UNESP - UNIVERSIDADE ESTADUAL PAULISTA

Faculdade de Odontologia de Araraquara

DÉBORA LOPES SALLES SCHEFFEL

Citotoxicidade Transdentinária e Efeito da Carbodiimida (EDC) na Biomodificação do Colágeno Dentinário e na Degradação da Interface Adesiva

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Tese apresentada ao Programa de Pós-Graduação em Ciências Odontológicas – Área de Odontopediatria da Faculdade de Odontologia de Araraquara da Universidade Estadual Paulista, como pré-requisito à obtenção do título de Doutor em Ciências Odontológicas.

Orientadora: Prof[a] Dr[a] Josimeri Hebling

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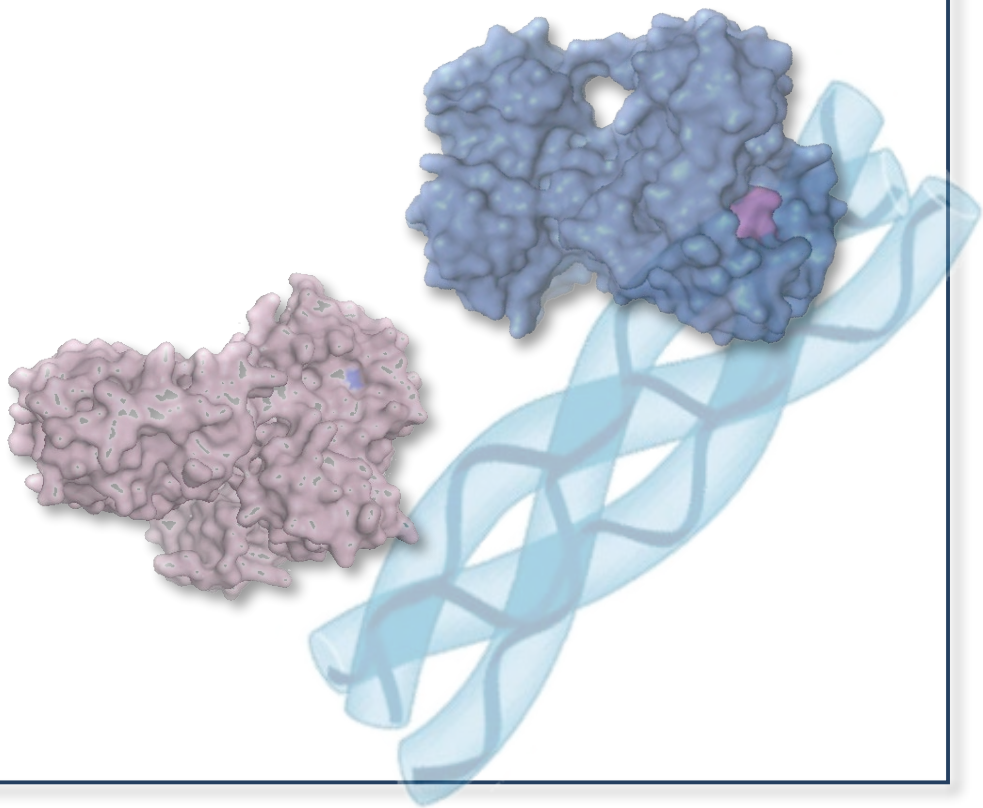
"Porque eu sei que meu Redentor vive, e que por fim se levantará sobre a terra." Jó 19:25

Dedicatória



Dedicatória

"A coisa mais indispensável a um homem é reconhecer o uso que deve fazer do seu próprio conhecimento."

Platão

À Deus, por me ouvir pacientemente, por cuidar dos detalhes da vida, por honrar os meus sonhos, por guiar os meus passos, por estar fielmente ao meu lado, pelas grandes e pequenas coisas que já fez em minha vida e pelas que com certeza Ele ainda fará.

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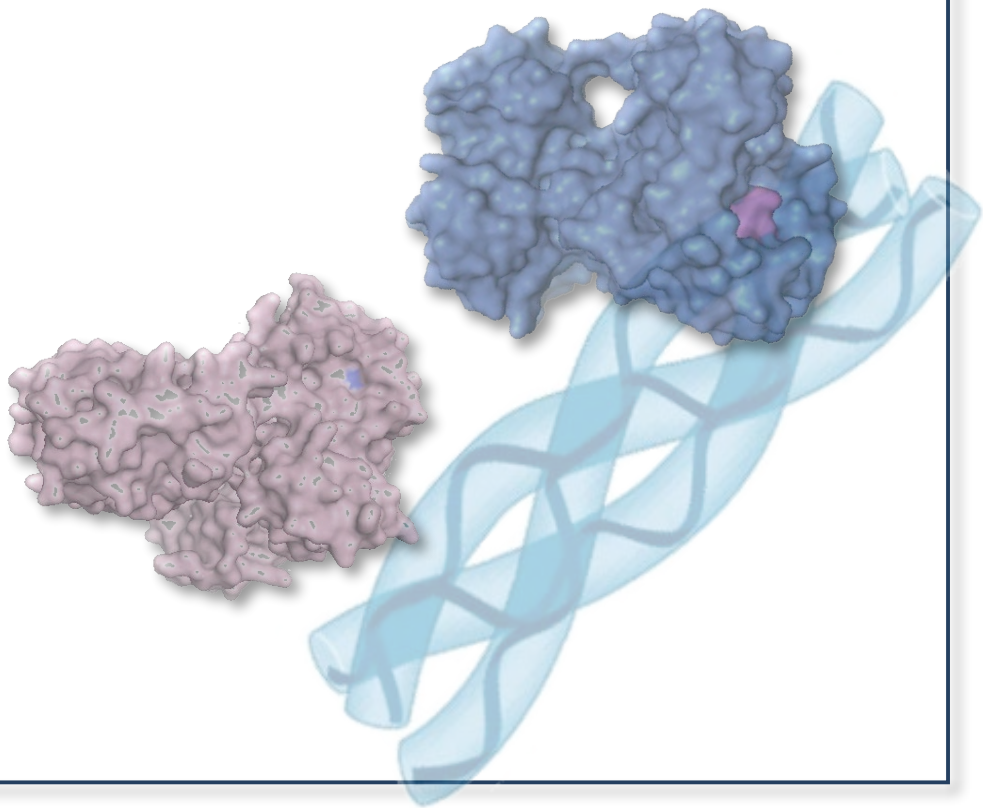

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"Cada um que passa em nossa vida, passa sozinho,
mas não vai só, nem nos deixa sós; leva um pouco
de nós mesmos, deixa um pouco de si mesmo."
Antoine de Saint-Exupéry

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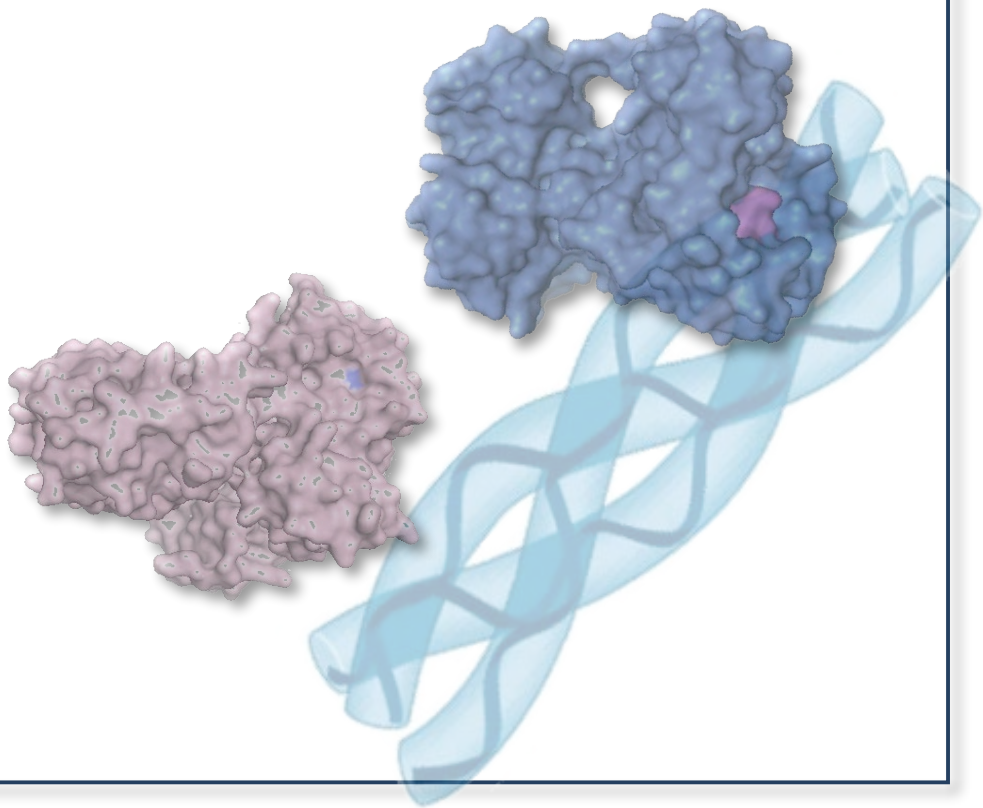

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"Devemos encontrar tempo para parar e agradecer as pessoas que fazem diferença em nossas vidas."

John F. Kennedy

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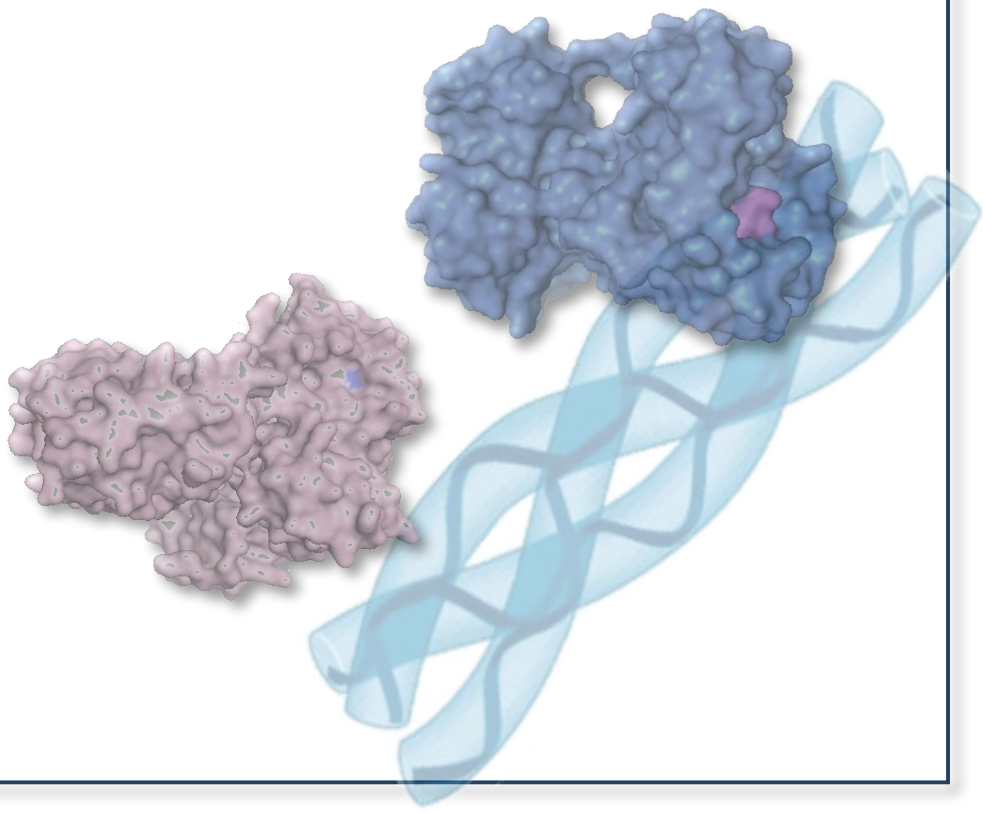
Sumário



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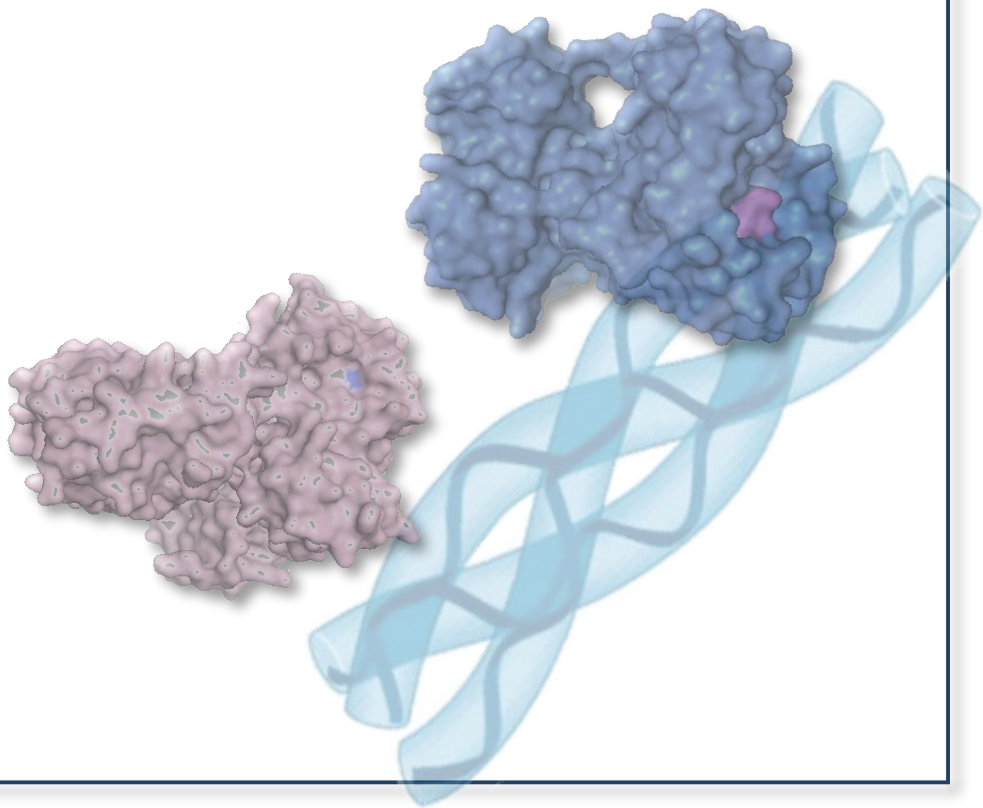


Scheffel DLS. Citotoxicidade transdentinária e efeito da carbodiimida (EDC) na biomodificação do colágeno dentinário e na degradação da interface adesiva [Tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2013.

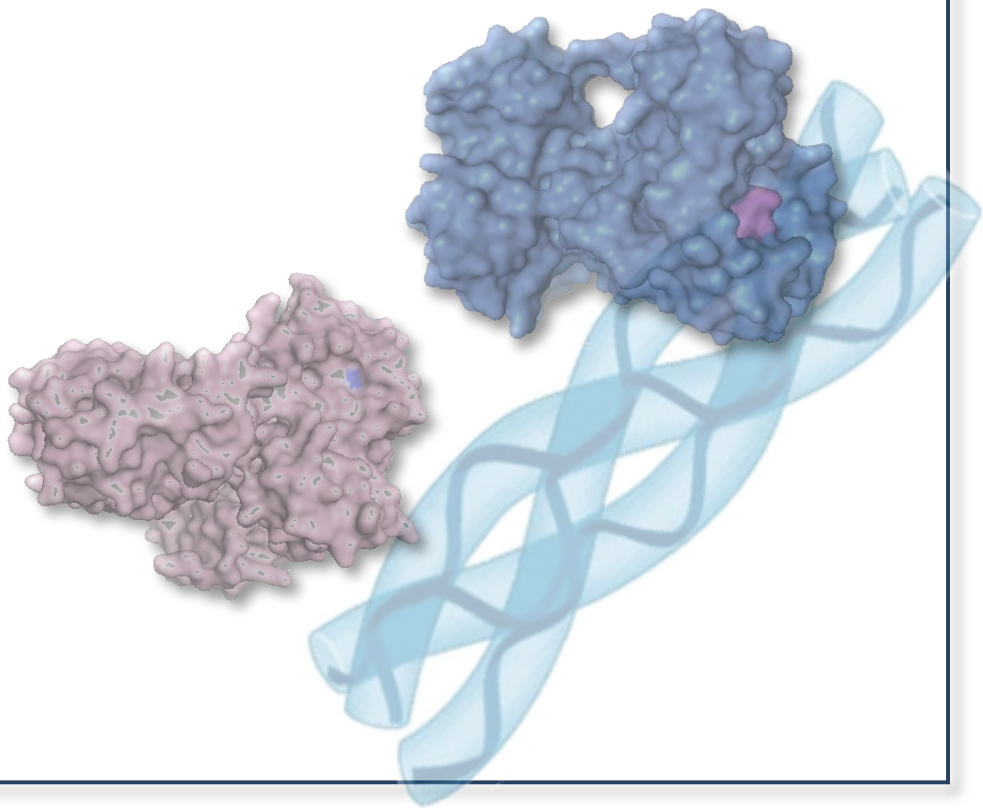
RESUMO

O objetivo geral deste trabalho foi avaliar a citotoxicidade transdentinária da carbodiimida (EDC), bem como sua influência na degradação do colágeno dentinário e na estabilidade da união resina-dentina. No estudo 1, células MDPC-23 foram plantadas na superfície pulpar de discos de dentina e a superfície oclusal foi tratada por 60s com uma das seguintes soluções: sem tratamento; EDC 0,1M; 0,3M ou 0,5M; glutaraldeído 5% (GA); tampão Sorensen ou H_2O_2 29%. A viabilidade e a morfologia celular foram analisadas pelos testes de MTT, Live/dead, produção de proteína total (PT), de colágeno e MEV. Os dados foram analisados pelos testes de Kruskal-Wallis e Mann-Whitney ($p<0,05$). O GA promoveu aumento do metabolismo celular. A morte por necrose e a morfologia celular não foram influenciadas pelos agentes *cross-linkers*. Não houve redução na produção de PT e colágeno após 7 dias. Para o estudo 2, espécimes de dentina foram completamente desmineralizados e a variação do módulo de elasticidade (E), inibição de MMP, perda de massa, liberação de hidroxiprolina (HYP) e degradação térmica do colágeno (DTC) foram analisados após tratamento com uma das seguintes soluções por 30s ou 60s: água deionizada (controle); EDC 0,5M; EDC 1M; EDC 2M e GA 10%. Os dados referentes ao E, atividade de MMP e liberação de HYP foram submetidos aos testes de Wilcoxon e Kruskal-Wallis ou Mann-Whitney. Os valores de perda de massa e DT foram analisados

pelos testes de ANOVA e Tukey ($p<0,05$). Os melhores resultados quanto ao E foram observados para o GA. Todos os *cross-linkers* reduziram a atividade de MMP e a liberação de HYP e aumentaram a temperatura de DT do colágeno. No estudo 3, sessenta palitos de dentina foram divididos em 6 grupos de acordo com a solução de tratamento: água deionizada (controle); EDC 0,1M; EDC 0,5M; EDC 0,5M + HEMA 35%; proantocianidina 5% (PA) ou clorexidina (CHX) 2%. Após condicionamento ácido os palitos foram tratados por 60s. A atividade de MMP foi analisada por ensaio colorimétrico. Os dados expressos em valores de absorbância e em equivalentes a atividade de MMP-9 foram submetidos aos testes de Kruskall-Wallis e Mann-Whitney ($p<0.05$). Todas as soluções inibiram a atividade de MMP. O EDC 0,5M e sua mistura com HEMA obtiveram os melhores resultados. Finalmente, no estudo 4, superfícies planas de dentina foram condicionadas e tratadas por 30 ou 60s com solução de EDC 0,5M ou água deionizada. Após o tratamento, o adesivo Single Bond 2 (SB2) foi aplicado e as coroas reconstruídas em resina composta. Espécimes foram produzidos e submetidos aos testes de microtração e nanoinfiltração após 24h, 6 ou 12 meses em saliva artificial. Os testes de ANOVA e Tukey ($p<0.05$) foram aplicados. O tratamento da dentina com EDC preveniu a redução da RU e o aumento da nanoinfiltração após 12 meses de armazenamento. Desta forma, pôde ser concluído que o pré-tratamento da dentina com agentes cross-linkers não exerceu efeito citotóxico trandentinário sobre células odontoblastóides, favoreceu as propriedades mecânicas do colágeno, foi capaz de inibir MMPs e prevenir a degradação da interface adesiva.

Palavras-chave: colágeno, dentina, resistência à tração, metaloproteinases da matriz

Abstract



Scheffel DLS. Trandentinal cytotoxicity and effect of carbodiimide (EDC) on dentin collagen biomodification and adhesive interface degradation [Tese de doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2013.

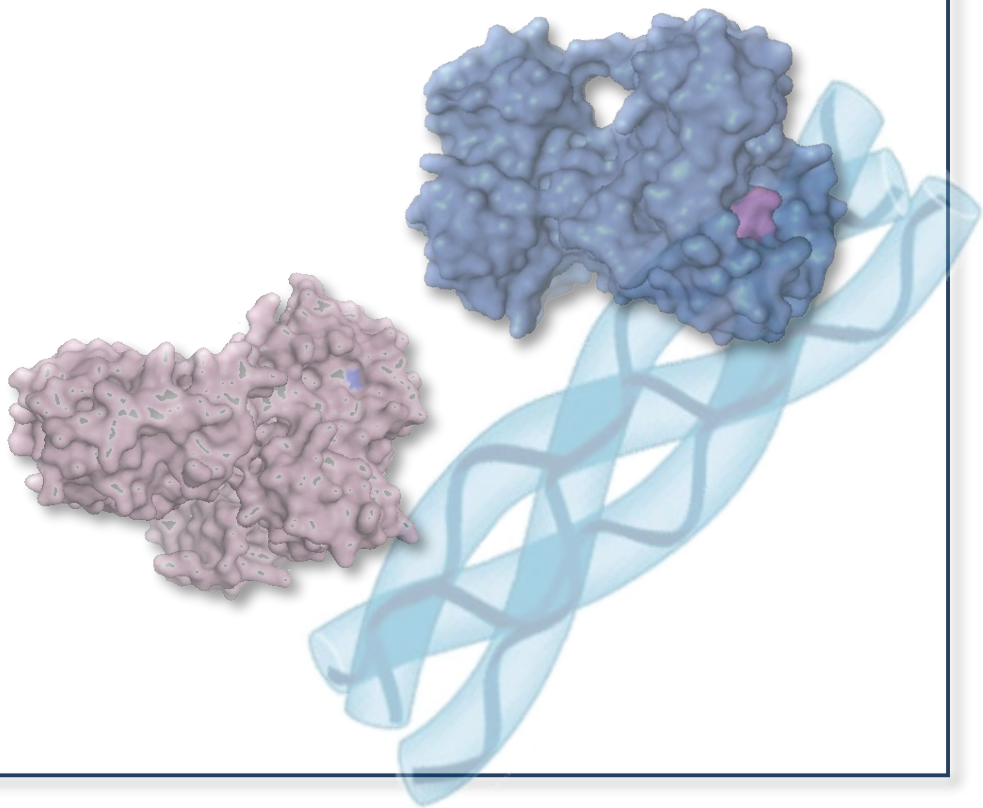
ABSTRACT

The purpose of this study was to evaluate the trandentinal cytotoxicity of carbodiimide (EDC), as well as its influence on dentinal collagen degradation and stability of resin-dentin bonds. In the first experiment, MDPC-23 cells were seeded on the pulp surface of the disks and one of the following solutions was applied on the occlusal surface for 60s: no treatment (negative control), 0.1M, 0.3M or 0.5M EDC; 5% glutaraldehyde (GA); Sorensen buffer or 29% hydrogen peroxide (positive control). Cell viability and morphology were analyzed by MTT, Live/Dead assays, total protein (TP) and collagen production and SEM. Data were analyzed by Kruskal-Wallis and Mann-Whitney tests ($p<0.05$). Only GA increased cellular metabolism. Cell death by necrosis and cell morphology were not affected by the cross-linker agents. There was no reduction in TP and collagen production after 7 days. For the second experiment, dentin beams were completely demineralized and the variation in elastic modulus (E), MMP activity, dry mass loss, hydroxyproline release (HYP) and collagen thermal degradation (CTD) were analyzed after the dentin treatment for 30s or 60s with the following solutions: water; 0.5M; 1M or 2M EDC and 10% GA. Data from E and MMP activity and HYP release were submitted to Wilcoxon and Kruskal-Wallis or Mann-Whitney tests. Dry mass loss and CTD data were analyzed by ANOVA and Tukey's tests ($p>0.05$). GA group obtained the highest E values. All cross-linking agents decreased MMP activity and HYP release and increased CTD. In the third

experiment, sixty dentin beams were randomly divided into 6 groups according to the treatment solution: deionized water (control), 0.1M EDC, 0.5M EDC, 0.5M EDC+35% HEMA, 5% proanthocyanidin (PA) or 2% chlorhexidine (CHX). The beams were acid etched and treated for 60s. The total MMP activity was analyzed by a colorimetric assay (Sensolyte®). Data were expressed as absorbance values at 412nm and MMP-9 equivalents and subjected to Kruskal-Wallis and Mann-Whitney tests ($p<0.05$). All cross-liking solutions inhibited MMPs. The 0.5 M EDC solution and its mixture with HEMA had the highest inhibition values. Finally, in experiment 4, flat dentin surfaces were etched and treated for 30 or 60s with 0.5M EDC or deionized water. After treatment, Single Bond 2 (SB2) was applied and the crowns were reconstructed with composite resin. Dentin specimens were produced and submitted to microtensile and nanoleakage tests after 24h, 6 or 12 months in artificial saliva. Bond strength (BS) data (MPa) were analyzed by ANOVA and Tukey tests ($p<0.05$). The dentin treatment with EDC did not affect SB2 immediate BS and prevented the degradation of the adhesive interface, even after 12 months of saliva storage. Thus, the dentin treatment with cross-linking agents did not exert transdentinal cytotoxic effects on odontoblast-like cells, increased collagen mechanical properties, was able to inhibit MMPs and prevent the degradation of the adhesive interface.

Keywords: collagen, dentin, tensile strength, matrix metalloproteinases

1. Introdução



INTRODUÇÃO

A camada híbrida resultante da impregnação monomérica dos espaços interfibrilares produzidos pela remoção dos minerais quando do condicionamento ácido, é considerada a principal responsável pela retenção micromecânica das restaurações resinosas e também responde, em grande parte, pelo selamento da dentina (Nakabayashi et al.[36], 1982). No entanto, esta importante estrutura é a zona mais vulnerável da interface adesiva, onde ocorre a maioria das falhas e a concentração de tensões (Van Noort et al.[61], 1989; Van Noort et al.[60], 1991; Reis et al.[44], 2013).

Além dos esforços mastigatórios, alterações de temperatura, umidade constante, e reações químicas diversas impostas pelo ambiente bucal (De Munck et al.[18], 2003), as interfaces adesivas também enfrentam obstáculos no momento de seu estabelecimento, inerentes às características do próprio substrato dentinário, como relevante heterogeneidade estrutural e composicional, além de umidade proveniente da exsudação do fluído dentinário. Assim a dentina é um grande desafio para a odontologia adesiva (De Munck et al.[18], 2003), principalmente ao que se refere a estabilidade longitudinal das interfaces produzidas neste substrato, tornando essa adesão menos previsível quando comparada àquela produzida com o esmalte (Reis et al.[45], 2008).

O tecido dentinário é complexo e altamente mineralizado, composto de 70%, em peso, de conteúdo mineral, 20% de conteúdo orgânico e 10% de água (Septier et al.[52], 2001; Embery et al.[19], 2001). O conteúdo orgânico da dentina apresenta 90% de colágeno do tipo I e 10% de proteínas não-colagenosas,

glicoproteínas e várias biomoléculas, as quais são expressas durante a síntese da matriz extracelular e aprisionadas no tecido dentinário após a finalização do processo de mineralização (Boskey[8], 1991; Marshall et al.[34], 1997).

Na dentina mineralizada, as fibrilas de colágeno são protegidas da ação enzimática pelo seu invólocro de cristais de hidroxiapatita. Uma vez destituídas dessa proteção, devido ao processo de cárie (Tjärderhane et al.[59], 1998), ou outro que envolva a remoção do conteúdo inorgânico, como o condicionamento ácido (Pashley et al.[43], 2004), essas fibrilas tornam-se susceptíveis à hidrólise e degradação enzimática mediada por proteases do próprio substrato, como metaloproteinases (MMPs) da matriz dentinária (Tjärderhane et al.[59], 1998; Fanchon et al.[21], 2004; Tezvergil-Mutluay et al.[56], 2013) e cisteínas catepsinas (Tersariol et al.[55], 2010), assim como pelas MMPs salivares e colagenases de origem bacteriana.

Após o condicionamento ácido, o encapsulamento total das fibrilas expostas por monômeros resinosos promoveria a proteção deste colágeno contra a degradação. No entanto, a profundidade de desmineralização da dentina excede a capacidade de infiltração monomérica, resultando na formação de uma zona de dentina desmineralizada não reforçada por resina na base da camada híbrida, (Nakabayashi et al.[37], 1998; Hashimoto et al.[26], 2000; Wang, Spencer[64], 2003; Spencer et al.[53], 2004; Scheffel et al.[51], 2010) observada tanto para sistemas convencionais (Wang, Spencer[64], 2003; Spencer et al.[53], 2004; Scheffel et al.[51], 2010) como para sistemas auto-condicionantes (Sano et al.[49], 1995; Oliveira et al.[41], 2004; Carvalho et al.[11], 2005).

Além disso, a eluição de hidrogéis poliméricos hidroliticamente instáveis, a qual ocorre durante a vida funcional dessa interface, contribui para o aumento das imperfeições no interior da camada híbrida, o que possibilita a ocorrência e manutenção da nanoinfiltração (De Munck et al.[18], 2003; Ferrari, Tay[22], 2003; Wang, Spencer[64], 2003; Pashley et al.[43], 2004). Este fenômeno propicia a penetração e circulação de fluidos e bioprodutos bacterianos (Sano et al.[49], 1995) e a subsequente degradação hidrolítica e enzimática (Hashimoto et al.[26], 2000; Pashley et al.[43], 2004; Carrilho et al.[10], 2009) dos constituintes da união resina-dentina, inclusive do colágeno, culminando em sua falência funcional. Diante destas evidências, vários estudos tem sido conduzidos na tentativa de modificar a matriz de colágeno para melhorar as propriedades mecânicas e a estabilidade das interfaces adesivas tornando-as menos susceptíveis a degradação (Cheung et al.[16], 1985; Nimni et al.[38], 1988; Bedran-Russo et al.[5], 2007; Bedran-Russo et al.[4], 2008; Walter et al.[63], 2008; Al-Ammar et al.[1], 2009; Castellan et al.[12], 2010; Castellan et al.[13], 2010; Green et al.[24], 2010; Bedran-Russo et al.[6], 2010; Bedran-Russo et al.[3], 2011; Pashley et al.[42], 2011; Chiang et al.[17], 2013).

Cada molécula de colágeno do tipo I é um bastão pequeno e rígido formado pelo entrelaçamento em trípla hélice de três cadeias polipeptídicas chamadas cadeias alfa, sendo elas: duas cadeias α1 e uma α2. Naturalmente, as moléculas de colágeno se unem, interna e externamente, por meio de ligações cruzadas (*cross-links*), para formar as fibrilas de colágeno. A presença de *cross-links* endógenos inter e intra-moleculares e inter-fibrilares promove a estabilidade, viscoelasticidade e resistência destas estruturas fibrilares (Yamauchi[66], 2000;

Charulatha, Rajaram[15] , 2003; Bedran-Russo et al.[6], 2010).

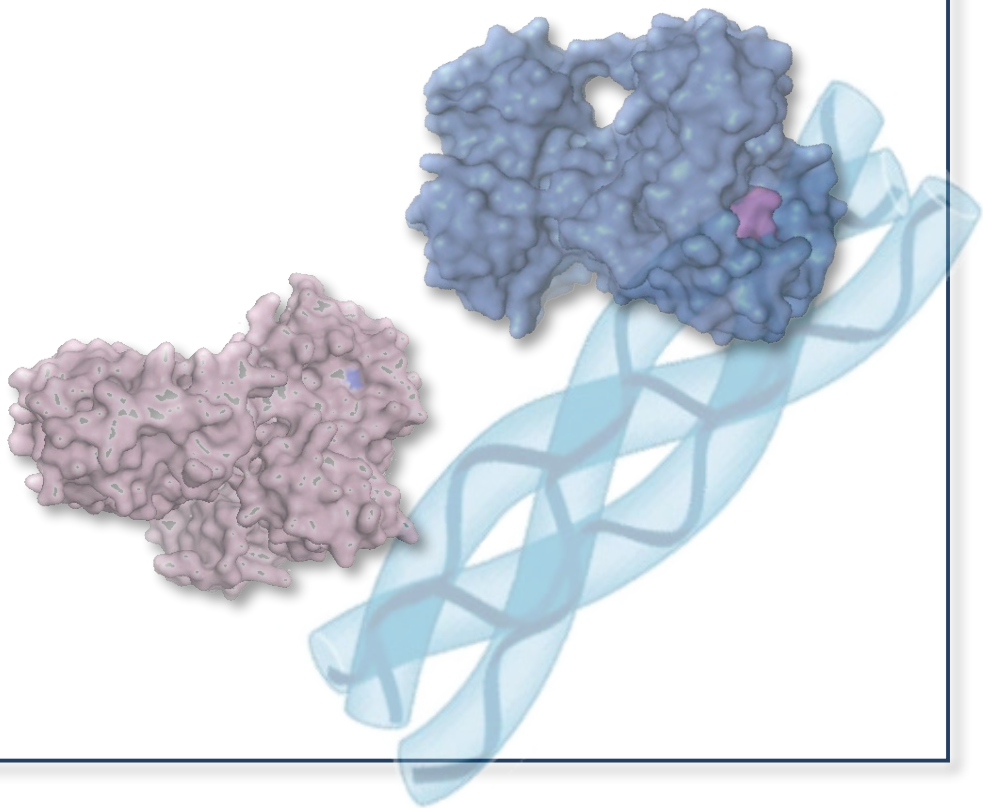
A engenharia tecidual tem utilizado algumas substâncias naturais e sintéticas para aumentar o número de *cross-links* (Sung et al.[54], 1999; Han et al.[25], 2003), visando melhorar as propriedades mecânicas e a resistência à degradação hidrolítica e enzimática das fibrilas de colágeno utilizadas como suporte para o estabelecimento das interfaces adesivas (Nimni et al.[38], 1988; Han et al.[25], 2003; Bedran-Russo et al.[5], 2007; Bedran-Russo et al.[3], 2011). Dentre estas substâncias, conhecidas como agentes de ligação cruzada (*cross-linkers)*, estão o glutaraldeido (GD), a proantocianidina (PA) e a carbodiimida (1-Etil-3-[3-dimetilaminopropil] hidroclorito de carbodiimida ou EDC) (Bedran-Russo et al.[4], 2008; Al-Ammar et al.[1], 2009; Bedran-Russo et al.[7], 2009; Bedran Russo et al.[6], 2010).

O EDC é o isômero de cianamida mais estável, é capaz de agregar aminoácidos em peptídeos, sendo um *cross-linker* inespecífico de capacidade única para ativar o grupo carboxila dos ácidos glutâmico e aspártico presentes nas cadeias proteicas (Timkovich[58], 1977; Zeeman et al.[67], 1999), resultando em *cross-links* sem grupos reativos residuais (Olde Damink et al.[39], 1996; Olde Damink et al.[40], 1996). Além disso, as carbodiimidas tem apresentado resultados superiores ao GD em termos de biocompatibilidade (van Wachem et al.[62], 1994; Chang et al.[14], 2001). No entanto, não existem na literatura trabalhos que avaliassem a citotoxicidade do EDC em células odontoblastóides, bem como sua eficácia na melhoria da resistência de união da interface resina-dentina em tempos menores que 10 min ou horas de aplicação (Bedran-Russo et al.[6], 2010) in vitro.

Dessa forma, o presente trabalho teve como objetivos (1) investigar o

potencial citotóxico do EDC sobre células odontoblastóides MDPC-23, (2) assim como o efeito da aplicação deste agente sobre as propriedades mecânicas do colágeno desmineralizado e sobre a atividade de MMPs in situ, além de (3) avaliar sua eficácia em prevenir a degradação imediata e em longo prazo da união resina-dentina.

2. Proposição



2 PROPOSIÇÃO GERAL

Avaliar a citotoxicidade transdentinária de agentes formadores de ligações cruzadas (*cross-linkers)* em células odontoblastóides e o efeito da aplicação destas substâncias na biomodificação do colágeno dentinário, na inativação de MMPs e na degradação da união resina-dentina.

Proposição Específica

Estudo 1: Transdentinal cytotoxicity of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and glutaraldehyde solutions on odontoblast-like cells

Avaliar a citotoxicidade transdentinária de três diferentes concentrações de EDC e glutaraldeído 5% em células odontoblastóides MDPC-23.

Estudo 2: Stabilization of dentin matrix after cross-linking treatments, in vitro

Investigar o efeito do tratamento da dentina completamente desmineralizada com EDC ou glutaraldeído nas propriedades mecânicas e na degradação do colágeno dentinário, bem como na inativação de MMPs.

Estudo 3: Inactivation of Matrix-bound MMPs by Cross-linking Agents in Acid Etched Dentin

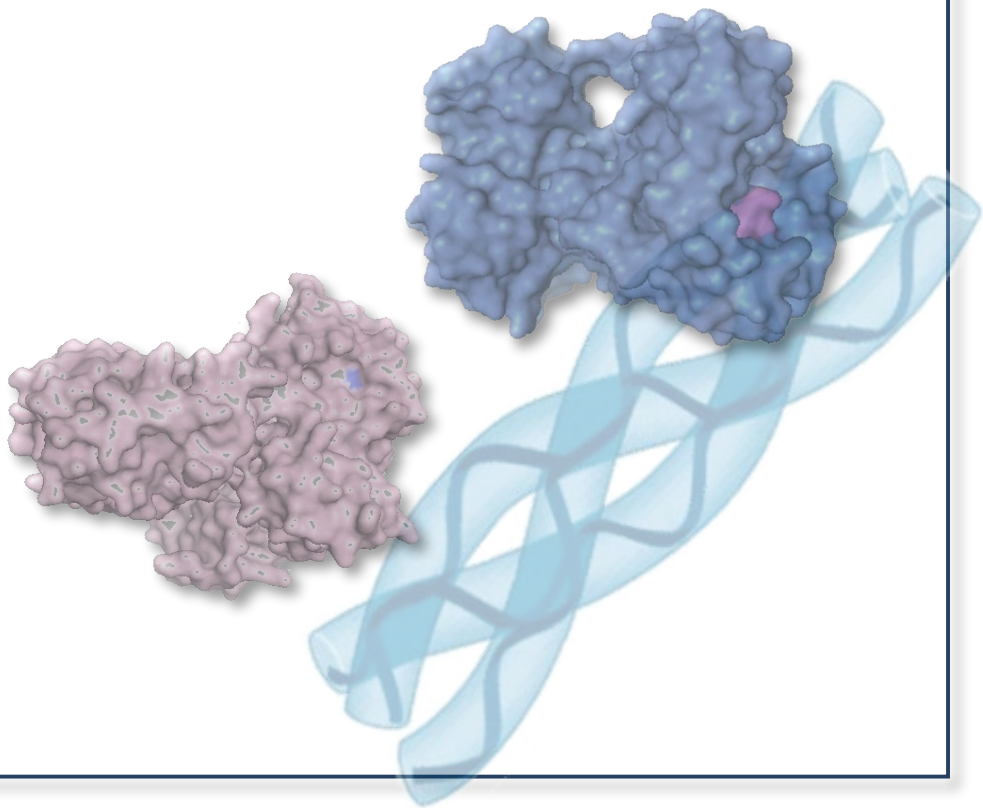
Avaliar a inativação de MMPs por EDC, proantocianidina ou clorexidina na dentina condicionada.

Estudo 4: Increased Durability of Resin-Dentin Bonds Following Cross-linking Treatment

Avaliar o efeito do tratamento da dentina condicionada com EDC na resistência de união imediata e em longo prazo e na ocorrência de nanoinfiltração utilizando um sistema adesivo simplificado.

Os dentes utilizados nos estudos que compõem esta tese foram doados pelo Banco de Dentes da Faculdade de Odontologia de Araraquara e utilizados após aprovação do Comitê de Ética em Pesquisa da mesma instituição (Anexo).

3. Estudo I



TRANSDENTINAL CYTOTOXICITY OF 1-ETHYL-3-(3-DIMETHYLAMINOPROPYL) CARBODIIMIDE HYDROCHLORIDE (EDC) AND GLUTARALDEHYDE SOLUTIONS ON ODONTOBLAST-LIKE CELLS

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Key words: cross-linking agents, cytotoxicity, dentin, odontoblasts

ABSTRACT

This study aimed to evaluate the transdentinal cytotoxicity of three different concentrations of EDC and 5% GA on MDPC-23 cells. Seventy 0.4mm-thick dentin disks obtained from sound human molars were adapted to artificial pulp chambers. Using a culture medium (DMEM) the cells ($3x10^4$) were seeded on the pulpal surface of the disks. After 48h, the occlusal dentin was acid-etched, rinsed, carefully dryed and treated for 60s with one of the following solutions (n=10): no treatment (negative control); 0.1M EDC; 0.3M EDC; 0.5M EDC; 5% Glutaraldehyde (GA); Sorensen´s buffer or 29% hydrogen peroxide (positive control). Cell viability and morphology were assessed by MTT assay and SEM, respectively. The eluates (DMEM + products that diffused through the disks) were collected after the treatments and applied on new MDPC-23 to analyze cell death, total protein (TP) and collagen production. The two last tests were performed 24h and 7 days after the contact. Data were analyzed by Kruskal-Wallis and Mann-Whitney tests ($p<0.05$). EDC did not reduce cell viability, which was increased by 5% GA. Cell death by necrosis was not elicited by EDC or 5% GA. At 24h period, 0.3M and 0.5M EDC reduced TP production by 18% and 26.8%, respectively. At 7 days, increased TP production was observed in all groups. Collagen production at the 24h period was reduced when 0.5M EDC was used. After 7 days, no difference was observed among the groups. SEM showed no alteration in cell morphology and number, except to positive control group. The treatment of acid-etched dentin with EDC or GA did not cause transdentinal cytotoxic effects on odontoblasts-like cells.

INTRODUCTION

The biodegradation of resin-dentin bonds is a complex process that involves the leaching of monomers that infiltrated the demineralized dentin matrix and the enzymatic cleavage of exposed collagen fibrils (Sano et al. 2006; Spencer et al. 2010). Therefore, the resistance of the adhesive interface components against degradation determines its stability and durability (Van Meerbeek et al. 2003; Hashimoto et al. 2000; Breschi et al. 2008; Sano et al. 2006).

Collagen fibrils that were not encased by monomers during the bonding procedure (Breschi et al. 2004; Spencer et al. 2004), as well as those exposed by the polymer degradation over time are highly susceptible to enzymatic hydrolysis (Tjärderhane et al. 1998; Fanchon et al. 2004; Tezvergil-Mutluay et al. 2013; Pashley et al. 2011; Mazzoni et al. 2012). Matrix metalloproteinases (MMPs) and cysteine cathepsins have been identified in sound and caries affected-dentin (Tjärderhane et al. 1998; Tersariol et al. 2010; Toledano et al. 2010; Nascimento et al. 2011; Tezvergil-Mutluay et al. 2013) and are alleged responsible for the disappearance of portions of the hybrid layers (Armstrong et al. 2004; Hebling et al. 2005; Mazzoni et al. 2012). Thus, improving collagen's resistance against enzymatic degradation and inactivating these proteases are important steps to enhance the quality and longevity of dentin bonding.

The treatment of demineralized dentin with EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) and glutaraldehyde (GA) increases dentin collagen mechanical properties (Bedran-Russo et al. 2010) and

inhibits MMPs (Tezvergil-Mutluay et al. 2012; Scheffel et al. 2013) by creating new cross-links among proteins peptide chains. EDC is capable of forming covalent peptide bonds between proteins by activating the free carboxyl group of glutamic and aspartic acids (Timkovich, 1977; Zeeman et al. 1999); while GA reacts with the ε-amino groups of lysyl or hydroxylysyl residues to induce the formation of intra and intermolecular cross-links (Sung et al. 1999; Nimni et al. 1988).

Although the effects of EDC and GA have been successfully demonstrated on collagen biomodification (Bedran-Russo et al. 2008; Bedran-Rsso et al. 2010), rendering collagen more resistant to degradation by improving its mechanical properties such as elastic modulus and ultimate tensile strength, and MMP inhibiton (Tezvergil-Mutluay et al. 2012; Scheffel et al. 2013; Scheffet et al. unpublished data), there is few information about their cytotoxicity. The carbodiimides have shown better results than GA in terms of biocompatibility on U937 macrophage-like cells (McDade et al. 2013), rats' ocular cells/tissue (Lai et al. 2010) and corneal endothelial cells (Lu et al. 2008). However, there are no studies that have tested the transdentinal cytotoxicity of these cross-linking agents on odontoblast-like cells and confirmed if these substances are safe when applied on acid-etched dentin.

Thus, the aim of this study was to evaluate the transdentinal cytotoxicity of different concentrations of EDC and 5% GA on odontoblast-like cells. The tested null hypothesis was that the treatment of acid-etched dentin with cross-

linkers for 60 s does not exert cytotoxic effect on the target cells.

MATERIALS AND METHODS

Preparation of dentin disks and permeability reading

Seventy sound human third molars were obtained upon approval by the Ethics Committee of the School of Dentistry at Araraquara – UNESP, and were stored in 0.12% thymol solution at 4^{o}C. The teeth were used within 3 months after extraction. One 0.5 mm-thick dentin disk was obtained from the mid-coronal dentin of each tooth using a precision cutting machine equipped with a water-cooled diamond saw (Isomet 1000, Buehler Ltda., Lake Bluff, IL, USA). The disks were carefully examined with a stereoscopic microscope (SZX7, Olympus, São Paulo, SP, Brazil) to confirm the absence of enamel islets and defects resulting from pulp horn projections. Then, the occlusal side of the disks was manually polished with wet 320-grit silicon carbide paper to reach a final thickness of 0.4 mm, as measured with a digital caliper accurate to the nearest 0.01 mm (Mitutoyo South Americana Ltd, Suzano, SP, Brazil).

Dentin permeability was determined to permit a homogeneous distribution of the dentin disks into the groups. The smear layer produced on both sides of the disks was removed by application of 0.5 M EDTA (pH 7.4) for 60 s, followed by abundant rinsing with deionized water. For determination of the hydraulic conductance, the disks were individually placed in *in vitro* pulp chambers (IVPCs) modified from Hanks et al. (1988). A metallic cannula connected the IVPC to a 180 cm column of water. The disk remained under this pressure for 5 min, after

which time the movement of a microbubble introduced through the cannula was recorded during 1 min and the obtained values transformed into conductance values. Then, the dentin disks were allocated into 7 groups (n=10), in such a way that the dentin permeability was statistically similar among the groups (ANOVA, $p>0.05$).

MDPC-23 Cell Seeding on Dentin Disks

MDPC-23 cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Aldrich Corp., St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Cultilab, Campinas, SP, Brazil), 100 IU/mL penicillin, 100 ìg/mL streptomycin and 2 mmol/L glutamine (Gibco, Grand Island, NY, USA) in an humidified incubator with 5% CO_2 and 95% air at 37^{o}C (Isotemp Fisher Scientific, Pittsburgh, PA, USA). The cells were sub-cultured each 3 days until reaching the number of cells necessary to perform the study.

To simulate clinical conditions, the smear layer was re-created on the occlusal side of each disk with a 600-grit silicon carbide paper for 10 s. Then, the disks were placed in modified IVPCs and both sterilized in ethylene oxide. MDPC-23 cells ($3x10^4$) were seeded on the pulpal side of the dentin disks (0.28 cm^2). In order to accomplish that, the IVPCs were placed in an inverted position (pulp side up) into the compartments of 24-well plates (COSTAR 3595 - Corning Incorporated, Corning, NY, USA) and maintained in an incubator with 5% CO_2 and 95% air at 37^{o}C for 48 h for adherence of cells. After this time, the IVPCs were carefully removed from the compartment and returned to the same compartment with the occlusal side up to receive the treatment solutions.

Application of the cross-linking solutions

Four treatment solutions (10 disks *per* solution) were prepared diluting the cross-linking agent in Sorensen buffer (pH 6.2): 0.1 M EDC (pH 6.04); 0.3 M EDC (pH 5.98); 0.5 M EDC (pH 5.93) and 5% GA (pH 5.8). In addition, three more conditions were tested (n=10): only Sorensen buffer, 29% hydrogen peroxide (positive control) and no treatment (negative control). The occlusal surface of the dentin disks was etched with 35% phosphoric acid (Scotchbond etchant, 3M ESPE. St. Paul, MN, USA) for 15 s, carefully rinsed with deionized water for 10 s and blot dried with sterilized cotton pellets. Then, 20 ìL of the pre-determined treatment solution (cross-linking solutions, Sorensen buffer and controls) were applied for 60 s, followed by water rinsing and blot drying. All procedures were performed in a vertical laminar flow chamber to prevent contamination and, immediately after, the IVPCs were placed again in a CO_2 incubator for additional 24 h.

Analysis of cell viability (MTT assay)

Eight out of the 10 disks of each group were randomly selected for cell viability analysis using the methyltetrazolium (MTT) assay. The eluates (DMEM + products that diffused through the dentin discs) from each well were collected and frozen for subsequent analysis of type of cell death as well as type 1 collagen and total protein production. After 24-hour incubation, the disks were removed from the IVPCs and individually placed in sterilized wells of new 24-well plates with the pulpal side containing the MDPC-23 cells turned upwards. Then, 900 µL of fresh DMEM and 100 µL of MTT solution (Sigma-Aldrich, St Louis, MO,

USA) (5 mg/mL sterile PBS) were placed in contact with the disks. The cells were incubated with the MTT solution at 37^{o}C for 4 h. Next, the MTT solution was aspirated and 400 ìL of acidified isopropanol solution were added (0.04 N HCl) in each well to dissolve the violet formazan crystals producing a homogeneous purple solution.

Three 100 ìL aliquots of each well were transferred to wells of 96-well plates (Costar Corp., Cambridge, MA, USA) and read at 570 nm wavelengths with an ELISA plate reader (Thermo Plate, Nanshan District, Shenzhen, Gandong, China). The values obtained from the three aliquots were averaged to provide a single value for each sample.

SEM cellular morphology analysis

Two disks from each group were randomly selected and prepared for SEM analysis. The cells were fixed in 2.5% glutaraldehyde (Sigma-Aldrich) in PBS for 1 h at room temperature. Next, the glutaraldehyde was aspirated and the cells were rinsed with PBS, post-fixed with 1% osmium tetroxide (Electron Microscopy Science, Fort Washington, PA, USA) for 1 h and rinsed again with PBS, followed by dehydration with ascending series of water–ethanol solutions (30%, 50%, 70%, 95%, and 100%) two times for 60 min each.

The cells were immersed for 60 minutes (three 20-minute changes) in 1,1,1,3,3,3-hexamethyldisilazane (HMDS, ACROS Organics, Morris Plains, NY, USA). Finally, the specimens were mounted on metallic stubs and stored in a desiccator for 24 hours, and sputter-coated with a gold layer (SDC 050; Bal-Tec

AG, Balzers, Germany), and their morphology was examined with a scanning electron microscope (DSM 960, Carl. Zeiss Inc., Oberkochen, Germany).

Cell Membrane Damage Measurements (cell death)

To analyze the cell membrane damage the Live/Dead Cell Viability/Cytotoxicity Kit (Invitrogen, San Francisco, CA, USA) was used. This assay uses the fluorescence probe Ethidium homodimer-1 (EthD-1) that binds to DNA bands only in cells with cell membrane rupture. The second probe is the Calcein AM (CA), which is hydrolyzed by cytoplasmic esterases in viable cells. MDPC-23 cells were seeded in a 24-wells plate (n=5) and exposed to the eluate collected after the dentin treatment for 24h. Then, the supernatant was centrifuged (4,000 rpm for 2 min), resuspended with DMEM and returned to its original well. The plate was centrifuged (4,000 rpm for 2 min) in order to allow the cells to precipitate, and the cells were incubated with 2 mM CA and 4 mM Eth-1, and Hoechst (1:5000) for nuclear staining, during 15 min. The fluorescence was analyzed by In Cell Analyzer 2000 (GE Healthcare Life Sciences, Freiburg, Germany) in 6 fields *per* well. The percentage of dead (positive Eth-1 staining) and live (positive CA staining) cells were calculated from the cells stained with Hoechst with the software In Cell Investigation (GE Healthcare Life Sciences), and the average value per well was used for statistical analysis.

Total Protein (TP) Production

Total protein (TP) production was evaluated according to the Read

and Northcote protocol (1981), as previously described by Basso et al. (2013). The eluate collected after dentin treatment (n=5) was placed in contact with MDPC-23 cells seeded in a 24-wells plate for 24h. Total protein production was also evaluated 7 days after had been placed in contact with EDC solutions. In both instances, the eluates were frozen for total collagen production detection and the cells were washed three times with 1 mL PBS at 37^{o}C and 1 mL of 0.1% sodium lauryl sulfate (Sigma-Aldrich Corp.) were added to each well for 40 min at room temperature to produce cell lysis. After homogenization an aliquot of 1 mL of each well was transferred to Falcon tubes while the blank tube received 1 mL of distilled water (TP protocol was performed in 24-wells plate, not in Falcon tubes). Next, the Lowry reagent solution (Sigma-Aldrich Corp.) was added (1 mL) to all samples, the tubes were agitated for 10 s and after 20 min, 500 mL of Folin-Ciocalteau's phenol reagent solution (Sigma-Aldrich Corp.) were added to each sample and homogenized. Three 100 µL aliquots of each tube were transferred to a 96-well plate after 30 min and the absorbance was read at 655 nm in an ELISA plate reader (Thermo Plate). The average of the three values was used for statistical analysis. Absorbance values were transformed into percentage, and the average of the control group was considered 100% of total protein production.

Collagen Production

Collagen production by MDPC-23 cells was evaluated 24 h and 7 days after contact with EDC and GA solutions. The 24 h and 7 days eluates were defrosted and a 200 µL aliquot from each tube was transferred to a sterilized well

of new 24-well plates. Then, 500 µL of Sirius Red dye (Sirius Red powder in picric acid, 0,1%, Sigma-Aldrich, St. Louis, MO, USA) were added to each well and the plate was incubated under agitation for 1 hour at 37^{o}C. The content of each well was put in 1.5 mL tubes, thus enabling samples centrifugation at 12000 rpm. Supernatant was discarded to subsequent addiction of 750 µL HCl to each tube. Once again, samples were centrifuged for 10 minutes, supernatant was discarded and 250 µL NaOH added. After that, three 100 µL aliquots were transferred to a 96-wells plate and read at 555 nm wavelengths with (Synergy H1 Hybrid Reader, Biotek, Winooski, VT, USA). The values obtained from the three aliquots were averaged to provide a single value for each sample. Total production of collagen was calculated based on standard curve performed with predetermined concentrations of this protein.

STATISTICAL ANALYSIS

Data from the response variables (cell viability, cell death by necrosis, production of total protein and type I collagen) were not normally distributed and were analyzed by the application of Kruskal-Wallis and Mann-Whitney non-parametric tests. The latter was used for pairwise comparison between groups. For all statistical tests $p<0.05$ was taken as statistically significant.

RESULTS

MDPC-23 cells viability and death by necrosis after contact with the transdentinal diffused components of different concentrations of EDC and 5%

glutaraldehyde (GA) are shown in Figure 1. None of the EDC concentrations differed from the negative control or Sorensen's buffer regarding cell viability (Figure 1a). Only 5% GA increased the cell viability, although it was not statistically different from the EDC solutions, irrespective of the concentration. Cell death by necrosis (Figure 1b) was not elicited by any of the investigated EDC concentrations or by 5% GA. The percentage of cell death varied from 0.5 to 1.9% for EDC groups. Variations on TP production are expressed as percentage in Table 1, all the values were calculated based on 24h-control. At 24-h, only 0.3 M and 0.5 M EDC negatively interfered with TP production. Compared to control (101.2%), the cell viability for these groups was 82.0% and 63.2%, respectively (Table 1, column). Lack of effect on TP production was seen for 0.1M EDC and 5% GA, which did not differ from the control. However, at 7 days, significant increase in TP production occurred in all groups compared to 24-hour period (Table 1, rows). Collagen production at 24-hour period was statistically significant reduced only when used 0.5 M EDC in comparison to the negative control. Significant increase in the production of collagen from 24 hours to 7 days was seen only for 0.5M EDC (Table 2, lines). The SEM analyses confirmed the results observed in the MTT and Live/Dead assays. A large number of odontoblast-like MDPC-23 cells remained adhered to the dentin disks in all groups (Figrues 2A, B, C, D, E, F and G), except in the positive control (29% hydrogen peroxide, Figure 2H). In the later, wide cell-free zones and large areas of residual fragments of dead cells were observed. In the negative control, EDC and GA groups, the cultured cells were near to confluence and exhibited abundant

cytoplasm with numerous thin cytoplasmic projections that seemed to be adhering the cells to the dentin substrate (Figure 2B).

DISCUSSION

This study evaluated the transdentinal cytotoxicity of EDC solutions on odontoblast-like cells. The IVPCs containing dentin disks were used in order to simulate extremely deep cavities in which MDPC-23 cells were indirectly exposed to the tested solutions. It approximates the *in vitro* test to the *in vivo* condition where the odontoblasts are the first cells to be in contact with components diffused through the dentin (Lanza et al., 2009). The sound dentin is a tubular and humid tissue capable of protect the pulp even at a thickness as thin as 0.5 mm (Lanza et al., 2009; Hanks et al., 1988). To increase cellular aggression and facilitate the evaluation of the cytotoxic potential of each solution, 0.4 mm-thick disks were used in the present study.

EDC has been investigated in dentistry as a cross-linking agent that applied directly on demineralized collagen increases its resistance against enzymatic degradation (Bedran-Russo et al., 2010) and inhibits MMPs (Tezvergil-Mutluay et al., 2013; Scheffel et al., 2013). In medical field, EDC has been used to reinforce different structures for tissue engineering applications, presenting satisfactory biocompatibility. This agent may support drug delivery systems development (Lai., 2013), bioprosthetic heart valves construction (McDade et al., 2013), preparation of collagen scaffold (Grant et al., 2013) and a large number of other purposes (Wu et al., 2013; Ruiz et al., 2013; Fan et al., 2013; Anisha et al.,

2013). However, these procedures use EDC to treat biomaterials previously to their contact with cells/tissues. The same is not observed when EDC is applied on dentin, since its tubular and porous structure allows this substance to diffuse and reach the odontoblast-like cells concomitant to its application.

Glutaraldehyde (GA) is used in many different processes as fixative, cross-linking and disinfecting agent. As EDC, GA participates in the construction of bioprosthetic heart valves (Mc Dade et al., 2013; Naso et al., 2013), inhibits proteases (Scheffel et al., unpublished data) and modifies gelatins and other materials and tissues (Lin and Tsai, 2013; McDade et al., 2013; Gomes et al., 2013). In dental treatments, GA has been used as a desensitizer that reacts with plasma proteins in dentin to precipitate them, blocking dentin tubules (Maita et al., 1991). Wiegand et al. (2008) showed that a GA-containing desensitizer applied on 1.0 mm-thick dentin specimens under simulated pulpal flow conditions did not present toxic effects on L-929 fibroblasts. The same was observed by Camps et al., (2002) using 0.5 mm-thick dentin slices. In agreement with these authors, the results of this study showed that 5% GA did not exert harmful effects on MDPC-23 cells when applied on 0.4 mm-thick dentin disks.

In the present study the cross-linking solutions were applied for 1 minute on the occlusal side of acid-etching dentin disks and then rinsed with deionized water prior to the analysis of the solutions cytotoxic effects. The MTT assay revealed that none of the EDC concentrations differed from the negative control or Sorensen's buffer regarding cell viability. While 5% GA was able to slightly increase the cellular metabolism. The good results obtained may be favored by the

water rinsing that removed the residual reagents after the treatment.

The cell death by necrosis occurs after irreversible cell damage generated by a chemical, physical or biological injury. The live/dead assay data showed that EDC and GA presented no difference regards to percentage of cell necrosis compared to the negative control group (no treatment). Oppositely, 29% hydrogen peroxide (positive control) induced between 90%-100% of cell death by necrosis. Corroborating cell viability and cell death findings, SEM that was used in this study as a descriptive and qualitative method, showed no significant alterations on the phenotypical characteristics and/or number of cells to all groups, except to positive control.

The total protein (TP) and collagen production (CP) were analyzed 24h and 7 days after the application of the treatment solutions. Only 0.3 M and 0.5 M EDC significantly reduced 24h-TP production compared to control group. However, this reduction was no longer significant after 7 days, what suggest that if EDC causes any alteration in the cell function it is reversible in a short period of time and does not affect cell viability. In spite of GA had been similar to the control group (no treatment) in 24h, it was not observed after 7 days when GA, 0.3 M EDC and Sorensen buffer treatments increased PT production. The analysis of collagen concentration in the eluate showed that 0.5M EDC reduced collagen production 24 h after its application. However, after 7 days, no difference was observed to any treated group when compared to control.

The results from the different tests are in agreement, showing that EDC or GA applied on acid-etched dentin for 60 s and then rinsed with deionized water

did not exert cytotoxic effect on odontoblasts-like cell MDPC-23 even when the remaining dentin is as thin as 0.4 mm. These finds requires the acceptance of the tested null hypothesis. In addition, MTT data associate with PT production seems to indicate that GA was able to slightly increase cell metabolism. This is a pioneering study in transdentinal cytotoxicity of cross-linking agents and showed that the use of these substances in dental practice to enhance resin-dentin bond quality may be safe. Future studies are necessary to better understand the interaction of these agents with pulp tissue and their behavior *in vivo*.

CONCLUSIONS

According to the methodology used in the present investigation, it may be concluded that the treatment of acid-etched dentin with EDC or GA for 60 s does not cause transdentinal cytotoxic effects on cultured odontoblasts-like cells.

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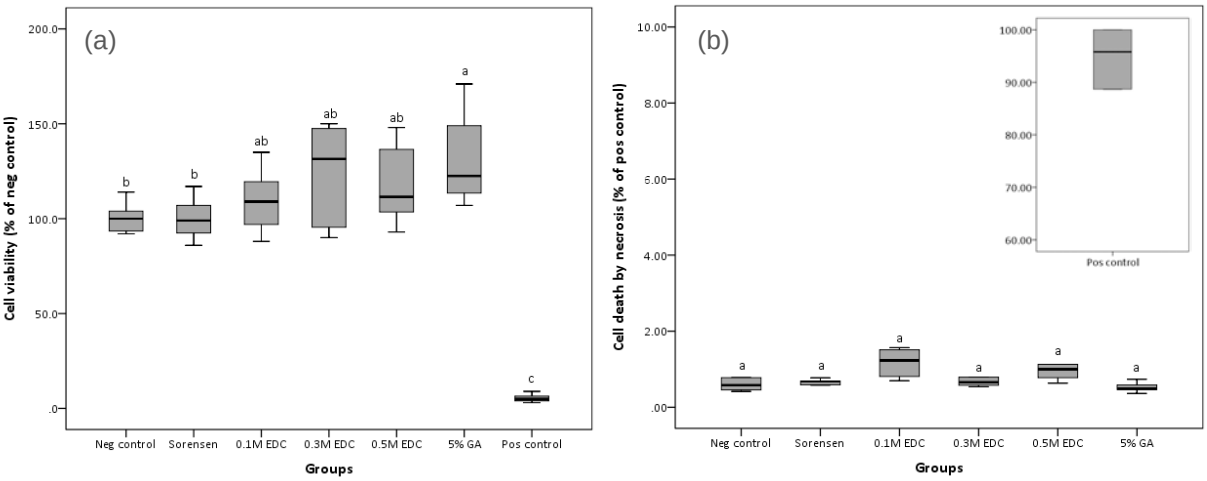
(a)
Cell viability (% of neg control)
200.0
150.0
100.0
50.0
.0
b
b
ab
ab
ab
a
c
Neg control
Sorensen
0.1M EDC
0.3M EDC
0.5M EDC
5% GA
Pos control
Groups
(b)
Cell death by necrosis (% of pos control)
10.00
8.00
6.00
4.00
2.00
.00
100.00
90.00
80.00
70.00
60.00
Pos control
a
a
a
a
a
a
Neg control
Sorensen
0.1M EDC
0.3M EDC
0.5M EDC
5% GA
Groups

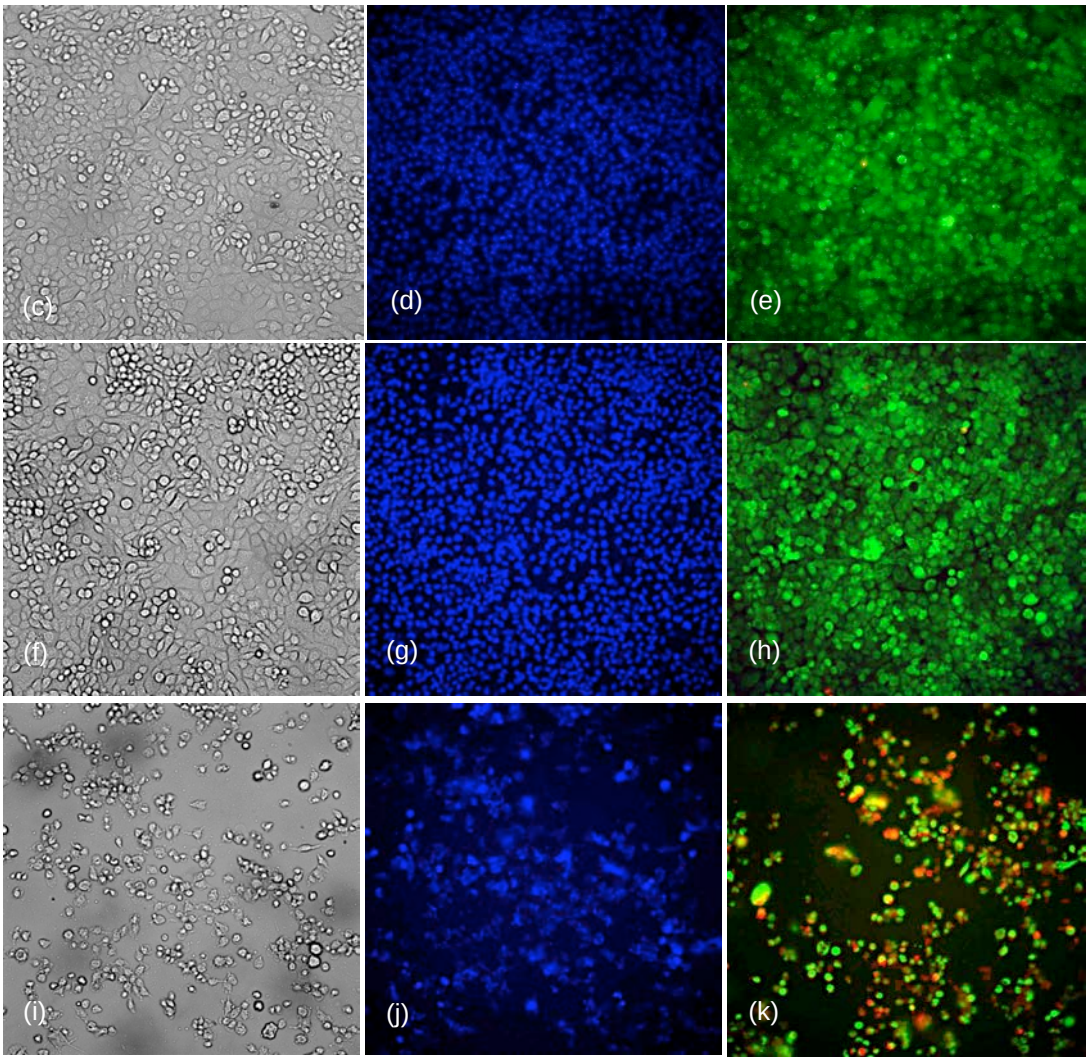
(c)
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Fig 1. Response of MDPC-23 cells after transdentinal contact with different concentrations of EDC. (a) Percentage of cell viability (MTT assay) related to the negative control (=100% of viable cells). (b) Percentage of necrotic dead cells related to the positive control (=100% of cell death). Groups identified by the same letter do not differ statistically (Mann-Whitney, $p>0.05$). (c) - (k) Representative confocal images of MDPC-23 cells co-stained with LIVE/DEAD® assay kit after contact with Sorensen's buffer (c, d and e), 0.5M EDC (f, g and h) and positive control (i, j and k). Live cells fluoresce green on Calcein dye uptake and necrotic cells fluoresce red on ethil homodimer-1 uptake. Hoechst dye (blue) shows cell nucleus, representing total cell number observed in the white field. Magnification x20.

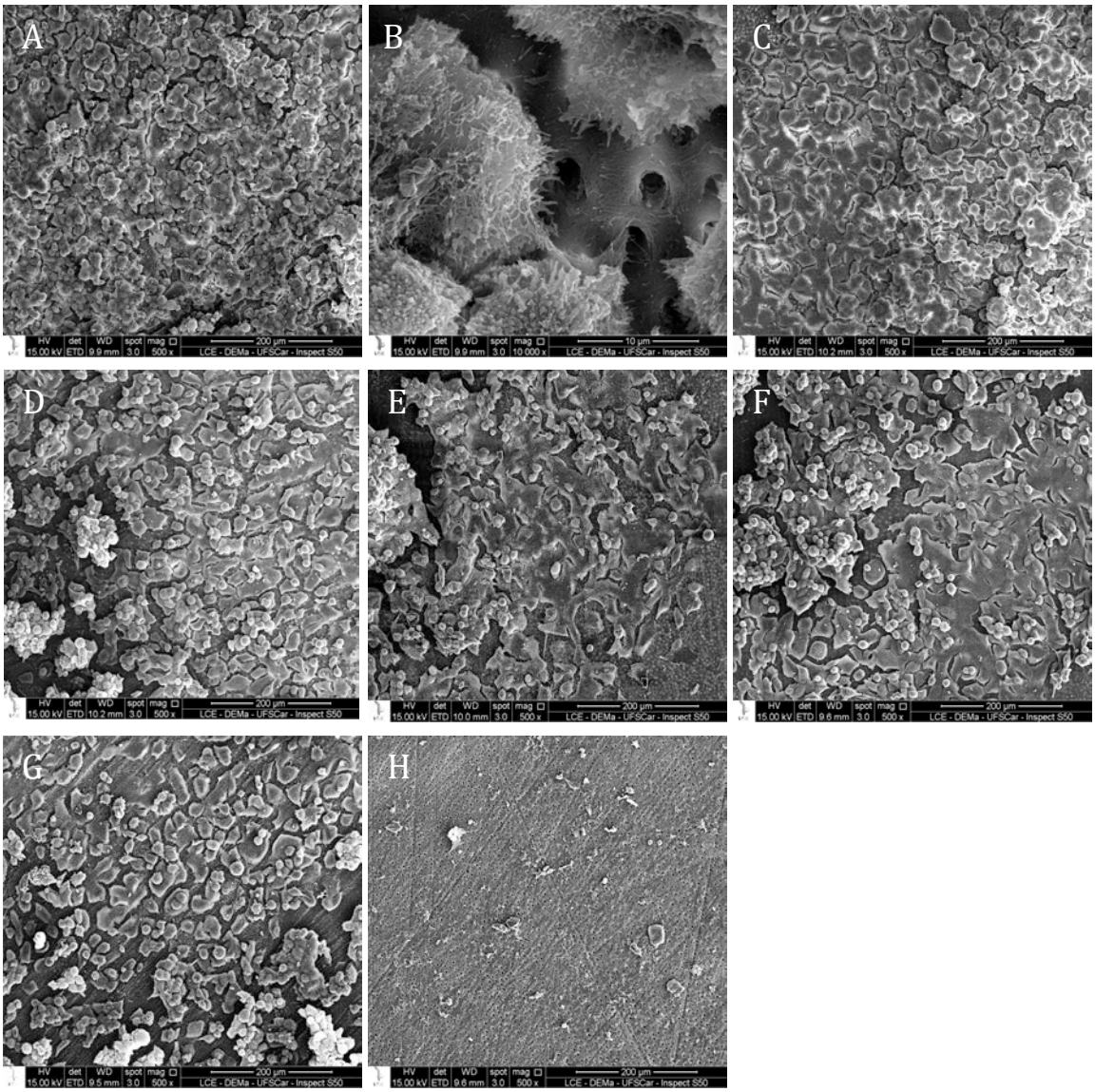


Fig 2. Composite figure of SEM micrographs representative of negative control (A and B), Sorensen buffer (C), 0.1 M EDC (D), 0.3 M EDC (E), 0.5 M EDC (F), 5% glutaraldehyde (G) and 29% hydrogen peroxide (positive control) (H). SEM x500. A large number of MDPC-23 cells remained attached to the dentin surface except in the positive control group (Fig. 2H). In that particular group most cells detached from the dentin and only residual fragments of dead cell are seen. In Fig. 2B is possible to observe the large number of micro-extensions on the cell membrane, responsible for the cell fixation to the dentin substrate seen in the background of the image. SEM x10,000

Table 1 Production of total protein (% of neg control at 24h) by MDPC-23 cells after transdentinal contact with different concentrations of EDC.

Groups	Period of evaluation		
	24 hours		7 days
Neg control	101.2 (100.7-101.2) [a]	*	250.4 (241.0-254.2) [bc]
Sorensen	108.8 (105.8-111.9) [a]	*	262.4 (254.8-263.6) [ab]
0.1M EDC	95.1 (81.5-97.7) [ab]	*	244.1 (243.5-245.4) [c]
0.3M EDC	82.0 (82.0-84.0) [bc]	*	263.6 (261.7-267.4) [ab]
0.5M EDC	63.2 (59.7-64.7) [c]	*	256.1 (248.5-262.4) [bc]
5% GA	87.0 (78.9-87.0) [ab]	*	271.8 (263.6-272.4) [a]

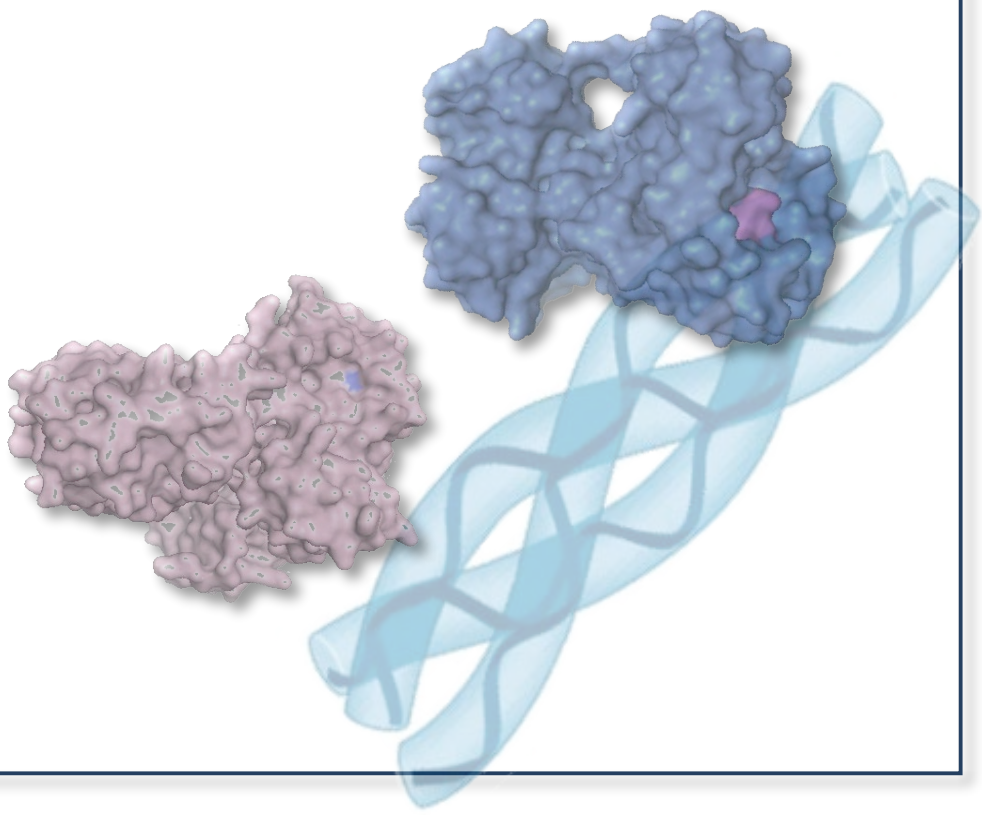
Numbers are median (percentile 25-percentile 75), n=5. Within each column, groups followed by the same letter are not statistically different (Mann-Whitney, p>0.05). *Indicates statistically significant difference between periods of evaluation (Mann-Whitney, p>0.05).

Table 2 Type I collagen production (% of neg control at 24h) by MDPC-23 cells after transdentinal contact with different concentrations of EDC.

Groups	Period of evaluation		
	24 hours		7 days
Neg control	100.4 (97.1-103.0) [a]	n.s.	100.3 (96.3-103.2) [ab]
Sorensen	102.0 (97.1-104.8) [a]	n.s.	94.9 (91.0-97.4) [b]
0.1M EDC	97.7 (91.1-104.7) [ab]	n.s.	89.7 (80.0-97.5) [b]
0.3M EDC	106.7 (103.1-110.7) [a]	n.s.	92.9 (92.4-93.3) [b]
0.5M EDC	83.8 (74.2-91.4) [b]	*	98.1 (96.3-100.8) [b]
5% GA	94.2 (87.2-102.6) [ab]	n.s.	105.3 (104.2-106.5) [a]

Numbers are median (percentile 25-percentile 75), n=5. Within each column, groups followed by the same letter are not statistically different (Mann-Whitney, $p>0.05$). *Indicates statistically significant difference between periods of evaluation while n.s. (not significant) indicates absence of significance (Mann-Whitney, $p>0.05$).

4. Estudo II



STABILIZATION OF DENTIN MATRIX AFTER CROSS-LINKING TREATMENTS, *IN VITRO*

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Short title: Stabilization of matrix by cross-linking

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Artigo formatado nas normas da Dental Materials

Stabilization of dentin matrix after cross-linking treatments, *in vitro*

Abstract

Objectives: To evaluate the effect of EDC on elastic modulus (E), MMPs activity, hydroxyproline (HYP) release and thermal degradation of demineralized dentin collagen. *Methods:* Dentin beams were obtained from human molars and completely demineralized in 10wt% H_3PO_4 for 18h. The initial E and MMP activity were determined with three-point bending and microcolorimetric assay, respectively. Extra demineralized beams were dehydrated and the initial dry mass (DM) was determined. All the beams were distributed into groups (n=10) and treated for 30s or 60s with: water, 0.5M, 1M or 2M EDC and 10% Glutaraldehyde (GA). After treatment, the new E and MMP activity were redetermined. The beams submitted to DM mesurements were storage for 1 week in artificial saliva, after that the mass loss and HYP release were evaluated. The collagen thermal degradation (CTD) was determined by DSC analysis. Data for E, MMP activity and HYP release were submitted to Wilcoxon and Kruskal-Wallis or Mann-Whitney tests. Mass loss and CTD data were submitted to ANOVA and Tukey tests at the 5% of significance. *Results:* EDC was able to significantly increase collagen stiffness in 60s. 10% GA groups obtained the highest E values after both 30 and 60 s. All cross-linking agents decreased MMP activity and HYP release and increased CTD temperature. Significant differences were identified among EDC groups after 30 or 60s of cross-linking, 1M or 2M EDC showed the lowest MMP activity. *Significance:* Cross-

linking agents are capable of preventing dentin collagen degradation. EDC treatment may be clinically useful to increase resin-dentin stability.

Key words: MMPs, collagen, dentin, cross-linkers, glutaraldehyde, EDC

Introduction

Contrary to stable resin-enamel bonds, effective, long-lasting dentin bonds remains a challenge to clinicians [1]. The hybrid layer is considered the main structure responsible for micromechanical retention of resin restorations and also responsible for sealing the dentin [2]. However, this important structure is the most vulnerable area of the adhesive interface [3-5].

Bond degradation has been attributed to hydrolytic breakdown of resin adhesive or dentin collagen, or both. Transmission electron microscopy (TEM) examination of the hybrid layers shows replacement of collagen fibrils by water [6]. This degradation is thought to be due to endogenous MMPs and cathepsins in acid-etched dentin. Demineralized dentin contains matrix-bound metalloproteinases-2, -3, -8, -9 and -20 (MMPs) and cathepsins [7,8] in their active forms. These enzymes are exposed and activated by acid-etching and can slowly degrade collagen fibrils [9-12] within the hybrid layer, resulting in a significant loss of bond strength of 36% to 70% within 12 to 14 months [13,14].

Cross-linking agents are capable of non-specifically cross-linking protein such as collagen and dentin proteases [15,16]. 1-Ethyl-3(3-Dimethylaminopropyl) carbodiimide (EDC) is a stable isomer of carbodiimide, capable of cross-linking

proteins by activating the carboxyl group of glutamic and aspartic acids and then reacting with ε-amino groups present in protein molecules, resulting in the creation of covalent cross-links [17,18]. Cross-linking improves the mechanical properties of dentin collagen and makes the fibrils more resistant to degradation [15]. Furthermore, EDC has no transdentinal cytotoxic effect (Scheffel et al., unpublished data). However, EDC has only been tested for such long time periods (10 min to 4 h) that are not clinically feasible [15].

Matrix-bound dentin proteases contain numerous residues of glutamic and aspartic acids in their structures. Thus, if EDC reacts with demineralized dentin collagen [15], it should also react with any proteases bound to collagen. Such protease cross-linking should reduce the molecular mobility of dentin MMPs and cathepsins, making it more difficult for them to bind to and cleave collagen [19]. Additionally, besides bonding between collagen polypeptide chains, it is plausible that EDC could cross-link the catalytic sites of dentin proteases, thereby blocking resin-dentin bond degradation. The purpose of this study was to evaluate the effect of EDC application on elastic modulus, total matrix-bound MMPs activity, hydroxyproline release and thermal degradation of completely demineralized dentin. The null hypotheses were that cross-linker-treated and untreated dentin do not differ regarding elastic modulus, total MMP activity, hydroxyproline release amount and thermal degradation temperature, and the time of application does not influence these properties.

Materials and Methods

Fifty extracted human third molars were obtained from 18-21 year-old patients with informed consent under a protocol approved by the Georgia Regents University. The teeth were stored frozen until required. After thawing, the enamel and superficial dentin were removed using an Isomet saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) under water cooling. One 1 mm-thick dentin disk was produced from the mid-coronal dentin of each tooth.

Elastic Modulus

One-hundred dentin beams (1x1x3 mm) were sectioned from the dentin disks. The beams were completely demineralized in 10 wt% H_3PO_4 (pH 1) for 18h at 25°C at the ratio of 1 beam per mL and rinsed with deionized water for 2 h at 3-4°C. The initial elastic modulus of each demineralized beam was determined by three-point flexure [20]. An aluminum testing jig with a 2.5 mm span between supports was fixed to the bottom of a glass Petri dish. Specimens were tested under compression, while immersed in distilled water, by means of a testing machine (Vitrodyne V1000, Liveco Inc., Burlington, VT, USA), with a 100 g load cell, at a crosshead speed of 1 mm/min. After maximum displacement, it was returned immediately to 0% stress to prevent creep of the demineralized collagen. Load-displacement curves were converted to stress-strain curves, and the apparent modulus of elasticity was calculated at 15% strain. Then the beams were randomly divided into 10 groups (n=10), so that the mean initial elastic modulus of each group was statistically

similar. To calculate elastic modulus of each specimen, the steepest slope of the linear portion of the stress–strain curve was placed in the following formula:

$$E = \frac{mL^3}{4bd^3}$$

m = slope (N/mm); L = support span (mm); d = thickness of beam (mm); b = width of beam (mm). Because specimen displacement was estimate from cross-head displacement, and the specimens thickness was not one-sixteenth of the length [20] the calculated elastic moduli are approximate. Although both the two supports and the third mid-beam compressive member may have slightly deformed the surface of the specimens, that deformation was the same before and after treatment. We were more interested in changes in modulus of elasticity, rather than their absolute values.

Pre-treatment MMP activity of dentin

To determine the initial total MMP activity, each beam was placed into 200 μL of a generic MMP substrate (Sensolyte Generic MMP colorimetric assay kit - catalog No. 72095, AnaSpec Inc. Fremont, CA, USA) for 60 min at 25^{o}C in a 96-well plate. At the end of 60 min, the total MMP activity was determined by measuring the absorbance of the wells at 412 nm in a plate reader (Synergy HT microplate reader, BioTek Instruments, Winooski, VT, USA) against blanks. The substrate is cleaved by MMPs 2, 8 and 9 in dentin and releases a sulfhydryl group that reacts with Ellman's Reagent (5,5'-dithiobis(2-nitrobenzoic acid). The final product of this reaction, 2-nitro-5-thiobenzoic acid (TNB), turns the medium yellow and can be read by a plate

reader. All chemicals were purchased from Sigma/Aldrich Chemical Co and used as received.

Post-treatment MMP activity of dentin

Each completely demineralized dentin beam was dipped for 30 or 60 s into 300 μL of the following solutions: water (positive control), 0.5 M, 1 M or 2 M EDC (EDC-HCl, ProteoChem, Denver, CO, USA) (pH 6.0) or freshly diluted 10 vol % (1 M) glutaraldehyde (GA) (negative control) made from 50 wt% Sigma-Alldrich (St. Louis, MO, USA), followed by abundant rinsing with deionized water for 30 s to dilute the cross-linking agents to near zero and to stop the cross-linking reaction. Immediately after treatment the new elastic modulus and residual total MMP activity were redetermined.

Hydroxyproline (HYP) assay and dry mass loss

To analyze hydroxyproline release and dry mass, loss one-hundred extra beams were prepared and demineralized as described above. The beams were placed in sealed containers of anhydrous calcium sulfate (Drierite, W.A. Hammond Drierite Company, Ltd., Xenia, OH, USA) overnight and the initial dry mass of each beam was measured using a microanalytical balance to the nearest 0.01 mg [21]. The beams were rehydrated in deionized water for 1 h before being treated according to the groups previously described to elastic modulus and MMP activity assay. Immediately after the treatments the beams were incubated for 1 week in 1ml of artificial saliva.

After 1-week incubation, the dry mass were re-measured for each beam and 200 µl of the 1 ml incubation medium was removed to perform the HYP assay. An equal volume of concentrated HCl (200 µl) was mixed to the artificial saliva to yield a final acid concentration of 6 N HCl in glass ampoules (Wheaton, Millville, NJ, USA). The vials were automatically sealed using an Ampulmatic Ampule Sealer (Bioscience, Inc., Allentown, PA, USA) and placed in an oil bath at 118°C for 18 h to hydrolyze the amino acids. After cooling, the glass vials were opened and placed in a glass dessicators with NaOH pellets to trap HCl vapor and anhydrous calcium sulfate to trap water vapor. After 3 days in the dry contents of the vials were analyzed for HYP using a colorimetric assay (modified from Jamall *et al.,* 1981) [22]. The absorbance of all specimens and standards was measured at 558 nm in a 96-well plate reader.

Differential scanning calorimetry (DSC) analysis

Dentin slabs (1 mm thick and 6 mm of diameter) were completely demineralized in 10% phosphoric acid aqueous solution for at least 18 h and then rinsed in Milli-Q water under agitation for 24 h. A FTIR-ATR (Fourier transform infra-red attenuated total reflectance, Nicolet 6700, Thermo scientific, Milan, Italy) was used to acquire the spectrum of each slab before and after the demineralization process to ensure that all mineral content was removed, verified by the disappearance of the PO_4^{3-} peak at 1004 cm^{-1}. Each demineralized slab was sectioned into cubic specimens (1x1x1 mm) using a sterile surgical blade. The dimensions of each

specimen were individually measured with a digital caliper to the nearest 0.01 mm. The dentin specimens were treated with the treatment solutions as described for the previous tests. Specimens immersed in the glutaraldehyde and EDC solutions were rinsed with Milli-Q water for at least 30 minute. The thermal degradation temperature (TDT) for each specimen was determined using a differential scanning calorimeter (DSC, Q10 TA Instruments, New Castle, DE, USA). All specimens were lightly blot dried and sealed in DSC aluminum pans. Specimens were heated from 30°C to 250°C at 10°C/min in a nitrogen atmosphere. Both the onset and the maximum signal of thermal degradation temperature were measured.

Statistical Analysis

Data sets for elastic modulus (E), MMP activity, mass loss, HYP release and termal degradation of demineralized dentin were evaluated regarding the normality of their distributions. Since the distributions of E, MMP activity and HYP release data sets were not normally distributed, nonparametric tests were selected to analyze these variables. All data sets were submitted to Wilcoxon non-parametric and Kruskal-Wallis tests (for tests involving more than two comparisons) or the Mann-Whitney test for comparing two groups. The percentage of total MMP activity inhibition was calculated based on the untreated water control group MMP activity. Data from % of mass loss and collagen thermal degradation (^{o}C) were submitted to ANOVA complemented by Tukey tests. All the statistical tests were performed at the 5% level of significance.

Results

Completely demineralized dentin beams dipped in water for 30 s or 60 s were used as controls for statistical compararsions and were considered as 100% to calculate percent changes in elastic modulus or percent decresases in MMP activity and dry mass in the results. Treatment of demineralized dentin with EDC, in concentrations between 0.5 M and 2 M, did not increase dentin elastic modulus after 30 s treatment. However, demineralized dentin beams treated with 1 M and 2 M for 60 s significantly ($p < 0.05$) increased their moduli of elasticity by 41.2% and 34.2%, respectively. When 10% GA was applied to demineralized dentin beams, the treatment significantly ($p < 0.05$) increased their elastic moduli at both time periods (123.5% for 30 s and 265.8% for 60 s) (Table 4). All cross-linking agents were capable of reducing the total MMP activity of demineralized dentin after 30 and 60 s of topical treatment (Table 3). After 30 s of EDC treatment, the total MMP inactivation among EDC groups was only 64.4%, 48.9% or 64.4% for the 0.5, 1 or 2 M groups and 65.3% in the 10% GA group. None of these values were significantly different from each other. After 60 s treatment of dentin with 1 M or 2 M EDC, the total MMP activity of dentin fell 77.6% and 81.9%, respectively (Table 3). No significant differences were observed between 0.5M EDC (58.6%) and 10% GA (55.4%) (Table 3). GA was more effective than EDC at increasing elastic moduli in 30 s or 60 s. That is, after 30 s of treatment, the modulus of elasticity increased 123.5%, while after 60 s of treatment, the modulus of elasticity increased 265.8%

(Table 4). However, GA was no more effective at inactivating the total MMP activity of demineralized dentin than was EDC.

Dry mass loss data showed that the treatment of dentin beams with EDC or GA was capable to significantly reduce mass loss (Table 5). After 1-week incubation the control group lost 6.39% ±0.67 (30 s) and 7.75% ±1.05 (60 s) of mass, while the beams treated with EDC lost between 2.77% ±1.25 (2 M EDC for 30 s) and 1.89% ±0.89 (0.5 M EDC for 60 s). No statistical difference was observed among EDC groups. The beams treated with 10% GA for 30 s and 60 s showed a increase of dry mass of 0.28% ±1.03 and 1.88% ±1.38, respectively. Hydroxyproline assay detected HYP release in all groups (Table 6). The highest HYP amount was observed to the uncrossed-linked control groups 2571.33 (30 s) and 3271.29 (60 s) ng HYP/mg dentin (90.8% reduction in HYP release in specimens treated with 10% GA for 60 s). EDC and GA treatments reduced HYP release to values between 445.49 (0.5 M EDC for 30 s or 60 s) and 299.75 (10% GA for 60 s) ng HYP/mg dentin. No difference was observed among the groups treated with EDC and GA and the HYP content decrease was not dependent on cross-linker application time.

The DSC analysis showed that at the same concentration the treatment time did not influence the collagen thermal degradation. The control group (water) exhibited a control onset denaturate temperature of 61.43 ± 2.39℃ and a registered significant lower onset (61.43 °C ± 2.39) maximum thermal denaturation temperature of 67.05 ± 3.87℃. The onset thermal denaturate of dentin treated with 10% GA for

60 s was 75.61 ± 1.54℃. However no difference was observed among this group and the specimens treated with 10% GA for 30 s (73.64 °C ± 3.97), 2 M EDC for 30 s (73.55 °C ± 1.07) and 60 s (75.22 °C ± 1.85) and 1 M EDC for 60 s (73.18 °C ± 1.38). Similar relation was observed to maximum denaturate temperature, with the highest values observed in the 10% GA group (60 s) (78.91 °C ± 2.28) which was not different from the groups treated with 2 M EDC (30 s and 60 s) or 1 M EDC (60 s).

Discussion

The degradation of resin-dentin bonds has been the subject of several recent studies. Host-derived proteases such as metalloproteinases (MMPs) and cysteine cathepsins plays an important role in collagen fibrils degradation via pepitide hydrolysis [23]. MMPs binding sites are located in a narrow cleft in collagen approximately 0.5 nm wide [24]. Hence to degrade collagen, MMPs must bind to collagen and unwind collagen molecules such a way that the enzyme's active site can react and attack the specific glycine-isoleucine peptide bond in peptide chains [25-27] culminating in cleavage of collagen peptides.

Carbodiimide has been investigated as a cross-linking agent that is capable of increasing the stiffness of dentin collagen fibrils, making them more difficult to unwind. Bedran-Russo et al. [15] demonstrated that the treatment of dentin collagen with EDC for periods of time between 10 min to 4 h, increases its mechanical properties. However such reaction times are not clinically applicable. The present

study tested much shorter clinically relevant time of EDC application on completely demineralized dentin, and its effects on elastic modulus, total MMP activity, release of hydroproline peptide fragments and thermal denaturation temperature.

Our results showed that in EDC concentrations between 0.5 and 2 M, applied for only 30 s significantly increase collagen stiffness. However, when the reaction time was increased to 60 s of treatment, EDC significantly ($p<0.05$) increased the modulus of elasticity of dentin when used at 1 M or 2 M concentrations. On the other hand, all these treatment times and concentrations were sufficient to significantly ($p<0.05$) reduce matrix-bound MMP activity. Thus, the results require rejection of the tested null hypotheses that cross-linker-treated and untreated dentin do not differ regarding elastic modulus and MMP activity, and that the time of application does not influence these properties.

GA was able to rapidly increase collagen stiffness (123.5% - 265.8%) more efficiently than EDC (34.2% - 41.2%). GA, being a dialdehyde, seems to react with amino acids faster than EDC. This may be because the mechanism of the GA reaction with proteins is direct and does not involve the production of an intermediate product as is seen in the EDC reactions [17]. However, GA is considered to be potentially cytotoxic. EDC did not present transdentinal cytotoxic effect on odontoblast-like cells (Scheffel et al., unpublished data). The fast reaction of GA was also observed in its ability to inactivate MMPs. GA was unable to inhibit more MMPs in 60 s than it was in 30 s, suggesting that it completely reacts with MMPs in the first seconds of application. However, the fact that it only inhibited the total MMP activity of dentin

65.3% (Table 3) indicates that cross-linkers may require longer diffusion times to diffuse to the center of 1.0 mm thick specimens.

EDC was a more effective MMPs inactivator, than it was as collagen cross-linker at both periods of time (i.e. 30 s and 60 s). EDC cross-links peptide chains in MMPs, thereby inactivating the enzyme by lowering the molecular mobility of the catalytic sites in these enzymes [19]. Moreover, MMPs-2, -8, -9 and -20 contain glutamic acid in their active sites allowing EDC to activate those free carboxyl groups not involved in peptide bonds. Despite the fact that cross-linking can be done rapidly in MMPs, it seems to occur more slowly in collagen. This suggests that carboxyl and amino groups in collagen may not be as accessible as those groups are in MMPs [28,29], allowing more rapid cross-linking in MMPs than collagen [23].

EDC requires a longer time to increase the elastic modulus of collagen. This study used completely demineralized 1.0 mm thick dentin beams. Such thick zones of demineralization are much deeper than those observed clinically in acid etched bonded teeth (8-10 μm). One mm thick completely demineralized beams must be infiltrated with reagents 500 μm from all sides of the beam to reach the center. We speculate that 10 micrometer-deep zones of demineralized dentin would be easily saturated by test solutions within seconds. We suggest that EDC could be used clinically to inactivate most of matrix-bound proteases and satisfactorily increase the stiffness of dentin collagen.

The dry mass and the hydroxyproline assay are indirect measures of dentin collagen degradation. Loss of dry mass over time indicates solubilization of collagen

matrix by activated endogenous MMPs. The mass loss in the control group was much higher than in the EDC pre-treated groups, while GA was able to completely stop mass loss after 1-week incubation. Likewise, all cross-linking solutions were able to reduce HYP release for both periods of treatment. These results agree with MMP activity assay that showed that EDC and GA inhibit MMPs, reducing cleavage and solubilization of collagen peptides.

DSC was used to investigate the thermal properties of dentin collagen. All the cross-linking solutions applied for 30 s or 60 s increased the thermal stability of demineralized collagen. Our results agree with those of Safandowska and Pietrucha (2013) [30], who treated collagen from fish skin with EDC to be used in medical applications. They observed that EDC was effective to cross-link fish collagen and increase the temperature required to thermal denaturation. In the present study the highest temperatures were observed to the groups treated with 10% GA (30 s and 60 s), 2 M EDC (30 s and 60 s) and 1 M EDC (60 s) similar to that showed by three point-bending test, especially after 60 s of treatment. Future studies will evaluate the effects of shorter application times of cross-linking agents over time *in vitro* and *in vivo*, in thinner more clinically relevant specimens.

Conclusion

The application of cross-linking agents to demineralized dentin was capable of inactivating matrix-bound MMPs, reducing dry mass loss, decreasing HYP release

and in increasing collagen E-modulus and denaturation temperature. The use of EDC in clinically applicable periods of time may prevent resin-dentin degradation. GA showed the highest values of elastic modulus and was more effective to prevent mass loss, although EDC was a better MMP inactivator in high concentrations.

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Table 1. Elastic modulus (MPa) of completely demineralized dentin specimens before and after the application of different cross-linking solutions for 30 s or 60 s.

Dentin Treatment	Cross-linking solution application time			
	30 s		60 s	
	Initial E-modulus	Final E-modulus	Initial E-modulus	Final E-modulus
Water	1.80 (1.69 – 2.09)[a]	1.80 (1.51 – 2.17)[a]	1.61 (1.37 – 2.32)[a]	1.62 (1.40 – 2.40)[a]
0.5M EDC	1.60 (1.52 – 2.36)[a]	1.51 (1.20 – 1.81)[a]	1.94 (1.87 – 2.02)[a]	1.80 (1.57 – 1.97)[a]
1M EDC	1.71 (1.38 – 2.41)[a]	1.62 (1.23 – 2.20)[a]	1.62 (1.33 – 1.85)[a]	2.29 (2.15 – 2.47)[b]
2M EDC	1.79 (1.50 – 2.13)[a]	1.82 (1.39 – 2.18)[a]	1.74 (1.63 – 2.03)[a]	2.17 (1.87 – 2.66)[b]
10% GA	1.94 (1.87 – 2.17)[a]	4.02 (3.50 – 5.41)[b]	1.94 (1.54 – 2.12)[a]	5.93 (4.56 – 7.25)[b]

[*] Values are expressed as MPa and represent median (25 percentile-75 percentile), n=10.

[a] Within each dentin treatment solution and application time (initial vs after-treatment MMP activity), medians identified by the same letter are not statistically different (Wilcoxon, $p>0.05$).

Table 2. Total MMP activity (absorbance) detected in completely demineralized dentin specimens before and after the application of different cross-linking solutions for 30 s or 60 s.

Dentin Treatment	Application time			
	30 s		60 s	
	Initial MMP activity	Post-treatment MMP activity	Initial MMP activity	Post-treatment MMP activity
Water	0.227 (0.146 – 0.279)[a]	0.265 (0.235 – 0.334)[a]	0.255 (0.225 – 0.302)[a]	0.232 (0.176 – 0.239)[a]
0.5M EDC	0.183 (0.157 – 0.229)[a]	0.095 (0.072 – 0.106)[b]	0.378 (0.332 – 0.426)[a]	0.100 (0.070 – 0.160)[b]
1M EDC	0.255 (0.240 – 0.319)[a]	0.136 (0.085 – 0.173)[b]	0.266 (0.236 – 0.311)[a]	0.052 (0.045 – 0.079)[b]
2M EDC	0.252 (0.236 – 0.279)[a]	0.095 (0.077 – 0.102)[b]	0.241 (0.194 – 0.260)[a]	0.042 (0.034 – 0.061)[b]
10% GA	0.183 (0.141 – 0.209)[a]	0.092 (0.078 – 0.108)[b]	0.384 (0.318 – 0.492)[a]	0.105 (0.100 – 0.120)[b]

[*] Values are expressed as absorbance at 412 nm after 60 min of incubation in SensoLyte substrate and represent median values (25 percentile-75 percentile), n=10.

[a] Within each dentin treatment and application time (initial vs after-treatment MMP activity), medians identified by the same letter are not statistically different (Wilcoxon, $p>0.05$)

Table 3. Percentage of total MMP inhibition in completely demineralized dentin specimens after the application of different concentrations of EDC and 10% glutaraldehyde (GA) for 30 s or 60 s.

Dentin Treatment	Application time		
	30 s		60 s
Water	0 (-26.0 – 11.3)[b]	n.s	-0.7 (-13.8 – 24.1)[c]
0.5M EDC	64.4 (60.0 – 72.8)[a]	n.s	58.6 (32.3 – 71.6)[ab]
1M EDC	48.9 (34.7 – 67.9)[a]	*	77.6 (65.9 – 80.6)[a]
2M EDC	64.4 (61.5 – 70.9)[a]	*	81.9 (73.3 – 85.3)[a]
10% GA	65.3 (59.2 – 70.6)[a]	n.s	55.4 (49.1 – 59.1)[b]

[*] Values are expressed as percentage absorbance related to the control and represent median (25 percentile-75 percentile), n=10

[a] Within each application time (columns), groups identified by the same letter are not statistically different (Mann-Whitney, $p>0.05$)

n.s – not statistically different, * statistically different

Table 4. Change in elastic modulus (E) of completely demineralized dentin specimens after the application of different concentrations of EDC and 10% glutaraldehyde (GA) for 30 s or 60 s.

Dentin Treatment	Application time		
	30 s		60 s
Water	0 (-16.4 – 20.7)[b]	n.s	0 (-13.6 – 48.1)[b]
0.5M EDC	-16.4 (-33.1 – 0.7)[b]	n.s	-11.1 (-3.2 – 21.5)[b]
1M EDC	-10.3 (-31.4 – 22.1)[b]	*	41.2 (-32.7 – 52.5)[b]
2M EDC	1.0 (-23 – 22.1)[b]	*	34.2 (15.7 – 64.4)[b]
10% GA	123.5 (94 – 200.6)[a]	*	265.8 (181.3 – 347.5)[a]

[*] Values are expressed as percentage change related to the control and represent median(25 percentile-75 percentile), n=10

[a] Within each application time (columns), groups identified by the same letter are not statistically different (Mann-Whitney, $p>0.05$)

n.s – not statistically different, * statistically different

Table 5. Mass loss (%) of total demineralized dentin specimens after treatment with cross-linkers for 30 or 60 seconds and incubation for one week.

Application time	Dentin treatment				
	Water	0.5M EDC	1M EDC	2M EDC	10% GA
30 s	-6.39±0.67^{d}	-2.48±0.65^{c}	-2.24±0.57^{c}	-2.77±1.25^{c}	0.28±1.03^{b}
60 s	-7.75±1.05^{d}	-1.89±0.89^{c}	-2.35±0.88^{c}	-2.17±0.70^{c}	1.88±1.38^{a}

Abbreviations = EDC: carbodiimida; GA: glutaraldehyde

Numbers are mean±standard-deviation, n=10.^{a} Means represented by the same letter do not differ statistically (Tukey, $p>0.05$)

Table 6. Hydroxyproline (ng HYP/mg dentin) detected after 7-day artificial saliva storage of total demineralized dentin specimens treated with cross-linkers for 30 or 60 seconds.

Application time	Dentin treatment				
	Water	0.5M EDC	1M EDC	2M EDC	10% GA
30 s	2571.33 (2073.11-3053.54)$^{a\ A}$	445.49 (292.71-479.19)$^{b\ A}$	437.47 (343.62-479.19)$^{b\ A}$	386.43 (347.19-450.56)$^{b\ A}$	371.21 (248.98-461.69)$^{b\ A}$
60 s	3271.29 (2390.33-3693.05)$^{a\ B}$	405.41 (351.11-442.39)$^{b\ A}$	383.65 (296.40-457.50)$^{b\ A}$	388.79 (244.09-462.87)$^{b\ A}$	299.75 (280.01-386.13)$^{b\ A}$

Abbreviations = EDC: carbodiimida; GA: glutaraldehyde

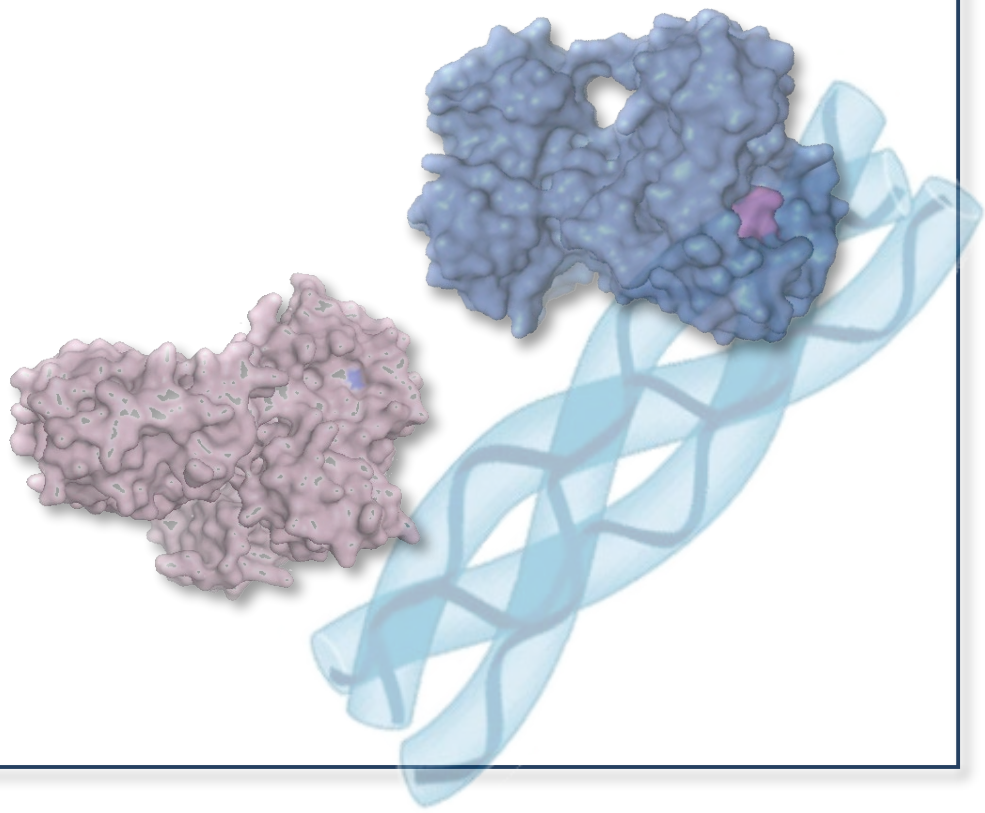
Numbers are median (percentile 25-percentile 75), n=10.^{a} Lowercase letters allow comparisons within application time (rows) while ^{A} uppercase letters allow comparison within treatment (columns). Medians indicated by the same letter do not differ statistically (Mann-Whitney, $p>0.05$).

Table 7. Increases in thermal degradation of completely demineralized dentin after the application of different concentrations of EDC and 10% glutaraldehyde (GA) for 30 s or 60 s.

Dentin Treatment	Onset Temperature		Signal Max Temperature	
	30 s	60 s	30 s	60 s
Water	61.43 °C ± 2.39 [e]		67.05 °C ± 3.87 [e]	
0.5M EDC	67.49 °C ± 1.66 [d]	69.79 °C ± 1.73 [cd]	73.57 °C ± 1.08 [d]	75.30 °C ± 0.70 [bcd]
1M EDC	71.13 °C ± 1.56 [bc]	73.18 °C ± 1.38 [ab]	74.69 °C ± 1.77 [cd]	77.23 °C ± 1.40 [abc]
2M EDC	73.55 °C ± 1.07 [ab]	75.22 °C ± 1.85 [a]	76.60 °C ± 0.30 [abcd]	78.48 °C ± 1.06 [ab]
10% GA	73.64 °C ± 3.97 [ab]	75.61 °C ± 1.54 [a]	76.20 °C ± 3.85 [abcd]	78.91 °C ± 2.28 [a]

Numbers are mean±standard-deviation, n=10.[a] Means represented by the same letter do not differ statistically (Tukey, p>0.05). EDC=carbodiimida; GA=glutaraldehyde

5. Estudo III



INACTIVATION OF MATRIX-BOUND MMPS BY CROSS-LINKING AGENTS IN ACID ETCHED DENTIN

Running title: Matrix-bound MMP inactivation of acid etched dentin

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Key words: MMPs, collagen, dentin, cross-linker

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Clinical significance statement

Cross-linking agents used in clinically applicable periods of time are capable of inactivating matrix-bound MMP in demineralized dentin. Such treatment may render the hybrid layer less prone to degradation over time and produce long lasting resin-dentin bonds.

ABSTRACT

Objectives: Published TEM analysis of *in vitro* resin-dentin bonds shows that in 44 months almost 70% of collagen fibrils from the hybrid layer disappear. Matrix metalloproteinases (MMPs) play an important role in that process and are thought to be the main factor responsible for the solubitization of dentin collagen. Therefore, this study aimed to evaluate the inactivation of matrix-bound MMPs by two different cross-linking agents, carbodiimide (EDC) or proanthocyanidin (PA), or the MMP-inhibitor, chlorhexidine (CHX), on acid-etched dentin using a simplified MMP assay method. **Methods:** Dentin beams (2x1x6mm) were obtained from mid-coronal dentin of sound third molars and randomly divided into 6 groups (G) according to the dentin treatment: G1: Deionized water (control), G2: 0.1M EDC, G3: 0.5M EDC, G4: 0.5M EDC+35% HEMA, G5: 5% Proanthocyanidin (PA) and G6: 2% CHX. The beams were etched for 15s with 37% phosphoric acid, rinsed and then immersed for 60s in one of the treatment solutions. The data were expressed both in absorbance values at 412 nm and in MMP-9 activity equivalents. The total MMP activity of dentin was analyzed for 1h by colorimetric assay (Sensolyte). Data were submitted to Wilcoxon non-parametric test and Mann-Whitney tests ($p>0.05$). **Results:** All experimental

cross-linking solutions significantly reduced MMP activity from 79.8% to 95.2% compared to the control group. No difference was observed among 0.1 M EDC (84.8%), 5% PA (87.6%) and 2% CHX (79.8%). Addition of 35% HEMA to 0.5M EDC produced inactivation (95.2%) that was similar to 0.5M EDC alone (92.7%). **Conclusion:** Dentin treatment with cross-linking agents is effective to significantly reduce MMP activity. Mixing 0.5M EDC and 35% HEMA did not influence EDC inhibitor potential.

INTRODUCTION

Since the introduction of the total-etching concept by Fusayama in 1980,[1] the effects of acid-etching of dentin has been subject to many studies. Etching dentin with 32-37% phosphoric acid removes the mineral content of the top 10 μm of dentin and exposes the collagen fibrils of the matrix, thereby creating space for monomer infiltration to achieve micromechanical retention of adhesive resins.[2] Although acid-etching of dentin provides satisfactory initial bond strength, those bond strengths fall over time, raising concerns about the long-term stability of adhesive-resin restorations.[3]

Resin/dentin bond degradation is a complex process that is not completely understood, involving the hydrolysis of both the resin and the collagen component of hybrid layers. Acid-etched dentin contains bound matrix metalloproteinases-2, -3, -8, -9 and -20 (MMPs) and cathepsins[4,5] in their active forms. These enzymes are exposed and activated by acid-etching and can slowly degrade collagen fibrils[6-9] within the hybrid layer, resulting in a significant bond strength loss of

36% to 70% between 12 to 14 months.[10,11]

In order to reduce the activity of these proteases and preserve the long term integrity of adhesive interfaces, chlorhexidine (CHX) has been used as a nonspecific inhibitor of MMPs. [6-9,11-13] CHX is also an effective inhibitor of cysteine cathepsins.[14] However, this substance is water-soluble and may undergo leaching from the hybrid layer, impairing its long-term anti-MMP effectiveness.[13]

A new alternative to the inhibition of proteases by inhibitors, is the treatment of demineralized dentin with cross-linking agents that can inactivate the catalytic site of these enzymes.[15] The cross-linker 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is capable of forming covalent peptide bonds between proteins by activating the free carboxyl groups of glutamic and aspartic acids present in protein molecules.[16-17] This results in the formation of a O-acylisourea intermediate that reacts with the epsilon amino group of lysine or hydroxylysine in an adjacent polypeptide chain to form a stable, covalent amide bond. The only byproduct of the reaction is urea,[18-19] which is water-soluble and easily removed from dentin by water rinsing. Furthermore, 0.5M EDC shows no transdentinal cytotoxicity on odontoblast-like cells (Scheffel et al. unpublished data) and is able to increase the mechanical properties of the collagen matrix.[20]

Other cross-linking agents, such as the proanthocyanidins (PA), are polyphenolic natural products composed of flavan-3-ol subunits linked mainly through C4– C8 (or –C6) bonds.[21] This substance is widely present in fruits, vegetables, nuts, seeds, flowers and barks, shows numerous biological activities,

such as antioxidant capacity,[22] anti-microbial effects,[23] anti-inflammatory properties,[24] positive effects on cardiovascular diseases[25] and anti-allergic activity.[26]

Thus, the purpose of this study was to evaluate the inactivation of matrix-bound MMPs by topical application of cross-linking agents on acid etched dentin. The null hypothesis was that cross-linker-treated and untreated dentin do not differ regarding MMP activity.

MATERIALS AND METHODS

Thirty extracted human third molars were obtained from 18-21 year-old patients with informed consent under a protocol approved by the Georgia Regents University. The teeth were stored frozen until required. After thawing, the enamel and superficial dentin were removed using an Isomet saw (Buehler Ltd., Lake Bluff, IL) under water cooling. One 1 mm-thick dentin disks were produced from the mid-coronal dentin of each tooth. Then, sixty dentin beams (2x1x6 mm) were sectioned from the dentin disks. One such beam was placed in each well. This represents 40 mm^2 of dentin which is equivalent to a class I cavity prepared in a mandibular first molar 2 mm into dentin and 3x4 mm in dimension. The beams were etched by dipping them into 37% phosphoric acid (pH -0.5) for 15 s and then copiously rinsed with deionized water for 15 s. The beams were randomly divided into 6 groups (n=10) according to the dentin treatment. G1: Deionized water (positive control) (pH 6.73), G2: 0.1 M 1-[3-dimethylaminopropyl) carbodiimide (EDC) (pH 6.07), G3: 0.5 M EDC (pH 6.24), G4: 35 vol%

hydroxyethylmethacrylate (HEMA) in water + 0.5 M EDC (pH 6.34), G5: 5% Proanthocyanidin (PA) (Polyphenolics Inc, Madera, California) (pH 5.2) in phosphate-buffered saline (pH 6.0) and G6: 2vol% CHX digluconate (negative control) in water (pH 6.43). All beams were dipped in the treatment solutions for 60 s and rinsed with distilled water for 10 s, except for 2% CHX where the beams were only blot dried. After the treatment, each beam was placed in a 200 µl/well of generic MMP substrate (Sensolyte Generic MMP colorimetric assay kit - catalog No. 72095, AnaSpec Inc. Fremont, CA) for 60 min at 25^{o}C in a 96-well plate. At the end of 60 min, the total MMP activity was determined by measuring the absorbance of the wells at 412 nm in a plate reader (Synergy HT microplate reader, BioTek Instruments, VT) against appropriate blanks. The absorbance of control the group was considered as 100% of the total MMP activity in the etched dentin and it was used to calculate the percentage of MMP activity inhibition of the investigated cross-linking agents and CHX. All chemicals were purchased from Sigma/Aldrich Chemical Co. The generic MMP assay uses a proprietary thiopeptide to assay MMP-1, 2, 3, 7, 8, 9, 12, 13 and 14. Thus, the kit measured the total endogenous MMP activity of dentin with the exception of MMP-20 (enamelysin). A standard curve of absorbance of the substrate vs. rh MMP-9 activity (ng) was constructed to permit expression of total MMP activity in MMP-9 equivalents. The rh MMP-9 was activated using trypsin at final concentration of 10 µg/ml, pH 7.4 at 37^{o}C for 2 h. Then the trypsin was inactivated by addition of trypsin inhibitor at final concentration of 100 µg/ml. Human recombinant MMP-9 was purchased from Calbiochem (cat. #PF038) Billerica, MA. Its specific activity

was 1300 pmoles/mg.

Statistical Analysis

For determination of MMP activity, the absorbance data set was submitted to Kruskal-Wallis and Mann-Whitney tests at 5% level of significance. The percentage of MMP activity inhibition was calculated based on the water control group MMP activity and MMP-9 equivalents (ng/well) based on rh MMP-9 curve.

RESULTS

When mineralized dentin beams were dipped in 37 wt% phosphoric acid for 15 s and then rinsed with water, the top 8-10 μm of the beams were completely demineralized (Fig. 1). When etched dentin beams were dipped in water (control) and then dropped into the generic MMP substrate, the absorbance at 412 nm gradually increased to 0.51(±0.138) over 60 min. A standard curve of substrate absorbance at 60 min vs. ng of rh MMP-9 is shown in figure 2. All cross-linking agents, significantly reduced MMP activity in acid etched dentin after 60 s of topical treatment (Table 1). The percentage of MMP inhibition for the EDC solutions, PA and CHX ranged from 79.8% for 2 wt% CHX to 95.2% for 0.5 M EDC + 35% HEMA (Table 1). There was no statistical difference in MMP activity when 0.1 M EDC, 5% PA and 2% CHX were compared (Table 1). When 0.5 M EDC was mixed with 35% HEMA to simulate the composition of an adhesive primer, the HEMA did not interfere with that cross-linker in inactivating the total MMP activity of acid-etched dentin. When the absorbances of the MMP

activity of acid etched dentin were expressed in MMP-9 activity equivalents, the total MMP activity of acid-etched dentin was equivalent to 6.10 (±1.93) ng of MMP-9 per 2x1x6 mm of acid-etched dentin.

DISCUSSION

The conventional method to analyze the total bound MMP activity using Sensolyte Generic MMP colorimetric assay kit includes the complete demineralization of dentin beams for 18 h with 10% phosphoric acid.[27] The current study used a simplified MMP assay method in which the dentin was acid etched for 15 s with 37% phosphoric acid. That avoids the complete dentin demineralization and reproduces more closely the surface demineralization of dentin that is done during etch-and-rinse bonding procedures. The complete demineralization of the dentin beam creates a much deeper collagen area (2x1x6mm) to be infiltrated by the cross-linking solutions and adhesive resins. Clinically, acid etching of dentin by 37 wt% phosphoric acid for 15 s only demineralizes dentin to a depth of 8-10 μm (Figure 1). Such relatively thin zones of demineralized dentin are easily saturated by test solutions within seconds. Nevertheless, this technique does not reproduce all *in vivo* conditions such as the presence of pulpal pressure and the outflow of dentinal fluid.

The hybrid layer is composed of 30 vol% collagen[28] (primarily type I), while the other 70% corresponds to resin and residual solvent[2]. The collagen fibril network acts as an anchorage to resin, enabling the retention of adhesive restorations. However, TEM analyses revealed that almost 70% of collagen from

the adhesive interface disappears after 44 months water storage.[29] Proteases such as metalloproteinases (MMPs) and cysteine cathepsins are thought to be responsible for collagen fibrils enzymatic degradation via hydrolysis.[30]

Exogenous MMP inhibitors have been tested in order to reduce protease activity and prolong the durability of resin-dentin bonds. Chlorhexidine was the first MMP inhibitor proposed for such a purpose during bonding to dentin.[31] It has been largely studied as a non-specific MMP[12] and cathepsin inhibitor.[14] CHX adsorbs on dentin and decreases hybrid layer degradation in vitro[7,32-34] and in vivo.[6,8,11,13] However this inhibitor is weakly bound to the collagen and can slowly be leached from the adhesive interface over time,[13] since no chemical bond is established between the CHX molecule and the collagen fibril.

One of the mechanisms proposed to explain how MMPs degrade collagen is that these proteases unwind collagen molecules when they bind to them. By doing that it allows the endogenous protease's active site sufficient space to attack the specific glycine-isoleucine peptide bond in peptide chains.[35-37] Cross-linking agents stiffens collagen polypeptides so that they can not unwind, and they can also inactivate the catalytic site of proteases[38] by creating a new peptide bond across adjacent peptides. Hence, it is reasonable to expect that MMP inactivation by cross-linking agents should last much longer than the inhibition of proteases by matrix-bound CHX. Carbodiimides (EDC) and proanthocyanidins (PA) were first used to increase the modulus of elasticity of collagen and make it more difficult to MMPs unwind the collagen triple-helix structure. However, EDC and PA are still

not capable of increasing the stiffness of collagen in clinically relevant periods of times, such as 30 s and 60 s (Scheffel et al., unpublished data).

Despite the long application times that cross-linking agents require to increase collagen stiffness,[20] they are effective against MMPs in 60 s. The results of this study require rejection of the tested null hypothesis. All investigated solutions significantly decreased MMP activity in acid etched dentin within 60 s. Both 0.1M EDC, 0.5M EDC and 5% PA were able to inactivate more than 84% of the total active MMPs. EDC activates the free carboxylic acid groups of glutamic and aspartic acids without introducing additional methylene groups. MMPs-2 (EC 3.4.24.24), -8 (EC 3.4.24.34), -9 (EC 3.4.24.35) and -20 (EC 3.4.24), the MMPs reported to be in dentin matrix, have glutamic acid in their active sites in position 404, 218, 402 and 227, respectively, allowing EDC to react to those sites. Additionally, the concentrations of EDC tested in this study did not produce any evidence of transdentinal cytotoxic effect on odontoblast-like cells in separate experiments (Scheffel et al., unpublished data), where they were also used at 0.1 M and 0.5 M, making EDC safe for *in vivo* application. The use of 0.5 M EDC was to accelerate its rate of diffusion into demineralized dentin. That EDC concentration is in far excess of the amount of protein in demineralized dentin. It is likely that only 1-2% of the EDC could react with proteins in 60 s. This would only generate 0.005-0.01 moles of urea, which is not sufficient to denature any proteins. Denaturing concentrations of urea require 2-8 moles[39,40]

Proanthocyanidins (PA) is a natural plant cross-linking agent. The mechanism of cross-linking is not completely understood. There are four different

theories to explain how PA interacts with proteins. They include covalent,[41] ionic,[42] hydrogen bonding,[43] and hydrophobic interactions.[44] This substance has been reported to increase the stiffness of demineralized dentin,[45] and to inhibit the progression of artificial root caries.[46,47] Additionally, scanning electron microscopy of demineralized dentin collagen treated with 15% PA for periods shorter than 120 s showed a homogeneous and regular collagen fibril arrangement, regardless of the surface moisture condition.[38] That result suggests that besides acting as MMP inhibitor cross-linking agents, it can stiffen demineralized dentin sufficiently to minimize the risk of collagen network collapse, resulted from air-drying. However, PA solution has a dark color, which stains the dentin despite water rinsing. That could be a drawback for the clinical use of this cross-linker. Its rapid, complete inactivation of matrix-bound MMPs in dentin suggests that more research should be done to try to isolate an uncolored fraction of the PA.

When 0.5 M EDC was solubilized in 35 vol% HEMA there was no reduction in its ability to inactivate all of the MMPs in dentin. That is, it was as effective as 0.5 M EDC alone. Since HEMA is an important component of adhesives, it may be possible to mix EDC with HEMA and other primer components in etch-and-rinse adhesive systems to inactivate MMPs during bonding. However, it is not known whether EDC influences adhesive polymerization. Further studies are still needed to demonstrate the effects of short-time application of cross-linking agents over time *in vitro* and *in vivo*.

CONCLUSIONS

Dentin treatment with cross-linking agents is effective to significantly reduce MMP activity; 0.5 M EDC showed the best results. Mixing 0.5 M EDC and 35% HEMA did not influence EDC crosslinking of MMPs, indicating that EDC could be added to primers in adhesive systems.

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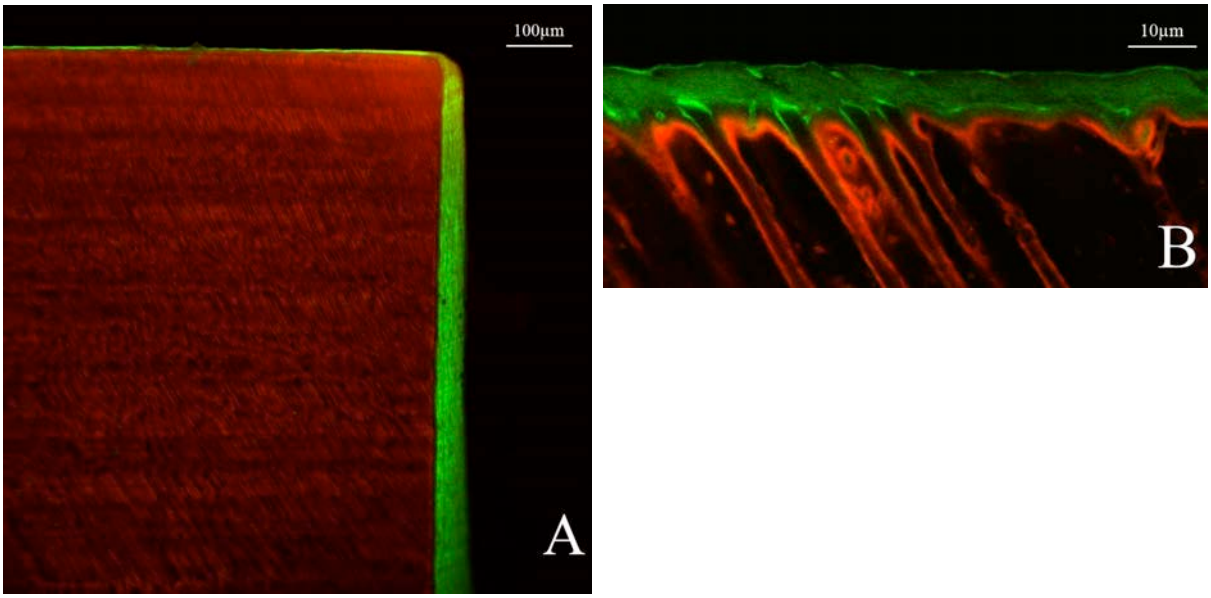


Figure 1. Confocal Laser Scanning Microscope (CLSM) images of the etched layer shown dyed green on the surface of a dentin beam. Dentin beams, were etched in 37% phosphoric acid for 15s and than rinsed with deionized water for 60s and labelled for 5 h respectively with 1 %w/v Fluorescein isothiocyanate (FITC) in anhydrous dimethyl sulfoxide (DMSO) and 1 %w/v Xylenol Orange (XO) in water. The two fluorochromes label collagen (FITC) and the mineralized matrix (XO), respectively. Prior to CLSM observation, the slabs were rapidly blotted with absorbent paper to remove the excess of fluorochrome, mounted on glass slides and promptly examined. Samples were scanned in two channels fluorescence mode with both 488nm excitation – 525nm emission (green channel) and 546nm excitation – 580nm emission (red channel), respectively, for FITC and XO labeling. (A) 10x projection of 53 images (final Z-stack thickness: 346µm), the sample was intentionally tilted to highlight the peripheral distribution of demineralized collagen. (B) 100x image of the border between demineralized surface collagen fibrils (etched layer) and underlying mineralized dentin matrix.

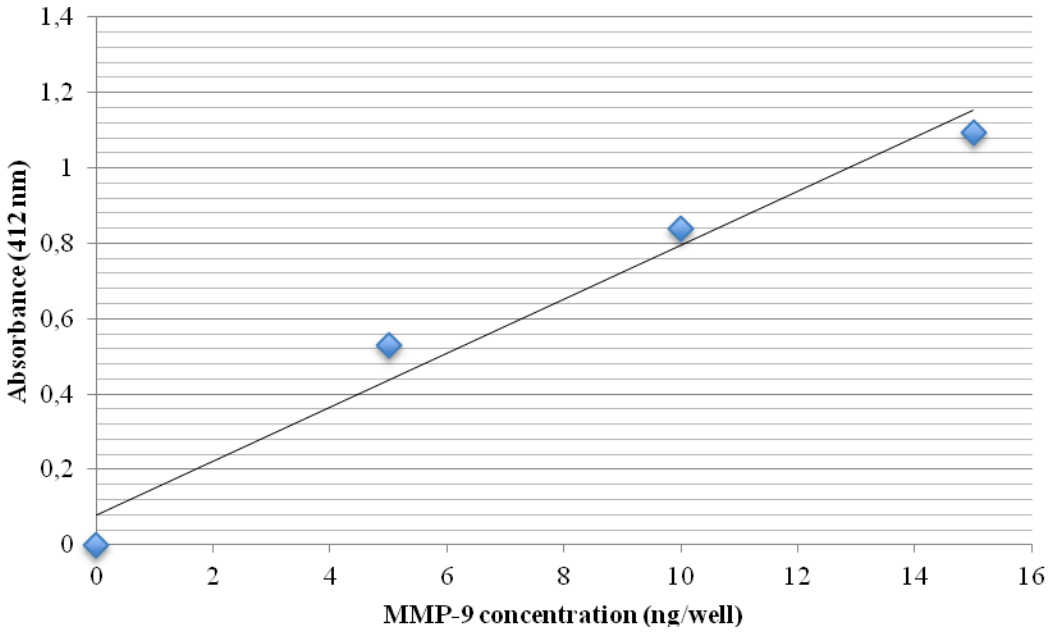


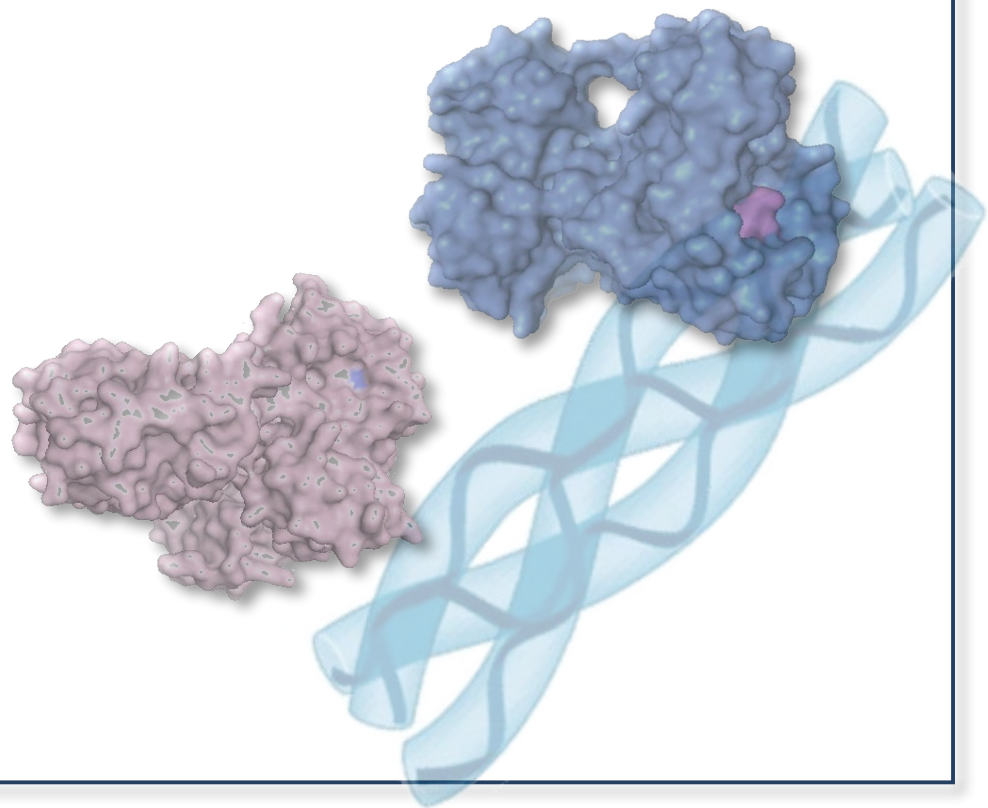
Figure 2. Standard curve of rh MMP-9 activity (ng/well) vs. absorbance at 412 nm after 60 min.

Table 1: Absorbance, percent inactivation/inhibition of total matrix-bound MMP activity in dentin and MMP9-equivalent (ng/dentin beam).

Demineralized dentin treatment	*Absorbance (412 nm)*	*MMP inhibition (%)*	*MMP-9 equivalent (ng)*
Water (control)	0.515 (±0.138) [a]	0 [d]	6.10 (±1.93) [a]
0.5 M EDC	0.038 (±0.014) [cd]	92.7 (±2.6) [ab]	0 (±0.19) [cd]
0.5 M EDC + 35% HEMA	0.025 (±0.016) [d]	95.2 (±3.0) [a]	0 (±0.22) [d]
0.1 M EDC	0.078 (±0.042) [bc]	84.8 (±8.2) [bc]	0 (±0.59) [bc]
5% Proanthocyanidin (PA)	0.064 (±0.035) [bc]	87.6 (±6.7) [bc]	0 (±0.49) [bc]
2% Chlorhexidine (CHX)	0.104 (±0.031) [b]	79.8 (±6.0) [c]	0.37 (±0.43) [b]

Values are mean (standard-deviation) absorbance, % inhibition of total MMP activity of dentin measured by SensoLyte substrate (AnaSpec Inc. Fremont, CA), and MMP9-equivalent (ng of MMP-9/dentin beam). EDC=1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride; HEMA=2-Hydroxyethyl methacrylate; PA=grape seed extract containing Proanthocyanidins; CHX=2wt% chlorhexidine digluconate. Within each columns, groups identified by different lower case letters are significantly different (Mann-Whitney, $p<0.05$).

6. Estudo IV



INCREASED DURABILITY OF RESIN-DENTIN BONDS FOLLOWING CROSS-LINKING TREATMENT

Running-tittle: Long-term effect of carbodiimide on bond strength

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INCREASED DURABILITY OF RESIN-DENTIN BONDS FOLLOWING CROSS-LINKING TREATMENT

ABSTRACT

Objectives: This study evaluated the long-term effect of carbodiimide treatments of acid-etched dentin on resin-dentin bond strength of a simplified etch-and-rinse adhesive system. **Methods**: Forty-eight sound third molars were divided into three groups (n=16) according to the dentin treatment: G1: Deionized water; G2: 0.5 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) applied for 30s and G3: 0.5 M EDC applied for 60s. Flat dentin surfaces were produced, etched with 37% phosphoric acid for 15s and then treated with deionized water for 60s or with 0.5 M EDC for 30s or 60s prior to the application of Single Bond 2. Crowns were restored with resin composite and beam specimens were prepared for microtensile testing. The beams from each group were tested 24h, 6 or 12 months after the adhesive procedures. One slab from each tooth was prepared and analyzed for nanolaekage. Bond strength (MPa) data were submitted to ANOVA and Tukey's test (α=0.05). **Results**: The treatment of dentin with 0.5 M EDC for 30 s (25.4±4.6 MPa) and 60 s (27.4±6.2 MPa) did not negatively affect the immediate bond strength of Single Bond 2 when compared to the control group (26.1±4.6 MPa). Additionally, EDC prevented resin/dentin bond degradation after 12 months in artificial saliva for both periods of treatment. An increased accumulation of silver ions was seen for the control group over time while a much lower amount of silver grains were observed for the EDC-treated groups. **Conclusions**: 0.5 M EDC was able to prevent resin/dentin bond degradation after 12 months even when applied for only 30 s.

Clinical significance statement: Topical treatments of acid-etched dentin with EDC increase resin-dentin bond stability and may provide better quality and durability to adhesive restorations. EDC used in short periods of time is able to prevent the degradation of the hybrid layer over time and produce long lasting resin-dentin bonds.

INTRODUCTION

Cross-linking agents have been reported to increase the stiffness of collagen making it more resistant to degradation.[1-3] These reagents link one peptide chain to another by covalent or ionic bonds.[1,4,5] Endogenous cross-links are naturally present in collagen structure and its mechanical properties depend on a highly regulated mechanism of intra and intermolecular cross-linking.[6] Increasing the number of cross-links in dentin collagen by applying exogenous cross-linking solutions prior to adhesive bonding or incorporating these agents into adhesive systems seems to enhance dentin-resin bond durability.[1,3,7,8]

Degradation of resin-dentin bonds is a complex process involving the deterioration of inorganic and organic portions of the hybrid layer.[9,10] Dentin matrix contains proteases, such as MMPs[11-15] that were secreted as inactive proenzyme forms during dental development and are released and activated after acid-etching during adhesive bonding.[16] The exposed MMPs on the collagen fibrils at the base of the hybrid layer slowly destroy the collagen fibrils to which they are bound, causing the loss of the anchoring function of hybrid layers and the loss of bond strength of resin composites.[17,18]

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) is a cross-linker that activates free carboxyl groups of glutamic and aspartic acids present in protein molecules[19,20] to form new peptide bonds. It is able to react with collagen[1] and to inactivate matrix-bound MMPs, even when applied on demineralized dentin for periods of time as short as 60 s.[23] In addition, EDC shows no transdentinal cytotoxicity on odontoblast-like cells when used in concentrations

between 0.1 M and 0.5 M (Scheffel et al., unpublished data). Therefore, EDC may provide long lasting adhesive bonds by inactivating MMPs[16,21] and by increasing collagen stiffness.[1]

The purpose of this study was to evaluate the long-term effect of EDC applied for short periods of time on dentin bond strength stability over a period of 12 months. The test null hypothesis was that 0.5 M EDC application has no significant effect on immediate and long-term dentin bond strength.

MATERIAL AND METHODS

Forty-eight sound human third molars were obtained under a protocol approved by the Ethics Committee of the Araraquara School of Dentistry (protocol #77/11). The occlusal enamel was completely removed from each tooth to obtain flat dentin surfaces using an ISOMET saw (Buehler Ltd., Lake Bluff, IL, USA) under water-cooling. Then the teeth were randomly divided into three groups, according to dentin treatment (n=16): G1: Deionized water applied for 60 s (control); G2: 0.5 M EDC applied for 30 s and G3: 0.5 M EDC applied for 60 s.

Bonding procedures

The dentin was etched with 35% phosphoric acid (Scotchbond etchant, 3M ESPE. St. Paul, MN, USA) for 15 s and then rinsed with deionized water for the same time. After blot-drying 20 µL of deionized water or 0.5 M EDC prepared in Sorensen's buffer (pH 6) were applied on demineralized dentin for 30 s or 60 s and then rinsed for 15 s. The excess of water was removed from the surface with

absorbent paper prior to bonding. The adhesive system Single Bond 2 (3M ESPE. St. Paul, MN, USA) was applied according to the manufacturer instructions, except for the dentin treatments, and photo-activated for 10 s (Radii Plus, SDI Limited, Bayswater, Victoria, Australia 1000±10 mW/cm^2). A 3 mm-high resin composite block (Z350. 3M ESPE. Dental Products. St Paul. MN) was built up incrementally and each increment was light cured for 20 s. The restored teeth were then stored in distilled water at 37^{o}C for 24 h.

Microtensile bond strength testing

Dentin beams with a cross-sectional area of 0.81 mm^2 were obtained in a high-precision cutting machine (Isomet 1000, Buehler, Lake Bluff, IL, USA) using a diamond saw (ISOMET, Buehler Ltd., Lake Bluff, IL, USA) under water-cooling. One third of the beams were tested 24h after the bonding procedures. The remaining specimens were storage in 3 mL of artificial saliva at 37^{o}C for 6 or 12 months before the microtensile test. The artificial saliva pH was monitored periodically to ensure that any significant changes occurred. The cross-sectional area of each beam was individually measured (Model 500-144B, Mytutoyo South America Ltda., SP, Brazil) and fixed to a testing device with cyanoacrylate glue (Super Bond Gel; Henkel Loctile. São Paulo, SP, Brazil) and subjected to the microtensile strength test in a mechanical testing machine (DL-Digital Line, EMIC, Parana, Brazil) equipped with a 100 N load cell running at a crosshead speed of 1.0 mm/min. Failures were classified as cohesive in resin or dentin, adhesive or mixed.

Nanoleakage analisys (SEM)

In each group, four 0.9 mm-thick slabs were randomly selected and immersed in 50 wt% ammoniacal $AgNO_3$ solution (pH 9.5) in the darkness for 24 h according to the protocol described by Tay et al.[22] After immersion in the tracer solution, followed by rinsing in deionized water for 5 min, the slabs were immersed in photo-developing solution for 8 h under a fluorescent light to reduce silver ions into metallic silver grain. The silver-stained specimens were polished with silicon carbide sandpaper with different grits (400, 600, 1200, 2400 and 4000). Then they were cleaned, mounted on aluminium stubs and placed in a desiccator for 24 h. Digital images were obtained using SEM (XL-30 FEG Scanning Electron Microscope; Philips, Eindhoven, The Netherlands) with a back-scattered electron detector at 10 kV at 2500 x magnification.

Statistical analysis

Bond strength recorded for specimens obtained from the same tooth were average in such a way that the tooth was used as the statistical unit of the study. Two-way ANOVA and Tukey tests were applied to analyze the effect of dentin treatment and storage period on microtensile bond-strength (MPa). The significance level was 5% for all analyses. Failure mode and nanoleakage data were analyzed descriptively.

RESULTS

Tensile bond strength

The ANOVA test showed a significant effect to dentin treatment ($p=0.0001$) and interaction between dentin treatment vs storage period ($p=0,035$). No significant effect was detected for storage period ($p=0,412$).

Bond strength data (MPa) are shown in Table 1 as mean and standard deviation. No statistically significant difference was observed among the immediate bond strength of Single Bond 2 after the treatment of dentin with deionized water (control) (26.1±4.6 MPa) or 0.5 M EDC 30 s (25.4±4.6 MPa) or 60 s (27.4±6.2 MPa).

After 6 months in artificial saliva, no significant bond strength decrease was observed for the control and EDC groups. However, bonds made to the EDC-treated dentin showed bond strength values that were significantly higher (29.6±4.0 MPa) than 6-month controls.

The beams treated with deionized water and stored for 12 months showed a significant reduction in bond strength (19.9±6.3 MPa) when compared to that observed after 24 h. The dentin treated with EDC for 30 s (22.2±5.1 MPa) or 60 s (29.2±6.5 MPa) presented no statistically significant decrease in bond strength of Single Bond 2 after 12 months of artificial saliva storage.

Failure modes and nanoleakage analysis

The distribution of failure modes is given in table 2 as absolute values and percentage of occurrence within the group. The largest percentages of failures involved the interface, adhesive and mixed fractures. Premature failures were observed in a small number and in all groups. EDC seems not influence resin/dentin bond failure mode.

Representative SEM photomicrographs of silver nanoleakage in adhesive bonds created by Single Bond 2 on dentin surfaces treated with water (control

group) or 0.5 M EDC for 30 s or 60 s and stored in artificial saliva for 24 h, 6 or 12 months at 37°C are presented in Figure 1. SEM images revealed the presence of silver deposits in all groups and storage periods. The specimens analyzed 24 h after the bonding procedures showed small silver accumulation. In the control group, a gradual increase in nanoleakage was observed over time. However, the groups treated with EDC for 30 s or 60 s showed much lower amounts of silver nanoleakage, even after 12 months in the artificial saliva. All the SEM images exhibited the presence of polyalkenoic acid copolymer globules in the adhesive layer of Single Bond 2, indicating the occurrence of phase separation.

DISCUSSION

Collagen fibrils in mineralized dentin are protected from hydrolytic and enzymatic degradation by hydroxyapatite crystals. However, after acid etching, collagen fibrils become uncovered and their bound proteases (MMPs and cathepsins) are activated[23] to cleave unprotected collagen, resulting in decrease in long-term bond strength[24] as was observed in the control group of this study.

In order to reduce the activity of these activated proteases and preserve the integrity and durability of the adhesive interfaces, chlorhexidine (CHX) has been used as a nonspecific MMP[18,24-28] and cathepsins[29] inhibitor. However, chlorhexidine is highly water-soluble and may be leached from the hybrid layer compromising its anti-protease efficacy.[24] The use of cross-linking agents instead of CHX has the advantage of inactivating endogenous MMPs[21] and

simultaneously increases collagen mechanical properties[1] by creating covalent cross-links that are stable over time.

The bio-modification of collagen by extrinsic cross-linkers can induce the formation of additional inter and intra-molecular cross-links,[30,31] increasing the ultimate tensile strength and elastic modulus of demineralized dentin.[32,33] Cross-linking agents such as proanthocyanidins, glutaraldehyde and tannic acid are able to[7,34] improve immediate resin-dentin bond strength after one-hour treatment, an observation that was not seen in our results. However, we used much shorter periods of treatment. The present study showed for the first time that 0.5 M EDC applied on dentin for short periods of time like 30 and 60 s was capable of preventing resin-dentin bond degradation up to 12 months of aging in saliva. These results require partial rejection of the tested null hypothesis.

To degrade the organic matrix of dentin, MMPs must link their narrow binding sites[35] to the substrate and unwind collagen molecules[36-38] culminating in collagen peptides cleavage. However, the treatment of demineralized dentin with cross-linking agents makes collagen more difficult to unwind, preventing the degradation by MMPs. EDC activates the free carboxylic groups of glutamic and aspartic acids present on collagen[1] and MMPs structures.[21] It increases collagen stiffness and inactivates MMPs activate sites.[21] Additionally, the cross-links created by EDC may reduce these enzymes mobility.

Although Scheffel et al. (unpublished data) demonstrated that EDC does not increase the elastic modulus of completely demineralized dentin beams (1x1x3 mm) when applied for 30 s and 60 s, we suggest that typical etch-and-

rinse 10 micrometer-deep zones of collagen exposed by acid-etching would be easily saturated by this cross-linking agent which may be effective to increase collagen mechanical properties.

Single Bond 2 is a two-step etch-and-rinse adhesive system that contains hydrophilic resin monomers to enhance the adhesive wetting properties and avoid phase changes observed when hydrophobic monomers are added to water.[39] Therefore, these adhesives have a high water affinity[40,41] that favors their degradation.[40] Over time, it is thought that infiltrated resins are extracted from dentin matrix[42,43] and uninfiltrated collagen fibrils are hydrolyzed and replaced by water.[44] The silver nanoleakage protocol fills water spaces with silver nitrate that is later photo-reduced to silver grains that can be analyzed by SEM.[22] The control group showed higher silver accumulation when compared to EDC treated groups after 6 and 12 months what demonstrates degradation of the hybrid layer. Conversely, the cross-linking agent applied for 30 s and 60 s on etched dentin was able to prevent the increase in nanoleakage.

Bond strength data and nanoleakage images showed that the treatment of demineralized dentin with EDC could be a simple, practical and clinically applicable method to reduce collagen degradation in the hybrid layer, being an efficient alternative to make resin-dentin bonds more durable. How much of the increase in durability is due to cross-linking of collagen versus collagen-bound matrix metalloproteases (MMPs) and cathepsins, is unclear. Further studies are still needed to better understand of the effects of EDC application *in vitro* and to demonstrate its efficacy *in vivo.*

CONCLUSION

The treatment of acid-etched dentin with 0.5 M EDC prior to bonding procedures was able to prevent resin-dentin bond degradation after up to 12 months even when applied for only 30 s.

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Table 1. Bond strength (MPa) of Single Bond 2 to dentin after treatment with 0.5 M EDC for 30 s or 60 s and storage in artificial saliva for up to 12 months.

Dentin Treatment	Storage Period		
	24 hours	6 months	12 months
Water (control)	26.1±4.6 [ab]	22.7±2.8 [bc]	19.9±6.3 [c]
EDC 30 s	25.4±4.6 [ab]	21.8±5.1 [bc]	22.2±5.1 [bc]
EDC 60 s	27.4±6.2 [a]	29.6±4.0 [a]	29.2±6.5 [a]

Numbers are mean±standard-deviation, n=16. [a] Groups identified by the same letter are statistically similar (Tukey, p>0.05).

Table 2. Distribution of failure types.

Dentin Treatment	Storage Period		
	24 hours	6 months	12 months
Water (control)	A=64 (88.9)[a]; M=1 (1.4); RC=1 (1.4); DC=1 (1.4); PF=5 (6.9)	A=47 (83.9); M=3 (5.4); PF=6 (10.7)	A=67 (82.7); M=2 (2.5); RC=1 (1.2); DC=1 (1.2); PF=10 (12.3)
EDC 30 s	A=78 (95.1); M=1 (1.2); DC=2 (2.4); PF=1 (1.2)	A=57 (75.0); M=15 (19.7); PF=4 (5.3)	A=76 (82.6); M=3 (3.3); RC=3 (3.3); DC=2 (2.2); PF=8 (8.7)
EDC 60 s	A=68 (95.8); DC=2 (2.8); PF=1 (1.4)	A=59 (83.1); M=8 (11.3); PF=4 (5.6)	A=63 (78.8); M=4 (5.0); RC=1 (1.3); DC=5 (6.3); PF=7 (8.8)

A = adhesive; M = mixed; CR = cohesive in resin; CD = cohesive in dentin; PF = premature failure. Values represent the absolute frequency (percentage of total specimens in the group).

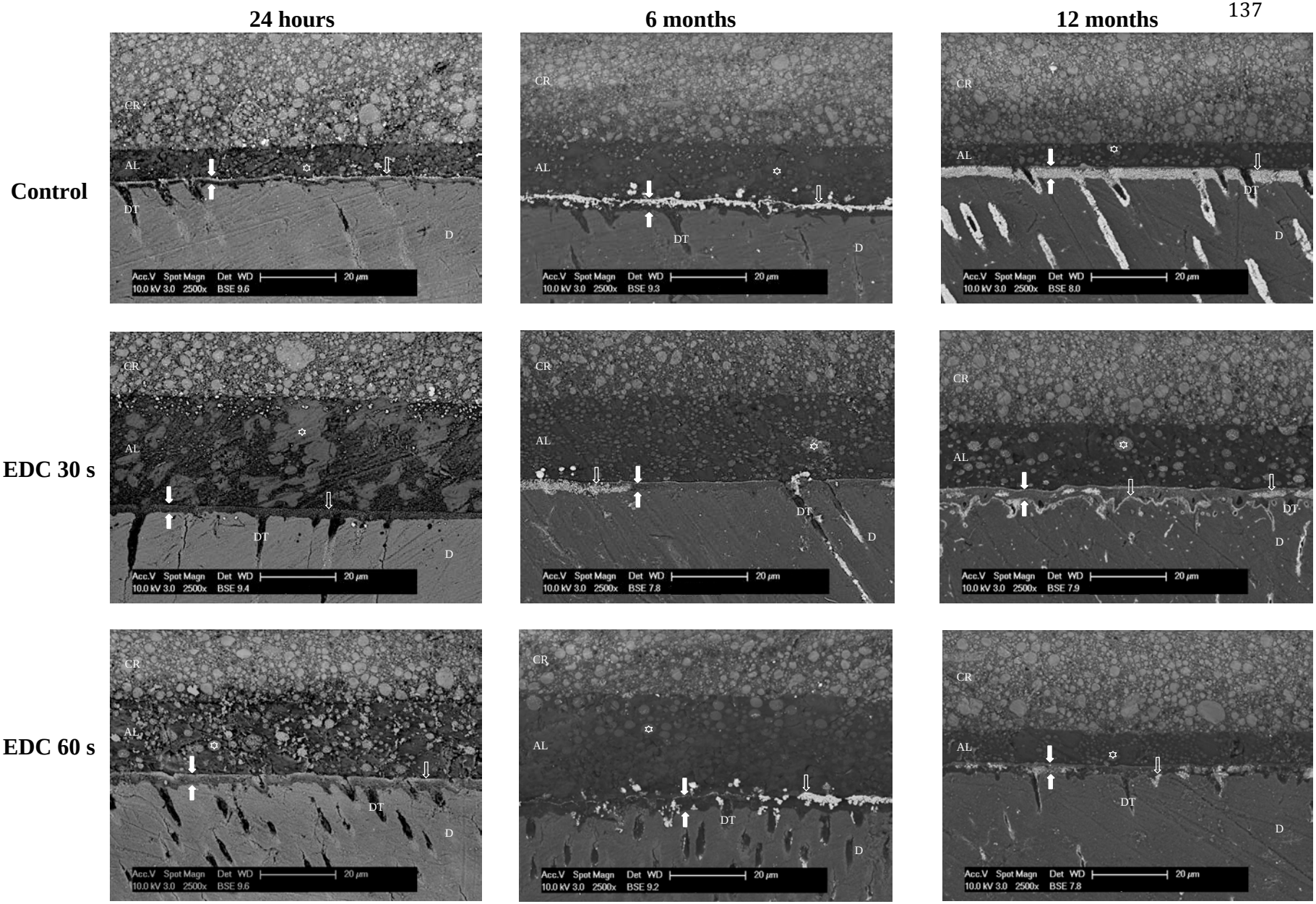
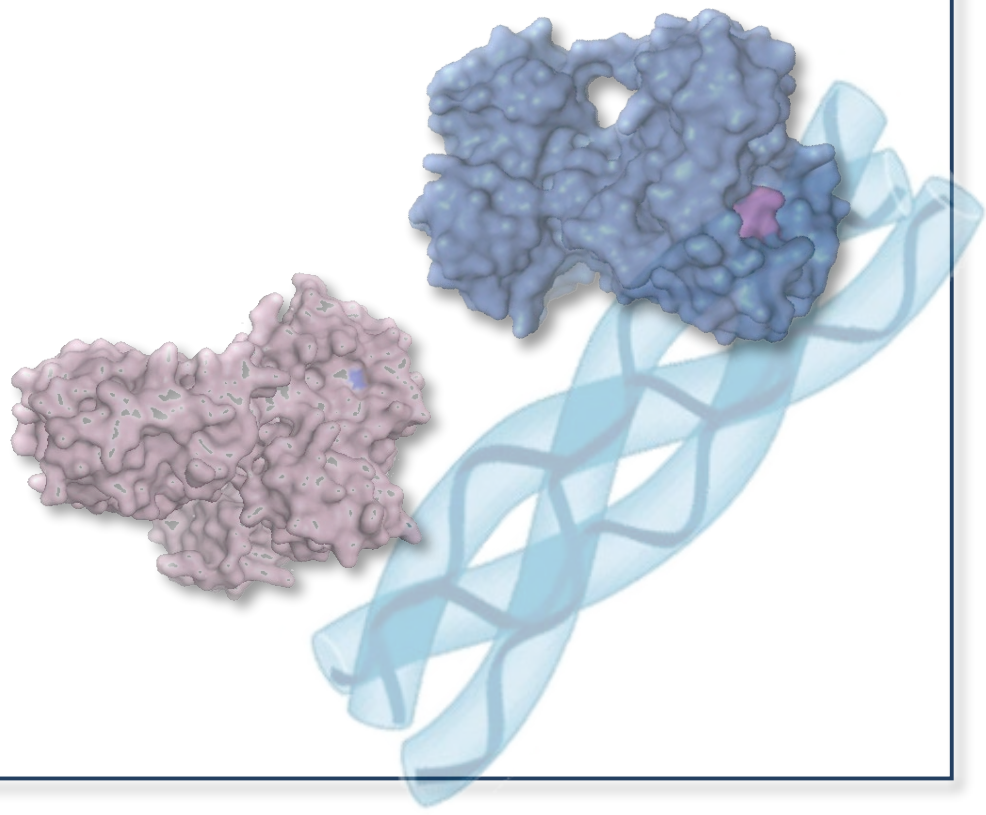


Figure 1: SEM photomicrographs of adhesive bonds created by Single Bond 2 on dentin surfaces pre-treated with water (control group) or 0.5 M EDC for 30 s or 60 s and stored in artificial saliva for 24 h, 6 or 12 months. The zone between white filled arrows represents the hybrid layer (HL). ⇧ shows silver deposits in the HL and = polyalkenoic acid copolymer globules. AL=adhesive layer, D=mineralized dentin, CR= composite resin and DT= dentin tubule. Magnification ×2,500.

7. Considerações Finais



7 CONSIDERAÇÕES FINAIS

Melhorar a qualidade e a estabilidade das interfaces adesivas tem sido o objetivo de vários estudos ao longo dos últimos anos. Técnicas clinicamente aplicáveis, como a utilização de clorexidina sobre a dentina condicionada (Hebling et al[27]., 2005; Carrilho et al[9]., 2010; Ricci et al[46]., 2010; Scaffa et al[50]., 2012) e técnicas que ainda permanecem restritas à aplicações in vitro, como a remineralização da camada híbrida (Mai et al[33]., 2010; Kim et al[28]., 2010; Kim et al[29]., 2010; Ryou et al[48]., 2011) vem sendo investigadas.

O tratamento da dentina desmineralizada com substâncias *cross-linkers* previamente a aplicação do sistema adesivo, surge como uma alternativa promissora para a redução da degradação da união resina-dentina. Os primeiros estudos que utilizaram estes agentes visando, direta ou indiretamente, a estabilidade das restaurações resinosas a partir do aumento das propriedades mecânicas do colágeno dentinário, obtiveram resultados positivos, porém, em tempos de aplicação clinicamente inviáveis (Bedran-Russo et al[6]., 2010; Al-Ammar et al[1]., 2009; Bedra-Russo et al[7]., 2009). Posteriormente, foi demonstrada a ação destas substâncias como inibidores inespecíficos de MMPs (Tezvergil-Mutluay et al[57]., 2012), abrindo a possibilidade de que os *cross-linkers* fossem capazes de agir concomitantemente, sobre as fibrilas de colágeno desmineralizadas e sobre as proteases presentes na dentina.

Dentre os diferentes *cross-linkers*, o EDC (1-Etil-3-[3-dimetilaminopropil] hidroclorito de carbodiimida) e o glutaraldeído (GA) tem sido os mais estudados principalmente, para aplicações médicas (Lu et al[32]., 2008; Lai et al[30]., 2010;

McDade et al[35]., 2013; Lai[31], 2013; Grant et al[23]., 2013; Wu et al[65]., 2013; Ruiz et al[47]., 2013; Fan et al[20]., 2013; Anisha et al[2]., 2013). No entanto, pouco se encontra na literatura quanto a resposta biológica, física e química destes materiais quando empregados no contexto odontológico.

Os estudos que constituem este trabalho objetivaram responder questões, que pairavam sobre a utilização destes agentes na prática restauradora como: o potencial citotóxico transdentinário destas substâncias sobre células odontoblastóides (Estudo 1), seus efeitos sobre a mecânica e degradação do colágeno dentinário (Estudos 2 e 3), sobre a inibição de MMPs ligadas a matriz de dentina em tempos clinicamente aplicáveis (Estudos 2 e 3) e como estes fatores refletiriam na degradação, em longo prazo, da interface adesiva (Estudo 4).

Os resultados obtidos, em conjunto, sugerem que a indução da formação de novas ligações cruzadas por meio da aplicação de agentes exógenos, representa uma forma simples e aplicável para a preservação da união adesiva de maneira eficaz e segura. A avaliação da citotoxicidade transdetinária de três diferentes soluções de EDC e GA 5% em células odontoblastóides MDPC-23 mostrou que, após o tratamento da dentina por 60 s com estas substâncias, a viabilidade, função e morfologia celular não foram negativamente alteradas. A partir destas observações, a concentração de EDC 0,5 M foi escolhida como a mínima concentração utilizada no estudo 2, bem como para ser aplicada sobre a dentina condicionada previamente ao sistema adesivo no estudo 4.

Uma vez demonstrada a ausência de efeitos tóxicos dos *cross-linkers* testados, estes agentes foram avaliados quanto ao seu potencial para inibir MMPs

e biomodificar o colágeno dentinário desmineralizado, melhorando suas propriedades mecânicas e resistência a degradação. Os resultados obtidos pelo estudo 2, revelaram que o EDC e o GA são capazes de aumentar a rigidez da matriz orgânica de dentina e simultaneamente inibir em até 81,9% a atividade de MMPs, reduzindo assim a perda de massa e a liberação de hidroxiprolina geradas pela biodegradação das fibrilas. Adicionalmente, o colágeno tornou-se mais resistente a degradação térmica. Contudo, altas concentrações de EDC (1 M e 2 M) e GA (10%) foram necessárias para a obtenção dos resultados acima descritos, principalmente pelo fato de que palitos de dentina com 1 mm de espessura e completamente desmineralizados foram utilizados. Desta forma, a menor concentração de EDC (0,5 M) utilizada neste estudo não atingiu resultados tão positivos quanto o EDC 1 M e 2 M.

Uma vez que 1 mm de colágeno desmineralizado representou uma espessura excessiva para a ação efetiva do EDC 0,5 M, o estudo 3 foi realizado buscando assemelhar-se mais a prática clínica. Sendo assim, ao invés de palitos completamente desmineralizados, o potencial do EDC como inibidor de MMPs foi avaliado em palitos condicionados com ácido fosfórico 37% por 15 s. Além disso, um *cross-linker* natural derivado de uvas foi testado (proantocianidina 5%), bem como a mistura do EDC com HEMA 35%, visando uma futura associação dos dois elementos para aplicação como primer, suprimindo desta forma, a necessidade da inclusão de mais um passo clínico (aplicação do *cross-linker*) no procedimento adesivo. Todas as soluções inibiram um maior percentual de MMPs quando comparadas a clorexidina 2%, sendo que os melhores resultados foram

observados para o tratamento da dentina com EDC 0,5 M associado ou não ao HEMA 35%.

Os achados favoráveis apresentados pelos três primeiros trabalhos culminaram na efetividade do EDC 0,5 M para preservar a interface adesiva ao longo do tempo. Quando aplicado sobre a dentina condicionada por 30 s ou 60 s no estudo 4, o EDC não prejudicou a resistência de união (RU) imediata do Single Bond 2 e preveniu sua redução após 12 meses de armazenamento em saliva artificial. A atenuação do processo de degradação da interface adesiva foi confirmada pela análise da nanoinfiltração presente na camada híbrida, a qual foi muito inferior nos grupos tratados com EDC, independente do tempo de aplicação.

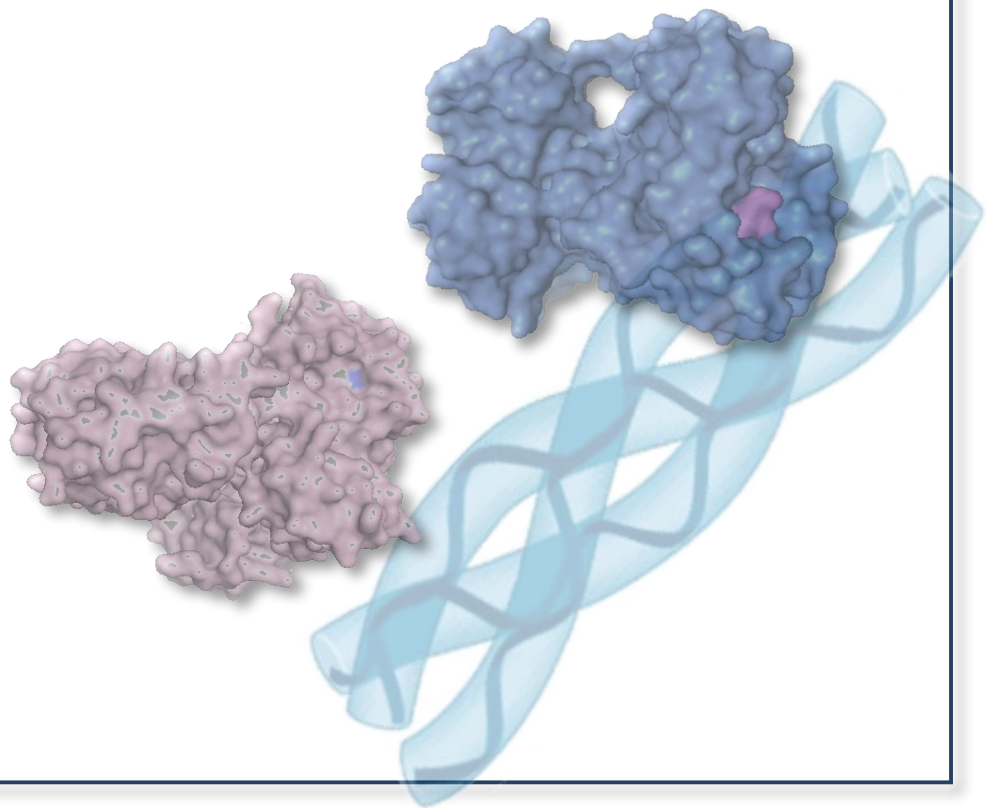
Todos os resultados obtidos nos quatro estudos que compõem esta tese sugerem que, o tratamento da dentina desmineralizada com EDC pode ser um método simples, prático e clinicamente viável que:

- Não apresenta efeito citotóxico sobre células MDPC-23.
- Favorece as propriedades mecânicas do colágeno dentinário.
- Inibe MMPs.
- Previne a degradação da interface adesiva.
- Torna a união resina-dentina mais estável e durável

No entanto, qual será a qualidade e estabilidade das interfaces adesivas criadas in vivo após a aplicação de EDC, como se comportará a associação desta substância com os sistemas adesivos e quão efetivo este agente será para inibir

outras proteases, são questões que permanecem para ser respondidas em estudos futuros.

8. Referências



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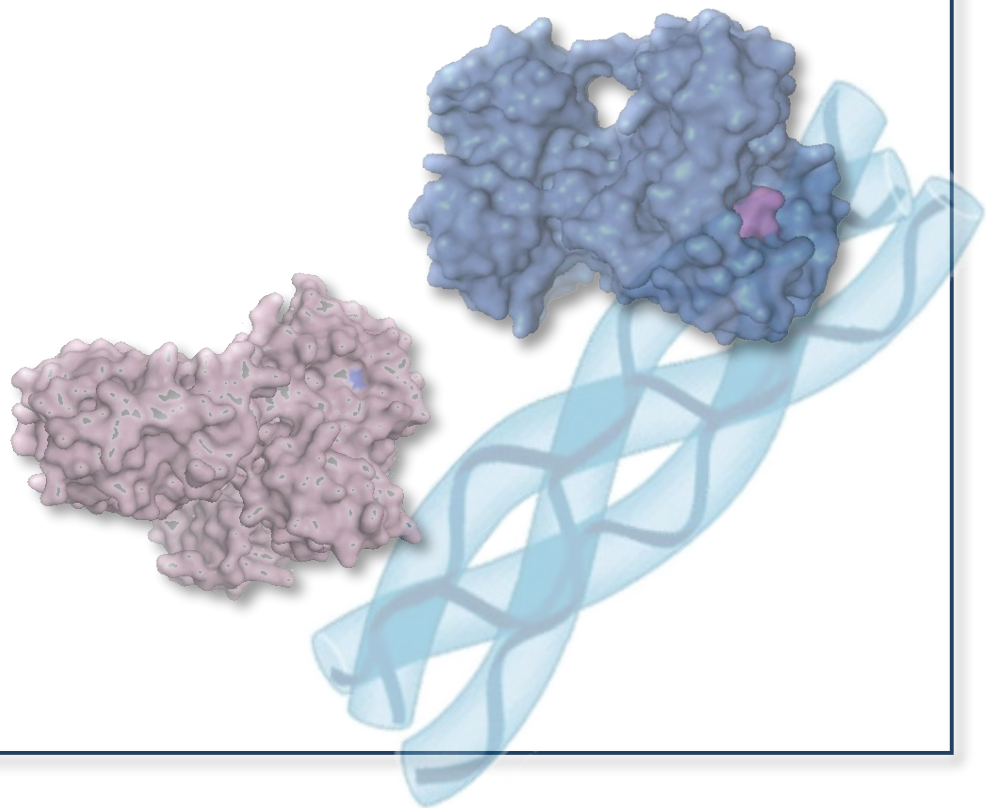
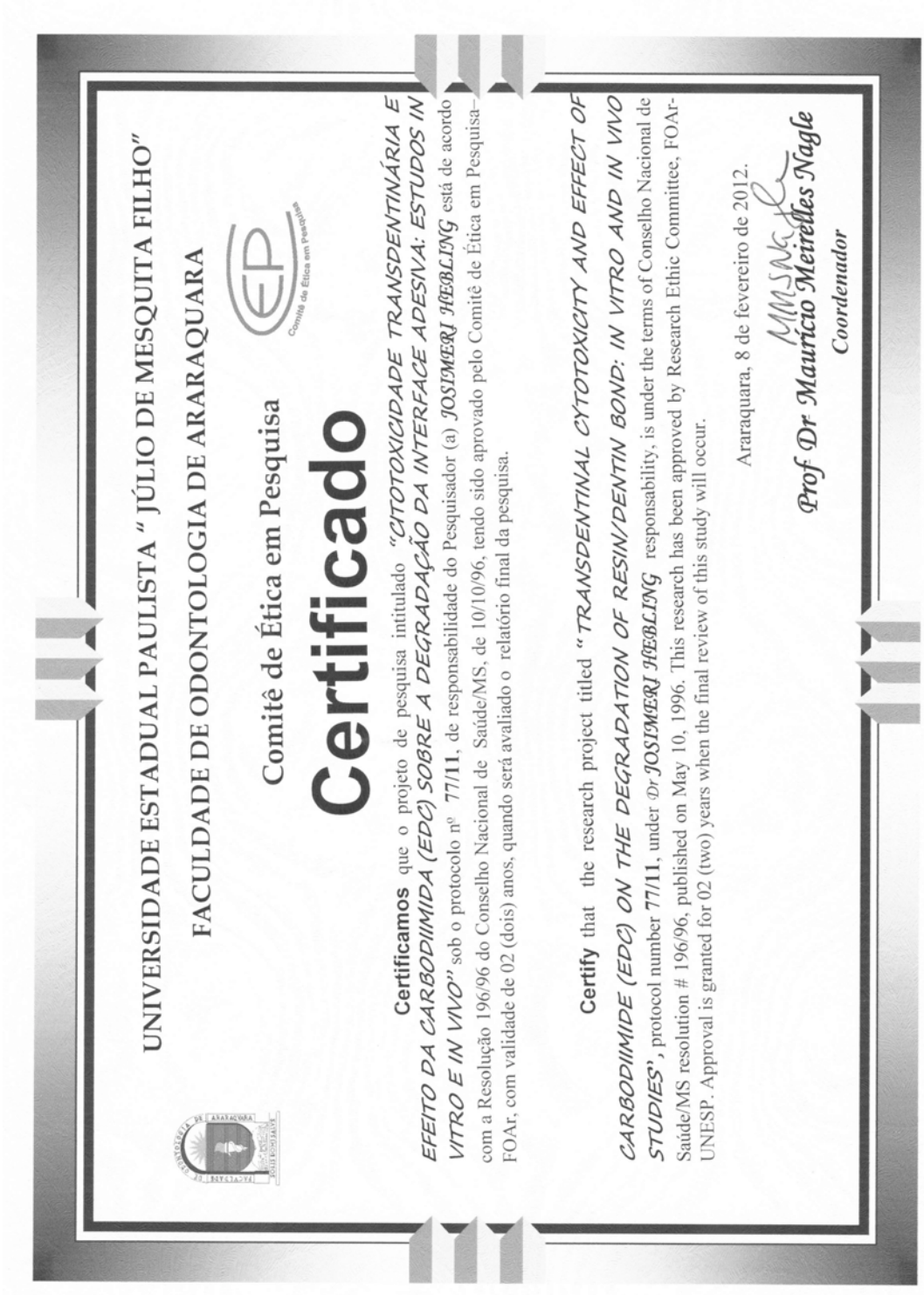
9. Anexo



Anexo - Certificado de aprovação do Comitê de Ética em Pesquisa (CEP) da Faculdade de Odontologia de Araraquara.



UNIVERSIDADE ESTADUAL PAULISTA " JÚLIO DE MESQUITA FILHO"

FACULDADE DE ODONTOLOGIA DE ARARAQUARA

Comitê de Ética em Pesquisa

Certificado

Certificamos que o projeto de pesquisa intitulado *"CITOTOXICIDADE TRANSDENTINÁRIA E EFEITO DA CARBODIIMIDA (EDC) SOBRE A DEGRADAÇÃO DA INTERFACE ADESIVA: ESTUDOS IN VITRO E IN VIVO"* sob o protocolo nº 77/11, de responsabilidade do Pesquisador (a) *JOSIMERI HEBLING* está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde/MS, de 10/10/96, tendo sido aprovado pelo Comitê de Ética em Pesquisa-FOAr, com validade de 02 (dois) anos, quando será avaliado o relatório final da pesquisa.

Certify that the research project titled *" TRANSDENTINAL CYTOTOXICITY AND EFFECT OF CARBODIIMIDE (EDC) ON THE DEGRADATION OF RESIN/DENTIN BOND: IN VITRO AND IN VIVO STUDIES"*, protocol number 77/11, under *Dr JOSIMERI HEBLING* responsability, is under the terms of Conselho Nacional de Saúde/MS resolution # 196/96, published on May 10, 1996. This research has been approved by Research Ethic Committee, FOAr-UNESP. Approval is granted for 02 (two) years when the final review of this study will occur.

Araraquara, 8 de fevereiro de 2012.

Prof Dr Maurício Meirelles Nagle

Coordenador

Autorizo a reprodução deste trabalho.

Araraquara, 12 de setembro de 2013.

DÉBORA LOPES SALLES SCHEFFEL