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PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA – DOUTORADO
ÁREA DE CONCENTRAÇÃO: DENTÍSTICA RESTAURADORA

VIVIANE HASS

Efeito do uso de agentes reticuladores de colágeno em procedimentos adesivos: estudos *in vitro* e *in situ*

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Tese apresentada para a obtenção do título de Doutor na Universidade Estadual de Ponta Grossa, no Curso de Doutorado em Odontologia - Área de Concentração Dentística Restauradora. Linha de pesquisa: Propriedades quimico físico e biológicas dos materias dentários

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Amo vocês...

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HASS V. **Efeito do uso de agentes reticuladores de colágeno em procedimentos adesivos: estudos *in vitro* e *in situ*.** [Tese] Doutorado em Dentística Restauradora. Ponta Grossa. Universidade Estadual de Ponta Grossa; 2015.

RESUMO

O objetivo desse trabalho foi verificar o efeito da aplicação de agentes reticuladores de colágeno em tempo e estratégia clinicamente relevantes, através de dois estudos laboratoriais *in vitro* e um estudo *in situ*. Para o experimento 1 *in vitro*, 52 dentes tiveram a superfície dentinária condicionada, e distribuídos entre 8 grupos experimentais pela combinação dos fatores: agente de tratamento (proantocianidina 6,5% [PA], riboflavina 0,1% ativada pela luz ultravioleta [RB], glutaraldeído [GA] e água destilada como grupo controle [CT]; e os sistemas adesivos (Adper Single Bond Plus [SB] e Tetric N-Bond [TN]. Os agentes foram aplicados com primers aquosos por 60 s, seguido da aplicação do sistema adesivo e restauração com resina composta. Depois foram seccionados e testados imediatamente (IM) e após 18 meses de armazenamento (18M) para análise da resistência de união à dentina (RU), nanoinfiltração (NI) e em IM para análise do grau de conversão (CG) e atividade de metaloproteinases (MMPs), dentro da camada híbrida. Outros 24 dentes foram utilizados para análise da citotoxicidade. Para o experimento 2 *in vitro*, foi utilizado 2 condicionadores ácidos (2%PA mais ácido 10% [ACPA] e ácido fosfórico 35% [CT]). Foi exposta a superfície dentinária de 16 dentes, realizado o condicionamento ácido, aplicados o adesivo SB e incrementos de resina composta. Esses dentes foram destinados para análise da RU, NI em IM e após 6 meses de armazenamento (6M) e atividade de MMPs dentro da camada híbrida IM. Outros 10 dentes tiveram planificadas as faces de esmalte para análise da resistência de união ao esmalte (RE), que também foi testada em IM e 6M. Para o experimento 3 *in situ*, foram utilizados 40 dentes, nos quais foram confeccionadas 2 cavidades em cada dente. Essas cavidades foram condicionadas, aplicados os mesmos agentes reticuladores de colágeno como no experimento 1, sistema adesivo SB e restauradas com resina composta. Uma das restaurações de cada dente foi testada IM e a outra foi fixada em um dispositivo palatino, que foi usado por 14 dias em ambiente cariogênico oral [ACO] por 10 voluntários. Esses dentes foram testados para análise da microdureza, RU, NI e análise histológica da morfologia da camada híbrida. Todos os dados foram submetidos a ANOVA 2 fatores de medidas repetidas e teste de Tukey (α=0,05). Para o experimento 1, todos os agentes foram eficazes para reduzir a degradação da interface de união após 18M e ausentes de citotoxicidade, exceto o GA ($p<0,05$). Para o experimento 2, a inclusão da PA no condicionamento ácido foi eficaz para reduzir a degradação em dentina ($p<0,05$) e não afetou a RE ($p>0,05$). Já para o experimento 3, *in situ*, apenas a PA e o GA foram eficazes para reduzir a degradação da interface de união à dentina em ACO ($p<0,05$). Os agentes reticuladores foram eficazes para reduzir a degradação da interface de união à dentina, em tempos e estratégia clinicamente relevantes. Contudo o GA foi considerado potencialmente citotóxico e deveria ser evitado para uso clínico.

Palavras-chave: colágeno dental, proantocianidina, riboflavina, glutaraldeído, adesão dental.

HASS V. **Effect of the use of collagen cross-link agents on bonding procedures: *in vitro* and *in situ* studies.** [Tese] Doutorado em Dentística Restauradora. Ponta Grossa. Universidade Estadual de Ponta Grossa; 2015.

ABSTRACT

This study evaluated the effect of application of collagen cross-link agents under time and clinically relevant strategy, through two in vitro and one in situ studies. For in vitro experiment 1, 52 third human molars had exposed the dentin surface, acid etched and distributed according the 8 experimental groups: collagen cross-link agent (6.5% pronthocyanidin [PA], 0.1% UVA-light actived riboflavin [RB], 5% glutaraldehyde [GA] and distilled water as control group [CT], and 2 simplified etch-and-rinse systems (Adper Single Bond Plus [SB] and Tetric N-Bond [TN]. The cross-link agentes were actively applied for 60 s, bonded and restorated with composite resin. Then, the teeth were sectioned to obtain specimens and tested in immediately [IM] and after 6 months [6M] storage time for microtensile bond strength (μTBS) and nanoleakage (NL), and IM for degree of conversion (DC) and MMP-activity within the hybrid layer. Additional 24 teeth were used by cytotoxicity assay. For in vitro experiment 2, 2 etchant agentes were tested (2%PA with 10% phosphoric acid [ACPA] and 35% phosphoric acid [CT]). Dentin of 16 teeth was exposed, etched, bonded using SB adhesive, and restored with composit resin. These teeth were tested by μTBS, NL in IM and 6M; and MMP-activity within the hybrid layer. Additional 10 teeth had flated the enamel surfaces and tested by enamel bond strength in IM and 6M. For the in situ experiment 3, 40 teeth were used, in which 2 cavities prepared. These cavities were etched, treated according the experiment 1, bonded and restored with composite resin. One restoration for each tooth was tested IM and another was included in a palatal device in cariogenic oral environment [COE] for 10 volunteers during 14 days. The restorations were sectioned and tested by microhardness, μTBS, NL and morphology of the hybrid layer for differential staining technique. All the data were analyzed by 2 way-ANOVA and Tukey's test ($\alpha = 0.05$). For experimente 1, all the collagen cross-link agentes were effective to reduce the resin-dentin degradation after 18M e did not exhibit cytotoxic potential, except the GA ($p<0.05$). For the experiment 2, the PA containing in etchant reduced the resin-dentin bond degradation ($p<0.05$) and did not affect the resin-enamel bond strentgh ($p>0.05$). However for in situ experiment 3, only the PA and GA reduced the resin-dentin degradation ($p<0.05$) in COE. The collagen cross-link agentes were effective to reduce the resin-dentin degradation, in time and relevant clinically strategy. The GA exhibited cytotoxic potential and should be avoided for clinical use.

Keywords: dentin collagen, proanthocyanidin, riboflavin, glutaraldehyde, dental bonding.

LISTA DE SÍMBOLOS E UNIDADES

%	porcentagem
s	segundos
ºC	Grau Celsius
±	Mais ou menos
MPa	Mega Pascal
<	Menor que
>	Maior que
α	Alfa (nível de significância)
p	probabilidade
n	Número amostral
mW/cm^2	MiliWatt por centímetro quadrado
λ	Lambda (comprimento de onda)
h	Hora
µm	Micrometro
mm^2	Milímetro quadrado (unidade de área)
min	Minuto
mm/min	Milímetro por minuto
nm	Nanometro (unidade de comprimento de onda)
x	Número de vezes
F/l	Flúor por litro
ml	Mililitro
M	Molar
mg	Miligrama
pH	Potencial hidrogeniônico
CO_2	Dióxido de carbono
µL	Microlitro

LISTA DE SIGLAS

MMP	Metaloproteinase
UVA	Ultravioleta
MTT	Metitetrazolium
ANOVA	Análise de variância
PVC	Policloreto de vinila
CLSM	Confocal Laser Microscopy (microscopia confocal a laser)

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1. INTRODUÇÃO

Sabe-se que durante a adesão à dentina, uma matriz colágena é exposta pela ação do condicionamento ácido. Após a infiltração de monômeros resinosos, as fibrilas colágenas são encapsuladas pelos monômeros promovendo um microembricamento mecânico que, após a fotopolimerização, forma a camada híbrida (NAKABAYASHI, KOJIMA e MASUHARA, 1982 [1]). Essa é a base que sustenta as restaurações adesivas. Assim, a estabilidade e manutenção das fibrilas colágenas são essenciais para que essa união seja eficaz e duradoura.

Infelizmente, a infiltração de monômeros não ocorre em toda a extensão desmineralizada pelo condicionamento ácido, gerando uma discrepância entre a profundidade de desmineralização e a de infiltração de monômeros (SPENCER e SWAFFORD, 1999 [2,] WANG e SPENCER, 2002 [3]). Além disto, com o passar do tempo há também degradação do polímero dentro da camada híbrida, causada pela eluição de monomeros hidrofílicos devido à sorção de água, que reduz as forças friccionais entre as cadeias poliméricas (MALACARNE, CARVALHO, DE GOES, et al., 2006 [4,] YIU, PASHLEY, HIRAISHI, et al., 2005 [5]). Isso amplia ainda mais a quantidade de fibrilas colágenas, que ficam sujeitas à degradação enzimática. Assim, a preservação da trama colágena é fundamental para melhorar a adesão e prevenir a degradação, aumentando assim a durabilidade da interface adesiva.

A deterioração das fibrilas colágenas por proteases endógenas tem sido considerada como uma possível responsável pelo mecanismo de degradação da interface de união à dentina (NISHITANI, YOSHIYAMA, WADGAONKAR, et al., 2006 [6,] PASHLEY, TAY, YIU, et al., 2004 [7,] TJADERHANE, LARJAVA, SORSA, et al., 1998 [8]).

O uso de inibidores de proteases endógenas tem sido investigado como uma alternativa de reduzir o potencial de degradação das fibrilas colágenas. Estudos *in vitro* e *in vivo* já demonstraram que a clorexidina utilizada como primer após o condicionamento ácido (CARRILHO, CARVALHO, DE GOES, et al., 2007 [9,] HEBLING, PASHLEY, TJADERHANE, et al., 2005 [10]), incorporada ao ácido fosfórico (STANISLAWCZUK, AMARAL, ZANDER-GRANDE, et al., 2009 [11,] STANISLAWCZUK, REIS e LOGUERCIO, 2011 [12]) e adesivo (ZHOU, TAN, CHEN, et al., 2009 [13]) foi capaz de preservar a interface de união após

envelhecimento. Além da clorexidina, a galardina, EDTA, cloreto de benzalcônio entre outras também têm sido investigados com resultados promissores no que tange a maior durabilidade da interface da união (CARRILHO, 2012 [14]). Contudo esses agentes são solúveis em água e podem ser lixiviados da interface de união ao longo do tempo, pelo fato de não estabelecerem ligações químicas com a dentina (RICCI, SANABE, DE SOUZA COSTA, et al., 2010 [15]).

Dessa maneira, o uso agentes reticuladores poderiam ser alternativas interessantes para aumentar a durabilidade da interface de união à dentina, já que podem estabelecer ligações químicas com o colágeno dental. Recentemente, esses agentes vêm sendo investigados por aumentar as propriedades mecânicas (BEDRAN-RUSSO, PASHLEY, AGEE, et al., 2008 [16,] BEDRAN-RUSSO, PEREIRA, DUARTE, et al., 2007 [17,] CASTELLAN, PEREIRA, GRANDE, et al., 2010 [18]) e resistência do colágeno à degradação enzimática (BEDRAN-RUSSO, VIDAL, DOS SANTOS, et al., 2010 [19,] CASTELLAN, BEDRAN-RUSSO, KAROL, et al., 2011 [20,] LIU, FANG, XIAO, et al., 2011 [21,] MACEDO, YAMAUCHI e BEDRAN-RUSSO, 2009 [22]). Eles são agentes de ligação cruzada, que podem ser quimicamente naturais ou sintéticos, e que têm capacidade de aumentar as ligações cruzadas inter e intramolecular das fibrilas colágenas (HAN, JAUREQUI, TANG, et al., 2003 [23,] SUNG, CHANG, CHIU, et al., 1999 [24]).

Dentre os agentes reticuladores investigados na Odontologia, o glutaraldeído (um agente sintético), as proantocianidinas do extrato de semente de uva (naturais) e a riboflavina-vitamina B2 (natural com ação combinada com a luz ultravioleta) são os mais investigados (BEDRAN-RUSSO, PEREIRA, DUARTE, et al., 2007 [17,] MACEDO, YAMAUCHI e BEDRAN-RUSSO, 2009 [22,] COVA, BRESCHI, NATO, et al., 2011 [25]). Contudo a diferença entre protocolos em relação aos tempos de aplicação que variam entre 10 min e 4 h os tornam impraticáveis para o uso clínico (BEDRAN-RUSSO, PASHLEY, AGEE, et al., 2008 [16,] CASTELLAN, BEDRAN-RUSSO, KAROL, et al., 2011 [20,] MACEDO, YAMAUCHI e BEDRAN-RUSSO, 2009 [22,] AL-AMMAR, DRUMMOND e BEDRAN-RUSSO, 2009 [26]), da mesma maneira, o tratamento com primers contendo agentes reticuladores requer a adição de um passo adicional no protocolo adesivo, contrariando a filosofia da simplificação técnica. Dessa

maneira, é interessante investigar formas de promover tratamento reticulador da dentina sem adicionar uma etapa adicional, por exemplo, a inclusão no condicionador ácido.

Ainda não está completamente elucidado o efeito direto desses agentes nas propriedades adesivas, como resistência de união e grau de conversão e seu efeito na preservação da interface de união, que pode variar de acordo com a forma de inclusão desses agentes dentro do protocolo adesivo (EPASINGHE, YIU, BURROW, et al., 2012 [27], GREEN, YAO, GANGULY, et al., 2010 [28]). Até o presente momento, poucos estudos avaliaram a longevidade das interfaces tratadas com esses agentes (COVA, BRESCHI, NATO, et al., 2011 [25], CASTELLAN, BEDRAN-RUSSO, ANTUNES, et al., 2013 [29]).

Também existe uma contradição na literatura em relação à compatibilidade biológica desses agentes (HAN, JAUREQUI, TANG, et al., 2003 [23], BOUILLAGUET, OWEN, WATAHA, et al., 2008 [30], SCHEFFEL, BIANCHI, SOARES, et al., 2015 [31], SCHEFFEL, SOARES, BASSO, et al., 2015 [32], WOLLENSAK, AURICH, PHAM, et al., 2007 [33]). Além disto, poucos destes estudos avaliaram o potencial citotóxico através do método transdentinário (SCHEFFEL, BIANCHI, SOARES, et al., 2015 [31], SCHEFFEL, SOARES, BASSO, et al., 2015 [32]), que simula melhor as condições nas quais estes agentes são aplicados numa situação clínica. Recentemente, discute-se o potencial destes agentes na inibição da atividade de metaloproteinases endógenas (MMPs) (COVA, BRESCHI, NATO, et al., 2011 [25], CHAUSSAIN, BOUKPESSI, KHADDAM, et al., 2013 [34], SCHEFFEL, HEBLING, SCHEFFEL, et al., 2014 [35]). Contudo até o presente momento, não existe evidência direta do efeito desses agentes na atividade enzimática por MMPs endógenas dentro da camada híbrida.

Outra lacuna, ainda não explorada por investigadores, se refere ao fato de que o conhecimento que temos neste assunto seja restrito a metodologias estritamente laboratoriais onde grande parte do complexo ambiente oral não é simulado.

Em função do exposto torna-se necessário a condução de estudos laboratoriais e *in situ* que comparem os diferentes agentes de ligação cruzada nas propriedades adesivas imediatas e ao longo do tempo, assim como seu

potencial de inibição de MMPs quando aplicados como *primers* aquosos ou associados com o condicionador ácido.

2. PROPOSIÇÃO

2.1 PROPOSIÇÃO GERAL

Investigar o efeito de agentes reticuladores de colágeno em diferentes veículos, na longevidade das interfaces adesivas produzidas nos substratos dentais com sistemas adesivos convencionais simplificados, por meio de dois estudos laboratoriais *in vitro* e um *in situ*.

2.2 PROPOSIÇÕES ESPECÍFICAS

2.2.1 Avaliar o efeito do uso de primers aquosos contendo agentes reticuladores de colágeno (proantocianidina, riboflavina e glutaraldeído) em propriedades adesivas à dentina como resistência de união, nanoinfiltração e grau de conversão, imediatamente e após 18 meses de armazenamento.

2.2.2 Avaliar a citotoxicidade através da viabilidade celular de diferentes agentes reticuladores de colágeno (proantocianidina, riboflavina e glutaraldeído).

2.2.3 Avaliar a atividade de MMPs 2 e 9 dentro da camada híbrida após tratamento com diferentes agentes reticuladores de colágeno (proantocianidina, riboflavina e glutaraldeído) por meio de zimografia *in situ*.

2.2.4 Avaliar o efeito do condicionamento ácido contendo proantocianidina na longevidade da adesão à dentina e ao esmalte através da resistência de união à dentina, nanoinfiltração à dentina e resistência de união ao esmalte.

2.2.5 Avaliar o efeito do condicionamento ácido contendo proantocianidina na atividade de MMPs 2 e 9 dentro da camada híbrida através da zimografia *in situ*.

2.2.6 Avaliar o efeito da aplicação de primers contendo agentes reticuladores de colágeno (proantocianidina, riboflavina e glutaraldeído) degradação da interface de união à dentina submetida em ambiente oral cariogênico, através

da microdureza, resistência de união, nanoinfiltração e análise morfológica da interface adesiva por histologia.

3. MATERIAL E MÉTODOS

Foram realizados três experimentos de acordo com a distribuição abaixo descriminada.

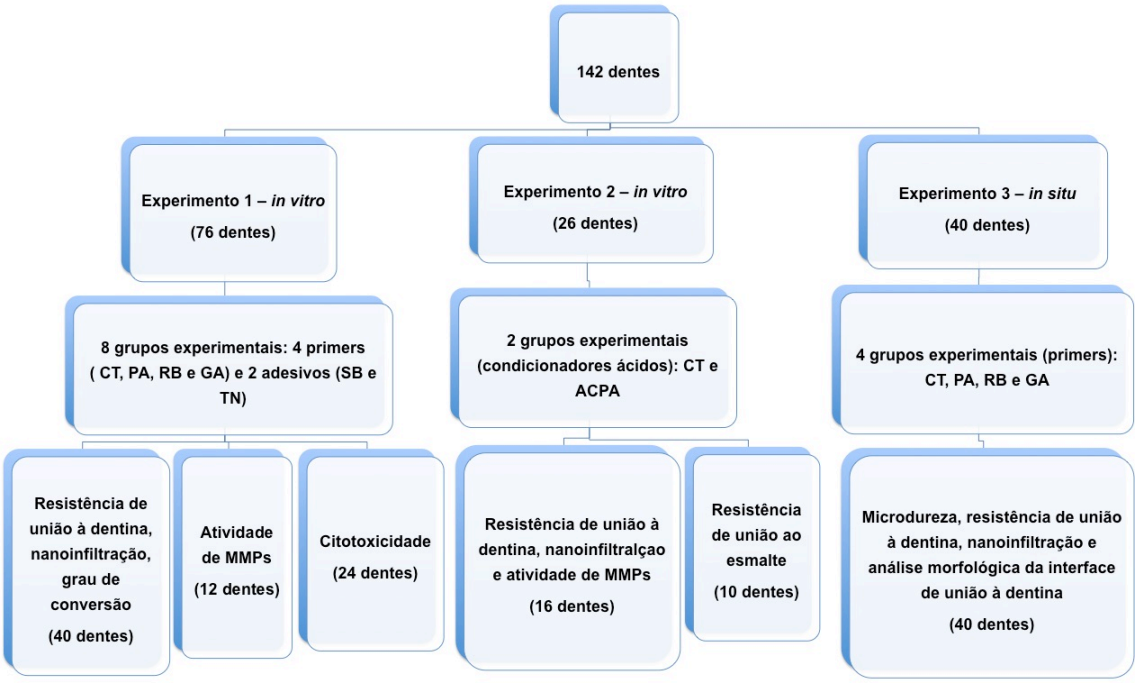

Figura 1. Organograma com a discriminação do número de dentes, experimentos, grupos experimentais e testes realizados.

3.1 EXPERIMENTO 1

"Agentes reticuladores de colágeno na adesão à dentina: estabilidade das interfaces adesivas, grau de conversão do adesivo na camada híbrida, citotoxicidade e inibição in situ de MMPs."

3.1.1 Delineamento experimental e preparo dos dentes

Este estudo foi aprovado pela Comissão de Ética em Pesquisa da Universidade Estadual de Ponta Grossa sob parecer 314.563. Setenta e seis terceiros molares humanos livres de cárie foram usados para esse experimento. Os dentes usados foram extraídos num período de até dois meses antes antes dos experimentos e foram armazenados em solução de cloramina 0,5%. A superfície do esmalte oclusal desses dentes foi removida

para exposição de uma superfície plana de dentina usando lixas carbeto de silício granulação 180 sob irrigação e bordas periféricas com fresa em alta rotação (**Figura 2**), e após isso criada uma smear layer por 60 s com lixas carbeto de silício com granulação 600.

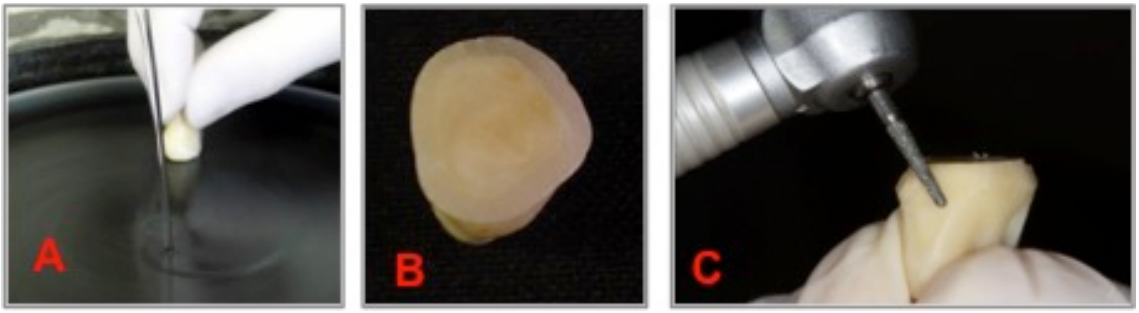

Figura 2. Preparação dos dentes. A: remoção do esmalte oclusal, B: superfície dentinária exposta e C: remoção das bordas periféricas de esmalte.

A dentina de 40 dentes foi condicionada com ácido fosfórico 35% gel (3M ESPE, St. Paul, EUA, lote N261433) durante 15 s, em seguida gentilmente lavados com água destilada durante 30 s e removidos o excesso de água com jato de ar por 5 s. Esses dentes foram então alocados entre os oito grupos experimentais de acordo com os quatro agentes de tratamento (proantocianidina do extrato de uva, riboflavina ativada por luz UVA, glutaraldeído e água destilada como grupo controle) e dois sistemas adesivos convencionais simplificados (Adper Single Bond Plus e Tetric N-Bond) (n=5) como detalhado na **Tabela 1 e Figura 3**.

Tabela 1. Descrição dos grupos experimentais, produtos, composição e modo de aplicação.

	Produto (Fabricante)	Composição	Modo de aplicação
AGENTES RETICULADORES	Proantocianidina primer (Mega Natural Gold, Madera, EUA) lote 05592502-01	Proantocianidina – extrato da semente de uva (90% de pureza) a 6,5 % peso, água destilada.	Após a etapa de condicionamento ácido, aplicação do primer aquoso contendo proantocianidina, por 60 s com um aplicador totalmente saturado. Forte jato de ar for 5 s mantendo a superfície levemente úmida.
	Riboflavina primer (Fisher Scientific GmbH, Schwerte, Alemanha) lote 070046	Riboflavina a 0,1 % em peso, água destilada.	Após a etapa de condicionamento ácido, aplicação do primer aquoso contendo riboflavina, por 60 s com um aplicador totalmente saturado. Forte jato de ar for 5 s mantendo a superfície levemente úmida.
	Glutaraldeído primer (Fisher Scientific GmbH, Schwerte, Alemanha) lote 186852	Glutaraldeído a 5 % em peso, água destilada.	Após a etapa de condicionamento ácido, aplicação do primer aquoso contendo glutaraldeído, por 60 s com um aplicador totalmente saturado. Forte jato de ar for 5 s mantendo a superfície levemente úmida.
	Groupo Controle	Água destilada	Após a etapa de condicionamento ácido, aplicação da água destilada por 60 s com um aplicador totalmente saturado. Forte jato de ar for 5 s mantendo a superfície levemente úmida.
SISTEMAS ADESIVOS	Single Bond Plus (SB) (3M ESPE, St. Paul, EUA) lote N531785	Álcool etílico, BisGMA, sílica silanizada (nanocargas), HEMA, copolímero de ácidos acrílico e itacônico, 1,3 glicerol dimetacrilato, água, UDMA, difeniliodonio, hexafluorfosfato, EDMAB.	Após tratamento de acordo com o agente de tratamento, aplicação de 2 camadas consecutivas de adesivo por 15 s, com agitação, usando um aplicador totalmente saturado de adesivo. Forte jato de ar por 5 s para evaporação de solvente. Fotoativação por 10 s.
	Tetric N-Bond (TN) (Ivoclar Vivadent, Schaan, Liechtenstein) lote L50568	Ácido fosfônico acrilato, HEMA, BisGMA, UDMA, etanol, nanocarga, aceleradores e estabilizadores.	Após tratamento de acordo com o agente de tratamento, aplicação de 2 camadas consecutivas de adesivo por 15 s, com agitação, usando um aplicador totalmente saturado de adesivo. Forte jato de ar por 5 s para evaporação de solvente. Fotoativação por 10 s.

BisGMA: bisfenol diglicidil éter dimetacrilato, *HEMA*: 2-hidroxietil metacrilato. *UDMA:* diuretano dimetacrilato, *EDMAB:* etil 4-dimetil aminobenzoato.

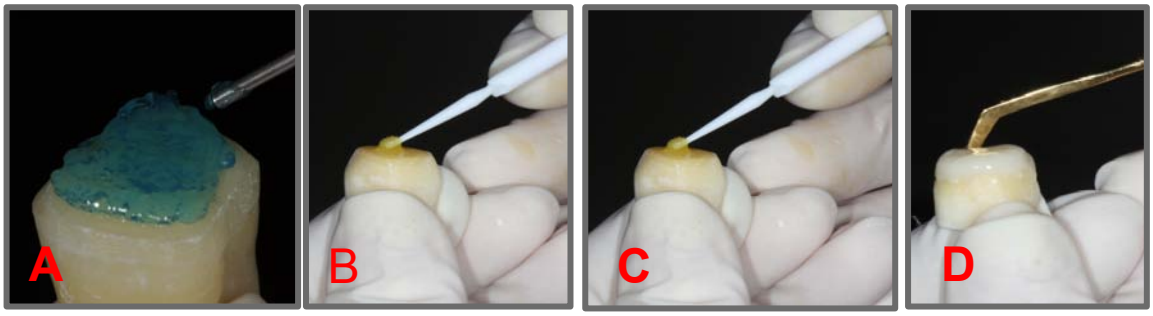

Figura 3. Condicionamento ácido e aplicação dos agentes reticuladores. Em A: condicionamento com ácido fosfórico 35%, B: aplicaçãoo ativa dos agentes reticuladores, C: aplicação do sistema adesivo e D: restauração com resina composta.

Após tratamento com os agentes reticuladores de colágeno (como detalhado na **Tabela 1**) foi aplicado o sistema adesivo de acordo com as recomendações dos fabricantes **(Figura 3)** e fotoativados com um aparelho fotopolimerizador Radii Cal (SDI, Bayswater, Victoria, Australia; 1,200 mW/cm^2) por 10 s. Para o grupo riboflavina, após o tratamento com o agente reticulador, foi realizada irradiação por 2 min com uma lâmpada ultravioleta - UVA (Philips, Hamburg, Alemanha; λ = 370 nm at 3 mW/cm^2) antes do jato de ar (COVA, BRESCHI, NATO, et al., 2011 [25]). Após isso, esses dentes foram incrementalmente restaurados com resina composta (Z250, 3M ESPE, cor A3, lote N549511) e fotoativadas as duas camadas por 40 s cada com o mesmo aparelho fotopolimerizador previamente descrito. Esses dentes então foram armazenados por 24 h em água destilada em uma estufa a 37°C.

3.1.2 Obtenção dos espécimes

As unidades experimentais foram fixadas em um dispositivo especial para máquina de corte (Isomet 1000, Buehler, Lake Bluff, IL, EUA) e seccionadas com um disco diamantado sob refrigeração, em ambos os sentidos mesiodistal e vestibulolingual, perpendiculares à interface adesiva, para obtenção de palitos como espécimes com aproximadamente 1mm^2 (**Figura 4**). O número de perdas prematuras por dente durante o seccionamento foi anotado. Metade dos espécimes obtidos por cada dente foi selecionada para serem testados imediatamente e a outra metade foi armazenada em *eppendorfs* contendo água destilada, em uma estufa a 37°C por um período de 18 meses. Em cada tempo de avaliação, previamente ao

teste de microtração, a área de cada espécime foi mensurada com o auxílio de um paquímetro digital (Absolute Digimatic, Mitutoyo, Tóquio, Japão).

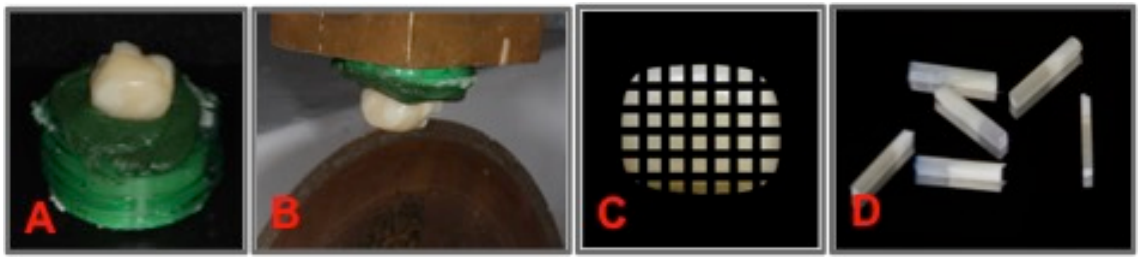

Figura 4. Seccionamento das unidades experimentais para obtenção dos espécimes. A: fixação das unidades experimentais no dispositivo para máquina de corte, B: seccionamento das unidades experimentais, C: unidade experimental seccionada e D: espécimes obtidos pelo seccionamento.

3.1.3 Teste de resistência de união à dentina por microtração

Após mensuração da área, cada espécime foi fixado com cola de cianoacrilato (Super Bonder Loctite, São Paulo, Brasil) em um dispositivo para microtração e sujeitos ao teste de microtração em uma máquina ensaios universal (Kratos IKCL 3-USB, Kratos Equipamentos Industriais Ltda, Cotia, São Paulo, Brasil) a 0,5 mm/min **(Figura 5)**. O modo de falha foi avaliado sob magnificação 40x e classificado como adesivo (falha na interface resina/dentina), adesivo/misto (falha na interface resina/dentina com falha parcial em substratos vizinhos) ou coesiva (falha dentro da dentina ou resina).

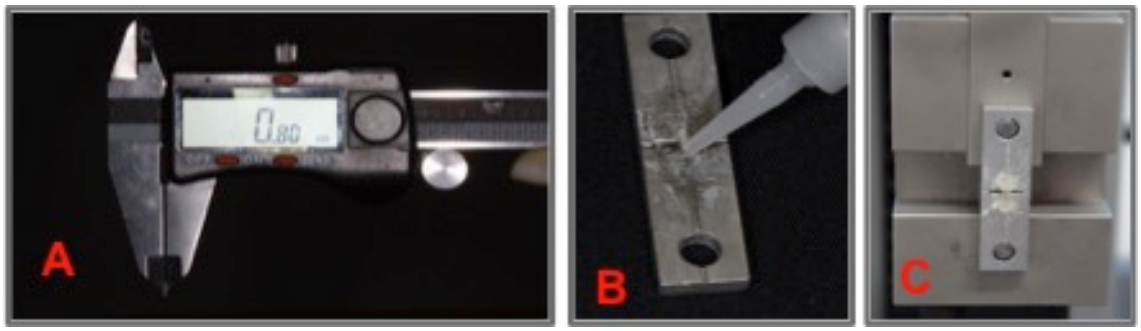

Figura 5. Teste de microtração. A: mensuração dos espécimes com paquímetro digital, B: fixação dos espécimes no dispositivo para microtração e C: tracionamento dos espécimes.

3.1.4 Análise da nanoinfiltração por microscopia eletrônica de varredura

Dois espécimes randomizados de cada dente, que não foram testados por RU, em cada tempo de avaliação, foram imersos em solução amoniacal de nitrato de prata a 50% em peso (TAY, PASHLEY e YOSHIYAMA, 2002 [36]) por 24 h em ambiente escuro protegido da luz. Então, os espécimes foram lavados em água destilada e armazenados em solução reveladora (Kodak, Rochester, Nova York, EUA) sob luz fluorescente por 8 h para que houvesse redução dos íons de prata ao longo das microporosidades na interface de união (**Figura 6**).

Figura 6. Imersão dos espécimes em solução de nitrato de prata e posterior imersão em solução reveladora sob luz fluorescente. Em A: imersão dos espécimes na solução de nitrato de prata, B: armazenamento dos espécimes com nitrato de prata em ambiente escuro e opaco, C: solução reveladora na qual os espécimes foram armazenados e D: espécimes imersos em solução reveladora sob luz fluorescente.

Depois disso, os espécimes foram fixados em stubs de alumínio e polidos com lixas de carbeto de silício de granulação 1200, 1500, 2000, 2500 e pastas diamantadas granulação 1 e 0,25 µm (Buehler Ltd., Lake Bluff, IL, EUA). Após os stubs contendo os espécimes foram lavados em cuba ultrassônica por 30 min, desidratados em ambiente contendo sílica e cobertos com carbono (MED 010, Balzers Union, Balzers, Liechtenstein). As interfaces foram observadas por microscopia eletrônica de varredura (MEV) no modo de elétrons retroespalhados a 12 kV (VEGA 3 TESCAM, Shimadzu, Tóquio, Japão) (**Figura 7**).

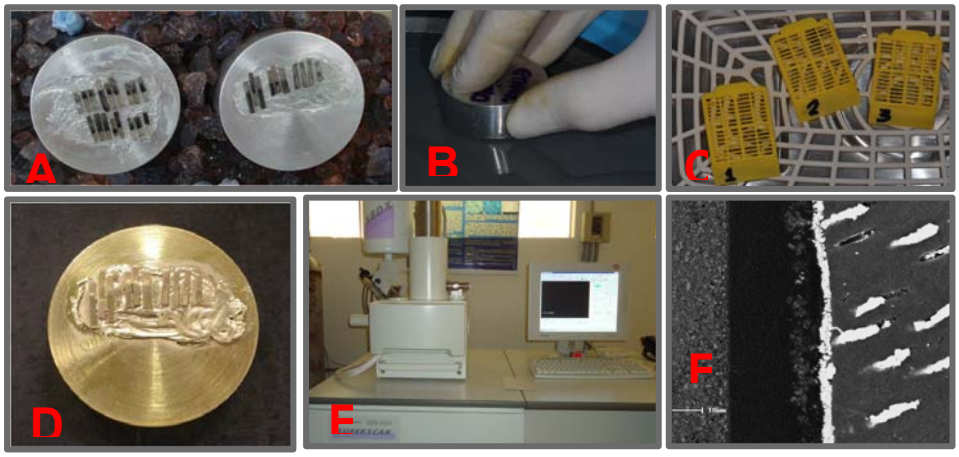

Figura 7. Preparo para microscopia eletrônica de varredura. A: fixação dos espécimes em stubs de alumínio, B: polimento, C: limpeza em cuba ultrassônica, D: metalização dos espécimes, E: equipamento de microscopia eletrônica de varredura e F: análise da interface de união.

Três microfotografias foram obtidas para cada espécime: a primeira ao centro, e as outras duas a 0,3 mm para direita e esquerda a partir da primeira microfotografia (REIS, GRANDE, OLIVEIRA, et al., 2007 [37]). Um total de seis microfotografias foram obtidas por cada dente em cada período de avaliação (3 microfotografias x 2 espécimes) e um total de 30 microfotografias foram obtidas por grupo experimental (6 microfotografias x 5 dentes) por um operador cego às condições experimentais. A porcentagem de nanoinfiltração dentro das camadas adesiva e híbrida foram mensuradas por um software específico (ImageTool 3.0, University of Texas Health Science Center, San Antonio, EUA) como previamente descrito (REIS, GRANDE, OLIVEIRA, et al., 2007 [37]).

3.1.5 Análise do grau de conversão (GC) dentro da camada híbrida

Dois outros espécimes randomizados, que não foram testados por RU no período imediato, foram submetidos à espectroscopia de micro-Raman (Senterra espectrofotômetro Bruker, Ettlingen, Baden-Württemberg, Alemanha) para análise do grau de conversão imediatamente após o polimento. Esses espécimes foram levemente polidos com lixas carbeto de sílicio granulação 1500 e 2000, e lavados em cuba ultrassônica por 10 min. As análises foram realizadas por uma magnificação de 100x (Olympus microscope, London, Reino Unido), com um laser de Neônio (1 μm) com 532 nm de comprimento de onda, com resolução espacial de 3 μm, resolução espectral de 5 cm^{-1}, num

tempo de 30 s com 5 coadições. Os espectros foram obtidos da interface adesivo/dentina, na região intertubular. Três sítios randomizados por espécime foram avaliados. Os espectros foram processados por um software específico (Opus Spectroscopy, versão 6.5). O conteúdo de duplas ligações carbônicas de monômero convertidas em polímero, no adesivo, foi calculado pela seguinte fórmula: GC (%) = (1 − [R polimerizado / R não-polimerizado]) x 100. Onde “R” é o conteúdo alifático e aromático dos picos 1639 cm^{-1} e 1609 cm^{-1} no adesivo polimerizado e não polimerizado **(Figura 8).**

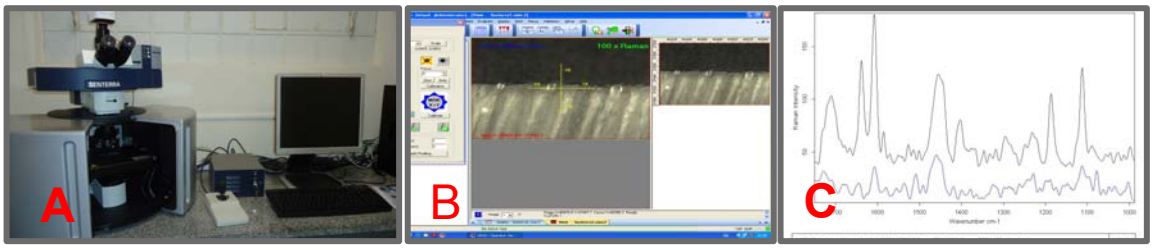

Figura 8. Análise do grau de conversão dentro da camada híbrida. A: espectrofotômetro micro-Raman, B: localização dos sítios intertubulares e C: obtenção do espectro.

3.1.6 Atividade de MMP por zimografia *in situ*

Três dentes por grupo tiveram a superfície de esmalte occlusal removida e padronizada uma smear layer na dentina da mesma maneira que para RU. Esses dentes foram condicionados com o mesmo ácido fosfórico previamente descrito.

Foi utilizado um corante específico em gel para MMP 2 e 9, preparado com uma gelatina hipersaturada de fluoresceina isotiocianato (FITC). Cinco mg de FITC foi dissolvido em 2 mL a 0,1 M de carbonato de sódio/tampão de bicarbonato (pH 9,0 Sigma Aldrich, Milwaukee, EUA). Este reagente foi dispersado em uma solução de gelatina 1mg/mL em um ambiente escuro e foi incubado à temperatura ambiente por 2 h. A gelatina conjugada de FITC reagida foi isolada a partir de um FITC não unido em uma coluna principal G-25M Sephadex. A proporção fluoresceína-proteína foi >15 e confirmada pelas leituras de absorbância em 495 nm e 280 nm respectivamente. Este corante para confocal foi dissolvido (0,3% em peso) em água destilada e aplicado ativamente por 60 s sobre a dentina previamente condicionada com ácido

fosfórico. Depois, foi usado um jato de ar por 5 s para evaporação do excesso de solvente e aplicado os primers contendo os agentes de reticulação, da mesma maneira que para resistência de união (**Tabela 1**) seguidos da aplicação do sistema adesivo e incrementos de resina composta como previamente descrito, depois armazenados por 24 h em estufa a 37°C. Esses dentes foram então seccionados perpendicularmente à interface de união, no sentido mesiodistal para obtenção de fatias como espécimes.

Os espécimes foram analisados por microscopia confocal a laser (Leica SP5 CLSM, Heidelberg, Alemanha) usando uma objetiva de imersão 63x/1,4 NA, com um laser de 468 nm. O escaneamento foi realizado a cada 1 µm e até 20 µm abaixo da superfície e foram compiladas em projeções únicas. Cada interface resina-dentina foi inteiramente caracterizada e as imagens representativas da atividade de MMPs foram capturadas ao longo das interfaces de união à dentina (**Figura 9**).

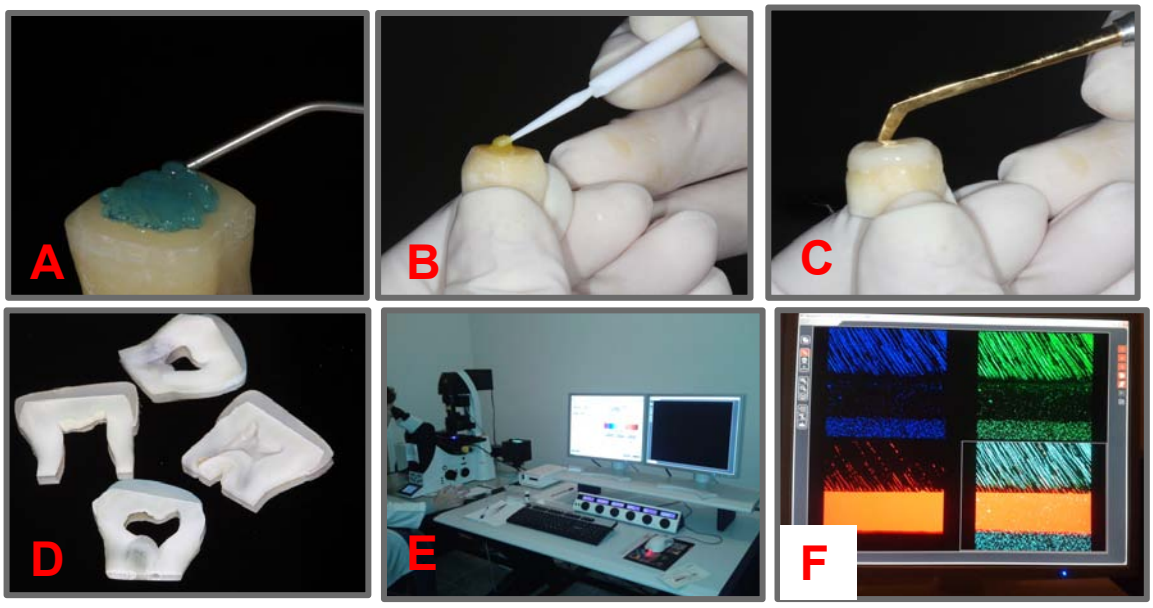

Figura 9. Preparo dos espécimes para microscopia confocal a laser. A: condicionamento ácido da superfície dentinária, B: aplicação de corante para MMPs, C: restauração, D: fatias obtidas para análise, E: microscópio confocal a laser e F: aquisição das imagens.

3.1.7 Avaliação da citotoxicidade através de cultura celular e MTT

Vinte e quatro dentes foram usados para esse teste. Esses dentes tiveram sua superficie de esmalte oclusal removida expondo uma superfície plana de dentina. Esses dentes foram seccionados (Isomet 1000, Buehler, Lake Bluff, EUA) para obtenção de discos de dentina com espessura de 0,6 mm. Os

discos foram cuidadosamente examinados com uma objetiva de 40x para confirmar a ausência de esmalte e exposição pulpar. Então, a superfície oclusal dos discos foram manualmente polidas com lixas carbeto de silício granulação 320 até atingir a espessura de 0,5 mm (Mitutoyo South Americana Ltd, Suzano, SP, Brasil). Depois disso, esses discos foram polidos para padronização de uma smear layer como descrito para RU. Esses discos foram limpos com solução de EDTA 0,5 M (pH 7,4) for 60 s e lavados abundantemente com água deionizada, então tiveram mensurada a permeabilidade através da condutância hidráulica (MENA-SERRANO, COSTA, PATZLAFF, et al., 2014 [38]) para permitir uma distribuição homogênea dos discos entre os grupos experimentais (n=6 discos por grupo). Os discos dentinários foram posicionados em uma cuba metálica e autoclavados 20 min/121°C). As superfícies oclusais dos discos dentinários foram condicionados com por 15 s com o mesmo ácido fosfórico previamente descrito, lavados com água deionizada por 10 s e secos com algodão estéril.

Fibroblastos da linhagem 3T3 foram cultivados em meio DMEM contendo soro fetal bovino a 10% e suplementado com penicilina/estreptomicina a 1% (110000U/100 µg/mL). As células foram mantidas em estufa a 37 ºC com 5% CO_2. Em suma, as células em uma concentração de 4 x 10^4 foram adicionadas em cada poço em uma placa de 24 poços. Após 24 horas, o meio foi removido e as células lavadas duas vezes em PBS. Adicionou-se meio DMEM contend soro fetal bovino a 1% e câmaras “trans-well” foram adicionadas em cada poço. Os discos de dentina foram cautelosamente adicionados e 10 µL de cada primer contendo os agentes de reticulação, foi aplicado na face oclusal. O grupo riboflavina foi irradiado com luz UVA através de uma lâmpada como previamente descrito, utilizando-se uma ponta de 10,4 mm que cobriu completamente cada poço. O protocolo seguido foi de acordo com Bouillaguet et al. 2008 (BOUILLAGUET, OWEN, WATAHA, et al., 2008 [30]), método que previne modificação da atividade mitocondrial celular. Após 24 horas, removeram-se as “trans-well” e as células foram lavadas em PBS. Ensaio MTT 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (Sigma Chemical Co., St. Louis, EUA) foi utilizado para determinar a viabilidade celular, com algumas modificações do método de Tada et al. 1986 (TADA, SHIHO, KUROSHIMA, et al., 1986 [39]). Adicionou-se 1,0 mg/mL de solução MTT e a

viabilidade celular foi mensurada através de ensaio colorimétrico, usando-se a atividade da dehidrogenase mitocondrial em células ativas para formar o formazan roxo. A absorbância de cada poço foi mensurada a 570 nm utilizando-se leior de placa (EL808B, BioTech Instruments Inc., Winooski, VT, EUA). A viabilidade celular foi expressa através da porcentagem dos valores ópticos de cada amostra *versus* o respectivo controle (sem tratamento), considerado 100%. Todos os experimentos foram realizados em triplicata.

3.1.8 Análise estatística dos dados

Foi realizada uma média dos valores obtidos da resistência de união à dentina (MPa) e nanoinfiltração (%) do mesmo dente em cada período de avaliação. Os espécimes com falhas prematuras e coesivas não foram incluídos na média de cada dente, devido ao pequeno número no experimento.

Primeiramente foi realizado o teste de Kolmogorov–Smirnov para verificar a distribuição normal de todos os dados (resistência de união à dentina, nanoinfiltração, grau de conversão e citotoxicidade). Depois foi realizado teste de Barlett para determinar a equalidade das variâncias. Depois de observada a normalidade e equalidade das variâncias, os dados da resistência de união à dentina (MPa) e nanoinfiltração (%) de cada adesivo foram submetidos a uma Análise de Variância-dois fatores de medidas repetidas (agente de tratamento X tempo de avaliação). Dados do grau de conversão (%) e citotoxicidade (%) de cada grupo foram sujeitos a uma Análise de Variância-um fator (agente de tratamento). Para todos os teste foi realizado Tukey para comparação das médias ($\alpha = 0,05$).

3.2 EXPERIMENTO 2

“Efeito da proantocianidina incluída no ácido fosfórico a 10% nas propriedades adesivas e inibição de MMPs in situ”

3.2.1 Delineamento experimental e preparo dos dentes

Da mesma forma que para o experimento 1, foram obtidos 26 dentes pela mesma aprovação pela Comissão de Ética em Pesquisa da Universidade

Estadual de Ponta Grossa. Em 16 dentes, a superfície de esmalte oclusal foi removida e padronizada a smear layer, da mesma maneira que para o experimento 1. Desses 16 dentes, 10 foram destinados para análise da resistência de união à dentina e nanoinfiltração, enquanto que os outros 6 dentes foram destinados para análise da atividade de MMPs.

Nos outros 10 dentes remanescentes, as raízes foram removidas pelo seccionamento da junção cemento-esmalte (Isomet 1000, Buehler, Lake Bluff, EUA). Depois as coroas dentais foram seccionadas novamente em duas diagonais ao longo eixo dos dentes, obtendo-se assim 4 partes (mesial, distal, vestibular e lingual). Dessa forma, pudemos obter 40 espécimes de esmalte. Esses 40 espécimes tiveram a superfície de esmalte planificada com uma lixa carbeto de silício de granulação 180. Depois essas superfícies foram polidas com as mesmas lixas, porém com granulação 600 durante 60 s e destinadas para avaliação da resistência de união ao esmalte por microshear.

3.2.2 Grupos experimentais e procedimento restaurador

Os espécimes foram randomizados e distribuídos entre os dois grupos experimentais por uma pessoa não envolvida no protocolo de pesquisa. No grupo controle a dentina e o esmalte de todos os espécimes foram condicionados com ácido fosfórico 35% por 15 s (3M ESPE, St. Paul, EUA, lote N261433). Para o grupo experimental, foi preparado um condicionador ácido experimental contendo: ácido fosfórico a 10%, proantocianidina do extrato de semente de uva 2% e etanol 20% (LIU, DUSEVICH e WANG, 2014 [40]). Esse condicionador ácido foi aplicado por 30 s. Todos os químicos foram obtidos da Sigma Aldrich (St. Louis, MO, EUA), menos o extrato de semente de uva (Mega Natural Gold Polyphenolics, Madera, EUA).

Após o condicionamento ácido, as superfícies foram lavadas com água destilada por 30 s, jato de ar por 5 s mantendo a superfície ligeiramente úmida para a aplicação do sistema adesivo (Adper Single Bond Plus, 3M ESPE, St. Paul, EUA, lote N531785) da mesma maneira que para o experimento 1. Para resistência de união à dentina e nanoinfiltração, coroas de resina composta foram realizadas em dois incrementos e fotoativadas por 40 s cada incremento (1,200 mW/cm^2; Radii-cal, SDI Limited, Bayswater, Victoria, Australia) e armazenados por 24 h a 37°C.

3.2.3 Resistência de união à dentina por microtração

Após armazenamento os dentes foram seccionados da mesma maneira que para o experimento 1, para obtenção de espécimes. Metade desses espécimes foi testada imediatamente e a outra metade após 6 meses de armazenamento em água destilada. A água destilada foi trocada semanalmente para maximizar o processo de degradação (SKOVRON, KOGEO, GORDILLO, et al., 2010 [41]). Assim como para o experimento 1, a área de secção transversal foi mensurada por um paquímetro digital (Absolute Digimatic, Mitutoyo, Tóquio, Japão). Cada espécime então foi fixado num dispositivo para microtração com uma resina adesiva a base de cianoacrilato (Super Bonder Gel, Loctite, São Paulo, Brasil) e submetidos à microtração (0,5 mm/min) em uma máquina de ensaios universais (Model 5565, Instron, Canton, OH, EUA). O modo de falha foi avaliado sob magnificação 100x e classificado como adesivo (falha na interface resina/dentina), adesivo/misto (falha na interface resina/dentina com falha parcial em substratos vizinhos) ou coesiva (falha dentro da dentina ou resina).

3.2.4 Nanoinfiltração

Dois espécimes de cada dente, que não foram testados por microtração, foram destinados a análise da nanoinfiltração, seguindo os mesmos procedimentos que para o experimento 1.

3.2.5 Atividade de MMPs por zimografia *in situ*

Esta etapa seguiu os mesmos passos que no experimento 1. Contudo o condicionamento ácido, previamente à aplicação do corante para MMPs, seguiu o protocolo de acordo com os grupos experimentais desse experimento.

3.2.6 Resistência de união ao esmalte por microshear

Previamente a aplicação do condicionamento ácido, cada espécime de esmalte foi montado em um anel de PVC contendo resina acrílica (AutoClear,

DentBras, Pirassununga, São Paulo, Brasil), de modo que a superfície de esmalte ficasse no topo do cilindro. Uma fita dupla face ácido-resistente (Adelbras Ind e Com Adesivos Ltda, São Paulo, Brasil) foi perfurada com um perfurador de lençol de borracha para delimitação das áreas (0,8 mm de diâmetro) de adesão. Após isso foi realizada a etapa de condicionamento ácido e sistema adesivo da mesma maneira como descrito anteriormente (SHIMAOKA, DE ANDRADE, CARDOSO, et al., 2011 [42]). Depois, tubos de polietileno (Tygon Medical Tubing Formulations 54-HL, Saint Gobain Performance Plastics, Akron, OH, EUA) com um diâmetro interno de 0,8 mm e altura de 0,5 mm foram posicionados sobre a fita dupla face com o lúmen coincidente com as perfurações. Um operador treinado posicionou de 7 a 9 tubos para cada superfície, usando lupas de magnificação. Após isso, resina composta foi inserida no interior de cada tubo e fotoativação por 40 s (1,200 mW/cm^2; Radii-cal, SDI Limited, Bayswater, Victoria, Australia).

Os espécimes então, foram armazenados em água destilada a 37ºC por 24 h, os tubos e a fita dupla face foram cuidadosamente removidos usando uma lâmina cirúrgica para exposição dos cilindros de resina. Cada espécime foi examinado sob magnificação de 10x, em caso da presença de bolhas de ar ou trincas o cilindro não era testado.

Depois, os espécimes contendo os cilindros foram fixados num dispositivo de microshear (Odeme Biotechnology) e então os cilindros de resina composta testados em uma máquina de ensaios universal (Kratos IKCL 3-USB, Kratos Equipamentos Industriais Ltda, Cotia, São Paulo, Brasil). Um fio de ortodôntico de aço (0,2 mm de diâmetro) foi posicionado de modo a circundar a base do cilindro de resina. Esse fio foi alinhado de modo a tracionar a interface resina/esmalte perpendicularmente ao centro da célula de carga induzindo a correta orientação das forças (SHIMADA, YAMAGUCHI e TAGAMI, 2002 [43]) sob a velocidade de 1 mm/min até a falha. Uma metade dos espécimes foi testada imediatamente e a outra foi armazenada por 6 meses como previamente descrito para resistência de união à dentina. Os valores da resistência de união ao esmalte foram calculados pela divisão da carga da fratura pela área da superfície (mm^2).

3.2.7 Análise estatística dos dados

Foi realizada uma média dos valores de resistência de união à dentina obtidos do mesmo dente, em cada período de avaliação, e também para os valores da resistência de união ao esmalte da mesma superfície de esmalte, para fins estatísticos. Espécimes que apresentaram falhas coesivas, bem como prematura, não foram incluídos na média dos dentes devido ao pequeno número obtido. Para a porcentagem de nanoinfiltração, também foi realizada uma média por dente e considerada para análise estatística.

Da mesma maneira que para o experimento 1, foi realizados teste de Kolmogorov-Smirnov para verificar a distribuição normal dos dados. E o teste de Barlett para avaliar a equivalência da variância. Após observado a equivalência e variância, os dados de resistência de união à dentina e resistência de união ao esmalte (MPa) e nanoinfiltração (%) foram submetidos a uma Análise de Variância-2 fatores de medidas repetidas e Teste de Tukey ($\alpha = 0,05$).

3.3 EXPERIMENTO 3

"Degradação da interface de união à dentina tratada com agentes reticuladores de colágeno em ambiente oral cariogênico: um estudo in situ"

3.3.1 Aspectos éticos

Este experimento foi aprovado juntamente com os outros experimentos, pela Comissão de Ética em Pesquisa da Universidade Estadual de Ponta Grossa sob parecer 314.563. Dez voluntários adultos, saudáveis com idade entre 21-30 anos, homens e mulheres, foram recrutados de acordo com os seguintes critérios de inclusão: boa saúde geral, fluxo salivar normal, não ter ingerido antibióticos nos últimos 2 meses antes do experimento, não ser usuário de prótese ou aparelho ortodôntico e disponibilidade de colaborar com o estudo. Todos os voluntários que aceitaram participar assinaram um termo de consentimento por escrito.

Um total de quarenta dentes, terceiros molares humanos e livres de cárie, foram usados nesse experimento. Assim como para os outros experimentos, os dentes foram obtidos após aprovação pela Comissão de Ética em Pesquisa da Universidade Estadual de Ponta Grossa, como previamente descrito. Os dentes foram esterilizados pelo armazenamento em solução tampão formalina 10%, pH=7, por 7 dias (DOMINICI, ELEAZER, CLARK, et al., 2001 [44]) e depois armazenados em água destilada por 2 meses após extração.

3.3.2 Desenho experimental

Foi um estudo *in situ*, boca dividida, delineado para acúmulo de biofilme dental sobre as restaurações em ambiente altamente cariogênico, promovido pela exposição à sacarose. Este protocolo foi realizado por 14 dias. Os fatores de avaliação foram: (1) agentes de tratamento reticuladores de colágeno-4 níveis (proantocianidina do extrato de uva, riboflavina ativada pela luz UVA, glutaraldeído e água destilada como grupo controle) e (2) tempo de avaliação-2 níveis (imediato - com procedimento adesivo realizado 24 h antes, e após 14 dias de degradação em ambiente cariogênico oral. Então, um total de 8 condições experimentais foram testadas.

3.3.3 Preparo e distribuição dos dentes

Em 20 dentes, uma superfície plana de esmalte foi realizada nas faces vestibular e lingual com lixas de carbeto de silício com granulação 180. Depois as raízes foram removidas pelo seccionamento na junção cemento-esmalte (Isomet 1000, Buehler, Lake Bluff, EUA). Então duas cavidades foram preparadas em cada dente, nessas superfícies (4 mm de largura, 4 mm de comprimento e 1,5 mm de profundidade) com uma broca carbide (# 330, KG Sorensen Ind. & Com. Ltda, Barueri, SP, Brasil), depois essa coroas contendo as cavidades foram novamente seccionadas, de modo soltar 2 blocos de dente (6 x 6 x 3 mm) contendo essas cavidades com bordas 0,3 a 0,5 mm em esmalte (**Figura 10**). Então, os blocos foram randomizados e distribuídos de

acordo com os grupos experimentais como previamente descrito, porém as duas cavidades do mesmo dente foram destinadas para o mesmo agente de tratamento, de modo que uma cavidade fosse testada imediatamente e a outra após 14 dias de degradação em ambiente oral cariogênico. Isso diminuiria a variabilidade intra-dentes.

3.3.4 Procedimento adesivo e restaurador para análise da microdureza, resistência de união à dentina, e nanoinfiltração

Todas as cavidades foram condicionadas com ácido fosfórico por 15 s, lavadas com agua destilada por 30 s, jato de ar por 5 s mantendo a superfície dentinária ligeiramente úmida. Após isso, as cavidades foram tratadas com os agentes reticuladores de colágeno, da mesma maneira que no experimento 1 (**Tabela 1**) e realizado o procedimento adesivo com o sistema Single Bond Plus (3M ESPE, St. Paul, MN, EUA) como descrito na **Tabela 1**. Depois as cavidades foram incrementalmente restauradas com resina composta, e fotoativadas por 40 s cada incremento, como descrito nos 2 primeiros experimentos (**Figura 10**).

3.3.5 Procedimento adesivo para análise da morfologia da interface de união por histologia

Outros 20 dentes foram preparados, distribuídos e tratados da mesma maneira que para a resistência de união à dentina (**Tabela 1**), contudo ao invés de restaurados com resina composta, duas camadas adicionais de sistema adesivo foram aplicadas para permitir o procedimento de microtomia. Entre essas camadas foi utilizado forte jato de ar e fotoativadas da mesma forma como previamente descrito. Todas as bordas de todos os blocos contendo as restaurações foram seladas com a aplicação de um esmalte para unhas ácido-resistente, exceto o topo da superfície. Esses blocos então foram armazenados em ambiente úmido por 24 h a 37°C (HARA, QUEIROZ, PAES LEME, et al.,

2003 [45]). Depois disso, para cada dente, uma das restaurações foi testada IM e a outra foi adaptada em um dispositivo palatino oral e submetidos ao desafio *in situ* por 14 dias em ambiente oral cariogênico.

3.3.6 Preparo do dispositivo palatino

Para cada voluntário, foi confeccionado um dispositivo palatino com quatro sítios (6,5 x 6,5 x 4 mm) nos quais as restaurações foram fixadas com cera pegajosa (**Figura 10**). Para permitir acúmulo de biofilme e proteção de distúrbios mecânicos, uma rede plástica foi fixada por resina acrílica deixando espaço de 1 mm da superfície do espécime (HARA, QUEIROZ, PAES LEME, et al., 2003 [45,] PAES LEME, DALCICO, TABCHOURY, et al., 2004 [46]). Dentro de cada sítio do dispositivo, as posições dos espécimes foram randomizadas.

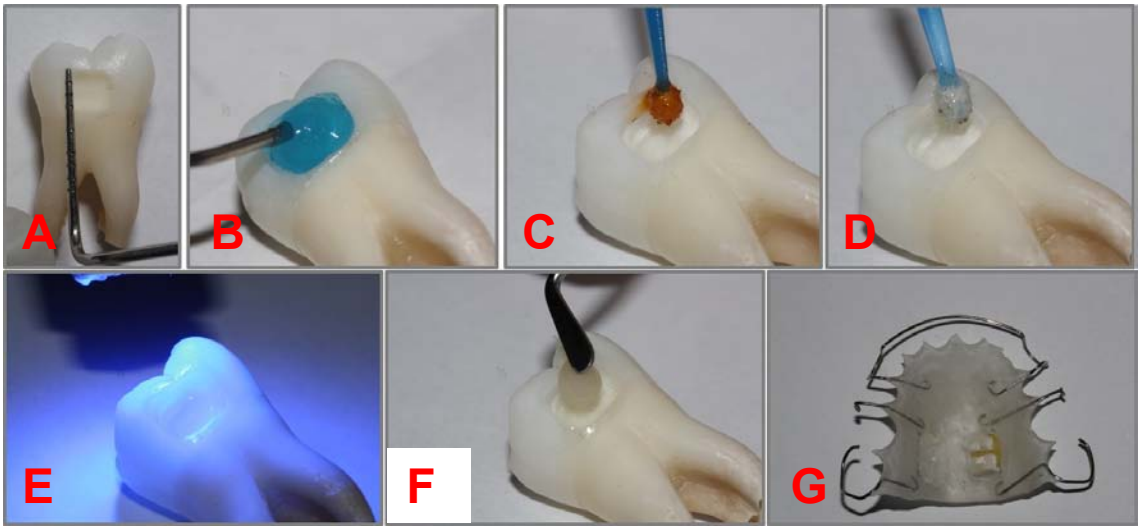

Figura 10. Experimento *in situ*. A: cavidades confeccionadas nos dentes, B: condicionamento ácido, C: aplicação dos agentes reticuladores, D: aplicação do sistema adesivo, E: fotoativação, F: restauração com resina composta e G: fixação no dispositivo palatino.

3.3.7 Fase intraoral

Durante 1 semana antes de iniciar a fase experimental, os voluntários deixaram de escovar seus dentes com dentifrício fluoretado, e passaram a

utilizar um dentifrício não-fluoretado (Fleming, Ponta Grossa, PR, Brasil) fornecido pela equipe de pesquisa.

Para induzir um desafio cariogênico em todos os espécimes, os voluntários foram instruídos a remover o dispositivo e gotejar solução de sacarose 20% (Fleming, Ponta Grossa, PR, Brasil) sobre todos os blocos, 4 vezes ao dia (8 h, 11 h, 15h30min e 19 h) durante 14 dias (CURY, FRANCISCO, DEL BEL CURY, et al., 2001 [47], REINKE, LAWDER, DIVARDIN, et al., 2012 [48]). Cinco minutos depois, o dispositivo era reinserido na cavidade bucal.

Todos os voluntários consumiram água fluoretada de abastecimento urbano (0,6-0,8 mg F/L) e comidas preparadas com essa água. Nenhuma restrição em relação à dieta foi determinada aos voluntários. Eles foram instruídos a usar o dispositivo oral o tempo todo durante esses 14 dias, removendo somente para higienização bucal e durante as refeições. A higienização do dispositivo era realizada extra-oralmente com escovação, exceto as restaurações, e os voluntários orientados para escovar cuidadosamente sobre a área do dispositivo, evitando remoção do biofilme sobre a rede plástica. No 15° dia de fase intra-oral, em torna das 12 h após a última aplicação da solução de sacarose, os voluntários pararam de utilizar o dispositivo, os mesmos foram recolhidos, as restaurações removidas, lavadas com água da torneira e longitudinalmente seccionadas para obtenção de uma fatia (1 mm) da interface restaurada para avaliação da microdureza Knoop. O remanescente da restauração foi utilizado para avaliação da resistência de união à dentina e nanoinfiltração.

3.3.8 Análise da microdureza

Em ambos os tempos de avaliação (imediato e após 14 dias de degradação em ambiente oral cariogênico, a fatia obtida pelo seccionamento foi embebida em resina acrílica, e polidas até exposição da superfície, e seguida de polimento com lixas carbeto de silício nas seguintes granulações: 1000, 1500, 2000 e 2500 sob irrigação e depois pastas diamantadas 1 e 0,25

μm (Buehler, Lake Bluff, IL, EUA). Depois os espécimes foram lavados em cuba ultrassônica por 20 min e mensurada a microdureza (HMV-2, Shimadzu, Tóquio, Japão) equipada com um identador Knoop (KHN) sob 15 g de carga por 5 s. Três linhas de identações foram realizadas, sendo uma delas a 20 μm de distância da margem da restauração, outra a 100 e outra a 200 μm de distância. As identações foram realizadas seguindo as profundidades de 5, 15, e 25 μm a partir da junção dentina-esmalte (REINKE, LAWDER, DIVARDIN, et al., 2012 [48]).

3.3.9 Análise da resistência de união à dentina

O remanescente da restauração foi submetido ao seccionamento para análise da resistência de união à dentina como previamente descrito nos experimento 1 e 2.

Os espécimes obtidos em cada tempo de avaliação (imediatamente e após 14 dias em ambiente oral cariogênico) foram testados por microtração como previamente descritos para os experimentos 1 e 2.

3.3.10 Análise da nanoinfiltração

Dois espécimes obtidos pelo seccionamento, e que não foram testados por microtração, foram submetidos à análise da nanoinfiltração. Esses espécimes foram preparados e testados da mesma maneira que para os experimentos 1 e 2, da mesma forma que conduzida a avaliação da porcentagem de nanoinfiltração.

3.3.11 Análise morfológica da interface de união por histologia

As restaurações somente com sistema adesivo, em cada tempo de avaliação, foram fixadas em um suporte acrílico, seccionadas (0,5 µm de espessura) com um micrótomo de tecidos duros e uma lâmina carbide tungstênio (Leica, Alemanha). Os cortes histológicos foram então montados em lâminas de vidro e a técnica histológica utilizada foi Tricrômico de Goldner (WANG e SPENCER, 2003 [49]). A lâminas histológicas foram examinadas por um microscópio de luz numa magnificação de 100x (Nikon E800). Para permitir uma comparação entre os grupos experimentais, as fotomicrografias foram obtidas com a mesma magnificação e estágio micrométrico.

3.3.12 Análise estatística dos dados

Foi realizada uma média para cada dente dos valores obtidos pela microdureza, resistência de união à dentina, nanoinfiltração para cada tempo de avaliação. As falhas prematuras e coesivas não foram incluídas no teste estatístico devido ao pequeno número neste experimento.

Assim como para nos Experimentos 1 e 2, foi realizado os testes para avaliar a normalidade dos dados e equivalência das médias de microdureza, resistência de união à dentina e nanoinfiltração. Após isso, os dados foram submetidos a um Análise de Variância-2 fatores de medidas repetidas (agente de tratamento x tempo de avaliação) e teste de Tukey para comparação das médias ($\alpha = 0,05$).

4. Artigos

4.1 Collagen cross-linkers on dentin bonding: stability of the adhesive interfaces, degree of conversion of the adhesive, cytotoxicity and in situ MMP inhibition

Status: submetido em maio/2015 no periódico Dental Materials.

4.2 The effect of a proanthocyanidin-containing 10% phosphoric acid on bonding properties and in situ MMP inhibition

Status: submetido em julho/2015 no periódico Dental Materials.

4.3 Degradation of dentin-bonding interfaces treated by collagen cross-links agents in a cariogenic oral environment: an *in situ* study.

Status: em processo de revisão.

Collagen cross-linkers on dentin bonding: stability of the adhesive interfaces, degree of conversion of the adhesive, cytotoxicity and in situ MMP inhibition.

Abstract

Objective: To investigate the effect of collagen cross-links on the stability of adhesive properties, the degree of conversion within the hybrid layer, cytotoxicity and the inhibition potential of the MMPs' activity.

Methods: The dentin surfaces of human molars were acid-etched and treated with primers containing: 6.5 wt% proanthocyanidin, UVA-activated 0.1 wt% riboflavin, 5 wt% glutaraldehyde and distilled water for 60 s. Following, dentin was bonded with Adper Single Bond Plus and Tetric N-Bond; and restored with resin composite. The samples were sectioned into resin–dentin "sticks" and tested for microtensile bond strength (μTBS) after immediate (IM) and 18-month (18M) periods. Bonded sticks at each period were used to evaluate nanoleakage and the degree of conversion (DC) under micro-Raman spectroscopy. The enzymatic activity in the hybrid layer was evaluated under confocal microscopy. Data from all tests were submitted to appropriate statistical analysis (α=0.05).

Results: All cross-linking primers reduced the degradation of μTBS compared with the control group after 18M ($p>0.05$). The DC was not affected ($p>0.213$). The NL increased after 18M for all experimental groups, except for proanthocyanidin with Single Bond Plus ($p>0.05$). All of the cross-link agents reduced the MMPs' activity, although this inhibition was more pronounced by PA. The cytotoxicity assay revealed reduced cell viability only for glutaraldehyde ($p<0.001$).

Significance: Cross-linking primers used in clinically relevant minimized the time degradation of the μTBS without jeopardizing the adhesive polymerization, as well as reduced the collagenolytic activity of MMPs. Glutaraldeyde reduced cell viability significantly and should be avoided for clinical use.

Keywords: dentin collagen, proanthocyanidin, riboflavin, glutaraldehyde, dental bonding.

1. Introduction

The longevity of hybrid layers depends upon the stability of their components, such as collagen fibrils and polymeric chains [1]. However, collagen fibrils are not completely infiltrated by resin monomers when exposed by acid etching [2, 3], thereby impeding optimal protection against denaturation challenges. Unprotected collagen is more prone to creep [4] and cyclic fatigue rupture [5] after prolonged function. Additionally, these resin-sparse collagen fibrils are also filled with and surrounded by water, which participates in the hydrolysis of resin matrices by esterases and collagen by collagenolytic enzymes [6].

Increasing the collagen's resistance against the degradation process may improve the stability of the resin–dentin bonded interface; this was the main purpose of incorporating collagen cross-linkers into the bonding process [7-9]. Collagen cross-linkers are effective in protecting collagen fibrils from degradation through enhancing the collagen's chemical and mechanical properties [10-13]. More recently, their benefits in dentin bonding have been credited to their ability to inhibit the activity of host-derived metalloproteinases [8, 14, 15]. However, literature showing direct evidence of the activity of endogenous dentin MMPs within the hybrid layer after treatment by cross-linkers agents is still scarce.

On the other hand, collagen degradation is only one part of the biodegradation process, and it is not clear how collagen cross-linkers can affect the adhesive properties of the hybrid layer. Collagen cross-linkers showed an inhibitory polymerization effect on dimethacrylates [16] that may impair the achievement of an adequate degree of conversion inside the hybrid layer and jeopardize the bonding effectiveness [4]. Incomplete polymerization of the adhesive monomers has been suggested as one reason for nanoleakage [17-19] due to the formation of a porous hybridoid structure with reduced sealing ability [20-22]. All of these factors can also affect the cytotoxicity of an adhesive interface [23, 24].

Although several collagen cross-linkers, such as glutaraldehyde and proanthocyanidins, have shown therapeutic effects on dentin collagen [25-28].

This requires their use for prolonged application times (10 min to 4 hours), which restricts their clinical applicability. The comparison of different, recently accredited collagen cross-linkers, applied at clinically relevant times, might be useful to select the most effective agent in preventing collagen degradation, while inducing low cytotoxicity, with low polymerization inhibition yet providing stable resin–dentin bond interfaces over time.

Thus, the aim of this study was to evaluate the transdentinal cytotoxicity, the stability of the resin-dentin interfaces by microtensile and nanoleakage tests, the degree of conversion for the adhesive by *in situ* micro-Raman spectroscopy and the collagenolytic activity of the adhesive interface using *in situ* zymography, both with and without the incorporation of cross-linkers into the dentin bonding protocol.

2. Materials and methods

2.1. Tooth preparation and experimental design

Seventy-six extracted, caries-free human third molars were used after approval of the Institutional Ethics Committee from the State University of Ponta Grossa, Paraná, Brazil (protocol 314.563). The teeth were stored in 0.5% chloramine solution and used within two months after extraction. A flat dentin surface was exposed after wet-grinding the occlusal enamel using 180-grit SiC paper and 600-grit SiC paper for 60 s.

The dentin of forty teeth was etched for 15 s with 35% phosphoric acid gel (Scotchbond etchant, 3M ESPE, St. Paul, USA, batch number N261433), rinsed with water (30 s), air-dried (5 s) and kept slightly moist. The specimens were then randomly allocated to eight groups according to the combination of the main factors: 1) collagen cross-linking treatment (6.5 wt% proanthocyanidin, ultra-violet activated-0.1 wt% riboflavin, 5 wt% glutaraldehyde and distilled water [control group]) and 2) two etch-and-rinse adhesive systems (Adper Single Bond Plus [SB] and Tetric N-Bond [TN], as detailed in Table 1. A total of five teeth were employed per experimental group.

The acid-etched dentin surfaces were primed according to the experimental groups (Table 1). For the riboflavin (RB), the dentin surfaces were

further exposed to ultraviolet light for 2 min with a UV lamp (Philips, Hamburg, Germany; λ = 370 nm at 3 mW/cm^2) before air-drying [8]. The light-curing steps were performed using an LED (Radii Cal, SDI, Bayswater, Victoria, Australia; 1,200 mW/cm^2). Resin composite build-ups (Z250, 3M ESPE, Shade A3, batch number N549511) were incrementally constructed, and each portion was light-cured (40 s). The bonded teeth were then stored for 24 h in distilled water at 37 °C.

Then, the specimens were longitudinally sectioned in both the mesiodistal and buccolingual directions across the bonded interface in a cutting machine (Buehler, Lake Bluff, USA), to obtain resin–dentin sticks (1 mm^2). The number of premature failures per tooth during specimen preparation was recorded. The cross-sectional area of each stick was measured with a digital caliper (Absolute Digimatic, Mitutoyo, Tokyo, Japan) to the nearest 0.01 mm.

2.2. Resin–dentin microtensile bond strength (μTBS)

For this test, a total of 40 teeth (n=5 teeth per group) were used. Each bonded stick was attached to a jig for microtensile testing with cyanoacrylate resin (Super Bonder Gel, Loctite, São Paulo, Brazil) and subjected to a tensile force in a universal testing machine (Kratos, São Paulo, SP, Brazil) at 0.5 mm/min. The failure modes were evaluated under stereomicroscopy at 40x magnification and classified as cohesive adhesive or adhesive/mixed.

2.3. Nanoleakage evaluation (NL)

Two resin-bonded sticks from each tooth at each storage period (not tested in *μTBS)* were randomly selected. The specimens were immersed in ammoniacal 50 wt% silver nitrate solution [22] in darkness for 24 h. Then, they were rinsed thoroughly in distilled water and photo-developed (8 h) under fluorescent light to reduce the silver ions into metallic silver grains. The specimens were polished down until 2500-grit SiC paper and 1 and 0.25 μm diamond paste (Buehler Ltd., Lake Bluff, IL, USA). They were ultrasonically cleaned, air-dried, mounted on stubs and coated with carbon (MED 010, Balzers Union, Balzers, Liechtenstein). The interfaces were observed in a scanning electron microscope

(SEM), in the backscattered mode at 12 kV (VEGA 3 TESCAM, Shimadzu, Tokyo, Japan). Three images were taken of each specimen: the first image was in the center of the stick, while the next two were obtained 0.3 mm left and right from the first picture, respectively [29].

A total of six images were obtained per tooth at each period (3 images x 2 bonded sticks). A total of 30 images were obtained per group (6 images x 5 teeth) by a blinded author. We measured the relative percentages of NL within the adhesive and hybrid layers with the ImageTool 3.0 software (University of Texas Health Science Center, San Antonio, USA), as described earlier [19, 30].

2.4. In situ degree of conversion (DC) within adhesive/hybrid layers

Two resin–dentin sticks were randomly selected from the immediate period and used to evaluate the DC immediately after sectioning. The sticks were wet polished using 1500- and 2000-grit SiC paper. The specimens were ultrasonically cleaned for 10 min and positioned into micro-Raman equipment (Senterra spectrophotometer Bruker, Ettlingen, Baden-Württemberg, Germany), which was first calibrated for zero and then for coefficient values using a silicon sample. The samples were analyzed using a 20 mW Neon laser with 532 nm wavelength, spatial resolution of 3 μm, spectral resolution 5 cm^{-1}, accumulation time of 30 s with 5 co-additions and 100x magnification (Olympus microscope, London, UK) with a 1-μm beam diameter. Spectra were obtained at the dentin-adhesive interface, at three random sites (per bonded stick) within intertubular-infiltrated dentin. Spectra of uncured adhesives were taken as references. Post-processing of the spectra was performed using the Opus Spectroscopy Software version 6.5. The ratio of double-bond content of monomer to polymer in the adhesive was calculated according to the following formula: DC (%) = (1 − [R cured / R uncured]) x 100, where ‘R’ is the ratio of aliphatic and aromatic peak intensities at 1639 cm^{-1} and 1609 cm^{-1} in cured and uncured adhesives.

2.5. In situ zymography

A dye-quenched MMP probe based on gelatin was prepared by means of a fluorescein isothiocyanate (FITC) hypersaturated gelatin. 5 mg of FITC was

dissolved in 2 ml 0.1M sodium carbonate/bicarbonate buffer (pH 9.0, Sigma Aldrich, Milwaukee, USA). This reactant was added dropwise to a 1mg/ml gelatin solution in the dark and was incubated at room temperature for 2 h. The reacted FITC–gelatin conjugate was isolated from unbound FITC by means of a G-25M Sephadex column. The fluorescein-to-protein ratio of >15 was confirmed from absorbance readings at 495 nm and 280 nm, respectively. This MMP-substrate confocal dye was dissolved (0.3wt%) in distilled water and actively applied for 60 s onto the phosphoric acid-etched dentin before the bonding agents and cross-linking primers were applied.

Three teeth per group (n = 3) were bonded as previously described and cut into resin–dentin slabs; their interfaces were observed by confocal laser scanning microscopy (CLSM), similarly to a previous study [31]. The specimens were examined using a CLSM (Leica SP5 CLSM, Heidelberg, Germany) equipped with a 63×/1.4 NA oil immersion lens using 468-nm laser illumination. The z-stack scans (one at each micrometer up to 20 μm below the surface) were compiled into single projections. Each resin–dentin interface was entirely characterized, and images representing the MMP-activity observed along the bonded interfaces were captured.

2.6. Cytotoxicity evaluation: cell culture and MTT assay

Twenty-four teeth were used in this test. Dentin disks with a thickness of 0.6 mm were obtained from the mid-coronal dentin of each tooth using a cutting machine (Isomet 1000, Buehler, Lake Bluff, USA). The disks were carefully examined with a stereomicroscope at 40X to confirm the absence of enamel and defects resulting from pulp horn projections. Then, the occlusal sides of the disks were manually finished with wet 320-grit silicon carbide paper to reach a final thickness of 0.5 mm (Mitutoyo South Americana Ltd, Suzano, SP, Brazil). Afterwards, a smear layer was produced with 600-grit SiC on both sides of the disks and immediately removed by 0.5 M EDTA (pH 7.4) for 60 s. After abundant rinsing with deionized water, the dentin's permeability was measured through a hydraulic conductance device [32] to permit a homogenous distribution of the dentin disks among the experimental groups (n=6 disks per group). The dentin disks were positioned in metallic devices and autoclaved (20

min/121° C). The occlusal surfaces of the dentin disks were etched with 35% phosphoric acid (15 s), carefully rinsed with deionized water (10 s) and blot dried with sterile cotton pellets.

The NIH/3T3 fibroblast cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin (10000U/100 µg/ml) at 37 ºC with 5% CO_2 in a humidified atmosphere. Briefly, 4 x 10^4 cells were added to each well of a 24-well plate. After 24 h, the medium was removed; the cells were washed twice with phosphate buffer solution (PBS). DMEM containing 1% fetal bovine serum and trans-well chambers were added into each well. The dentin disks were carefully added, and 10 µL of each cross-linking primer was applied onto the occlusal surface. For the riboflavin group, the UVA-light was irradiated by a lamp, as previously described, using a 10.4 mm tip that completely covered the culture well (24-well format). This protocol followed Bouillaguet *et al.* 2008 [33] to prevent modifications to the cell mitochondrial activity.

The trans-well was removed and the cells were washed with phosphate-buffered saline (PBS) 24 h later. MTT cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, USA) according to the method of Tada *et al.* 1986 [34] with some modifications. MTT solution was added (1.0 mg/mL), and cell viability was then assessed in a colorimetric assay using mitochondrial dehydrogenase activity in active mitochondria to form purple formazan. The absorbance of each well was read at 570 nm using a plate reader (EL808B, BioTech Instruments Inc., Winooski, VT, USA). Cell viability was expressed as the percentage of optical values in the treated samples versus the concurrent control (no treatment), considered as 100%. All of the experiments were performed in triplicate.

2.7. Statistical analysis

The µTBS (MPa) and NL (%) from the same experimental unit were averaged for statistical purposes at each storage time. The bonded sticks with premature and cohesive failures were not included in the tooth mean, due to their low frequency in the experiment.

The Kolmogorov–Smirnov test was employed to assess whether the data from each test (µTBS, NL, DC and cytotoxicity) followed a normal distribution.

Barlett's test was performed to determine if the assumption of equal variances was valid. After observing the data's normality and equality of the variances, the data from the μTBS (MPa) and NL (%) of each adhesive were subjected to a two-way repeated measure ANOVA (on solutions and storage time). The data from the DC (%) and cytotoxicity (%) of each adhesive were subjected to a one-way ANOVA (on solutions). For all of the test Tukey's test was used for pairwise comparisons (α=0.05).

3. Results

3.1. Microtensile bond strength

Approximately 29–37 bonded sticks could be obtained per tooth, including the pre-test failures. The mean cross-sectional area was 0.9 ± 0.13 mm^2, and no differences among the groups were detected (data not shown; $p>0.05$). Most of the failures were mixed (data not shown). None of the cross-linker primers affected the immediate μTBS of either adhesive (Table 2). For SB, all of the primers except the control group produced stable μTBS after 18 months of storage. A similar trend was observed for TN, except that degradation was also observed in the glutaraldehyde group (Table 2).

3.2. Nanoleakage

For SB, reduced nanoleakage was observed at the immediate period for all of the primers (Table 3). This increased significantly after 18 months for all of the primers ($p<0.001$), except proanthocyanidin. For TN, a more pronounced nanoleakage was observed at the immediate period for the control and glutaraldehyde groups ($p<0.001$). This increased significantly after 18 months of water storage for all of the groups ($p<0.001$) (Table 3).

3.3. Degree of conversion

No statistically significant differences ($p > 0.213$) were observed among the experimental groups for both of the adhesive systems (Table 4).

3.4. In situ zymography

In the control groups of both of the adhesive systems, the in situ zymography revealed an intense green fluorescence at the hybrid layer, indicating strong activity of MMPs (Fig. 1). The MMP activity for both adhesives was completely

eliminated with the preliminary application of the proanthocyanidin primer. In the UVA-riboflavin and glutaraldehyde groups, an expressive reduction of MMP activity was also observed for both adhesives, although not to the same extent as that achieved with proanthocyanidin (Fig. 1).

3.5. Cell viability

The treatment with glutaraldehyde reduced 81.1 ± 4% of cell viability in which was statistically different than the other groups ($p<0.001$). UVA-riboflavin (0.1%) and proanthocyanidin (6.5%) did not alter the cell viability, compared with the control group (Fig.2).

4. Discussion

This study evaluated the use of aqueous primers containing different collagen cross-links in the bonding protocol applied on demineralized dentin under clinically relevant time periods. Although some studies have had the same aim [8, 12, 35], they did not compare the most effective agents in a single experimental design. We observed that all of the cross-linking agents employed produced stable bond strength after 18 months of water storage for both adhesives, which is in agreement with previous studies [8, 27].

The chemical and structural characteristics of each cross-linking agent determine its ability to interact and modify the dentin matrix and the consequent impact on the stability of the collagen, the vulnerability of the adhesive interfaces to degradation and its cytotoxic potential [36, 37]. For instance, glutaraldehyde has a five-carbon aliphatic molecule with an aldehyde at each end of the chain, rendering it bifunctional. The aldehyde group is able to interact chemically with the amino groups of collagen [38], thus increasing the strength of the collagen [39] and minimizing the degradation of the adhesive interface [28]. The disadvantage of this agent is that it promotes rapid surface cross-linking of the tissue, generating a barrier that impedes its further diffusion into the tissue bulk, jeopardizing the fixation of the tissue as the depth of the tissue increases [37, 40]. This might be one of the reasons why this agent was not capable of completely inhibiting the activity of MMPs in the deeper region of the hybrid layer (Fig. 1).

Riboflavin (vitamin B2) was also shown to reduce the degradation of the adhesive interface in both the present and earlier studies [8, 35]. The high energy of UVA-light (365 nm) breaks down weak and intrinsic cross-links among collagen fibrils and generates free oxygen radicals. The reactive oxygen species can induce the formation of new covalent and strong cross-links within collagen [41]. This occurs through the binding of the functional hydroxyl groups in riboflavin to proline and/or lysine in the collagen [42].

Proanthocyanidins are natural polyphenolic compounds that are widely used as food supplements. Due to their free-radical scavenging capacity, high affinity to protein and antioxidant potential, these materials have been extensively studied for enhancing dentin bonds [36, 43, 44]. The stabilization of the dentin bonding after water storage can be explained by four interaction mechanisms between the proanthocyanidin and proteins, including covalent, ionic and hydrogen bonding, as well as hydrophobic interactions [36, 44, 45]. Proline-rich proteins like collagen have an extremely high affinity to proantocyandin [46], yielding strong bonds. This interaction primarily occurs through hydrogen bonding between the protein amide carbonyl group of the collagen and the phenolic hydroxyl group of the cross-linking agent [47].

Riboflavin and proanthocyanidin are very safe agents, as they did not reduce cell viability in the cytotoxicity test, thus overcoming some of the drawbacks that are typically encountered with synthetic cross-linking agents such as glutaraldehyde. The high cytotoxicity of glutaraldehyde might be because this agent suffers from depolymerization [40, 48]. The residues from this depolymerization along with uncured molecules render it very cytotoxic, as reported based on the MTT assay of the present investigation and in previous investigations [36, 49, 50]. These two disadvantages make glurahaldehyde the least adequate cross-linking agent for incorporation into the bonding protocol.

The literature about the cytotoxic potential of riboflavin is controversial [33, 37, 42, 51]. We used the transdentinal method in the present study's MTT assay to better simulate the clinical situation. In this case, the product is applied onto the dentin surface, where it is capable of protecting the underlying cells. The other studies reporting that riboflavin is cytotoxic applied the product directly onto the cell culture [33, 37]. On the other hand, proanthocyanidin's lack of cytotoxicity is in agreement with other studies [36, 48, 52]. The high affinity of

proanthocyanidin to the organic matrix of collagen may lead to effective cross-linking without the production of cytotoxic residual molecules, rendering it a safe product for use in dentin bonding.

A high degree of conversion for the adhesive is critical to produce a high cross-linked polymer within the hybrid layer and also yield adhesive interfaces that is less prone to degradation [17-19]. Faithfully, the use of the cross-linking agents did not significantly affect the degree of conversion within the hybrid layer, which suggests that none of the agents jeopardized the polymerization of adhesive system when applied like primers.

Although proanthocyanidins were reported to have the potential to reduce the polymerization efficacy of resins, this seems to depend on the concentration of the agent [9, 53] as well as how this cross-linking is incorporated into the bonding process. For instance, the incorporation of proanthocyanidin into a simplified etch-and-rinse adhesive system in concentrations equal to or higher than 2% resulted in inadequate polymerization of the adhesive and the formation of microvoids within the adhesive layer. [53, 54] In the present study, the polymerization of the adhesive was not jeopardized, even when applying proanthocyanidin at higher concentrations (6.5 wt%). Perhaps the only substrate that the proanthocyanidin has for bonding is collagen when applied as an aqueous primer, before applying the adhesive [36, 37, 44, 47]. Therefore, by the time the adhesive is applied, there is no longer proanthocyanidin available to compete for binding with the free radicals generated during the adhesive's polymerization.

It is quite difficult to produce nanoleakage-free interfaces. Nanoleakage reveals the locations of defects at the resin–dentin interface that could work as the pathway for the resin–dentin bonds' degradation over time. These defects result in areas in which resin failed to infiltrate, residual water/solvent had not been displaced by adhesive resin [55] or fluid transudation occurred through the dentin to the adhesive layer [21, 56-60]. Although these nanometer-sized spaces are too small to allow for bacterial penetration, they are large enough to work as channels for water sorption [18, 20, 61] and leaching of uncured water-soluble monomers.

We cannot expect the application of aqueous solutions of cross-linkers to produce nanoleakage-free interfaces, since the protocols tested herein cannot

alter the hydrophilic nature of the adhesive, improve water/solvent evaporation or produce less permeable adhesive interfaces. Thus, the similar nanoleakage among the experimental and control groups, both immediately and at 18 months, can be seen with enthusiasm. This is evidence that the infiltration of monomers, solvent evaporation and polymerization of the adhesive were not jeopardized by the preliminary step of collagen cross-linking. If this had occurred, a more pronounced and intense nanoleakage, like those observed in the poorly polymerized conditions of the adhesive interfaces [17, 19, 62], would have been observed.

Interestingly, the application of proanthocyanidin and riboflavin produced interfaces that were less prone to nanoleakage over time for both adhesives. For SB system, the proanthocyanidin group did not show a significant increase of nanoleakage over time. The exogenous cross-link of the dentin matrix by proanthocyanidin leads to dehydration on the collagen fibrils, which decreases the swelling ratio and the water absorption [6, 12, 45]. This suggests that the application of proanthocyanidin can minimize the risk of collagen network collapse resulting from air drying and therefore minimize the technique sensitivity of the wet bonding [6, 43].

All previous attempts to inhibit MMPs with different chemicals required that these inhibitors bind to MMPs irreversibly; however, there is no evidence of such long-term binding. As riboflavin—the unique cross-linking that is applied—is associated with high energy of UVA light, we speculate that, despite an immediate limited effect in terms of MMP inhibition, this effect could be maintained for a long time.

Obviously, we cannot rule out the fact that the biodegradation of the resin–dentin interface is quite complex and involves a cascade of events, starting with the extraction of resins that have infiltrated the dentin matrix, followed by an enzymatic attack on exposed collagen fibrils [63, 64]. Proteases such as metalloproteinases (MMPs) and cysteine cathepsins are thought to be responsible for enzymatic degradation of the collagen fibrils via hydrolysis [65].

All of the cross-linking agents reduced or eliminated the MMPs' activity in the dentin-bonding interface [8, 15, 35]. This is the first laboratory study that demonstrated such inhibition potential in situ for these tested agents. On the dentin collagen, the triple-helical conformation makes the interstitial collagens

resistant to most proteinases; however, when collagenases bind to collagen, they unwind the triple-helix, allowing single peptides to enter the substrate binding and catalytic domains [66]. Cross-linking agents produce cross-links in collagen that stiffen it enough that it cannot unwind its structure. In addition, cross-linking agents can cross-link proteases, which directly interferes with their molecular mobility [67]. They can inactivate C-terminal telopeptidases, thereby maintaining the telopeptides' ability to sterically block collagenase binding to the critical peptide bond [67, 68]. Hence, it is reasonable to expect that cross-linking agents are capable of inhibiting the MMP activity. This is in agreement with previous findings reporting that cross-linked collagen exhibited reduced digestibility by collagenase [8, 12, 15, 28, 69].

Among all of the cross-linking agents, proanthocyanidin was the most effective agent in reducing the MMP activity. Again, the very effective chemical interaction of proanthocyanidin and high reticulation ability throughout the whole collagen length [36] make it very effective. It is speculated that proanthocyanidin has stronger interaction ability with collagen than glutaraldehyde and riboflavin do. Also, the dehydration of the collagen fibrils and the hydrophobic effect (as previously discussed) produced by proanthocyanidin result in less water and fluid sorption, better adhesive infiltration and consequently fewer denuded fibrils within the hybrid layer. Altogether, these factors result in a hybrid layer that is less prone to the MMPs' activity [4, 62].

5. Conclusions

Under a standardized application time of 60 s, the use of all of the cross-linking primers before applying the adhesive was effective in minimizing the degradation of the μTBS of both adhesives, without jeopardizing the polymerization of the resin monomers. The stable μTBS of the adhesives can be attributed to the reduced in situ collagenolytic activity of the MMPs, which was much more pronounced with the use of proanthocyanidin. Glutaraldeyde reduced cell viability significantly and should be avoided for clinical use.

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Table 1. Description of all experimental groups, composition and application mode.

Product (Company)	Composition	Application mode
Proanthocyanidin (PA) primer (Mega Natural Gold, Madera, USA) Batch number 05592502-01	Proanthocyanidin-Grape seed extract 6.5 % weight, distilled water.	After acid etching step, application for 60 s with gentle agitation using a fully satured applicator. Gently air-drier for 5 s and kept slightly moist the surface.
Riboflavin (RB) primer (Fisher Scientific GmbH, Schwerte, Germany) Batch number 070046	Riboflavin 0.1 % weight, distilled water.	After acid etching step, application for 60 s with gentle agitation using a fully satured applicator. After that, irradiation using UVA-light for 2 min. Gently air-drier for 5 s and kept slightly moist the surface.
Glutaraldehyde (GA) primer (Fisher Scientific GmbH, Schwerte, Germany) Batch number 186852	Glutaraldehyde 5 % weight, distilled water.	After acid etching step, application for 60 s with gentle agitation using a fully satured applicator. Gently air-drier for 5 s and kept slightly moist the surface.
Control Group (CT)	Distilled water	After acid etching step, application for 60 s with gentle agitation using a fully satured applicator. Gently air-drier for 5 s and kept slightly moist the surface.
Single Bond Plus (SB) (3M ESPE, St. Paul, USA) Batch number N531785	Ethyl alcohol, BisGMA, silane treated silica (nanofiller), HEMA, copolymer of acrylic and itaconic acids, glycerol 1,3-dimethacrylate, water, UDMA, diphenyliodonium hexafluorophosphate, EDMAB.	After treatment according the experimental groups, application 2 consecutive coats of adhesive for 15 s with gentle agitation using a fully saturated applicator. Gently air thin for 5 s to evaporate solvent. Light-cure for 10 s.
Tetric N-Bond (TN) (Ivoclar Vivadent AG,Scha Liechtenstein) Batch number L50568	Phosphonic acid acrylate, HEMA, BisGMA, UDMA, ethanol, nanofiller, catalysts and stabilizer.	After treatment according the experimental groups, application 2 consecutive coats of adhesive for 15 s with gentle agitation using a fully saturated applicator. Gently air thin for 5 s to evaporate solvent. Light-cure for 10 s.

BisGMA: bisphenol a diglycidyl ether dimethacrylate, *HEMA*: 2-hydroxyethyl methacrylate. *UDMA:* diurethane dimethacrylate, *EDMAB:* ethyl 4-dimethyl aminobenzoate.

Table 2. Means and standard deviations of μTBS (MPa) for all experimental groups.

Groups	SB		TN	
	Immediate	18-month	Immediate	18-month
Control	39.5 ± 7.9 a	13.9 ± 1.8 b	36.8 ± 4.7 A	13.9 ± 1.8 D
Proanthocyanidin	36.2 ± 5.5 a	31.9 ± 4.3 a	29.2 ± 1.2 ABC	27.6 ± 6.3 BC
UVA- riboflavin	37.1 ± 9.7 a	31.6 ± 3.5 a	31.5 ± 6.9 AB	25.1 ± 1.3 BC
Glutaraldehyde	38.5 ± 2.4 a	29.7 ± 2.6 a	35.7 ± 1.9 AB	24.2 ± 1.4 C

Comparisons are only valid within the same adhesive system. Means identified with the same letter are statistically similar (Tukey's test; p>0.05).

Table 3. Means and standard deviations of nanoleakage (%) for all experimental groups.

Groups	SB		TN	
	Immediate	18-month	Immediate	18-month
Control	7.2 ± 4.5 Aa	20.3 ± 4.6 Bb	19.5± 3.8 Ff	25.9 ± 3.5 Eg
Proanthocyanidin	4.6 ± 2.1 Aa	8.0 ± 0.9 Aa	13.2 ± 4.8 Df	15.7 ± 1.9 Cg
UVA-riboflavin	4.5 ± 2.6 Aa	11.6 ± 1.6 Ab	13.9 ± 1.9 Df	20.0 ± 2.1 Dg
Glutaraldehyde	6.1 ± 2.3 Aa	14.7± 2.4 Bb	17.6 ± 7.2 Ef	24.2 ± 2.3 Eg

Comparisons are only valid within the same adhesive system. In each column, means identified with the same uppercase letter are statistically similar. In each row (for each adhesive), means identified with the same lowercase letters are statistically similar (Tukey's test; p>0.05).

Table 4. Means and standard deviations of the degree of conversion (%) for all experimental groups.

Groups	SB	TN
Control	79.4 ± 7.2 A	77.6 ± 6.8 a
Proanthocyanidin	79.7 ± 6.0 A	77.7 ± 7.8 a
UVA-riboflavin	73.7 ± 5.8 A	71.9 ± 7.4 a
Glutaraldehyde	77.5 ± 9.0 A	72.7 ± 8.8 a

Comparisons are only valid within the same adhesive system. No significant difference was observed among groups (Tukey's test; p>0.05).

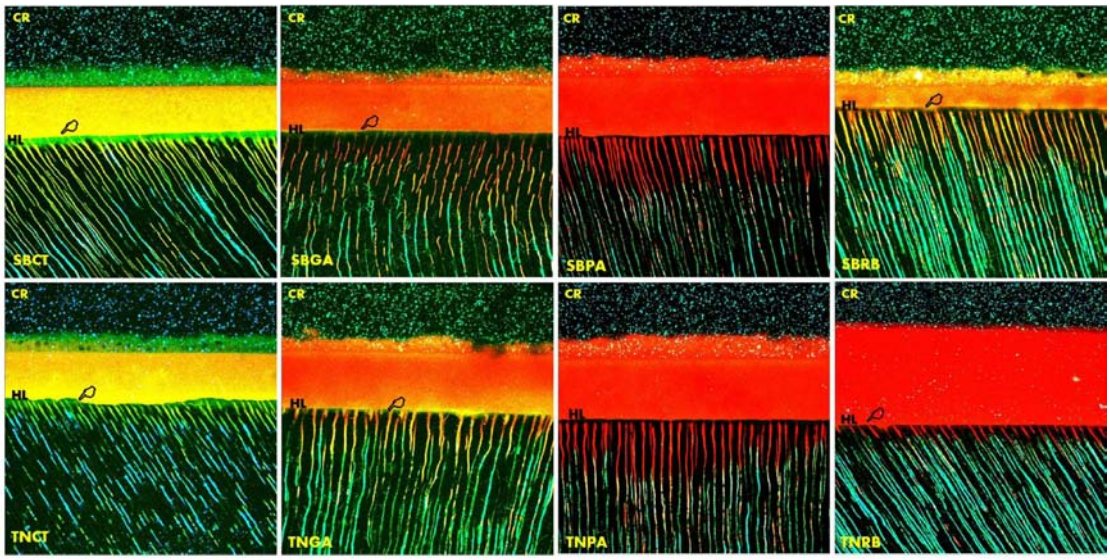

Figure 1. The confocal micrographs of the *in situ* MMP-activity at the dentin-adhesive interface after incubation for 24 h. The figures SBCT and TNCT represent the control groups and show the intense activity of MMPs (green staining) at the hybrid layer (black hand) and beneath it. The figures SBGA and TNGA represent the glutaraldehyde groups and exhibit the considerable reduction of the intensity of the active MMP-binding fluorescent dye. However, there is still moderate green staining (enzymatic activity) at the top of the hybrid layer. In contrast, the cross-link treatment using proanthocyanidin (Figure PA) eliminated almost entirely the MMP-activity represented by the lack of green staining at the thick hybrid layer. In the case of UVA-activated riboflavin the green staining was somewhat between the control group and the treatment with

glutaraldehyde. The pre-treatment using riboflavin reduced the MMP activity but not in a greater extent as proanthocyanidin. CR: composite-resin and HL: hybrid layer.

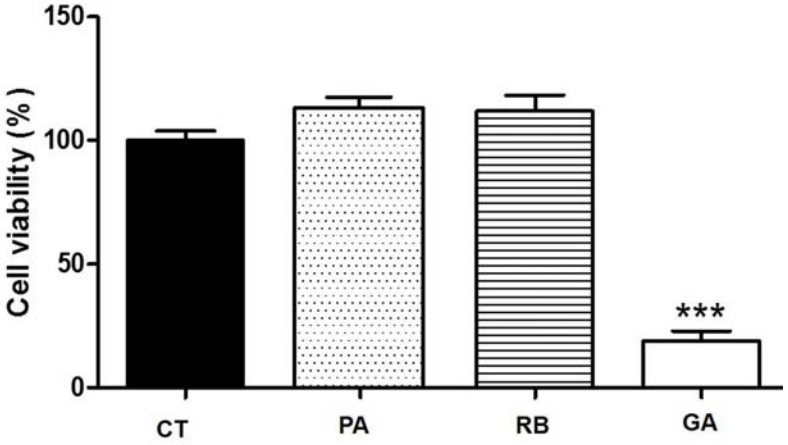

Figure 2. Graphic representation of the effect of on 3T3 cells after 24 h of exposure in the MTT assay. *** Indicates statistical difference compared to the other groups (*Tukey's test;* $p < 0.001$).

The effect of a proanthocyanidin-containing 10% phosphoric acid on bonding properties and in situ MMP inhibition

Abstract

This study evaluated the effect of etching using 2% proanthocynidin-containing 10 % phosphoric acid (2% PA) and 35% phosphoric acid (CT) on immediate (IM) and 6-months (6M) resin-enamel microshear bond strength (μSBS), resin–dentin microtensile bond strength (μTBS), nanoleakage (NL) and as well as in situ MMP inhibition potential.

Methods: Human molars had exposed the dentin surface and were etched using 35%phosphoric acid for 15 s or 2% PA for 30 s. After that, the bonding procedure was performed (Single Bond Plus, 3M ESPE) and composite build-ups were constructed followed polymerization. The teeth were sectioned and tested by microtensile bond strength for μTBS analysis and by SEM for NL analysis in IM and 6M. For MMP activity, resin-dentin slices were analyzed under confocal microscopy. For μSBS, others teeth had etched the enamel surface according the experimental groups and prepared to microshear procedure. The specimens were tested in IM and after 6M by microshear bond strength. The data were submitted to two-way repeated measures ANOVA and Tukey´s test (α = 0.05).

Results: The etching using the 2% PA did not affect the μTBS in IM ($p > 0.05$) comparing to the CT group, however after 6M only the 2% PA etching remained stable the resin-dentin bond strength ($p<0.05$), as well for NL, only the CT showed increase the NL% after 6M ($p <0.05$). The MMP activity within the resin-dentin interface was almost completely reduced after 2% PA etching, while the CT exhibited intense MMP activity. For μSBS, the type of etchant and the storage period did not affect the resin-enamel bond strengths ($p>0.05$).

Significance: The etching acid containing proanthocyanidin can be a relevant alternative for produce stable resin-dentin and enamel-resin interfaces, without requires additional steps in the bonding procedure. Future studies for longer evaluation time are required.

Key words: dentin collagen, acid etching, crosslinking reagents, proanthocyanidins, demineralized dentin layer.

1. Introduction

There is a general consensus that the resin-dentin bonds created with contemporary hydrophilic dentin bonding systems deteriorate over time [1, 2]. For etch-and-rinse adhesives, there is a decreasing gradient of resin monomer diffusion within the hybrid layers [3, 4]. This results in incomplete resin infiltration at the bottom the hybrid layer, leaving denuded collagen fibrils [3, 5] that are susceptible to enzymatic degradation by host-derived collagen-bound matrix metalloproteinases (MMPs) and cysteine cathepsins [6-9]. Additionally, simplified etch-and-rinse systems are capable to activate these matrix metalloproteinases (MMPs) [6, 10]. Consequently, procedures that enhance dentin collagen's resistance towards collagenolytic activity of host-derived enzymes have great potential to improving the longevity of dentin bonding.

To increase the collagen stability, one might employ MMP inhibitors and collagen cross-linking agents. Exogenous MMP inhibitors, such as chlorhexidine, are capable to reduce the protease activity and to prolong the durability of resin-dentin bonds [11-13] but they lack chemical bond with the collagen fibrils. Collagen cross-linkers establish chemical bonds with the collagen and may also increase the collagen resistance against the effect of host-derived proteases [14-16].

The delivery of these agents to the demineralized dentin can perform as primer they can be incorporated into one of the components of the bonding protocol [17]. The application of the agents as a primer is hampered by the fact that this procedure adds another step to the bonding protocol, which is against the clinician's preference for simplification. This fact has motivated some authors to combine MMPS inhibitors or cross-linking agents in the etchants [13, 18, 19].

Among the cross-linking agents, proanthocyanidin (PA)–rich grape seed extract (GSE) is a promising agent due to its effectiveness under shorter treatment times [20] and its absence of cytotoxicity [21]. Additionally, PA can stabilize the resin-dentin bond strength of the adhesive interface and decrease the dentin-bound MMPs activity [22].

Following the trend of simplicity, the incorporation of PA into a 10% phosphoric acid showed promising results, rendering the demineralized dentin collagen inert to bacterial collagenase digestion [18]. However, the authors did not evaluate the PA-etchant into a clinically relevant bonding procedure, which prevent us from knowing whether or not the low concentrated PA-containing phosphoric acid is capable to promote an effective etching in enamel and dentin substrates and also produce stable resin-dentin bonds after water storage.

Therefore, the aim of this study was to evaluate the immediate and 6-month effectiveness of this modified phosphoric acid etchant in dentin and enamel through resin-dentin microtensile bond strength, resin-enamel microshear bond strength and nanoleakage. Additionally, the in situ MMP inhibition potential was also evaluated through in situ zymography.

2. Material and Methods

2.1 Specimen preparation

A total of 26 extracted, caries-free, human third molars were used. The teeth were collected after obtaining the patients' informed consent under a protocol approved by the Ethics Committee Review Board from the State University of Ponta Grossa (Parana, Brazil). The teeth were disinfected in 0.5% chloramine, stored in distilled water, and used within six months after extraction.

In 16 teeth, a flat occlusal dentin surface was exposed after wet grinding the occlusal enamel with #180-grit silicon-carbide (SiC) paper for 60 s. The exposed dentin surfaces were further polished with wet #600-grit SiC paper for 60 s to standardize the smear layer. Ten teeth were used for evaluation of the resin-dentin microtensile bond strength (μTBS) and nanoleakage, while the remaining six was used for the evaluation of the MMP-activity.

In 10 teeth, the roots of all teeth were removed by sectioning at the enamel-cementum junction. The dental crowns were then sectioned in the diagonals across the long axis of teeth to produce four enamel specimens (buccal, lingual, and proximals). Forty enamel specimens were ground wet with # 180 and 600-grit SiC paper for 60 s each and used for evaluation of resin-enamel microshear bond strength (μSBS).

2.2 Experimental groups and restorative procedure

The specimens were randomly distributed into the control and experimental groups by a person not involved in the research protocol using computer-generated tables. In the control group, the dentin and enamel surfaces of all specimens were conditioned with a 35% phosphoric acid for 15 s (Scotchbond etchant, 3M ESPE, St. Paul, USA, batch number N261433). In the experimental group, previously to apply on dentin and enamel surfaces for 30 s, 2% PA-containing 10% phosphoric acid was prepared by mixing GSE powder, ethanol, distilled water, and 85% phosphoric acid to final concentrations (weight percentage with respect to total mass) of 2% PA-rich GSE, 20% ethanol and 10% phosphoric acid [18]. The chemicals herein used were purchased from Sigma-Aldrich (St. Louis, MO) unless the GSE which was purchased from Mega Natural Gold (Polyphenolics, Madera, USA).

After conditioning, the surfaces were rinsed off with distilled water for 30 s, air-dried for 5 s and kept slightly moist for the application of the two-step etch-and-rinse adhesive (Adper Single Bond Plus, 3M ESPE, St. Paul, USA, batch number N531785) using double application layer according to the manufacturer's instructions. A composite resin restoration (Filtek Z350, 3M ESPE, St Paul, MN, USA) was build up in two 2-mm increments and each one was light-cured for 40 s using an LED light curing (1,200 mW/cm^2; Radii-cal, SDI Limited, Bayswater, Victoria, Australia). A radiometer (Demetron LED Radiometer, Kerr Sybron Dental Specialties, Middleton, WI, USA) was used to check the light intensity every five restorations.

2.3 Resin-dentin microtensile bond strength (μTBS)

After water storage for 24 h at 37ºC, the restored teeth were longitudinally sectioned in both mesio-to-distal and buccal-to-lingual directions across the bonded interface, using a diamond saw mounted in a cutting machine (Isomet 1000, Buehler, Lake Bluff, USA). This procedure was performed to obtain resin-dentin sticks with a cross-sectional area of approximately 1 mm^2. They were either tested immediately of after 6 months of storage in distilled water for 37°C.

The distilled water was changed weekly to maximize the degradation process [23].

The cross-sectional area of each stick was measured with a digital caliper (Absolute Digimatic, Mitutoyo, Tokyo, Japan) to the nearest 0.01 mm. Each bonded stick was attached to a jig for microtensile testing with cyanoacrylate resin (Super Bonder Gel, Loctite, São Paulo, Brazil) and subjected to a tensile force in a universal testing machine (Model 5565, Instron, Canton, OH, USA) at a crosshead speed of 0.5 mm/min. The failure modes were evaluated under stereomicroscopy at 100x magnification and classified as cohesive (within dentine or resin composite), adhesive (failure at resin/dentine interface, or adhesive/mixed (failure at resin/dentine interface with partial cohesive failure of the neighboring substrates).

2.4 Nanoleakage evaluation

Two resin-bonded sticks from each tooth at each storage period, and not used for microtensile testing, were randomly selected for nanoleakage evaluation. The sticks were immersed in 50 wt% ammoniacal silver nitrate solution in total darkness for 24 h. Thereafter, they were rinsed thoroughly in distilled water, and immersed in a photo-developing solution for 8 h under fluorescent light to reduce silver ions into metallic silver grains within voids along the bonded interface. Specimens were polished using 1000-, 1500-, 2000- and 2500-grit SiC papers and 1 and 0.25 μm diamond paste (Buehler Ltd., Lake Bluff, IL, USA) on polishing clothes. They were ultrasonically cleaned, air-dried, mounted on stubs and coated with evaporated carbon (MED 010, Balzers Union, Balzers, Liechtenstein).

The interfaces were observed in a scanning electron microscope (SEM) in the backscattered mode at 12 kV (VEGA 3 TESCAM, Shimadzu, Tokyo, Japan). Three images were taken from each specimen. The first image was obtained in the center of the stick, while the further two were obtained 0.3 mm left and 0.3 mm right from the first picture. A total of six images were obtained per tooth at each period (3 images x 2 bonded sticks). Thus, for each experimental condition, 30 images were evaluated per group (6 images x 5

teeth) [24]. A blinded author to the experimental conditions took the pictures. The relative percentage of silver nitrate uptake within the hybrid layer was measured in all pictures using the ImageTool 3.0 software (Department of Dental Diagnostic Science, University of Texas Health Science Center, San Antonio, USA).

2.5 In situ zymography by CLSM

A dye quenched MMP probe based on gelatin was prepared by means of a fluorescein isothiocyanate (FITC) hypersaturated gelatine. Five mg of FITC was dissolved in 2 mL 0.1 M sodium carbonate/bicarbonate buffer (pH 9.0, Sigma Aldrich, Milwakee, USA). This reactant was added dropwise to a 1 mg/mL gelatine solution in the dark and was incubated at room temperature for 2 h. The reacted FITC-gelatine conjugate was isolated from unbound FITC by means of a G-25M Sephadex column. The fluorescein to protein ratio of > 15 was confirmed from absorbance readings at 495 nm and 280 nm, respectively. This MMP-substrate confocal dye was dissolved (0.3wt%) in distilled water and this solution was actively applied for 60 s on the acid-etched dentin before the application of the bonding agents. Three teeth per group (n = 3) were bonded as previously described and cut into resin-dentin slabs where the interfaces were observed by confocal laser scanning microscopy (CLSM) similarly to a previous study [25]. The specimens were examined using a CLSM (Leica SP5 CLSM, Heidelberg, Germany) equipped with a 63×/1.4 NA oil immersion lens using 468-nm laser illumination. The z-stack scans (one at each micrometer up to 20 μm below the surface) were compiled into single projections. Each resin-dentin interface was entirely characterized and the images were captured representing the MMP-activity observed along the bonded interfaces.

2.6 Resin-enamel microshear bond strength (μSBS)

Prior to applying the adhesive, each enamel specimen was mounted in a polyvinyl chloride ring filled with acrylic resin (AutoClear, DentBras, Pirassununga, São Paulo, Brazil), in a way that the enamel surface was on the top of the cylinder. An acid-resistant, double-faced adhesive tape (Adelbras Ind

e Com Adesivos Ltda, São Paulo, Brazil) was perforated with a Hygenic Ainsworth-style rubber-dam punch (Coltene, Alstätten, Switzerland) and bonded to the enamel surface Shimaoka and others [26].

The enamel surfaces were acid-etched with their respective etchants, rinsed off, and the adhesive applied according to the manufacturer's directions. Then, polyethylene Tygon tubes (Tygon Medical Tubing Formulations 54-HL, Saint Gobain Performance Plastics, Akron, OH, USA) with an internal diameter of 0.8 mm and a height of 0.5 mm were positioned over the double-faced tape with the lumen coincident with the perforations. An operator trained in the μSBS technique positioned seven to nine tubes per surface, using magnifying loupes. Resin composite was carefully packed inside each tube, pressed gently into place and light-cured for 40 s using an LED light-curing (1,200 mW/cm^2; Radii-cal, SDI Limited, Bayswater, Victoria, Australia).

After 24 h of storage in distilled water at 37ºC, the Tygon tubes and the double-faced adhesive tape were carefully removed with a surgical blade to expose the composite resin cylinders. Each specimen was examined under a stereomicroscope at 10X magnification. Specimens with evidence of air bubbles or gaps at the interface were discarded.

The specimens were attached to a shear-testing fixture (Odeme Biotechnology) so that each composite resin cylinder was tested in a universal testing machine (Kratos IKCL 3-USB, Kratos Equipamentos Industriais Ltda, Cotia, São Paulo, Brazil). A thin wire (0.2 mm diameter) was looped around the base of each composite cylinder to maintain the setup aligned (resin-enamel interface, the wire loop, and the center of the load cell) and ensure correct orientation of the shear forces [27]. The shear load was applied at a crosshead of 1 mm/min until failure. One half of specimens were tested immediately and another half after 6 months of storage, as described previously for μTBS. The μSBS values were calculated by dividing the load at failure by the surface area (mm^2).

2.7 Statistical analysis

The μTBS values from the same dentin surface at each storage period and the μSBS values from the same enamel surface were averaged and only one value per tooth was taken to the statistical analysis. Specimens that showed cohesive failures as well as the premature failures were not included in the tooth mean due to the low frequency of this fracture mode. The percentage of nanoleakage observed in specimens from the same tooth at each storage period was also averaged and only one value per tooth was taken to the statistical analysis.

The Kolmogorov-Smirnov test was performed to assess whether the data followed a normal distribution. The Barlett's test was performed to evaluate the equality of variances. After observing the normality of the data distribution and the equality of the variances, the μTBS and the μSBS (MPa) and nanoleakage (%) data were submitted to a two-way repeated measures ANOVA and Tukey´s test. For all tests, the level of significance was pre-set in 5%.

3. Results

3.1 Resin-dentin microtensile bond strength

The mean cross-sectional areas of the resin-dentin bonded sticks ranged from 0.7 to 1.05 mm^2 (0.92 ± 0.15 mm^2). Most of the resin-dentin bonded sticks showed mixed failures and a low number of cohesive and premature failures were observed (Table 1). A statistically significant cross-product interaction etchant vs. storage period was observed (Table 1, $p = 0.002$). Both etchants yielded similar bond strength values at the immediate period; however stable bond strengths was only observed for the PA-containing 10% phosphoric acid (Table 1).

3.2 Nanoleakage evaluation

In none of the conditions, a nanoleakage-free interface was observed (Figure 1). The cross-product interaction etchant vs. storage period was statistically significant (Table 1, $p = 0.002$). Both etchants yield similar nanoleakage at the immediate period; however this nanoleakage increased significantly after 6 months only for the conventional 35% phosphoric acid etchant (Table 1).

3.3 In situ zymography by CLSM

The confocal micrographs of *in situ* MMP-activity at the resin-dentin interface after incubation for 24 h can be seen in Figure 2. A more intense fluorescence (yellow hand), which represents intense MMP activity, can be seen in cyan (overlay of the fluorescein green and the reflection at blue) within the hybrid layer of the 35% phosphoric acid group. Although the MMP activity was almost disappeared at the GSE group the presence of MMP even at small proportion appears just inside the tubules (yellow pointer). Suggesting the MMPs unquenched were highly inhibited by the GSE group.

3.4 Resin-enamel microshear bond strength

Most of the failures of the resin-enamel specimens were adhesive/mixed (Table 2). Neither the cross-product interaction etchant vs. storage period ($p = 0.274$) nor the main factors etchant ($p = 0.107$) and storage period ($p = 0.601$) were statistically significant (Table 2). The type of etchant and the storage period did not affect the resin-enamel bond strengths.

4. Discussion

The similar immediate bond strengths produced with the 35% phosphoric acid and the PA-containing 10% phosphoric acid on enamel are in agreement with earlier studies. It was already demonstrated that low and high concentrations of phosphoric acids (from 2.5% to 40%) [28, 29] as well as varying etching times (15 to 120 s) [30, 31] did not alter significantly the resin-enamel strength. Indeed, the concentration of phosphoric acid used in the PA-containing acid seems to be already enough to produce selective enamel dissolution [28, 29, 31]. Although we have not evaluate the etching pattern of the enamel surfaces after conditioning with the different etching protocols, the present results suggest that both etching agents were able to promote selective dissolution of the enamel surface to allow inter-crystallite resin penetration and result in a strong adhesive interface on the enamel substrate [28].

After 6-month water storage, no deterioration of the resin-enamel bond strength was observed for either etchants. This was expected in light that previous studies revealed that enamel bonding does not deteriorate as well as dentin [32, 33] and therefore the short 6-month aging period was not long enough to induce significant changes in the resin-enamel adhesive interface as already demonstrated in other investigations [34]. Lack of organic component (collagen) part may be likely one of the reasons for the increased resistance of the bond produced by the enamel substrate

PA-etchant and commercial etchant produced similar **immediate** bond strength to dentin. Coupled with similar immediate bond strength to enamel, 10% acid for 30 s seems to be fine. Earlier pubs claimed that low acid concentrations generates precipitates with different phases of calcium phosphate salts, as brushite and thus affecting the immediate bonding in enamel [35]. But recently pubs found good results for low acid concentrations using phosphoric acid in this substrate [28], as well for dentin in immediate and after storage time [36]. Actually, the mineral precipitation under the surface is dependent on the pH, buffer capacity and number of hydrogen ions available for deprotonisation [37], at pH bellow 3, such as in phosphoric acid, brushite is mainly precipitated [38]. Due to low solubility of brushite along the with the volume of crystallites, could contribute to the entrapment of minerals within the demineralized dentin collagen and less water content within the etched-dentin substrate, which could facilitate the diffusion and greater polymerization of more hydrophobic resin monomers within the bonding interface [36]. However, the possible formation of precipitates from the brushite within the resin-interface requires further investigation and cannot be confirmed by our findings. Then, within the limitation of current study, low acid concentration was not a concern regardless of bonding to dentin or enamel.

After water storage, control decrease by half. Water storage accelerated bond degradation. At this accelerated bond degeneration condition, the PA-containing 10% phosphoric acid produced stable resin-dentin interfaces after 6 months of water storage, a finding not observed for the 35% phosphoric acid group. This agrees with earlier discovery that the effectiveness of the PA was not affected by the low pH of the phosphoric acid [18]. Additionally, the collagen

cross-linking leads to dehydration of the collagen fibrils, making it less susceptible to collagen network collapse after air-drying [39]. As a consequence, better adhesive infiltration may occur resulting in an adhesive interface less prone to hydrolytic and enzymatic breakdown.

Other cross-link agents, such as glutaraldehyde, are incompatible with the phosphoric acid as they depend on deprotonated amines to form covalent bonds. At low pH, amines are predominantly protonated and not reactive for covalent bonding [40]. PA cross-link collagen on a non-covalent basis [41-43] the reason of why PA can remain active in a low pH environment [18].

PA has a high affinity and specificity for interaction with the collagen proteins. This is achieved through several chemical mechanisms such as hydrogen bonding, hydrophobic interactions, covalent and ionic bonds [21, 42, 44] producing collagen cross-linking. This improves the mechanical properties of the demineralized dentin matrix, making it less prone to digestion by host-derived MMPs [22, 45, 46].

In the present study, we observed reduced MMP activity in hybrid layer of dentin interfaces treated with the PA-containing 10% phosphoric acid when compared to 35% phosphoric acid. PA can reduce the MMP activity by stiffening the collagen polypeptides so that they cannot be unwind; furthermore they also inactivate the catalytic site of the host-derived proteases by creating a new peptide bond across adjacent peptides [39].

Therefore, the stability of the resin-dentin interfaces produced with a PA-containing 10% phosphoric acid after 6 months of water storage can be attributed to several factors that work synergistically. The ability of PA to interact with collagen even in an acid environment, its ability to inactive the catalytic site of proteases and the increase in the strength of collagen fibrils that makes it less susceptible to digestion by proteases are the most important ones. However, clinical studies are still needed to clarify whether the use of a PA-containing 10% phosphoric acid preserves resin-dentin interface after long-term function.

In summary, the application of a PA-containing 10% phosphoric acid for 30 s yielded immediate nanoleakage, resin-enamel and resin-dentin bond

strength similar to the control 35% phosphoric acid with the advantage of producing stable resin-dentin bond strength after 6 months which may be probably attributed to collagen cross-linking and the reduction of the MMP activity.

5. Conclusion

The use of a PA-containing 10% phosphoric acid did not jeopardize the bonding effectiveness on enamel and dentin and also produced stable resin-dentin bond strengths after 6-month water aging.

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Table 1. Means and standard deviations of the resin-dentin microtensile bond strength (μTBS) and nanoleakage for both groups as well as the percentage of fracture pattern of the resin-dentin sticks [adhesive and mixed/cohesive/premature failures].

Groups	μTBS (MPa) [fracture mode]		Nanoleakage (%)	
	Immediate	6 months	Immediate	6 months
35% etchant	41.5 (2.1) a [97/3/0]	21.9 (1.0) b [100/0/0]	31.7 (4.7) A	67.7 (6.5) B
2%PA-containing, 10% etchant	47.4 (2.9) a [84/16/0]	48.7 (4.8) a [82/18/0]	36.4 (5.0) A	33.8 (4.1) A

**Means identified with the same letter are statistically similar (p > 0.05).*

Table 2. Means and standard deviations of the resin-enamel microshear bond strength (μSBS) for both groups as well as the percentage of fracture pattern of the resin-dentin specimens [adhesive and mixed/cohesive/premature failures].

Groups	μSBS (MPa) [fracture mode]	
	Immediate	6 months
35% etchant	18.9 (2.5) a [87.5/7.5/5]	20.7 (0.8) a [79.1/4.3/16.6]
2%PA-containing, 10% etchant	18.6 (2.6) a [95/2.5/2.5]	17.9 (1.2) a [75/4.2/20.8]

**Means identified with the same letter are statistically similar*

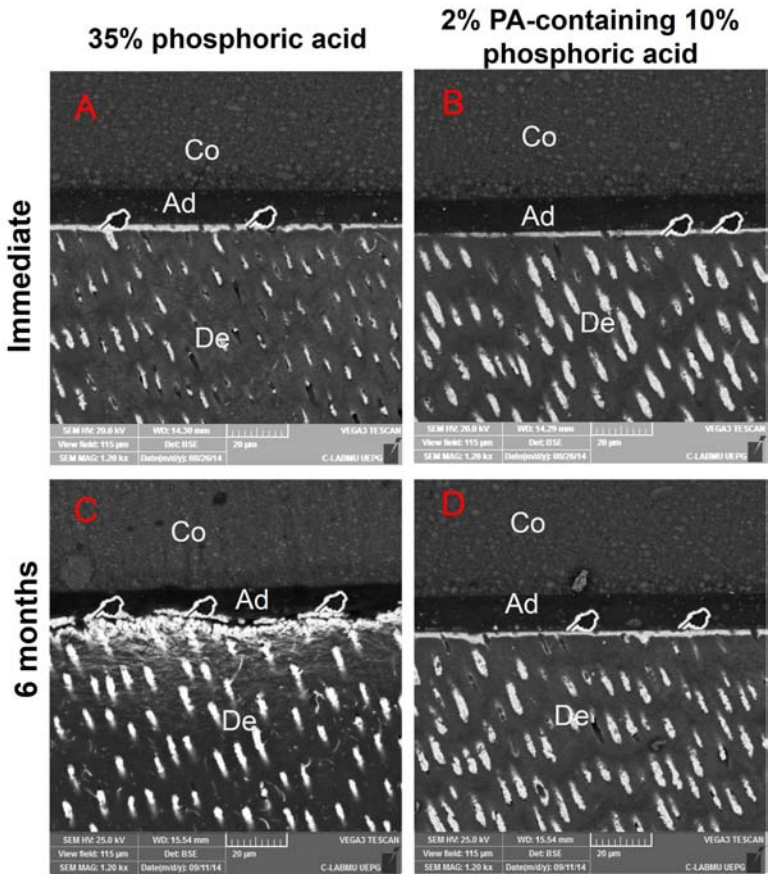

Figure 1. Representative backscattered SEM images of the resin-dentin interfaces for all experimental groups. After 6 months storage time, the amount of silver penetration (white hand)

for the 35% phosphoric acid group (C) was higher than the 2% PA-containing 10% phosphoric acid (D). Co: composite resin; Ad: adhesive layer; and De: dentin.

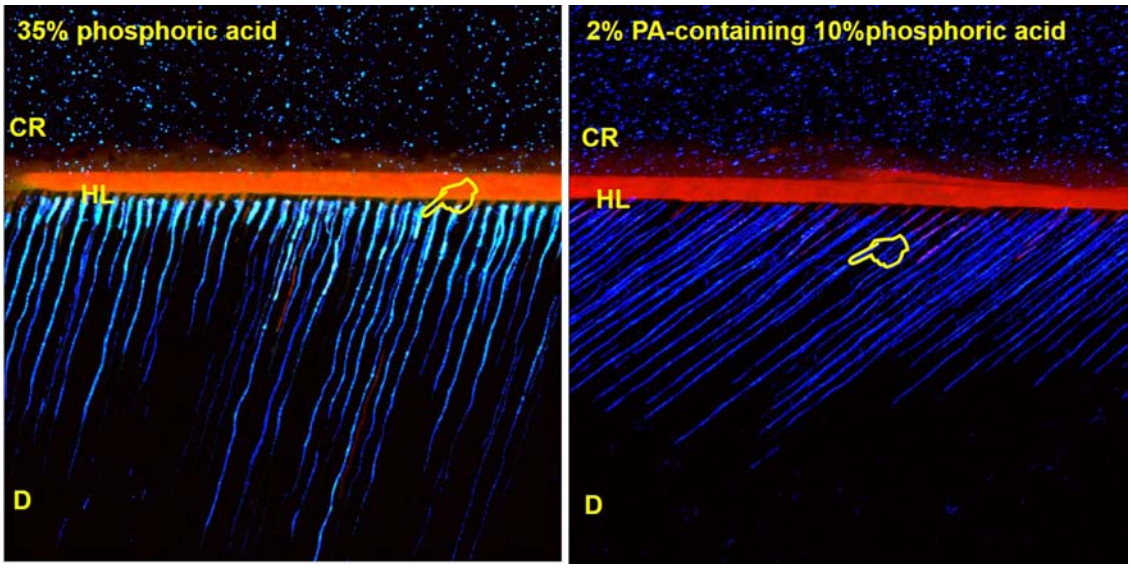

Figure 2. Confocal Laser Scanning Microscopy. The figure 35% phosphoric acid represents the control group and shows the intense activity of MMP (cyan staining) at the hybrid layer and beneath it (yellow hand). In contrast to the control group, the 2% PA-containing 10% phosphoric acid group eliminated almost entirely the MMP-activity represented by the lack of cyan staining at the hybrid layer and some activity within the dentinal tubules (yellow hand) but these are not in contact the hybrid layer. CR: composite resin; HL: hybrid layer; D: dentin.

Degradation of dentin-bonding interfaces treated by collagen cross-links agents in a cariogenic oral environment: an in situ study.

Abstract

The aim of this in situ study was to evaluate o effect of treatment using collagen cross-link agents as primer on resin–dentin bond interfaces submitted in cariogenic oral environment. Forty human teeth had two cavities (4 mm wide, 4 mm long, and 1.5 mm deep) prepared with enamel margins. These cavities were acid-etched and treated by primers contained the treatment agents (6.5% proanthocyanidin, 0.1% riboflavin-UVA activated light, 5% glutaraldehyde and distilled water as control group). After that the cavities were bonded (Adper Single Bond Plus-3M) and restored with composite resin. The teeth were sectioned and taken the restorations. One restoration for each tooth was tested immediately (IM) and another included in an intra-oral palatal device that was used for 10 adult volunteers for 14 days in cariogenic oral environment (COE). Each restoration was sectioned to obtain a slice for cross-sectional Knoop microhardness evaluation and resin–dentin bonded sticks (0.8 mm^2) for microtensile bond strength (μTBS) and nanoleakage (NL) evaluation. For differential staining technique the adhesive/dentin interfaces were sectioned and stained by Goldner's Trichrome. Data were evaluated by two-way ANOVA and Tukey's tests (α=0.05). After COE the microhardness was reduced for all experimental groups, except for glutaraldehyde, however the proanthocyanidin remained the higher means in IM and COE ($p<0.05$). The μTBS was not reduced after COE for proanthocyanidin and glutaraldehyde groups, however only the proantocyanidin did not increase the NL after COE ($p>0.05$). ***Significance:*** The in situ study model seems to be a suitable short-term methodology to investigate the degradation of the resin–dentin bonds under a more realistic condition. Under cariogenic oral environment, the proanthocyanidin produced stable resin-dentin interfaces and is worth further clinical investigation. The glutaraldehyde was effect for stabilization of resin-dentin interfaces but its use clinically is of concern due to its cytotoxicity potential.

Keywords: dentin collagen, proanthocyanidin, riboflavin, glutaraldehyde, *in situ* model, dental bonding.

1. Introduction

The hybrid structure formed during the dental bonding procedure occurs by demineralization of the surface and subsurface, followed by infiltration and subsequent polymerization of monomers around the collagen fibrils [1]. Therefore, to achieve an effective and stable bonding, the preservation of the dentin collagen is critical, since collagen represents the major organic component of the dentin matrix.

Unfortunately this is not an easy task. During the bonding procedure, the demineralized collagen fibrils are not completely infiltrated by resin monomers [2, 3] and these denuded collagen fibrils are more prone to denaturation challenges. Resin-dentin interfaces are vulnerable to challenging conditions in an oral environment. Fluctuations of pH produced by different pHs of foods and drinks as well as the that induced by bacterial acid may increase the amount of exposed organic matrix to breakdown by bacterially derived enzymes. Additionally, by host-derived enzymes [4] such as matrix metalloproteinases (MMPs) and cysteine catepsins [5-8] presented in the dentin matrix and in the gingival crevicular fluid also play a role on resin-dentin degradation.

It was demonstrated that these host-derived proteases are involved in the breakdown of the collagen matrices during the pathogenesis of dentin caries [9, 10], periodontal disease [11] and degradation of resin-dentin bonded interfaces [12]. Then, measures that enhance dentin resistance toward collagenolytic activities have great potential to improving the longevity of the dentin bonding.

The use of collagen cross-linking agents have been investigated as dentin biomodifiers, due to their ability to interact with various extracellular matrix components and induce increases in the mechanical properties of the tissue, decreases in the biodegradation rates and possibly induction of mineral nucleation [13-15]. Therefore, the incorporation of cross-linking agents into the bonding protocol appeared as a promising solution for preservation of resin-dentin bonded interfaces [16, 17].

However, most of the studies that support the benefits of cross-linking agents are performed in the laboratory where the challenging conditions of the oral environment are barely reproduced. Although randomized clinical studies are the best study design to evaluate both the performance and longevity of restorative materials, they are time demanding, costly and depend on the approval by a local Ethics Committee. Under this scenario, the conduction of in situ studies may gather important information to the field, as it resembles the challenging clinical conditions that resin-dentin interfaces are prone to better than in vitro studies. Therefore, *in situ* studies may be considered an intermediate stage between in vitro and clinical studies. Therefore, the aim of this study was investigate the degradation of resin-dentin interfaces bonded treated by different collagen cross-links agents after simulated cariogenic challenge *in situ*, using microhardness, bond strength, nanoleakage and differential staining technique.

2. Material and Methods

2.1 Ethical aspects

The study protocol was approved by the Local Ethics Committee Review Board under protocol number 314.563. Ten healthy adult volunteers (aged 21–30 years, female and male) were selected according to the following inclusion criteria: good general and oral health, normal salivary flow rate, not taken antibiotics during 2 months before the experiment, not wearing prosthesis or orthodontic devices and ability to comply with the study. All volunteers agreed to participate and signed an informed written consent.

A total of forty extracted, non-erupted human third molars were used. The teeth were collected after obtaining the patients' informed consent under a protocol approved by the Ethics Committee previously described. Teeth free from cracks or any other kinds of structural defects were selected. The teeth were sterilized by storage in 10% buffered formalin solution, pH 7, for 7 days [18] and stored in distilled water for up 2 months after extraction.

2.2 Experimental design

This in situ, split-mouth study was designed for accumulation of a plaque-like biofilm on the restorations in a high cariogenic challenge promoted by sucrose exposure. This protocol was performed for 14 days. The factors under evaluation were: (1) collagen cross-linking agents with four levels (proanthocyanidins from grape seed extract, UVA-activated riboflavin, glutaraldehyde and distilled water as control group); and (2) evaluation time – 2 levels (immediate, with the bonding performed 24 h after the bonding procedure and 14 days after degradation in a cariogenic oral environment. Then, a total of eight experimental conditions were tested.

2.3 Teeth preparation and bonding procedures

2.3.1 Microtensile bond strength (μTBS), microhardness and nanoleakage evaluation

In twenty tooth, a flat and superficial enamel surface was exposed on each tooth after wet grinding the occlusal, buccal and lingual enamel on # 180-grit SiC paper. On each tooth, two dental blocks (6 x 6 x 3 mm) were obtained from the buccal and lingual surfaces. In each dental block a standardized rectangular cavity were prepared (4 mm wide, 4 mm long, and 1.5 mm deep) with a carbide bur (# 330, KG Sorensen Ind. & Com. Ltda, Barueri, SP, Brazil), so that the axial wall was located in dentin and the thickness of enamel border ranged from 0.3 to 0.5 mm. After that, the teeth were randomized by lottery and distributed within the different levels of the cross-linking agents (n = 5 specimens per group), so that the two cavities from the same tooth could be evaluated at baseline and after 14 days in a paired design to reduce the intra-tooth variability.

All cavities were etched with 35% phosphoric acid gel for 15 s, water rinsed (30 s), air-dried (5 s) and kept slightly moist (details in **Table 1**). After that, the different collagen cross-linking primers (**Table 1**) were applied for 60 s under agitation. The RB group was irradiated with UVA-light for 2 min (Philips, Hamburg, Germany; λ = 370 nm at 3 mW/cm^2) after the priming step [17].

The cavities were incrementally filled with a composite resin (Z250, 3M ESPE, Shade A3, batch number N549511) and each increment light cured for 40 s. The light curing procedures were performed using a LED light curing unit (Radii Cal, SDI, Bayswater, Victoria, Australia) set at 1,200 mW/cm^2.

2.3.2 Differential staining technique

Additional twenty teeth were prepared and distributed within the study groups as previously described (**Table 1**), except that two consecutives and additional adhesive layers were applied instead of performing a composite resin restoration. This procedure was done to allow for the cutting procedures in the microtome. Between the additional adhesive layers we used gentle air-drying and they were light-cured using the same light-curing unit previously described.

All borders of the dental blocks were coated with an acid resistant nail varnish, except from the top surface. These blocks were stored in a moist environment at 37°C for 24 h [19]. After that, for each tooth, one of the cavities was tested immediately and the other was placed in a palatal appliance for in situ challenge.

2.4 Palatal device preparation

For each volunteer, acrylic custom-made palatal devices were made with four sites (6.5 x 6.5 x 4 mm) in which the dental slabs were positioned and fixed with wax. To allow for plaque accumulation and for protection from mechanical disturbance, a plastic mesh was fixed to the acrylic resin, leaving a 1 mm space from the surface of the specimen [19, 20]. Within each side of the palatal device, the positions of the specimens were randomly determined by lottery.

2.5 Intra-oral phase

During a 1-week lead-in period, and throughout the entire experimental phase, the volunteers brushed their teeth with a non-fluoride silica based dentifrice formulation (Fleming, Ponta Grossa, PR, Brazil) prepared for this study. To provide a cariogenic challenge in all four specimens, the volunteers were instructed to remove the device and drip 20% sucrose solution (Fleming) onto

all blocks four times a day (8 and 11 am and 3:30 and 7 pm) during 14 days [21, 22]. Five minutes later, the device was re-inserted in the mouth.

All volunteers consumed city fluoridated water (0.6-0.8 mg F/l) and foods prepared with it. No restriction was made with regard to diet of the volunteers. They were instructed to wear the intraoral devices the whole time for 14 consecutive days, removing them only for dental hygiene and during the meals. The appliances were extra-orally brushed, except the restorations, and the volunteers were asked to brush carefully over palatal area, to avoid disturbing the biofilm covering the mesh. They were asked to brush their teeth and appliance for up to 5 min. On the day 15th of the oral phase, around the 12 h after the last application of the sucrose solution, the volunteers stopped wearing the intraoral device.

The restorations were then removed, washed in tap water and longitudinally sectioned to obtaion a thin slice from the resin-dentin restoration for cross-sectional Knoop microhardness. The remaining of the cavity was used for resin-dentin μTBS and nanoleakage evaluation.

2.6 Cross-sectional microhardness

The thin restoration slab was embedded in acrylic resin, the cut surface being exposed, for subsequent flattening and polishing with 1000, 1500, 2000, and 2500-grit SiC paper and 1 and 0.25 lm diamond paste (Buehler, Lake Bluff, IL, USA) using a polish cloth. After ultrasonic cleaning, cross-sectional microhardness measurements were made in dentin with a microhardness tester (HMV-2, Shimadzu, Tokyo, Japan) equipped with a Knoop indenter (KHN) under a 15 g load for 5 s. Three lines of three indentations each were made, one lane being 20 μm distant from the restoration margin and the other, 100 and 200 μm distant. The indentations were made at the following depths from the enamel–dentin junction: 5, 15, and 25 μm [22].

2.7 Microtensile bond strength evaluation

The remaining restoration was submitted to the μTBS test. The restorations were longitudinally sectioned in both “x” and “y” directions across the bonded

interface with a diamond saw. This procedure was performed to obtain resin-dentin sticks with a cross-sectional area of approximately 1 mm^2. They were either tested immediately or after 14 days of cariogenic challenge in the in situ model.

The cross-sectional area of each stick was measured with a digital caliper (Absolute Digimatic, Mitutoyo, Tokyo, Japan) to the nearest 0.01 mm. Each bonded stick was attached to a jig for microtensile testing with cyanoacrylate resin (Super Bonder Gel, Loctite, São Paulo, Brazil) and subjected to a tensile force in a universal testing machine (Model 5565, Instron, Canton, OH, USA) at a crosshead speed of 0.5 mm/min. The failure modes were evaluated under stereomicroscopy at 100x magnification and classified as cohesive (within dentine or resin composite), adhesive (failure at resin/dentine interface, or adhesive/mixed (failure at resin/dentine interface with partial cohesive failure of the neighboring substrates).

2.8 Nanoleakage evaluation

Two resin-bonded sticks from each restoration, not used for microtensile testing, were randomly selected for nanoleakage evaluation. The sticks were immersed in 50 wt% ammoniacal silver nitrate solution in total darkness for 24 h. Thereafter, they were rinsed thoroughly in distilled water, and immersed in a photo-developing solution for 8 h under fluorescent light to reduce silver ions into metallic silver grains within voids along the bonded interface. Specimens were polished using 1000-, 1500-, 2000- and 2500-grit SiC papers and 1 and 0.25 μm diamond paste (Buehler Ltd., Lake Bluff, IL, USA) on polishing clothes. They were ultrasonically cleaned, air-dried, mounted on stubs and coated with evaporated carbon (MED 010, Balzers Union, Balzers, Liechtenstein).

The interfaces were observed in a scanning electron microscope (SEM) in the backscattered mode at 12 kV (VEGA 3 TESCAM, Shimadzu, Tokyo, Japan). Three images were taken from each specimen. The first image was obtained in the center of the stick, while the further two were obtained 0.3 mm left and 0.3 mm right from the first picture. A total of six images were obtained per tooth at each period (3 images x 2 bonded sticks). Thus, for each

experimental condition, 30 images were evaluated per group (6 images x 5 teeth) [23]. A blinded author to the experimental conditions took the pictures. The relative percentage of silver nitrate uptake within the hybrid layer was measured in all pictures using the ImageTool 3.0 software (Department of Dental Diagnostic Science, University of Texas Health Science Center, San Antonio, USA).

2.9 Differential staining technique

Resin-dentin slabs were prepared in the teeth used for the differential staining technique. These slabs were mounted on an acrylic support and 5 μm thick sections were cut from the slab using a tungsten carbide knife mounted on a Polycut S “sledge” microtome (Leica, Germany). Differential staining was accomplished with Goldner’s trichrome [24] and the sections were examined and photographed at 100× magnification with a Nikon E800 light microscope. To allow for comparison among the experimental groups, photomicrographs with exact magnification was established with the stage micrometer.

2.10 Statistical analysis

The μTBS (MPa), nanoleakage (%) and microhardness from the same experimental unit were averaged for statistical purposes at each storage time interval. The bonded sticks with premature and cohesive failures were not included in the tooth mean due to their low frequency in this experiment.

The Kolmogorov–Smirnov test was employed to assess whether the data from each test (μTBS, nanoleakage and microhardness) followed a normal distribution. Barlett’s test was performed to determine if the assumption of equal variances was valid. After observing the data normality and equality of the variances, the data from μTBS (MPa), nanoleakage (%) and microhardness (KHN) were subjected to a two-way repeated measures ANOVA (cross-linking agents and evaluation time) and Tukey’s test for pair wise comparisons ($\alpha = 0.05$).

3. Results

3.1 Microhardness

The statistical analysis revealed that the main factors cross-linking agents and evaluation time were statistically significant (**Table 2,** $p < 0.05$). Significant decreases in the microhardness values were observed after 14 days in a cariogenic oral environment for all groups, except the glutaraldehyde ($p=0.002$). The proanthocyanidin group showed the highest microhardness values in the immediate period and after 14 days of cariogenic challenge. The control group (distilled water) showed the lowest microhardness values in both evaluation periods ($p=0.002$).

3.2 Microtensile Bond Strength

Approximately 15-20 bonded sticks could be obtained per tooth including the pre-test failures. The mean cross-sectional area was 0.8 ± 0.12 mm^2 and no difference among groups was detected (data not shown; $p > 0.05$). Most of the failures were mixed (data not shown). None of the cross-linking primers affected the immediate μTBS (**Table 3**) ($p > 0.05$), however after 14 days of cariogenic challenge, only the proanthocyanidin and gluraldehyde groups showed stable μTBS ($p < 0.05$).

3.3 Nanoleakage

Nanoleakage-free interfaces were never seen. However, reduced nanoleakage expression was observed at the immediate period for all tested agents (**Table 4**). This increased significantly after 14 days in cariogenic oral environment ($p < 0.001$), except for the proanthocyanidin group.

3.4 Differential staining technique

The representative light micrographs of Goldner's Trichrome stained sections of resin-dentin interfaces for all experimental groups are shown in **Figure 1**. Due the experimental design and position of restorations, the sections were made in parallel orientation to dentinal tubules. In the Goldner-trichrome-stained sections of the resin-dentin interfaces exposed protein stained red, mineral

appeared green, protein that was partially coated with adhesive stained orange, and pure adhesive stained beige [2].

At the immediate period, the stained dentin interface for the control group (distilled water) showed wider and more intense red color zone that further intensified after 14 days of cariogenic challenge. For both riboflavin and glutaraldehyde groups, some orange areas were detected indicating collagen partially encapsulated for adhesive system at the immediate period. However after cariogenic challenge, we observed an increase in the red zones for the riboflavin group, while for the gluraraldehyde these zones remained practically unchanged. For the proanthocyanidin group, a distinct dark brown color, due the PA pigments, was observed at the immediate period, and the pattern of staining did not change after cariogenic oral environment.

4. Discussion

The present study was the first that evaluated the application of collagen cross-link agents in resin-dentin interfaces submitted to aging in oral environment. So far, it was the first study that evaluated the incorporation of cross-linking agents in an in situ model.

After 14 days of cariogenic challenge in oral environment one could observe significant reductions of the resin-dentin bond strength, microhardness as well as increase in the nanoleakage deposition for the control group. This means that the in situ model with a cariogenic challenge is a useful method to age the resin-dentin interfaces [22]. This is very relevant for further studies on dentin bonding, since this model resembles better the oral environment compared to in vitro studies and also provide clinically relevant information in a relatively short period of time [25].

The preservation of the resin-dentin interfaces from degradation was dependent on the type of cross-linking agent used. In the oral environment, the dynamic process of demineralization occurs with minerals being solubilized by organic acids produced by oral bacterial and it is balanced by the buffering potential of the saliva that allows remineralization to occur [10, 26]. The demineralization process may be followed by degradation of the exposed

organic dentin matrix that consists mainly of collagen type I. If the balance between de- and remineralization is lost, pathological factors predominate [27]. This may induce several modifications of the dentin (reduction of mineral content, increase in micro- and nano-porosities due to changes in dentin collagen structure and distribution and noncollagenous protein), synergistically contributing to reductions in physical and mechanical dentin properties [8].

The cariogenic environment promoted in our in situ model can explain the reductions of microhardness values after aging when compared to the immediate period. A higher rate of organic acids produced by oral bacteria induced more solubilization of mineral, which affected the microhardness values. The control group exhibited the lowest microhardness mean as well as intense degradation of resin-dentin interface. This lower microhardness reflects lower mineral content in the intertubular dentin [28-30] and the biochemical and structural changes of the dentin matrix produced by these pH fluctuations that compromised the mechanical properties of the tissue [31].

Although the degradation of the hybrid layer has not been completely understood yet, it seems that first stage of degradation involves the elution of the hydrophilic resins that had infiltrated the dentin by water sorption and solubility phenomena [32]. Water sorption reduces the frictional forces between the polymer chains, which decrease the mechanical properties of the polymeric material due to polymer swelling [33] However, fourteen days is a very short period of time to allow the polymeric degradation to occur. Perhaps, the demineralization of the enamel margins may have enhanced gap formation at the interface and increased the flow of fluids and bacteria through the adhesive interface. This could have led to fast undesirable consequences on the bond strength of adhesive systems.

This was also observed in the differential staining technique **(Figure 1)**. The higher intensity of red zones after 14 days of cariogenic challenge is further evidence that there is an increase in the exposure of collagen fibrils in the control group. It is known that the degradation of the organic component of the hybrid layer of resin-dentin interfaces is mediated by host-derived MMPs [9] and cysteine cathepsins [34, 35].

The further demineralization of the collagen matrix in the acid environment might have led to the cleavage of prodomains of proteases,

facilitating the functional activity of MMPs [9] in the cleavage of organic matrix components [9, 36, 37].

Curiously, the collagen cross-link agents produced highest microhardness values compared to the control group, except for the glutaraldehyde group. Indeed, the exogenous collagen cross-link agents can acts as biomodifiers, resulting in dentin with higher mechanical strength, improved stability and reduced rate of biodegradation than those of natural tissues [13, 14, 17, 38]. The glutaraldehyde is widely known as a fixation agent. Its interaction mechanism occurs through of reactions from aldehyde groups containing in the glutaraldehyde with the ε-amino groups of lysyl (or hydroxysyl) residues on collagen [39, 40]. However, this agent can promote rapid surface cross-linking of the tissue, generating a barrier that impedes its further diffusion into the tissue bulk [41, 42]. This may have affected the depth of fixation, limiting its ability to increase the strength of the bulk of the dentin substrate.

Interestingly, this agent did not exhibit reduction of the microhardness after 14 days of cariogenic challenge. Actually, this could be expected due to a strong antibacterial activity [43-45] that could influence the bacterial growth, and consequently the organic acid production and the subsequent demineralization. Then, the synergic effect between the antibacterial activity (reducing indirectly the demineralization and consequently less exposed collagen matrix) with exogenous collagen cross-link effect (increasing the stability of collagen fibrils and decreasing the MMPs potential activity), could explain the stable resin-dentin bond strength [46]. The differential staining technique revealed stability of resin-dentin interface **(Figure 1)**. However, due to the fact that glutaraldehyde has been associated with a high cytotoxic potential in previous investigations [47-49] this agent seems to be the least adequate cross-linking agent for incorporation into the bonding protocol.

Although reductions of the microhardness values were observed for the proanthocyanidin group after 14 days of cariogenic challenge, the microahardness values were the highest among all groups. This fact can be explained for the very effective chemical interaction of proanthocyanidin with collagen matrices. The proanthocyandin can interact with dentin collagen, through four distinct mechanisms. Ionic interaction, hydrogen and hydrophobic bonding interactions along with covalent bonding produced with proline-rich

proteins [13, 50] are listed as the possible methods of interaction of proanthocyanidin with the organic substrate. Therefore, its ability to establish a strong cross-linking with the proline-rich proteins, like collagen, demonstrates an extremely high affinity with this substrate [13, 51].

With the Goldner's Trichrome differential staining technique it is possible to identify the presence of protein (collagen) within the hybrid layer [2]. In the present investigation (**Figure 1**) the formation of dark brown interfaces in the proanthocyandin group make it difficult to identify the presence of exposed and encapsulated collagen fibrils. This could be due to the strong reaction between the proanthocyanidin with the collagen fibrils, and this remained bonded even after 14 days of cariogenic challenge, what could be confirme the strong cross-linking with this substrate. The great stabilization of collagen matrices due to increases of the biomechanical and biochemical properties of collagen [14, 15, 38, 52] and the interaction of proanthocyanidin with non-collagenous proteins could have increased the ability for mineral nucleation [13], which could explain the highest microhardness obtained for this agent and the stable bond strength after 14 days of cariogenic challenge. Apart from the biochemical and biomechanical modification of collagen fibrils, the inactivation of MMPs by proanthocyanidin made the resin-dentin interface more resistant toward the endogenous proteolytic activity [38, 53, 54].

The riboflavin (vitamin B2) associated with the high energy of UVA-light (365 nm) breaks down weak and intrinsic cross-links among collagen fibrils and generates free oxygen radicals. The reactive oxygen species can induce the formation of new covalent and strong cross-links within collagen [55]. This occurs through binding of the functional hydroxyl groups in riboflavin to proline and/or lysine in the collagen [56]. Therefore the increase of the dentin properties might have contributed to the increase of the microhardness in the immediate period.

In previous studies, the riboflavin showed promising results for preservation of resin-dentin bonding interfaces. This was attributed to improvements of the collagen properties and its inhibitory potential under MMPs [17, 57]. Unfortunately, this was not observed in the present study. Perhaps for this agent, depends of a physical method to promote its action mechanism (UVA-light) to induce the exogenous cross-links on dental collagen, but the UVA

irradiation is absorbed into the superficial layers, thereby protecting the underlying cells [58, 59], differently of PA that is a chemical agent with diffusion potential in depth tissues [47]. Thus, probably, likewise the CT group, after solubilized the dentin mineral contend in COE, the adjacent collagen fibrils exposed would be more prone to endogenous proteolytic activity by MMPs and cysteine cathepsins [8], contributing for the decrease of dentin properties, as well of resin-dentin bond strength.

The nanoleakage was not affect by the exogenous collagen cross-links in the immediate time. Actually, we could not expect that the application of aqueous solutions of the cross-linkers would produce nanoleakage-free interfaces since the protocols herein tested cannot alter the hydrophilic nature of the adhesive, improve water/solvent evaporation and/or produce less permeable adhesive interfaces, which are important parameters for nanoleakage [60-63].

However, interestingly, the application of PA produced resin-dentin interfaces less prone to nanoleakage after 14 days of cariogenic challenge. The proanthocyanidin induces exogenous cross-link in dentin matrix, it leads to dehydration of the collagen fibrils, with decrease in its swelling ratio and water absorption [53, 64, 65]. This can minimize the risk of collagen network collapse, resulted from air drying [13, 64] and allow better adhesive infiltration, reducing the space for silver nitrate deposition in the nanoleakage test.

5. Conclusion

The in situ model seems to be a suitable short-term methodology to investigate the degradation of the resin–dentin bonds. Under the protocol of 14 days of cariogenic oral environment, the use of proanthocyandin as primer produced stable resin-dentin interfaces and it is worth further clinical investigation. The glutaraldehyde was effective for stabilization of resin-dentin interfaces but its use clinically is of concern due to its cytotoxicity potential.

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Table 1. Description of products, composition and application mode.

Product (Company)	Composition	Application mode
Scotchbond etchant (3M ESPE, St. Paul, USA) batch number N261433	Phosphoric acid 35%, water and poly (vinyl alcohol).	Application on dentin surface. Wait 15 s. Rinsing for 10 s. Blot excess water using a cotton pellet.
Single Bond Plus (3M ESPE, St. Paul, USA) batch number N531785	Ethyl alcohol, BisGMA, silane treated silica (nanofiller), HEMA, copolymer of acrylic and itaconic acids, glycerol 1,3-dimethacrylate, water, UDMA, diphenyliodonium hexafluorophosphate, EDMAB.	After treatment according the experimental groups, application 2 consecutive coats of adhesive for 15 s with gentle agitation using a fully saturated applicator. Gently air thin for 5 s to evaporate solvent. Light-cure for 10 s.
Proanthocyanidin (PA) primer (Mega Natural Gold, Madera, USA) Batch number 05592502-01	Proanthocyanidin-Grape seed extract 6.5 % weight, deionized water.	After acid etching step, application for 60 s with gentle agitation using a fully satured applicator. Gently air-drier for 5 s and kept slightly moist the surface.
Riboflavin (RB) primer (Fisher Scientific GmbH, Schwerte, Germany) Batch number 070046	Riboflavin 0.1 % weight, deionized water.	After acid etching step, application for 60 s with gentle agitation using a fully satured applicator. After that, irradiation using UVA-light for 2 min (Philips, Hamburg, Germany; λ = 370 nm at 3 mW/cm^2). Gently air-drier for 5 s and kept slightly moist the surface.
Glutaraldehyde (GA) primer (Fisher Scientific GmbH, Schwerte, Germany) Batch number 186852	Glutaraldehyde 5 % weight, deionized water.	After acid etching step, application for 60 s with gentle agitation using a fully satured applicator. Gently air-drier for 5 s and kept slightly moist the surface.
Control Group (CT)	Distilled water	After acid etching step, application for 60 s with gentle agitation using a fully satured applicator. Gently air-drier for 5 s and kept slightly moist the surface.

BisGMA: bisphenol a diglycidyl ether dimethacrylate, *HEMA*: 2-hydroxyethyl methacrylate. *UDMA:* diurethane dimethacrylate, *EDMAB:* ethyl 4-dimethyl aminobenzoate.

Table 2. Means and standard deviations of the Knoop microhardness for all experimental groups.

Group	Immediate	14 days
Control	25.2 ± 4.3 c	16.7 ± 2.2 c
Proanthocyanidin	48.0 ± 12.2 a	33.6 ± 11.5 abc
Riboflavin	44.2 ± 6.8 ab	27.1 ± 4.0 bc
Glutaraldehyde	25.9 ± 1.9 bc	28.5 ± 4.7 bc

Means identified with the same letter are statistically similar (Tukey's test; $p > 0.05$).

Table 3. Means and standard deviations of the μTBS (MPa) for all experimental groups.

Group	Immediate	After 14 days
Control	39.2 ± 3.4 Aa	29.1 ± 3.7 Bb
Proanthocyanidin	36.4 ± 6.5 Aa	36.7 ± 7.1 Ba
Riboflavin	37.6 ± 3.7 Aa	29.7 ± 4.4 Bb
Glutaraldehyde	34.1 ± 2.5 Aa	30.5 ± 1.2 Ba

** In each column, means identified with the same uppercase letter are statistically. In each row, means identified with the same lowercase letters are statistically similar (Tukey's test; p > 0.05).*

Table 4. Means and standard deviations of nanoleakage (%) for all experimental groups.

Group	Immediate	After 14 days
Control	5.9 ± 2.4 Aa	13.8 ± 3.9 Bb
Proanthocyanidin	6.1 ± 1.9 Aa	9.3 ± 4.1 Ba
Riboflavin	6.3 ± 2.4 Aa	11.4 ± 1.4 Bb
Glutaraldehyde	6.7 ± 3.4 Aa	10.5 ± 2.0 Bb

In each column, means identified with the same uppercase letter are statistically similar. In each row, means identified with the same lowercase letters are statistically similar (Tukey's test; p > 0.05).

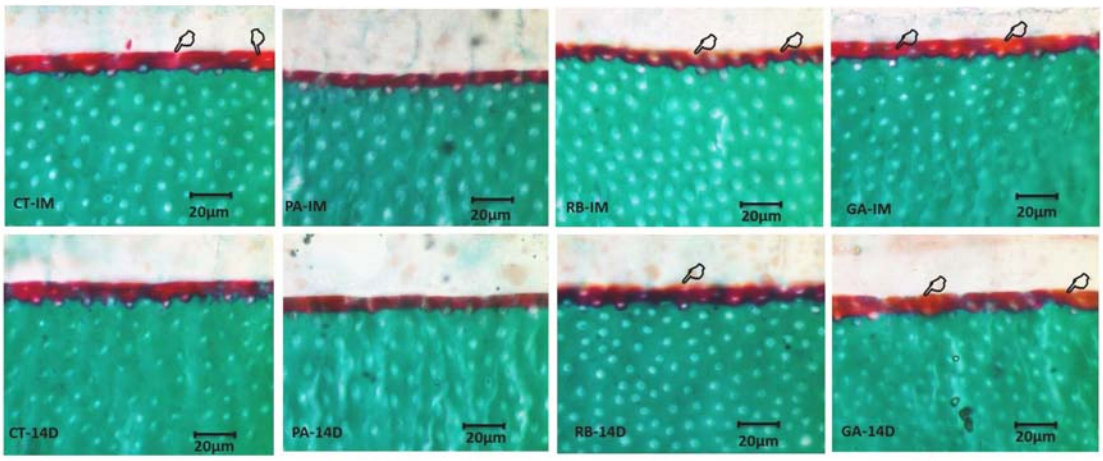

Figure 1. Representative Goldner's trichrome stains for all experimental groups. Green color represents mineralized dentin, beige color represents adhesive, red color represents exposed collagen fibrils and orange color represents partially encapsulated collagen fibrils. At the immediate (IM) period for the control group (CT), one can see small orange zones (black hands) showing the collagen encapsulated for the adhesive system, which disappeared after 14 days of cariogenic challenge (intense red zone). For riboflavin group (RB) few encapsulated fibrils zones (black hands) can be seen at the immediate time, which reduced after 14 days of cariogenic challenge. The glutaraldehyde group exhibited a hybrid layer partially encapsulated for adhesive system, which remained stable after 14 days. The proanthocyandin group exhibited a characteristic dark brown colored hybrid layer (PA reacted with the collagen) and after 14 days of cariogenic challenge it is possible to detect a strong reaction with the dentin collagen in the hybrid layer.

5. DISCUSSÃO

Foi especulado através desses experimentos que a incorporação de agentes reticuladores de colágeno no protocolo adesivo em tempo e protocolo clinicamente relevante, na tentativa de reduzir a degradação das interfaces adesivas. Essa seria a grande importância dos nossos experimentos, tendo em vista que a maioria dos estudos prévios não os fizeram sob condições clinicamente realísticas e também não avaliaram a longevidade ao longo do tempo (BEDRAN-RUSSO, PASHLEY, AGEE, et al., 2008 [16,] BEDRAN-RUSSO, PEREIRA, DUARTE, et al., 2007 [17,] MACEDO, YAMAUCHI e BEDRAN-RUSSO, 2009 [22]).

Esses estudos também não compararam em único desenho experimental a performance desses agentes (COVA, BRESCHI, NATO, et al., 2011 [25,] CHIANG, CHEN, CHUANG, et al., 2013 [50]) e tampouco utilizaram metodologias que simulem melhor o complexo ambiente oral (mimetizado neste estudo com o experimento *in situ*). Em um ambiente oral as interfaces estão propensas a condições desafiadoras: existe a presença de ácidos orgânicos produzidos pelas bactérias orais, há mudanças constantes de pH e o efeito da capacidade tampão salivar e ainda, o efeito do complexo endógeno (CHAUSSAIN-MILLER, FIORETTI, GOLDBERG, et al., 2006 [51,] MAZZONI, TJADERHANE, CHECCHI, et al., 2015 [52,] VAN STRIJP, JANSEN, DEGROOT, et al., 2003 [53]).

De acordo com nossos resultados, pudemos perceber que as características químicas e estruturais de cada agente testado foi fundamental para explicar seu mecanismo de ação e o efeito direto no processo de preservação da interface de união. O glutaraldeído é um conhecido agente fixador e antibacteriano (ANDRE, GOMES, DUQUE, et al., 2015 [54,] ERGUCU, HILLER e SCHMALZ, 2005 [55]), que possui em sua composição 5 carbonos e dois grupos aldeído no final da cadeia. Basicamente, os grupamentos amino presentes na lisina e hidroxilisina do colágeno estão aptos a interagir quimicamente com os grupos aldeído presentes no glutaraldeído (BOWES e CATER, 1968 [56]) gerando a a reticulação química por ligações covalentes, o que aumenta a resistência do colágeno (SUNG, CHANG, CHIU, et al., 1999 [24])

e diminui a degradação da interface adesiva (MACEDO, YAMAUCHI e BEDRAN-RUSSO, 2009 [22]), **Figura 11**.

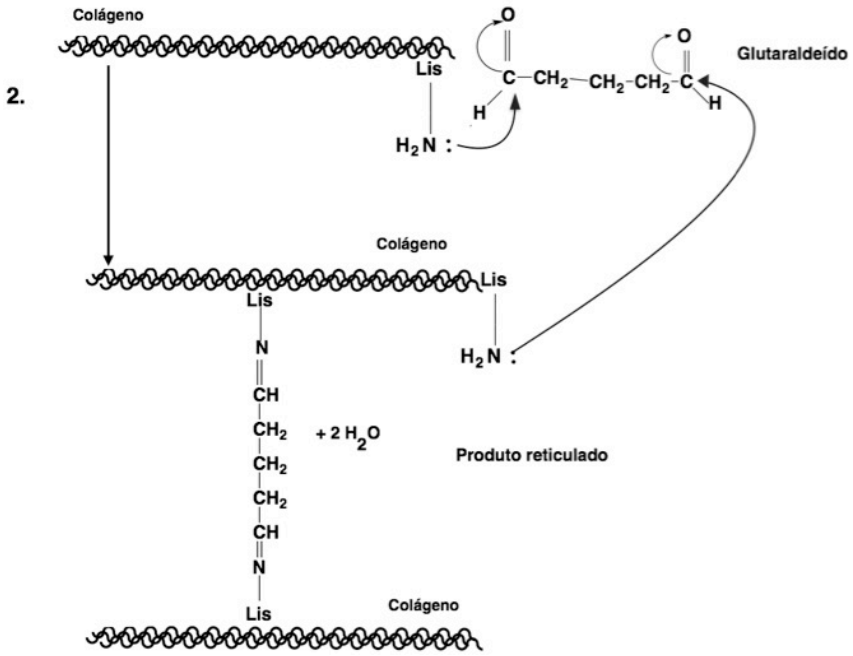

Figura 11. Representação esquemática da reticulação química entre o glutaraldeído e moléculas de colágeno.

A desvantagem desse agente é que ele promove a rápida reticulação na superfície do substrato a ser reticulado, podendo gerar uma barreira que impede a sua difusão para as camadas mais profundas (KHOR, 1997 [57], SATO, TAGUSHI, TOMOKO, et al., 1995 [58]). Isso pode explicar porque este agente não foi capaz de inibir MMPs em toda a profundidade da camada híbrida e ainda, não aumentar a microdureza no experimento *in situ*, já que esse experimento mensurou também regiões mais distantes da camada híbrida.

Outra desvantagem é o potencial de despolimerização desse agente (KHOR, 1997 [57], GENDLER, GENDLER e NIMNI, 1984 [59]), indiretamente observado no nosso estudo pelo seu maior potencial citotóxico e que está de acordo com estudos prévios na literatura (HAN, JAUREQUI, TANG, et al., 2003 [23], KHOR, 1997 [57], GENDLER, GENDLER e NIMNI, 1984 [59], VAN WACHEM, VAN LUYN, OLDE DAMINK, et al., 1994 [60], XU, LI, WANG, et al., 2013 [61]). Esta citotoxicidade está relacionada à indução de morte celular por apoptose, que

ao contrário da necrose, a apoptose produz fragmentos celulares chamados corpos apoptóticos, que as células fagocíticas são capazes de engolir e remove-las rapidamente antes do conteúdo celular espalhar-se para células vizinhas e causar danos (GOUGH, SCOTCHFORD e DOWNES, 2002 [62]). Entretanto, a citotoxicidade deve ser sempre a primeira consideração em relação ao desenvolvimento de materias biomédicos (HAN, JAUREQUI, TANG, et al., 2003 [23]), a aplicação de agente na concentração de 5%, deveria ser desaconselhada para investigações clínicas.

A riboflavina (vitamina B2) é também um conhecido agente da indústria alimentícia e da Medicina na área oftalmológica. Na Odontologia, a riboflavina age como um agente cromóforo, sendo que seus elétrons absorvem energia para formar radicais livres. Basicamente, a combinação da riboflavina com a luz UVA permite a formação de radicais livres a partir dos grupos funcionais hidroxila da riboflavina, que interagem com os aminoácidos histidina, prolina e/ou lisina do colágeno e, por mecanismo radicalar, induzem a formação de novas ligações covalentes e forte reticulação intra e intermolecular no colágeno (SIONKOWSKA, 2006 [63]) como esquematicamente demonstrado na **Figura 12**. Recentemente seu uso na Odontologia vem sendo promissor na preservação das interfaces adesivas em estudos *in vitro* (COVA, BRESCHI, NATO, et al., 2011 [25,] CHIANG, CHEN, CHUANG, et al., 2013 [50]).

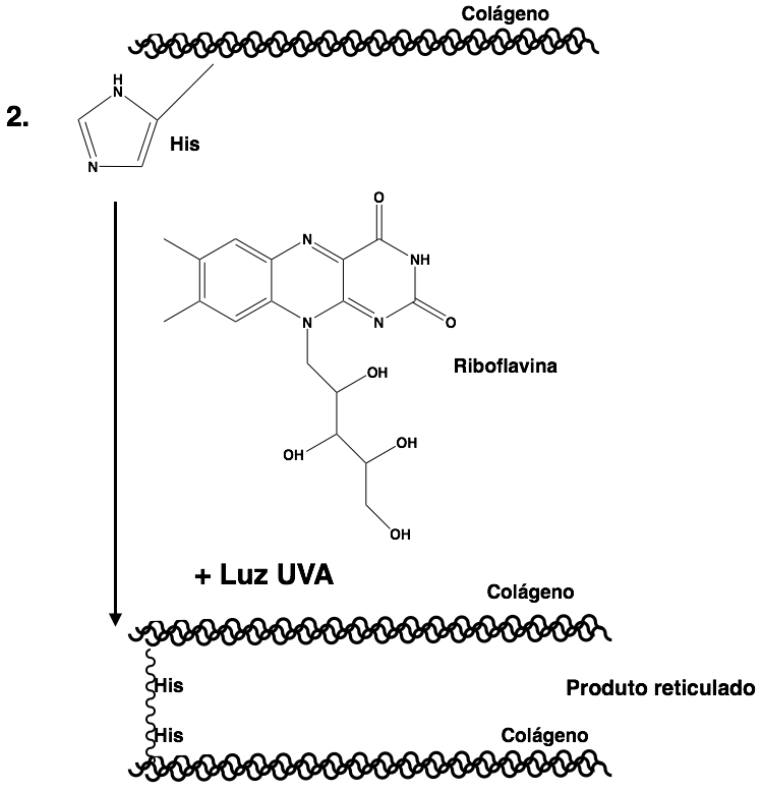

Figura 12. Representação esquemática da reticulação química entre a riboflavina e moléculas de colágeno.

A riboflavina reduziu a degradação da interface de união, bem como reduziu a atividade de MMPs em relação ao controle. Infelizmente quando submetida em ambiente oral cariogênico, ela não apresentou resultados satisfatórios, talvez pelo fato desse ambiente proporcionar uma condição mais desafiadora do que o armazenamento em água destilada. Talvez em ambiente oral, seu efeito reticulador por meio de ligações covalentes pode não ter sido suficientemente efetivo para evitar o processo de degradação.

Já a proantocianidina apresentou superior eficiência para estabilizar as interfaces adesivas. Elas são compostos polifenólicos, antioxidantes, muito usados em suplementos alimentares, indústria de cosmético e também para o desenvolvimento de bioproteses na Medicina. Em função da sua capacidade de aumentar as propriedades do colágeno, recentemente ela vem sendo investigada como agente biomodificador dentinário (BEDRAN-RUSSO, CASTELLAN, SHINOHARA, et al., 2011 [64], BEDRAN-RUSSO, PAULI, CHEN, et al., 2014 [65]).

A superior estabilização da interface adesiva pode ser devido a sua maior disponibilidade em interagir com o colágeno dental, ao contrário do glutaraldeído e da riboflavina, a proantocianidina pode interagir através de 4 mecanismos distintos: ligações covalentes, interações iônicas, pontes de hidrogênio e ainda, interações hidrofóbicas (HAN, JAUREQUI, TANG, et al., 2003 [23], HE, MU, SHI, et al., 2011 [66], MILES, AVERY, RODIN, et al., 2005 [67]). O mecanismo de interação da proantocianidina com o colágeno dental é principalmente por meio de interações iônicas e/ou pontes de hidrogênio, que podem ocorrer entre os grupos carbonila dos aminoácidos do colágeno, como a lisina, e os grupamentos hidroxila da proantocianidina (HAGERMAN e KLUCHER, 1986 [68]), **Figura 13**.

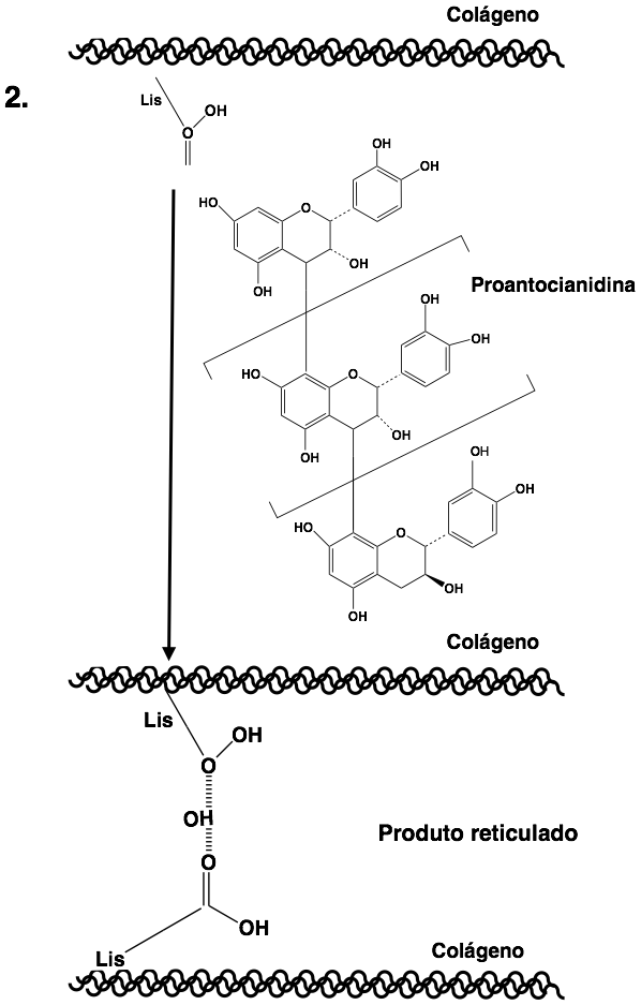

Figura 13. Representação esquemática da reticulação física por pontes de hidrogênio entre a proantocianidina e moléculas de colágeno.

Interessante também é que esse agente é capaz de exercer seu mecanismo de ação em meio ácido, já que a base de sua interação não ocorre normalmente por ligações covalentes (HAGERMAN e KLUCHER, 1986 [68], HASLAM, 1996 [69], HAGERMAN e BUTLER, 1981 [70]), ao contrário dos outros agentes reticuladores testados (MIGNEAULT, DARTIGUENAVE, BERTRAND, et al., 2004 [71]). Devido à coloração escura da proantoacianidina, e isso ainda é um fator limitante na utilização como primer, a inclusão desse agente no condicionador ácido é interessante, já que essa susbtância é lavada após a etapa de condicionamento. Em relação à sua compatibilidade biológica, ela demonstrou ser segura em nosso estudo, e isto está de acordo com a literatura (HAN, JAUREQUI, TANG, et al., 2003 [23], GENDLER, GENDLER e NIMNI, 1984 [59], YAMAKOSHI, SAITO, KATAOKA, et al., 2002 [72]).

Realmente o processo de degradação das interfaces adesivas envolve uma cascata de eventos, que até o momento ainda não podem ser completamente compreendidos. Contudo, sabemos que esses eventos dependem da degradação polimérica (e isso é inerente da natureza dos sistemas adesivos) seguida da degradação da matriz colágena (REIS, CARRILHO, BRESCHI, et al., 2013 [73]). Por mais que o uso dos agentes reticuladores não altere na natureza dos sistemas adesivos, a sua inclusão parece interessante tanto pelo aumento das propriedades do colágeno como também pela forma com que eles reduzem a atividade colagenolítica por proteases, o que minimiza e retarda a degradação destas interfaces ao longo do tempo.

Futuros estudos clínicos devem ser conduzidos para averiguar o potencial destes agentes em aumentar a longevidade de restaurações adesivas.

6. CONCLUSÕES

O uso de agentes reticuladores de colágeno em tempo clinicamente relevante mostrou-se efetivo na redução da degradação das interfaces adesivas e da atividade de MMPs, com variação de grau entre os diferentes agentes, sendo a proantocianidina o agente que, no somatório das propriedades, mostrou-se mais promissor. O glutaraldeído apresentou potencial citotóxico e deve ser evitado para uso clínico.

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ANEXO A

TERMO DE APROVAÇÃO DA COMISSÃO DE ÉTICA EM PESQUISA

UNIVERSIDADE ESTADUAL DE PONTA GROSSA - UEPG

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Efeito de agentes reticuladores de colágeno na adesão à dentina com sistemas adesivos convencionais simplificados

Pesquisador: Alessandra Reis

Área Temática:

Versão: 1

CAAE: 15478113.0.0000.0105

Instituição Proponente: Universidade Estadual de Ponta Grossa

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 314.563

Data da Relatoria: 27/06/2013

Apresentação do Projeto:

Efeito de agentes reticuladores de colágeno na adesão à dentina com sistemas adesivos convencionais simplificados

Objetivo da Pesquisa:

Avaliar a longevidade da adesão à dentina, o potencial citotóxico, as interações químicas com o colágeno e o potencial inibitório de proteases endógenas ao associar o uso de agentes reticuladores de colágeno na adesão à dentina.

Avaliação dos Riscos e Benefícios:

Riscos:

Como potencial risco desta pesquisa pode citar:- Possível desmineralização dos dentes pela aplicação de solução de sacarose (Os voluntários serão
instruídos e monitorados durante toda a pesquisa. Procederão à sua higienização de maneira habitual e este procedimento da pesquisa durará no
máximo quatorze dias e após este período os voluntários não utilizarão mais o dispositivo intra-oral);- Dispositivo intra-oral causar incômodo ao
voluntário (Todos os voluntários da pesquisa receberão instruções de como proceder com sua utilização; caso o desconforto persista, os voluntários

Endereço: Av. Gen. Carlos Cavalcanti, nº 4748 bl M sala 12
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ANEXO B

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)

UNIVERSIDADE ESTADUAL DE PONTA GROSSA

PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMISSÃO DE ÉTICA EM PESQUISA - COEP

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (T.C.L.E)
PESQUISAS COM SERES HUMANOS

Eu,_____portador(a) do RG nº _____, participo de livre e espontânea vontade, da pesquisa intitulada "***Efeito de agentes reticuladores de colágeno na adesão à dentina com sistemas adesivos convencionais simplificados***", sob a responsabilidade dos cirurgiões-dentistas Viviane Hass - doutoranda em Odontologia pela Universidade Estadual de Ponta Grossa, Douglas Francisco e Thalita Paris de Matos - graduandos em Odontologia desta instituição e o Dr. Alessandro Dourado Loguercio, tendo como orientadora a Dra. Alessandra Reis, professores do Departamento de Odontologia desta instituição.

A degradação da união das restaurações em resina composta e o dente é o principal responsável por problemas como infiltração, recorrência de cárie, manchamento das margens e perda das restaurações, e até agora, ainda representa um grande desafio para a Odontologia. Assim, o objetivo dessa pesquisa é investigar o efeito de diferentes agentes reticuladores de colágeno dentário na preservação dessa união em um modelo *in situ*.

O trabalho será constituído de exame clínico dos dentes; confecção de dispositivos palatinos e palestra de 5 minutos com instruções quanto ao uso destes dispositivos. Durante toda a pesquisa, os voluntários serão acompanhados pelo pesquisador responsável para avaliação de qualquer efeito adverso (Caso isso venha ocorrer, os voluntários receberão tratamento adequado). Como potenciais riscos desta pesquisa podemos citar:

- Possível desmineralização dos dentes pela aplicação de solução de sacarose. Os voluntários serão instruídos e monitorados durante toda a pesquisa. Procederão à sua higienização de maneira habitual e este procedimento da pesquisa durará no máximo quatorze dias e após este período os voluntários não utilizarão mais o dispositivo intra-oral;
- Dispositivo intra-oral causar incômodo ao voluntário. Os voluntários da pesquisa receberão instruções de como proceder com sua utilização; caso exista o desconforto poderão suspender o seu uso e abandonar a pesquisa a qualquer momento, sem que isto cause danos ou prejuízos ao voluntário.

A pesquisa contribuirá para um melhor entendimento sobre o efeito dos agentes reticuladores de colágeno sobre a degradação da interface de união dente/restauração, consequentemente, preservação de estrutura dental em detrimento da necessidade de troca de restaurações em um modelo *in situ*. Os benefícios para os voluntários pesquisados são:

- Os voluntários serão acompanhados e receberão esclarecimentos do pesquisador responsável sobre eventuais dúvidas durante toda a pesquisa;

- Receberão uma profilaxia prévia a colocação do dispositivo intra-oral e outra profilaxia após a retirada deste dispositivo (este procedimento será realizado pelos demais pesquisadores juntamente com o pesquisador responsável);

- Os voluntários receberão tratamento odontológico quando necessário durante todo o período da pesquisa.

Garanto cumprir todos os itens acima mencionados:

Assinatura_____

Profa. Dra Alessandra Reis

Pesquisadora Responsável

Certifico que, tendo lido as informações acima e sendo suficientemente esclarecido (a) de todos os itens pelo pesquisador responsável Alessandra Reis, estou plenamente de acordo com a realização do experimento. Assim, eu concordo em participar como voluntário do trabalho de pesquisa, exposto acima.

Ponta Grossa, ____ de _____ de 2013.

1ª via da instituição, 2ª via do sujeito da pesquisa

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