Universidade Estadual de Campinas
Faculdade de Odontologia de Piracicaba

Raquel Viana Rodrigues

ABORDAGENS ALTERNATIVAS PARA REDUZIR A DEGRADAÇÃO DO COLÁGENO TIPO I

ALTERNATIVE APPROACHES TO REDUCE TYPE I COLLAGEN DEGRADATION

Piracicaba
2017

Raquel Viana Rodrigues

ABORDAGENS ALTERNATIVAS PARA REDUZIR A DEGRADAÇÃO DO COLÁGENO TIPO I

ALTERNATIVE APPROACHES TO REDUCE TYPE I COLLAGEN DEGRADATION

Tese apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do Título de Doutora em Materiais Dentários.

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Dental Materials.

Orientador: Profa. Dra. Fernanda Miori Pascon
Coorientador: Prof. Dr. Marcelo Giannini

Este exemplar corresponde à versão final da Tese defendida pela aluna Raquel Viana Rodrigues, e orientada pela Profa. Dra. Fernanda Miori Pascon.

Piracicaba
2017

Agência(s) de fomento e nº(s) de processo(s): CAPES, 99999.010711/2014-07; CAPES, 1777-2014

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Odontologia de Piracicaba
Marilene Girello - CRB 8/6159

R618a

Rodrigues, Raquel Viana, 1988-
Abordagens alternativas para reduzir a degradação do colágeno tipo I / Raquel Viana Rodrigues. – Piracicaba, SP : [s.n.], 2017.

Orientador: Fernanda Miori Pascon.
Coorientador: Marcelo Giannini.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Catepsinas. 2. Cloreto férrico. 3. Colágeno tipo I. 4. Dentina. 5. Ferro. I. Pascon, Fernanda Miori,1977-. II. Giannini, Marcelo,1969-. III. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. IV. Título.

Informações para Biblioteca Digital

Titulo em outro idioma: Alternative approaches to reduce type I collagen degradation
Palavras-chave em inglês:
Cathepsins
Ferric chloride
Collagen type I
Dentin
Iron
Área de concentração: Materiais Dentários
Titulação: Doutora em Materiais Dentários
Banca examinadora:
Fernanda Miori Pascon [Orientador]
Ana Cecília Correa Aranha
Fábio Dupart Nascimento
Sergio Roberto Peres Line
Mario Alexandre Coelho Sinhoreti
Data de defesa: 22-02-2017
Programa de Pós-Graduação: Materiais Dentários

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 22 de Fevereiro de 2017, considerou a candidata RAQUEL VIANA RODRIGUES aprovada.

PROFª. DRª. FERNANDA MIORI PASCON

PROFª. DRª. ANA CECÍLIA CORREA ARANHA

PROF. DR. FÁBIO DUPART NASCIMENTO

PROF. DR. SERGIO ROBERTO PERES LINE

PROF. DR. MARIO ALEXANDRE COELHO SINHORETI

A Ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

DEDICATÓRIA

Aos meus pais, **Antônio Tadeu Rodrigues e Cecilia Brandão Viana Rodrigues**, por serem responsáveis pelo caráter em mim imputado por todo o apoio e suporte dado para que fosse possível a realização desse sonho, além de todos os outros já realizados e também os que ainda virão, muitas vezes deixando de realizar os seus próprios. Pela educação e esforço sem medida. Agradeço ao amor incondicional, em todos os momentos. Por me mostrarem um modelo maior que vocês, JESUS.

À minha irmã **Esther Rodrigues Prado**, por sempre acreditar em mim. Pela presença em todos os momentos de minha vida, pelos abraços e sorrisos, por todo amor. Também pela proteção na tenra infância. E por cuidar sempre de mim.

Ao meu marido **Rafael Fávaro**, pela colaboração extrema, sendo corresponsável pelo sucesso deste trabalho. Pela paciência quando eu estava tomada de afazeres nos finais de semana, férias e feriados, estando sempre ao meu lado. Agradeço o incentivo e por ser minha motivação para iniciar e prosseguir em minha caminhada. Agradeço pelo companheirismo, amizade e amor eternos.

Amo vocês! Obrigada por tudo...

AGRADECIMENTOS ESPECIAIS

A minha orientadora, Profa. Dra. **Fernanda Miori Pascon**, inicialmente por acreditar em meu potencial e oportunidade desde a iniciação cientifica completando assim 9 anos de trabalho em equipe. Sou grata por todo conhecimento adquirido, atenção, confiança e ensino passado. Agradeço pelo convívio agradável, e pelos laços criados, sendo uma pessoa que admiro de maneira pessoal. Muito obrigada!

Ao meu co-orientador, Prof. Dr. **Marcelo Giannini**, que confiou em meu trabalho e proporcionou a oportunidade de fazer parte do seu grupo de trabalho, indicando-me também para meu doutorado-sanduíche. Sou grata por todo conhecimento adquirido, atenção, confiança e ensino passado.

Ao Prof. Dr. **Ricardo Marins de Carvalho,** por todo conhecimento compartilhado, convívio agradável e oportunidades ofertadas. Sua humildade e busca de conhecimento contínuo é fonte de estímulo na caminhada diária. Obrigado por acreditar em minha capacidade, comprometimento e por contribuir para o meu crescimento pessoal e profissional.

Meu reconhecimento e gratidão pela orientação
Obrigada!

AGRADECIMENTOS

A **Deus** por todo amparo e proteção em todos os momentos de minha vida. Ensinando-me a ser paciente e persistente com as dificuldades do caminho. Por ser minha esperança e dirigir minha de vida de maneira cuidadosa e afetiva.

A **Faculdade de Odontologia de Piracicaba – UNICAMP**, na pessoa do seu Diretor **Prof. Dr. Guilherme Elias Pessanha Henriques** pela oportunidade da realização do Curso de Doutorado nesta instituição.

A Coordenadoria da Pós-Graduação em nome da **Profa. Dra. Cínthia Pereira Machado Tabchoury** e ao Programa de Pós-Graduação em Materiais Dentários em nome da coordenadora **Profa. Dra. Regina Maria Puppin Rontani**.

A **Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES** pela concessão da bolsa de doutorado e oportunidade de realizar parte do meu doutorado em uma universidade estrangeira, com a concessão da bolsa de Doutorado Sanduíche na **University of British Columbia**.

Aos meus amigos e colegas de sanduíche**, Bernardo Ubanetto Peres, Luana Dutra de Carvalho, Prof. Dr. Sérgio Lima Santiago, Xianjya Huang, Hazuki Maesono, Dayse Dai,** pelos momentos compartilhados e colaborações e melhores desejos de sucesso profissional. Em especial a **Eveline Turatti e Elaine Carbonero**, pelo e auxílio com os testes laboratoriais durante a realização deste trabalho.

A **Profa. Dra. Adriana Manso**, pela receptividade e preocupação. Pelo conhecimento partilhado e oportunidade de trabalharmos juntas durante minha estadia na Universidade British Columbia, e pelos almoços veganos personalizados.

Aos **docentes do Curso de Pós-Graduação em Materiais Dentários**, pelos ensinamentos e experiências cotidianas fundamentais para minha formação. Em especial a professora **Profa. Dra. Regina Maria Puppin Rontani,** que acompanha minha trajetória desde a iniciação científica acreditando em meu potencial.

Aos técnicos do departamento de Materiais Dentários, engenheiro **Marcos Blanco Cangiani** e **Selma Segalla** pela disponibilidade e auxílio quando solicitado. Ao funcionário do laboratório da Área de Odontopediatria, **Marcelo Correa Maistro**, pela disponibilidade e colaboração. Aos técnicos do laboratório de

Microscopia eletrônica de Varredura **Eliene Narvaes Romani e Adriano Luis Martins**, pela prontidão e boa vontade em ensinar.

Aos Profs. Drs. **Vanessa Cavalli Gobbo**, **Américo Bortolazzo Correr** e **Rafael Pino Vitti** pela significante contribuição para o aprimoramento dessa tese quando do exame de qualificação.

A todos os professores da FOP-UNICAMP que contribuíram para minha formação, em especial aos professores da área de Materiais Dentários pelos ensinamentos e conhecimentos transmitidos, por toda a atenção e incentivo durante o curso. Agradeço a oportunidade proporcionada e pela grande contribuição para o meu desenvolvimento profissional e pessoal.

À secretária da pós-graduação nas pessoas da **Érica A. Pinho Sinhoreti**, **Raquel Q. Marcondes Cesar Sacchi**, **Ana Paula Carone**, **Claudinéia Prata Pradella** e **Leandro Viganó** pelo comprometimento com que realizam seus trabalhos, pela prontidão em ajudar e atenção dispensada a todos os alunos de pós-graduação.

Aos funcionários da Faculdade de Odontologia de Piracicaba (FOP/Unicamp) por estarem sempre prontos a ajudar.

Aos amigos de doutorado: **Renata Fernandes, Eveline Soares, Camila Sobral, Tales Candido Garcia, Rafael Pacheco, Caio Vinícius, Valéria Bisinoto, Pedro Freitas, Tóride Cellegati, Ana Paula Ayres e Dayane Oliveira**.

Em especial aos amigos:

Tales Candido Garcia pelo apoio e ajuda, por ter tido a oportunidade de caminhar pelos mesmos caminhos, e nunca estar sozinha por estar acompanhada por vocês meus amigos.

Camila Sobral pela parceria em trabalhos, companheirismos, ajuda, conversas e risadas, e por poder compartilhar essa etapa de nossas vidas.

Eveline Soares e Camila Bortoleto Schoba amigas que escolhi como irmãs. Agradeço pelo companheirismo, compaixão, pelas caronas, por poder dividir conversar, choros, angustias, e ainda sorrir da vida! Agradeço por fazer parte da vida de vocês, e lhes desejo todo sucesso do universo.

A todos os **amigos da área de Materiais Dentários** e também **amigos de outras áreas**: enfrentarmos momentos de dificuldades e conquistas. A nossa amizade e troca de experiências foi essencial para o crescimento pessoal e profissional de cada um.

A todos **meus amigos**, estivessem eles perto ou longe, presentes ou apenas em pensamento, se fizeram sempre presentes me auxiliando a ser mais forte quando necessário, principalmente nos momentos de distância e solidão. Vocês alegram meus dias e me ajudaram a tornar possível a conclusão de mais esta etapa.

Agradeço aos **meus preciosos avós, Leonilda Brunheroto Garcia, Sávio Viana e Emiliana Brandão Viana** por todo cuidado, incentivo e amor a minha vida. Sempre preocupados com minha felicidade e com o sucesso das minhas atividades. Amo muito vocês meus queridos!

Aos meus **queridos tios**, **Maria de Lourdes, Edemir, Neris, Laudemir, Ceris e Ademir, João Batista, José Maria, Helena, Sávio, Gláucia, Márcia, João e Marina**, que sempre contribuíram me apoiando, incentivando e com muitas ideias construtivas.

Agradeço em especial também **aos meus tios, Sávio, Gláucia e Márcia** que apoiaram durante minha estadia no Canadá, ajudando constantemente, incentivando meu desenvolvimento e proporcionando ótimos momentos.

A meus tios sou grata por todo amor dedicado em toda minha vida. Também responsáveis pela minha criação e meu senso crítico. Amo-os de todo meu coração.

Aos meus **queridos primos Barbara, André, Felipe, Renata, Ana Paula, João Pedro, Rebeca, Helen, Karen, Raquel, Alexandre, Andrea, Márcio, Nélio, Maria Rita, Maria Carolina,** por todo companheirismo e apoio durante essa jornada de estudos, sendo eles pessoas de grande importância na minha vida e que desejo muito sucesso e bênçãos de Deus, AMO VOCÊS!

A todos que direta ou indiretamente contribuíram para a realização deste trabalho.

Obrigada!

EPÍGRAFE

“Porque sou eu que conheço os planos que tenho para vocês", diz o Senhor, "planos de fazê-los prosperar e não de lhes causar dano, planos de dar-lhes esperança e um futuro.

Então vocês clamarão a mim, virão orar a mim, e eu os ouvirei. Vocês me procurarão e me acharão quando me procurarem de todo o coração...”

Jeremias 29:11-13

RESUMO

Os objetivos nesta tese foram: (1) investigar a capacidade do $FeCl_3$ prevenir degradação do colágeno solúvel (CS) e insolúvel (CI); (2) comparar os efeitos do ácido fosfórico a 35% (AF) e soluções condicionantes contendo ácido cítrico (AC) e $FeCl_3$ na resistência da união a dentina, degradação do colágeno e o efeito de $FeCl_3$ na atividade da Catepsina K (CT-K). A tese foi apresentada em dois artigos: (1) Para avaliar a degradação do CS e CI (fibras de tendões de cauda de camundongo - FTCCs), os substratos foram incubados com CT-K e concentrações de 0,02% a 2% de $FeCl_3$. O sobrenadante foi submetido à eletroforese de proteínas. Os experimentos foram feitos em triplicata. As FTCCs foram caracterizadas em microscopia eletrônica de varredura (MEV) e a quantidade de Fe absorvida foi determinada pela espectroscopia de energia dispersiva de raios-X (EDX). Os dados foram submetidos à análise de variância (Brown-Forsythe), normalidade (Shapiro-Wilk ou Anderson-Darling), ANOVA um fator + Holm-Sidak ou Tukey (α=5%). (2) Para avaliar a resistência da união a microtração (μTBS), 78 dentes foram distribuídos em 13 grupos de soluções contendo AC e/ou $FeCl_3$ nas concentrações: 10%-1,8%, 10%-0,6%, 10% AC, 5%-1,8%, 5%-0,6%, 5% AC, 1%-1,8%, 1%-0,6%, 1% AC, 1,8% $FeCl_3$, 0,6% $FeCl_3$ (aplicadas por 15 segundos) e com AF (aplicado por 5 ou 15 segundos). Utilizou-se o sistema adesivo Adper Scotchbond Multi-Purpose, fotoativado por 20 segundos com o aparelho Bluephase G2 (1200Mw/cm^2). A μTBS foi avaliada em 24 horas e 9 meses de armazenamento. Para a degradação do colágeno dentinário, fatias de dentina (n=3) foram tratadas com e sem $FeCl_3$ e incubadas com CT-K. A dentina foi caracterizada após a incubação utilizando MEV. Outras fatias foram submetidas às soluções condicionantes por 15 segundos e analisadas utilizando EDX. Para avaliar a atividade enzimática da CT-K e efeito de $FeCl_3$ utilizou-se a espectrofluorimetria. Os dados foram submetidos aos testes: normalidade, ANOVA dois fatores + Holm-Sidak, ANOVA um fator + Tukey ou Fisher (α=5%). $FeCl_3$ foi capaz de prevenir a degradação do CS e CI contra CT-K para as FTCCs e para a dentina desmineralizada. As micrografias revelaram desenovelamento das FTCCs na ausência de $FeCl_3$ e integridade quando expostas a concentrações de 0,02%-0,8% de $FeCl_3$. Observou-se interação Fe/colágeno. Os grupos 5-0,6 e 5-1,8 de AC/$FeCl_3$ apresentaram maiores valores de μTBS comparados ao AF, independentemente do tempo de armazenamento. Observou-se redução da μTBS aos 9 meses para todos os grupos. A solução contendo 1,8% de

$FeCl_3$ preservou a estrutura da dentina. Observou-se pela análise de EDX que a imersão nas soluções condicionantes com $FeCl_3$ por 15 segundos foi suficiente para que as fibrilas de colágeno adsorvessem Fe. A análise por espectrofluorimetria revelou que o $FeCl_3$ inibiu a atividade de CT-K nas concentrações 0,005%-0,08%e suprimiu a atividade na concentração 0,08%. Concluiu-se que o $FeCl_3$ agiu como um indutor de reticulação do colágeno e como inibidor da atividade CT-K e estas duas habilidades atuaram em conjunto para a preservação do colágeno tipo I nas FTCCs e na dentina desmineralizada.

Palavras-chave: Catepsinas. Cloreto Férrico. Colágeno Tipo I. Dentina. Ferro.

ABSTRACT

The aim of this thesis was (1) to investigate the $FeCl_3$ ability to prevent degradation of soluble (SC) and insoluble collagen (IC), and (2) to compare the effects of phosphoric acid 35% and conditioning solutions containing citric acid (CA) and/or $FeCl_3$ in dentine bond strength, collagen degradation and the effect of $FeCl_3$ on Cathepsin K (CT-K) activity. The thesis was presented in two articles: (1) To evaluate SC and IC (mouse tail tendon fibers-MTTF) degradation, the substrates were incubating with CT-K with different concentrations of FeCl3 (0.02% to 2%). The supernatant was submitted to protein electrophoresis. The experiments were done in triplicate. The fibers were characterized after incubation by scanning electron microscopy (SEM). The amount of Fe absorbed was determined by X-ray dispersive energy spectroscopy (EDX). Data were submitted to analysis of variance (Brown-Forsythe), normality test (Shapiro-Wilk or Anderson-Darling), and one-way ANOVA (Holm-Sidak or Tukey post-test) (α=5%). (2) For the microtensile bond strength (μTBS), 78 teeth were distributed into 13 groups of solutions containing citric acid (AC) and/or $FeCl_3$ in different concentrations: 10%-1,8%, 10%-0,6%, 10% AC, 5%-1,8%, 5%-0,6%, 5% AC, 1%-1,8%, 1%-0,6%, 1% AC, 1,8% $FeCl_3$, 0,6% $FeCl_3$ (applied for 15 seconds) e com AF (applied for 5 and 15 seconds). The Adper Scotchbond Multi-Purpose adhesive system was used and light/cured for 20 seconds with Bluephase G2 (1200Mw/cm^2). The μTBS was evaluated at 24 hours and 9 months of storage. For the dentine collagen degradation, dentine slices (n=3) were treated with and without $FeCl_3$ were incubated with CT-K. Dentine was characterized after enzymatic treatment by SEM, and other slices were submitted to conditioning solutions and analyzed by EDX. Spectrofluorimetry was used to evaluate the $FeCl_3$ effect against CT-K enzymatic activity. The data were submitted to the tests: normality, two–way ANOVA and post-test Holm-Sidak, one–way ANOVA and Tukey or Fisher tests (α=5%). $FeCl_3$ was able to prevent the degradation of soluble collagen and collagen fibers against CT-K for the MTTF and the demineralized dentine. The micrographs revealed unraveling of collagen fibers in the absence of $FeCl_3$ and showed fiber integrity exposed to 0.02%-2% $FeCl_3$ concentrations. The Fe/collagen interaction was observed. AC/$FeCl_3$ 5-0.6 and 5-1.8 solutions had higher μTBS values compared to the AF, regardless of storage time. A reduction of μTBS was observed at 9 months for all groups. The treatment solution 1.8% $FeCl_3$ preserved the dentine structure. The EDX analysis indicated that the 15 second immersion in

the $FeCl_3$ conditioning solutions was sufficient for the collagen fibrils to adsorb Fe. Spectrofluorimetry analysis revealed that $FeCl_3$ inhibited CT-K activity at concentrations 0.005%-0.08% and suppressed activity when using 0.08%. It was concluded that $FeCl_3$ acted both as an inducer of collagen cross-linking and as inhibitor of CT-K activity, both abilities acted together for the preservation of collagen type I on the MTTF and on the demineralized dentine.

Keywords: Cathepsins. Collagen type I. Dentine. Ferric Chloride. Iron.

SUMÁRIO

1 INTRODUÇÃO 16

2 ARTIGOS

2.1 Artigo 1: Does Ferric Chloride prevent enzymatic collagenase activity? 21

2.2 Artigo 2: Effect of conditioning solutions containing ferric chloride on dentine bond strength and collagen degradation* 43

3 DISCUSSÃO 70

4 CONCLUSÃO 75

REFERÊNCIAS 76

APÊNDICE - Figuras ilustrativas com a descrição da metodologia empregada nos estudos 84

ANEXOS

ANEXO 1 - Certificado de submissão para a Dental Materials 94

ANEXO 2 - Certificado do Comite de Ética e Pesquisa da Universiade de British Columbia 95

1 INTRODUÇÃO

O colágeno é o principal componente do tecido conjuntivo, sendo também a proteína mais abundante em mamíferos, representando 25% aproximadamente em massa corporal (Dang e Leong, 2006; Bertassoni et al., 2012; Bertassoni et al., 2016). Ainda, é composto por mais de 30 cadeias polipeptídicas distintas e pelo menos 25 tipos distintos de colágeno foram identificados em tecidos humanos (Dang e Leong, 2006). Do ponto de vista molecular, o colágeno é formado por três cadeias polipeptídicas em forma de tripla hélice. O colágeno tipo I, por exemplo, é formado por duas cadeias α 1 e uma cadeia α 2. Cada uma destas 3 cadeias de proteínas são formadas por glicina (que representa 1/3 da sequência), prolina e lisina, e por mais dois aminoácidos que são modificados pelos ribossomos: a hidroxiprolina e a hidroxilisina Cada cinco moléculas tri-helicoidais adjacentes, por sua vez, formam uma estrutura que se assemelha a uma hélice, chamada microfibrila (Dang e Leong, 2006; Bertassoni et al., 2012).

Na dentina, o colágeno tipo I apresenta-se em feixes concêntricos, medindo cerca de 100 nm de diâmetro (Bertassoni et al., 2012), os quais se auto-organizam hierarquicamente para formar as fibrilas de colágeno (Bertassoni et al., 2016). O colágeno tipo I representa 90% das proteínas encontradas neste substrato (Tjäderhane et al., 2015). Outros componentes, como proteoglicanos, fosfolipídeos e proteases endógenas, as quais estão ligadas a processos de degradação da matriz orgânica extracelular são observados na dentina (Tjäderhane et al., 2015; Mazzoni et al., 2015). Entre as proteinases endógenas, as metaloproteinases de matriz (MMPs) (Shimada et al., 2009; Mazzoni et al., 2012 [a]; Tjäderhane et al., 2015) desempenham funções fisiológicas e patológicas (Mazzoni et al., 2015). Quanto às funções fisiológicas, acredita-se que as MMPs participam do processo de formação da dentina peritubular e dentina terciária, e também na liberação de fatores de crescimento durante o processo da formação da cárie, regulando as respostas de proteção da polpa (Mazzoni et al., 2012; Tjäderhane et al., 2013 [a]; Tjäderhane, 2015). Quanto às patológicas, participam do processo de formação da cárie dentária, erosão e degradação da camada híbrida (Tjäderhane et al., 2015).

Outra importante família de proteases, as cisteíno catepsinas (CTs) foram observadas em dentina (Nascimento et al., 2011) e a expressão das mesmas (A, B, C, F, H, L, O, Z, D, E, K, L2 e S) foi identificada em odontoblastos de tecido pulpar (Tersariol et al., 2010). AS CTs, assim como as MMPs, também participam dos

processos fisiológicos da dentina, e patológicos estando associadas à progressão de lesão de cárie e degradação da interface adesiva ao longo do tempo (Mazzoni et al., 2012; Vidal et al., 2014; Tjäderhane et al., 2015; Tjäderhane, 2015).

A CT-K, recentemente encontrada em dentina sadia e cariada (Vidal et al., 2014) é caracterizada como uma das mais efetivas colagenases, capaz de hidrolisar o colágeno do tipo I, II e IV. A atividade colagenolítica da CT-K é direcionada tanto para fora da região de hélice da molécula como também em vários locais dentro da região helicoidal, do colágeno tipo I em pH ácido (5,5), sendo que esta propriedade única entre as proteinases dos mamíferos (Garnero et al., 1998), comparadas as MMPs agem em pH neutro (7-8).

Sendo assim diante de situações de desequilíbrio metabólico, a matriz orgânica dentinária é exposta à degradação por essas duas classes de enzimas proteolíticas (MMPs e CTs). Essas enzimas se tornam ativas no momento em que estão livres do componente mineral e, portanto, aptas a degradar o colágeno e outros componentes da matriz dentinária que compõe a camada híbrida. Assim ocorre desestruturação do colágeno exposto e parcialmente infiltrado pelo sistema adesivo, explicando parcialmente à prematura degradação das restaurações adesivas (Tjäderhane et al., 2013 [a]; Tjäderhane et al*.,* 2013 [b]; Tjäderhane et al., 2015). Isto levou os pesquisadores a buscarem estratégias para preservar a matriz do colágeno, prevenir a atividade enzimática na dentina e manter a resistência da união em longo prazo (Tjäderhane et al*.,* 2013 [b]; Tjäderhane et al*.,* 2015).

Essas estratégias envolvem o uso de inibidores enzimáticos, que podem ser incorporados em adesivos ou usados como um passo extra de aplicação. Ainda, podem ser utilizados agentes de ligação cruzada, artificiais e naturais, com o objetivo de aumentar a resistência do colágeno a degradação enzimática, e diminuir a presença da água na interface, com o objetivo de diminuir a velocidade da degradação hidrolítica dos componentes da camada híbrida (Tjäderhane, 2015).

A abordagem mais comum, baseada na inibição de proteases endógenas é o uso a clorexidina. Esta substância tem se mostrado capaz de inibir colagenases e gelatinases presentes na dentina (Pashley et al., 2004, Garcia et al., 2009; Zhou et al., 2011; Scaffa et al., 2012; Sabatini et al., 2013). Entretanto, uma desvantagem clínica do uso da clorexidina é a necessidade de um passo extra de aplicação antes da aplicação do sistema adesivo (Tjäderhane; 2015). Além disso, dificuldades foram encontradas na adição da clorexidina a um adesivo, pois a concentração afetou a

sorção e solubilidade do adesivo, o grau de conversão e as propriedades mecânicas deste (Hiraishi et al., 2008; Pallan et al.,2012; Nishitani et al., 2013). Estudos acerca de um condicionador ácido contendo clorexidina também apresentaram resultados positivos na redução da nanoinfiltraçao e também na perda de resistência de união (Stanislawczuk et al., 2009 e 2011). Porém, por ser uma molécula grande e insolúvel em água ela pode se lixiviada da camada híbrida e perder seu efeito inibitório em longo prazo (Ricci et al., 2010). Assim outros produtos foram testados quanto à habilidade de inibir MMPs: tetraciclinas; bisfosfonatos; íons catiônicos (Zn, Fe); EDTA; Dimetil Sulfóxido (DMSO) e compostos de amônia quaternária como cloreto de benzalcônio e brometo de metacriloíloxidodecilpiridínio (MDPB) (Chaussain et al., 2013; Tjäderhane et al., 2013[b]; Buzalaf et al., 2015; Tjäderhane, 2015).

A estratégia da utilização de substâncias químicas sintéticas como reticuladores não enzimáticos também tem sido estudada e mostrado resultados positivos (Tjadehane, 2015; Buzalaf et al., 2015). O uso do glutaraldeído, cloridrato de carbodiimida (EDC) induziram ligações cruzadas mesmo em tecidos altamente reticulados como a dentina. Contudo, o uso clínico permanece incerto devido à citotoxidade dos compostos (Sung et al., 2003; Han et al., 2003; Bedran-Russo 2010). Também foi demonstrado que agentes derivados de plantas, as proantocianidinas (PACs), interagem fortemente com tecidos biológicos, aumentando as propriedades mecânicas e diminuindo a degradação da dentina através da interação com colágeno tipo I (Han et al., 2003; Vidal et al., 2016). Glutaraldeído, EDC e PACs e riboflavina associada à luz ultravioleta são compostos capazes de inibir proteases endógenas e induzir a reticulação do colágeno (Han et al., 2003; Scheffel et al., 2014; Seseogullari-Dirihan et al., 2015 [a]; Seseogullari-Dirihan et al., 2015 [b]; Seseogullari-Dirihan et al., 2016; Vidal et al. 2016; Seseogullari-Dirihan et al., 2017).

Essas abordagens foram descritas na literatura mais recentemente, entretanto, no inicio do desenvolvimento da “Odontologia Adesiva”, em sua proposta original, Nakabayashi et al. (1982) utilizaram uma solução ácida composta de ácido cítrico (AC) a 10% e cloreto férrico hidratado ($FeCl_3.6H_2O$) a 3%, solução essa conhecida como 10-3. Segundo os autores, o emprego do $FeCl_3$ seria importante para preservar a estrutura do colágeno exposto, evitando desnaturação, o que era uma preocupação diante do uso de ácidos sobre o colágeno (Nakabayashi et al., 1992 [a]; Nakabayashi et al., 1992 [b]; Nakabayashi & Hiranuma, 2000).

A definição precisa dos benefícios da adição do $FeCl_3$ à solução de condicionamento da dentina nunca foi esclarecida. Os estudos apresentam discrepâncias sobre os mecanismos envolvidos na melhoria da adesão quando esse componente foi adicionado ao agente de condicionamento, ora como agente de prevenção da desnaturação do colágeno, ora como agente de melhoria da polimerização do adesivo (Imai et al*.,* 1991; Nakabayashi et al*.,* 1992 [a]; Nakabayashi & Hiranuma, 2000).

Independente dos mecanismos de ação, a solução 10-3 foi adotada como agente de condicionamento ácido de dentina por vários produtos japoneses na década de 90 e segue até hoje sendo empregada no cimento resinoso Super Bond C&B (Sun Medical, Japan). Entretanto, esta solução não recebeu atenção por parte dos pesquisadores e fabricantes. Após muita relutância para a aceitação da técnica de condicionamento ácido total devido aos potenciais efeitos deletérios sobre a polpa, pesquisadores e clínicos do ocidente se renderam ao emprego do ácido fosfórico como agente de condicionamento da dentina, seguido do emprego da técnica úmida de adesão para se preservar os espaços interfibrilares e favorecer a infiltração da resina adesiva (Kanca, 1991; Gwinnett, 1994; Carvalho et al., 1996; Hitmi et al*.,* 2012). A aceitação geral do uso do ácido fosfórico se deu ao fato da ausência de evidências demonstrando seu efeito lesivo ao colágeno (quando aplicado dentro dos parâmetros clínicos aceitos atualmente) (Tezvergil-Mutluay et al*.,* 2013). Além da facilidade de fabricação e estabilidade de armazenagem e favorável relação custo-eficácia. Com isso, os estudos acerca da solução 10-3 ou mais especificamente do $FeCl_3$, como agente de condicionamento da dentina, são escassos e há lacunas no conhecimento sobre os potenciais benefícios sobre a estabilidade da camada híbrida quando do emprego dessa solução.

O ferro ganhou destaque em estudos arqueológicos, os quais investigaram como ocorreu a manutenção do colágeno tipo I e proteínas em fósseis (Schweitzer et al*.,* 2009; San Antonio et al*.,* 2011; Schweitzer et al*.,* 2013; Boatman et al*.,* 2014). Estes estudos sugerem que a preservação de tecidos moles encontrados em fósseis foi resultado da ação do ferro. Além disso, foi observado que o colágeno tipo I hiper-reticulado estava presente em fósseis de Tyrannosaurus rex com associação aos nanocristais à base de Fe com o colágeno dos vasos sanguíneos. Indicando dessa forma que o componente férrico [FeO(OH)] derivados da hemoglobina através da formação de ferritina contribuiu para a preservação do tecido (Schweitzer et al*.,*

2013; Boatman et al., 2014). Além disso, o uso de $FeSO_4$ mostrou resultados promissores em relação à inibição da atividade das MMP-2 e -9 (Kato et al., 2010, Kato et al., 2012).

Assim como vários produtos químicos naturais e sintéticos possuem capacidade de aumentar a reticulação do colágeno inter e intramolecular (Seseogullari-Dirihan et al., 2015 [a]; Vidal et al., 2016), o que reflete no aumento das propriedades mecânicas e efeito potencial na conservação da estrutura colágena contra a degradação (Seseogullari-Dirihan et al., 2015 [a]), o emprego do Fe pode atuar como forma de estabilizar o colágeno (Fathima et al., 2006). Contudo, a maioria dos produtos descritos anteriormente é utilizada adicionalmente ao condicionamento ácido e aplicação do sistema adesivo. Assim, a vantagem da utilização de soluções alternativas contendo AC e $FeCl_3$ seria a utilização de um único composto para condicionamento ácido da dentina.

Entretanto, até o momento não foi realizada qualquer investigação sobre possíveis interações do $FeCl_3$ com CT-K e a capacidade deste composto férrico de prevenir a degradação do colágeno solúvel e insolúvel, bem como os efeitos na dentina e por consequência na camada híbrida e resistência de união. Sendo assim, os objetivos da presente tese, a qual foi apresentada em dois artigos, foram investigar a interação do $FeCl_3$ com o colágeno solúvel e insolúvel frente a degradação pela CT-K (Artigo 1) e comparar os efeitos do ácido fosfórico a 35% e soluções condicionantes alternativas contendo AC e $FeCl_3$ na resistência da união a dentina, degradação do colágeno dentinário e o efeito do $FeCl_3$ sobre a atividade da CT-K (Artigo 2).

2 ARTIGOS

2.1 Artigo 1

Does Ferric Chloride prevent enzymatic collagenase activity?

Raquel Viana Rodrigues [a1], Marcelo Giannini [b], Fernanda Miori Pascon [c], Preety Panwar [ade], Dieter Brömme [adf], Ricardo Marins de Carvalho [a]

Raquel Viana Rodrigues

[a] Department of Oral Biological and Medical Sciences, Faculty of Dentistry, University of Columbia, 368-2199 Wesbrook Mall Vancouver, BC V6T 1Z3 Canada. e-mail: rquelrodrigues@hotmail.com

[1] Present address - Department of Restorative Dentistry, Dental Materials Division, Piracicaba Dental School, UNICAMP. Av. Limeira, 901, CEP 13414903 Piracicaba, SP, Brazil. Tel.: +55 19 21065200; Fax: +55 19 34210144

Marcelo Giannini

[b] Department of Restorative Dentistry, Piracicaba Dental School, UNICAMP, Av. Limeira, 901, CEP 13414903 Piracicaba, SP, Brazil. Tel.: +55 19 21065200; Fax: +55 19 34210144/ e-mail: giannini@fop.unicamp.br

Fernanda Miori Pascon

[c] Department of Pediatric Dentistry, Piracicaba Dental School, UNICAMP, Av. Limeira, 901, CEP 13414903 Piracicaba, SP, Brazil. Tel.: +55 19 21065200; Fax: +55 19 34210144/ e-mail: fmpascon@yahoo.com

Preety Panwar

[a] Department of Oral Biological and Medical Sciences, Faculty of Dentistry

[d] Center for Blood Research, University of British Columbia, 4540, Life Sciences Centre 2350 Health Sciences Mall Vancouver, BC, V6T 1Z3 Canada.

[e] ClinicalCell Biology, Vejle Hospital/Lillebaelt Hospital, Institute of Regional Health Research, University of Southern Denmark, Vejle, Denmark. e-mail: preity.pan@gmail.com

Dieter Brömme

[a] Department of Oral Biological and Medical Sciences, Faculty of Dentistry, [d] Center for Blood Research, [f] Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of British Columbia, 2350 Health Sciences Mall, Vancouver, BC, V6T 1Z3 Canada. e-mail: dbromme@dentistry.ubc.ca

Corresponding Author:

Ricardo Marins de Carvalho

[a] Department of Oral Biological and Medical Sciences, Faculty of Dentistry, University of British Columbia, 368-2199 Wesbrook Mall Vancouver, BC V6T 1Z3 Canada.

Phone: +1-604-827-0566/ e-mail: rickmc@dentistry.ubc.ca

ABSTRACT

Collagen is the most abundant protein in mammals and a biomaterial with application in regeneration field. Cathepsin-K (CT-K) it is the most potent collagenase, and it has an important role on degradation of collagen. This study investigated the effect of $FeCl_3$ ability to prevent degradation of soluble and insoluble collagen. Soluble and insoluble collagen (mouse tail tendon fibers-MTTF) degradation at the presence of $FeCl_3$ against CT-K was measured through electrophoresis. Substrates were incubated with CT-K and different concentrations of $FeCl_3$ (0.02%; 0.04%; 0.06%; 0.08%; 0.1%; 0.2%; 0.4%; 0.8%; 1%; 2%). The supernatant was subjected to SDS/PAGE. The collagenase activity was evaluated by the lost or generation of α bands. The MTTF were characterized after enzymatic treatment using scanning electronic microscopy (SEM) and energy-dispersive X-ray (EDX) evaluation. Data were submitted to the normality test and one-way ANOVA + Holm/Sidak were applied (α=5%). $FeCl_3$ was able to prevent soluble and insoluble collagen degradation, and inhibited the enzyme starting at 0.06% of $FeCl_3$. SEM observation revealed unraveling of collagen fibers in $FeCl_3$ absence. It was observed integrity of the fiber exposed to low concentrations of $FeCl_3$ (0.06%-0.4%), and interaction between Fe/Collagen. It can be concluded that $FeCl_3$ showed to be a CT-K inhibitor and cross-linking inducer reducing the collagen enzymatic degradation.

Keywords: Cathepsins. Collagen type I. Ferric Chloride. Ferric Compounds. Iron.

1. INTRODUCTION

Enzymatic activity play crucial role in several biological systems in the human body. The excessive activity of collagenolytic and elastolytic cathepsins (CTs) has been associated with bone and cartilage erosion in osteoporosis and arthritis [1], as well as plaque and blood vessel destabilization in atherosclerosis and aneurysms [2,3]. In dentistry, dental tissue collagenolytic CTs and matrix metalloproteinases (MMPs) have been associated with caries, dental erosion and degradation of resin-dentin bonds [4,5]. Together, these findings make collagenolytic CTs an important drug target [4,6].

Cathepsin K (CT-K) represents the most potent mammalian collagenase [6,7]. It can degrade collagen at various sites of the helical region [8]. CT-K is also a pH-dependent and has its best activity at a relatively low pH (5.5) [9]. The progression of dental erosion and caries and at the onset of clinical adhesive procedures, the surrounding environment usually faces a pH drop. During the bond procedure, acidic conditioners or acidic monomers are used to etch the surface. The acidic condition is required to remove minerals and expose the interfibrillar collagen network to allow for resin infiltration and secure bonding. However, the acidic environment also exposes and activates endogenous proteolytic enzymes (MMPs and CTs) that are considered to be a long-term threat to the stability of the newly formed bond to dentin [4,10,11]. They may be responsible for endogenous proteolytic activity of dentin, contributing not only to collagen degradation at resin-dentin interfaces but in caries progression, since both are present in human dentin [10,12–14].

Because of that, most of the recent research in resin-dentin adhesion has focused on ways to inhibit or reduce enzymatic activity at the interface in order to preserve the exposed collagen and extend the durability of the bonds [4,15].

The interaction of collagen with metal ions such as Fe^{2+} Fe^{3+} is one of the known methods to stabilize collagen. Metal ions find predominant application in cross-linking of collagen for various end uses [16]. Cross-link process could promote tissue stability, strength and restore function in connective tissues such as skin, bone and dentin which are mainly composed of type I collagen [16,17].

Some metal salts can inhibit enzymes that collectively target collagen (such as MMPs), and $FeSO_4$ showed promising result against MMP-2 and -9 activity [18,19]. Since ferric chloride ($FeCl_3$) has been used as part of dental conditioner, in the past [20], and seems to have a especial interaction with dentin collagen [21,22], it

becomes interesting to investigate the ability of $FeCl_3$ to potentially protect collagen against collagenases. Therefore, the aim of the study was to evaluate the effect of $FeCl_3$ on CT-K activity and the interaction with the soluble and insoluble collagen. The hypothesis tested was that $FeCl_3$ would be able to prevent soluble and insoluble collagen degradation.

2. MATERIALS AND METHODS

2.1 Materials

All chemicals and solvents used in the study: chondroitin 4-sulfate (CSA), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), glutaraldehyde, dimethylmethylene blue, N, N-dimethylformamide (DMF), - ferric chloride ($FeCl_3$), carboxy-trans-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane (E-64) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

30% acrylamide and bis-acrylamide solution, 37.5:1 (Bio-Rad Laboratories, Mississauga, ON, Canada) was purchased from the supplier Bachem (Weil Amrhein, Germany). Mouse tail tendons from unrelated study that would be otherwise disposed, and CT-K was kindly provided by the Department of Biochemistry and Molecular Biology, University of British Columbia, CA.

2.2 Interaction enzyme/ substrate/ inhibitors

2.2.1Soluble Collagen degradation assay

Bovine type 1 soluble collagen (0.6 mg/ml) (MSJ BioLynx Inc.) was incubated for 4 h at 28◦C in assay buffer (100 mM acetate buffer, pH 5.5, containing 2.5 mM DTT and EDTA 2 5 mM) containing 400 nM CT-K wild type in the presence of 200 nM CAS with addition of the inhibitors in solution form ($FeCl_3$ in concentrations of 0.02% to 2% (Table 1). The reactions were stopped by the addition of 10μM E-64 and subsequently the reaction mixtures were subjected to 10% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS/PAGE). Bands were visualized by Coomassie Brilliant Blue R-250 (CBB) and the staining quantified using densitometrically analysis program Image J (National Institutes of Health). The collagenase activity was quantified based on the loss of α1- and α2 bands after SDS/PAGE. Three individual experiments were performed.

Table 1. Groups inserted at the lanes and respective $FeCl_3$ concentrations on soluble collagen assays.

Lanes	Groups	CT-K	% $FeCl_3$ in weight
1	⊕ control collagen soluble	NO	0
2	soluble collagen + CT-K	YES	0
3	⊖ control soluble collagen + CT-K+ CSA	YES	0
4	soluble collagen + CT-K+ CSA+ 0.02% $FeCl_3$	YES	0.02%
5	soluble collagen + CT-K+ CSA+ 0.04% $FeCl_3$	YES	0.04%
6	soluble collagen + CT-K+ CSA+ 0.06% $FeCl_3$	YES	0.06%
7	soluble collagen + CT-K+ CSA+ 0.08% $FeCl_3$	YES	0.08%
8	soluble collagen + CT-K+ CSA+ 0.1% $FeCl_3$	YES	0.1%
9	soluble collagen + CT-K+ CSA+ 0.2% $FeCl_3$	YES	0.2%
10	soluble collagen + CT-K+ CSA+ 0.4% $FeCl_3$	YES	0.4%
11	soluble collagen + CT-K+ CSA+ 0.8% $FeCl_3$	YES	0.8%
12	soluble collagen + CT-K+ CSA+ 1% $FeCl_3$	YES	1%
13	soluble collagen + CT-K+ CSA+ 2% $FeCl_3$	YES	2%

⊕ Positive ⊖ Negative

CT-K: Catephin K

CSA: chondroitin 4-sulfate

2.2.2 Insoluble Collagen assay

2.2.2.1Isolation of Collagen Fibers

Freshly isolated collagen fibers were used for the present experiments. Type I collagen fibers were isolated from mouse tail tendons. The fibers were pulled out from the distal end of mouse tail using surgical clamps and collected in PBS. These fibers were disinfected with 70% ethanol for five seconds, air-dried, and transferred to a sterile container for further use.

2.2.2.2 In Vitro Collagen Fiber Degradation

Insoluble type I collagen fibers (1 mg) were incubated with wild type CT-K 1μM concentration in 100 mM sodium acetate buffer, pH 5.5, containing 2.5 mM DTT and EDTA and the inhibitors in solution form ($FeCl_3$ in concentrations of 0.02% to 2% (Table 2) for 20 hours at 28 °C. The reaction was stopped by the addition of 10 M E-64. Subsequently, the reaction mixture was centrifuged for 20 minutes, and the supernatant was taken and subjected to SDS/PAGE analysis using 10% Tris/glycine gels. Bands were visualized by Coomassie Brilliant Blue R-250 staining and quantified using densitometrically analysis program Image J. The collagenase activity was evaluated on the basis of the generation and loss of α1 and α2 bands after SDS-PAGE [23]. Three individual experiments were performed.

Table 2. Groups inserted at the lanes and respective $FeCl_3$ concentrations on insoluble collagen assays.

Lanes	Groups	CT-K	% $FeCl_3$ in weight
1	⊕ control insoluble collagen	NO	0
2	⊖ control insoluble collagen + CT-K	YES	0
3	insoluble collagen + CT-K + 0.02% $FeCl_3$	YES	0.02%
4	insoluble collagen CT-K+ 0.04% $FeCl_3$	YES	0.04%
5	insoluble collagen + CT-K+ 0.06% $FeCl_3$	YES	0.06%
6	insoluble collagen + CT-K + 0.08% $FeCl_3$	YES	0.08%
7	insoluble collagen + CT-K+ 0.1% $FeCl_3$	YES	0.1%
8	insoluble collagen + CT-K+ 0.2% $FeCl_3$	YES	0.2%
9	insoluble collagen + CT-K+ 0.4% $FeCl_3$	YES	0.4%
10	insoluble collagen + CT-K+ 0.8% $FeCl_3$	YES	0.8%
11	insoluble collagen + CT-K+ 1% $FeCl_3$	YES	1%
12	insoluble collagen + CT-K+ 2% $FeCl_3$	YES	2%

⊕ Positive ⊖ Negative

CT-K: Catephin K

2.2.2.3 Electron Microscopy Imaging and Energy-Dispersive X-ray Measurement

Scanning electron microscopy (SEM) was used to characterize collagen fibers after enzymatic treatment. Collagen fibers were incubated under the conditions as described above. The treated fibers were processed for imaging as described previously [9]. After drying procedure, fibers were positioned on acrylic stub with double-sided carbon adhesive tape and sputter-coated with carbon (MED 010 Baltec, Balzers, Liechtenstein). Images were examined under SEM (JEOL, JSM-5600LV, Tokyo, Japan) and energy-dispersive X-ray (EDX) spectrometry analysis (Vantage, NORAN Instruments, Middleton, WI, USA) used to identify the percentage of elements and quantify Fe% in weight. Each spectrum was acquired for 100 seconds (voltage 15 kV, working distance 20 mm). Images showing the identified chemical elements were obtained from ten distinct areas of each treatment group. Micrographs were taken from different regions of the fibers to evaluate morphological changes.

2.3 Statistical Analysis

The data for soluble and insoluble collagen degradation assay were evaluated to check the equality of variance (Brown-Forsythe) and to confirm a normal distribution (Shapiro-Wilk). So, data were submitted to one-way ANOVA and all

pairwise multiple comparison procedures (Holm-Sidak method) (SigmaPlot 11, Systat Software Inc.).

EDX data were tested to confirm a normal distribution (Anderson-Darling Test). Data were submitted to one-way ANOVA all pairwise multiple comparison procedures (Tukey) (Minitab express, version 1.5.0, Minitab Inc.). The significance level was pre-set to all analysis $\alpha = 5\%$.

3. RESULTS

3.1 Degradation of soluble Type I Collagen by CT-K

Soluble collagen assay showed that significant reduction of CT-K activity and consequent reduction of collagen degradation was observed beyond 0.04% and up to 0.1% $FeCl_3$ (Fig. 1). As concentration of $FeCl_3$ increased from 0.04% to 0.1%, the inhibition of CT-K activity gradually diminished, but were still significantly different from control and from solutions containing 0.02% and 0.04% $FeCl_3$. When soluble collagen was mixed with different concentrations of $FeCl_3$ without adding the enzyme and run through the gel, it was noted that at higher concentrations of $FeCl_3$ there was collagen precipitation caused by iron cross-link with soluble collagen. This cross-link generates high weight molecules that are not able to go through the gel during the electrophoresis (Fig. 2).

Because of this technical limitation, we subtracted the precipitation rate from the CT-K activity results to obtain the actual, relative percentage of CT-K activity as a function of $FeCl_3$ concentration (Fig. 3). From that, the results indicated that there was a significant reduction of approximately 50-55% of CT-K activity when as little as 0.02-0.04% $FeCl_3$ was added to the solution and further reductions of CT-K activity to approximately 80-90% was observed beyond the threshold of 0.06% $FeCl_3$ concentration (Fig. 3).

3.2 Degradation of Insoluble Type I Collagen Fibers by CT-K

Fig. 4A showed the results collagenase activity when the substrate is insoluble collagen. At 0.02% $FeCl_3$ is was observed a significant reduction of CT-k activity dropping around 11.05% ($\pm$5.33), at 0.04% $FeCl_3$ CT-k activity was 7.75%($\pm$2.2) and starting at 0.06% $FeCl_3$ it is consider that no degradation occurs ($p < 0.05$). The visibility of α1 and α2 bands represents the collagenase activity, considering lane 2 (no inhibitors) as 100% of CT-K activity Fig. 4B.

3.2.1 EDX *Measurement*

It was noted by EDX analysis that, after the 20 hours incubation, the fibers adsorbed Fe on the structure. It was found in all groups higher concentration of Fe (%wt.) attached to the fibers than the initial solution concentration, as showed in Fig. 4C. Also Fe % wt found in the fibers it is not directed proportional to the $FeCl_3$ reaction solutions. Groups 11(1% $FeCl_3$) and 12 (2% $FeCl_3$) presented a lower amount of Fe compared to 7 (0.1% $FeCl_3$).

3.2.2 SEM micrographs analysis

CT-K incubation with collagen revealed a dramatic degradation resulting in the disruption of the arrangement of fibrils with significant structural changes were observed as showed in Fig 4. D1. The iron exercised a degradation preventive effect on the fibers (Fig 4. D2-9). It was observed too the effect of the long ionic exposition on the groups 10,11,12 micrographs, where the fibers were denatured assuming a flat shape.

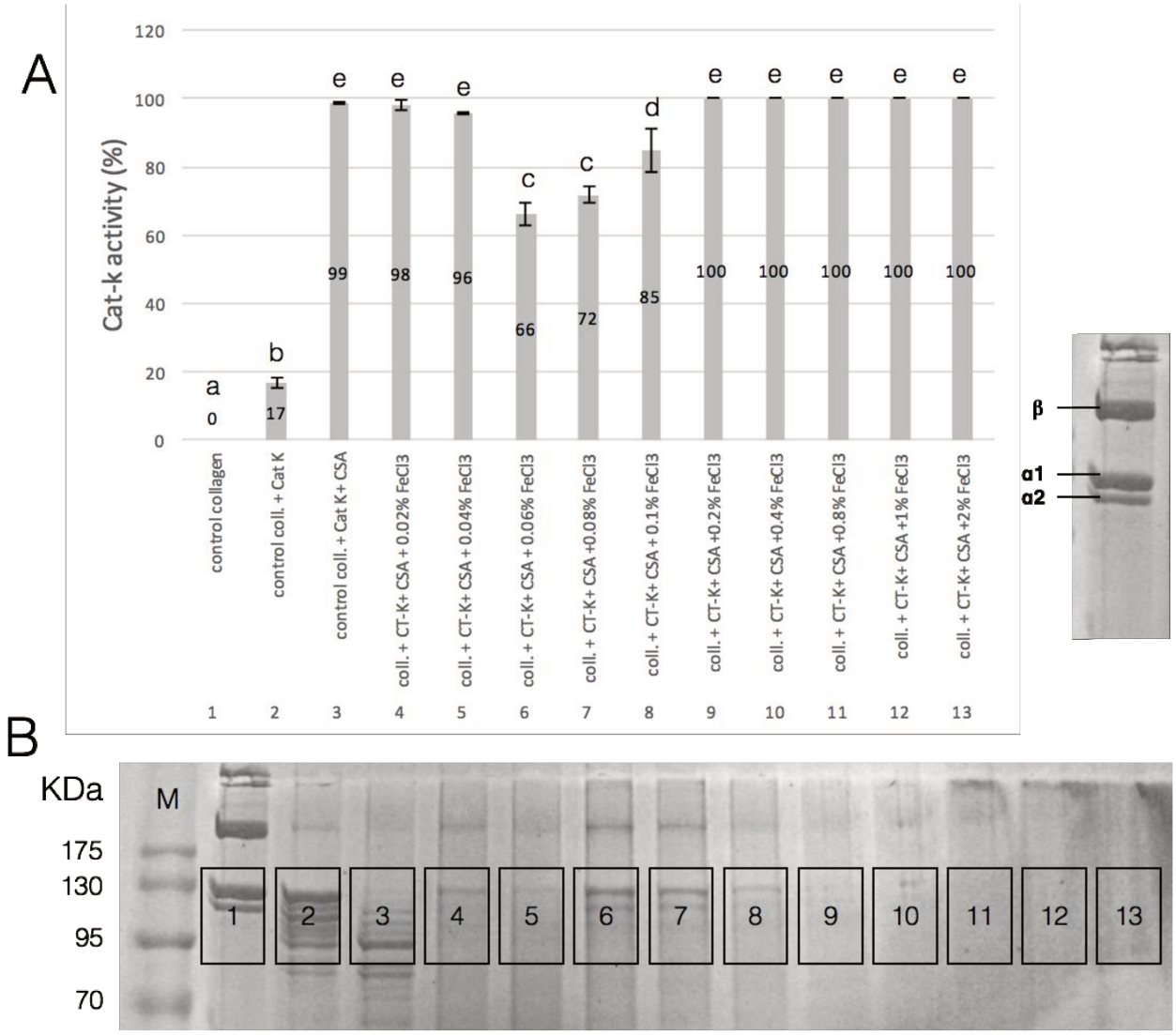


Fig. 1. Effects of $FeCl_3$ on inhibition of CT-K activity and degradation of soluble collagen. (A) CT-K activity (%) as function of $FeCl_3$ concentration. The apparent lack of inhibition of enzymatic activity at and beyond 0.2% $FeCl_3$ was due to precipitation of collagen (see Results and Fig. 2). Different letters indicate statistically significant differences ($p<0.05$). (B) Representative SDS/PAGE analysis of soluble collagen degradation products. Soluble bovine collagen was incubated with 400 nM enzyme at pH 5.5 and 28◦C for 4 h in the presence of CSA. Bands α1 and α 2 (130-100 kDa) as showed in the rectangular area, were investigate by densitometric analysis, considering control collagen (lane 1) as no degradation. Molecular weight Marker (M) (PiNK Plus Prestained Protein Ladder - Genedirex ladder).

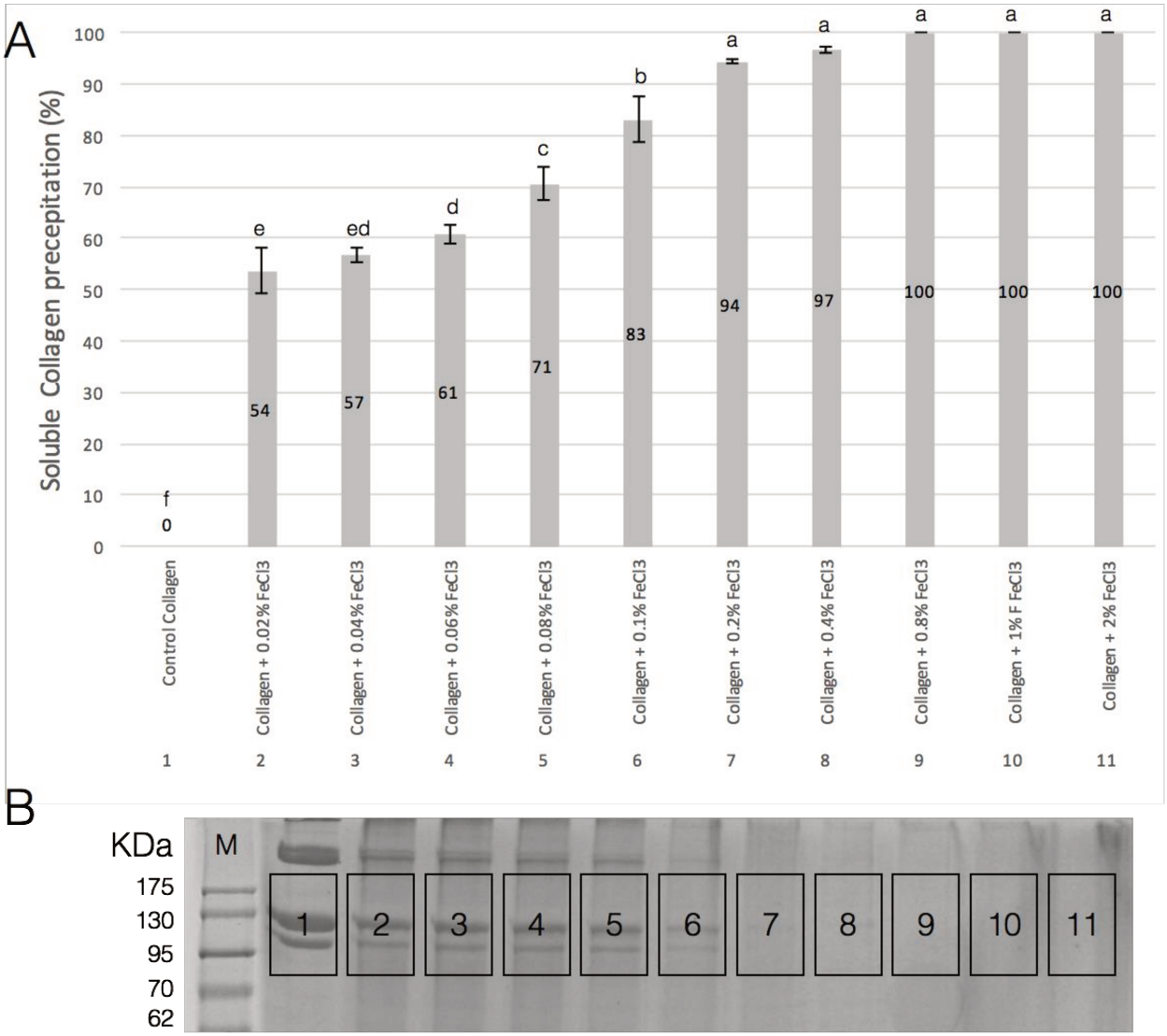


Fig. 2**.** Collagen precipitation caused by iron cross-link. (A) Collagen precipitation increased with increased $FeCl_3$ concentration reaching above 95% when $FeCl_3$ concentration was 0.2% and beyond. (B) Precipitation quantification was based on the loss of the bands α1 and α2 by densitometric analysis as showed in the rectangular area, considering control collagen (lane 1) as no precipitation. Different letters indicate statistically significant differences ($p<0.0001$). Molecular weight Marker (M) (PiNK Plus Prestained Protein Ladder - Genedirex ladder).

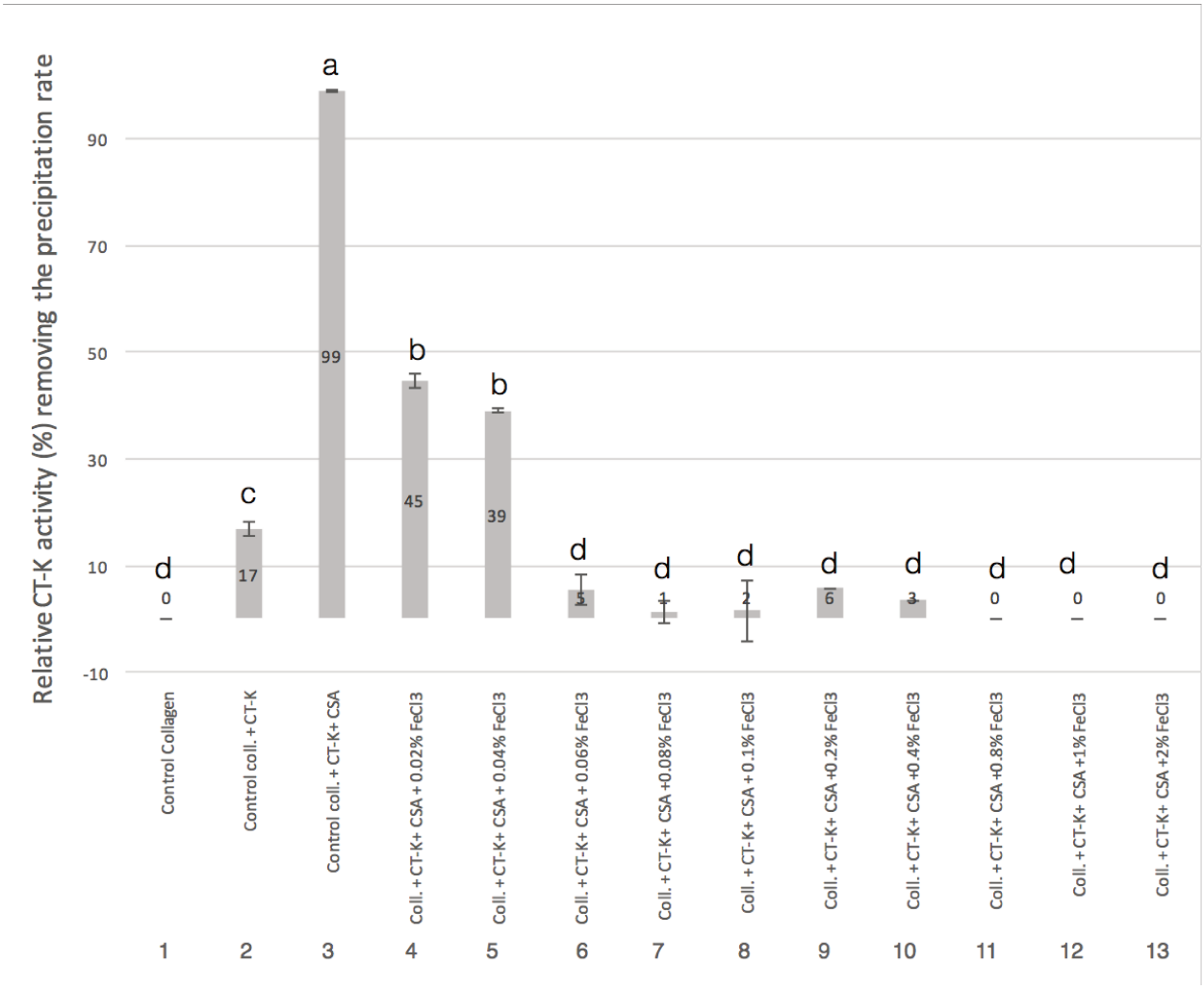
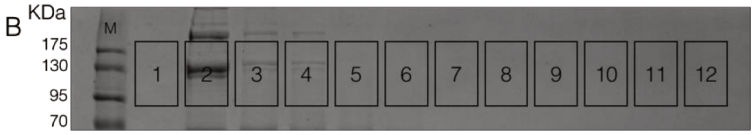
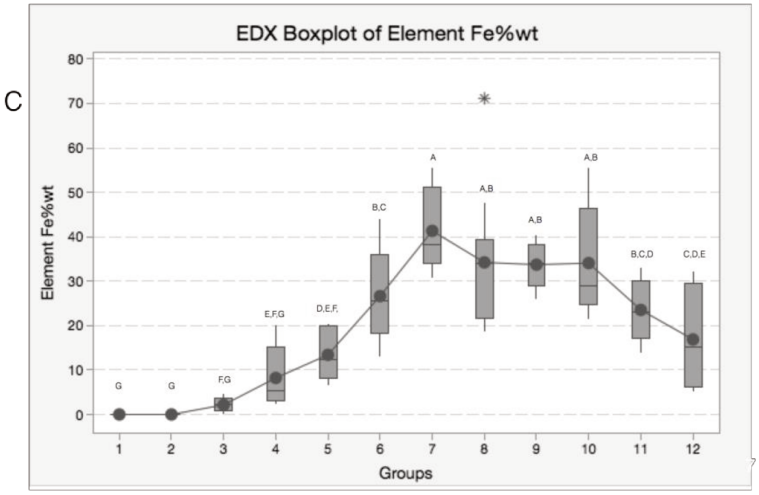


Fig. 3. Relative CT-K activity after removing the precipitation rate caused by collagen cross-linking. Significant reductions of CT-K activity could be observed with as little as 0.02% $FeCl_3$ in the solution, with further reductions as the concentration of $FeCl_3$ increased.

A

Lanes	Groups	CT-K Aticity (%) ± SD
1	control insoluble	0.78 ± 0.394 c
2	control insoluble coll.+ CT-K	100 ± 0 a
3	Insoluble coll.+ CT-K+ 0.02% $FeCl_3$	11.037 ± 5.33 b
4	Insoluble coll.+ CT-K+ 0.04% $FeCl_3$	7.751 ± 2.244 b
5	Insoluble coll.+ CT-K+ 0.06% $FeCl_3$	1.712 ± 2.956 c
6	Insoluble coll.+ CT-K+ 0.08% $FeCl_3$	0 ± 0 c
7	Insoluble coll.+ CT-K+ 0.1% $FeCl_3$	0 ± 0 c
8	Insoluble coll.+ CT-K+ 0.2% $FeCl_3$	0 ± 0 c
9	Insoluble coll.+ CT-K+ 0.4% $FeCl_3$	0 ± 0 c
10	Insoluble coll.+ CT-K+ 0.8% $FeCl_3$	1.128 ± 1.953 c
11	Insoluble coll.+ CT-K+ 1% $FeCl_3$	0.121 ± 0.209 c
12	Insoluble coll.+ CT-K+ 2% $FeCl_3$	0 ± 0 c





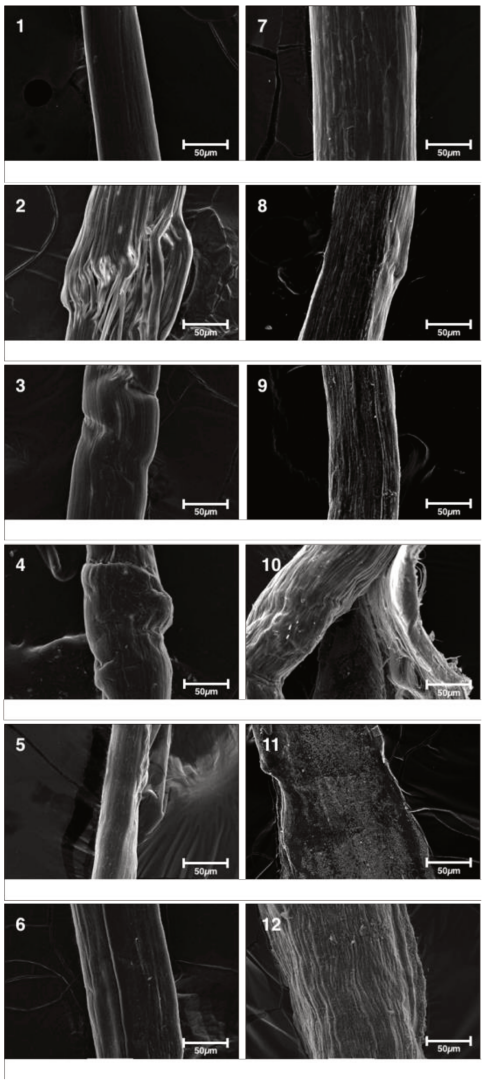


Fig. 4. $FeCl_3$ action on the degradation of type I collagen fibers by CT-K. (A) Quantitative analysis of collagenase activity by SDS/PAGE based on released products (α-Chains) of degradation from fibers upon digestion. The results of three independent experiments are expressed in mean standard deviation (SD) ($p < 0.05$). The statistic difference is showed by lowercase letters. (B) Representative collagenase activity by SDS/PAGE gel. Quantification of collagenase activity is based on the presence of bands α1 and α2 (130-100 kDa) by densitometric analysis as showed in the rectangular area. Considering lane 1 as a control (no degradation), and lane 2 as the maximum of collagen fiber degradation and products formation. (M) Molecular weight Marker (PiNK Plus Prestained Protein Ladder - Genedirex ladder). (C) EDX results showed in boxplot graphic about the percentage in weight (wt%) of iron in the collagen fibers after incubation. The statistic difference was showed by uppercase letters ($p<0.05$). (D) Representative images corresponding to 1-13

incubated fibers treatments ($FeCl_3$ ≠concentration) and gel lanes. The micrographs (500X) were taken from different fibers spots. The morphology of fibers after 20 hours CT-K incubation was observed (bars represent 50μm), on 2 CT-K unrevealing effect it is observed. CT-K partially degrade the collagen fiber at its surface on 3, 4. Note the collagen fibers incubated with activity buffer in the absence of CT-K reveal no structural changes after 20 hours (D.1) as well as 5, 6, 7, 8, 9 (0.06%- 0.4% $FeCl_3$). The long ionic exposition on the groups 10,11,12 micrographs, cause denaturation, changing the cylindrical morphology of the fibers flattening them.

4 DISCUSSION

The tested hypothesis that $FeCl_3$ would be able to prevent soluble and insoluble collagen degradation was confirmed. It was demonstrated a direct relationship between $FeCl_3$ concentration and inhibition of CT-K activity. Our study showed the effect of $FeCl_3$ on CT-K activity and the interaction with soluble and insoluble collagen, preventing their degradation (Fig. 3, Fig 4 A).

CT-K is a thiol-dependent cathepsins, so it has a cysteine residue in its active site [24]. As Fe^{3+} Fe^{2+} ions generate reactive oxygen species, they could cause oxidation of the thiol groups of cysteine residues and result in enzyme inhibition [25–27]. Also, the thiol group (–C–SH or R–SH) has a high affinity for heavy metals. So, the formation of irreversible covalent bond between iron-sulfur, could block the active site of the enzyme [28,29]. As well the Fe^{3+} could change the collagen enzyme site inhibiting the degradation, as collagen became unrecognizable to collagenase. [16]. All the three alternatives that probably occur simultaneously will consequently inactivate enzymes catalytic functions [16,25,27,26,30,28,29,31], and result in reduced activity of the CT-K at concentrations of 0.06% of FeCl3 (Fig. 3).

Complexation of metal ions with EDTA leads to stabilization of proteins. Similarly, destructive oxidative reactions of thiol groups of cysteine can also be prevent by adding reducing agents such as β-mercaptoethanol or DTT [26]. The activity buffer used on assays contains 2.5 mM DTT and EDTA. So, if we consider that EDTA is a chelator, the activity buffer was prepared in the absence of EDTA certainly the $FeCl_3$ inhibition concentration would be lower. However, DTT in the presence transition metals and O_2, can induce oxidative damage in biomolecules , and the combination of EDTA ,DTT and Fe^{3+} ions showed to be involved in several phases with radicals (O^{2-}), O_2 and H_2O_2 formation that could interact with

biomolecules degradation [32]. As well as EDTA stimulates the iron-catalyzed oxidation of DTT probably by accelerating Fe^{3+} reduction [32].

Soluble collagen is a pre-fragmented molecule (α1 and α2) that is easily broken into smaller parts. Fig. 1B showed the presence of the band α1 and α2 (100–130 kDa, Lane 1) indicated no degradation, and the loss of the bands α1 and α2 indicated the increase of collagenase activity, demonstrating by the presence of sub sequential bands of low molecular weight fragments [23]. In our study, it was observed a high rate of collagen precipitation (Fig. 2) caused by iron cross-linking with soluble collagen. Ferric ions could do the deeds of lysyl oxidase inducing the collagen cross-linkage. Ions might act on lysine and hydroxylysine aminoacids at the telopeptides regions producing aldehyde groups, resulting in the formation of inter-, and intra-molecular and inter-microfibrillar [33,34]. Iron form covalent complex with collagen [16]. So the cross-link bond takes place between the iron complex and collagen [34], drawing the collagen molecules closer together and increase stability [34–36], improve the mechanical properties and decrease degradation rates [33]. Because of the collagen cross-link we showed the results as “Relative CT-K activity removing the precipitation rate” with the goal to reveal the real enzymatic action (Fig. 3). So the apparent lack of CT-K inhibition at 0.2% $FeCl_3$ and beyond was due to collagen precipitation caused by cross-linking with iron (see Fig. 2) [9].

A differential in soluble collagen degradation assay was the addition of chondroitin sulfate (CSA) on the reaction. CT-K activity depends on the formation of a complex glycosaminoglycans(GAGs) such as CSA [37]. CSA it is present on nature tissues as bone, cartilage, tendon [9,37] and dentin [38]. Because of the importance of the GAG complex, as was used processed collagen, it is necessary to add CSA into reactions in order to active the enzyme as we observed on Fig. 1, lane 2 without CSA, and lane 3 with CSA exhibited a significant increase on CT-K activity.

Morphology of collagen fibers is considered a sequential arrangement of fibrils that through GAG mediated proteoglycan interactions were put together [39,40]. When the degradation occurs by the action of CT-K, the product formation was characterized by the release of collagen α -chains from the fiber [9,23], as showed in Fig. 4A, B (lane 2) and D micrograph 2, where was possible to see the fiber degradation, and unraveling process [41]. CT-K is the only CT able to release significant amounts of tropocollagen fragments from insoluble collagen fibers [9], as CT-K it is the unique enzyme holding the ability to cleave at multiple sites the triple

helical region of tropocollagen [7].

CT-K is regulated by sulphated glycosaminoglycans (GAG) when connected with exosites [40]. It is possible that cross-linking agents such as Fe^{3+}ion alter the GAG-CK-T exosite interaction, changing its conformation and consequently inactivate the catalytic function. Cross-linking also decreases enzymatic degradation by altering the enzyme binding site on the collagen molecule [17,42–45]. Since EDX analysis demonstrated higher concentration of Fe (%wt.) attached to the fibers (Fig. 4C) the alteration on enzyme binding site on the collagen molecule and GAG-CK-T exosite interaction probably were the two protect mechanism on insoluble collagen assay. However, Fe % wt. found in the fibers did not increase as $FeCl_3$ concentrations at reaction solutions. Insoluble collagen groups 11 (1% $FeCl_3$) and 12 (2% $FeCl_3$) presented a lower amount of Fe compared to 7 (0.1% $FeCl_3$). It is probably related to ionic degradation as visualized in Fig. 4 D micrographs 11 and 12. So the formed Fe/collagen complex is well documented pointing an increase of enzymatic stability [16,18,19].

Micrographs reveal iron exercised a preventive effect of degradation on the fibers (Fig. 4 D2-9). But also the effect of the high long ionic exposition (20h) on the groups 10,11,12 micrographs, causing denaturation of the fibers. Denaturation by ionic forces act on secondary and / or tertiary structure, that is, the three-dimensional arrangement of the polypeptide chain is broken, almost always losing its characteristic biological activity. But the primary structure it is kept intact. There is no disruption of covalent bonds of the polypeptide chain, preserving the amino acid sequence. So the ionic degradation did not affect the soluble collagen since it is formed by simplified chains [46]

The use of protease inhibitor or cross-link inducer can increase collagen properties and reduce its enzymatic degradation [33]. As we showed, $FeCl_3$ act in both outcomes. This is an important field where a more detailed picture of the actions will further improve our understanding of the collagen degradation processes and the participants involved.

5 CONCLUSION

Based on the in vitro conditions of the present study, it could be concluded that $FeCl_3$ inhibited CT-K activity starting at 0.06% concentrations. In addition, $FeCl_3$ was able to prevent soluble and insoluble collagen fibers degradation by CT-K, while

it was observed unraveling of collagen fibers in $FeCl_3$ absence. The use of $FeCl_3$ formed a cross-linked complex with collagen that increases stability against collagenolytic enzymes.

Statement of Significance

Collagen is the main component of connective tissue and the most abundant protein in mammals, making up about 25% of the total body mass. Improvements on collagen stability against collagenases could be useful in multiples areas of science and health. CT-K it is the most potent mammalian collagenase and its hyperactivity is related to several implications related to skeletal and vascular diseases, caries, dental erosion and hybrid layer degradation. This is the first report to show the inhibition effect of $FeCl_3$ against CT-K, and its preventive effect on collagen type I, as well its ability to induce collagen cross-link.

Acknowledgements

This study was partially supported by: Coordination for the Improvement of Higher Education Personnel (Capes #1777-2014 and 999990107), National Counsel of Technological and Scientific Development (CNPq #30217-2014-0), UBC Start-Up research funding to RMC and AMP, and by Canadian Institutes of Health Research Grants MOP-8994 (DB) and a Canada Research Chair award (DB).

6 REFERENCES

[1] Y. Yasuda, J. Kaleta, D. Br??mme, The role of cathepsins in osteoporosis and arthritis: Rationale for the design of new therapeutics, Adv. Drug Deliv. Rev. 57 (2005) 973–993. doi:10.1016/j.addr.2004.12.013.

[2] I. Podgorski, Future of anticathepsin K drugs: dual therapy for skeletal disease and atherosclerosis?, Future Med. Chem. 1 (2009) 21–34. http://www.ncbi.nlm.nih.gov/pubmed/20126511 (accessed January 8, 2017).

[3] S.P.M. Lutgens, K.B.J.M. Cleutjens, M.J.A.P. Daemen, S. Heeneman, Cathepsin cysteine proteases in cardiovascular disease, FASEB J. 21 (2007) 3029–3041. doi:10.1096/fj.06-7924com.

[4] L. Tjäderhane, M.A.R. Buzalaf, M. Carrilho, C. Chaussain, Matrix metalloproteinases and other matrix proteinases in relation to cariology: The era of “dentin degradomics,” Caries Res. 49 (2015) 193–208. doi:10.1159/000363582.

[5] M.A.R. Buzalaf, S. Charone, L. Tjäderhane, Role of host-derived proteinases in dentine caries and erosion., Caries Res. 49 Suppl 1 (2015) 30–7. doi:10.1159/000380885.

[6] A.G. Costa, N.E. Cusano, B.C. Silva, S. Cremers, J.P. Bilezikian, Cathepsin K: its skeletal actions and role as a therapeutic target in osteoporosis., Nat. Rev. Rheumatol. 7 (2011) 447–56. doi:10.1038/nrrheum.2011.77.

[7] P. Garnero, O. Borel, I. Byrjalsen, M. Ferreras, F.H. Drake, M.S. McQueney, N.T. Foged, P.D. Delmas, J.M. Delaissé, The collagenolytic activity of cathepsin K is unique among mammalian proteinases, J. Biol. Chem. 273 (1998) 32347–32352. doi:10.1074/jbc.273.48.32347.

[8] A. Mazzoni, L. Breschi, M. Carrilho, F.D. Nascimento, G. Orsini, A. Ruggeri Jr, P. Gobbi, L. Manzoli, F.R. Tay, D.H. Pashley, L. Tjäderhane, A review of the nature, role, and function of dentin non-collagenous proteins. Part II: enzymes, serum proteins, and growth factors, Endod. Top. (2012) 19–40. doi:10.1111/j.1601-1546.2012.00268.x.

[9] P. Panwar, X. Du, V. Sharma, G. Lamour, M. Castro, H. Li, D. Br??mme,

Effects of cysteine proteases on the structural and mechanical properties of collagen fibers, J. Biol. Chem. 288 (2013) 5940–5950. doi:10.1074/jbc.M112.419689.

[10] L. Tjäderhane, F.D. Nascimento, L. Breschi, A. Mazzoni, I.L.S. Tersariol, S. Geraldeli, A. Tezvergil-Mutluay, M. Carrilho, R.M. Carvalho, F.R. Tay, D.H. Pashley, Strategies to prevent hydrolytic degradation of the hybrid layer - A review, Dent. Mater. 29 (2013) 999–1011. doi:10.1016/j.dental.2013.07.016.

[11] L. Tjäderhane, F.D. Nascimento, L. Breschi, A. Mazzoni, I.L.S. Tersariol, S. Geraldeli, A. Tezvergil-Mutluay, M.R. Carrilho, R.M. Carvalho, F.R. Tay, D.H. Pashley, Optimizing dentin bond durability: Control of collagen degradation by matrix metalloproteinases and cysteine cathepsins, Dent. Mater. 29 (2013) 116–135. doi:10.1016/j.dental.2012.08.004.

[12] C. Vidal, L. Tjäderhane, P. Scaffa, I. Tersariol, D. Pashley, H. Nader, F. Nascimento, M. Carrilho, Abundance of MMPs and cysteine cathepsins in caries-affected dentin., J. Dent. Res. (2014) 269–274. doi:10.1177/0022034513516979.

[13] I.L. Tersariol, S. Geraldeli, C.L. Minciotti, F.D. Nascimento, V. Pääkkönen, M.T. Martins, M.R. Carrilho, D.H. Pashley, F.R. Tay, T. Salo, L. Tjäderhane, Cysteine Cathepsins in Human Dentin-Pulp Complex, J. Endod. 36 (2010) 475–481. doi:10.1016/j.joen.2009.12.034.

[14] a. Mazzoni, F.D. Nascimento, M. Carrilho, I. Tersariol, V. Papa, L. Tjaderhane, R. Di Lenarda, F.R. Tay, D.H. Pashley, L. Breschi, MMP Activity in the Hybrid Layer Detected with in situ Zymography, J. Dent. Res. 91 (2012) 467–472. doi:10.1177/0022034512439210.

[15] L. Tjäderhane, Dentin Bonding: Can We Make it Last?, Oper. Dent. 40 (2015) 4–18. doi:10.2341/14-095-BL.

[16] N.N. Fathima, M.C. Bose, J.R. Rao, B.U. Nair, Stabilization of type I collagen against collagenases (type I) and thermal degradation using iron complex, J. Inorg. Biochem. 100 (2006) 1774–1780. doi:10.1016/j.jinorgbio.2006.06.014.

[17] Y. Liu, L. Tjäderhane, L. Breschi, A. Mazzoni, N. Li, J. Mao, D.H. Pashley, F.R.

Tay, Limitations in bonding to dentin and experimental strategies to prevent bond degradation., J. Dent. Res. 90 (2011) 953–968. doi:10.1177/0022034510391799.

[18] M.T. Kato, A.L. Leite, A.R. Hannas, R.C. Oliveira, J.C. Pereira, L. Tjäderhane, M.A.R. Buzalaf, Effect of iron on matrix metalloproteinase inhibition and on the prevention of dentine erosion, Caries Res. 44 (2010) 309–316. doi:10.1159/000315932.

[19] M.T. Kato, M.A.R. Buzalaf, Iron supplementation reduces the erosive potential of a cola drink on enamel and dentin in situ, J. Appl. Oral Sci. 20 (2012) 318–322. doi:10.1590/S1678-77572012000300004.

[20] Y. Imai, Y. Kadoma, K. Kojima, T. Akimoto, K. Ikakura, T. Ohta, Importance of polymerization initiator systems and interfacial initiation of polymerization in adhesive bonding of resin to dentin., J. Dent. Res. 70 (1991) 1088–91. doi:10.1177/00220345910700071401.

[21] N. Nakabayashi, K. Kojima, E. Masuhara, The promotion of adhesion by the infiltration of monomers into tooth substrates, J. Biomed. Mater. Res. 16 (1982) 265–273. doi:10.1002/jbm.820160307.

[22] N. Nakabayashi, K. Hiranuma, Effect of etchant variation on wet and dry dentin bonding primed with 4-META/acetone., Dent. Mater. 16 (2000) 274–279. doi:10.1016/S0109-5641(00)00017-8.

[23] V. Sharma, P. Panwar, A.J. O'Donoghue, H. Cui, R.V.C. Guido, C.S. Craik, D. Brömme, Structural requirements for the collagenase and elastase activity of cathepsin K and its selective inhibition by an exosite inhibitor., J., Biochem. 465 (2015) 163–173. doi:10.1042/BJ20140809.

[24] E. Godat, V. Hervé-Grvépinet, F. Veillard, F. Lecaille, M. Belghazi, D. Brömme, G. Lalmanach, Regulation of cathepsin K activity by hydrogen peroxide., Biol. Chem. 389 (2008) 1123–6. doi:10.1515/BC.2008.109.

[25] K. Kim, S.G. Rhee, E.R. Stadtman, Nonenzymatic cleavage of proteins by reactive oxygen species generated by dithiothreitol and iron, J. Biol. Chem. 260 (1985) 15394–15397.

[26] R. Wünschiers, M. Jahn, D. Jahn, I. Schomburg, S. Peifer, E. Heinzle, H. Burtscher, J. Garbe, A. Steen, M. Schobert, D. Oesterhelt, J. Wachtveitl, A. Chang, Metabolism, in: Biochem. Pathways, John Wiley & Sons, Inc., Hoboken, NJ, USA, 2013: pp. 37–209. doi:10.1002/9781118657072.ch3.

[27] E.R. Stadtman, Mechanism and Biological Consequences, 9 (1990) 315–325.

[28] R. Lill, U. Mühlenhoff, Iron-sulfur protein biogenesis in eukaryotes: components and mechanisms., Annu. Rev. Cell Dev. Biol. 22 (2006) 457–86. doi:10.1146/annurev.cellbio.22.010305.104538.

[29] D.H. Baker, G.L. Czarnecki-Maulden, Pharmacologic role of cysteine in ameliorating or exacerbating mineral toxicities., J. Nutr. 117 (1987) 1003–10. http://www.ncbi.nlm.nih.gov/pubmed/3298579 (accessed January 8, 2017).

[30] A. Domsalla, M.F. Melzig, Occurrence and properties of proteases in plant latices, Planta Med. 74 (2008) 699–711. doi:10.1055/s-2008-1074530.

[31] M.L. Mei, L. Ito, Y. Cao, Q.L. Li, C.H. Chu, E.C.M. Lo, The inhibitory effects of silver diamine fluorides on cysteine cathepsins, J. Dent. 42 (2014) 329–335. doi:10.1016/j.jdent.2013.11.018.

[32] L.E.S. Netto, E.R. Stadtman, The Iron-Catalyzed Oxidation of Dithiothreitol Is a Biphasic Process: Hydrogen Peroxide Is Involved in the Initiation of a Free Radical Chain of Reactions, Arch. Biochem. Biophys. 333 (1996) 233–242. doi:10.1006/abbi.1996.0386.

[33] C.M.P. Vidal, W. Zhu, S. Manohar, B. Aydin, T.A. Keiderling, P.B. Messersmith, A.K. Bedran-Russo, Collagen-collagen interactions mediated by plant-derived proanthocyanidins: A spectroscopic and atomic force microscopy study, Acta Biomater. 41 (2016) 110–118. doi:10.1016/j.actbio.2016.05.026.

[34] N.N. Fathima, J.R. Rao, B.U. Nair, Effect of UV irradiation on the physico-chemical properties of iron crosslinked collagen, J. Photochem. Photobiol. B Biol. 105 (2011) 203–206. doi:10.1016/j.jphotobiol.2011.09.003.

[35] N.N. Fathima, M. Baias, B. Blumich, T. Ramasami, Structure and dynamics of water in native and tanned collagen fibers: Effect of crosslinking, Int. J. Biol.

Macromol. 47 (2010) 590–596. doi:10.1016/j.ijbiomac.2010.08.003.

[36] C.A. Miles, N.C. Avery, V. V Rodin, A.J. Bailey, The increase in denaturation temperature following cross-linking of collagen is caused by dehydration of the fibres., J. Mol. Biol. 346 (2005) 551–6. doi:10.1016/j.jmb.2004.12.001.

[37] Z. Li, Y. Yasuda, W. Li, M. Bogyo, N. Katz, R.E. Gordon, G.B. Fields, D. Brömme, Regulation of Collagenase Activities of Human Cathepsins by Glycosaminoglycans, J. Biol. Chem. 279 (2004) 5470–5479. doi:10.1074/jbc.M310349200.

[38] G. Orsini, A.R. Jr, A. Mazzoni, F. Nato, L. Manzoli, A. Putignano, R. To, D.I. Lenarda, L.E.O. Tjäderhane, L. Breschi, A review of the nature , role , and function of dentin non-collagenous proteins . Part 1 : proteoglycans and glycoproteins, Endod. Top. (2012) 1–18. doi:10.1111/j.1601-1546.2012.00268.x.

[39] L.E. Bertassoni, J.P.R. Orgel, O. Antipova, M. V. Swain, The dentin organic matrix - Limitations of restorative dentistry hidden on the nanometer scale, Acta Biomater. 8 (2012) 2419–2433. doi:10.1016/j.actbio.2012.02.022.

[40] J.P.R.O. Orgel, T.C. Irving, A. Miller, T.J. Wess, Microfibrillar structure of type I collagen in situ., Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 9001. doi:10.1073/pnas.0502718103.

[41] L.E. Bertassoni, M. V. Swain, 5323 Removal of Dentin Non-collagenous Structures Results in the Unraveling of Microfibril Bundles in Collagen Type I, Connect. Tissue Res. 8207 (2016) 03008207.2016.1235566. doi:10.1080/03008207.2016.1235566.

[42] P. Panwar, G. Lamour, N.C.W. Mackenzie, H. Yang, F. Ko, H. Li, D. Brömme, Changes in structural-mechanical properties and degradability of collagen during aging-associated modifications, J. Biol. Chem. 290 (2015) 23291–23306. doi:10.1074/jbc.M115.644310.

[43] M. Novinec, L. Kovacic, B. Lenarcic, A. Baici, Conformational flexibility and allosteric regulation of cathepsin K., Biochem. J. 429 (2010) 379–89. doi:10.1042/BJ20100337.

[44] D.L.S. Scheffel, J. Hebling, R.H. Scheffel, K.A. Agee, M. Cadenaro, G. Turco, L. Breschi, A. Mazzoni, C.A. De Souza Costa, D.H. Pashley, Stabilization of dentin matrix after cross-linking treatments, in vitro, Dent. Mater. 30 (2014) 227–233. doi:10.1016/j.dental.2013.11.007.

[45] A.K. Bedran-Russo, G.F. Pauli, S.-N. Chen, J. McAlpine, C.S. Castellan, R.S. Phansalkar, T.R. Aguiar, C.M.P. Vidal, J.G. Napotilano, J.-W. Nam, A.A. Leme, Dentin biomodification: strategies, renewable resources and clinical applications, Dent. Mater. 30 (2014) 62–76. doi:10.1016/j.dental.2013.10.012.

[46] D.L. Nelson, M.M. Cox, Lehninger Principles of Biochemistry, 6th ed., W.H. Freeman and Company, New York, 2012. https://www.amazon.com.br/Lehninger-Principles-Biochemistry-David-Nelson/dp/1429234148/ref=sr_1_sc_1?ie=UTF8&qid=1485207236&sr=8-1-spell&keywords=Lehninger+Principles+of+Biochemstry (accessed January 23, 2017).

2.2 Artigo 2

Effect of conditioning solutions containing ferric chloride on dentine bond strength and collagen degradation*

* Artigo submetido para publicação no periódico Dental Materials

Raquel Viana Rodrigues [a1], Marcelo Giannini [b], Fernanda Miori Pascon [c], Dieter Brömme [ade], Adriana Pigozzo Manso [a], Ricardo Marins de Carvalho [a]

Raquel Viana Rodrigues
[a] Department of Oral Biological and Medical Sciences, Faculty of Dentistry, University of Columbia, 368-2199 Wesbrook Mall Vancouver, BC V6T 1Z3 Canada. e-mail: rquelrodrigues@hotmail.com
[1] Present address - Department of Restorative Dentistry, Dental Materials Division, Piracicaba Dental School, UNICAMP. Av. Limeira, 901, CEP 13414903 Piracicaba, SP, Brazil. Tel.: +55 19 21065200; Fax: +55 19 34210144
Marcelo Giannini
[b] Department of Restorative Dentistry, Piracicaba Dental School, UNICAMP, Av. Limeira, 901, CEP 13414903 Piracicaba, SP, Brazil. Tel.: +55 19 21065200; Fax: +55 19 34210144/ e-mail: giannini@fop.unicamp.br
Fernanda Miori Pascon
[c] Department of Pediatric Dentistry, Piracicaba Dental School, UNICAMP, Av. Limeira, 901, CEP 13414903 Piracicaba, SP, Brazil. Tel.: +55 19 21065285; Fax: +55 19 34210144/ e-mail: fmpascon@fop.unicamp.br
Dieter Brömme
[a] Department of Oral Biological and Medical Sciences, Faculty of Dentistry, [d] Center for Blood Research, [e] Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of British Columbia, 2350 Health Sciences Mall, Vancouver, BC, V6T 1Z3 Canada. e-mail: dbromme@dentistry.ubc.ca
Adriana Pigozzo Manso
[a] Department of Oral Biological and Medical Sciences, Faculty of Dentistry, University of Columbia, 368-2199 Wesbrook Mall, Vancouver, BC V6T 1Z3 Canada.
Corresponding Author:
Ricardo Marins de Carvalho
[a] Department of Oral Biological and Medical Sciences, Faculty of Dentistry, University of Columbia, 368-2199 Wesbrook Mall, Vancouver, BC V6T 1Z3 Canada.
Phone: +1-604-827-0566/ e-mail: rickmc@dentistry.ubc.ca

ABSTRACT

Objectives - To investigate the effects of conditioning solutions containing ferric chloride ($FeCl_3$) on resin-dentine bondstrength; on protection of dentine collagen against enzymatic degradation and on cathepsin-K (CT-K) activity. Methods - Conditioning solutions were prepared combining citric acid (CA) and anhydrous ferric chloride ($FeCl_3$) in different concentrations. The solutions were applied to etch flat dentine surfaces followed by bonding with adhesive resin. Phosphoric acid (PA) gel etchant was used as control. The microtensile bond strength (μTBS) was tested after 24 hours and 9 months of storage in phosphate buffer saline. Dentine slabs were demineralized in 0.5M EDTA, pretreated or not with $FeCl_3$ and incubated with CT-K. The collagenase activity on dentine collagen matrix was examined and characterized by SEM. Additional demineralized dentine slabs were treated with the conditioning solutions, and the amount of Fe bound to collagen was determined by EDX. The activity of CT-K in the presence of $FeCl_3$ was monitored fluorimetrically. Data were analyzed by ANOVA followed by post-hoc tests as required (α = 5%). Results – Slightly higher bond strengths were obtained when dentine was conditioned with 5%CA/0.6% FeCl3 and 5%CA-1.8% $FeCl_3$ regardless of storage time. Bond strengths reduced significantly for all tested conditioners after 9 months of storage. Treating dentine with 1.8% $FeCl_3$ was effective to preserve the structure of collagen against CT-K. EDX analysis revealed binding of Fe-ions to dentine collagen after 15 seconds immersion of demineralized dentine slabs into $FeCl_3$ solutions. $FeCl_3$ at concentration of 0.08% was able to suppress CT-K activity. Significance – This study shows that $FeCl_3$ binds to collagen and offers protection against Cat-K degradation. Mixed solutions of CA and $FeCl_3$ may be used as

Keywords: dentin, cathepsins, ferric compounds, collagen

1 INTRODUCTION

In 1982, Nakabayashi et al. [1], proposed the used of 10-3 solution, containing 10% citric acid (CA) and 3% ferric chloride hydrate ($FeCl_3.6H_2O$) to prepare dentine for infiltrating resin monomers. According to reports, $FeCl_3$ was useful to preserve the exposed collagen structure against denaturation, which was a concern associated with the use of acids on collagen at that time [2–4]. Conversely, other authors suggested that $FeCl_3$ was important as an interfacial initiator for improved curing of the 4-META/MMA-TBB adhesive [5]. Despite the claims above, the precise definition of the benefits, or the role of $FeCl_3$ in resin-dentine bonding has never been clarified. Additionally, no investigation has followed to investigate any possible interaction of $FeCl_3$ with enzymatic activities in resin-dentine bonding. That was probably because evidences of the role of host-derived enzymes in resin-dentine bonding were not available at that time; and also because current use of such dentine conditioner (i.e., 10-3 solution) is limited to few products available, such as the resin cement Super Bond C & B (Sun Medical, Japan), thus catching little attention from researchers.

The degradation of collagen fibrils and hydrophilic components of adhesive resins are considered the determining factors of destruction of the hybrid layer and consequent reduction of bond strength to dentine over time [6]. The mineral removal by acid etching exposes collagen fibrils to degradation by two classes of proteolytic enzymes: metalloproteinases (MMPs) and cathepsins (CTs) [7–11]. Among the CTs present in dentin [12], Cathepsin-K (CT-K) deserves special attention for its ability to degrade collagen quickly [10]. The collagenolytic activity of CT-K is directed to the cleavage of the non-helical telopeptides of collagen and cleavages within the helical region, being the activity higher at an acid pH [13]. Thus, it is likely that CT-K could start the collagen degradation process once exposed by the etching procedure in a typical dentin bonding procedure. With time, both families of enzymes (MMPs and CTs) work together to degrade exposed collagen [14]. In concert, they exert a significant role in the degradation of exposed collagen in incompletely infiltrated hybrid layers [6,9,14,15], and also participate in the progression of dentinal caries and erosion [7–10,16–18].

Despite of the successful performance in the 90s, no further attention has been given to 10-3 solution by researchers or manufacturers. While being almost forgotten in Dentistry, the Fe-ion gained recent prominence in archaeological science, in studies investigating the preservation of type I collagen and other proteins

in fossils [19,20]. These studies suggest that the intriguing preservation of fossil soft tissues were a result of the action of ferric ions (likely from decaying red blood cells) interacting with collagen and making it resistant to the challenge of time. The researchers found that cross-linked type I collagen was present in fossil tissues of *Tyrannosaurus rex* in close association with iron nanocrystals [20,21].

It is known that various naturals and synthetic chemical products have the ability to increase the number of intramolecular cross-links in collagen [22], and increased cross-links in dentine collagen improve the mechanical properties and allows for potential protection against degradation [23]. Assuming the preservation of the collagen fibrils in the hybrid layer is essential for the preservation of the resin-dentine interface, the archaeological finding renewed interest in the study of agents based on iron and how it may play a role in preserving resin-dentin bonding against degradation. It has been observed, for instance, that iron showed inhibitory effect against MMP-2 and -9 activity [24,25]. We speculate that iron in conditioning solutions can interact with dentine collagen and offers protection against enzymatic degradation. We also speculate that Fe may also present inhibitory effect on CT-K. In concert, these mechanisms could inhibit collagenase activity and preserve collagen structure, thus preserving the resin-dentin bonds against degradation.

Therefore, the aim of this study was to evaluate the effects of conditioning solutions containing CA and/or $FeCl_3$ in different concentrations on long-term dentine bond strength and dentine collagen degradation. This study investigated the hypotheses that; (1) the use of conditioning solutions with $FeCl_3$ will result in stable resin-dentine bond strength over time; (2) that $FeCl_3$ can inhibit CT-K activity; and (3) that dentine collagen exposed to $FeCl_3$ will bind Fe-ions and be more resistant to degradation against the collagenase CT-K.

2 MATERIALS AND METHODS

2.1 Teeth

A total of 80 human caries-free third molars were used in this study. The study was approved by the local Ethics Board (approval # H15-02264). Seventy eight teeth were used for microtensile bond strength (μTBS) test and 2 teeth were used for collagen degradation and Fe-ion binding examination.

2.2 μTBS Test

2.2.1 Teeth Preparation

After extraction, the teeth were stored in tap water at 4°C for no longer than 3 months. They were scrapped to remove organic debris and pumiced with rubber cup (KG Sorensen, Barueri, SP, Brazil). The roots were sectioned 1 mm below the cementum/enamel junction and a parallel cut made to expose flat mid-coronal dentine surface using a diamond saw (EXTEC Corporation, Enfield, CT, USA) coupled to a cutting machine (Isomet 1000, Buehler Ltd., Lake Bluff, Il, USA). The surrounding enamel was removed by grinding on 180-grit SiC paper and the teeth were kept in tap water until used.

2.2.2 Preparation of Conditioning Solutions

The CA and/or $FeCl_3$ powder (Sigma/Aldrich Corporation, St. Louis, Missouri, USA) were dissolved in w/w% ratios in distilled water according to different concentration ratios (Table 1). The solutions were prepared from anhydrous $FeCl_3$ (Sigma/Aldrich Corporation, St. Louis, Missouri, USA). The concentration ratios of CA and $FeCl_3$ were chosen to be variants of similar solutions previously described in the literature as 10-3 and 1-1 [1,26]. For instance, the 1.8% $FeCl_3$ used in this study equals in Fe amount to that of 3% hydrated $FeCl_3$ ($FeCl_3.6H_2O$) used in previous studies (10-3 solution) [1–3]. The conditioning solutions were labeled and kept refrigerated in sealed vials until used. A commercially available 35% phosphoric acid gel (Select HV Etch, Bisco Inc., Schaumburg, IL, USA) was also used in the study. All conditioning solutions had their pH measured with a pH-meter (model mPA-210p, MS Tecnopon Equipamentos Especiais LTDA, SP, Brazil) (Table 1).

Table 1. Description of the experimental groups and composition of conditioning solutions used in the study

Experimental Groups (abbreviation)	Conditioning Solutions w/w (etching time)	pH
10-1.8	10% Citric Acid / 1.8% Ferric Chloride (15 seconds)	0.57
10-0.6	10% Citric Acid / 0.6% Ferric Chloride (15 seconds)	0.84
10-0.0	10% Citric Acid (15 seconds)	1.42
5-1.8	5% Citric Acid / 1.8% Ferric Chloride (15 seconds)	0.65
5-0.6	5% Citric Acid / 0.6% Ferric Chloride (15 seconds)	0.94
5-0	5% Citric Acid (15 seconds)	1.87
1-1.8	1% Citric Acid /1.8% Ferric Chloride (15 seconds)	0.85
1-0.6	1% Citric Acid / 0.6% Ferric Chloride (15 seconds)	0.98
1-0	1% Citric Acid (15 seconds)	1.96
0-1.8	1.8% Ferric Chloride (15 seconds)	1.58
0-0.6	0.6% Ferric Chloride (15 seconds)	1.80
PA5	35% Phosphoric Acid (5 seconds)	0.1-0.4
⊕ Control PA15	35% Phosphoric Acid (15 seconds)	0.1-0.4

⊕ Positive

2.2.3 Bonding Procedures

The dentine surface was polished with 320-grit SiC paper under water-cooling for 10 seconds to standardize the smear layer. The surface was rinsed with water and air-dried to remove excess water without desiccating the surface. The crown segments were randomly allocated to 13 groups (Table 1).

Twenty μL of each conditioning solution were dispensed to the dentine surface followed by spreading with disposable applicators (Vigodent, Rio de Janeiro, RJ, Brazil) for 15 seconds. The surface was then rinsed for 15 seconds with distilled water and blot/dried with tissue paper (Kimwipes, Kimberly-Clark Global Sales, Inc, GA, USA), keeping the surface visibly moist. This procedure was reproduced for all groups, except for the PA5 and PA15 groups, in which the phosphoric acid was applied for 5 and 15 seconds, respectively (see Table 1).

The Adper Scotchbond Multi-Purpose adhesive system (3M ESPE, St. Paul, MN, USA) was applied according to the manufacturer's recommendations to all groups, and light/cured for 20 seconds at 1,200 mW/cm^2 using Bluephase G2 (Ivoclar Vivadent, Schaan, Liechtenstein). After bonding, 4 mm composite (Aelite™ All/Purpose Body, Bisco Inc., Schaumburg, IL, USA) block was incrementally built-up. Each 2mm increment was light-cured for 40 seconds using Bluephase G2.

2.2.4 Specimens Preparation for μTBS Test

The bonded teeth were stored in water at 37°C for 24 hours and sectioned perpendicular to the adhesive-dentine interface (Isomet 1000, Buehler Ltd., Lake Bluff, IL, USA) with double/sided diamond saw (EXTEC Corporation, Enfield, CT, USA) under water cooling to obtain beams of approximately 1 mm^2 of cross-sectional area. Half of the beams obtained from each tooth were randomly selected and tested immediately after sectioning, while the remaining were kept in a sealed vial containing Phosphate Buffered Saline (PBS, Sigma Aldrich, St. Louis, MO, USA) at 37°C for nine months. The PBS was renewed monthly and preservatives and/or antimicrobial agents were not added to the PBS in this study.

2.2.5 Bond Strength Test

Bonded beams were mounted on a microtensile testing jig (Odeme Dental Research, Luzerna, SC, Brazil) with cyanoacrylate glue (Super Glue Gel Control, Loctite, Mississauga, ON, Canada) and tested until failure at 1.0 mm/min. The load (N) at failure was divided by the cross-sectional area of the beam (mm^2) measured with a digital caliper to the nearest 0.01 mm (Fisher Scientific, Chicago, IL, USA) to calculate the microtensile bond strength that was expressed in MPa. Beams were carefully removed from the jig, and failure modes were evaluated under 40X using light microscope (Olympus, Tokyo, Japan).

2.2.6 Failure Mode Analysis

Fractures were classified as cohesive, adhesive or mixed. When it occurred exclusively in either dentine or composite, it was classified as cohesive in dentine (CD) or cohesive in composite resin (CC); when occurred at dentin/resin bonded interface as adhesive (A), and mixed (M) when two modes of failures occurred simultaneously. To observe the ultrastructure of the failing sites, three selected beams of each failure mode were examined at 80X and 3000X under Scanning Electron Microscopy (SEM) (JSM/5600LV, JEOL, Tokyo, Japan) operating at 15 kV. For that, the beams were positioned side-by-side on metallic stub with carbon tape, dried overnight in a desiccator and sputter-coated with gold (MED 010 Baltec, Balzers, Leichtenstein).

2.3 Dentine Slabs Preparation for EDX and SEM analysis of collagen

The roots of two teeth were transversally cut 1 mm below the cementum-enamel junction and then coronally to expose mid-coronal dentin. The resultant tooth segment was fixed in an acrylic base with wax and sectioned perpendicularly in both X and Y directions to obtain rectangular dentine slabs (2 mm x 3 mm x 0.5 mm) using the same cutting machine under water cooling. The dentine slabs were then demineralized in 0.5 M ethylenediaminetetraacetic acid (EDTA) for 3 days followed by rinsing with distilled water for 2 hours. A total of 42 demineralized dentine slabs were obtained and randomly divided into two groups, one for energy-dispersive X-ray spectroscopy (*EDX)* analysi*s* of iron binding to collagen (n=27) and another (n=15) for SEM examination of dentine collagen surface features after exposure to CT-K (Fig. 1).

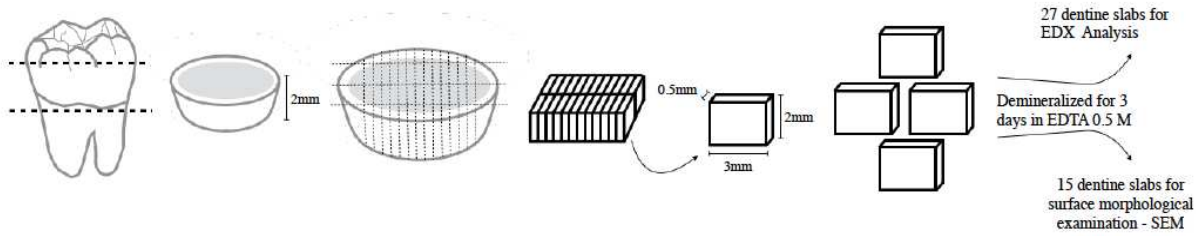


Fig.1. Schematic of specimen preparation for EDX and collagen degradation experiments. Two teeth were used to produce 42 dentine slabs.

2.3.1 EDX Analysis of Fe-ion binding to collagen

Twenty seven dentine slabs were divided into nine groups. Three dentine slabs were used for each of the nine conditioning solutions: 10% CA (as negative control, no $FeCl_3$); 10% CA-1.8% $FeCl_3$; 10% CA-0.6% $FeCl_3$; 5% CA-1.8 %$FeCl_3$; 5% CA-0.6% $FeCl_3$; 1% CA-1.8% $FeCl_3$; 1% CA-0.6% $FeCl_3$; 1.8% $FeCl_3$; and 0.6% $FeCl_3$. The slabs were immersed for 15 seconds in the conditioning solution followed by immersion in distilled water for 15 seconds. This procedure was done to mimic the exposure time of conditioning and rinsing of a typical etch-and-rinse bonding approach. The specimens were then dried at 37°C in a desiccator with silica gel for 24 hours. Afterwards, the slabs were fixed on acrylic stubs with carbon tape and sputter-coated with carbon (MED 010 Baltec, Balzers, Liechtenstein) prior to EDX. Spectrometry analysis (Vantage, NORAN Instruments, Middleton, WI, USA) coupled

to a SEM (JEOL, JSM/5600LV, Tokyo, Japan) was performed in order to identify the elements and quantify the Fe weight percent. Each specimen was examined at 400X and selected areas of approximately 490 μm^2 were scanned for elements. Each spectrum was acquired for 100 seconds (voltage 15 kV, working distance 20 mm). Quantitative data were obtained from 10 analyzed areas of each treatment group and averaged for analysis.

2.3.2 SEM analysis of collagen exposed to CT-K

Fifteen demineralized dentine slabs were divided into 5 groups. Three slabs per group were immersed for 15 seconds in either 0.06% $FeCl_3$; 0.08% $FeCl_3$ or 1.8% $FeCl_3$; and rinsed for 15 seconds with distilled water before incubation with CT-K. Two additional control groups were created. One was exposed to neither $FeCl_3$ solutions nor incubation with CT-K; the other was not exposed to $FeCl_3$ solutions, but was exposed to CT-K incubation. After exposure to the conditioning solutions, exposed and control specimens were dried at 37°C with silica gel in a desiccator for 24 hours before being prepared for CT-K incubation. We used recombinant, human CT-K that was prepared as previously described elsewhere [27].

The dried specimens were weighted and incubated at a ratio of 1 mg collagen/ 1 μM CT-K in 50 μl of activity buffer (100 mM sodium acetate buffer, pH 5.5, containing 2.5 mM DTT (Dithiothreitol) and EDTA) for 20 hours at 28°C. The reaction was stopped by the addition of 10 μM E-64 (carboxy-trans-2,3-epoxypropionyl-leucylamido-(4-guanidino)). After centrifuging, the specimens were removed from solution, rinsed with water, fixed in 2.5% glutaraldehyde, rinsed with water five times, dehydrated in ascending ethanol and dried with HMDS (hexamethyl di-silizane) before taken to sputter-coating with gold (MED 010 Baltec, Balzers, Leichtenstein) prior to SEM (JEOL, JSM/5600LV, Tokyo, Japan) observation [28]. Micrographs were taken (voltage 15 kV) from randomly selected areas of the collagen matrix surface to assess the morphological changes using magnifications of 2,000x, 6,000x and 15,000x.

2.4 Effect of $FeCl_3$ on CT-K activity

CT-K activity was monitored with fluorimetric analysis (Luminescence Spectrometer, PerkinElmer, UK) by the degradation of Z-FR-AMC (Carbobenzoxy-

phenylalanine-arginine-7 amino-4-methylcoumarin; Weil am Rhein, Germany) substrate in the presence of $FeCl_3$ at 0.005%; 0.01%; 0.02%; 0.04%; 0.08% concentrations. Solutions were prepared by adding 5 µL of 2 mM Z-FR-MCA dissolved in DMSO (Dimethyl sulfoxide) to 990 µL of acetate buffer (100 mM sodium acetate buffer, pH 5.5, containing 2.5 mM DTT and EDTA) containing or not $FeCl_3$ at the above concentrations. The reaction was initiated by adding 5 µL of CT-K at a final concentration of 5 nM to the solution and the hydrolysis rate of the Z-FR-AMC substrate was monitored by measuring the rate of increase in fluorescence as a function of time (30 seconds). Control assays were performed without $FeCl_3$ and it was considered the total hydrolysis of fluorescent substrate (100% enzymatic activity) against which the tested solutions were compared. The percentage of substrate degradation was plotted against the different concentrations of $FeCl_3$ and analyzed by best-fit regression analysis. The experiments were conducted in triplicate.

.*2.6 Statistical Analysis*

Bond strength data were analyzed by 2-Way ANOVA (conditioning solutions vs. storage time) followed by Holm/Sidak *post hoc* multiple comparisons. Significance level was pre-set to $\alpha = 5\%$. Standard error of the mean (SEM) was given by Least-square means (LSM) analysis. (SigmaPlot 11, Systat Software Inc.). For EDX data (Fe-ion weight%), One-Way ANOVA followed by all pairwise multiple comparison procedures (Fisher) under grouping information using LSD (Least Significant Difference) method was used. Non-linear regression analysis was used to investigate the relationship between $FeCl_3$ concentration and CT-K activity. All statistical analyses were done at 95% of confidence.

3 RESULTS

Two-way ANOVA indicated significant effect of conditioning solutions ($F= 18.231$, $p<0.001$) and storage time ($F= 277.652$, $p<0.001$) on bond strength, but the interaction of factors was not significant ($F= 1.456$, $p = 0.135$). Bond strength values were significantly higher at 24 hours than those obtained after 9 months storage for all conditioning solutions (Fig. 2). By looking at the different conditioning solutions within each storage period, no clear trend was observed, except that consistently higher bond strengths were observed for 5-0.6 and 5-1.8 for both storage periods,

being the values for these groups significantly higher than PA15 (control); and that 0-1.8 presented the lowest bond strength of all in both storage periods (Fig. 2). Meanwhile, 10-1.8; 10- 0.6; 10-0; 1-0.6; 5-0; 0-0.6; PA5 did not significantly differ from PA15 (control), and the solutions with the lower or no CA concentrations (i.e., 0-1.8, 1-0 and 1-1.8) all presented the lowest bond strength values recorded in both storage periods (Fig. 2).

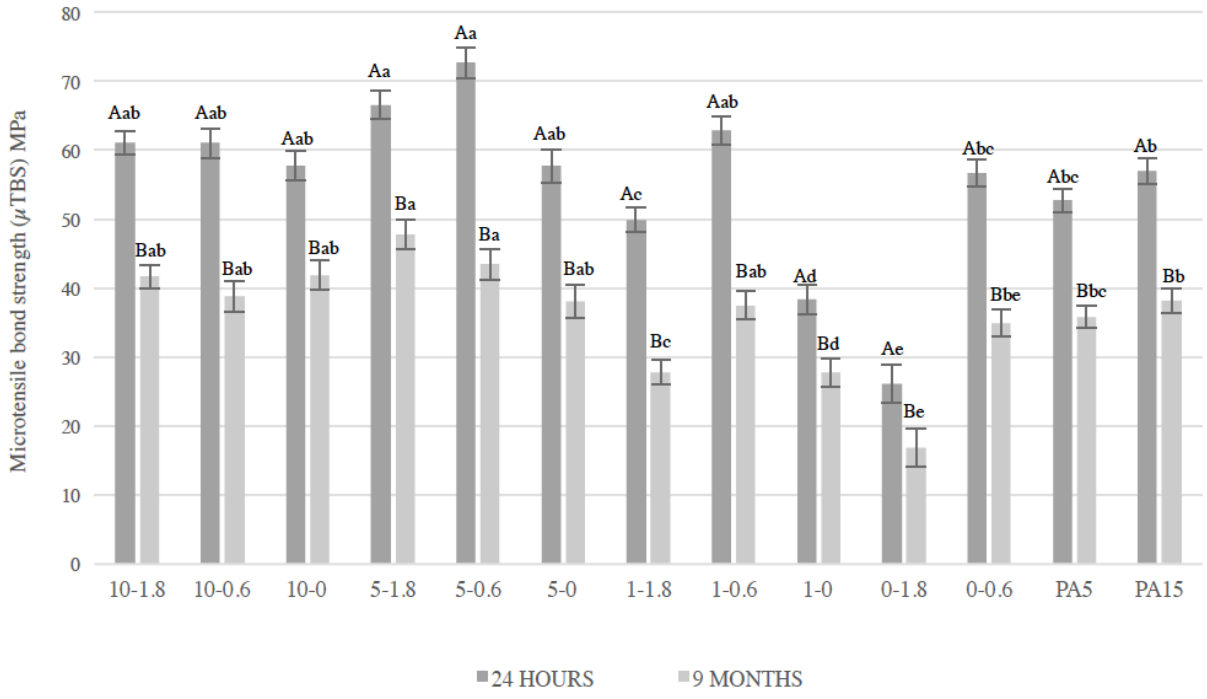


Fig. 2. Means and standard error (error bars) of dentine bond strength (MPa) at 24 hours and 9 months. Different uppercase letters indicate statistic difference between the different storage times for each solution ($p<0.0001$). Different lowercase letters indicate the statistic difference between the solutions within the same storage time ($p<0.0001$).

Scanning Electron Microscopy (SEM) analysis of failure mode reveled that the most of failures were adhesive and mixed in all groups, regardless of storage time (Fig. 3A). The solutions with 0% CA (i.e. 0-0.6 and 0-1.8) and the solution with 1%CA and no $FeCl_3$ (i.e., 1-0) presented relatively higher incidence of adhesive failures when compared to the other groups. These groups are also amongst the lowest bond strength values observed for both periods. Representative SEM images (Fig. 3B)

illustrate the failure mode of selected specimens. Mixed (Fig. 3B, c and d) and adhesive failures (Fig. 3B, a and b) together represented up to 60% of all failures for all groups in both storage times (Fig. 3A).

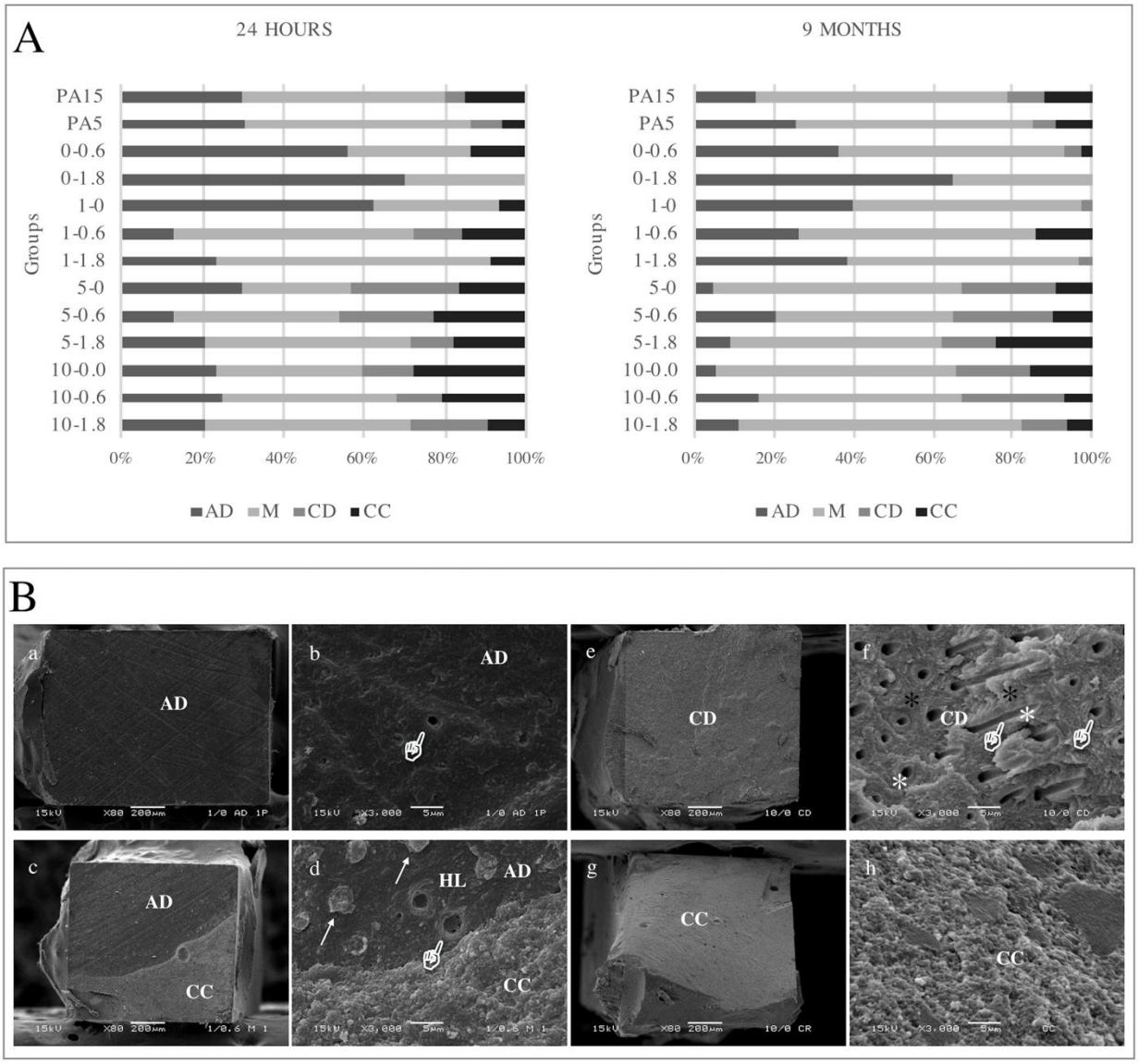


Fig. 3. A: Distribution (in percentage) of failure modes for all groups. Cohesive fractures within dentine (CD), cohesive within composite resin (CC), adhesive (AD) that occurred at dentin-resin bonded interface, and mixed (M) when two modes of failures, adhesive and cohesive occurred simultaneously; B: Representative SEM images of fractured beams with different failure modes. Pairs represent different failure mode (80X/3000X): a/b = Adhesive; c/d = Mixed (interfacial + cohesive in composite resin or dentine); e/f =Cohesive in dentine; g/h = cohesive in composite resin. (AD) adhesive resin; (HL) hybrid layer; (CC) composite resin; (white arrows) dentinal tubules filled by adhesive resin tags; (white asterisk) peritubular dentin; (black asterisk) intertubular dentin; (pointer) dentinal tubule.

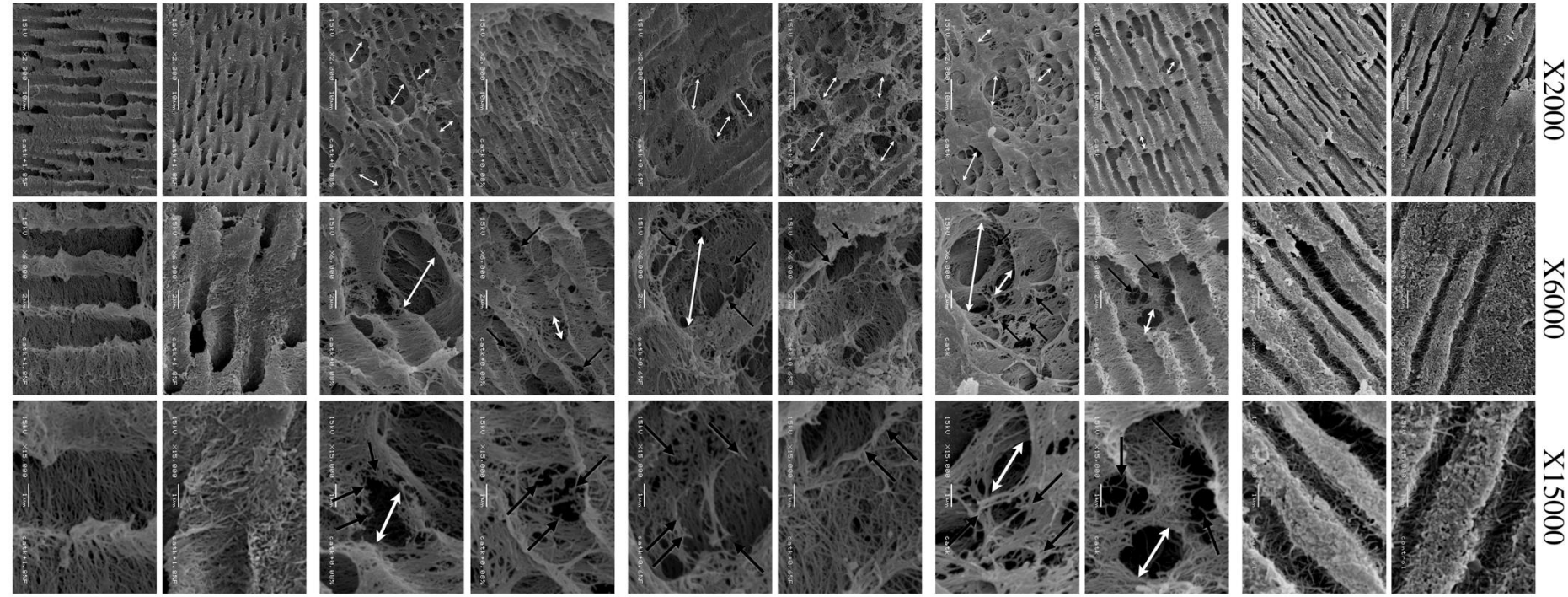

X2000
X6000
X15000
T-1.8% FeCl3
T-0.08% FeCl3
T-0.06% FeCl3
T-0
Control
Cat-k Incubation

Fig. 4. Representative SEM micrographs of the morphological features of the dentine collagen matrix after CT-K challenge. Two different areas per group are presented in three different magnifications. Control (no FeCl3 and no CT-K) images show the typical aspect of the EDTA-demineralized dentine with the absence of the peritubular dentine along the dentinal tubules and the exposure of collagen fibrils. When dentine matrix was exposed to CT-K without pre-treatment with FeCl3, visible signs of surface degradation could be observed. Several pores (white arrows) originated on the surface and disappearance of the intertubular collagen was readily observed in all specimens. Rupture of individual fibrils could be spotted (black arrows) and the consequent disruption of the intertubular dentine matrix resulting in merging of dentinal tubules into enlarged orifices. The overall appearance suggested a progressive process of destruction of the collagen matrix. Similar features of collagen matrix degradation were observed when the

specimens were treated with either 0.06% or 0.08% FeCl3 and challenged with CT-K, thus indicating a lack of protection against enzymatic activity. Conversely, morphological

features appeared intact and similar to control when specimens were pre-treated with 1.8% FeCl3 prior to CT-K. This suggested a protective effect against the enzymatic action on the fibrils.

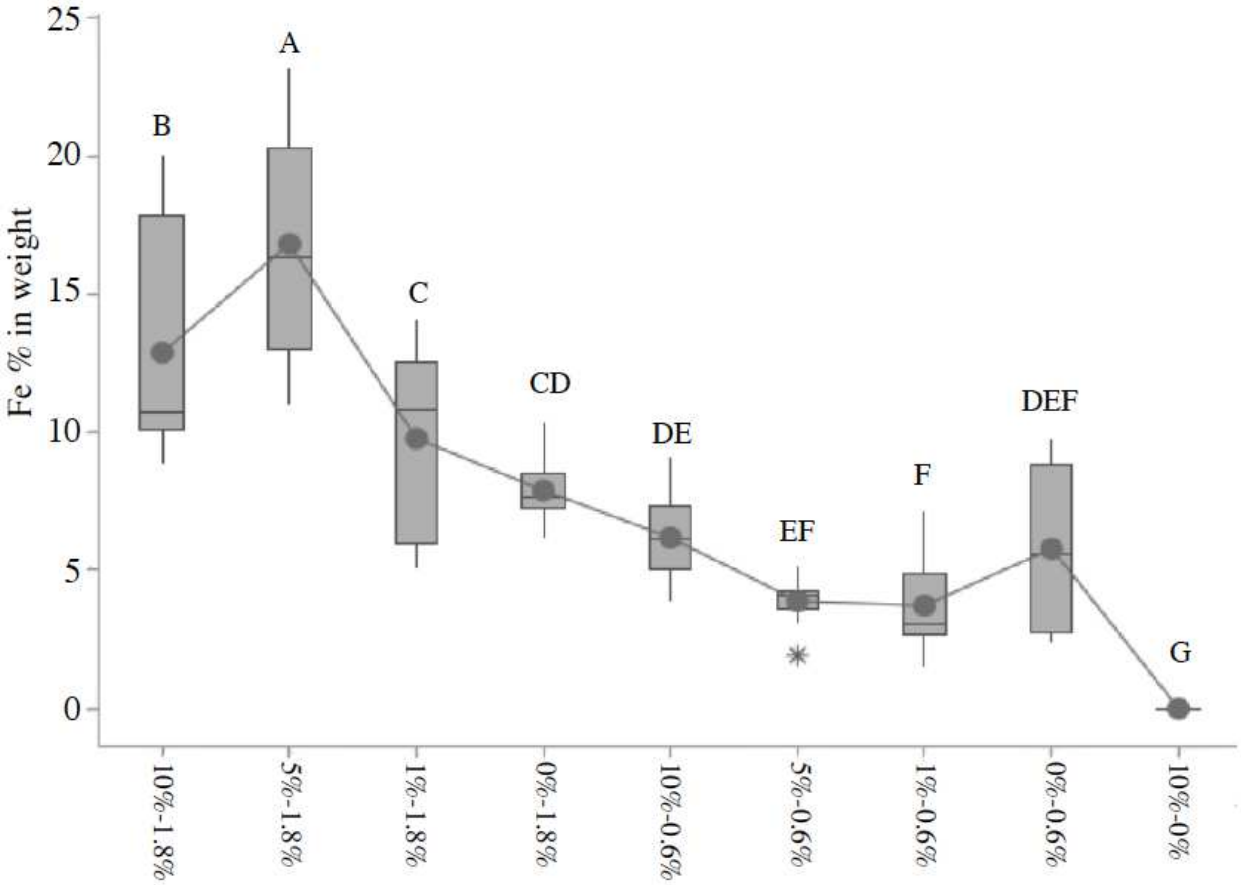


Fig. 5. Boxplot of the weight % of Fe-ion detected on the surface of dentine samples after immersion in the conditioning solutions. Different uppercase letters indicate statistically significant differences (p<005).

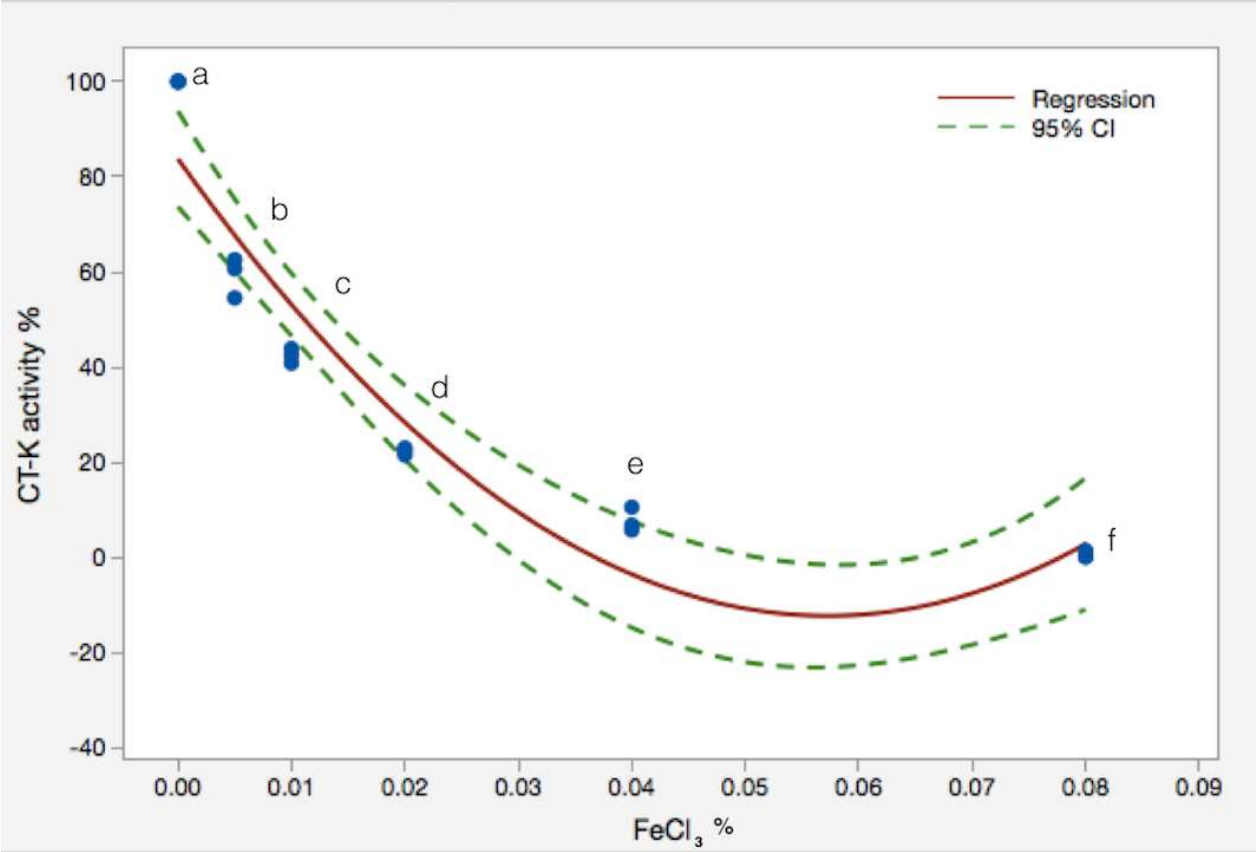


Fig. 6. Relationship between FeCl3 concentration and CT-K activity. There was a significant reduction of CT-K activity as the concentration of FeCl3 increased in the solution (R2 = 0.907; P< 0.0001). CT-K was suppressed when FeCl3 reached 0.08% concentration in solution.

Loss of the original dentinal collagen scaffold morphology, including the presence of enlarged holes and segmented dentine matrix were observed when the specimen was exposed to CT-K in the absence of $FeCl_3$ (Fig. 4, T-0). Similar features were also observed in specimens pre-treated with low iron content 0.06% and 0.08% $FeCl_3$ solutions. However, the morphology of the collagen matrix was maintained similar to the control group (not exposed to CT-K) when the samples were pre-treated with 1.8% $FeCl_3$ prior to CT-K exposure (Fig. 4).

EDX analysis showed that 15 seconds immersion in $FeCl_3$ -containing solutions was effective to result in iron binding to the collagen matrix (Fig. 5). The presence of quantifiable iron concentrations could be observed in all groups and it varied according to the ratio of CA and $FeCl_3$ in each solution. In general, higher iron binding was observed with the solutions with higher concentration of $FeCl_3$. Although not significant, there was a trend that, within each concentration of $FeCl_3$, higher

concentration of CA in the solution resulted in higher amount of iron bound to the surface. The highest concentration of iron was observed with the 5% CA / 1.8 $FeCl_3$ solution ($p<0.05$), and no iron was detected on the surface treated with 10% CA alone.

Regression analysis indicated that increasing $FeCl_3$ concentrations in solution (0-0.08%) resulted in significant reductions ($R^2 = 0.907$; $p < 0.001$) of CT-K activity (Fig. 6). At 0.08%, $FeCl_3$ was able to completely inhibit CT-K activity.

4 DISCUSSION

Inspired by the archaeological findings that iron was responsible to preserve soft tissues in fossils [19–21], this study aimed to revisit the role of $FeCl_3$ in resin-dentin bonding. Important to this approach was the demonstration that conditioning solutions containing CA and $FeCl_3$ resulted in iron binding to the collagen matrix after an exposure time of 15 seconds followed by 15 seconds rinsing that simulates a typical etch-&-rinse procedure. Not surprisingly, conditioning solutions with higher $FeCl_3$ content resulted in increased Fe binding to collagen. The contribution of CA % in the mixture on the ability of iron binding to collagen remains unknown. Different concentrations of CA in the solutions did not seem to have a significant impact on Fe-binding when the lower concentration of $FeCl_3$ (i.e. 0.6%) was used. Some effect of CA concentration was observed when $FeCl_3$ was present at 1.8% (Fig. 5). We speculate that the influence of CA concentration is more related to the increased ability of CA to better dissolve and disperse the highest concentrated 1.8% $FeCl_3$ into solution, thus yielding more Fe-ions to bind to collagen. Apart from the mechanism involved, it was clear that the presence of CA at either 5% or 10% in the solution resulted in the most effective binding of iron to collagen. Saeki et al. [29] reported that dentine treated with solutions containing more than 1% $FeCl_3.6H_2O$ were more capable to re-expand when rehydrated after being air-dried, recovering more completely the original dimension than when treated with solutions containing less than 1% $FeCl_3.6H_2O$. That fact suggested a distinct interaction of $FeCl_3$ with collagen that promoted the integrity of the structure. It has been demonstrated that iron can result in cross-linking of collagen [30,31]. This fact was reported to be responsible for the preservation of soft tissues in fossils [19–21]. Fe^{3+} and Fe^{2+} act on lysine and hydroxylysine producing aldehyde groups that will eventually undergo covalent bonding among tropocollagen molecules [31]. Chemical cross-linking of collagen

improves its structural stability, increases mechanical properties and makes it more resistant to degradation [22,31–34].

In this study, we did not attempt to determine whether there was an increase in collagen cross-link caused by $FeCl_3$. However, it became evident from our SEM observations that the pre-treatment of dentine with 1.8% $FeCl_3$ resulted in preservation of the morphological features of the dentine collagen matrix after exposure to CT-K challenge (Fig. 4). The resistance of the dentine collagen structure treated with 1.8% $FeCl_3$ against the potent collagenase CT-K was remarkable, and it is plausible to assume that Fe-ions may have increased the cross-linking of collagen and turned it resistant to the action of CT-K. CT-K is regulated by sulphated glycosaminoglycans (GAG) when connected with exosites [40]. It is possible that cross-linking agents such as Fe-ion alter the GAG-CK-T exosite interaction, changing its conformation and consequently inactivate the catalytic function. Cross-linking also decreases enzymatic degradation by altering the enzyme binding site on the collagen molecule or by binding to the exosite of the enzyme [35–39]. There must be a threshold concentration of $FeCl_3$ necessary to protect dentine collagen against CT-K and that should be above 0.08% $FeCl_3$ according to our findings. It is assumed that the protection provided by increased cross-linking of collagen should be permanent and make the structure resistant to several other potential agents of degradation, as demonstrated with the preserved soft tissues in fossils [20,21].

In addition to the direct protection of the collagen structure, we also demonstrated that $FeCl_3$ is an effective inhibitor of CT-K activity at concentrations as low as 0.08% (Fig. 6). Dentine collagen fibrils are organized in a hierarchical order starting from tropocollagen units, where five adjacent triple/helical molecules form a microfibril, which are bundled into fibrils measuring about 100 nm in diameter [40]. Thus, the morphology of collagen is considered a sequential arrangement of fibrils, which are hold together by proteoglycans (PG) and cross-links [40–44]. When the degradation occurs by the action of CT-K, PGs are removed [28] and as a consequence the collagen microfibril bundles unravel [42] and tropocollagen fragments are released after CT-K-mediated cleavage in the N- and C-terminal helical regions of collagen [45]. Thiol-dependent cathepsins, such as CT-K, have a cysteine residue in their active site, which is prone to oxidation [46]. As Fe-ions generate reactive oxygen species, they could cause oxidation of the thiol groups of cysteine residues [47–49] and result in reduced activity of the enzyme to the point of

irreversible and almost complete inhibition of CT-K at concentrations of 0.08% of $FeCl_3$. Together, the ability of Fe-ions derived from $FeCl_3$ to bind to dentine collagen, protects against CT-K digestion and they act as an inhibitor of CT-K activity. This may make Fe-ions a desirable component of conditioning solutions to treat dentin prior to bonding. These findings support acceptance of hypotheses 2 and 3.

Although the findings of this study support the potential benefits of $FeCl_3$ to the preservation of collagen against degradation, such effects did not translate into stability of resin-dentin bond strengths after 9 months of storage. Bond strengths were significantly reduced for all groups after storage (Fig. 2), thus indicating that $FeCl_3$ at either 0.6% or 1.8% was not effective to make bonds stable over time. This requires rejection of hypothesis 1. The overall reduction of bond strengths can be attributed to the degradation of the adhesive polymer as a supplementary mechanism of degradation of resin-dentin interfaces [6]. In that realm, it is desirable to increase the degree of conversion of adhesives to improve their resistance to water sorption and consequent hydrolysis. This could, in turn, increase the durability of bonds [35]. $FeCl_3$ has been suggested to be a promoter of polymerization [5,50,51]. This hypothesis is based on the ability of Fe^{3+} and Fe^{2+} ions to act as a reducing agent and when in contact with an initiator, the polymerization can be initiated and may be accelerated [5,50]. These possibilities, however, have been demonstrated using a 4-META/MMA-TBB adhesive system, which relies on chemical curing only and differs significantly from the adhesive used in this study. It is unknown if $FeCl_3$ has had any effect on the curing and properties of the adhesive used in this study.

A general observation from the bond strength data indicate that conditioning solutions of CA- $FeCl_3$ mixes are acceptable alternatives to traditional PA etchants. CA seems to be an important component in the solution, particularly when the $FeCl_3$ concentration is high (i.e., 1.8%). It was noted in the preparation that 1.8% $FeCl_3$ solutions were less soluble in water or in 1%CA. At higher CA concentrations, $FeCl_3$ readily dissolved. The findings also showed that 5% CA seems to be an adequate concentration to mix with $FeCl_3$ at either 0.6% or 1.8% where maximum bond strengths were observed for these solutions in both periods when compared with the control PA15. Etching dentine with PA has been shown to promote the MMP-mediated release of C-terminal telopeptides (ICTP) and the release of the CT-K-specific C-terminal peptide (CTX) fragments [52]. Thus, when exposed to

demineralizing etchants, collagen fibrils become more vulnerable to the subsequent attack of proteolytic enzymes. However, based on the findings of this study, the presence of $FeCl_3$ may immediately protect collagen structure by binding to its surface and inducing cross-linking [30,31] while simultaneously acting to inhibit CT-K and MMPs [24,25]. Since we expected that CA-$FeCl_3$ solutions would present a lower etching capacity than PA (see pH values, Table1), we shortened the etching time of PA from 15 seconds to 5 seconds to produce less demineralization of the dentine surface. It was interesting to note that etching with PA for only 5 seconds resulted in bond strength values that were not different from the control PA for 15 seconds and from most of the experimental solutions used in the study. This suggests that weaker etchants or shorter application time of PA can produce outcomes of bond strength that are similar to those obtained with typical PA for 15 seconds. This is worth to consider for future studies.

In summary, the results of this study demonstrate the effects of $FeCl_3$ on the protection of the dentine collagen structure against the strong CT-K collagenase activity and its inhibitory effect on the enzyme. The findings add knowledge and clarify the long-standing question of the role of $FeCl_3$ on resin-dentin bonding. Based on our findings, we believe that the use of $FeCl_3$ in dentine conditioning solutions is a viable option to incorporate potential benefits of protection of collagen against the degradation caused by collagenases. Further studies are warranted to investigate the role of $FeCl_3$ on the polymerization of current adhesive systems.

5 CONCLUSION

With few exceptions, the bond strength of the experimental solutions were not significantly different among each other and from the control group PA15. The bond strength of all tested solutions significantly reduced after storage in PBS for 9 months. $FeCl_3$ at concentrations as low as 0.08% presented effective inhibition of CT-K. Fe-ions can bind to dentine collagen from solutions containing different concentrations of $FeCl_3$ and CA. At 1.8%, $FeCl_3$ was capable of protecting dentine collagen against degradation promoted by CT-K.

Conflict of interest

All authors declare no financial and personal conflict of interest.

Acknowledgements

This study was partially supported by: Coordination for the Improvement of Higher Education Personnel (Capes # 1777-2014 and 999990107), National Counsel of Technological and Scientific Development (CNPq # 30217-2014-0), UBC Start-Up research funding to RMC and AMP, and by Canadian Institutes of Health Research Grants MOP-8994 (DB) and a Canada Research Chair award (DB).

6 REFERENCES

[1] Nakabayashi N, Kojima K, Masuhara E. The promotion of adhesion by the infiltration of monomers into tooth substrates. J Biomed Mater Res 1982;16:265–73. doi:10.1002/jbm.820160307.

[2] Nakabayashi N, Watanabe A, Gendusa NJ. Dentin adhesion of “modified” 4-META/MMA-TBB resin: function of HEMA. Dent Mater 1992;8:259–64. doi:10.1016/0109-5641(92)90096-U.

[3] Nakabayashi N, Takarada K. Effect of HEMA on bonding to dentin. Dent Mater 1992;8:125–30. doi:10.1016/0109-5641(92)90067-M.

[4] Nakabayashi N, Hiranuma K. Effect of etchant variation on wet and dry dentin bonding primed with 4-META/acetone. Dent Mater 2000;16:274–9. doi:10.1016/S0109-5641(00)00017-8.

[5] Taira Y, Matsumura H, Yoshida K, Tanaka T, Atsuta M. Adhesive bonding to dentin with ferrous chloride primers and tri-n-butylborane-initiated luting agents. J Dent Res 1996;75:1859–64.

[6] Tjäderhane L. Dentin Bonding: Can We Make it Last? Oper Dent 2015;40:4–18. doi:10.2341/14-095-BL.

[7] Tjäderhane L, Buzalaf MAR, Carrilho M, Chaussain C. Matrix metalloproteinases and other matrix proteinases in relation to cariology: The era of “dentin degradomics.” Caries Res 2015;49:193–208. doi:10.1159/000363582.

[8] Shimada Y, Ichinose S, Sadr A, Burrow MF, Tagami J. Localization of matrix metalloproteinases (MMPs-2, 8, 9 and 20) in normal and carious dentine. Aust Dent J 2009;54:347–54. doi:10.1111/j.1834-7819.2009.01161.x.

[9] Mazzoni a., Nascimento FD, Carrilho M, Tersariol I, Papa V, Tjaderhane L, et al. MMP Activity in the Hybrid Layer Detected with in situ Zymography. J Dent Res 2012;91:467–72. doi:10.1177/0022034512439210.

[10] Vidal C, Tjäderhane L, Scaffa P, Tersariol I, Pashley D, Nader H, et al. Abundance of MMPs and cysteine cathepsins in caries-affected dentin. J Dent Res 2014:269–74. doi:10.1177/0022034513516979.

[11] Tersariol IL, Geraldeli S, Minciotti CL, Nascimento FD, Pääkkönen V, Martins MT, et al. Cysteine Cathepsins in Human Dentin-Pulp Complex. J Endod 2010;36:475–81. doi:10.1016/j.joen.2009.12.034.

[12] Vidal CMP, Tjaderhane L, Scaffa PM, Tersariol IL, Pashley D, Nader HB, et al.

Abundance of MMPs and Cysteine Cathepsins in Caries-affected Dentin. J Dent Res 2014;93:269–74. doi:10.1177/0022034513516979.

[13] Garnero P, Borel O, Byrjalsen I, Ferreras M, Drake FH, McQueney MS, et al. The collagenolytic activity of cathepsin K is unique among mammalian proteinases. J Biol Chem 1998;273:32347–52. doi:10.1074/jbc.273.48.32347.

[14] Tjäderhane L, Nascimento FD, Breschi L, Mazzoni A, Tersariol ILS, Geraldeli S, et al. Optimizing dentin bond durability: Control of collagen degradation by matrix metalloproteinases and cysteine cathepsins. Dent Mater 2013;29:116–35. doi:10.1016/j.dental.2012.08.004.

[15] Tjäderhane L, Nascimento FD, Breschi L, Mazzoni A, Tersariol ILS, Geraldeli S, et al. Strategies to prevent hydrolytic degradation of the hybrid layer - A review. Dent Mater 2013;29:999–1011. doi:10.1016/j.dental.2013.07.016.

[16] Buzalaf MAR, Charone S, Tjäderhane L. Role of host-derived proteinases in dentine caries and erosion. Caries Res 2015;49 Suppl 1:30–7. doi:10.1159/000380885.

[17] Zarella BL, Cardoso CAB, Pelá VT, Kato MT, Tjäderhane L, Buzalaf MAR. The role of matrix metalloproteinases and cysteine-cathepsins on the progression of dentine erosion. Arch Oral Biol 2015;60:1340–5. doi:10.1016/j.archoralbio.2015.06.011.

[18] Nascimento FD, Minciotti CL, Geraldeli S, Carrilho MR, Pashley DH, Tay FR, et al. Cysteine cathepsins in human carious dentin. J Dent Res 2011;90:506–11. doi:10.1177/0022034510391906.

[19] San Antonio JD, Schweitzer MH, Jensen ST, Kalluri R, Buckley M, Orgel JPRO. Dinosaur peptides suggest mechanisms of protein survival. PLoS One 2011;6. doi:10.1371/journal.pone.0020381.

[20] Schweitzer MH, Zheng W, Cleland TP, Goodwin MB, Boatman E, Theil E, et al. A role for iron and oxygen chemistry in preserving soft tissues, cells and molecules from deep time. Proc Biol Sci 2014;281:20132741. doi:10.1098/rspb.2013.2741.

[21] Boatman EM, Goodwin MB, Holman H-Y, Fakra S, Schweitzer MH, Gronsky R, et al. Synchrotron Chemical and Structural Analysis of Tyrannosaurus rex Blood Vessels: The Contribution of Collagen Hypercrosslinking to Tissue Longevity. Microsc Microanal 2014;20:1430–1. doi:10.1017/S1431927614008885.

[22] Vidal CMP, Zhu W, Manohar S, Aydin B, Keiderling TA, Messersmith PB, et al. Collagen-collagen interactions mediated by plant-derived proanthocyanidins: A spectroscopic and atomic force microscopy study. Acta Biomater 2016;41:110–8. doi:10.1016/j.actbio.2016.05.026.

[23] Seseogullari-Dirihan R, Mutluay MM, Pashley DH, Tezvergil-Mutluay A. Is the inactivation of dentin proteases by crosslinkers reversible? Dent Mater 2017;33:e62–8. doi:10.1016/j.dental.2016.09.036.

[24] Kato MT, Leite AL, Hannas AR, Oliveira RC, Pereira JC, Tjäderhane L, et al. Effect of iron on matrix metalloproteinase inhibition and on the prevention of dentine erosion. Caries Res 2010;44:309–16. doi:10.1159/000315932.

[25] Kato MT, Buzalaf MAR. Iron supplementation reduces the erosive potential of a cola drink on enamel and dentin in situ. J Appl Oral Sci 2012;20:318–22. doi:10.1590/S1678-77572012000300004.

[26] Piemjai M, Nakabayashi N. Effect of dentin conditioners on wet bonding of 4-META/MMA-TBB resin. J Adhes Dent 2001;3:325–31.

[27] Linnevers CJ, Mcgrath ME, Armstrong A, Mistry FR, Barnes MG, Klaus JL, et al. Expression of human cathepsin K in Pichia pastoris and preliminary crystallographic studies of an inhibitor complex. Protein Sci 1997;6:919–21. doi:10.1002/pro.5560060421.

[28] Panwar P, Du X, Sharma V, Lamour G, Castro M, Li H, et al. Effects of cysteine proteases on the structural and mechanical properties of collagen fibers. J Biol Chem 2013;288:5940–50. doi:10.1074/jbc.M112.419689.

[29] Saeki K, Marshall SJ, Gansky S a, Marshall GW. Etching characteristics of dentin: effect of ferric chloride in citric acid. J Oral Rehabil 2001;28:301–8.

[30] Fathima NN, Bose MC, Rao JR, Nair BU. Stabilization of type I collagen against collagenases (type I) and thermal degradation using iron complex. J Inorg Biochem 2006;100:1774–80. doi:10.1016/j.jinorgbio.2006.06.014.

[31] Fathima NN, Rao JR, Nair BU. Effect of UV irradiation on the physico-chemical properties of iron crosslinked collagen. J Photochem Photobiol B Biol 2011;105:203–6. doi:10.1016/j.jphotobiol.2011.09.003.

[32] Seseogullari-Dirihan R, Mutluay MM, Pashley DH, Tezvergil-Mutluay A. Is the inactivation of dentin proteases by crosslinkers reversible? Dent Mater 2017;33:e62–8. doi:10.1016/j.dental.2016.09.036.

[33] Seseogullari-Dirihan R, Apollonio F, Mazzoni A, Tjaderhane L, Pashley D,

Breschi L, et al. Use of crosslinkers to inactivate dentin MMPs. Dent Mater 2016;32:423–32. doi:10.1016/j.dental.2015.12.012.

[34] Fathima NN, Baias M, Blumich B, Ramasami T. Structure and dynamics of water in native and tanned collagen fibers: Effect of crosslinking. Int J Biol Macromol 2010;47:590–6. doi:10.1016/j.ijbiomac.2010.08.003.

[35] Liu Y, Tjäderhane L, Breschi L, Mazzoni A, Li N, Mao J, et al. Limitations in bonding to dentin and experimental strategies to prevent bond degradation. J Dent Res 2011;90:953–68. doi:10.1177/0022034510391799.

[36] Panwar P, Lamour G, Mackenzie NCW, Yang H, Ko F, Li H, et al. Changes in structural-mechanical properties and degradability of collagen during aging-associated modifications. J Biol Chem 2015;290:23291–306. doi:10.1074/jbc.M115.644310.

[37] Novinec M, Kovacic L, Lenarcic B, Baici A. Conformational flexibility and allosteric regulation of cathepsin K. Biochem J 2010;429:379–89. doi:10.1042/BJ20100337.

[38] Scheffel DLS, Hebling J, Scheffel RH, Agee KA, Cadenaro M, Turco G, et al. Stabilization of dentin matrix after cross-linking treatments, in vitro. Dent Mater 2014;30:227–33. doi:10.1016/j.dental.2013.11.007.

[39] Bedran-Russo AK, Pauli GF, Chen S-N, McAlpine J, Castellan CS, Phansalkar RS, et al. Dentin biomodification: strategies, renewable resources and clinical applications. Dent Mater 2014;30:62–76. doi:10.1016/j.dental.2013.10.012.

[40] Bertassoni LE, Orgel JPR, Antipova O, Swain M V. The dentin organic matrix - Limitations of restorative dentistry hidden on the nanometer scale. Acta Biomater 2012;8:2419–33. doi:10.1016/j.actbio.2012.02.022.

[41] Bertassoni LE, Kury M, Rathsam C, Little CB, Swain M V. The role of proteoglycans in the nanoindentation creep behavior of human dentin. J Mech Behav Biomed Mater 2015;55:264–70. doi:10.1016/j.jmbbm.2015.10.018.

[42] Bertassoni LE, Swain M V. 5323 Removal of Dentin Non-collagenous Structures Results in the Unraveling of Microfibril Bundles in Collagen Type I. Connect Tissue Res 2016;8207:03008207.2016.1235566. doi:10.1080/03008207.2016.1235566.

[43] Orsini G, Jr AR, Mazzoni A, Nato F, Manzoli L, Putignano A, et al. A review of the nature , role , and function of dentin non-collagenous proteins . Part 1 : proteoglycans and glycoproteins. Endod Top 2012:1–18. doi:10.1111/j.1601-

1546.2012.00268.x.

[44] Mazzoni A, Breschi L, Carrilho M, Nascimento FD, Orsini G, Ruggeri Jr A, et al. A review of the nature, role, and function of dentin non-collagenous proteins. Part II: enzymes, serum proteins, and growth factors. Endod Top 2012:19–40. doi:10.1111/j.1601-1546.2012.00268.x.

[45] Aguda AH, Panwar P, Du X, Nguyen NT, Brayer GD, Brömme D. Structural basis of collagen fiber degradation by cathepsin K. Proc Natl Acad Sci U S A 2014;111:17474–9. doi:10.1073/pnas.1414126111.

[46] Godat E, Hervé-Grvépinet V, Veillard F, Lecaille F, Belghazi M, Brömme D, et al. Regulation of cathepsin K activity by hydrogen peroxide. Biol Chem 2008;389:1123–6. doi:10.1515/BC.2008.109.

[47] Kim K, Rhee SG, Stadtman ER. Nonenzymatic cleavage of proteins by reactive oxygen species generated by dithiothreitol and iron. J Biol Chem 1985;260:15394–7.

[48] Stadtman ER. Mechanism and Biological Consequences 1990;9:315–25.

[49] Wünschiers R, Jahn M, Jahn D, Schomburg I, Peifer S, Heinzle E, et al. Metabolism. Biochem. Pathways, Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2013, p. 37–209. doi:10.1002/9781118657072.ch3.

[50] Imai Y, Kadoma Y, Kojima K, Akimoto T, Ikakura K, Ohta T. Importance of polymerization initiator systems and interfacial initiation of polymerization in adhesive bonding of resin to dentin. J Dent Res 1991;70:1088–91. doi:10.1177/00220345910700071401.

[51] Bowen RL. Adhesive bonding of various materials to hard tooth tissues. XXII. The effects of a cleanser, mordant, and polySAC on adhesion between a composite resin and dentin. J Dent Res 1980;59:809–14. doi:10.1177/00220345800590051001.

[52] Tezvergil-Mutluay A, Mutluay M, Seseogullari-Dirihan R, Agee KA, Key WO, Scheffel DLS, et al. Effect of Phosphoric Acid on the Degradation of Human Dentin Matrix. J Dent Res 2013;92:87. doi:10.1177/0022034512466264.

3 DISCUSSÃO

De acordo com os objetivos propostos e as metodologias empregadas no presente estudo considerando a interação com o substrato, o colágeno solúvel é uma molécula pré-fragmentada formado de cadeias α1 e α2, que são facilmente quebradas em partes menores. Assim a diminuição das bandas α1 e α 2 indicam o aumento da atividade da colagenase (Sharma et al*.,* 2015).

Na presente tese, observou-se alta taxa de precipitação de colágeno solúvel causada pela reticulação induzida pelos íons Fe^{3+}. Estes íons podem atuar como "lisil oxidase" induzindo a reticulação do colágeno (Fathima et al*.,* 2011) agindo nos terminais dos aminoácidos de lisina e hidroxilisina, produzindo grupos aldeídos, nas regiões de telopeptidios resultando na formação de ligações inter e intramolecular e intermicrofibrilar (Vidal et al*.,* 2016). Desta forma, o complexo covalente Fe/colágeno dá lugar às ligações cruzadas aproximando as moléculas de colágeno (Fathima et al*.,* 2006; Fathima et al*.,* 2010), melhorando as propriedades mecânicas e diminuindo as taxas de degradação (Fathima et al*.,* 2011; Vidal et al*.,* 2016).

Mesmo com a limitação técnica causada pelo efeito da reticulação do colágeno causando a precipitação do colágeno solúvel impedindo que este fosse capaz de atravessar o gel de sulfato de poliacrilamida a 10% (SDS-PAGE), o $FeCl_3$ foi capaz de prevenir a degradação do colágeno solúvel contra a CT-K, e inibir totalmente sua atividade a partir 0.06% de $FeCl_3$. Ainda, a ligação entre Fe/colágeno foi demonstrada por meio da análise por espectroscopia de energia dispersiva de raios-X (EDX), tanto nas fatias de dentina desmineralizadas quanto nas fibras colágenas de tendão de camundongo.

Apesar de diferenças importantes acerca da metodologia entre os dois estudos (Artigos 1 e 2), as imagens em MEV revelaram que o Fe exerceu um efeito preventivo da degradação, tanto na dentina quanto na fibra de tendão da cauda de camundongo. No caso das fibras de tendão o tempo de exposição foi longo (20 horas) e a exposição do inibidor e substrato à enzima foi simultânea. Nas fatias de dentina desmineralizadas a exposição ao inibidor foi prévia à incubação enzimática, simulando a aplicação clínica. É claramente observado o efeito da CT-K na estrutura colágena, causando ruptura das fibrilas que levam a formação de vazios e perda da morfologia, e também o "desnovelamento" das microfibrilas (Bertassoni et al*.,* 2016). O tratamento com 1,8% de $FeCl_3$ foi efetivo na preservação da dentina, apesar, da pequena liberação de cadeias α1 e α2, a morfologia do arcabouço das cadeias de

colágeno estava intacta. Ainda, pelo tempo curto de aplicação no pré-tratamento (15 segundos), não foi observada degradação iônica, como o caso das fibras colágenas do tendão quando do uso das concentrações de 0,8; 1 e 2% de $FeCl_3$.

Além disso, outra diferença na metodologia dos estudos é que as fatias de dentina desmineralizadas foram imersas nas soluções condicionantes (combinações de AC/$FeCl_3$) por 15 segundos e lavadas com água deionizada pelo mesmo tempo, enquanto as fibras colágenas do tendão foram imersas, por 20 horas, apenas nas soluções tampão de ativação enzimática com adição das diversas concentrações de $FeCl_3$. Essas divergências afetaram a quantidade final de $FeCl_3$ encontrado no colágeno, observados nos dois estudos. Assim pode-se afirmar que mesmo com a utilização de um tempo curto de aplicação das soluções foi possível observar a interação do Fe/colágeno. Ainda, o passo da lavagem pode ter contribuído para a diminuição da porcentagem de Fe nas fatias de dentina. Além disso, o tempo de exposição ao Fe pode ter sido fator diferencial entre os dois experimentos. No entanto, em ambos se observou maior concentração de ferro (% em peso) ligado às fibras que nas soluções experimentais de imersão ou incubação.

Para as fibras, a porcentagem de Fe (em peso) encontrada não foi proporcional ao aumento das concentrações de $FeCl_3$ nas soluções de reação. Os grupos 1% e 2% de $FeCl_3$ apresentaram menor quantidade de Fe em relação a 0,1%. Isto provavelmente está relacionada à degradação iônica devido ao longo período de exposição (20 horas). Além disso, a porcentagem de Fe encontrada nas fatias de dentina parece estar relacionada à solubilidade dele na solução, aumentada ou diminuída pela quantidade de AC. Parece que existe um balanço entre o efeito do AC, desmineralizador de superfície e desnaturador do colágeno, com o efeito indutor de reticulação do $FeCl_3$. A solução 5-1,8% apresentou o melhor resultado de absorção de Fe, sendo assim a concentração 5% de AC proporcionou a melhor solubilidade de 1,8% do $FeCl_3$.

Importante ressaltar que a característica de um tecido depende de como as fibrilas se organizam e como continuam o arranjo sequencial, que é mediado por meio de interações com as glicosaminoglicanas (Orge et al., 2006; Bertassoni et al., 2012). Na dentina, o colágeno insolúvel é organizado em ordem hierárquica: tropocolágeno, microfibrilas e fibrilas (Bertassoni et al., 2012) e para a fibra do tendão da cauda de camundongo: tropocolágeno, microfibrilas, fibrilas e fibras formando a estrutura do tendão (Orgel et al., 2006; Gautieri et al., 2011). Assim a

degradação das fibras colágenas pela ação de CT-K é caracterizada pela liberação de cadeias α1 e α2 (Panwar et al., 2013; Sharma et al., 2015). CT-K é a única catepsina capaz de libertar quantidades significativas de fragmentos de tropocolágeno a partir de fibras de colágeno insolúveis (Panwar et al., 2013), por causa da sua habilidade para clivar a tripla hélice em múltiplos sítios (Garnero et al., 1998).

Como já descrito na literatura, a hiperatividade da CT-K pode levar a várias implicações relacionadas a doenças esqueléticas e vasculares (Podgorski, 2009; Fonovic & Turk, 2014), cárie, erosão dentária e degradação da camada híbrida (Tjäderhane et al., 2015, Buzalaf et al., 2015). Dessa forma, ambos os trabalhos apresentados na presente tese demonstraram a habilidade do $FeCl_3$ em prevenir a degradação do colágeno insolúvel, tanto na dentina desmineralizada como em fibras provindas de tendões da cauda de camundongos, como também observou-se que o 0,06% de $FeCl_3$ apresentou capacidade inibitória da atividade da CT-K, uma vez que foi observada relação direta entre a concentração de $FeCl_3$ e inibição da atividade da CT-K.

Para o segundo estudo, foi proposto o uso de diferentes concentrações de AC e $FeCl_3$ nas soluções condicionantes, em relação à solução tradicional 10/3 sugerida por Nakabayashi et al. (1982). No estudo de Piemjai & Nakabayashi (2001) foram observados resultados positivos em relação à resistência de união à dentina também quando soluções com 1% de AC associada a 1% de $FeCl_3.6H_2O$ foi utilizada.

De acordo com os resultados apresentados na presente tese, observa-se um claro benefício do uso da solução AC-$FeCl_3$ de acordo com os resultados de obtidos pelo teste da resistência de união. Grupos com diferentes concentrações de $FeCl_3$ apresentaram melhores resultados do que aqueles obtidos com condicionamento realizado com ácido fosfórico a 35%. No caso as soluções condicionantes 5-1,8 e 5-0,6, estas apresentaram maiores valores de resistência de união, comparados aos grupos AF15, AF5, 0-0,6, 1-0, 0-1,8, 1-1,8. Isso pode ser atribuído a vários fatores, como a solubilidade $FeCl_3$ que é aumentada na presença de AC. Assim, as concentrações 0-1,8 e 0-0,6 poderiam apresentar deposição cristalina de ferro na superfície da dentina. Além disso, a ausência de AC para alguns grupos não permitiu um condicionamento adequado da dentina, comprometendo a formação da camada híbrida. Um condicionamento insuficiente provavelmente também ocorreu

com soluções 1-0 e 1-1,8. Contudo, 1,8% de $FeCl_3$ pareceu ser menos solúvel na presença de água ou 1% de AC, fato que foi observado durante a preparação da solução. Além disso, as soluções de 0-1,8 e 1-1,8 produziram menor resistência de união com possível precipitação de $FeCl_3$ na superfície da dentina que geraram falhas mistas e adesivas. Sendo assim, em tecido mineralizado a atuação do $FeCl_3$ é dependente da solubilidade, capacidade juntamente com o AC em formar uma solução homogênea (sem precipitados) e da sua capacidade em desmineralizar a adequadamente a superfície dentinária.

As combinações 5-1,8, 5-0,6 e 5-0, intermediárias entre 10-3 e 1-1 também foram selecionadas e não haviam sido relatadas na literatura. As soluções 5-1,8 e 5-0,6 apresentaram melhores resultados de resistência de união, independentemente do tempo de armazenamento. Parece que as soluções 5-1,8 e 5-0,6 apresentaram uma solubilidade e pKa adequados, que proporcionou condicionamento formação de camadas híbridas de qualidade. Todas as soluções com 5% de AC (5-1,8; 5-0,6; 5-0) mostraram-se semelhantes as 10-1,8, 10-0,6, 10-0, 1-0,6.

Tezvergil-Mutluay et al. (2013) demonstraram que condicionamento com ácido fosfórico pode promover a liberação de telopeptídeos reticulados do terminal C (ICTP) pela ação de MMPs, bem como a liberação de fragmentos de peptídeos do terminal C (CTX) pela atividade de CT-K endógena sobre a dentina (Tezvergil-Mutluay et al*.,* 2013). Assim, quando privados de minerais pelo ataque com ácido fosfórico, o colágeno das microfibrilas é mais vulnerável ao ataque das enzimas proteolíticas (Pashley et al*.,* 2004; Tezvergil-Mutluay et al*.,* 2013). Assim, é provável que a CT-K que atua em pH 5,5 possa iniciar o processo de degradação do colágeno após o processo de ataque ácido, uma vez que ambas as famílias de enzimas (MMPs e CTs) trabalham em conjunto, mas em diferentes períodos metabólicos que dependem das mudanças de pH locais (Tjäderhane et al*.,* 2013 [a]; Tjäderhane et al*.,* 2013 [b]).

Com relação ao tempo de armazenamento, a redução da resistência da união dentinária após nove meses pode ser explicada em parte pela composição do adesivo, que contém grande quantidade de monômeros hidrófilos, principalmente na solução do “primer” (Tjäderhane, 2015; Tjaderhane et al., 2013 [b]).

O “primer” do Adper Scotchbond multiuso tem 35-45% em peso de HEMA na sua composição e 40-50% em peso de água, enquanto que o agente de união tem 30-40% em peso de HEMA. A hidrofilia é necessária, porque os monômeros devem

ser capazes de se infiltrar pelas fibrilas colágenas (Tjäderhane, 2015). Porém a água inibe o processo de polimerização (Cardoso et al., 2011), e se não evaporada adequadamente, o excesso de umidade pode causar separação de fases entre monômeros hidrófobos e hidrófilos, resultando em infiltração irregular dos monômeros na camada híbrida (Cardoso et al., 2011; Tjäderhane et al., 2013 [b]; Tjäderhane, 2015). Isso pode levar a diminuição das propriedades mecânicas do adesivo e degradação hidrolítica (Van Landuyt et al., 2007), o que reduz a longevidade da adesão dentinária (Tjäderhane, 2015). Assim, mesmo que a degradação enzimática seja paralisada e o colágeno tenha sido preservado, uma elevada característica hidrófila do adesivo testado pode ser responsável pela redução da resistência da união na dentina (Tjäderhane, 2015). Por conseguinte, a escolha de um adesivo mais hidrófobo poderia produzir resultados de resistência de união diferentes.

O aumento das propriedades biomecânicas do colágeno, inibição das proteases e remineralização biomimética são as principais estratégias para proteger a integridade da matriz de colágeno dentinária (Liu et al., 2011). O colágeno é um importante biomaterial com imensa aplicação na área de regeneração de tecidos. Assim mantas e arcabouço de colágeno têm sido estudados como substitutos para a matriz extracelular (Powell et al., 2006). Sendo que tecidos conjuntivos como pele, osso e dentina são compostos principalmente de colágeno tipo I, o uso de inibidores ou indutores de reticulação pode aumentar as propriedades biomecânicas e reduzir a sua degradação enzimática (Vidal et al., 2016). O íon Fe^{2+} já mostrou efeito inibitório contra a atividade de MMP-2 e -9 (Kato et al., 2010; Kato et al., 2012), e como mostrado no presente estudo, $FeCl_3$ pode inibir a atividade de CT-K e atuar como escudo para as fibras de colágeno aumentando a taxa de reticulação, e por consequência a estabilidade química e mecânica (Fathima et al., 2006; Fathima et al., 2011).

4 CONCLUSÃO

Baseado nas condições e nos resultados do presente estudo pode-se concluir que:

- $FeCl_3$ foi capaz de prevenir a degradação do colágeno solúvel pela CT-K;
- $FeCl_3$ foi capaz de prevenir a degradação das fibras colágenas pela CT-K tanto para as fibras do tendão de cauda de camundongo, como para a dentina desmineralizada;
- A aplicação de 1,8% de $FeCl_3$ na dentina desmineralizada foi capaz de prevenir a degradação da dentina pela CT-K, sem causar degradação iônica.
- $FeCl_3$ aumentou a estabilidade do colágeno tipo I contra enzima colagenolítica.
- As soluções de AC/$FeCl_3$ demonstraram resultado favorável quanto a μTBS, sendo que as soluções 5-0,6 e 5-1,8 apresentaram melhores, independente do tempo de armazenamento e aplicação.
- $FeCl_3$ foi capaz de inibir a atividade da CT-K;

REFERÊNCIAS[1]

1. Baker DH, Czarnecki-Maulden GL. Pharmacologic role of cysteine in ameliorating or exacerbating mineral toxicities. J Nutr. 1987 Jun;117(6):1003-10
2. Bedran-Russo AK, Vidal CM, Dos Santos PH, Castellan CS. Long-term effect ofcarbodiimide on dentin matrix and resin-dentin bonds. J Biomed Mater Res B Appl Biomater. 2010 Jul;94(1):250-5. doi: 10.1002/jbm.b.31649.
3. Berg JM, Tymoczko JL, Gatto GJ, Stryker L. Biochemistry. 8th ed. New York: W. H. Freeman; 2015.
4. Bertassoni LE, Orgel JP, Antipova O, Swain MV. The dentin organic matrix / limitations of restorative dentistry hidden on the nanometer scale. Acta Biomater. 2012 Jul;8(7):2419/33. doi: 10.1016/j.actbio.2012.02.022.
5. Bertassoni LE, Swain MV. Removal of dentin non/collagenous structures results in the unraveling of microfibril bundles in collagen type I. Connect Tissue Res. 2016 Sep 22:1-10.
6. Boatman EM, Goodwin MB, Holman H,Fakra S, Schweitzer MH, Gronsky R, Horner JR. Synchrotron Chemical and Structural Analysis of Tyrannosaurus rex Blood Vessels: The Contribution of Collagen Hypercrosslinking to Tissue Longevity. Microsc Microanal. 2014; 20(03): 1430-31.
7. Buzalaf MA, Charone S, Tjäderhane L. Role of host-derived proteinases in dentine caries and erosion. Caries Res. 2015;49 Suppl 1:30-7. doi: 10.1159/000380885.
8. Cardoso MV, Neves A, Mine A, Coutinho E, Van Landuyt K, De Munck J, & Van Meerbeek B. Current aspects on bonding effectiveness and stability in adhesive dentistry. Aust Dent J. 2011 Jun;56 Suppl 1:31-44. doi: 10.1111/j.1834-7819.2011
9. Carvalho RM, Yoshiyama M, Pashley EL, Pashley DH. In vitro study on the dimensional changes of human dentine after demineralization. Arch Oral Biol. 1996 Apr;41(4):369-77.

[1] De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors - Vancouver Group. Abreviatura dos periódicos em conformidade com o PubMed.

10. Chaussain C, Boukpessi T, Khaddam M, Tjaderhane L, George A, Menashi S. Dentin matrix degradation by host matrix metalloproteinases: inhibition and clinical perspectives toward regeneration. Front Physiol. 2013 Nov 1;4:308. doi: 10.3389/fphys.2013.00308.
11. Dang JM, Leong KW. Natural polymers for gene delivery and tissue engineering. Adv Drug Deliv Rev. 2006 Jul 7;58(4):487-99.
12. Domsalla A, Melzig MF. Occurrence and properties of proteases in plant latices. Planta Med. 2008 Jun;74(7):699-711. doi: 10.1055/s-2008-1074530.
13. Fathima NN, Baias M, Blumich B, Ramasami T. Structure and dynamics of water in native and tanned collagen fibers: effect of crosslinking. Int J Biol Macromol. 2010 Dec 1;47(5):590-6. doi: 10.1016/j.ijbiomac.2010.08.003.
14. Fathima NN, Bose MC, Rao JR, Nair BU, Stabilization of type I collagen against collagenases (type I) and thermal degradation using iron complex. J Inorg Biochem. 2006 Nov;100(11):1774-80. Epub 2006 Jul 11.
15. Fathima NN, Dhathathreyan A, Ramasami T, Kragel J, Miller R. Degree of crosslinking of collagen at interfaces: adhesion and shear rheological indicators. Int J Biol Macromol. 2011 Jan 1;48(1):67-73. doi: 10.1016/j.ijbiomac.2010.09.017.
16. Fathima NN, Rao JR, Nair BU. Effect of UV irradiation on the physico-chemical properties of iron crosslinked collagen. J Photochem Photobiol B. 2011 Dec 2;105(3):203-6. doi: 10.1016/j.jphotobiol.2011.09.003.
17. Fonović M, Turk B.Cysteine cathepsins and extracellular matrix degradation. Biochim Biophys Acta. 2014 Aug;1840(8):2560-70. doi: 10.1016/j.bbagen.2014.03.017.
18. Garcia MB, Carrilho MR, Nör JE, Anauate-Netto C, Anido-Anido A, Amore R, Tjäderhane L, Bretz WA. Chlorhexidine Inhibits the Proteolytic Activity of Root and Coronal Carious Dentin in vitro. Caries Res. 2009;43(2):92-6. doi:10.1159/000209340.
19. Garnero P, Borel O, Byrjalsen O, Ferreras M, Drake FH, Mc- Queney MS, Foged NT, Delmas PD, Delaissé JM. The collagenolytic activity of cathepsin K is unique among mammalian proteinases. J Biol Chem. 1998 Nov 27;273(48):32347-52.
20. Gautieri A, Vesentini S, Redaelli A, Buehler MJ. Hierarchical structure and

nanomechanics of collagen microfibrils from the atomistic scale up. Nano Lett. 2011 Feb 9;11(2):757-66. doi: 10.1021/nl103943u. Epub 2011 Jan 5.

21. Gwinnett AJ. Dentin bond strength after air drying and rewetting. Am J Dent. 1994 Jun; 7(3):144-8.
22. Han B, Jaurequi J, Tang BW, Nimni ME. Proanthocyanidin: a natural crosslinking reagent for stabilizing collagen matrices. J Biomed Mater Res A. 2003 Apr 1;65(1):118-24. PubMed PMID: 12635161.
23. Hiraishi N, Yiu CK, King NM, Tay FR, Pashley DH. Chlorhexidine release and water sorption characteristics of chlorhexidine-incorporated hydrophobic/hydrophilic resins. Dent Mater. 2008 Oct;24(10):1391-9. doi: 10.1016/j.dental.2008.03.011.
24. Hitmi L, Bouter D, Degrange M. Influence of drying and HEMA treatment on dentin wettability. Dent Mater. 2002 Nov;18(7):503-11.
25. Imai Y, Kadoma Y, Kojima K, Akimoto T, Ikakura K, Ohta T. Importance of polymerization initiator systems and interfacial initiation of polymerization in adhesive bonding of resin to dentin. J Dent Res 1991 Jul; 70:1088/1091.
26. Kanca J. A method for bonding to tooth structure using phosphoric acid as a dentin-enamel conditioner. Quintessence Int. 1991 Apr; 22(4): 285-90.
27. Kato MT, Leite AL, Hannas AR, Calabria MP, Magalhães AC, Pereira JC, Buzalaf MA. Impact of protease inhibitors on dentin matrix degradation by collagenase. J Dent Res. 2012 Dec;91(12):1119-23. doi: 10.1177/0022034512455801.
28. Kato MT, Leite AL, Hannas AR, Oliveira RC, Pereira JC, Tjäderhane L, Buzalaf MA. Effect of iron on matrix metalloproteinase inhibition and on the prevention of dentine erosion. Caries Res. 2010;44(3):309-16. doi: 10.1159/000315932.
29. Kim K, Rhee SG, Stadtman ER. Nonenzymatic cleavage of proteins by reactive oxygen species generated by dithiothreitol and iron. J Biol Chem. 1985 Dec 15;260(29):15394-7.
30. Lill R, Mühlenhoff U. Iron-sulfur protein biogenesis in eukaryotes: components and mechanisms. Annu Rev Cell Dev Biol. 2006;22:457-86.
31. Lippard SJ, Berg JM. Principles of Bioinorganic Chemistry. Mill Valley, CA: University Science Books; 1994. ISBN 0-935702-73-3.

32. Liu Y, Tjäderhane L, Breschi L, Mazzoni A, Li N, Mao J, Pashley DH, Tay FR. Limitations in bonding to dentin and experimental strategies to prevent bond degradation. J Dent Res. 2011 Aug;90(8):953-68. doi: 10.1177/0022034510391799.
33. Lundblad RL. Chemical Reagents for Protein Modification CRC Press Inc; 2004. ISBN 0-8493-1983-8
34. Mazzoni a., Nascimento FD, Carrilho M, Tersariol I, Papa V, Tjaderhane L, et al. MMP Activity in the Hybrid Layer Detected with in situ Zymography. J Dent Res 2012;91:467–72. doi:10.1177/0022034512439210
35. Mazzoni A, Breschi L, Carrilho M, Nascimento FD, Orsini G, Ruggeri A, Gobbi P, Mazzotti G, Tay FR, Pashley DH, Tjäderhane L: A review on nature, role and functions of dentin non-collagenous proteins. Part II: enzymes, serum proteins and growth factors. Endod Topics 2012a;21: 19–40. [b]
36. Mazzoni A, Nascimento FD, Carrilho M, Tersariol I, Papa V, Tjäderhane L, et al. MMP activity in the hybrid layer detected with in situ zymography. J Dent Res. 2012 May;91(5):467-72. doi: 10.1177/0022034512439210. [a]
37. Nakabayashi N, Hiranuma K. Effect of etchant variation on wet and dry dentin bonding primed with 4/META/acetone. Dent Mater. 2000 Jul; 16(4): 274-9.
38. Nakabayashi N, Kojima K, Masuhara E. The promotion of adhesion by infiltration of monomers into tooth substrates. J Biomed Mater Res 1982 May; 16(3): 265–73.
39. Nakabayashi N, Watanabe A, Gendusa NJ. Dentin adhesion of "modified" 4-META/MMA-TBB resin: function of HEMA. Dent Mater. 1992 Jul;8(4):259-64. [a]
40. Nakabayashi N, Takarada K. Effect of HEMA on bonding to dentin. Dent Mater. 1992 Mar;8(2):125-30. [b]
41. Nishitani Y, Hosaka K, Hoshika T, Yoshiyama M, Pashley DH. Effects of chlorhexidine in self-etching adhesive: 24 hours results. Dent Mater J. 2013;32(3):420-4.
42. Orgel JP, Irving TC, Miller A, Wess TJ. Microfibrillar structure of type I collagen in situ. Proc Natl Acad Sci U S A. 2006 Jun 13;103(24):9001-5.
43. Pallan S, Furtado Araujo MV, Cilli R, Prakki A. Mechanical properties and characteristics of developmental copolymers incorporating catechin or

chlorhexidine. Dent Mater. 2012 Jun;28(6):687-94. doi: 10.1016/j.dental.2012.03.003.

44. Panwar P, Du X., Sharma V, Lamour G, Castro M, Li H, Brömme D. Effects of cysteine proteases on the structural and mechanical properties of collagen fibers. J Biol Chem. 2013 Feb 22;288(8):5940-50. doi: 10.1074/jbc.M112.419689.
45. Pashley DH, Tay FR, Yiu C, Hashimoto M, Breschi L, Carvalho RM, Ito S. Collagen degradation by host-derived enzymes during aging. J Dent Res. 2004 Mar; 83(3): 216-21.
46. Piemjai M, Nakabayashi N. Effect of dentin conditioners on wet bonding of 4/META/MMA/TBB resin. J Adhes Dent. 2001 Winter;3(4):325/31.
47. Podgorski, I. Future of anticathepsin K drugs: dual therapy for skeletal disease and atherosclerosis? Future Med Chem. 2009 Apr;1(1):21-34.
48. Powell HM, Boyce ST, EDC cross-linking improves skin substitute strength and stability. Biomaterials. 2006 Dec;27(34):5821-7.
49. Ricci HA, Sanabe ME, de Souza Costa CA, Pashley DH, Hebling J. Chlorhexidine increases the longevity of in vivo resin-dentin bonds. Eur J Oral Sci. 2010 Aug;118(4):411-6. doi: 10.1111/j.1600-0722.2010.00754.x. Erratum in: Eur J Oral Sci. 2010 Oct;118(5):535.
50. Sabatini C. Effect of a chlorhexidine-containing adhesive on dentin bond strength stability. Oper Dent. 2013 Nov Dec;38(6):609-17. doi: 10.2341/12-239-L.
51. San Antonio JD, Schweitzer MH, Jensen ST, Kalluri R, Buckley M, Orgel JP. Dinosaur peptides suggest mechanisms of protein survival. PLoS One. 2011; 6(6): e20381.
52. Scaffa PM, Vidal CM, Barros N, Gesteira TF, Carmona AK, Breschi L, Pashley DH, Tjäderhane L, Tersariol IL, Nascimento FD, Carrilho MR. Chlorhexidine inhibits the activity of dental cysteine cathepsins. J Dent Res. 2012 Apr;91(4):420-5. doi: 10.1177/0022034511435329.
53. Scheffel DL, Hebling J, Scheffel RH, Agee K, Turco G, de Souza Costa CA, Pashley D. Inactivation of matrix-bound matrix metalloproteinases by cross-linking agents in acid-etched dentin. Oper Dent. 2014 Mar-Apr;39(2):152-8. doi: 10.2341/12-425-L.

54. Schweitzer MH, Zheng W, Cleland TP, Goodwin MB, Boatman E, Theil E, Marcus MA, Fakra SC. A role for iron and oxygen chemistry in preserving soft tissues, cells and molecules from deep time. Proc Biol Sci. 2013 Nov 27;281(1775):20132741. doi: 10.1098/rspb.2013.2741
55. Schweitzer MH, Zheng W, Organ CL, Avci R, Suo Z, Freimark LM, Lebleu VS, Duncan MB, Vander Heiden MG, Neveu JM, Lane WS, Cottrell JS, Horner JR, Cantley LC, Kalluri R, Asara JM. Biomolecular characterization and protein sequences of the Campanian hadrosaur B. canadensis. Science. 2009; 324(5927): 626-31.
56. Seseogullari-Dirihan R, Apollonio F, Mazzoni A, Tjaderhane L, Pashley D, Breschi L, Tezvergil-Mutluay A. Use of crosslinkers to inactivate dentin MMPs. Dent Mater. 2016 Mar;32(3):423-32. doi: 10.1016/j.dental.2015.12.012.
57. Seseogullari-Dirihan R, Mutluay MM, Pashley DH, Tezvergil-Mutluay A. Is the inactivation of dentin proteases by crosslinkers reversible? Dent Mater. 2017 Feb;33(2):e62-e68. doi: 10.1016/j.dental.2016.09.036.
58. Seseogullari-Dirihan R, Mutluay MM, Vallittu P, Pashley DH, Tezvergil-Mutluay A. Effect of pretreatment with collagen crosslinkers on dentin protease activity. Dent Mater. 2015 Aug;31(8):941-7. doi: 10.1016/j.dental.2015.05.002. [a]
59. Seseogullari-Dirihan R, Tjäderhane L, Pashley DH, Tezvergil-Mutluay A. Effect of ultraviolet A-induced crosslinking on dentin collagen matrix. Dent Mater. 2015 Oct;31(10):1225-31. doi: 10.1016/j.dental.2015.08.145. [b]
60. Severino RP, Busca de produtos naturais como inibidores específicos de enzimas[tese]. Centro de Ciências Exatas e de Tecnologia, Universidade Federal de São Carlos; 2008.
61. Sharma V, Panwar P, O'Donoghue AJ, Cui H, Guido RV, Craik CS, Brömme D. Structural requirements for the collagenase and elastase activity of cathepsin K and its selective inhibition by an exosite inhibitor. Biochem J. 2015 Jan 1;465(1):163-73. doi: 10.1042/BJ20140809.
62. Shimada Y, Ichinose S, Sadr A, Burrow MF, Tagami J. Localization of matrix metalloproteinases (MMPs-2, 8, 9 and 20) in normal and carious dentine. Aust Dent J. 2009 Dec;54(4):347-54. doi: 10.1111/j.1834-7819.2009.01161.x.
63. Stadtman ER. Metal ion-catalyzed oxidation of proteins: biochemical

mechanism and biological consequences. Free Radic Biol Med. 1990;9(4):315-25.

64. Stanislawczuk R, Amaral RC, Zander-Grande C, Gagler D, Reis A, Loguercio AD. Chlorhexidine-containing acid conditioner preserves the longevity of resin-dentin bonds. Oper Dent. 2009 Jul-Aug;34(4):481-90. doi: 10.2341/08-016-L.
65. Stanislawczuk R, Reis A, Loguercio AD. A 2-year in vitro evaluation of a chlorhexidine-containing acid on the durability of resin-dentin interfaces. J Dent. 2011 Jan;39(1):40-7. doi: 10.1016/j.jdent.2010.10.001.
66. Sung HW, Chang WH, Ma CY, Lee MH. Crosslinking of biological tissues using genipin and/or carbodiimide. J Biomed Mater Res A. 2003 Mar 1;64(3):427-38.
67. Tersariol IL, Geraldeli S, Minciotti CL, Nascimento FD, Pääkkönen V, Martins MT, et al. Cysteine cathepsins in human dentin-pulp complex. J Endod. 2010 Mar;36(3):475-81. doi: 10.1016/j.joen.2009.12.034.
68. Tezvergil-Mutluay A, Mutluay M, Seseogullari-Dirihan R, Agee KA, Key WO, Scheffel DL, Breschi L, Mazzoni A, Tjäderhane L, Nishitani Y, Tay FR, Pashley DH. Effect of phosphoric acid on the degradation of human dentin matrix. J Dent Res. 2013 Jan;92(1):87-91. doi: 10.1177/0022034512466264.
69. Tjäderhane L, Buzalaf MA, Carrilho M, Chaussain C. Matrix metalloproteinases and other matrix proteinases in relation to cariology: the era of 'dentin degradomics'. Caries Res. 2015;49(3):193-208. doi: 10.1159/000363582.
70. Tjäderhane L, Nascimento FD, Breschi L, Mazzoni A, Tersariol IL, Geraldeli S, Tezvergil-Mutluay A, Carrilho MR, Carvalho RM, Tay FR, Pashley DH. Optimizing dentin bond durability: Control of collagen degradation by matrix metalloproteinases and cysteine cathepsins. Dent. Mater. 2013 Jan;29(1): 116–135 (2013). [a]
71. Tjäderhane L, Nascimento FD, Breschi L, Mazzoni A, Tersariol ILS, Geraldeli S, et al. Strategies to prevent hydrolytic degradation of the hybrid layer - A review. Dent Mater 2013;29:999–1011. doi:10.1016/j.dental.2013.07.016.[b]
72. Tjäderhane L. Dentin bonding: can we make it last? Oper Dent. 2015 Jan-Feb;40(1):4-18. doi: 10.2341/14-095-BL.

73. Van Landuyt KL, Snauwaert J, De Munck J, Peumans M, Yoshida Y, Poitevin A, Coutinho E, Suzuki K, Lambrechts P, Van Meerbeek B (2007) Systematic review of the chemical composition of contemporary dental adhesives Biomaterials 28(26) 3757-3785.
74. Vidal CM, Tjäderhane L, Scaffa PM, Tersariol IL, Pashley D, Nader HB, et al. Abundance of MMPs and cysteine cathepsins in caries-affected dentin. J Dent Res. 2014 Mar;93(3):269-74. doi: 10.1177/0022034513516979
75. Vidal CM, Zhu W, Manohar S, Aydin B, Keiderling TA, Messersmith PB, et al. Collagen-collagen interactions mediated by plant-derived proanthocyanidins: A spectroscopic and atomic force microscopy study. Acta Biomater. 2016 Sep 1;41:110-8. doi: 10.1016/j.actbio.2016.05.026.
76. Wünschiers R, Jahn M, Jahn D, Schomburg I, Peifer S, Heinzle E, et al. Metabolism. Biochem. Pathways, Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2013, p. 37–209. doi:10.1002/9781118657072.ch3.
77. Zhou J, Tan J, Yang X, Xu X, Li D, Chen L. MMP-inhibitory effect of chlorhexidine applied in a self-etching adhesive. J Adhes Dent. 2011 Apr;13(2):111-5. doi: 10.3290/j.jad.a18783.

APÊNDICE

Figuras ilustrativas com a descrição da metodologia empregada nos estudos

Figura 1. Esquema (modificado de Severino, 2008) do ensaio enzimático com o substrato fluorogênico Z-FR-MCA (Cbz-Phe-Arg-MCA ou carbobenzoxi-fenilalanina-arginina-7-amino-4-metilcumarina ou benziloxicarbonil-fenilalanina-arginina-4-metil-7-coumarilamida), mostrando o ponto de clivagem pela CT-K e a formação do MCA livre de alta fluorescência.

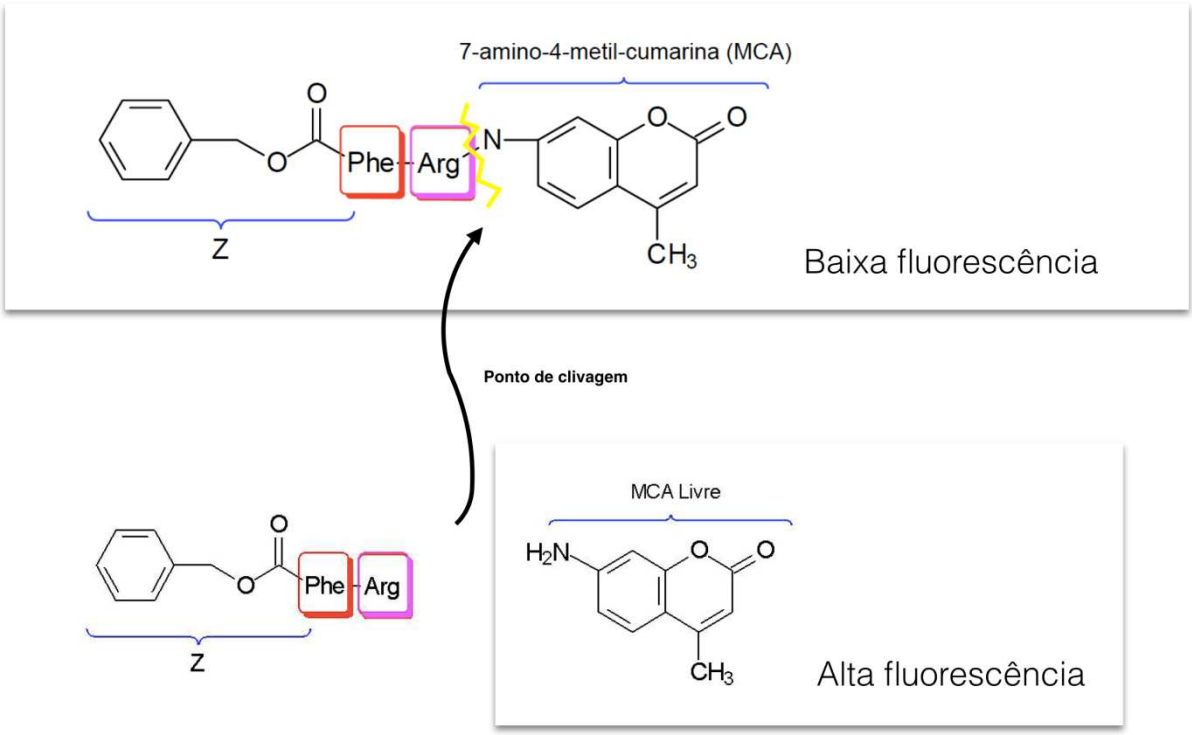


Figura 2. (A) Espectrofluorometro (Luminescence Spectrometer, PerkinElmer, UK) utilizado no presente estudo. (B) Curvas do aumento da fluorescência em função do tempo, obtidas por meio da quebra do substrato Z-FR-MCA, sendo que a fluorescência foi monitorada por 30 segundos, considerando controle (100% de atividade enzimática) quando a ausência dos inibidores (curva azul), e as demais curvas quando na presença das diferentes concentrações de $FeCl_3$ (0,005% a 0,08%).

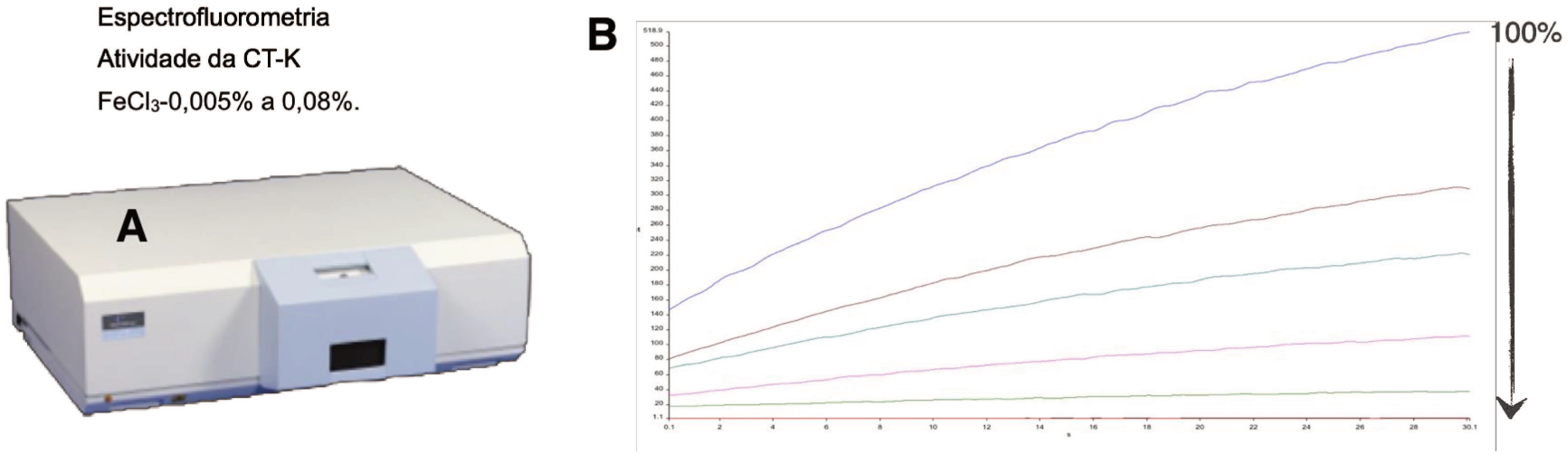


Figura 3. Esquema explicativo da incubação do colágeno solúvel e insolúvel. (A) O colágeno solúvel (0,6 mg/ml) (MSJ BioLynx Inc.) foi incubado por 4 horas a 28°C em tampão de acetato (100 mM, pH 5,5, contendo DTT 2,5 mM e EDTA 2,5 mM), com 400 nM de CT-K na presença de CAS 200 nM com adição dos inibidores na forma de solução (0,02% a 2% de $FeCl_3$). O colágeno insolúvel (fibras de tendões de cauda de camundongo) (1 mg) foi incubado por 20 horas a 28°C em tampão acetato (100 mM, pH 5,5, contendo DTT 2,5 mM e EDTA 2,5 mM), com 1μM de CT-K com adição dos inibidores na forma de solução (0,02% a 2% de $FeCl_3$). As reações foram interrompidas pela adição de E-64 10μM. (B) e subsequentemente o sobrenadante das misturas reacionais foram submetidas à eletroforese em gel de sulfato de poliacrilamida a 10% (SDS-PAGE). (C) Demonstração de como o gel separa as moléculas de proteína por tamanho (Modificado de Berg et al., 2015).

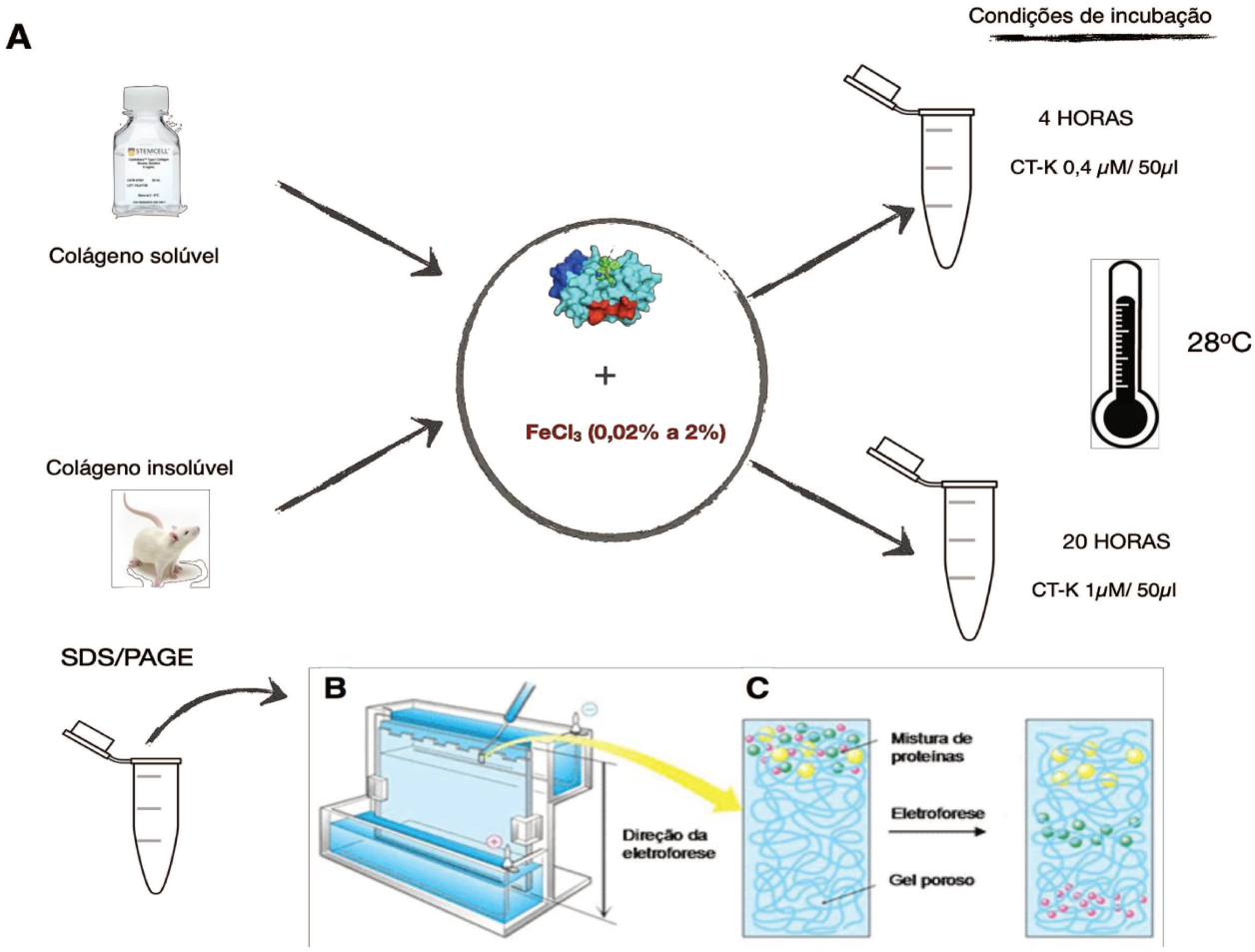


Figura 4. Descrição e imagens representativas da metodologia utilizada. (A) Setenta e oito terceiros molares hígidos foram divididos aleatoriamente em 13 grupos. O esmalte oclusal foi removido e a superfície da dentina foi lixada (SiC 320-grit) sob refrigeração por 10 segundos para padronizar a lama dentinária. Os espécimes de dentina coronária foram condicionados (B, C, D) com soluções a base de água deionizada contendo ácido cítrico (AC) e/ou $FeCl_3$ nas concentrações: 10%-1,8%, 10%-0,6%, 10% AC, 5%-1,8%, 5%-0,6%, 5% AC, 1%-1,8%, 1%-0,6%, 1% AC, 1,8% $FeCl_3$, 0,6% $FeCl_3$ por 15 segundos e com ácido fosfórico (AF) (5 e 15 segundos) (E). Todos os grupos foram lavados por 15 segundos cada (F). Foi utilizado o sistema adesivo Adper Scotchbond Multi-Purpose (3M ESPE, St. Paul, MN, USA) (G) conforme as recomendações do fabricante, aplicação ativa do primer seguido de leve jato de ar por 5 segundos, aplicação ativa do componente *bond*, fotoativado por 20 segundos (1,200Mw/cm^2) com o aparelho Bluephase G2 (Ivoclar Vivadent, Schaan, Liechtenstein). Camadas de resina composta (Aelite^{TM} All/Purpose Body, Bisco Inc., Schaumburg, IL, USA) foram colocadas (I), e sobre a superfície adesiva foi construído um bloco de resina de 5mm de altura com incrementos de 2 mm de espessura os quais foram fotoativados por 40 segundos (J e K). Os espécimes foram mantidos a 37°C por 24 horas. Cortes perpendiculares ao longo eixo do dente foram realizadas para se obter palitos de 1mm^2 de secção transversal (L) (área aderida). Metade dos palitos de cada dente foi testado (M) após 24 horas ou 9 meses de armazenamento.

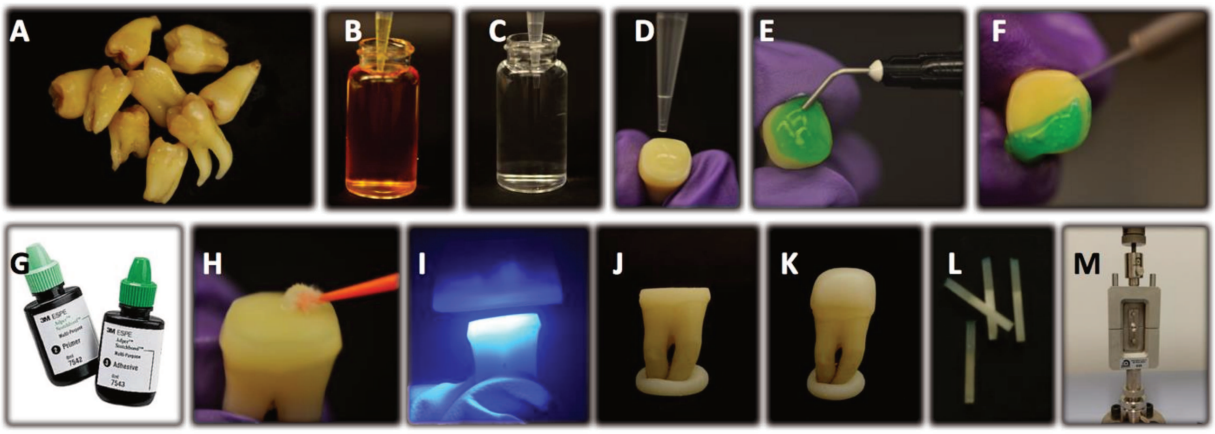


Figura 5. (A) Metade dos palitos de cada dente foi testado após 24 horas ou 9 meses de armazenamento. (B) Dispositivo de teste de microtração (Odeme Dental Pesquisa, Luzerna, SC, Brasil). (C) Palitos fixados no dispositivo de teste com cola de cianoacrilato (Super Glue Gel Control, Loctite, Mississauga, ON, Canadá) e posicionado na maquina de ensaio. (D) Máquina de ensaio (AGS-X Shimadzu, Tóquio, Japão) numa velocidade de 1.0 mm/min até a falha.

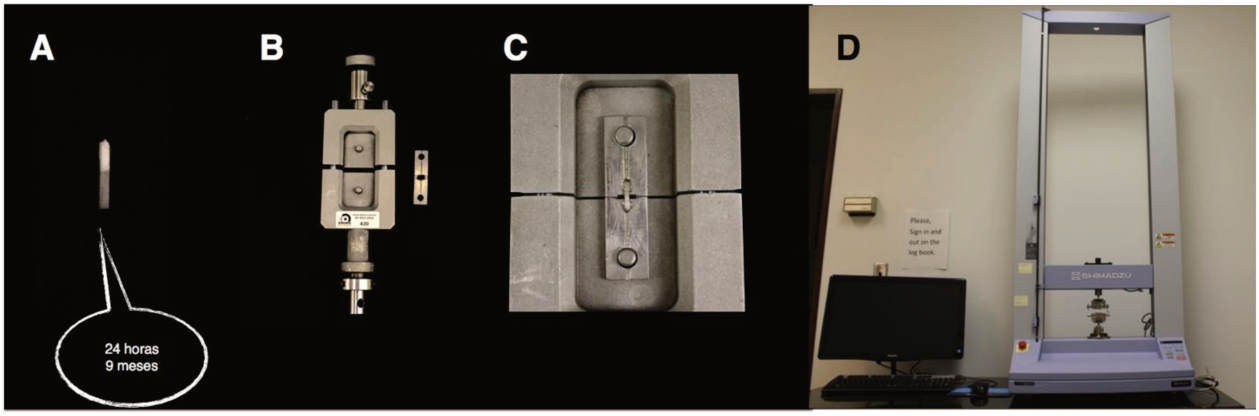


Figura 6. Esquema explicativo da separação das fatias de dentina obtidas a partir da secção de dois terceiros molares hígidos. As fatias foram dividas para as análises a serem realizadas. Sendo que para a degradação do colágeno foram utilizadas 15 fatias de dentina divididas em 5 grupos (n=3), e para a análise em EDX 27 fatias divididas em 9 grupos (n=3).

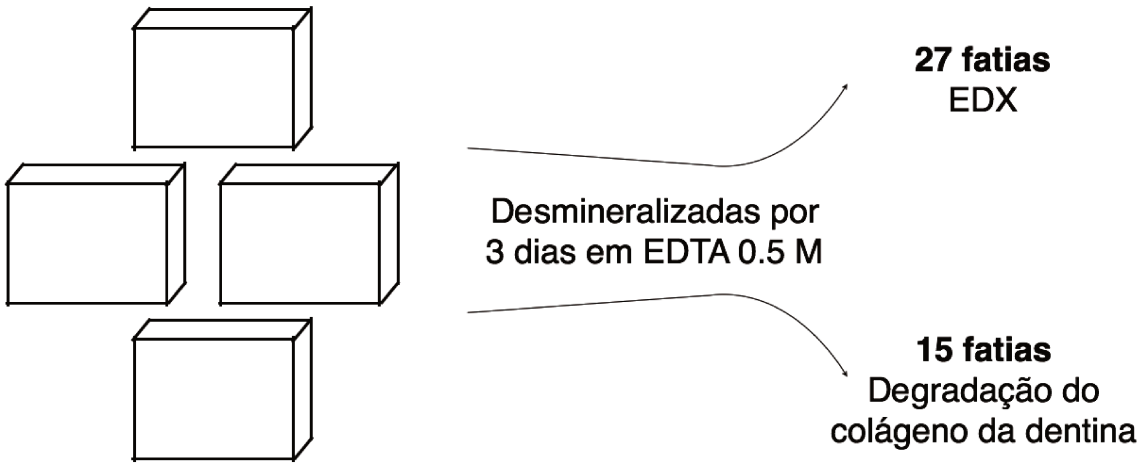


Figura 7. Esquema explicativo da análise da degradação *in vitro* do colágeno dentinário. Fatias de dentina foram tratadas com e sem $FeCl_3$ (0,06 0,08 e 1,8 % de $FeCl_3$) por 15 segundos (A), lavadas por 15 segundos (B), desidratadas e pesadas (C, D). Em seguida, 1 mg de dentina desmineralizada foi incubada por 20 horas a 28°C em tampão de acetato (100 mM, pH 5,5, contendo DTT 2,5 mM e EDTA 2,5 mM), com 1μM de CT-K (E). As reações foram interrompidas pela adição de E-64 (10μM). O conteudo foi centrifugado e as fatias de dentina foram preparadas para microscopia eletrônica de varedura (MEV).

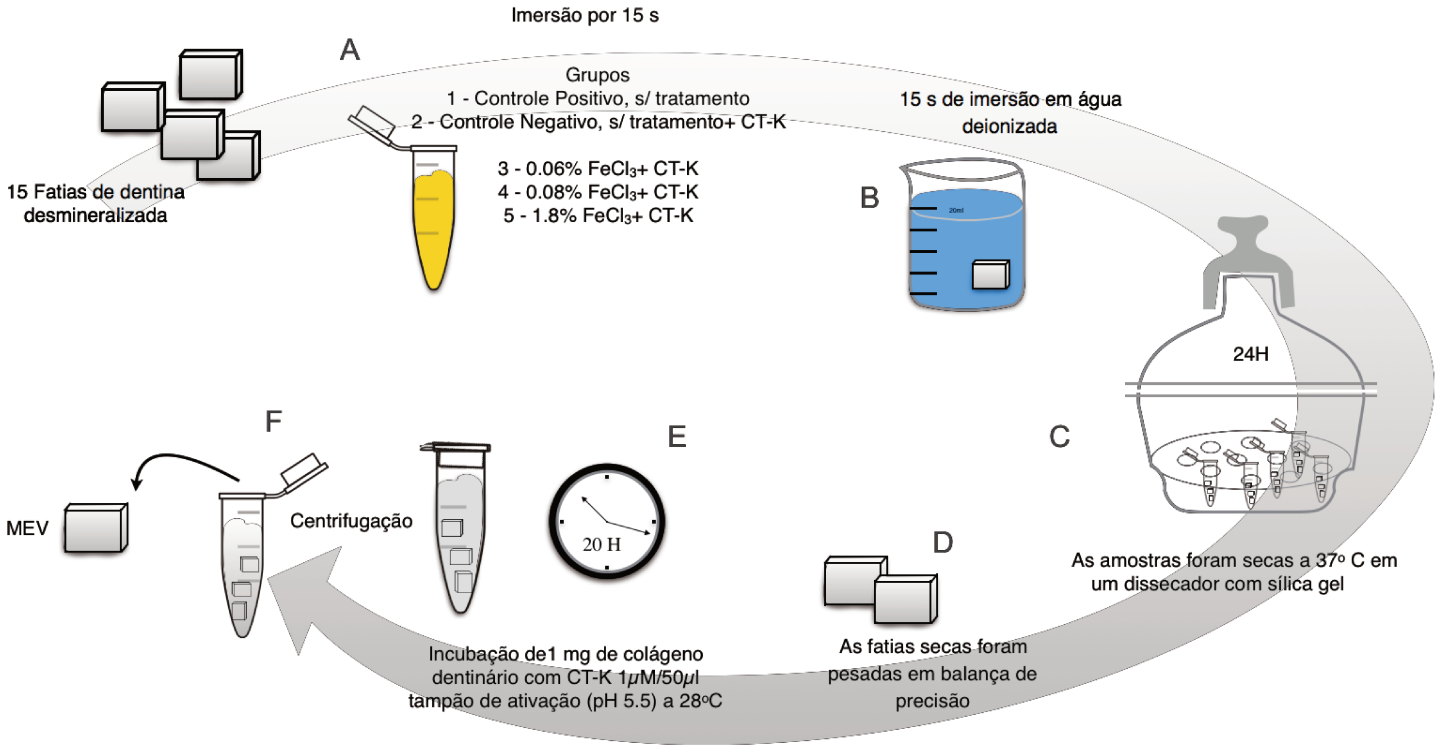


Figura 8. Esquema explicativo da preparação dos espécimes para observação em Microscopia Eletrônica de Varredura (MEV) após a incubação enzimática. Fatias de dentina desmineralizadas foram lavadas com água, fixadas em glutaraldeído por 30 minutos, lavadas com água, desidratadas com concentrações crescentes de etanol. Após a imersão em 100% de etanol, os espécimes foram transferidos para HDMS (Hexametildisilazano) para que as fibras mantivessem sua estrutura 3D. Então, os espécimes foram fixados em *stubs* metálico, cobertos por ouro e observados em MEV (JEOL, JSM-5600LV, Tokyo, Japan) 15 kV em aumentos de 2000x, 6000x, 15000x.

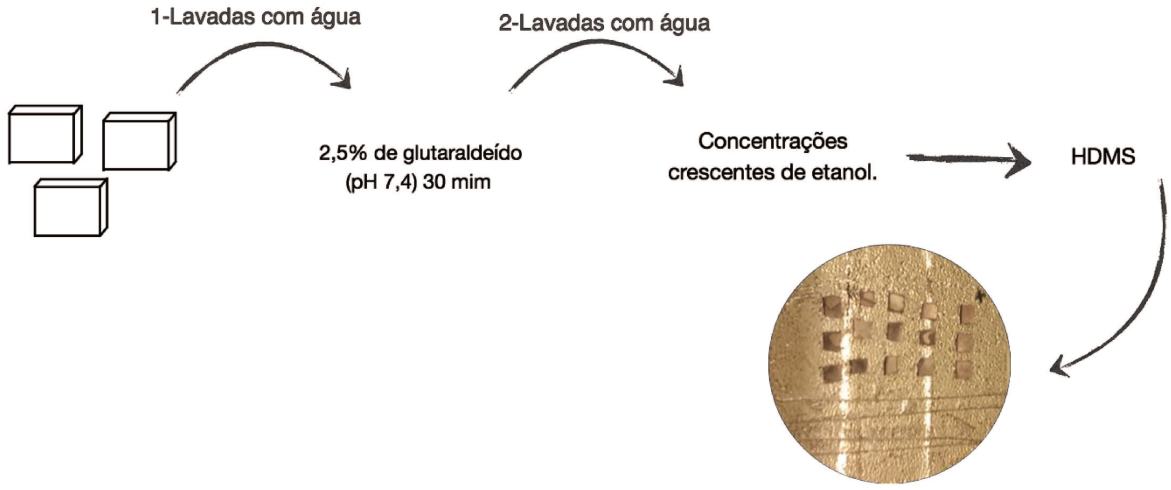


Figura 9. Esquema explicativo da preparação das amostras para análise por meio da EDX. Fatias de dentina desmineralizadas foram submetidas a soluções condicionadoras por 15 segundos, lavadas por 15 segundos e foram secas sob *stubs* acrílicos acondicionados em recipientes com sílica gel, em temperatura ambiente. Em seguida, os *stubs* foram cobertos com carbono (MED 010 Baltec, Balzers, Liechtenstein) e os espécimes foram analisados.

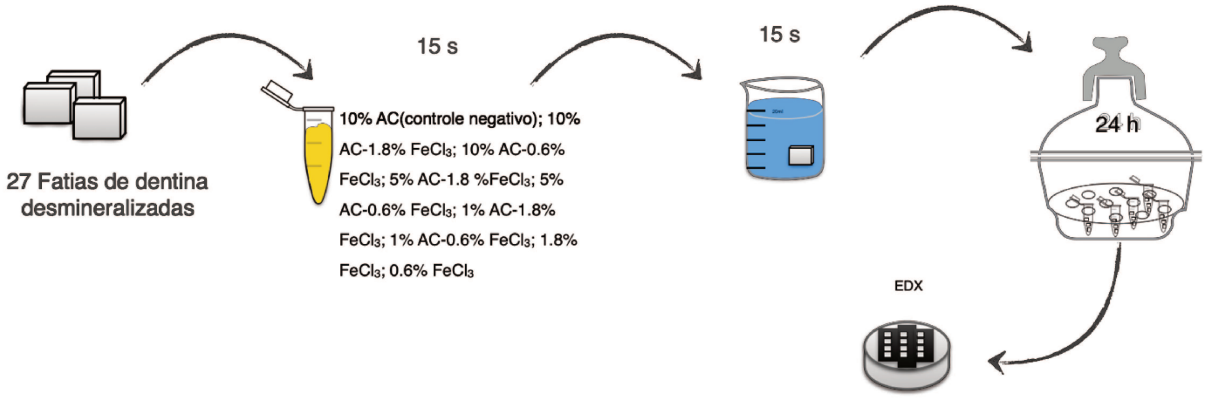
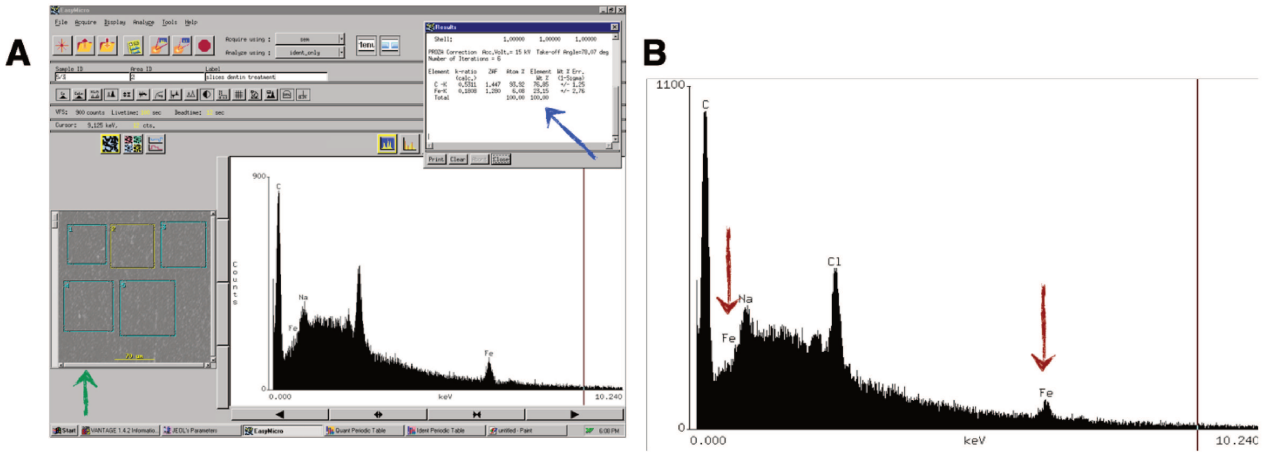


Figura 10. (A) Imagens representativas das janelas do programa do Vantage (NORAN Instruments, Middleton, WI, USA). A seta em verde indica a seleção das áreas de análise (quadrados). Seta azul mostra a janela do programa que fornece os valore de porcentagem de Fe em peso e também a relação da porcentagem atômica do elemento (B) Gráfico do espectro da superfície dentinária, as setas vermelhas no gráfico indicam a identificação da presença do Fe.



ANEXOS

ANEXO 1

Certificado de submissão para a Dental Materials

Your co-authored submission

Dental Materials <EviseSupport@elsevier.com>

qua 22/02/2017 01:28

Para:rquelrodrigues@hotmail.com <rquelrodrigues@hotmail.com>;

Dear Dr. VIANA RODRIGUES,

You have been listed as a Co-Author of the following submission:

Journal: Dental Materials

Title: Effect of conditioning solutions containing ferric chloride on dentine bond strength and collagen degradation

Corresponding Author: Ricardo Carvalho

Co-Authors: RAQUEL VIANA RODRIGUES, Marcelo Giannini, Fernanda Miori Pascon, Preety Panwar, Dieter Bromme, Adriana Pigozzo Manso

Ricardo Carvalho submitted this manuscript via Elsevier's online submission system, EVISE®. If you are not already registered in EVISE®, please take a moment to set up an author account by navigating to http://www.evise.com/evise/faces/pages/navigation/NavController.jspx?JRNL_ACR=DEMA

If you already have an ORCID, we invite you to link it to this submission. If the submission is accepted, your ORCID will be transferred to ScienceDirect and CrossRef and published with the manuscript.

To link an existing ORCID to this submission, or sign up for an ORCID if you do not already have one, please click the following link: Link ORCID

What is ORCID?

ORCID is an open, non-profit, community-based effort to create and maintain a registry of unique researcher identifiers and a transparent method of linking research activities and outputs to these identifiers.

More information on ORCID can be found on the ORCID website, http://www.ORCID.org, or on our ORCID help page: http://help.elsevier.com/app/answers/detail/a_id/2210/p/7923

If you did not co-author this submission, please contact the Corresponding Author directly at rickmc@dentistry.ubc.ca.

Thank you,
Dental Materials

This message was sent automatically. Please do not reply

ANEXO 2

Certificado do Comite de Ética e Pesquisa da Universiade de British Columbia

The University of British Columbia
Office of Research Ethics
Clinical Research Ethics Board – Room 210, 828 West 10th Avenue, Vancouver, BC V5Z 1L8

ETHICS CERTIFICATE OF EXPEDITED APPROVAL

PRINCIPAL INVESTIGATOR:	INSTITUTION / DEPARTMENT:	UBC CREB NUMBER:
Adriana Pigozzo Manso	UBC/Dentistry/Oral Biological & Medical Sciences	H15-02264

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

Institution	Site
UBC	Vancouver (excludes UBC Hospital)

Other locations where the research will be conducted:
N/A

CO-INVESTIGATOR(S):
N/A

SPONSORING AGENCIES:
N/A

PROJECT TITLE:
Alternative approaches to reduce collagen degradation in resin-dentin bonds

THE CURRENT UBC CREB APPROVAL FOR THIS STUDY EXPIRES: January 23, 2018

The UBC Clinical Research Ethics Board Chair or Associate Chair, has reviewed the above described research project, including associated documentation noted below, and finds the research project acceptable on ethical grounds for research involving human subjects and hereby grants approval.

This approval applies to research ethics issues only. The approval does not obligate an institution or any of its departments to proceed with activation of the study. The Principal Investigator for the study is responsible for identifying and ensuring that resource impacts from this study on any institution are properly negotiated, and that other institutional policies are followed. The REB assumes that investigators and the coordinating office of all trials continuously review new information for findings that indicate a change should be made to the protocol, consent documents or conduct of the trial and that such changes will be brought to the attention of the REB in a timely manner.

DOCUMENTS INCLUDED IN THIS APPROVAL:			APPROVAL DATE:
Document Name	Version	Date	
Protocol:			
Alternative approaches to reduce collagen degradation in resin-dentin bonds	2	January 6, 2017	**January 23, 2017**
Other Documents:			
Provisos	1	January 6, 2017	

CERTIFICATION:
In respect of clinical trials:
1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The documentation included for the above-named project has been reviewed by the UBC CREB, and the research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved by the UBC CREB.

Approval of the Clinical Research Ethics Board by:
Dr. Stephen Hoption Cann,
Chair