factor in the failure of endodontic treatments (Lin *et al.* 1992, Siqueira 2001). *Enterococcus faecalis* has frequently been detected in teeth with apical periodontitis (Molander *et al.* 1998, Penas *et al.* 2013), and *Candida albicans* is the most prevalent species of fungi associated with persistent endodontic infections (Waltimo *et al.* 1997, Persoon *et al.* 2016). Although these microorganisms are not always among the colonisers of the root canal system with necrotic tissue, they may invade the root canal at any time during or after treatment via a communication with the oral cavity, such as through a defective coronal seal, and form biofilms that cause secondary infections (Zehnder & Guggenheim 2009, Persoon *et al.* 2016, Delboni *et al.* 2017).

The irreversible adhesion of microorganisms to surfaces is the first step in colonisation and biofilm formation and can result in persistent and chronic infections (Odds 1988, Costerton *et al.* 1999, Baca *et al.* 2011). The mechanism for this attachment seems to involve the interaction of the microorganisms' cell surface proteins with the substratum (McCourtie & Douglas 1984, Calderone & Braun 1991, Nallapareddy *et al.* 2000a, Nallapareddy *et al.* 2000b, Modrzewska & Kurnatowski 2015). The physicochemical properties of the substratum such as roughness, surface free energy, wettability and composition are believed to influence this initial adhesion (Quirynen & Bollen 1995, Teughels *et al.* 2006, Tang *et al.* 2011). Studies have shown that surfaces with high roughness and free energy provide more favourable sites for colonisation and biofilm formation (Quirynen & Bollen 1995, An & Friedman 1998, Teughels *et al.* 2006, Subramani *et al.* 2009). However, conflicting evidence exists because recent papers have reported that surface free energy appears to have more influence on initial fungal biofilm formation than roughness (Burgers *et al.* 2010) and that the increase in roughness does not stimulate the initial adhesion of microorganisms (do Nascimento *et al.* 2013, Ferreira Ribeiro *et al.* 2016). Regarding the substratum composition, *E. faecalis* showed a high affinity for exposed unmineralised collagen fibrils (Nallapareddy *et al.* 2000a, Love 2001, Kishen *et al.* 2008) and *C. albicans* presented an adhesion enhancement in the presence of calcium (Holmes *et al.* 1991, Klotz *et al.* 1993), while both highly adhered on dentin surfaces covered by smear layer (Klotz *et al.* 1993, Sen *et al.* 1997a, Sen *et al.* 2003, Kishen *et al.* 2008).

The irrigants used in endodontic procedures can modify the physicochemical characteristics of dentin (Ari *et al.* 2004, Ari & Erdemir 2005, Ballal *et al.* 2010, Hu *et al.* 2010a, Hu *et al.* 2010b, Cobankara *et al.* 2011, Tartari *et al.* 2013a, Tartari *et al.* 2013b, Tartari *et al.* 2017b) and consequently influence the adhesion of microorganisms to this substratum in cases of secondary infections (Ates *et al.* 2005, Kishen *et al.* 2008, Jaramillo *et al.* 2012). Once there is no single irrigation solution that has all the desired properties of an ideal irrigant, it is necessary to use more than one solution to better clean and disinfect the root canal system

during an endodontic treatment (Zehnder 2006, Haapasalo *et al.* 2014). The solution of chlorhexidine (CHX) seems to be a promising final irrigant in these associations, because of its adsorption to the dentin (Rölla *et al.* 1970) and slow release due to a phenomenon known as substantivity (Rölla & Melsen 1975, Parsons *et al.* 1980) that extends its antimicrobial residual activity (Komorowski *et al.* 2000, Zamany *et al.* 2003, Kishen *et al.* 2008). However, there is a lack of studies assessing the effects of different combinations of irrigants on dentin roughness and wettability, particularly pertaining to the effects of peracetic acid (PAA) and etidronate (HEDP), which were proposed as substitutes for EDTA (Zehnder *et al.* 2005, Lottanti *et al.* 2009). These studies should also address the effects of these agents on adhesion of microorganisms and biofilm.

Therefore, the objectives of this study were to evaluate the roughness and wettability of the dentin surface after the use of different combinations of irrigation solutions in irrigation protocols and to quantify the adhesion of *E. faecalis* and *C. albicans* on the dentin surface subjected to the same protocols. In addition, the adsorption of CHX to mineralised dentin and to dentin previously demineralised by different irrigation protocols was quantified using attenuated total reflectance (ATR) of Fourier transform infrared spectroscopy (FTIR). The null hypothesis tested were that there are no differences among the different combinations of irrigants on the roughness and wettability of the dentin surface and on the adhesion of microorganisms and adsorption of CHX to the dentin.

MATERIAL AND METHODS

Solutions

For this study, 0.9% saline solution (LBS Laborasa, São Paulo, SP, Brazil), 17% EDTA (Biodinâmica Química e Farmacêutica Ltda., Ibiporã, PR, Brazil) and 2% CHX solution (Maquira, Maringá, PR, Brasil) were purchased from commercially available sources. Sodium hypochlorite at 2.5% and 5% (NaOCl, Sigma-Aldrich, St. Louis, MO, USA), 18% HEDP (Zschimmer & Schwarz, Burgstädt, Germany) and 0.5% PAA (Sigma-Aldrich) solutions were prepared with distilled water. All solutions were stored at 4°C in air-tight dark containers until the use. For all experiments, irrigation solutions were taken from the refrigerator and placed for 60 min at room temperature prior to being used.

Roughness and wettability analyses

A diamond disc was used to remove incisal boards and the roots of bovine incisors teeth. The crowns obtained were fixed in an apparatus and separated into buccal and lingual portions using an IsoMet precision saw (Buehler Ltd., Lake Bluff, IL, USA). Then, dentin slices with parallel surfaces and approximately 1.5 mm thickness were obtained from the buccal and lingual portions of the crowns also using an IsoMet precision saw. The enamel surrounding the slices was eliminated with a diamond disc, and the slices were cut in samples with

approximately 7 × 7 × 1.5 mm (length x width x thickness). All cuts were performed under water cooling. The specimens were wet polished with 600-, 1200- and 2000-grit silicon carbide abrasive papers (3M do Brasil Ltda., Sumaré, SP, Brazil), and then ultrasonicated in distilled water to remove residual particles.

The surface roughness of each specimen was determined using the parameter Ra (arithmetic average roughness - μm) with the MarSurf XR 20 roughness measuring station (Mahr, Göttingen, Germany). Five tracings with a cut-off of 2.5 mm and 1 mm distance from each other were made on the dentin surface. The specimens were considered prepared for the experiments when they presented a roughness standard average between 0.10 and 0.15 Ra; the samples that did not present this average value were polished again until this value was reached.

The specimens were them randomly distributed in the following groups (n = 10): G1- 0.9% saline solution (5 min); G2- 2.5% NaOCl (5 min); G3- 2.5% NaOCl (5 min) + 17% EDTA (1 min); G4- 2.5% NaOCl (5 min) + 0.5% AP (1 min); G5- 2.5% NaOCl (5 min) + 9% HEDP (5 min); G6- 2.5% NaOCl (5 min) + 17% EDTA (1 min) + 2% CHX (2 min); G7- 2.5% NaOCl (5 min) + 0.5% AP (1 min) + 2% CHX (2 min); G8- 2.5% NaOCl (5 min) + 9% HEDP(5 min) + 2% CHX (2 min); and G9- mixture of 5% NaOCl + 18% HEDP in equal proportions (5 min). Each specimen was immersed in 2 mL of every solution in the irrigation protocols for the pre-established time, with sonication performed for 15 s/min. After the use of each irrigant, the specimens were rinsed in 2 mL of distilled water for 1 min. When the irrigation sequence was completed, the specimens were dried with absorbent paper and new roughness measurements were taken as previously described.

Following the roughness measurements, the same specimens had their water contact angles evaluated by the sessile drop technique using the goniometer Optical Contact Angle OCA20 (DataPhysics Instruments GmbH, Filderstadt, Germany). Three drops (5 μL/drop) of Milli-Q water (Billerica, MA, USA) were automatically deposited on the surface of the dentin specimens with a microsyringe attached to the equipment. Images were captured 30 s after water deposition using a microvideo system. The acquired images were analysed using the software SCA 20 (DataPhysics Instruments GmbH), and static contact angles between the treated dentin surfaces and water were determined. The three values obtained from each dentin specimen were used to determine the average water contact angle of each sample. All measurements were performed in a closed environment with a controlled room temperature (22°C).

Assays of adhesion of microorganisms

For these experiments, dentin samples of approximately 5 x 5 x 1.5 mm (length x width x thickness) size were prepared as previously described, including the standardisation of the surface roughness. After sterilisation, the samples were distributed in the same nine groups (n

= 10) tested in the roughness and wettability assays and irrigated in an identical manner, but inside a laminar flow chamber. All samples were dried with sterilised absorbent paper and stored until experimentation.

Strains of *E. faecalis* OG1RF and *C. albicans* SC5314 were used in the assays of adhesion of microorganisms. Cells of *E. faecalis* from glycerol stocks were streaked out on brain–heart infusion (BHI) agar (Sigma-Aldrich) plates and cultivated. Single colonies were inoculated in BHI bouillon (Sigma-Aldrich) and allowed to grow overnight (14 h) in an orbital incubator at 200 rpm, 37°C and 5% CO_2. *C. albicans* was cultivated in Sabouraud Dextrose agar and bouillon (Sigma-Aldrich). The optical density (OD600) of *E. faecalis* was measured using a spectrophotometer (Shimadzu Kyoto, Japan) and adjusted to 0.25 to obtain a bacterial suspension. The concentration of *C. albicans* was determined using a Neubauer counting chamber and adjusted to 1×10^7 cells/mL colony-forming units. The concentrations of the microorganisms to be used in the experiments were determined in pilot studies.

The dentin samples were individually inserted into wells of 12-well plates, and 2 mL of *E. faecalis* or *C. albicans* suspensions were added to each well. Dentin blocks of each group were inoculated with 2 mL of BHI or Sabouraud bouillon to serve as sterility controls. The samples were incubated at 37°C for 2 h with constant swirling. After the incubation period, the samples were taken out of the wells, rinsed with PBS to remove the nonadherent microorganisms and stained with Live/Dead *Bac*Light (Invitrogen, Molecular Probes, Carlsbad, CA) for 15 min. The specimens were then washed again with PBS and observed with a confocal laser scanning microscope (Nikon Eclipse 80i; Tokyo, Japan). Each specimen was scanned at six different locations, and standard images were obtained with a ×100 objective under oil immersion. The adhering microorganisms in each field were counted with the aid of ImageJ software, and the average number of adherent cells in each specimen was determined.

FTIR analysis

Dentin specimens from bovine crowns with dimensions of 4 x 4 x 0.8 mm (length x width x thickness) were obtained the same way as that described before. One of the sample's surfaces was sequentially polished with 4000-grit silicon carbide abrasive paper (Buehler, Lake Bluff, IL, USA) and alpha alumina suspensions with 1 and 0.3 microns (Struers, Ballerup, Denmark) until it became flat and smooth.

Then, the samples were ultrasonicated in distilled water for 1 min to remove cutting and polishing residues. The samples were dried with absorbent paper, and the compositional analysis of the polished surface was performed using ATR technique with a diamond crystal detector coupled to the FTIR Spectrometer Nicolet 380 (Thermo Fisher Scientific Inc.; Waltham, MA, USA). The initial infrared absorbance spectrum of the samples was obtained over the range of 4000–400 cm^{-1} at 1 cm^{-1} resolution using 32 scans and was recorded using the OMNIC Spectra Software. Thereafter, the samples were distributed into four groups (n =

5) and were subjected to irrigation protocols as previously described elsewhere (Tartari *et al.* 2016). In the first set of experiments, the samples of one group were dipped individually in microtubes containing 1.5 mL of 2% CHX solution for 30 s and ultrasonicated for 15 s. Then, they received a flush in a microtube with 1.5 mL of distilled water for 1 min of which 15 s were under ultrasonic agitation. They were then dried with absorbent paper, and the ATR-FTIR spectra were again recorded. This procedure was sequentially repeated with samples exposed to CHX solution at time intervals of 0, 0.5, 1, 2, 5 and 10 min. After achieving the spectrum of 1 min, the ultrasonic agitation passed to be performed for 15 s/min of immersion in CHX.

In the second set of experiments, the dentin slices of the other three groups were subjected to one of the following irrigation protocols (n = 5): A- 2.5% NaOCl (5 min) + 17% EDTA (1 min) + 2% CHX (2 and 5 min); B - 2.5% NaOCl (5 min) + 0.5% PAA (1 min) + 2% CHX (2 and 5 min); and C - 2.5% NaOCl (5 min) + 9% HEDP (5 min) + 2% CHX (2 and 5 min). Absorption spectra were collected using ATR-FTIR after each immersion of the samples in the different solutions.

The band at 1492 cm^{-1} assigned to the functional group methylene (C=C) of CHX appeared in the infrared spectra of dentin samples after immersion in this irrigant and was observed between 1479 and 1500 cm^{-1} of the spectral range.

Statistical analyses

Values offor the roughness, wettability, adhesion of microorganisms and area of the CHX band obtained presented normal distribution on the Shapiro–Wilk test. Based on this, the values of roughness, wettability, adhesion of microorganisms and area of the CHX band were analysed using one-way analysis of variance (ANOVA) with Tukey's multiple-comparison test ($\alpha < 0.05$) for intergroup comparisons. The values of the CHX band were also subjected to one-way repeated measures ANOVA with Tukey's multiple-comparison test ($\alpha < 0.05$) for intragroup comparisons over different time intervals.

RESULTS

Roughness, Wettability and Adhesion of Microorganisms

The values of the average (X) and standard deviation (SD) of roughness and wettability of the dentin surface and the number of cells of *E. faecalis* and *C. albicans* adhered on the samples are presented in Table 1. The initial roughness of the dentin surface was standardised, and all groups were statistically similar (one-way ANOVA, $P > 0.05$, data not shown). The saline solution did not alter the surface compared with the initial value ($P > 0.05$, data not shown) and served as the control for the comparisons with all other groups. The groups that used 2.5% NaOCl alone (G2) and HEDP as the chelating solution isolated or mixed with NaOCl (G5, G8 and G9) were similar among each other and with the control group of saline solution ($P > 0.05$). The highest modifications on the surface roughness were observed

with the use of 17% EDTA, followed by 0.5% PAA. The use of 2% CHX solution as the final irrigant (G6, G7 and G8) did not induce major alterations on the surface when comparing to the protocols with the same sequence of NaOCl + chelating agent but without the use of CHX (G3, G4 and G5).

The values for contact angles were significantly reduced ($P < 0.05$) after the treatment of dentin samples with all irrigation protocols tested when compared to the saline solution (G1). The use of HEDP as the final irrigant in the groups G5 (NaOCl + HEDP) and G9 (Mixture of NaOCl + HEDP) promoted significant increases in the wettability of dentin compared with the other groups ($P < 0.05$). The contact angles in the groups with CHX as the final irrigant (G6, G7 and G8) had significantly increased ($P > 0.05$) when compared with the counterpart groups without CHX (G3, G4 and G5).

Regarding the adhesion of microorganism, all negative controls showed no growth. For *E. faecalis*, the groups G1 (saline solution), G2 (NaOCl), G3 (NaOCl + EDTA), G4 (NaOCl + PAA) and G5 (NaOCl + HEDP) significantly favoured the adhesion of the bacteria when compared to all other groups ($P < 0.05$). The use of the mixture of NaOCl + HEDP in G9 and CHX as the final irrigant in G6 (NaOCl + EDTA + CHX), G7 (NaOCl + PAA + CHX) and G8 (NaOCl + HEDP + CHX) resulted in lower initial adherence of *E. faecalis*.

For *C. albicans*, the results showed a significantly greater affinity ($P > 0.05$) to the surfaces of G1 (saline solution), statistically followed by G2 (NaOCl) and G9 (mixture of NaOCl + HEDP). The use of decalcifying agents as the final irrigant in G3 (NaOCl + EDTA), G4 (NaOCl + PAA) and G5 (NaOCl + HEDP) significantly reduced the quantities of microorganisms adhering ($P < 0.05$). The final flushes with CHX in G6 (NaOCl + EDTA + CHX), G7 (NaOCl + PAA + CHX) and G8 (NaOCl + HEDP + CHX) reduced the number of *C. albicans* adhered to the dentin surface, but no differences were identified between these groups with the counterparts G3 (NaOCl + EDTA), G4 (NaOCl + PAA), and G5 (NaOCl + HEDP) that were not irrigated with CHX ($P > 0.05$).

Table 1 – Average (X) and standard deviation (SD) values for the surface roughness before and after dentin submission to the different irrigation sequences and for the dentin surface contact angle, Enterococcus faecalis, Candida albicans adhesion after dentin submission to the different irrigation sequences.

Group	**Roughness final (Ra) X ± SD**	**Contact angle (degrees) X ± SD**	**E. faecalis (nº of cells) X ± SD**	**C. albicans (nº of cells) X ± SD**
G1 – 0.9% saline solution (5')	0.136 ± 0,01^{E}	59.4 ± 10.8^{A}	277 ± 92^{A}	98 ± 18^{A}
G2 – 2.5% NaOCl (5')	0.154 ± 0,01^{E}	37.6 ± 10.5^{BC}	260 ± 108^{A}	61 ± 16^{B}
G3 – 2.5% NaOCl (5') + 17% EDTA (1')	0.396 ± 0,12^{A}	33.7 ± 3.5^{C}	271 ± 88A^{A}	36 ± 16^{C}
G4 – 2.5% NaOCl (5') + 0.5% PAA (1')	0.250 ± 0.05^{BCD}	33.5 ± 5.2^{C}	249 ± 89^{AB}	31 ± 14^{C}
G5 – 2.5% NaOCl (5') + 9% HEDP (5')	0.201 ± 0.02^{CDE}	20.7 ± 3.7^{D}	161 ± 29^{B}	37 ± 12^{C}
G6 – 2.5% NaOCl (5') + 17% EDTA (1') + 2% CHX (2')	0.321 ± 0.06^{AB}	42.6 ± 3.4^{B}	47 ± 14^{C}	19 ± 10^{C}
G7 – 2.5% NaOCl (5') + 0.5% PAA (1') + 2% CHX (2')	0.275 ± 0.03^{BC}	44.3 ± 3.1^{B}	51 ± 17^{C}	19 ± 9^{C}
G8 – 2.5% NaOCl (5') + 9% HEDP (5') + 2% CHX (2')	0.194 ± 0.03^{DE}	39.2 ± 3.6^{BC}	38 ± 14^{C}	24 ± 8^{C}
G9 – Mixture of 5% NaOCl and 18% HEDP (5')	0.212 ± 0.02^{CDE}	16.0 ± 3.0^{D}	65 ± 14^{C}	61 ± 21^{B}

*One-way Anova p-value < 0.05; Different capital letters in the same column indicate statistically significant intergroup differences.

FTIR analysis

Figure 1 presents the infrared absorption spectra of CHX and mineralised dentin. The band associated with the functional group methylene of CHX that is located at 1479–1500 cm^{-1} with peak at 1492 cm^{-1} does not overlap with the bands of the dentin. This band passed to be observed on the absorption spectra collected from dentin samples after immersion in CHX.

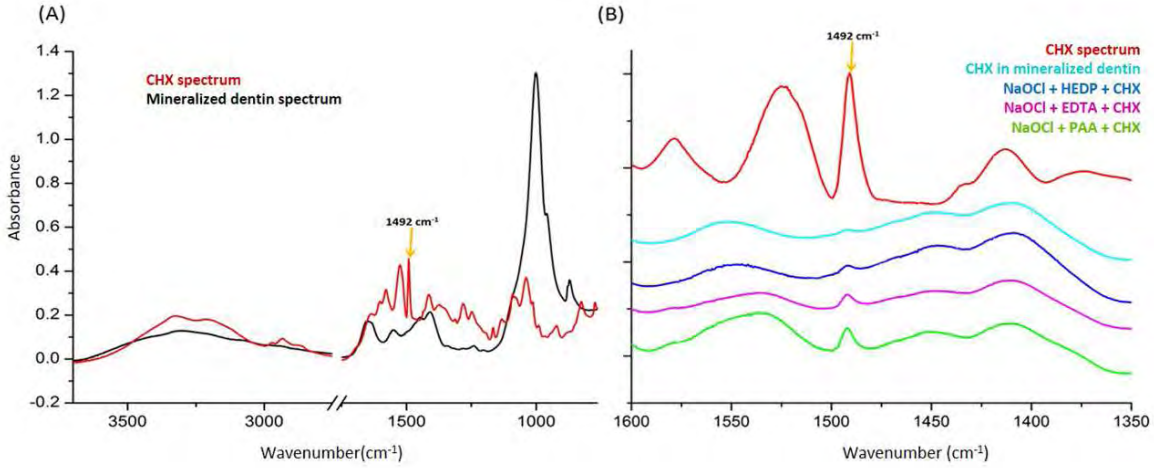

Figure 1 (A) Absorption spectra of CHX and mineralised dentin. The band associated with CHX located at 1492 cm^{-1} does not overlap with the bands of dentin. It is observed in (B) a magnification of the spectral region from 1600–1350 cm^{-1} to better visualise the band associated with CHX in mineralised dentin and in dentin subjected to the different irrigation protocols after immersion in 2% CHX for 2 min.

Table 2 presents the X and SD values for the area of the CHX band at 1479–1500 cm^{-1} in the spectra obtained from mineralised dentin samples immersed in CHX and from dentin samples subjected to the use of different irrigation solutions prior to CHX. In the mineralised dentin samples, the area of the CHX band presented a significant value after 1 min of immersion in the irrigant ($P < 0.05$). The values of the band continued to increase in the subsequent periods of immersion (2, 5 and 10 min), but there were no statistical differences among them ($P > 0.05$). In the irrigation protocols, the band at 1492 cm^{-1} was identified just after immersion in CHX. In the intragroup comparison, differences in the area of this band between 2 and 5 min of immersion in CHX were not found in any group ($P > 0.05$). In the intergroup comparison, the values of this band were higher for group B (NaOCl + AP + CHX), followed by group A (NaOCl + AP + CHX), group C (NaOCl + HEDP + CHX) and mineralised dentin with significant differences among all of them in 2 min ($P < 0.05$). This same rank was maintained in 5 min of immersion in CHX; however, there was no difference between group C (NaOCl + HEDP + CHX) and mineralised dentin.

Table 2 – Mean (X) and standard deviation (SD) values for the area of CHX band at 1479 to 1500 cm^{-1} on mineralized dentin surface after immersion in the CHX for different periods of time and on dentin surface immersed previously in different irrigation sequences.

GROUPS	**T0** **X ± SD**	**CHX (0.5')** **X ± SD**	**CHX (1')** **X ± SD**	**CHX (2')** **X ± SD**		**CHX (5')** **X ± SD**	**CHX (10')** **X ± SD**
2% Chlorexidine on mineralized dentin	0.0 ± 0.0^{b}	0.0034 ± 0.0034^{b}	0.0263 ± 0.0062^{a}	$0.0334 \pm 0.0115^{D,a}$		$0.0660 \pm 0.0265^{C,a}$	0.0705 ± 0.0231^{a}
		NaOCl (5') **X ± SD**	**Quelator (1 or 5')** **X ± SD**	**CHX (2')** **X ± SD**	**Increase in the mean of CHX band in 2' compared to mineralized dentin**	**CHX (5')** **X ± SD**	**Increase in the mean of CHX band in 5' compared to mineralized dentin**
A – 2.5% NaOCl (5') + 17% EDTA (1) + 2% CHX (2 and 5')	$0^{A,c}$	$0^{A,c}$	$0^{A,c}$	$0.1587 \pm 0.0318^{B,a}$	4.7 x	$0.2174 \pm 0.0292^{B,a}$	3.3 x
B – 2.5% NaOCl (5') + 0.5% PAA (1') + 2% CHX (2 and 5')	$0^{A,b}$	$0^{A,b}$	$0^{A,b}$	$0.2765 \pm 0.0366^{A,a}$	8.2 x	$0.3148 \pm 0.0513^{A,a}$	4.7 x
C – 2.5% NaOCl (5') + 9% HEBP (5') + 2% CHX (2 and 5')	$0^{A,b}$	$0^{A,b}$	$0^{A,b}$	$0.0810 \pm 0.0117^{C,a}$	2.4 x	$0.1073 \pm 0.0151^{C,a}$	1.6 x

*One-way repeated measures Anova p-value <0.05; Different lowercase letters in the same row indicate statistically significant intragroup differences; *One-way Anova p-value < 0.05; Different capital letters in the same column indicate statistically significant intergroup differences.

DISCUSSION

In this study, the different chemical solutions tested when applied in protocols altered unequally the roughness and wettability of the substrate and resulted in different quantities of microorganisms adhering and CHX adsorbing to the surfaces treated. Thus, the null hypothesis tested needed to be rejected.

In the experiments, the solution of 2% CHX was not used after NaOCl and the mixture of NaOCl + HEDP because in the pilot studies, the flush with distilled water after the use of these irrigants was not effective in preventing the formation of a brown precipitated that stained the dentin, as previously observed (Magro *et al.* 2015). Because tooth discoloration should be avoided in a clinical scenario, these protocols were excluded from the tests.

The initial surface roughness of the dentin specimens of all groups was standardised to avoid bias in the results of the experiments because it offers a controlled reference point to assess the modifications induced by the chemical irrigants (El Feninat *et al.* 2001). The solutions of NaOCl and HEDP do not alter the surface roughness. These results are similar to those of previous papers that already showed that NaOCl does not increase dentin roughness (Tartari *et al.* 2013b, Pascon *et al.* 2014) and that HEDP is a weak chelating agent that causes minor alterations (Tartari *et al.* 2013b). The higher values presented in the mixture of NaOCl + HEDP and EDTA in a former study (Tartari *et al.* 2013b) might be explained by the longer times of immersion of the samples in the solutions. Although CHX was used as a final irrigant in G6 (NaOCl + EDTA + CHX), G7 (NaOCl + PAA + CHX) and G8 (NaOCl + HEDP + CHX), the comparison with the counterpart other groups without the use of CHX solution (G3, G4 and G5) allows us to conclude that this irrigant does not alter the surface roughness as previously shown (Ari *et al.* 2004, Patil & Uppin 2011).

Dentin roughness significantly increased after the removal of the smear layer and demineralisation of the surface by EDTA and PAA. EDTA induced considerable major increases on the roughness in the protocols it was applied, and these greater effects of EDTA were already observed (Ari *et al.* 2004, Eldeniz *et al.* 2005, Ballal *et al.* 2010, Patil & Uppin 2011, Pascon *et al.* 2014). However, PAA caused major demineralisation of the dentin surface compared with EDTA in other studies (Cobankara *et al.* 2011, Tartari *et al.* 2017a), but this effect might not be expressed in the dentin roughness the same way because the decalcification kinetics of PAA and EDTA seem to be different (Kawasaki *et al.* 2000).

Wettability is an important physicochemical property of dentin that might influence the adhesion and is determined by the measurement of contact angles of the substances with the substratum. Low contact angles are formed on surfaces with greater wettability (Marshall *et al.* 2010). All protocols tested in this study had contact angles that were significantly less than the saline solution, indicating an increase in the dentin wettability. The increase in the wettability promoted by NaOCl agrees with a previous study (Hu *et al.* 2010a, Yilmaz *et al.* 2011) and

might be the result of deproteination of dentin collagen that makes the surface more hydrophilic (Panighi & G'Sell 1992, Attal *et al.* 1994). Although NaOCl just removed the organic components of the smear layer, the surfaces of the samples of G2 (NaOCl) are cleaner than those of G1 (saline solution), and these results support the concept that low contact angles are formed on cleaner surfaces with high energy (Marshall *et al.* 2010). However, other authors reported a decrease or no change on the surface wettability with the use of NaOCl (Attal *et al.* 1994, Dogan Buzoglu *et al.* 2007). Some studies reported reductions in the wettability of dentin surface after the use of EDTA (Attal *et al.* 1994, Dogan Buzoglu *et al.* 2007, Hu *et al.* 2010a), which was not observed in this study. The use of all decalcifying agents after NaOCl showed increases in the values of wettability compared with the group that used just NaOCl (G2), although differences were just significant with the use of HEDP. These findings concur with those of previous papers that reported a reduction in the contact angles of dentin surface with the use of EDTA and other decalcifying agents (Rosales *et al.* 1999, Toledano *et al.* 1999, Yilmaz *et al.* 2011), and it might be the result of surface cleanliness due to the removal of the smear layer, which seems to have a character mainly hydrophobic (Attal *et al.* 1994), and the increases in the surface roughness.

It has also been postulated that the wettability of a surface is dependent on many other factors, such as roughness, hydration state and chemical composition (Rosales *et al.* 1999), and the standardisation of the initial roughness of the dentin surface before subjection to the different irrigation protocols is also important to avoid bias in the wettability experiments. Although it is difficult to determine the contribution of surface roughness to surface wettability (Attal *et al.* 1994, Al-Omari *et al.* 2001) based on the results of this study, it is possible to conclude that the components of the dentin surface exert more effects than the roughness. An example is the groups with HEDP as a final irrigant (G5 and G9); although the samples had minor changes in roughness, the strong adsorption of this irrigant to the hydroxyapatite crystals increased the surface Gibbs free energy (Francis & Valent 2007). The high surface Gibbs free energy gives rise to low contact angles, which express high wettability of the dentin that facilitates molecular attraction between materials. This explains the lowest gap size values and better marginal adaptation of AH Plus observed on the dentin walls irrigated with HEDP compared with other irrigants (Ulusoy *et al.* 2017), as well as the high push-out bond strength values for the groups irrigated with HEDP (De-Deus *et al.* 2008a, Neelakantan *et al.* 2012). Another example that the components of the dentin surface influence the wettability more than the roughness is the groups with CHX final flushes (G6, G7 and G8). Even though the CHX does not modify dentin roughness, its adsorption increased the contact angles of the groups G6 (NaOCl + EDTA + CHX), G7 (NaOCl + PAA + CHX) and G8 (NaOCl + HEDP + CHX) compared with the counterpart groups G3 (NaOCl + EDTA), G4 (NaOCl + PAA) and G5 (NaOCl + HEDP), reflecting a reduction in the wettability of the dentin, although it is still greater

than the wettability of the control group G1 (saline solution). A previous article also observed a smaller wettability for the group without dentin treatment compared with the group that used CHX aqueous solution after acid etching with phosphoric acid (Ricci *et al.* 2014). However, in that same study, no differences were identified between the groups of acid etching and acid etching + CHX. Aqueous solution of CHX increased the wettability of root canal dentin by endodontic sealers (de Assis *et al.* 2011, Prado *et al.* 2011), but in these studies, the values of the contact angles were also influenced by the hydrophobic or hydrophilic nature of the sealers.

In the experiments of adhesion of microorganisms, the adherence of *E. faecalis* was favoured by the presence of the smear layer on the surface of dentin samples in G1 (saline solution) and G2 (NaOCl) and the collagen matrix exposed in G3 (NaOCl + EDTA), G4 (NaOCl + PAA) and G5 (NaOCl + HEDP). The results of this study corroborate previous findings in which the groups of NaOCl and NaOCl + EDTA were similar between each other and promoted small inhibition in the percentage of biofilm formation (Baca *et al.* 2011). Other studies also emphasised the role of the smear layer (Yang *et al.* 2006, Kishen *et al.* 2008) and collagen in the adherence of *E. faecalis* (Love 2001, Hubble *et al.* 2003, Kowalski *et al.* 2006, Kishen *et al.* 2008). Therefore, it is possible to conclude that the exposure of collagen by the decalcifying agents creates an ideal substrate for *E. faecalis*. It might be because of the virulence factors of this microorganism, such as collagen binding protein and serine protease, that mediate its adherence to the dentin (Nallapareddy *et al.* 2000a, Hubble *et al.* 2003) and allow the invasion of the dentinal tubules (Love 2001). The groups with collagen fibrils denuded but that received final flushes with CHX, which were G6 (NaOCl + EDTA + CHX), G7 (NaOCl + PAA + CHX) and G8 (NaOCl + HEDP + CHX), presented a significant reduction in the quantities of adherent cells, probably due to the residual antimicrobial action of this irrigant. These findings agree with previous studies that reported reductions or complete inhibition of *E. faecalis* adherence or biofilm formation with the use of CHX (Komorowski *et al.* 2000, Yang *et al.* 2006, Kishen *et al.* 2008, Baca *et al.* 2011). Another group that had small quantities of *E. faecalis* adhered was G9 (mixture of NaOCl + HEDP), and this can be justified by the presence of clean surfaces that are rich in minerals and lack collagen (De-Deus *et al.* 2008b, Tartari *et al.* 2017a).

Regarding *C. albicans*, the results showed a greater affinity to the surfaces covered by the smear layer of G1 (saline solution) and G2 (NaOCl) and surfaces rich mineral content of G9 (mixture of NaOCl + HEDP). The group G2 could also be included in this last case, because NaOCl denatures the collagen of the smear layer and dentin, leaving the inorganic components of the smear layer and unbounded hydroxyapatite crystals on the surface (Di Renzo *et al.* 2001, Tartari *et al.* 2017a). Previous studies with *C. albicans* showed that the presence of smear layer increased its adhesion to the dentin and resulted in the formation of dense biofilms (Sen *et al.* 1997a, Sen *et al.* 2003, Turk *et al.* 2008). Magnesium and calcium ions have a

critical role in *C. albicans* growth and adherence to extracellular matrix proteins (Holmes *et al.* 1991, Klotz *et al.* 1993) that can justify the major adherence to mineral-rich surfaces. *C. albicans* seem to show a poor attachment to clean surfaces (Cannon *et al.* 1995, Sen *et al.* 1997b, Sen *et al.* 2003), and a slow and limited penetration by both hyphae and yeasts was reported on dentin that had the smear layer removed by EDTA (Waltimo *et al.* 2000). The results of this study showed a significant reduction in the quantity of *C. albicans* adhered to the surfaces demineralised by the decalcifying agents in G3 (NaOCl + EDTA), G4 (NaOCl + PAA) and G5 (NaOCl + HEDP). These results reinforce that this microorganism can use the dentin as a source of nutrition (Sen *et al.* 1997b) by digesting the collagen (Kaminishi *et al.* 1986, Hagihara *et al.* 1988) and releasing the calcium necessary for their calcium-dependent surface proteins with adherence activity (Klotz *et al.* 1993). The final flushes with CHX reduced the quantity of cells adhered in G6 (NaOCl + EDTA + CHX), G7 (NaOCl + PAA + CHX) and G8 (NaOCl + HEDP + CHX) compared with G3 (NaOCl + EDTA), G4 (NaOCl + PAA) and G5 (NaOCl + HEDP); however, there were no statistical differences among all these groups.

An important fact to be reported is that in the groups that CHX was used as the final irrigant, a high quantity of *E. faecalis* and *C. albicans* that adhered to the surfaces were stained in red by the Live/Dead Baclight stain, which was not observed in the other groups. This fact might be related to the residual antimicrobial effect of CHX that occurs as a result of its adsorption to the surface of the dentin samples, as shown in the ATR-FTIR experiments, and the subsequent release (Parsons *et al.* 1980). This CHX release, besides to interfere with the adhesion of microorganisms, probably caused damage to the cytoplasmic membrane of the microorganisms that adhered, resulting in the microleakage and precipitation of the intracellular components and cell death (Hugo & Longworth 1964, 1965, Davies 1973, Yang *et al.* 2006).

Statistical correlation tests were not performed with the experimental data from the measurements of roughness, wettability and adhesion of microorganisms because the dentin samples used in the different sets of experiments were not the same, although they were identically prepared. The increase in roughness and wettability is shown to increase the adhesion of microorganisms on surfaces (Mei *et al.* 2011, Tang *et al.* 2011, Petrackova *et al.* 2013). In this study, the effects of roughness on adhesion of *E. faecalis* can be observed when comparing the results of G3 (NaOCl + EDTA) and G5 (NaOCl + HEDP). Although these groups have a similar surface composition with collagen matrix exposed, the group G3 had a greater roughness than G5 and also a higher quantity of *E. faecalis* adhered. For the *C. albicans*, the influence of roughness on adhesion could not be established by this study, as previously described (Burgers *et al.* 2010, do Nascimento *et al.* 2013). Regarding the wettability, it was not possible to observe a pattern between the values obtained and the number of both microorganisms that adhered. For groups with similar roughness and wettability but different

compositions, such as G5 (NaOCl + HEDP) and G9 (mixture of NaOCl + HEDP), the adhesion was greater on the surfaces that offered a better chemical substrate for each type of microorganism. For *E. faecalis*, the adhesion was higher in G5 because of the presence of exposed collagen matrix, and for *C. albicans*, it was higher in G9 probably due to the surface rich in mineral (Tartari *et al.* 2017a).

In ATR-FTIR, the adsorption of CHX on the dentin surface (Rölla *et al.* 1970) and the superimposition of CHX absorption infrared bands with the bands of the dentin were detected (Figure 1), agreeing with a previous study (Kim *et al.* 2010) and shifting the bands of amide III and carbonate of the dentin due to chemical interaction. Intensity change of some absorption bands were also observed by Botta *et al.* (2012). However, a band associated with the functional group methylene of this irrigant, which has the peak located in 1492 cm^{-1}, does not present superimposition with dentin bands. The spectra analysis of the present study demonstrated that on the mineralised dentin samples, the adsorption of CHX was immediately detected in 30 s of immersion in the irrigant and increased over time. Moreover, there were no statistical differences among the time intervals of 1, 2, 5 and 10 min of immersion and the area of the 1492 cm^{-1} infrared band in 5 min was more than twice the area obtained in 1 min. However, between the 5- and 10-min periods, the value of the area was almost the same, suggesting that the adhesion of the irrigant to the surface reached a plateau. On the dentin samples previously subjected to irrigation protocols that employed different decalcifying agents, the area of the CHX infrared band was significantly higher in 2 and 5 min when compared to the mineralised dentin, except for group C (NaOCl + HEDP + CHX) in 5 min. These results showed a greater CHX binding to the demineralised dentin, agreeing with a previous study (Kim *et al.* 2010).

It was suggested that CHX binding to mineralised and demineralised dentin occurs via different mechanisms. On mineralised surfaces, it reacts with phosphate of the hydroxyapatite crystals (Misra 1994, Kim *et al.* 2010). The positive charges of CHX were electrostatically attracted to the negative charges of the phosphate groups (Kim *et al.* 2010). On the demineralised dentin, this interaction seems to occur with the collagen (Kim *et al.* 2010, Botta *et al.* 2012). The CHX charges might bind electrostatically or by hydrogen bond to the negative charges of the carboxyl groups of glutamic and aspartic amino acids of the collagen and associated noncollagenous exposed proteins (Kim *et al.* 2010). Moreover, on the demineralised dentin, the CHX can diffuse through the collagen matrix and remain trapped within the spaces between the collagen fibrils (Kim *et al.* 2010). This penetration and permanence of the irrigant adhered to the collagen matrix explains the differences in the values of CHX binding among the groups A, B and C. Once the degree of demineralisation of the dentin surface seems to be bigger for PAA, followed by EDTA and HEDP (Tartari *et al.* 2017a), it is possible to conclude that the higher the demineralisation capacity, the higher is the area

of collagen matrix exposed for CHX to penetrate and consequently the higher is the quantity of CHX adhered. These results also showed that the flush with distilled water to remove the excess irrigants from the samples was not able to completely remove CHX that was adhered to the dentin. The water might have removed some CHX that was inside the dentin tubules and collagen matrix, but it was not effective to unbind all the irrigant, an observation that disagrees with that of another study that recommended avoiding water rinses after the use of CHX (Kim *et al.* 2010).

Within its limitations, this study started to fill a gap in the literature regarding the effects of different irrigation solutions when applied in protocols on dentin surface roughness and wettability, in the adsorption of CHX and the consequences in the initial adhesion of *E. faecalis* and *C. albicans*. The results suggested that to prevent the invasion and biofilm formation of both species tested, the protocols with final flushes with CHX are good options. However, little is known about how long the effects of CHX will last to prevent secondary colonisation by microorganisms. An alternative to prevent the initial adhesion of *E. faecalis* and *C. albicans* is the mixture of NaOCl + HEDP because although it did not cause higher reduction in the *C. albicans* adherence, this protocol was able to reduce the adhesion of both species. Future studies should be directed to determine the long-term effects of the protocols tested regarding the adhesion of microorganisms and root canal sealers to the dentin layer.

CONCLUSION

The irrigation protocols investigated differently change the roughness and wettability of the surfaces. In surfaces with similar composition, the increases in the roughness seem to potentiate adhesion of *E. faecalis*, while the wettability did not influence the quantities of both microorganisms that adhered to the surface. The adhesion of *E. faecalis* and *C. albicans* to the dentin were highly influenced by the composition of the surface. The final flushes with CHX reduced the adhesion of microorganisms and the risk of secondary contamination of the root canal system. The use of chelating solutions previously to CHX potentiates the adhesion of this irrigant to the dentin.

ACKNOWLEDGMENTS

This study was supported by State of São Paulo Research Foundation, FAPESP (2012/02460-6, 2013/19789-3, and 2015/09958-8) and UTHSC School of Dentistry Startup funds to Dr. Renato Silva.

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2.6 Article 6 - **Different Irrigation Protocols Promote Stable Long-Term Push-out Bond Strength of AH Plus to Dentin**

The article presented in this thesis was written according to the Journal of Endodontics instructions and guidelines for article submission

Abstract

Introduction: This study analyzed the impact of different irrigation protocols on the dentin surface and on bond strength of AH Plus 7 days and 20 months after obturation.

Methods: Canals of bovine incisors were prepared and after irrigated with (n=21): G1 – 2.5% sodium hypochlorite (NaOCl); G2 – 2.5% NaOCl + 17% EDTA; G3 – 2.5% NaOCl + 17% EDTA + 2.5% NaOCl; G4 – 2.5% NaOCl + 17% EDTA + 2% chlorhexidine (CHX); G5 – mixture 5% NaOCl + 18% etidronate (HEBP); and G6 – mixture 5% NaOCl + 10% tetrasodium EDTA ($EDTANa_4$). After the irrigation 1 root/group was split and images were obtained by scanning electron microscopy (SEM). The other 20 roots/group were filled with AH Plus sealer. In 7 days, transversal cuts were performed in 10 roots/group with 2 mm of distance between each other. Three slices per root were used for the push-out assessment on a universal testing machine. The other 10 roots/group were stored for 20 months and after were cut and the samples analyzed the same way. One-way ANOVA and Tukey ($\alpha < 0.05$) were used to compare the results among the experimental groups and unpaired t-test ($\alpha < 0.05$) to compare the results of the same group over time.

Results: The photomicrographs showed that, except for G1, all groups removed the smear layer from the dentin surfaces. In G2 and G4 there was enlargement of the opening of dentin tubules and in G3 erosion can be observed in the peritubular and intertubular dentin. The values of the push-out bond strength 7 days after obturation were G2=G3=G4=G5>G1=G6. In 20 months, the values of the bond strength were G3=G5>G6=G4>G1=G2. The groups G3, G5 and G6 presented values of push-out bond strength in 20 months similar to the values in 7 days ($P > 0.05$).

Conclusions: The irrigation protocols tested produced dentin surfaces with different characteristics and the dentin bond strength values for AH Plus were high and stable over time just in the protocols that removed the smear layer and did not leave collagen exposed on the dentin surface.

Keywords: Resin cements, Root canal irrigants, Root canal obturation, Scanning electron microscopy, Surface properties, Time factors

Introduction

Adhesion is a complex process involving molecular interactions at the interface of an adherend, which in dentistry can be the dentin, with an adhesive, such as the endodontic sealers. It can be obtained by physical, chemical, and/or mechanical bonding between the materials, being the latter the most efficacious to create strong joints (1). In the field of Endodontics, the adhesion of the root canal sealers to the dentine walls is important in static situations to eliminate spaces that allow the percolation of fluids (2), prevent microbial reinfection and trap remaining bacteria (3). On the other hand, in dynamic situations, this adhesion should avoid the displacement of the obturation during a subsequent handling (4, 5). However, the achievement of a strong and long-term stable adhesion between the endodontic sealers and the root canal walls still a challenge nowadays.

The properties of the dentin surface are crucial to reaching good interaction with the dental materials and among the relevant features of this substrate are the cleanliness, roughness, and wettability (1). Studies showed that irrigation solutions applied in the process of shaping and cleaning of the root canal system might alter the characteristics of dentin substrate (6-10) and consequently interfere with the adhesion of the endodontic sealers (11-15). The main irrigant in Endodontics is the sodium hypochlorite (NaOCl) that is used due to its antimicrobial action and ability to dissolve the organic matter (16). However, it denatures the collagen of dentin (6, 9) and its oxidizing effect can compromise the polymerization of the resin sealers (15, 17, 18). The EDTA solutions used to remove the smear layer increase the roughness of the surface (8, 10, 19) and produces a large demineralized dentine zone with great amounts of collagen fibrils exposed (6). These fibrils can contribute to the adhesion of endodontic sealers, such as AH Plus (20). However, an incomplete infiltration of the dental materials into the full extension of the exposed collagen matrix results in a weak bond interface that is degraded over time (21-24). This degradation is caused by host-derived proteolytic enzymes, called metalloproteinases, on the denuded collagen fibrils (21, 24).

In theory, there are some irrigation protocols that could help to overcome the problem of the adhesive interface degradation providing stronger, favorable and stable bonding of endodontic sealers over time. One of the protocols proposes the application of chlorhexidine (CHX) after the use of the decalcifying agent, with the intent to inhibit the matrix metalloproteinases (25). Another option is to use weaker chelating agents, such as etidronate (HEDP) or tetrasodium EDTA ($EDTANa_4$), because they promote moderate demineralizing effects that supposedly allow an adequate infiltration of the filling material throughout the depth

of exposed collagen matrix (11). The third alternative is to remove the exposed collagen fibrils with the use of a deproteinizing agent, such as NaOCl, after the chelating agents (6, 9, 26).

Once there is a lack of researches that evaluated the long-term effects of different irrigation protocols on the dentin bond strength of endodontic sealers, this paper presents the impact of different irrigation protocols in the bond strength of AH Plus to dentin in 7 days and 20 months after the obturation. In addition, scanning electron micrographs were obtained from 1 sample per group for analysis of the dentin surface. This study was designed to test the null hypotheses that the different irrigation protocols do not have a significant impact on the immediate and long-term bond strength of AH Plus root fillings and produce similar dentin surfaces for the interaction with the endodontic sealers.

Material and Methods

Specimen preparation

Bovine incisors were sectioned transversally at the cementoenamel junction and apically at the root end to obtain a root segment with approximately 14 mm of length. The cuts were performed using a diamond disc, under water cooling. Following a size 80-K file was used to determinate the diameter of the root canals and 120 roots with the canals of diameters compatible with this instrument or lesser were selected for the study. After, the canals were entirely prepared using the Gates-Glidden drills #3, #4 and #5 and Largo drills #4 and #5 (Dentsply/Maillefer, Rio de Janeiro, RJ, Brazil) to obtain standardized samples with parallel-sided cavities in all groups (27). To keep the roots exactly in the same position during the canal preparation, the contra-angle was mounted in a parallelometer and the samples positioned in a fixed base. The root canals were irrigated with 2 mL of distilled water after the use of each instrument to remove the dentin residues. Irrigation was performed by using a 10 mL disposable plastic syringe attached to a polypropylene capillary tip (Ultradent Products Inc., South Jordan, UT, USA). After the final irrigation, the root canals were completely dried with absorbent paper points. The roots were then distributed into 6 groups (n=21) according to the final irrigation protocols: G1 –2.5% NaOCl (5 min); G2 – 2.5% NaOCl (5 min) + 17% EDTA (1 min); G3 – 2.5% NaOCl (5 min) + 17% EDTA (1 min) + 2.5% NaOCl (1 min); G4 – 2.5% NaOCl (5 min) + 17% EDTA (1 min) + 2% CHX (2 min); G5 – Mixture 5% NaOCl + 18% HEBP (5 min); and G6 – Mixture 5% NaOCl + 10% $EDTANa_4$ (5 min). The apex portion of the canals was sealed with wax to allow the irrigation solutions to stay inside of the canals. For the irrigation procedure, 1 mL of the irrigant was used per min of the time set per solution. After the use of each irrigant, the solutions were aspirated and the canals were rinsed with 2 mL of distilled

water for 1 min to remove the remnants of the irrigators. At the end, the canals were dried with absorbent paper (Dentsply Maillefer, Ballaigues, Switzerland).

Push-out test

Following, the AH Plus sealer (Dentsply DeTrey, Konstanz, Germany) was prepared according to the manufacturer instructions. To obtain a similar composition of the sealer in all groups only the middle portion of the tubes was used (28). The root canals of 20 samples/group were filled using only the sealer to have exclusively the results of the bond strength between the sealer and the dentin and avoid confounding factors (12, 29). The AH Plus was inserted into canals with the aid of a lentulo spiral #40 (Dentsply Maillefer) until the entire canal was filled to the orifice. The roots were radiographed at two angulations and the ones with voids or bubbles were discarded. The roots of all groups were stored at 37°C and 100% humidity, and on the day after the obturation the wax on the apical portion of the roots was removed and the coronal and apical extremities of the canals were sealed with glass ionomer cement.

After 1 week, 10 roots of each group were sectioned in a precision cutting machine (Isomet, Buehler, Lake Bluff, IL, USA) under constant water coolant. To remove the glass ionomer cement from the extremities of the canal, one slice of 1 mm was cut at the cervical and one at the apical portion of the roots and discarded. Then, cuts were performed with 2 mm of distance between each other, so six slices were obtained from each root (2 per root third) and the most cervical slice of each third was selected for the push-out test. The other 10 roots/group were stored for 20 months and after this time they were cut in slices the same way.

Each slice was marked on its apical side, and the cervical and apical diameters of the cross-section of the canal were measured using the ***Zeiss AxioCam MRm*** camera (Carl ***Zeiss*****,** ***Oberkochen*****,** ***Germany)*** attached to the stereomicroscope Stemi 2000-C (Carl ***Zeiss)*****.** The thickness of the specimens was determined with a digital caliper with 0.02 mm accuracy (Mitutoyo, Tokyo, Japan). For the push-out assessment the slices were placed on a base with a central hole. The tests were performed by applying a compressive load to the apical-coronal direction by using 1.3 mm-diameter cylindrical plunger tip. The plunger tip was positioned centered on the filling material without contact the surrounding dentine surface. Loading was performed on a universal testing machine (Instron 3342 – Instron Corporation, Canton, MA, USA) at a speed of 0.5 mm/min until bond failure occurred. The Megapascals (MPa) value of push-out bond strength of each specimen was calculated as the maximum failure load (N) divided by the bonding surface area of the root canal filling (mm^2) determined by the formula of a conical frustum.

SEM analysis

The only specimen of each group that was not obturated was used to obtain images of the dentin surface by scanning electron microscopy (SEM). The specimens were split longitudinally and mounted on SEM stubs using a double-faced carbon tape, then they were sputter coated with platinum and palladium, and evaluated under the FEI Nova NanoSEM 230 ultra-high resolution scanning electron microscope (FEI Europe, Eindhoven, Netherlands). Photomicrographs were taken from the middle portion of the root fragments at 25000× magnification.

Statistical analysis

The data of push-out bond strength obtained exhibited a normal distribution in the preliminary normality test ($P > 0.05$); therefore, the one-way analysis of variance ANOVA and Tukey post-hoc tests were used to compare the results among the different irrigation protocols at the same experimental time. The unpaired T-test was used to compare the results of the same irrigation protocol 7 days and 20 months after the obturation. The confidence level of all tests was set at 95%.

Results

Push-out analyses

The effect of root canal thirds was assessed, however, significant differences among them were not found in all groups in both time periods ($P > 0.05$). Thus, the data from the 3 slices of the 10 roots of each group, totalizing an n of 30 slices/group/period of time, were used for the statistical comparison.

Table 1 displays the average and standard deviation of the dentin bond strength of the AH Plus sealer for each of the tested irrigation protocols 7 days and 20 months after the obturation. The one-way ANOVA demonstrated that 7 days after obturation the values of the push-out bond strength of the groups G2, G3, G4, and G5 were statistically similar to each other ($P > 0.05$) and higher than the other groups ($P < 0.01$). The groups G1 and G6 presented the lower values of the bond ($P < 0.01$) being also similar to each other ($P > 0.05$). Regarding the values obtained 20 months after the root canal fillings, G3 and G5 presented similar values of bond strength between each other ($P > 0.05$), and higher from all other groups ($P < 0.01$). They were followed by G4 and G6 that were also similar between each other ($P > 0.05$). The lower bond values were associated with G1 and G2 ($P < 0.01$) that were statistically similar ($P > 0.05$).

The comparison of the values of the same group over time by the unpaired t-test revealed that only the groups G3, G5 and G6 presented results of push-out bond strength in 20 months similar to the values obtained in 7 days ($P > 0.05$).

Table 1 – Average (X) and standard deviation (SD) for the push-out bond strength (MPa) of the epoxy resin sealer AH Plus 7 days and 20 months after the root canal filling according the irrigation protocols employed.

Groups	**MPa 7 days** **X ± SD**	**MPa 20 months** **X ± SD**
G1 – NaOCl 2.5% (5 min)	15.6 ± 3.9^{Ba}	11.7 ± 3.5^{Cb}
G2 – NaOCl 2.5% (5 min) + EDTA 17% (1 min)	19.4 ± 4.0^{Aa}	10.4 ± 4.6^{Cb}
G3 – NaOCl 2.5% (5 min) + EDTA 17% (1 min) + NaOCl 2.5% (1 min)	18.8 ± 3.5^{Aa}	20.0 ± 3.8^{Aa}
G4 – NaOCl 2.5% (5 min) + EDTA 17% (1 min) + CHX 2% (2 min)	19.0 ± 2.7^{Aa}	15.3 ± 3.4^{Bb}
G5 – Mixture NaOCl 5% + HEBP 18% (5 min)	19.7 ± 2.6^{Aa}	19.5 ± 3.4^{Aa}
G6 – Mixture NaOCl 5% + $EDTANa_4$ 10% (5 min)	14.1 ± 3.2^{Ba}	15.7 ± 4.5^{Ba}

*One-Way Anova with Tukey post-hoc p-value < 0.01; Different capital letters in columns indicate statistically significant intergroup differences in the same time period; *Unpaired t-test p-value < 0.05; Different lowercase letters in rows indicate statistically significant intragroup differences.

SEM analyses

The SEM images taken from all groups showed that, except for the use of NaOCl alone, all irrigation protocols removed the smear layer produced by the root canal preparation (Figure 1). In the groups of NaOCl + EDTA and NaOCl + EDTA + CHX, it is possible to observe a discrete enlargement of the dentin tubules opening. In the group of NaOCl + EDTA + NaOCl, the erosive effect of the irrigation sequence can be observed in the peritubular and intertubular dentin.

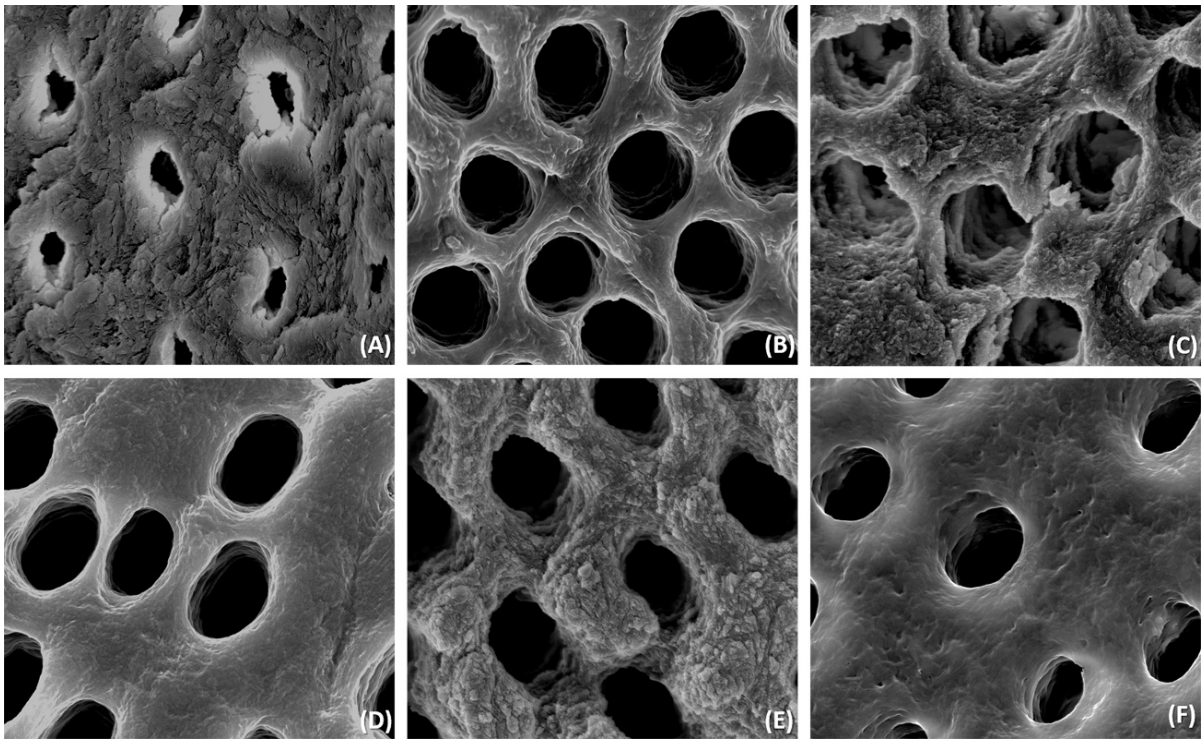

Figure 1- Scanning electron microscopic images of the dentin surface in the middle third of the root segments after treatment with the irrigation protocols (magnification of 25.000x). (A) The organic components of smear layer were removed by the 2.5% NaOCl, but the inorganic phase still partially covering the entrance of the dentin tubules; (B) The smear layer was completely removed by the use of 2.5% NaOCl followed by 17% EDTA and the opening of the dentin tubules seems to be enlarged; (C) Erosion of peritubular and intertubular dentin with the dentin tubule orifices irregularly enlarged can be observed after the use of 2.5% NaOCl + 17% EDTA + 2.5% NaOCl; (D) Image of the group of 2.5% NaOCl + 17% EDTA + 2% CHX that presents a clean dentin surface very similar with the one observed in the group of 2.5% NaOCl + 17% EDTA; (E) A clean dentin surface was promoted by the use of the mixture of 5% NaOCl + HEDP and the entrance of the dentin tubules were not enlarged by the use of these irrigants; (F) similar to the previous group, in the group of the mixture of 5% NaOCl + 10% EDTANa4 the dentin surface is clean and the opening of the dentin tubules were not enlarged.

Discussion

The results of this study showed that the different irrigation protocols tested produced different dentin surfaces for the interaction with the endodontic sealers and they have a significant impact on the immediate and long-term dentin bond strength of AH Plus. Therefore, the null hypothesis has to be rejected. Although there are many studies that evaluated the long-term stability of fibre-post bonding to dentin, the authors failed to find studies that analyzed the

impact of different irrigation protocols on the bond strength of endodontic sealers over time, except for one study that presented the results after 3 months of the obturation (30).

The images obtained by the SEM showed different characteristics on the dentin surface according to the irrigation protocols tested (Figure 1). Once the NaOCl just have effect on the organic matter (16, 31), the smear layer was not completely removed by this protocol and it is possible to observe inorganic matter covering the surface of the dentin on the photomicrography, agreeing with previous studies (32-34). The use NaOCl associated with chelating solutions in G2 (NaOCl + EDTA), G3 (NaOCl + EDTA + NaOCl), G4 (NaOCl + EDTA + CHX), G5 (mixture of NaOCl + HEDP) and G6 (mixture of NaOCl + $EDTANa_4$), promoted the completely removal of the smear layer in these groups. However, probably due to the higher demineralization capacity of 17% EDTA compared to $EDTANa_4$ and HEDP (6, 35, 36) it is possible to observe an enlargement of the entrance of dentin tubules in G2 (NaOCl + EDTA), G3 (NaOCl + EDTA + NaOCl) and G4 (NaOCl + EDTA + CHX). In the group G3 (NaOCl + EDTA + NaOCl), the intercalated use of NaOCl and EDTA resulted in dentin erosion, and irregular and rough tubules orifices, as previously described (32, 37). In the SEM images, the microtopography appears to be rougher and irregular in G1 (NaOCl), G3 (NaOCl + EDTA + NaOCl), and G4 (mixture of NaOCl + HEDP). This feature might be the result of the higher amounts of mineral on the surface of these groups due to the collagen deproteination by the NaOCl (6, 9), once the exposition of the collagen has been associated with smooth and plane images (32). However, this appearance is not related to major values of surface roughness (19).

The best results of initial bond strength were obtained by groups G2 (NaOCl + EDTA), G3 (NaOCl + EDTA + NaOCl) and G4 (NaOCl + EDTA + CHX) that were similar to each other. These groups presented a clean surface with high roughness due to the use of the EDTA (7, 8, 10, 19, 35), which favoured the micromechanical interlocking of the endodontic sealers (1, 38). The use of CHX after the decalcifying agent in G4 (NaOCl + EDTA + CHX) did not improve the initial bond strength when compared to the group G2 (NaOCl + EDTA), as observed in previous studies related to adhesion (39-42). In G2 (NaOCl + EDTA) and G4 (NaOCl + EDTA + CHX), the exposed collagen on the dentin surfaces probably contributed to the adhesion of the AH Plus, once it was reported that the amino groups of the collagen fibers can bond chemically with this sealer (20). However, this contribution was not significant to produce statistical differences among these groups and G3 (NaOCl + EDTA + NaOCl) that had the collagen matrix deproteinated by the final irrigation with NaOCl (6). Another research did not find differences in the bond strength of other sealers with the use of NaOCl after the EDTA

when comparing to the group that applied EDTA as the last irrigant as well (30). These results reinforce the concept that the strong joints are most effectively created by the mechanical bonding (1).

The specimens treated with the mixture of NaOCl and HEDP (G5) had initial bond strength similar to the groups that employed the EDTA as the decalcifying agent (G2, G3, and G4). However, previous studies obtained values of bond strength for the mixture of NaOCl and HEDP higher compared to the conventional irrigation protocol of NaOCl followed by EDTA (11, 13, 30). A paper on FTIR analysis showed that after the use of the mixture of NaOCl and HEDP there is more mineral than collagen on the surface of the substrate (6). Consequently, the high push-out bond strength obtained in this group might have resulted from the cleanliness of the surface and other reasons than the interaction of the collagen amino groups with AH Plus (13). Once the roughness of the dentin surface is not increased with the use of HEDP as much as when EDTA is used (7, 19), an important factor to be considered is that HEDP is a bisphosphonate that strong adsorb to hydroxyapatite surface and increase de surface free energy (43). In the root dentin, this increase in the surface free energy results in a very high wettability of the canal walls (19), providing the necessary proximity between the materials to facilitate the molecular attraction. It results in a major penetration of the sealer into the irregularities of the surface, observed through low gap distance between the filling material the root dentin (44), and in an enhancement of the mechanical interlocking of endodontic sealers to dentin when HEDP is used to irrigate the root canals (11, 13).

Amongst the six irrigating protocols tested, G1 (NaOCl) was one of the groups that presented the lowest initial push-out strength value. This result agrees with previous studies that obtained lower values of bond strength for the group that used only the NaOCl as the irrigant in comparison with groups that employed the NaOCl associated with EDTA and/or HEDP (11, 13, 14). The possible reason for this outcome was the sealer adhesion to the inorganic components of the smear layer reducing the sealer resistance to the dislodgement forces (11). However, this find disagrees with other research that observed an enhancement of the sealers adhesion to dentin in the presence of smear layer (45). Once the mechanical bonding is the most effective mean to create a strong joint (1) and the NaOCl does not significantly increase the roughness of the dentin surface (7, 19), this irrigation protocol does not favour a mechanical interlocking of the sealer to the dentin. Another reason that could be suggested for the low bond strength values is the oxidizing effect of the NaOCl that remain inside of the root canal and negatively affect the bond strength of the resin based sealers (15, 17). However, this effect might have been insignificant in this study because of the final rinse with distilled water

(11). Besides, in G3 (NaOCl + EDTA + NaOCl) the last irrigant used was also NaOCl and the push-out bond strength values of this group were significantly higher than the values of G1 (NaOCl).

The other group that presented the lowest initial push-out bond strength values was G6 (Mixture of NaOCl + $EDTANa_4$). In this group, the smear layer was completely removed after the 5 min of the use of the mixture and the chemical composition of the surface is similar to a surface not treat with irrigants (6). However, since this is the first study that tested the effects of this irrigation protocol on the bond strength of sealers to dentin, comparisons of these results with literature were not possible.

After 20 months of aging, the rank of irrigation protocols from the higher to the lowest values of the push out bond strength was G3=G5>G6=G4>G1=G2. An important fact observed in the groups G3 (NaOCl + EDTA + NaOCl) and G5 (Mixture of NaOCl + HEDP), was the high occurrence of fractures of the dentin slices.

When analysing the results of the same group over time, the groups that had bond strength preserved (G3, G5 and G6) were smear layer free and did not present the collagen matrix exposed on the dentin surface (6). In G1 (NaOCl), the values of bond strength reduced after 20 months of the obturation when comparing to the initial values, suggesting that the presence of smear layer on the surface can negatively affect the adhesion over time. Although the groups G2 (NaOCl + EDTA) and G4 (NaOCl + EDTA + CHX) presented initial high of bond strength values, a reduction occurred after 20 months of the obturation. The likely main reason for this find was a suboptimal infiltration of the sealer in the demineralized collagen matrix, followed by the degradation of the denuded collagen fibers by the action of metalloproteinases (21). The group G4 (NaOCl + EDTA + CHX) had a significantly better preservation of the bond strength than G2 (NaOCl + EDTA) after aging, agreeing with previous studies that reported some long-term stability of bond strength with the use of the CHX (39, 46). Probably, in G4 (NaOCl + EDTA + CHX) the action of the metalloproteinases was partially suppressed by the protease inhibitor effect of CHX (21, 25). However, the protective effects of CHX do not remain for long aging periods (46).

Within the limitations of the present study, it was possible to rank the irrigation protocols tested that promote strong and stable dentin bond strength of AH Plus in a long-term. It also reinforced the importance of the achievement of surfaces that are clean and present high roughness and wettability to obtain good interfacial interaction between the adherend and the adhesive (1). However, more studies should be performed with the aim to understand the

adhesive interface and the changes that occur in this region over time when different irrigation protocols and different types of endodontic sealers are used.

Acknowledgments

This study was supported by State of São Paulo Research Foundation, FAPESP (2013/19789-3).

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3 DISCUSSION

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The success of endodontic treatments is dependent on the disinfection of the root canal system and prevention of the recontamination (HAAPASALO et al., 2010). The main objective of the mechanical preparation of the root canal is to create a space for the effective delivery of antimicrobial substances and adequate root canal filling (HAAPASALO et al., 2010; ZEHNDER, 2006). The instrumentation associated with inert irrigant solutions is not able to reduce the quantities of the microorganisms in infected teeth to levels considered adequate (BYSTRÖM; SUNDQVIST, 1981; GRAHNEN; KRASSE, 1963) and cannot prevent the smear layer formation (MAYER; PETERS; BARBAKOW, 2002). Based on this, it is possible to affirm that the active irrigation solutions play an important role in the outcome of the endodontic treatments since they have chemical, mechanical and biological functions and the irrigation is the only way to reach the areas not cleaned by the instruments (HAAPASALO et al., 2014).

However, since there is no single solution that has all necessary properties required from an ideal irrigant, it's necessary to combine 2 or more substances in sequence to obtain an effective irrigation (HAAPASALO et al., 2014; ZEHNDER, 2006). An irrigation protocol has been recommended to optimize the cleanliness and disinfection of the root canal system (YAMADA et al., 1983). This protocol suggests the use of NaOCl during the canal instrumentation to dissolve the organic matter and promote the elimination of microorganisms, then a decalcifying agent should be used to remove the smear layer, clean the root canal walls and dentin tubules entrance. Following, another solution with antimicrobial action, such as NaOCl or CHX, is employed to act in the areas not reached when dentin was covered by residues and potentiate the disinfection.

The NaOCl remain the main irrigant in endodontics, due to its antimicrobial effects and capability to dissolve organic matter (ZEHNDER, 2006). The results of all tissue dissolution assays described on this thesis showed that among the irrigants tested, only the NaOCl was able to dissolve the organic matter, agreeing with previous studies (IRALA et al., 2010; NAENNI; THOMA; ZEHNDER, 2004; STOJICIC et al., 2010). The importance of promoting the dissolution and removal of the organic tissue from the root canals is in the fact that it can be used as a substrate for bacteria that

remained in the root canal after the biomechanical preparation, allowing their growth (DELANY et al., 1982). One of the ways to increase the tissue dissolution inside of the root canal is by increasing the concentration of the NaOCl solutions, but the toxicity to the periapical tissues increase together (GERNHARDT et al., 2004). However, the irrigation solutions are used inside of the root canal and with adequate technique minimal amounts are extruded to the periapical area (RODRIGUEZ-FIGUEROA; MCCLANAHAN; BOWLES, 2014). The experiments performed to compare the capability of 1%, 2.5%, and 5% NaOCl to dissolve fragments of bovine muscle tissue showed that the dissolution was time and concentration dependent, agreeing with previous studies (ALMEIDA et al., 2013; CHRISTENSEN; MCNEAL; ELEAZER, 2008; DUMITRIU; DOBRE, 2015; HAND; SMITH; HARRISON, 1978; STOJICIC et al., 2010). Therefore, once the biomechanical preparation is becoming faster with the development of new instruments, the solutions of 2.5% and 5% NaOCl seems to be more indicated to promote adequate tissue dissolution and do not compromise the quality of the root canal treatment.

Since the phosphate, carbonate, organic matter and water are dentin components that strongly absorb infrared radiation, the ATR-FTIR is a technique highly indicated to determinate the quantities of these compounds on dentine (BACHMANN et al., 2003; BACHMANN; ZEZELL, 2010; BOTTA et al., 2012; ZHANG; KIM; et al., 2010). Since previous studies described that irrigation solutions can also affect the dentin physicochemical properties (ARI; ERDEMIR, 2005; BALLAL et al., 2010; COBANKARA et al., 2011; DOĞAN; ÇALT, 2001; HU; LING; et al., 2010; TARTARI; DE ALMEIDA RODRIGUES SILVA; et al., 2013; TARTARI; DUARTE JUNIOR; et al., 2013) analysis of dentin surface components were performed with ATR-FTIR to verify the effects of the use of 1%, 2.5% and 5% NaOCl. For these analyses, the parameter of amide III/phosphate ratio was suggested (TARTARI; BACHMANN; et al., 2017) as a substitute for the parameter of amide I/phosphate ratio, which was commonly used (ATABEK et al., 2014; HU; PENG; et al., 2010). The objective of this new parameter is to avoid the influence of bands overlapping in the results, once the band of amide I at 1645 cm^{-1} overlapping with water bands (BACHMANN et al., 2003). In the experiments, NaOCl caused a degradation of dentin collagen that was time and concentration-dependent, such as in the experiments of tissue dissolution, but statistical differences were found only between 5% NaOCl and saline solution,

agreeing with previous papers that already reported major collagen removal for higher concentrations of NaOCl (ATABEK et al., 2014; HU; PENG; et al., 2010; ZHANG; TAY; et al., 2010). When considering the effects of the different concentrations of NaOCl on dentin collagen and organic tissue, the concentration of 2.5% seems to be more indicated to obtain a good dissolution of organic matter in a short working time, without cause many alterations on dentin structure.

Recently, other decalcifying solutions were pointed as substitutes to EDTA, with the objective to simplify the ideal irrigation protocol previously described (LOTTANTI et al., 2009; TARTARI; ODA; et al., 2017). Among them is the HEDP, a substance that can be used mixed with NaOCl without short term loss of the antimicrobial action (ZEHNDER et al., 2005) and organic matter dissolution capability of the NaOCl, as showed in one of the articles of this thesis (TARTARI et al., 2015). When the mixture of NaOCl and HEDP is used during the biomechanical preparation it reduces the accumulation of debris in the isthmus area of the canals (PAQUÉ; RECHENBERG; ZEHNDER, 2012). Although the HEDP has a weak chelating capability (DE-DEUS; NAMEN; et al., 2008), its mixture with NaOCl has been suggested to be used during and after instrumentation with the intent to avoid the smear layer formation and increase the disinfection (ARIAS-MOLIZ et al., 2014; LOTTANTI et al., 2009).

The solutions of $EDTANa_4$ were suggested during the development of this thesis with the same proposal of being used mixed with NaOCl as the HEDP (TARTARI; ODA; et al., 2017). To verify the compatibility of the irrigants, experiments of iodometric titration of free chlorine and capability to dissolve organic matter of NaOCl in mixtures with $EDTANa_4$ and $EDTAHNa_3$ were performed. The results showed that $EDTANa_4$ solutions caused minor reductions in the free available chlorine content and did not affect the NaOCl organic matter dissolution capability. On the contraty, the 17% $EDTAHNa_3$ solutions caused almost a complete loss of free chlorine immediately upon mixing and prevented the tissue dissolution by the NaOCl, as previously reported (GRAWEHR et al., 2003; TARTARI et al., 2015; ZEHNDER et al., 2005). In relation to the smear layer removal, the $EDTANa_4$ showed to be a weak chelating agent, such as HEDP (DE-DEUS; NAMEN; et al., 2008), needing 5 min to completely clean the dentin surface at concentrations of 10% and 20% isolated or mixed with 5% NaOCl. A study evaluating the effects of mixing the salt of 3% $EDTANa_4$ directly in the solution of 1%

NaOCl reported that the mixtures were stable for about 30 min after the preparation (BIEL et al., 2017). Another recent study to determine the antimicrobial activity of the mixture of NaOCl and $EDTANa_4$ against 3-week E. faecalis biofilm found that alkaline $EDTANa_4$ with and without the addition of cetramide do not interfere with the antimicrobial activity of NaOCl (SOLANA et al., 2017).

PAA was the other decalcifying agent suggested as a substitute for EDTA tested in this study. It is a substance with a strong disinfection capability that can simplify the ideal irrigation protocol for dispensing the final flushes with an antimicrobial agent (LOTTANTI et al., 2009). This substance has sporicidal, bactericidal, virucidal and fungicidal actions in concentrations below 0.5%, even in the presence of protein (LENSING; OEI, 1985). A previous study showed that 4% PAA as similar effects as 2.5% and 5.25% NaOCl in biofilms, being able to kill and dissolve biofilms from dentin species infected intra-orally (ORDINOLA-ZAPATA et al., 2013).

ATR-FTIR experiments performed to determine the effects of $EDTAHNa_3$, PAA, HEDP and $EDTANa_4$ solutions at different concentrations in the composition of the dentin surface showed that for the same decalcifying agent, the higher the concentration and immersion time the greater the demineralization and exposure of the collagen matrix (TARTARI; BACHMANN; et al., 2017). The PAA caused greater demineralization followed by $EDTAHNa_3$, $EDTANa_4$, and HEDP. The demineralization promoted by the 2% PAA was very high compared to the other solutions, suggesting that this concentration should be used with caution, as previously reported (COBANKARA et al., 2011). Due to this result, the 0.5% PAA, which had effects similar to $EDTAHNa_3$, was chosen to be used in the irrigation protocols applied in following experiments of this thesis. The low chelating capability of $EDTANa_4$ and HEDP observed by ATR-FTIR, are in agreement with previous studies (DE-DEUS; NAMEN; et al., 2008; DE-DEUS; ZEHNDER; et al., 2008; TARTARI; ODA; et al., 2017).

The effects of irrigation protocols in which NaOCl was used associated with decalcifying agents were also analyzed by ATR-FTIR (TARTARI; BACHMANN; et al., 2017). In these irrigation sequences, even with the use of NaOCl before of decalcifying agents, the rank of demineralization capability was maintained by PAA, $EDTAHNa_3$, $EDTANa_4$, and HEDP. The results also showed that final flushes with 2.5% NaOCl required approximately 0.5 min to completely remove all collagen matrix exposed by

EDTAHNa$_3$ and PAA. These flushes return the dentin surface to its natural composition in a process called deproteination (DI RENZO et al., 2001). For all irrigation protocols tested, the last irrigant used defined the final dentine amide III/phosphate and carbonate/phosphate ratios. The ATR-FTIR analyses showed that carbonate ions of dentin were removed faster than phosphate ions by all solutions tested even if they are employed alone or in irrigation protocols These results confirmed that the carbonate group is more soluble than phosphate group (OTSUKA et al., 2012; YAO; LEGEROS; LEGEROS, 2009).

Due to the adsorption of CHX to the dentin surface and the superimposition of the bands of this solution with dentin bands, it was not possible to determine the effects of the CHX on dentin composition using the parameters amide III/phosphate and carbonate/phosphate (TARTARI et al., 2016). These superimpositions resulted in modifications in the intensity of adsorption bands, such as amide III and carbonate, and were also observed by BOTTA et al. (2012). However, it was possible to quantify the adsorption of the CHX to dentin by determining the area of the band associated with the functional group methylene (C=C) of the irrigant, which has the peak located at 1492 cm^{-1} and does not present superimposition with dentin bands. The results obtained showed that the adsorption of this irrigant to dentin was much higher when demineralizing agents were used prior to the CHX, agreeing with a previous paper (KIM et al., 2010). The quantity of CHX adsorbed to the dentin surface in the irrigation protocols followed the order of demineralization capability of decalcifying agents (TARTARI; BACHMANN; et al., 2017), showing that CHX adhesion to the surface is higher as higher the demineralization previously promoted.

The roughness of a surface is a property that can be measured by many types of instruments, like computerized roughness tester, profilometer, and more recently atomic force microscope (BALLAL et al., 2010; HU; LING; et al., 2010). In this study, the analyses were performed with a benchtop computerized roughness tester because in the pilot studies with the atomic force microscope, due to the small size of the tips, the entrance of dentin tubules was included in the measurement of the surface roughness providing wrong results about the topography. In the experiments, the saline solution, NaOCl, HEDP, and CHX did not alter the roughness of the dentin surface as previously observed (ARI et al., 2004; PASCON et al., 2014; PATIL; UPPIN, 2011; TARTARI; DUARTE JUNIOR; et al., 2013). The solutions of EDTAHNa$_3$ and

PAA caused significant increases on the surface roughness. Although the PAA showed to cause a major demineralization of the dentin surface than $EDTAHNa_3$ (COBANKARA et al., 2011; TARTARI; BACHMANN; et al., 2017), the increases on the roughness were higher for the latter. Probably this contrast between the results of the experiments happened because of the differences in decalcification kinetics of PAA and $EDTAHNa_3$ (KAWASAKI et al., 2000).

Wettability is an important physicochemical property related with surface free energy that has been reported to influence the adhesion of dental materials (MARSHALL et al., 2010) and microorganisms to dentin (BURGERS et al., 2010). It is determined by measurement of contact angles of substances with substratum, being low contact angles formed on surfaces with greater wettability (MARSHALL et al., 2010). The measurements of the contact angles showed major values of wettability for all irrigation protocols tested compared to the control saline solution. The most important result observed was the high enhancement in surface wettability promoted by HEDP. This find is related with the adsorption of the irrigant to hydroxyapatite, which increases the surface Gibbs free energy and consequently the wettability of dentin (FRANCIS; VALENT, 2007). This explain the low gap size values and better marginal adaptation of AH Plus observed on dentin walls irrigated with HEDP compared to other irrigants (ULUSOY; ZEYREK; CELIK, 2017), as well as the high push-out bond strength values of endodontic sealers for the groups irrigated with HEDP (DE-DEUS; NAMEN; et al., 2008; NEELAKANTAN et al., 2012).

The alterations produced by irrigation protocols on the dentin surface, previously described in this thesis, were analyzed in relation to the influences in adhesion of microorganisms and AH Plus sealer to dentin. For the microorganisms assays, *Enterococcus faecalis* and *Candida albicans* were chosen because *E. faecalis* has been frequently detected in teeth with apical periodontitis (MOLANDER et al., 1998; PENAS et al., 2013) and *Candida albicans* is the most prevalent specie of fungi associated with persistent endodontic infections (PERSOON; CRIELAARD; OZOK, 2016; WALTIMO et al., 1997). Once these microorganisms are not among the colonizers of the root canal system with necrotic tissue, they may invade the root canal at any time during or after treatment via a communication with the oral cavity, such as through a defective coronal seal, and form biofilms that cause secondary infections (DELBONI et al., 2017; PERSOON et al., 2016; ZEHNDER; GUGGENHEIM, 2009).

In the assays of microorganisms' adhesion, after the microbial contact with dentin for 2 hours, the samples were analyzed by CLSM. The adhesion of *E. faecalis* was favored on surfaces with smear layer or collagen exposed by chelating agents, agreeing with other papers (HUBBLE et al., 2003; KISHEN et al., 2008; KOWALSKI et al., 2006; YANG et al., 2006). *C. albicans* adhesion was major on surfaces with smear layer or more mineral. Previous studies with *C. albicans* showed that the presence of smear layer increased its adhesion (SEN et al., 2003; SEN et al., 1997; TURK et al., 2008) and that presence of magnesium and calcium ions has a critical role in the morphogenesis and adherence of this microorganisms to extracellular matrix proteins (HOLMES et al., 1991; KLOTZ et al., 1993). Final flushes with CHX reduced the adhesion of both microorganisms probably because of the residual antimicrobial effect of this irrigant due to its adsorption to dentin (DAVIES, 1973; PARSONS et al., 1980; YANG et al., 2006). When analyzing the results of wettability, roughness and adhesion of microorganisms together, it was possible to observe that wettability did not influence the adhesion of microorganisms. However, increases on the roughness seem to potentiate the adherence of *E. faecalis*. The influence of surface roughness on *C. albicans* adhesion was not detected in this thesis, reinforcing previous finds (BURGERS et al., 2010; DO NASCIMENTO et al., 2013).

In the push-out assessment of the adhesion of AH Plus to dentin, the bond strength results showed that irrigation with NaOCl + $EDTAHNa_3$ + NaOCl and mixture of NaOCl + HEDP promoted the higher and more stable values over time. In the group of the mixture of NaOCl + $EDTANa_4$, the initial push-out bond strength was not among the higher values obtained, however, they didn't change in 20 months. The groups with smear layer on the surface or collagen matrix exposed, the push-out bond strength values, although were initially high, reduced over time. The main reason for this find in groups with collagen exposed is a suboptimal sealer infiltration in the demineralized collagen matrix, followed by the degradation of the denuded fibers by the action of metalloproteinases (PASHLEY et al., 2004). The residual effects of CHX adsorption to the dentin surface on metalloproteinases of the matrix were observed in the group of NaOCl + $EDTAHNa_3$ + CHX. Although there was a reduction in the push-out values after 20 months compared to the initial values, this reduction was not as higher as the one observed for the group of NaOCl + $EDTAHNa_3$. However, due to the instability of CHX (CAMARA DE BEM et al., 2014; SARKAR; BHATTACHARJEE; CURCIO, 2015;

ZONG; KIRSCH, 2012), little is known about how long will last its effects on the prevention of metalloproteinases action.

Within its limitations, this thesis showed that irrigation solutions used in endodontics are able to cause different types of modifications on dentin physicochemical properties when used isolated or associated in irrigation protocols. It was also possible to conclude that the alterations produced on dentin can influence the adhesion of *E. faecalis* and *C. albicans* to the surface and affect the long-term stability of AH Plus adhesion to root canal walls. Results suggested that among the protocols tested, the ones that employed final flushes with CHX can reduce the adherence of microorganisms and the effects of matrix metalloproteinases on the adhesion of AH Plus. However, once the residual effects of CHX to not last forever, irrigation with the mixture of NaOCl + HEDP seems to be the more promisor irrigation protocol to obtain a long-term stability of the adhesion of AH Plus to dentin and avoid the adhesion of microorganisms in cases of recontaminations.

4 CONCLUSIONS

4 CONCLUSIONS

- The solution of 2.5% NaOCl promotes a good tissue dissolution without cause high modifications on dentin composition, being a good concentration to be used during the biomechanical preparation.

- Alkaline $EDTANa_4$ and HEDP solutions are alternatives to the 17% $EDTAHNa_3$, with the intent to reduce the irrigation protocol of 3 steps. They should be mixed with NaOCl solutions immediately before use and the mixtures can be applied during and after root canal instrumentation to dissolve organic matter, kill microorganisms and remove smear layer.

- HEDP and EDTANa4 solutions cause minor demineralization whilst EDTAHNa3 and PAA caused a greater demineralization of dentine, both time and concentration dependent.

- The combined and sequential treatment of dentine with NaOCl and different decalcifying agents can be used to obtain a dentine surface rich or poor in collagen or to restore its organic/inorganic natural composition.

- The use of chelating solutions previously to CHX potentiates the adsorption of this irrigant to dentin.

- Alterations on surface roughness are higher in irrigation protocols with $EDTAHNa_3$ and wettability of dentin surface increases significantly with the use of HEDP.

- To prevent the invasion and biofilm formation for *E. faecalis* and *C. albicans* in case of failure in coronal seal it is desirable to use irrigation protocols that reduce the initial adhesion of microorganisms, such as

the ones with final flushes with CHX. A second option is the mixture of NaOCl + HEDP.

- High bond strength values of AH Plus to dentin that were also more stable over time were obtained in the groups of NaOCl + $EDTAHNa_3$ + NaOCl and the mixture of NaOCl + HEDP followed by the mixture of NaOCl + $EDTANa_4$ and NaOCl + $EDTAHNa_3$ + CHX.

- The irrigation protocols that seems to be more indicated to obtain a good adhesion of AH Plus sealer to dentin over time and also avoid adhesion of microorganisms is the mixture of NaOCl + HEDP, once the effects of CHX will not last forever.

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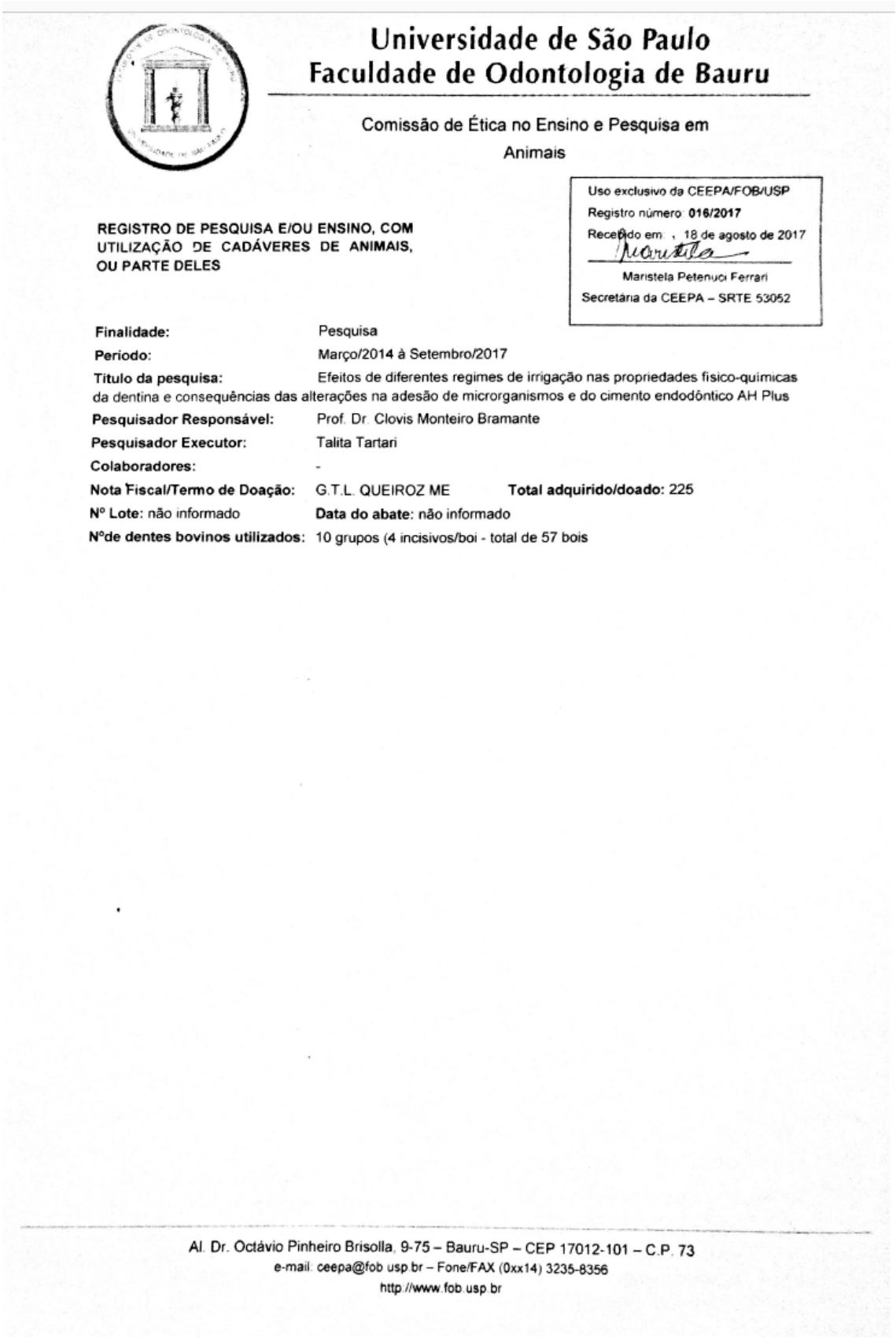

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Ensino e Pesquisa em Animais

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Registro número: **016/2017**
Recebido em: , 18 de agosto de 2017
Maristela Petenuci Ferrari
Secretária da CEEPA – SRTE 53052

REGISTRO DE PESQUISA E/OU ENSINO, COM UTILIZAÇÃO DE CADÁVERES DE ANIMAIS, OU PARTE DELES

Finalidade: Pesquisa
Período: Março/2014 à Setembro/2017
Título da pesquisa: Efeitos de diferentes regimes de irrigação nas propriedades físico-químicas da dentina e consequências das alterações na adesão de microrganismos e do cimento endodôntico AH Plus
Pesquisador Responsável: Prof. Dr. Clovis Monteiro Bramante
Pesquisador Executor: Talita Tartari
Colaboradores: -
Nota Fiscal/Termo de Doação: G.T.L. QUEIROZ ME **Total adquirido/doado:** 225
Nº Lote: não informado **Data do abate:** não informado
Nºde dentes bovinos utilizados: 10 grupos (4 incisivos/boi - total de 57 bois

Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP 17012-101 – C.P. 73
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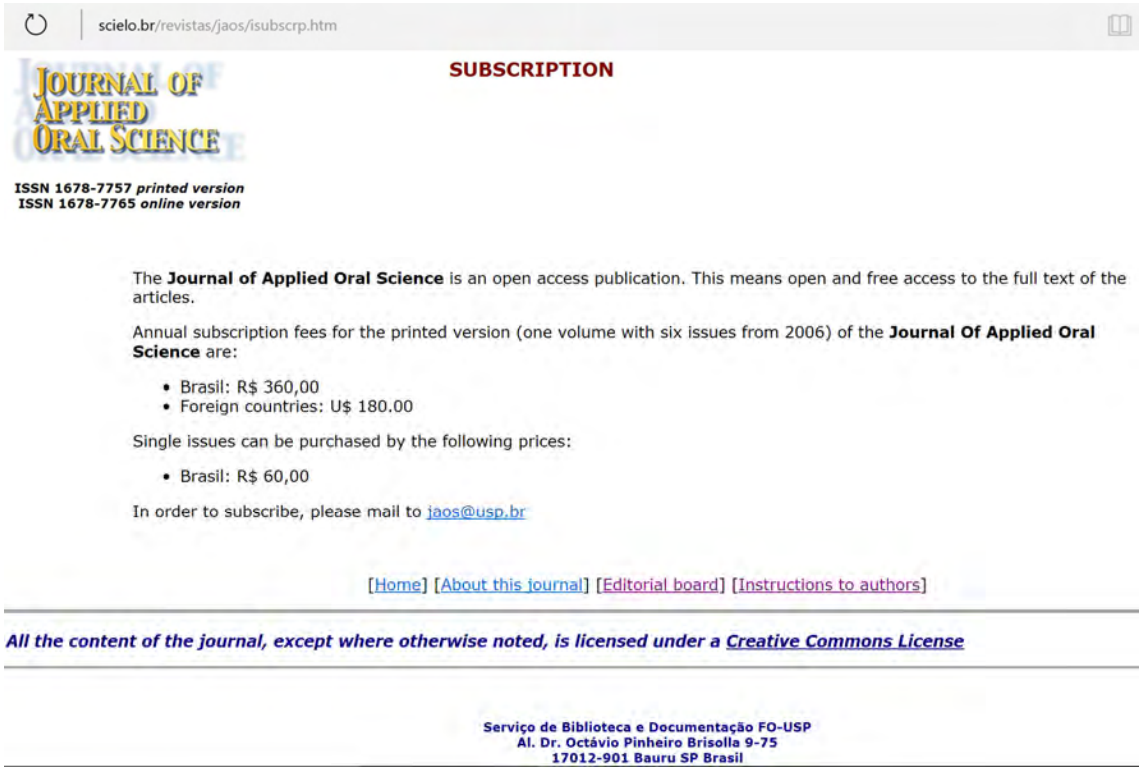

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JOURNAL OF APPLIED ORAL SCIENCE

ISSN 1678-7757 *printed version*
ISSN 1678-7765 *online version*

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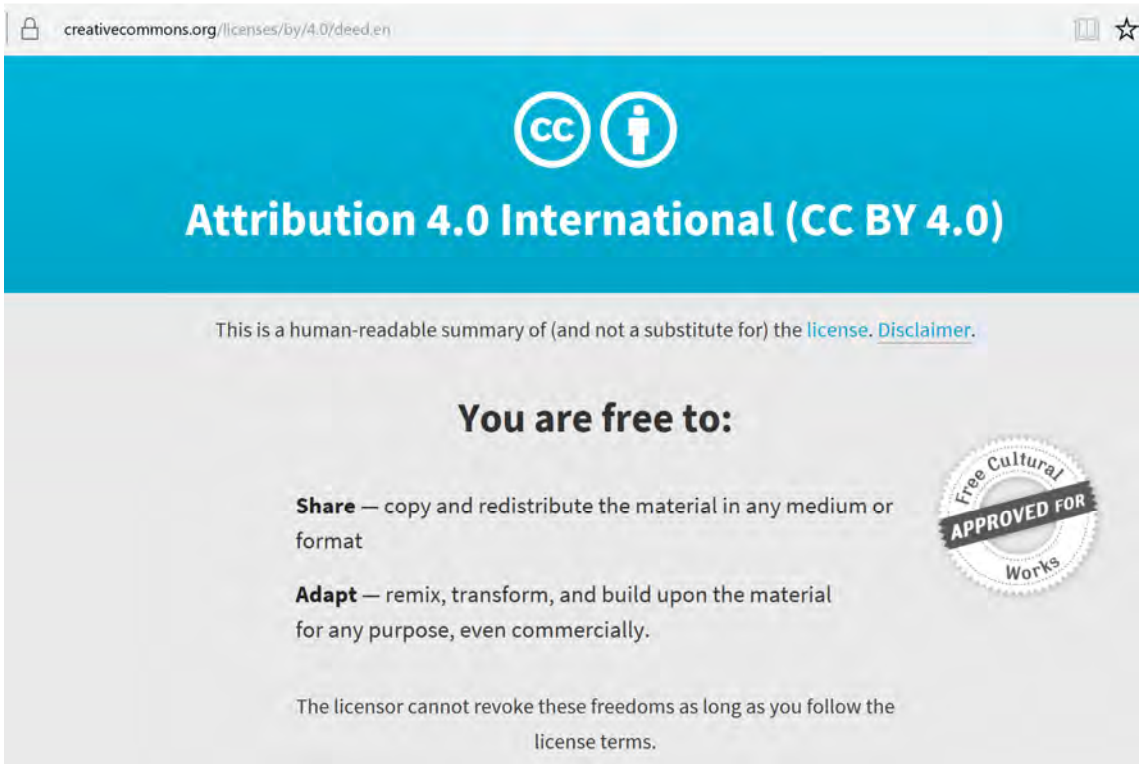

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