UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

MARINA CICCONE GIACOMINI

Interaction between dentin bonding agents and dentin: from in situ proteolytic activity to mechanical test

A interação de agentes adesivos à dentina: da ação proteolítica in situ aos testes mecânicos de adesão

BAURU
2019

MARINA CICCONE GIACOMINI

Interaction between dentin bonding agents and dentin: from in situ proteolytic activity to mechanical test

A interação de agentes adesivos à dentina: da ação proteolítica in situ aos testes mecânicos de adesão

Tese apresentada a Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Doutor em Ciências no Programa de Ciências Odontológicas Aplicadas, na área de concentração Dentística.

Orientador: Profª. Drª. Linda Wang

BAURU

2019

Giacomini Ciccone, Marina
Interaction between dentin bonding agents and dentin: from in situ proteolytic activity to mechanical test / Marina Giacomini Ciccone - Bauru, 2019.
88p. : il. ; 31cm.

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

Orientador: Profª. Drª. Linda Wang

Comitê de Ética da FOB-USP
Protocolo nº:
59799916.5.0000.5417
Data: 06/10/2016

FOLHA DE APROVAÇÃO

DEDICATÓRIA

À **Deus**, por estar presente e sempre guiar meus passos e decisões ao decorrer da vida.

Aos meus pais, **Graziela e José Eduardo**, pelo constante incentivo e apoio em todas as decisões que tomei sempre levando os valores de casa para me tornar uma profissional de sucesso.

Ao meu marido **Felipe**, por estar ao meu lado, me ajudando, me incentivando, nos todos os momentos.

À minha família, a minha **tia Tania, ao meu avô Adalto** (in memoriam), a **minha avó Neusa**, ao **meu avô Gerson** (in memoriam) e a **minha avó Vera** (in memoriam) por serem os anjos da guarda, tornando essa caminhada mais agradável possível.

À **Profª. Drª. Linda Wang**, que me acolheu e me lapidou desde a graduação com sua ética impar e generosidade. Acompanhou e auxiliou no meu crescimento pessoal e profissional, se tornando uma pessoa especial e inesquecível, a qual levarei para o resto da vida.

AGRADECIMENTOS

À **Deus,** com saúde e fé, vivenciar e aprender diante de todas essas experiências que me foram proporcionadas.

Aos meus pais, **Graziela e José Eduardo**, por desde sempre me incentivar e me amparar durante os tropeços da vida, por acreditarem no meu potencial e serem meu porto seguro.

Ao meu marido **Felipe,** obrigada pela compreensão, pelo apoio e por ser acima de tudo meu amigo e companheiro de vida.

À minha **tia Tânia** pelos conselhos e incentivo.

À minha **avó Neusa** por ser uma segunda mãe e por fazer meus dias mais felizes e doces.

Às Tias **Marisa, Maria Nair e Renneé** pelo incentivo, pelas risadas e por sempre estarem presentes.

À minha segunda família, **Cibele, Luiz Claudemir e Laura,** por estarem presentes, tornando a caminhada mais alegre e acolhedora.

À família **Elias e Belei,** sou grata por fazer parte dessa família há 11 anos. Obrigada sempre pelo incentivo e risadas.

À minha amiga **Izabella Rosso,** por estar sempre presente desde a graduação.

À **Maria Angélica,** obrigada por ser a calma e o equilíbrio diante de tantas situações e por seu jeito mãe de ser. E agradeço a família **Silvério Agulhari**, por participar e conviver com uma criança tão especial como o **Lucas,** que fez meus dias mais alegres.

À **Juliana,** obrigada por sua amizade, sinceridade e por ser minha irmã caçula.

À **Tamires,** obrigada pela amizade, conversas e pelas risadas, independente de distância física.

À **Giovanna,** obrigada por ser essa pessoa especial e determinada.

À **Genine** e **Alyssa**, obrigada pelas conversas, amizade e por compartilharmos de tantas coisas em comum.

Ao grupo de pesquisa **Letícia, Rafael Simões e Marília Velo** obrigada pela oportunidade de trabalharmos e aprendermos juntos.

À **minha turma de doutorado,** obrigada pela convivência e aprendizado compartilhado.

À **turma de mestrado de 2017,** obrigada por me acolherem e me receberem nessa turma tão querida.

À **turma de doutorado de 2016,** obrigada pela convivência e companheirismo.

Às **turmas da graduação,** obrigada pela paciência e oportunidade de aprender com vocês.

À **Faculdade de Odontologia de Bauru – Universidade de São Paulo** por agradecimento por todo ambiente inspirador e pela oportunidade de aprender e crescer como pessoa e profissional.

Aos professores **Prof. Dr. Adilson Yoshio Furuse, Profa. Dra. Ana Flávia Sanches Borges, Prof. Dr. Aquira Ishikiriama, Profa. Dra. Diana Gabriela Soares dos Passos**, **Prof. Dr. José Mondelli**, **Profª. Dr.ª Juliana Fraga Soares Bombonatti**, **Profª. Dr.ª Linda Wang**, **Profª. Drª. Maria Fidela de Lima Navarro**, **Profª. Drª. Maria Teresa Atta**, **Prof. Dr. Rafael Francisco Lia Mondelli**, **Prof. Dr. Paulo Afonso Silveira Francisconi** e **Prof. Dr. Sérgio Kiyoshi Ishikiriama** obrigada pela orientação incansável, o empenho e a confiança

Aos professores da disciplina de bioquímica **Profa. Dra. Ana Carolina Magalhães, Profa. Dra. Marília Afonso Rabelo Buzalaf e Prof. Dr. Rodrigo Cardoso de Oliveira** pela oportunidade e apoio em trabalhar no laboratório do departamento.

Ao **Prof. Dr. Heitor Marques Honório,** pela disponibilidade e colaboração.

À **Profa. Dr. Cristina Vidal e Profa. Dra. Marcela Carrilho,** por ser uma pessoa prestativa e sempre estar disposta à ajudar.

Aos funcionários **Alcides**, **Audria, Charlene, Elízio, Natália, Nelson e Rita** por toda a ajuda, amparo e cafés ao longo desses anos.

Aos funcionários do laboratório de bioquímica, **Aline, Larissa e Thelma** pelo auxílio, receptividade e um sorriso em meio aos experimentos.

Aos funcionários da pós graduação, **Letícia, Leila, Fátima, Margareth e Hebe,** obrigada pelo auxílio em diversas situações.

AGRADECIMENTOS ESPECIAIS

À minha querida orientadora, **Linda Wang**, serei eternamente grata por tudo o que fez e faz por mim desde 2009. Acredito que na vida tudo acontece por um propósito e ao longo dessa caminhada pude compreender o motivo para eu entrado na FOB em 2009. Te agradeço por cada oportunidade, por cada elogio e sorrisos, mas principalmente por todas as críticas construtivas que só realmente uma mãe pode fazer à um filho com o objetivo sempre de faze-lo crescer. Tenho muito orgulho em ser sua aluna e hoje me vejo, muitas vezes, fazendo as coisas do jeito que você faria e fico muito feliz por ter uma professora tão ética e justa como você. Além de orientadora, ganhei uma amiga e uma mãe-científica. Saiba que levarei seus ensinamentos para sempre e estarei sempre aqui para retribuir tudo o que você já fez e faz por mim.

AGRADECIMENTOS ESPECIAIS

À minha co-orientadora **Polliana Scaffa**, que desde sempre chamo de Polli. Obrigada por toda a ajuda, por toda a positividade e persistência. Sem você este trabalho não teria acontecido. Você é uma pessoa especial, de uma simplicidade impar e uma capacidade excepcional. Desejo que você seja muito feliz! Te admiro muito e estarei sempre aqui se precisar de qualquer coisa.

AGRADECIMENTOS INSTITUCIONAIS

Ao Prof. Dr. **Vahan Agopyan** digníssimo reitor da Universidade de São Paulo.

Ao Prof. Dr. **Carlos Ferreira dos Santos** , diretor da Faculdade de Odontologia de Bauru – Universidade de São Paulo .

À Prof. Dra. Profa. **Izabel Regina Fischer Rubira de Bullen**, presidente da Comissão de Pós Graduação da Faculdade de Odontologia de Bauru – Universidade de São Paulo.

Ao **Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)** pelo suporte financeiro fundamento pelos processos: (163402/2015-9 e 142065/2018-8).

O presente trabalho foi realizado com apoio da **Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES)** - Código de Financiamento 001.

“Você tem um brilho intelectual

E energia nas suas veias

Você chegará mais alto do que eles jamais foram”

Chris Martin

RESUMO

A interação de agentes adesivos à dentina: Da ação proteolítica in situ aos testes mecânicos de adesão

A utilização dos versáteis sistemas adesivos universais tem por objetivo melhorar à adesão à dentina e simplificar o procedimento adesivo. A associação entre monômeros funcionais como o 10-Metacriloiloxidecil dihidrogênio fosfato (10-MDP) e inibidores proteolíticos tende a ser uma estratégia promissora para melhorar a longevidade da camada hibrida. Desta forma, este trabalho tem por objetivo avaliar o desempenho do Adper Single Bond Universal (SU) combinado com inibidores proteolíticos, especialmente a clorexidina (CHX), em diferentes substratos dentinários ao longo do tempo. No artigo 1, a interação entre CHX e E-64 com SU (modo convencional) foi investigada em dentina sadia, artificialmente cariada e erodida em 18 meses de envelhecimento. Foi encontrado que o substrato cariado foi o mais afetado e nenhum dos inibidores testados foram capazes de manter a estabilidade ao longo de 18 meses. Além do mais, observou-se que a CHX impactou de forma negativa independente do substrato avaliado, levando a hipótese de uma possível competição entre ela e o 10-MDP, visto que ambos envolvem íons Ca em seus mecanismos de ação. Para uma melhor compreensão, no artigo 2 foi proposto testes para avaliação de atividade proteolítica e resistência de união à dentina, com foco no desempenho do SU nos dois modos (convencional e autocondicionantes) comparado a um convencional de 2 passos livre de MDP (Adper Single Bond 2), associados com CHX em 6 meses de envelhecimento. Foi observado que a atividade proteolítica foi evidente em todos os sistemas adesivos (SA). O SU no modo autocondicionantes apresentou os maiores valores de resistência de união. A CHX foi capaz de reduzir a atividade proteolítica, independente dos SA mesmo em 6 meses de envelhecimento. Além disso, a CHX não afetou negativamente as propriedades mecânicas. A CHX é capaz de reduzir a atividade proteolítica, no entanto não perdura até 18 meses.

Palavras-Chaves: Adesivos Dentinários; Camada Híbrida; Dentina; Inibidores de Proteases.

ABSTRACT

Interaction between dentin bonding agents and dentin: from in situ proteolytic activity to mechanical test

The use of the versatile universal adhesive systems aims to improve adhesion to dentin and simplify the bonding procedure. The association between functional monomer as 10-methacryloyloxydecyl-dihydrogen phosphate (10-MDP) and proteolytic inhibitors seems to be a promising strategy to improve the longevity of hybrid layer. Therefore, this study aimed to evaluate the performance of Adper Single Bond Universal (SU) combined with proteolytic inhibitors, especially chlorhexidine (CHX), in different dentin substrates overtime. In article 1, the interaction between CHX and E-64 with SU (in etch-and-rinse mode) was investigated in sound, artificial carious and eroded dentin over 18 months aging. It was found that carious substrate was the most affected and none of the inhibitors tested were able to maintain stability over 18 months. Furthermore, it was observed that CHX negatively impacted regardless of the substrate, leading to the hypothesis of possible competition between CHX and 10-MDP, since both involve calcium ions in their mechanism of action. For a better comprehension, article 2 purposed the evaluation of proteolytic activity and bonding to dentin tests, focusing on the performance of SU in both modes (etch-and-rinse and self-etching) compared to a conventional MDP-free 2-step adhesive system (Adper Single Bond 2), associated with CHX over 6 months aging. It was observed that proteolytic activity was evidenced when all dentin bonding systems (DBS) was used. SU in self-etching mode showed the highest values of microtensile bond strength. CHX was able to reduce proteolytic activity, regardless of DBS even in 6 months aging. Moreover, CHX did not affect negatively mechanical properties. In conclusion, CHX is capable of reduce proteolytic activity, however it did not provide long lasting up to 18 months.

Key words: Dentin. Dentin Bonding Agents. Hybrid Layer. Proteases Inhibitor.

LIST OF ILLUSTRATIONS

- ARTICLE 1
    - Figures

- ARTICLE 2
    - Figures

LIST OF TABLES

- ARTICLE 1

- ARTICLE 2

LIST OF ABREVIATIONS AND ACRONYMS

MMP	Matrix Metalloproteinases
CCs	Cysteine Cathepsins
CAD	Carious affected dentin
DBS	Dentin bonding system
ER	Etch and rinse dentin bonding system
SE	Self-etching dentin bonding system
MDP	10-methacryloyloxy-decyl-dihydrogen phosphate
W	Water
CHX	Chlorhexidine
E64	trans-Epoxysuccinyl-L-Leucylamido-(4-guanidino) butane
ACD	Artificial caries dentin
DE	Demineralizing solution
RE	Remineralizing solution
ERO	Artificial erosion dentin
µM	Micro Molar
mm^2	Square millimetre
mm	Millimetre
mm/min	Millimetre per minute
mW/cm^2	MiliWatt per square centimetre
µm	micrometre
au	Arbitrary Unit
°C	Celsius

N	Newton
MDI	Minimal Dentistry Intervention
μTBS	Microtensile bond strength
MPa	Mega Pascal unit
Ca	Calcium
ANOVA	Analysis of variance
BS	Bond Strength
SU	Adper Single Bond Universal
SB	Adper Single Bond

SUMMARY

1 Introduction

1 INTRODUCTION

Since its introduction, the adhesive concept has driven a revolution on Dentistry, from minimal interventions to rehabilitation procedures. Regardless of them, the basis for a successful performance relies on the establishment of an effective resin-dentin interface, based mostly on the formation of a hybrid layer. (Nakabayashi et al., 1991; Tjaderhane et al., 2013).

Dentin adhesion relies on in situ tissue engineering, which is designed to create a resin-enveloped collagen scaffold that, ideally, will remain in place for decades (Pashley et al., 2011; Frassetto et al., 2016). Despite of all efforts to create a stable and long-lasting hybrid layer, it still persists on a limitation (Breschi et al., 2018). Since dentin is a more complex tissue, bonding is feasible but more susceptible to be degraded overtime, especially due to the exposition of denuded collagen fibril and the hydrophilic characteristic of the resin monomers. It negatively favors for the activation and degradation of organic matrix by both hydrolytic and enzymatic actions (Pashley et al., 2011; Tjaderhane et al., 2013; Breschi et al., 2018).

Dentin is mineralized and hydrated leading to, a complex and dynamic substrate (Pashley et al., 1989; Carvalho et al., 2012). Its morphology and physiology affect directly the bonding procedure (Carvalho et al., 2012). Besides that, endogenous proteases, such as matrix metalloproteinases (MMPs) and cysteine cathepsins (CCs) are present within dentin matrix and in saliva (Tjaderhane et al., 1998; Mazzoni et al., 2006). In dental tissue, these proteases are present in inactive pro-forms, interlocking and protected by dentin minerals (Mazzoni et al., 2006; Nishitani et al., 2006). They are activated by an acid event; the low-pH environment leads to minerals loss unprotecting the MMPs and CCs, provoking their exposition and activation (Tjaderhane et al., 1998; Mazzoni et al., 2006; Scaffa et al., 2012; Vidal et al., 2014).

Dentin is severally challenged by demineralized events, such as dental caries and dental erosion. New concepts in cariology provoked modification into clinical protocols, involving the concept of Minimal Dentistry Intervention (MDI) (Innes et al., 2016; Schwendicke et al., 2016; Isolan et al., 2018). This technique consists on exclusive removal of the infected dentin layer, maintaining the carious affected dentin

(CAD) layer (Innes et al., 2016; Schwendicke et al., 2016), thus the bonding procured will be performed above CAD.

On the other hand, dental erosion consists on a non-bacterial chemical process of dental structure dissolution with two remarkable phases due to the interaction between dental structure and an acid substance (Magalhães et al., 2009; Huysmans et al., 2011). The first phase corresponds to the softening of surface due to mineral loss followed by the second phase based on the mechanical removal of this softening layer (Magalhães et al., 2009). In consequence, it leads to a partially demineralized dentin the exposure of denuded collagen fibrill (Magalhães et al., 2009; Huysmans et al., 2011; Francisconi-dos-Rios et al., 2015a; Machado et al., 2018).

Furthermore, the MMPs are more expressive in human dentin than CCs (Scaffa et al., 2017) and play the main role in dental erosion (Buzalaf et al., 2015; Zarella et al., 2015). In carious dentin, a significant increase in MMP and CCs levels and activities occurs in caries-infected and -affected dentine and in dentinal fluid (Buzalaf et al., 2015; Tjaderhane et al., 2015). When restorations of both carious and erosion lesion are required, the first choice of material mostly is based on bonding procedure. During it, an acid-etching step is performed regardless of the dentin bonding system (DBS) used.

On the adhesive surface, the MMPs and CCs roles are synergistically through the hydrolytic degradation of collagen fibrils (Mazzoni et al., 2006; Nishitani et al., 2006; Tjaderhane et al., 2013). Clinically, partially demineralized dentin is formed using etch-and-rinse (ER) and self-etching (SE) adhesives (Wang and Spencer, 2002) and evidences pointed that collagenolytic and gelatinolytic activities occur regardless the etching bonding strategy, either ER or SE (Mazzoni et al., 2006; Nishitani et al., 2006; Mazzoni et al., 2013). For ER, there are discrepancies between gradient of resin monomer diffusion within the acid-etched dentin and infiltrated zones, a large penetration of resin monomers on the top of hybrid layer, whereas a low gradient was founded at its bottom. The non-infiltrated zone contains denuded collagen fibrils, which turns the interface more fragile (Wang and Spencer, 2002; Hashimoto et al., 2002). On the other hand, as self-etching adhesives present the ability to etch and prime simultaneously, nanoleakage within hybrid layers was observed is less observed in consequence (Pashley et al., 2004; Van Merbeek et al., 2011).

The association between a proteolytic inhibitor during the bonding procedure is an interesting strategy to reduce or paralyze the proteolytic activity (Carrilho et al., 2007; Carrilho et al., 2010) Chlorhexidine is a well-known proteolytic inhibitor, mainly due to its non-specific action, able to reduce activity of both MMPs (-2, -8 and -9) and CCs (-B, -K and -L), even at low concentrations (Gendron et al. 1999). Furthermore, it presents practical use, substantivity in dentin and inexpensive cost (Gerdron et al., 1999; Carrilho et al., 2010). In dentistry, digluconate of chlorhexidine 2% is the useful form and shows an interesting strategy to preserve bonding durability (Hebling et al., 2005; Carrilho et al., 2010; Francisconi-dos-Rios et al., 2015a). Evaluating the positive correlation between MMPs and CCs (Scaffa et al., 2012; Tjaderhane et al., 2013; Vidal et al., 2014), the use of a specific inhibitor for CCs is pertinent. E64 ((trans-Epoxysuccinyl-L-Leucylamido-(4-guanidino) butane) is a specific inhibitor irreversible for CCs, soluble in water and 5μM concentration was adopted in dentistry (Tersariol et al., 2010; Nascimento et al., 2011). When proteases inhibitors are associated to bonding procedure, they are applied after acid etching and before bonding application.

In relation to DBS development, the main target is to form a stable adhesive interface, promoting strength retentive, marginal seal and clinical durability (Tjaderhane et al., 2013; Breschi et al., 2018). A new DBS category, denominated “universal or multi-mode” intended to be more versatile, simplifying the bonding procure and minimizing difficulties (Montagner et al., 2015; Isolan et al., 2018). Universal DBSs present a complex composition and different between them (Rosa et al., 2015), but all are based on acidic functional monomers, especially 10-MDP (10-methacryloyloxydecyl-dihydrogen phosphate). 10-MDP is a phosphate and bifunctional monomer, able to bind chemically to dental structure through calcium ions present in hydroxyapatite (Yoshida et al., 2004; Hanabusa et al., 2012; Montagner et al., 2015). Some studies demonstrated a negative interaction between 10-MDP and CHX, seeing that both depends on calcium ions to act effectively (Di Hipólito et al., 2012; Francisconi-dos-Rios et al., 2015a; Francisconi-dos-Rios et al., 2015b; Giacomini et al., 2017).

Up to now, there is no consensus about the most appropraite technique (ER or SE) (Hanabusa et al., 2012; Marchesi et al., 2014; Wagner et al., 2014; Rosa et al., 2015) and if the use of protease inhibitor is actually a positive association (Göstemeyer et al., 2016). According to the exposed above, the aim of this study was

to evaluate and elucidate the performance overtime of a MDP-based adhesive system before different dentin substrates, associated with different proteolytic inhibitor by *in situ* and bond strength on dentin tests.

2 Articles

2 ARTICLES

The articles below were written according to the Operative Dentistry and Journal of Dentistry instructions and guideline for article submission, respectively.

2.1 Article 1 - MDP-based adhesive and protease inhibitors association: 18-month exploratory study

2.2 Article 2 - Profile of a multi-mode MDP-based adhesive system: *in situ* zymography and bond strength on dentin performance

2.1 Article 1

Title Page

MDP-based Adhesive and Protease Inhibitor Association: 18-month Exploratory Study

Running title: Performance of universal bonding system with proteolytic inhibitors over time.

Marina Ciccone Giacomini[1]
giacominimarina@hotmail.com
Alameda Octávio Pinheiro Brisolla, 9-75, Bauru, SP, Brazil/17012-901
DDS, MS, PhD student
[1]Department of Operative Dentistry, Endodontics and Dental Materials, Bauru School of Dentistry, University of São Paulo.

Polliana Mendes Candia Scaffa[2]
polli_scaffa@yahoo.com.br
Alameda Octávio Pinheiro Brisolla, 9-75, Bauru, SP, Brazil/Zip Code:17012-901
DDS, MS, PhD, PostDoc
[2]Department of Biological Science, Bauru School of Dentistry, University of São Paulo.

Rafael Simões Gonçalves[1]
rafael895@hotmail.com
Alameda Octávio Pinheiro Brisolla, 9-75, Bauru, SP, Brazil/Zip Code:17012-901
DDS, MS, PhD
[1]Department of Operative Dentistry, Endodontics and Dental Materials, Bauru School of Dentistry, University of São Paulo.

Juliana Carvalho Jacomine[1]
juliana.jacomine@usp.br
Alameda Octávio Pinheiro Brisolla, 9-75, Bauru, SP, Brazil/Zip Code:17012-901
DDS, MS Student

[1]Department of Operative Dentistry, Endodontics and Dental Materials, Bauru School of Dentistry, University of São Paulo

Giovanna Speranza Zabeu[1]
giovanna.zabeu@gmail.com
Alameda Octávio Pinheiro Brisolla, 9-75, Bauru, SP, Brazil/ Zip Code: 17012-901
DDS, MS, PhD student
[1]Department of Operative Dentistry, Endodontics and Dental Materials, Bauru School of Dentistry, University of São Paulo.

Marcela Rocha de Oliveira Carrilho[3]
marcelacarrilho@gmail.com
555 31st St. Downers Grove, IL, USA/ Zip Code: 60515
DDS, MS, PhD, PostDoc, Associate professor
Midwestern University, College of Dental Medicine Illinois (CDMI)
[3]Biomaterials in Dentistry Program, Anhanguera University São Paulo

Linda Wang[1]
wang.linda@usp.br
Alameda Octávio Pinheiro Brisolla, 9-75, Bauru, SP, Brazil/Zip Code:17012-901
DDS, MS, PhD, Associate Professor
[1]Department of Operative Dentistry, Endodontics and Dental Materials, Bauru School of Dentistry, University of São Paulo.

Keywords: dentin bonding agents; dental caries; dental erosion; enzymes inhibitors.

Corresponding author: Prof. Dr. Linda Wang
Department of Operative Dentistry, Endodontics and Dental Materials
Bauru School of Dentistry-FOB-USP
Alameda Octávio Pinheiro Brisolla, 9-75, Bauru, SP, Brazil/17012-901
Phone: +55-14-3235-8323/ 8480
Fax: +55-14-3235-8323
E-mail:wang.linda@usp.br

MDP-based Adhesive and Protease Inhibitors Association: 18-month Exploratory Study

Giacomini MC, Scaffa PMC, Gonçalves RS, Jacomine JC, Zabeu GS, Carrilho MRO, Wang L.

Abstract

The aim of this study was to explore the interaction between two calcium-dependent active ingredients 10-methacryloyloxydecyl-dihydrogen phosphate (MDP) and 2% digluconate chlorhexidine (CHX) on the longevity of bonding-restoration into demineralized dentin up to 18 months. Ninety sound human third molars were randomly distributed into three groups according to the substrate: N- no challenges (stored in artificial saliva), ACD- artificial caries dentin (6h DE+ 18h-RE/ 5 days + 48h RE) and ERO-artificial erosion dentin (3x5min/5 days with orange juice). They were redivided according to dentin pretreatment: W- water, CHX- 2% digluconate chlorhexidine and E64- 5μM E64 inhibitor, which resulted in the following 9 groups (n=10): N-W, N-CHX, N-E64, ACD-W, ACD-CHX, ACD-E64, ERO-W, ERO-CHX and ERO-E64. All specimens were restored with Adper Single Bond Universal (Etch-and-rinse mode)/Filtek Z250. Sticks (0.64 mm^2) were obtained and subjected to the microtensile test (μTBS) in a universal testing machine at 0.5 mm/min for 7-day, 6 and 18-month analyses. Failure modes were classified using optical microscopy (40X). Data was statistically analyzed with three-way ANOVA and Tukey tests ($p<0.05$). All individual factors ($p<0.0001$) and interaction between factors were statistically significant (substrate X pretreatment ($p=0.00093$); substrate X time ($p=0.01035$) and pretreatment X time ($p=0.0035$). Caries substrate was the most affected substrate, disregarding the pretreatment. Even no inhibitors stabilized the bond strength up to 18 months; CHX notably early compromised bonding strength to dentin, which was attributed to its interaction with calcium, which negatively impacted the MDP reaction to dentin, as both ingredients competed for calcium.

Key words: Dentin Bonding Agents, Dental Caries, Dental Erosion, Enzyme inhibitors

Clinical Relevance Statement

Carious and eroded dentin represents the most common affected dental tissues that call for restorative approach. Even universal dental system has been advocated as a friendly and effective system, when MDP-based, they must be avoided to be associated with calcium-dependent proteolytic inhibitors. CHX and E64 did not improve the bonding stability overtime, regardless dentin condition.

Regulatory Statement

This study was conducted in accordance with all provisions of the local human subjects oversight committee guidelines and polices of the Ethic Committee for Human Studies of Bauru School of Dentistry, University of São Paulo, Brazil. The approval code for this study is: 16558913.2.0000.5417.

INTRODUCTION

In clinical routine, dental caries and tooth erosive wear mostly affect dentin causing alterations that calls for restorative procedure. These events promote alterations on organic and inorganic matrices of dentin, which in turns modify the interaction with dentin bonding systems (DBSs) that need to be taken into account to promote a satisfactory adhesion.[1-3] Systems and strategies had been developed not only for immediate effectiveness but mainly to improve their durability overtime. The adjunctively use of DBS with proteolytic inhibitors have been a feasible trend strategy in the last years.[4-7]

One of the most relevant concerns relies on the comprehension of the reminiscent tissue. In this scenario, Minimal Dentistry Intervention (MDI) concept in dental caries has addressed to the selective removal of carious tissue.[3,8,9] As this approach consists on the removal of infected dentin and maintenance of affected dentin[8,9] probably a less mineralized and disorganized collagen fibril mesh will be infiltrated by the bonding systems,[1] changing the substrate to be restored being a challenge for bonding procedure.[3]

On the other hand, dental erosion is a chemical process that involves a non-bacterial acid agent (extrinsic and/or intrinsic) leading to enamel and dentin dissolution.[10,11] This process occurs through the sequential removing of dental layers from outer surface, which can expose dentin, resulting in less mineralized surface with no exacerbated alteration of collagen fibrils. [5,10,11,12]

For the clinical approach, as both dental caries and dental erosion are dependent on patient behavior, strong guidance need to be informed to them simultaneously to the lesions management.[10] Besides that, both conditions frequently require restorations, facing these altered substrates.[3,13,14] Therefore, the mechanisms behind these processes must sustain the researches to look for the improvement of long-term performance of bonding to these substrates, as resin-based materials are the most advocated materials for the treatment of these events. [2,13,14]

The sound dentin is a heterogeneous, dynamic, permeable and complex substrate, which varies according to depth and clinical conditions.[15-17] Up to last two decades, clinical strategies were based exclusively in remineralization of dentin.[8,9,18] From the early 2000', intensified focus on the role of organic matrix opened a new rationale. Demineralized exposed dentin is degraded by dentin host-derived

enzymes, as matrix metalloproteinases (MMPs) and cysteine cathepsins (CCs).[1,19-22] These enzymes play important role on degradation on poorly resin-infiltrated dentin matrices in adhesive restorations.[16] Therefore, one of the strategies to favor an adequate bonding and improve their durability is the association of dentin bonding agents with proteolytic inhibitors, especially when associated with etch-and-rinse systems.[4-6,14,23]

Giacomini et al., 2017[6] analyzed the interaction of a MDP-based universal dentin system to dentin previously associated with aqueous solution of chlorhexidine and E64, as dentin matrix metalloproteinase (MMP) and cysteine-cathepsin (CC) inhibitors, respectively up to 6 months.[1,20,22] The authors observed that CHX negatively affected the bonding ability, suggesting that as MDP and CHX are both calcium-dependent for their reactions, probably it could influence. This performance along time was not reported yet. Since we know that water and enzymatic degradation continue overtime, longer assessment would be interesting.

Up to 6 months, most studies demonstrate that chlorhexidine (CHX) is able to inhibit MMP. [24-26] Furthermore, CHX has practical use, easy access and low cost. Other alternatives as E64 (trans-Epoxysuccinyl-L-Leucylamido-(4-guanidino) butane) as a synthetic, water soluble and specific irreversible protease inhibitor for CCs was introduced to optimize the inhibitory effect against them.[27,28] These agents were also previously investigated in this time by Giacomini et al. 2017[6] trough means of microtensile bond strength tests in combination with an MDP-universal dentin system. Chaves et al. 2018[7] also tested this agent on bonding to root canal, which is not directly calcium-dependent. Its mechanism is based on its ability to link to collagen fibrils and then, protect the degradation. However, longer effects need to be better investigated and support the comprehension of the involved mechanisms.

Among the DBSs, universal systems are generally based on a complex composition based on an acidic functional monomer, mostly a bifunctional and phosphate monomer, 10-MDP (10-methacryloyloxydecyl-dihydrogen phosphate). It is able to bind chemically to dental substrate.[29-33] In this study, universal DBS was applied in etch-and-rinse mode, since it was the most active host-enzymes due to acid etching [19,20] and there is no difference on hybrid layer formation between self-etching mode. [32,34]

The aim of this study was to explore the interaction between two calcium-dependent active ingredients 10-methacryloyloxydecyl-dihydrogen phosphate (MDP)

and 2% chlorhexidine (CHX) on the longevity of bonding restoration into demineralized dentin up to 18 months. The null hypotheses tested were: 1) there is no difference in dentin bond strength to normal, carious, and eroded dentin; 2) there is no difference in dentin bond strength after treating the affected dentin with CHX or E64; and 3) there is no difference on bond strength over time (24hours, 6 months and 18 months) regardless of the substrate and pretreatment.

MATERIALS AND METHODS

The Ethic Committee for Human Studies of Bauru School of Dentistry, University of São Paulo, Brazil approved this study (CAAE 16558913.2.0000.5417) before its beginning. Ninety extracted healthy third molars were selected and stored in 0.1% thymol solution at room temperature.

The methodology was used by Giacomini et al. 2017[6]. The crowns were separated from the root sectioning it 3 mm below the cement–enamel junction. The occlusal enamel was cut horizontally using a water-cooled diamond-impregnated disc (Extec Corp, Enfield, CT, USA) to expose medium dentin surface. This substrate was polished to obtain a flat surface. Smear layer was standardized using 600-grit SiC paper under running water for 60 seconds[35] (Politriz APL-4 AROTEC, Cotia, SP, Brazil). The specimens were distributed according to the pretreatment of the dentin before the bonding procedures as: normal dentin (control [N]), artificial carious dentin (ACD) or erosion (ERO). The specimens from normal dentin group were stored in artificial saliva (1.5 mM $Ca[NO_3]_2{\cdot}4H_2O$, 0.9 mM $NaH_2PO_4{\cdot}2H_2O$, 150 mM KCl, 0.1 mol/L Tris, 0.03 ppmF, pH 7.0) at 37°C for 7 days. For artificial carious dentin, the lesions were formed following Vieira et al., 2005[36] protocol, which consists of cycles of 6 hours of demineralization (2 mM $Ca[NO_3]_2{\cdot}4H_2O$, 2 mM $NaH_2PO_4{\cdot}2H_2O$, 0.075 mM acetate buffer, 0.02 ppm F, pH 4.6), followed by 18 hours of incubation in artificial saliva and the solutions were renewed daily. Cycles were performed for five days, followed by 48 hours of incubation in artificial saliva.

The erosive challenge involved the immersion of the specimens in industrialized orange juice (Suco Del Valle do Brasil, Coca-Cola, Americana, SP, Brazil), composed by water, sugar, orange juice concentrate, natural flavor, citric acid, and antioxidant ascorbic acid and pH 3.1, for 5 minutes, 3 times per day, for 5

days. Between each erosive cycle up to its end, the specimens were stored in artificial saliva, which were daily renewed.

Both substrates were evaluated by transverse microradiography, after all challenged cycles were completed, to validate the formation of artificial carious dentin and eroded dentin. For the artificial carious dentin substrate, a demineralized subsurface layer with the preservation of the outer enamel surface was observed. For the artificially eroded substrate, a slight superficial layer was noted.

All specimens were etched with 37% phosphoric acid (Dentsply, Catanduva, SP, Brazil) for 15 seconds. Specimens from each dentin substrate groups were subdivided according to the three pretreatment (n=10), including application of distilled water (W), 2% chlorhexidine digluconate aqueous solution pH 5.8 (CHX, Pharmácia Específica, Bauru, SP, Brazil), and 5 μM E64 aqueous solution (E64, Sigma-Aldrich, St Louis, MO, USA) pH 5.5. After passive application for 60 seconds, excess was removed with absorbent paper. After that, the dentin-bonding agent (Adper Single Bond Universal, 3M ESPE, St Paul, MN, USA) was applied according to the manufacturer's instructions and light cured using a 1,000 mW/cm^2 LED unit (Radical, SDI, Bayswater, VIC, Australia). Two increments of 2-mm layers of the resin-based composite (Filtek Z350 Universal Restorative, 3M ESPE) were layered and light cured for 20 seconds each. The specimens were stored in artificial saliva for 24 hours at 37°C and then longitudinally sectioned, perpendicularly to the bonding interface, using an Isomet 1000 digital saw (Buehler, Lake Bluff, IL, USA) to obtain beams of ≅ 0.64 mm^2 area (0.8×0.8 mm). Each beam was measured at the dentin–adhesive interface using a digital caliper (Mitutoyo America, Aurora, IL, USA) to obtain the surface area of interface, which was fixed to the Bencor Multi-T testing apparatus (Danville Engineering Co, Danville, CA, USA) with cyanoacrylate resin (Super Bonder Flex Gel-Loctite, Henckel Ltda, Itapevi, SP, Brazil) and tested in tension in a universal testing machine (Instron 3342, Instron Co., Canton, MA, USA) at a 0.5 mm/min crosshead speed and with a 500 N load cell. Each tooth was considered the experimental unit while the beams are categorized as repetitions.[37] From each specimen (tooth), an average of 8 to 10 beams was obtained for each time (after 24 hours, 6 months and 18 month). During the 6 and 18 months aging period, all beams were stored in a weekly renewed artificial saliva at 37 °C.

Microtensile bond strength (μTBS) values were obtained in MPa and failures mode of each beam were analyzed with a handheld digital microscope (DINO-

LITE^{plus} digital microscope, AnMo Electronics Corp, Hsinchu- China) at 40× magnification, and classified in adhesive (failure in the adhesive interface), cohesive in dentin, cohesive in composite resin, or mixed. For the statistical analysis, as the tooth was the experimental unit, an average of all beams per tooth was performed to determine the tooth μTBS for each time. Data were calculated and statistically analyzed with Statistica software (Statsoft, Tulsa, OK, USA). Assumptions of a normal distribution and equality of variance were tested for all the variables using the Kolmogorov-Smirnov and the Levene tests, respectively. As the assumptions were satisfied, the data were subjected to three-way ANOVA ($p \leq 0.05$), followed by the Tukey test ($p<0.05$) for individual comparisons.

RESULTS

The comparison of the groups is shown in Figure 1.

All tested individual factors (substrate, pretreatment and time) were statistically significant ($p<0.0001$), although the interaction between all of them simultaneously were not ($p=0.68091$). Also, significant interactions were detected between substrate and time ($p=0.01035$), substrate and pretreatment ($p=0.00093$) and pretreatment and time ($p=0.0035$), which are presented in tables 1 to 3.

Data indicated that bond strength was committed in relation to three factors: artificial carious dentin condition, chlorhexidine as a pretreatment, and periods of aging. Bond strength trended lower in the artificial carious dentin condition, even treated with chlorhexidine.

It was notable in table 1 that ACD was the most affected substrate disregarding the time up to 6 months. However, after 18 months, there is no difference among substrate x time. In table 2, it is observed that the control group N-W presented the highest value of BS, while ACD-CHX showed the lowest values. As no statistically difference between N-W and N-CHX was detected, it could suggest an interaction between substrate, dentin bonding agents and pretreatment.

For eroded substrate, no statistically difference was observed for ERO-W and ERO-E64 compared to N-W, while ERO-CHX presented the lowest value among eroded group.

In table 2, normal substrate treated with water showed the highest values, which presented no difference to eroded substrate, while carious substrate showed

the worst values. Groups treated with CHX showed the same pattern. When treated with E64, no significant statistical differences were detected for the comparison among all the conditions.

In table 3, groups treated with water in initial time presented the highest value, followed by E64. Groups pretreated with CHX up 6 months aging showed the lowest values. After 18 months aging, all pretreatments showed the worst performance for all pretreatments with no significant statistical difference.

The distribution of failure mode analysis is presented in Figure 2, demonstrated that adhesive and mixed failures were predominant in all groups, regardless substrate, pretreatment and aging.

DISCUSSION

Most studies are performed in sound dentin, so it is more appropriate to investigate the interaction between altered substrates and dentin bonding systems for more realistic interpretation. In this study, artificial challenges were performed to create substrates commonly founded clinically. Since the study of Giacomini et al., 2017[6], the interaction of substrate, enzymatic agent and time was reported, impacting the bonding performance up to 6 months. In this study, data revealed some distinct performance, likely impacted overtime when assessed up to 18 months. These performance calls for new interpretation of the involved mechanism.

The data showed significant differences in adhesion according to all tested conditions regardless dentin substrate. Normal dentin substrate presented the highest values, which was not different from eroded dentin, while artificial carious was clearly the most impaired substrate. Therefore, the first hypothesis was rejected. These results are in agreement with previous studies, which showed that in carious dentin, occurs an unfavorable resin bonding infiltration, [6,19,20] leading to alterations in hybrid layer.

Dentin caries leads to a disorganization of fibrils collagen and loss of mineral[1,20,22] especially calcium, which is essential to hybrid layer formation associated with 10-MDP.[30,32,34] This could explain low values obtained on initial aging time on carious dentin substrate.

Eroded tooth wear consists on progressive process based of two phases.[10] The first one is denominated “softening”, which corresponds to softening of the outer

surface, followed by a mechanical loss of dental structure, which is the second phase.[11]

Overall results revealed that for all eroded test conditions, it was similar to normal substrate performance. Laboratory protocols have been investigated that the industrialized orange juice impact seeing that the consumption of this beverage is increasing.[38] In this study, it was used orange juice to simulate dental erosion, which contain vitamin C in its composition. Vitamin C is an important antioxidant and has been investigating to improve resistance of hybrid layer against degradation[39] through the reduction of hydrolysis and the degradation of free radicals.[39] Likely, its effect what may explain the similarity between the normal and eroded values in most of the tested conditions tested up to 18 months.

Regarding carious dentin, it can be supposed that the proteolytic enzimes were more aggressive. MMPs and CCs co-exist in normal dentin[1,19-22,40] however in carious substrate, they play an important role in exacerbate degradation of the dentin organic matrix. [1,22] Furthermore, MMPs and CCs may have synergistic and dependent activities, indicated by the increase of CC in carious dentin with increasing depth toward the pulp.[19]

According to statistical analysis, it was verified that there was interaction between substrate and pretreatment. Normal dentin pretreated with water presented the highest values, corresponding to the control group. On the other hand, artificial carious dentin treated with CHX presented the lowest values. CHX impaired on bonding only for carious dentin, while E64 impaired the bond strength for normal dentin. None of them impacted on the performance of eroded surface. For these reason the second hypothesis was rejected.

The benefit of CHX use in preserving the integrity of resin-dentin bonds was related to its ability to inhibit proteolytic activity of MMPs, but it was also shown to be effective in controlling the activity of dentinal CCs.[19] However, its mechanism of action is through calcium ion chelation.[24] Calcium also participates on the formation of MDP-Ca salt, providing a satisfactory bonding stability[1,32, 34] Therefore, the simultaneous presence of CHX and MDP might establish a competition for calcium between them, leading to an interaction and a negative effect on adhesion.[6]

Adper Single Bond Universal is classified as “mild” self-etching DBS,[41] remaining part of the smear layer in dentinal tubules, providing calcium for 10-MDP

bonding.[29] The carious substrate is the most challenged regardless the strategy adopted[3] and, calcium is devoided, which could compromise it more intensively.

Chaves et al., 2018[7] demonstrated that CHX associated with MDP-free three-step dentin bonding system for glass-fiber posts in root canals was able to promote beneficial bond strength after 6 months, which was not noted for any other tested enzyme inhibitors. As no MDP was present, no competition for Ca occurred, which reinforce this likely mechanism.

Regarding the time of aging, statistical difference was observed among initial, 6 and 18 months groups, and then the third hypothesis was rejected. On initial time, normal dentin presented the highest values, which was not different from eroded dentin. ACD dentin was already affected since the initial time. Taking the pretreatment into account, this fact may be related to loss of the substantively of CHX and loss of E64 action. According to substrate, it is observed that normal dentin presented the highest values on initial time, following by a huge reduction after both 6 and 18 months. This pattern of reduction was different for carious and eroded dentin, since both showed initial lower values and a small reduction after 6 and 18 months.

The bonding procedure is based on the micromechanical interlocking between bonding agent and collagen structure.[1] In carious and eroded dentin, the collagen matrix is initially altered, leading to a non-adequate hybrid layer formation. Although, this hybrid layer is able to be maintained over time.

Based on the interaction between pretreatment and time, water and E64 showed reduced bond strength over time, while CHX did not. It was shown because of the initial reduction performed by CHX. It was also impaired from the beginning. Therefore, one need to be aware on the interpretation of literature regarding the association of MDP based materials and other functional monomer-based system associated with anti-enzymatic agent, especially when it is calcium dependent-on the MDP, as CHX.

The CHX acts through chelation of calcium ions[24] from hydroxiapatatite, as well as, MDP interacts with the same calcium ions, [30,42]allowing the chemical bonding to dental structure. Giacomini et al., 2017[6] speculated that both competed for calcium ions, leading to a substantial decrease on initial values of BS. However, overtime CHX is able to play it role. Another possibility is that CHX cations binds to 10-MDP anions impairing it bonding stability.[43]

In this scenario, this study highlighted that when a MDP-based universal system used in etch- and-rinse is used on dentin, CHX is not able to provide benefits up to 18 months. Substrate qualities do influence the interaction of this system to dentin in terms of bonding and needs to be considered for a long-term successful performance.

CONCLUSION

The use of any proteolytic inhibitors to treat affected dentin seems not to improve BS associated with a universal bonding system after 18 months. Caries substrate was the most affected substrate Controversially, CHX associated with 10-MDP based dentin-bonding system notably compromised bonding durability overtime, suggesting a negative interference between these agents.

References

1. Tjaderhane L, Buzalaf MA, Carrilho M & Chaussain C (2015) Matrix metalloproteinases and other matrixproteinases in relation to cariology: The era of ‘‘dentin degradomics’’ *Caries Research* **49(3)** 193-208.

2. Oliveira B, Ulbaldini A, Sato F, Baesso ML, Bento AC, Andrade L, Lima SM & Pascotto RC (2017) Chemical Interaction Analysis of an Adhesive Containing 10-Methacryloyloxydecyl Dihydrogen Phosphate (10-MDP) With the Dentin in Noncarious Cervical Lesions *Operative Dentistry* **42(4)** 357-366.

3. Isolan CP, Sarkis-Onofre R, Lima GS & Moraes RR (2018) Bonding to sound and caries-affected dentin: a systematic review and meta-analysis *Journal of Adhesive Dentistry* **20(1)** 7-18

4. Carrilho MR, Geraldeli S, Tay F, de Goes MF, Carvalho RM, Tjäderhane L, Reis AF, Hebling J, Mazzoni A, Breschi L, & Pashley D (2007) In vivo preservation of the hybrid layer by chlorhexidine *Journal of Dental Research* **86(6)** 529-533.

5. Francisconi-dos-Rios LF, Calabria MP, Casas-Apayco LC, Honório HM, Carrilho MR, Pereira JC, & Wang L (2015a) Chlorhexidine does not improve but preserves bond strength to eroded dentin *American Journal of Dentistry* **28(1)** 28-31.

6. Giacomini MC, Scaffa P, Chaves LP, Vidal C, Machado TN, Honório HM, Tjäderhane L & Wang L (2017) Role of Proteolytic Enzyme Inhibitors on Carious and Eroded Dentin Associated With a Universal Bonding System *Operative Dentistry* **42(6)** E188-E196.

7. Chaves LP, Ciantelli TL, Araújo DFG, Giacomini MC, Tjäderhane L, Scaffa PMC, Honório HM & Wang L (2018) How proteolytic inhibitors interact with dentin on glass-fiber post luting over 6 months *Journal of the Mechanical Behavior of Biomedical Materials* **79** 348-353.

8. Innes NP, Frencken JE, Bjørndal L, Maltz M, Manton DJ, Ricketts D, Van Landuyt K, Banerjee A, Campus G, Doméjean S, Fontana M, Leal S, Lo E, Machiulskiene V, Schulte A, Splieth C, Zandona A & Schwendicke F (2016) Managing Carious Lesions: Consensus Recommendations on Terminology *Advances in Dental Research* **28(2)** 49-57.

9. Schwendicke F, Frencken JE, Bjørndal L, Maltz M, Manton DJ, Ricketts D, Van Landuyt K, Banerjee A, Campus G, Doméjean S, Fontana M, Leal S, Lo E, Machiulskiene V, Schulte A, Splieth C, Zandona AF & Innes NP (2016) Managing Carious Lesions: Consensus Recommendations on Carious Tissue Removal *Advances in Dental Research* **28(2)** 58-67.

10. Tjäderhane L, Buzalaf MA, Carrilho M, Chaussain C (2015) Matrix metalloproteinases and other matrix proteinases in relation to cariology: the era of 'dentin degradomics' *Caries Research* **49(3)** 193-208.

11. Magalhães AC, Wiegand A, Rios D, Honório HM & Buzalaf MA (2009) Insights into preventive measures for dental erosion *Journal of Applied Oral Science* **17(2)** 75-86.

12. Huysmans MC, Chew HP & Ellwood RP (2011) Clinical studies of dental erosion and erosive wear *Caries Research* **45(1)** 60-68.

13. Machado CM, Bim Júnior O, Giacomini MC, Graeff MSZ, Garcia FCP, Rios D, Honório HM & Wang L (2018) Effect of ethanol-dissolved rhodamine B marker on mechanical properties of non-simplified adhesives *Journal of the Mechanical Behavior of Biomedical Materials* **84** 145-150

14. Reis A, Higashi C & Loguercio AD (2009) Re-anatomization of anterior eroded teeth by stratification with direct composite resin *Journal of Esthetic Restorative Dentistry* **21(5)** 304-316.

15. Komori PC, Pashley DH, Tjäderhane L, Breschi L, Mazzoni A, de Goes MF, Wang L, & Carrilho M (2009) Effect of 2% chlorhexidine digluconate on the bond strength to normal versus caries-affected dentin *Operative Dentistry* **34(2)** 157-165.

16. Perdigão J (2010) Dentin bonding-variables related to the clinical situation and the substrate treatment *Dental Materials* **26(2)** e24-37.

17. Pashley DH, Tay FR, Breschi L, Tjäderhane L, Carvalho RM, Carrilho M & Tezvergil-Mutluay A (2011) State of the art etch-and-rinse adhesives. *Dental Materials* **27(1)** 1-16

18. Carvalho RM, Manso AP, Geraldeli S, Tay FR & Pashley DH (2012) Durability of bonds and clinical success of adhesive restorations *Dental Materials* **28(1)** 72-86.

19. Momoi Y, Hayashi M, Fujitani M, Fukushima M, Imazato S, Kubo S, Nikaido T, Shimizu A, Unemori M, & Yamaki C (2012) Clinical guidelines for treating caries in adults following a minimal intervention policy—Evidence and consensus based report *Journal of Dentistry* **40(2)** 95-105.

20. Scaffa PM, Vidal CM, Barros N, Gesteira TF, Carmona AK, Breschi L, , Pashley DH, Tjäderhane L, Tersariol IL, Nascimento FD & Carrilho MR (2012) Chlorhexidine inhibits the activity of dental cysteine cathepsins *Journal of Dentistry Research* **91(4)** 420-425.

21. Tjäderhane L, Nascimento FD, Breschi L, Mazzoni A, Tersariol IL, Geraldeli S, Tezvergil-Mutluay A, Carrilho MR, Carvalho RM, Tay FR & Pashley DH (2013) Optimizing dentin bond durability: Control of collagen degradation by matrix metalloproteinases and cysteine cathepsins *Dental Materials* **29(1)**116-135.

22. Vidal CM, Tjäderhane L, Scaffa PM, Tersariol IL, Pashley D, Nader HB, Nascimento FD & Carrilho MR (2014) Abundance of MMPs and cysteine cathepsins in caries-affected dentin *Journal of Dentistry Research* **93(3)** 269-274.

23. Buzalaf MA, Charone S & Tjäderhane L (2015) Role of host-derived proteinases in dentine caries and erosion *Caries Research* **49(1)** 30-37.

24. Francisconi-dos-Rios LF, Casas-Apayco LC, Calabria MP, Francisconi PA, Borges AF, & Wang L (2015b) Role of chlorhexidine in bond strength to artificially eroded dentin over time *Journal of Adhesive Dentistry* **17(2)** 133-139.

25. Gendron R, Grenier D, Sorsa T & Mayrand D (1999) Inhibition of the activities of matrix metalloproteinases 2, 8, and 9 by chlorhexidine *Clinical and Diagnostic Laboratory Immunology* **6(3)** 437-439.

26.Carrilho MR, Carvalho RM, Sousa EN, Nicolau J, Breschi L, Mazzoni A, Tjäderhane L, Tay FR, Agee K & Pashley DH (2010) Substantivity of chlorhexidine to human dentin *Dental Materials* **26(8)** 779-785.

27. Breschi L, Maravic T, Cunha SR, Comba A, Cadenaro M, Tjäderhane L, Pashley DH, Tay FR & Mazzoni A (2018) Dentin bonding systems: From dentin collagen structure to bond preservation and clinical applications *Dental Materials* **34(1)** 78-96.

28. Tersariol IL, Geraldeli S, Minciotti CL, Nascimento FD, Pääkkönen V, Martins MT, Carrilho MR, Pashley DH, Tay FR, Salo T & Tjäderhane L (2010) Cysteine cathepsins in human dentin-pulp *Journal of Endodontics* **36(3)** 475-481.

29. Nascimento FD, Minciotti CL, Geraldeli S, Carrilho MR, Pashley DH, Tay FR, Nader HB, Salo T, Tjäderhane L & Tersariol IL (2011) Cysteine cathepsins in human carious dentin *Journal of Dental Research* **90(4)** 506-511.

30. Yoshida Y, Nagakane K, Fukuda R, Nakayama Y, Okazaki M, Shintani H, Inoue S, Tagawa Y, Suzuki K, De Munck J & Van Meerbeek B (2004) Comparative study on adhesive performance of functional monomers *Journal of Dental Research* **83(6)** 454-458.

31. Hanabusa M, Mine A, Kuboki T, Momoi Y, Van Ende A, Van Meerbeek B & De Munck J (2012) Bonding effectiveness of a new “multi-mode” adhesive to enamel and dentine *Journal of Dentistry* **40(6)** 475-484.

32. Matsui N, Takagaki T, Sadr A, Ikeda M, Ichinose S, Nikaido T & Tagami J (2015) The role of MDP in a bonding resin of a two-step self-etching adhesive system *Dental Materials Journal* **34(2)** 227-233.

33. Muñoz MA, Luque-Martinez I, Malaquias P, Hass V, Reis A, Campanha NH, & Loguercio AD (2015) In vitro longevity of bonding properties of universal adhesives to dentin *Operative Dentistry* 2015 **18(3)** 282-292.

34. Wang R, Shi Y, Li T, Pan Y1, Cui Y & Xia W (2017) Adhesive interfacial characteristics and the related bonding performance of four self-etching adhesives with different functional monomers applied to dentin *Journal of Dentistry* **62** 72-80.

35. Scotti N, Cavalli G, Gagliani M & Breschi L (2017) New adhesives and bonding techniques. Why and when? The *International Journal of Esthetic Dentistry* **12(4)** 524-535.

36. Pashley DH, Tao L, Boyd L, King GE & Horner JA (1988) Scanning electron microscopy of the substructure of smear layers in human dentine *Archives Oral Biol*ogy **33(4)** 265-270.

37. Vieira AE, Delbem AC, Sassaki KT, Rodrigues E, Curya JA & Cunha RF (2005) Fluoride dose response in pH-cycling models using bovine enamel *Caries Research* **39(6)** 514-520.

38. Armstrong S, Breschi L, Özcan M, Pfefferkorn F, Ferrari M & Van Meerbeek B (2017) Academy of Dental Materials guidance on in vitro testing of dental composite bonding effectiveness to dentin/enamel using micro-tensile bond strength (μTBS) approach *Dental Materials* **33(2)** 133-143.

39. Scaramucci T, Sobral MA, Eckert GJ, Zero DT & Hara AT (2012) In situ evaluation of the erosive potential of orange juice modified by food additives *Caries Research* **46(1)** 55-61.

40. Gotti VB, Feitosa VP, Sauro S, Correr-Sobrinho L, Leal FB, Stansbury JW & Correr AB (2015) Effect of antioxidants on the dentin interface bond stability of adhesives exposed to hydrolytic degradation *Journal of Adhesive Dentistry* **17(1)** 35-44.

41. Scaffa PM, Breschi L, Mazzoni A, Vidal CM, Curci R, Apolonio F, Gobbi P, Pashley D, Tjäderhane L, Tersariol IL, Nascimento FD & Carrilho MR (2017) Co-distribution of cysteine cathepsins and matrix metalloproteases in human dentin *Archives Oral Biol*ogy **74** 101-107.

42. Rosa WL, Piva E, Silva AF (2015) Bond strength of universal adhesives: A systematic review and meta-analysis *Journal of Dentistry* **43(7)** 765-776.

43. Yoshida Y, Yoshihara K, Nagaoka N, Hayakawa S, Torii Y, Ogawa T, Osaka A & Meerbeek BV (2012) Self-assembled nano-layering at the adhesive interface *Journal of Dental Research* **91(4)** 376-381.

44. da Rosa LS, Follak AC, Lenzi TL, Rocha RO & Soares FZM (2018) Phosphoric Acid Containing Chlorhexidine Compromises Bonding of Universal Adhesive *Journal of Adhesive Dentistry* **20(3)** 243-247.

Acknowledgements

This study was performed by MCG as fulfillment of her phD`s degree at the University of São Paulo, Brasil. The authors are grateful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001 and CNPq (163402/2015-9 and 408865/2016-4); FAPESP (2013/12203-3) for the financial support.

Table 1- Mean values of the interaction between substrate X time

	7 days	6 Months	18 Months
N	30.87a	23.87bc	21.94cd
ACD	22.09cd	19.20d	19.68d
ERO	27.54ab	24.09bc	23.04cd

N=10 p<0.05
Different letters indicate significant statistical differences

Table 2- Mean values of the interaction between substrate X pretreatment

	W	CHX	E64
N	26.06a	24.97abcd	23.45bcd
ACD	21.44d	17.42e	22.09cd
ERO	25.83abc	21.98cd	26.86ab

N=10 p<0.05
Different letters indicate significant statistical differences

Table 3- Mean values of the interaction between pretreatment X time (p=0.0035)

	7 days	6 months	18 months
W	29.53a	24.60bc	21.20cd
CHX	23.07cd	19.74d	21.56cdc
E64	27.69ab	22.82cd	21.90cd

N=10 p<0.05
Different letters indicate significant statistical differences

Figure 1: Mean values and standard deviation (MPa) of bond strength and comparison between all groups.

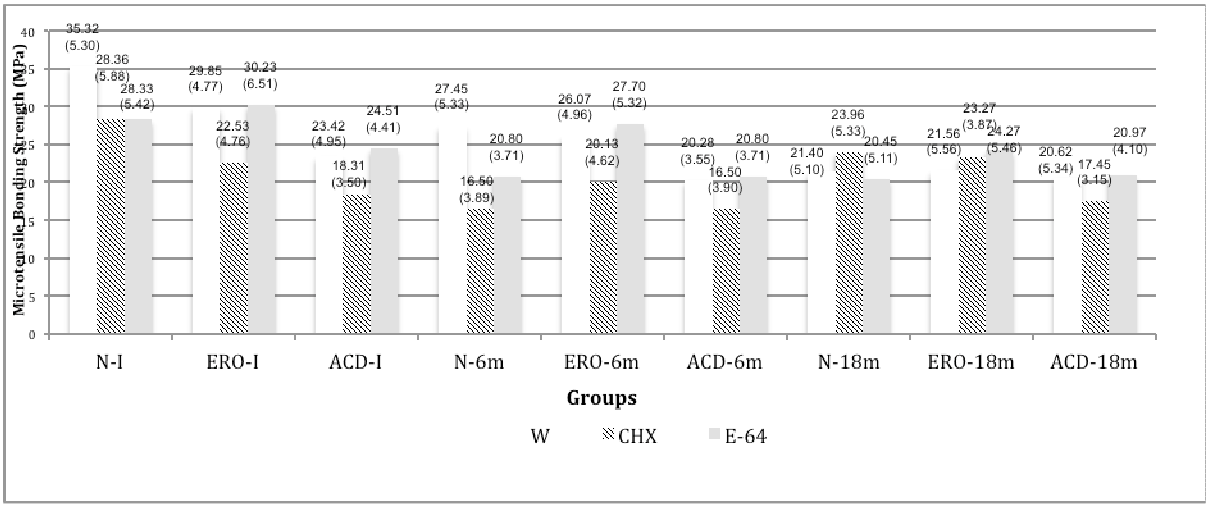
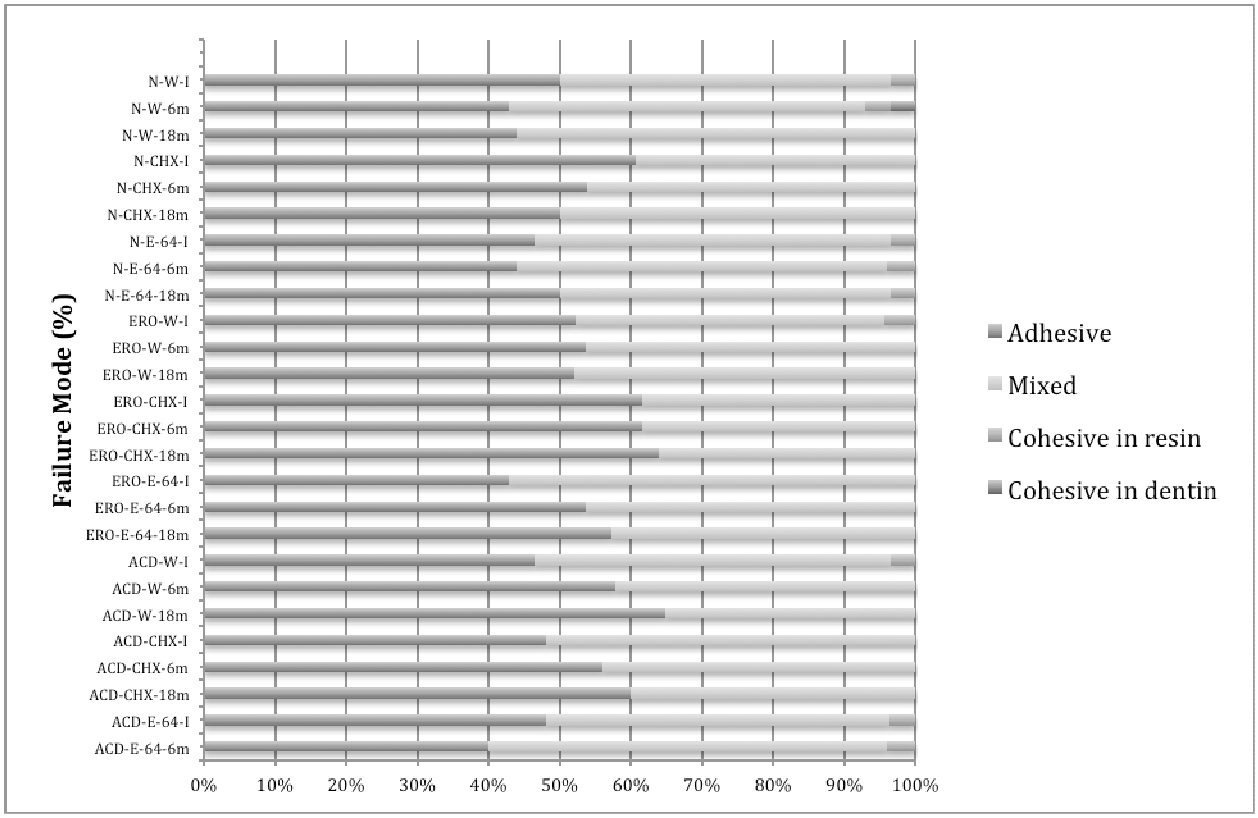


Figure 2 – Failure mode distribution (%) regarding all substrates and pretreatment with inhibitors in initial, 6 and 18 months time.



2.2 Article 2

Profile of a multi-mode MDP-based adhesive system: *in situ* zymography and bond strength on dentin performance

Short title: Profile of a multi-mode MDP-based adhesive on dentin

Marina Ciccone Giacomini[1], Polliana Mendes Candia Scaffa[2], Rafael Simões Gonçalves[3], Giovanna Speranza Zabeu[4], Cristina de Mattos Pimenta Vidal[5], Marcela Rocha de Oliveira Carrilho[6], Heitor Marques Honório[7], Linda Wang[8].

[1]PhD student, Department of Operative Dentistry, Endodontics and Dental Materials, Bauru School of Dentistry, University of São Paulo, Bauru, SP, Brazil. marina.giacomini@usp.br

[2]Post doctor, Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo, Bauru, SP, Brazil. polli_scaffa@yahoo.com.br

[3]PhD student, Department of Operative Dentistry, Endodontics and Dental Materials, Bauru School of Dentistry, University of São Paulo, Bauru, SP, Brazil. rafael895@hotmail.com

[4]PhD student, Department of Operative Dentistry, Endodontics and Dental Materials, Bauru School of Dentistry, University of São Paulo, Bauru, SP, Brazil. giovanna.zabeu@gmail.com

[5]Assistant professor, Department of Operative Dentistry, College of Dentistry, University of Iowa, Iowa City, IA, USA. cristina-vidal@uiowa.edu

[6]Assistant professor, College of Dental Medicine Illinois, Midwestern University, Chicago, IL, USA. mcarri@midwestern.edu

[7]Associate Professor, Department of Pediatric Dentistry, Orthodontics and Collective Health, Bauru School of Dentistry, University of São Paulo, Bauru, SP, Brazil. heitorhonorio@usp.br

[8]Associate Professor, Department of Operative Dentistry, Endodontics and Dental Materials, Bauru School of Dentistry, University of São Paulo, Bauru, SP, Brazil. wang.linda@usp.br

Corresponding author

Dra. Linda Wang

Bauru School of Dentistry, University of São Paulo – FOB/USP
Alameda Dr. Otávio Pinheiro Brisolla, 9-75
Vila Nova Cidade Universitária
Zip code: 17012-901
Bauru – São Paulo / Brazil
Tel: +55 14 3235-8480

Keywords: Dentin. Dentin bonding systems. Hybrid layer. MDP. Protease inhibitors.

Title: Profile of a multi-mode MDP-based adhesive system: *in situ* zymography and bond strength on dentin performance

Authors: Giacomini MC, Scaffa PMC, Gonçalves RS, Zabeu, GS, Vidal CMP, Carrilho MRO, Honório HM, Wang L.

ABSTRACT

Objectives: The aim of this study was to assess the *in situ* gelatinolytic activity profile of Adper Single Bond Universal (SU), a multi-mode MDP-based dentin bonding system (DBS) combined with chlorhexidine (CHX) as a proteolytic inhibitor on dentin up to 6 months. Methods: 102 sound human third molars were prepared and randomly divided into 3 groups according to the DBS: SB- Adper Single Bond 2 (control MDP-free group); SUER- SU on etch-and-rinse mode and SUSE- SU on self-etching mode. The groups were redivided into two subgroups according to the dentin pretreatment: W- water or CHX- 2% digluconate chlorhexidine aqueous solution (SB-W, SB-CHX, SUER-W, SUER-CHX, SUSE-W, SUSE-CHX) and subsequently restored according to the manufacturer's instructions. For *in situ* zymography (n=5), 0.1mm-thickness samples were incubated with fluorescein-conjugated gelatin for 24h at 37°C and analyzed by confocal laser scanning microscopy. Hybrid layer zone and adjacencies were assessed by Image J. For microtensile bond test (μTBS) (n=12/ tooth as experimental unit), beams (0.64mm^2) were obtained and tested (500N/0.5mm/min). Data was analyzed by three-way ANOVA and Tukey tests ($p<0.05$). Results: For *in situ* zymography, all DBS demonstrated proteolytic activity and CHX was able to reduce it in all conditions. For μTBS, SUSE showed the highest values and SB and SUER were not statically different. Conclusion: In conclusion, the use of CHX leads to a reduction of the proteolytic activity independent of the DBS used and maintained μTBS over 6 months aging.

Clinical Significance: Adper Single Bond Universal in self-etching mode reached the best performance in terms of bonding strength and its use associated with CHX did not impair it and leaded to a reduction of proteolytic activity.

Key Words: Dentin bonding systems. Hybrid layer. Protease inhibitors.

INTRODUCTION

Certainly, “bonding era” has increased the possibility of restorative procedures from minimal to extensive dental reconstructions, serving a variety of clinical resolutions involving esthetic and/or functional demand [1].

In this scenario, the challenge now relies on the durability of these procedures overtime. Even most of the studies and marketing have been addressed for the development of new materials and technologies that support these improvements, we cannot ignore the better comprehension of the dentin, a dynamic and complex substrate that is involved in this process for the optimized bonding strategies [1,2].

Therefore, since the nineties of the last century [3], many studies have been addressed focusing in strategies to inhibit dentin matrix intrinsic proteolytic enzymes [4,5,6] and not only regarding the mineral content. Direct and indirect analyzes regarding the role of these non-collagenous proteins, mainly metalloproteinases (MMPs) and cysteine cathepsins (CCs) have been extensively investigated. By now, some consensus states that inhibitors of them, mainly chlorhexidine is useful up to 6-12 months [1,4,7,8]. Most of them were developed in laboratory and even systematic reviews conclude based on these reports [9]. There is still a lack of information clinically assessed [9,10,11].

One more realistic option is the assessment of the performance of materials and technical steps using in situ analyzes, as it is closer to clinical situation [12,13]. A multi-mode/universal adhesive system was developed to facilitating the applicability in clinical routine. The universal DBS can be used as etch-and-rise or self-etching and in dry and wet dentin conditions [14,15,16]. The best choice still is controversial and not clear, in particular considering dentin, there is no evidence about acid strategy [17]. Some studies pointed out that etch-and-rinse mode was more susceptible to degradation in dentin [14,15], while other investigations supported no differences between etch-and-rinse and self-etch modes [16,18]. Most of them are based on functional monomers, specially 10-methacryloyloxydecyl-dihydrogen phosphate (MDP), which is a phosphate and bifunctional monomer that is able to bind chemically to dental substrate [14,15,16,19].

The 10-MDP presents a high chemical bonding potential to hydroxyapatite within a clinically adequate application time [19] besides these chemical bonds are stable in aqueous environment. CHX is water-soluble and binds to the inorganic

components of dentin and when applied, the concentration of ions calcium decrease [20]. The mechanism of action of 10-MDP and CHX are calcium-dependent leading to a possible competition between them [7,20].

In this study, a MDP-based universal adhesive system was used in both modes to compare proteolytic activity between both strategies and also compared with an etch-and-rinse two-steps adhesive system. As few studies associate universal adhesive system with chlorhexidine, this association was also purposed.

Therefore, an *in situ* zymography was chosen to verify the gelatinolytic activity in the hybrid layer comparing the strategies of etch-and-rinse and self-etching modes of a MDP-based universal DBS with a no functional DBS. Also, its impact on the bond strength by means of microtensile along 24 hours and 6 months were also purposed.

The null hypotheses tested for proteolytic activity by *in situ* zymography and bond strength to dentin were: 1) There is no difference using a MDP- based universal adhesive system in etch-and-rinse and self-etch mode compared with a conventional two-step adhesive system; 2) There is no difference between the groups pretreated with water or chlorhexidine; 3) There is no difference overtime (initial and 6 months).

MATERIAL AND METHODS

The Ethic Committee for Human Studies of Bauru School of Dentistry, University of São Paulo, Brazil approved this study (59799916.5.0000.5417) before its beginning.

In situ zimography

The methodology of the *in situ* zimography adopted in this study was based on Mazzoni et al., 2012 [13] with some modifications.

Thirty extracted freshly and non-carious third molars were collected and stored for a maximum of one month in 0.1% in thymol saline solution. The roots were sectioned 3 mm below the cement-enamel junction. The occlusal enamel was removed horizontally using a water-cooled diamond disc (Extec Corp., Enfield, CT, USA) to expose a level medium dentin surface. The dentin surface was ground flat and a smear layer was standardized using 600-grit SiC paper under running water for 60 s (APL-4 Arotec, Cotia, SP, Brazil) [21].

The specimens were divided into 3 groups according to DBS: Adper Single Bond 2 (SB) (3M ESPE, St. Paul, MN, USA), pH around 4; Adper Single Bond Universal (3M ESPE, St. Paul, MN, USA), pH around 3 in etch-and-rinse (SUER) and self-etch (SUSE) mode. The pH was measured by pH strips (ColorpHast pH Test Strips, Sigma-Aldrich, Saint Louis, MN, USA) immersed in adhesive bottle. Table 1 presents the bonding procedures and composition of each adhesive.

Specimens from each DBS were subdivided into 2 pretreatment groups (n=5) including: application of distilled water (W) and 2% chlorhexidine digluconate (CHX) aqueous solution pH 5.0 (Sigma-Aldrich, Saint Louis, MN, USA). All DBS were applied according to the manufacturer's instructions.

The dentin of SB and SUER groups were etched with 37% phosphoric acid (Dentsply, Catanduva, SP, Brazil) for 15s, while SUSE, as self-etch mode did not apply phosphoric acid on dentin. Thereafter, pretreatments with either W or CHX were applied during 30s and then the excess was removed with absorbent paper. The DBSs were applied according to manufacturer and light-cured using a 1,000 mW/cm^2 LED unit (Radical, SDI, Bayswater, VIC, Australia). Two increments of 2 mm of the resin-based composite (Filtek^{TM} Z250 Universal Restorative®, 3M ESPE, St. Paul, MN, USA) were layered and light cured for 20 s each.

The specimens were stored in artificial saliva for 24 hours at 37°C and then longitudinally sectioned, perpendicularly to the bonding interface, using an Isomet 1000 digital saw (Buehler, Lake Bluff, IL, USA) to obtain slices of ≅0.1 mm^2 of thickness. Slices were demineralized by 1% phosphoric acid solution (Sigma-Aldrich, Saint Louis, MN, USA) for 30 s and rinse for 60 s with distilled water.

In situ zymography was realized with self-quenched fluorescein-conjugated (EnzChek gelatinase/colagenase assay kit, Molecular Probes, Eugene, OR, EUA) acting as enzymes substrate. For each slice, 15μL gelatin conjugated with fluorescein stock solution was diluted in 120 μL 1:8 in a buffer (NaCl 150 mM, CaCl2 5 mM, Tris-HCl 50 mM, pH 8.0) with 15 μL of anti-fading agent (Mounting Medium with Dapi H-1200, Vectashield, Vector Laboratories LTD, Cambridgeshire, UK). Slices were placed on a culture plate; one per window and 150 μL of the mixture was placed on top of each slice, completely covering the adhesive/dentin interface and incubated in a dark humid chamber at 37 °C for 24 h.

When bound to gelatin, the fluorophore (fluorescein) does not emit fluorescence, but then gelatin was hydrolyzed by gelatinase, the substrate For

internal negative control group, one slice for each group were incubated with non-fluorescence gelatin, validating that the fluorescence presented on images was provided from proteolytic activity.

After 24 h, the samples were analyzed by laser confocal microscopy (Microscope Leica TCS SPE, Leica Microsystems, Mannheim, BW, GER).

Microtensile bond strength

For microtensile bond strength, seventy-two (n=12) extracted and non-carious third molars were collected and followed the same steps until bonding procedure as *in situ* Zymography. After restoration, specimens were stored in artificial saliva for 24 hours at 37°C and sectioned using an Isomet 1000 digital saw (Buehler, Lake Bluff, IL, USA), perpendicularly to the bonding interface, into beams of ≅0.64 mm^2 area (0.8mm x 0.8mm). To obtain the area, each beam was measured at the dentin-adhesive interface using a digital caliper (Mitutoyo digital caliper, Mitutoyo America, Aurora, IL, USA) and fixed to a jig, with cyanoacrylate glue (Super Bonder Flex Gel-Loctite, Henckel Ltda, Itapevi, SP, Brazil) and tested in tensile in a universal testing machine (Instron 3342, Instron Co., Canton, MA, USA) at a 0.5 mm/min crosshead speed and a 500 N load cell. μTBS (MPa) was calculated through maximum load (kgf) divided by the specimen cross-sectional area (mm^2). The failure mode was analyzed by a hand-held digital microscope (DINO-LITEplus digital microscope, AnMo Electronics Corporation, Hsinchu- China) at 40x magnification and failure was classified in the adhesive, mixed, cohesive in dentin or cohesive in composite resin.

Statistical analyzes

For *In situ* zymography, three images were collected from each sample. The first one was obtained only by fluorescence, where an intense green fluorescence in mineralized dentin and within hybrid layer can be noted. A second image was obtained by reflectance to observe resin composite and adhesive layer dentin. The third image was formed from the overlap of first and second images. 10 μm-thick optical sections were obtained from different focal planes. The images obtained through overlapping were used to assess the fluorescence using Image J (National Institutes of Health, Bethesda, MD, USA). 5 images per groups were selected and a rectangular standardize area was determined to measure the fluorescence intensity

of each image obtaining means of standardized arbitrary measurements. After that, it was performed ANOVA three-way criteria ($p \leq 0.05$) and Tukey test.

For microtensile bond strength, each tooth was considered the experimental unit and one third of beams obtained were used for calculate an average for each specimen. Three-way ANOVA criteria and Tukey tests were applied ($p \leq 0.05$).

RESULTS

In situ zymography

The images below were divided according to aging time and pretreatment and signed with: CR: Composite Resin; HL: Hybrid Layer and D: dentin.

All individual factors (dentin bonding system, pretreatment and time) tested were statistically significant ($p<0.0001$). There was statistically interaction between DBS and pretreatment ($p=0.0223$); DBS and time ($p=<0.0001$) and DBS x pretreatment x time ($p=0.029$). According to DBS, SB showed performed equally with higher fluorescence intensity, regardless time and pretreatment; SUER and SUSE demonstrated an increase in fluorescence overtime aging. For groups pretreated with water, in initial time, no statically difference was observed between DBS. However, after 6 months of aging, SUER showed the highest values and SUSE was not statically different from it. SB presented the lowest fluorescence.

All groups pretreated with CHX demonstrated a significant reduction in fluorescence values, regardless DBS and time. SB-CHX and SUER-CHX demonstrated no difference between initial and 6 months. However, SUSE-CHX showed increased values, showing the highest fluorescence in 6 months.

Microtensile bond strength

Dentin bonding systems was the only significant factor ($p <0.0001$), while pretreatment ($p=0.0917$) and time ($p=0.547$) were not significant. The interaction between the factors ($p> 0.05$) was not significant. In relation to DBS, SUSE presents the highest values and SB and SUER the lowest.

The failure mode distribution analysis is presented in Figure 5, demonstrated that adhesive and mixed failures were predominant in all groups, regardless pretreatment and aging.

DISCUSSION

Overall results indicate that the MDP-based DBS and acid strategy impacted on the performance on bonding to dentin overtime and depends on its interaction with chlorhexidine.

In situ data reveal interesting evidences related to the proteolytic activities in hybrid layer. Analyzing the initial time of all DBS on their control group (water), there was no significant difference in this comparison as attested in table 2 and figures 1a-1c. Although, it is possible to observe different patterns in each DBS. Analyzing the both etch-and-rinse systems, SB presented an intense fluorescence in the hybrid layer and SUER showed a small proteolytic activity, besides a thin layer of adhesive. On the other hand, SUSE presented an evident fluorescence in hybrid layer and larger adhesive layer. This difference between thicknesses of adhesive layers is expected, seeing that in self-etching adhesives the phosphoric acid etching was not performed, consequently the adhesive penetrated superficially in dentin, with a possibility of accumulation on dentin surface [22]. .

Mazzoni et al., 2006 [12] described that both etch-and-rinse and self-etch adhesive systems are capable of increasing the gelatinolytic activity of MMPs present in dentin. Since self-etch adhesive systems could present less proteolytic activity due to lower MMP exposure [12, 23], which is according to the images (Figure 1a-1c).

When associated with CHX, all tested DBS showed reduced proteolytic activities. It is accordance with table 2 and figures 2a-2c. Based on this analyzes, it is relevant to highlight that all of them showed a notable inhibition zone of the proteolytic activity, concentrated mainly in the hybrid layer zone, specially in initial time. For SB-CHX and SUER-CHX, this inhibition zone is evident, while for SUSE-CHX it presented some areas without fluorescence all long the hybrid layer, but did not show an inhibition zone. As SB and SUER are etch-and-rinse, acid etching promotes a demineralization of 5-8μM [22] facilitating CHX penetration [20,24,25].

After 6 months, regarding the same volume and area of analyzes involving hybrid layer and its immediate sublayer, it is possible to note that SB presented similar pattern to its initial condition. However, SUER and SUSE showed increased

fluorescence intensity in this specific zone, which is compatible to the data presented in the table 2 and figures 3a-3c. Adper Single Bond Universal has a complex composition [9,17], particularly due to 10-MDP and polyalkenoic acid copolymer (so-called Vitrebond Copolymer) and a pH around 3. This low pH could activated MMPs and CCs over time, leading to increase the proteolytic activity [4,5,6].

Associating CHX with 6 months aging, SB and SUER performance was similar to their initial condition. On the other hand, this activity seems to be increased for SUSE. A precipitated is observed inside dentinal tubules is observed in all groups treated with CHX after 6 months (Figures 4a-4c). However, the precipitated occurs in Adper Single Bond Universal treated with water: SUER-W-6m and SUSE-W-6m (Figures 3b-3c). This found could be related to calcium interaction between CHX and MDP. In SB-CHX-6m, precipitated can be attributed to CHX, seeing the chemical bind Ca-CHX and the SB did not present MDP in its composition. On the other hand, the precipitated was observed in SUER-W-6m and SUSE-W-6m, may be related to interaction between MDP-Ca, since that there is no CHX application. Whereas SUER-CHX-6m and SUSE-CHX-6m allowed the association MDP-Ca-CHX, probably it may lead to a minimized precipitated formation when compared to SB-CHX-6m, SUER-W-6m. For SUSE-W-6m, it might be due to chemical competition or the chemical bind between MDP-CHX [25]. Da Rosa et al., 2018 [25] described that CHX cations could bind chemically to 10-MDP anions affecting on the bonding process.

In relation to aging over 6 months, SB demonstrated more intense fluorescence with minimal changes overtime, showing no difference between initial and 6 months groups, regardless pretreatment. SUER-W demonstrated an increase on proteolytic activity as well as SUSE-W, however CHX was able to reduce fluorescence in all conditions. Up to 6 months, it is possible to attribute this performance to substantivity of CHX. However, CHX is water-soluble and might be removed from hybrid layer over time [10, 24].

For a better understanding of the association between CHX and MDP, a mechanical test, micro tensile bond, was performed. Data showed that only DBS was a statistically significance factor, then the first null hypothesis was rejected. SUSE showed the highest value while and SB and SUER demonstrated the lowest.

Adper Single Bond Universal is considered a “mild” self-etching DBS, what means that it is capable to partially remove smear layer, remaining hydroxyapatite around exposed collagen [17,19,26].10-MDP depends on calcium release to promote

a satisfactory adhesion, then in a self-etching mode, more calcium is available consequently 10-MDP performed an adequate bonding [15,17,19].. This action may explain its better performance compared to the etch-and-rinse systems, regardless the presence of MDP.

As no statistically difference was found between initial and 6 months aging time, the third hypothesis was accepted. CHX was able to maintain μTBS stable and did not provoke a negative effect, regardless DBS used, just as demonstrated by zymography *in situ* result. Several studies demonstrated that the association between CHX and universal adhesives systems could be a promising strategy to increase the bonding durability [14,15,27,28]. Tekçe et al., 2016 [28] showed that chlorhexidine could increase the bond durability of mild universal adhesives. On the other hand, Giacomini et al., 2017 [7] founded that collagenolytic enzyme inhibition, such as chlorhexidine, did not improve the durability of bond strength associated with the SU and these results may derive from an interaction between CHX and MDP.

Although, these negative results could be related to time and active mode application of CHX. Di Hipólito et al., 2012 [20] also applied CHX for 60s and the results was not satisfactory. In this study, CHX was applied on a passive mode and may not lead to erosion on dentin surface. Besides interaction CHX and 10-MDP, CHX persists on dentin surface, impairing in hybrid layer formation, seeing that SU has a complex composition [9,25]. It has been speculated that this negative correlation between CHX and multi-modes adhesives could be directly related to their composition [9,25].

Based on the overall performance of both methodologies, self-etching MDP-based universal dentin system seems to be promissory overtime, while CHX can be adjunctively associated for the reduction of proteolytic activity.

CONCLUSION

MDP-based multimode system demonstrated an improvement on dentin bonding performance, especially in the self-etching mode, compared to an MDP-free ER system. CHX did not negative affect bond strength of any of the tested DBSs and an impact between MDP and CHX was not proven.

Acknowledgements

The authors are grateful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001 and CNPq (163402/2015-9 and 408865/2016-4) for the financial support.

References

1. L. Breschi, T. Maravic, S.R. Cunha, A. Comba, M. Cadenaro, L. Tjäderhane, D.H. Pashley, F.R. Tay, A. Mazzoni, Dentin bonding systems: From dentin collagen structure to bond preservation and clinica l applications, Dent. Mater. 34 (2018) 78-96. https://doi.org/10.1016/j.dental.2017.11.005.

2. G. Göstemeyer, F. Schwendicke, Inhibition of hybrid layer degradation by cavity pretreatment: Meta- and trial sequential analysis, J. Dent. 49 (2016) 14-21. https://doi.org/10.1016/j.jdent.2016.04.007

3. R. Gendron, D. Grenier, T. Sorsa, D. Mayrand, Inhibition of the activities of matrix metalloproteinases 2, 8, and 9 by chlorhexidine, Clin. Diagn. Lab. Immunol. 6 (1999) 437-439.

4. P.M. Scaffa, C.M. Vidal, N. Barros, T.F. Gesteira, A.K. Carmona, L. Breschi, D.H. Pashley, L. Tjäderhane, I.L. Tersariol, F.D. Nascimento, M.R. Carrilho, Chlorhexidine inhibits the activity of dental cysteine cathepsins, J. Dent. Res. 91 (2012) 420-425. https://doi.org/10.1177/0022034511435329.

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

6. C.M. Vidal, L. Tjäderhane, P.M. Scaffa, I.L. Tersariol, D. Pashley, H.B. Nader, F.D. Nascimento, M.R. Carrilho, Abundance of MMPs and cysteine cathepsins in

caries-affected dentin, J. Dent. Res. 93 (2014) 269-274. https://doi.org/10.1177/0022034513516979.

7. M.C. Giacomini, P. Scaffa, L.P. Chaves, C. Vidal, T.N. Machado, H.M. Honório, L. Tjäderhane, L. Wang, Role of Proteolytic Enzyme Inhibitors on Carious and Eroded Dentin Associated With a Universal Bonding System, Oper. Dent. 42 (2017) E188-E196. https://doi.org/10.2341/16-178-L.

8. L.P. Chaves, T.L. Ciantelli, D.F.G. Araújo, M.C. Giacomini, L. Tjäderhane, P.M.C. Scaffa, H.M. Honório, L. Wang, How proteolytic inhibitors interact with dentin on glass-fiber post luting over 6 months, J. Mech. Behav. Biomed. Mater. 79 (2018) 348-353 https://doi.org/10.1016/j.jmbbm.2018.01.011.

9. A.F. Montagner, T. Pereira-Cenci, M.S. Cenci, Influence of cariogenic challenge on bond strength stability of dentin, Braz. Dent. J. 26 (2015) 128-134. https://doi.org/10.1590/0103-6440201300348.

10. M.R. Carrilho, R.M. Carvalho, M.F. de Goes, V. di Hipólito, S. Geraldeli, F.R. Tay, D.H. Pashley, L. Tjäderhane, Chlorhexidine preserves dentin bond in vitro, J. Dent. Res. 86 (2007) 90-94. https://doi.org/10.1177/154405910708600115.

11. M. Favetti, T. Schroeder, A.F. Montagner, M.B. Correa, T. Pereira-Cenci, M.S. Cenci, Effectiveness of pre-treatment with chlorhexidine in restoration retention: A 36-month follow-up randomized clinical trial, J. Dent. 60 (2017) 44-49 https://doi.org/10.1016/j.jdent.2017.02.014.

12. A. Mazzoni, D.H. Pashley, Y. Nishitani, L. Breschi, F. Mannello, L. Tjäderhane, M. Toledano, E.L. Pashley, F.R. Tay, Reactivation of inactivated endogenous proteolytic activities in phosphoric acid-etched dentine by etch-and-rinse adhesives, Biomaterials 27 (2006) 4470-4476. https://doi.org/10.1016/j.biomaterials.2006.01.040.

13. A. Mazzoni, F.D. Nascimento, M. Carrilho, I. Tersariol, V. Papa, L. Tjäderhane, R. Di Lenarda, F.R. Tay, D.H. Pashley, L. Breschi, MMP activity in the hybrid layer detected with in situ zymography, J. Dent. Res. 91 (2012) 467-472. https://doi.org/10.1177/0022034512439210.

14. M. Hanabusa, A. Mine, T. Kuboki, Y. Momoi, A. Van Ende, B. Van Meerbeek, J. De Munck, Bonding effectiveness of a new 'multi-mode' adhesive to enamel and dentine. J. Dent. 40 (2012) 475-484. https://doi.org/10.1016/j.jdent.2012.02.012.

15. G. Marchesi, A. Frassetto, A. Mazzoni, F. Apolonio, M. Diolosà, M. Cadenaro, R. Di Lenarda, D.H. Pashley, F. Tay, L. Breschi, Adhesive performance of a multi-mode adhesive system: 1-year in vitro study, J. Dent. 42 (2014) 603-612. https://doi.org/10.1016/j.jdent.2013.12.008.

16. A. Wagner, M. Wendler, A. Petschelt, R. Belli, U. Lohbauer, Bonding performance of universal adhesives in different etching modes, J. Dent. 42 (2014) 800-807. https://doi.org/10.1016/j.jdent.2014.04.012.

17. W.L. Rosa, E. Piva, A.F. Silva, Bond strength of universal adhesives: A systematic review and meta-analysis, J. Dent. 43 (2015) 765-776. https://doi.org/10.1016/j.jdent.2015.04.003.

18. A.A. Elkaffas, H.H.H. Hamama, S.H. Mahmoud, Do universal adhesives promote bonding to dentin? A systematic review and meta-analysis, Restor. Dent. Endod. 43 (2018) e29. https://doi.org/10.5395/rde.2018.43.e29.

19. Y. Yoshida, K. Nagakane, R. Fukuda, Y. Nakayama, M. Okazaki, H. Shintani, S. Inoue, Y. Tagawa, K. Suzuki, J. De Munck, B. Van Meerbeek, Comparative study on adhesive performance of functional monomers, J. Dent. Res. 83 (2004) 454-458. https://doi.org/10.1177/154405910408300604.

20. V. Di Hipólito, F.P. Rodrigues, F.B. Piveta, C. Azevedo Lda, R.C. Bruschi Alonso, N. Silikas, R.M. Carvalho, M.F. De Goes, P.H. Perlatti D'Alpino,

Effectiveness of self-adhesive luting cements in bonding to chlorhexidine-treated dentin, Dent. Mater. 28 (2012) 495-501. https://doi.org/10.1016/j.dental.2011.11.027.

21. D.H. Pashley, L. Tao, L. Boyd, G.E. King, J.A. Horner JA, Scanning electron microscopy of the substructure of smear layers in human dentine. Arch Oral Biol. 33 (1988) 265-270.

22. D.H. Pashley, F.R. Tay, C. Yiu, M. Hashimoto, L. Breschi, R.M. Carvalho. S. Ito, Collagen degradation by host-derived enzymes during aging, J. Dent. Res. 83 (2004) 216-221. https://doi.org/10.1177/154405910408300306.

23. Y. Nishitani, M. Yoshiyama, B. Wadgaonkar, L. Breschi, F. Mannello, A. Mazzoni, R.M. Carvalho, L. Tjäderhane, F.R. Tay, D.H. Pashley, Activation of gelatinolytic/collagenolytic activity in dentin by self-etching adhesives, Eur. J. Oral Sci. 114 (2006) 160-166. https://doi.org/10.1111/j.1600-0722.2006.00342.x

24. A. Frassetto, L. Breschi, G. Turco, G. Marchesi, R. Di Lenarda, F.R. Tay, D.H. Pashley, M. Cadenaro, Mechanisms of degradation of the hybrid layer in adhesive dentistry and therapeutic agents to improve bond durability--A literature review, Dent. Mater. 32 (2016) e41-53. https://10.1016/j.dental.2015.11.007

25. L.S. da Rosa, A.C. Follak, T.L. Lenzi, R.O. Rocha, F.Z.M. Soares, Phosphoric Acid Containing Chlorhexidine Compromises Bonding of Universal Adhesive, J. Adhes. Dent. 20 (2018) 243-247. https://doi.org/10.3290/j.jad.a40631.

26. S. Inoue, M.A. Vargas, Y. Abe, Y. Yoshida, P. Lambrechts, G. Vanherle, H. Sano, B. Van Meerbeek, Micro-tensile bond strength of eleven modern adhesives to dentin, J. Adhes. Dent. 3 (2001) 237-246.

27. M.A. Muñoz, I. Luque-Martinez, P. Malaquias, V. Hass, A Reis, N.H. Campanha, A.D. Loguércio, In vitro longevity of bonding properties of universal adhesives to dentin, Oper. Dent. 18 (2015) 282-292. https:// doi: 10.2341/14-055-L.

28. N. Tekçe, S. Tuncer, M. Demirci, S. Balci, Do matrix metalloproteinase inhibitors improve the bond durability of universal dental adhesives? Scanning. 38 (2016) 535-544. https://doi.org/10.1002/sca.21293.

Tables

Table 1: Protocols of application of each DBS and their composition

	Water (W)	**2% Chlorhexidine Digluconate (CHX)**	**Composition**
Adper Single Bond 2 (SB)	37% phosphoric acid etching for 30s + Rinse for 15s + drying with absorbent paper + Active DBS application + Solvent evaporation for 5s + Light cure for 10s.	37% phosphoric acid etching for 15s + Rinse for 30s + drying with absorbent paper + Passive application of CHX for 30s + Removal excess with absorbent paper + Active DBS application + Solvent evaporation for 5s + Light cure for 10s.	Dimethacrylate resins, HEMA, Vitrebond™ Copolymer, Filler, Ethanol, Water, Initiators
Adper Single Bond Universal – Etch and Rinse Mode (SUER)	37% phosphoric acid etching for 15s + Rinse for 30s + drying with absorbent paper + Active DBS application + Solvent evaporation for 5s + Light cure for 10s.	37% phosphoric acid etching for 15s + Rinse for 30s + drying with absorbent paper + Passive application of CHX for 30s + Removal excess with absorbent paper + Active DBS application + Solvent evaporation for 5s + Light cure for 10s.	MDP Phosphate Monomer, Dimethacrylate resins, HEMA Vitrebond™ Copolymer, Filler, Ethanol, Water, Initiators, Silane
Adper Single Bond Universal – Self Etching Mode (SUSE)	37% phosphoric acid selective enamel etching Rinse for 15s + Rinse for 30s + drying with absorbent paper + Active DBS application + Application of a second DBS layer + Solvent evaporation for 5s + Light cure for 10s.	37% phosphoric acid selective enamel etching + Rinse for 15s + Rinse for 30s + drying with absorbent paper + + Passive application of CHX for 30s + Removal excess with absorbent paper Active DBS application + Application of a second DBS layer + Solvent evaporation for 5s + Light cure for 10s.	

Table 2: Mean values (au) of fluorescence according to DBS, pretreatment and time

	W-I	CHX-I	W-6m	CHX-6m
SB	110.395EF	56.321D	105.883EFG	56.616D
SUER	125.611BE	83.184CG	164.171A	87.004CFG
SUSE	106.171EFG	69.729CD	145.693AB	105.346EFG

N=3, p<0.05; SB= Adper Single Bond 2; SUER= Adper Single Bond Universal- Etch-and-rinse mode; SUSE= Adper Single Bond Universal- Self-etching mode
Different letters indicate statistical differences (p<0.05)

Table 3: Mean values (MPa) and standard deviation of bond strength to dentin of DBS pretreated with water or CHX on initial and 6 months aging time.

Dentin Bonding System	Pretreatment	Time	
		Initial	6 Months
SB	Water	33.35±9.01b	32.59± 9.44b
	CHX	28.41±7.64b	31.55± 6.15b
SUER	Water	31.62±8.20b	32.05± 7.04b
	CHX	33.66±7.79b	33.79± 6.24b
SUSE	Water	45.62±12.39a	40.15± 14.77a
	CHX	37.47±10.68a	34.25± 11.21a

N=12, p<0.05; SB= Adper Single Bond 2; SUER= Adper Single Bond Universal- Etch-and-rinse mode; SUSE= Adper Single Bond Universal- Self-etching mode
Different lowercase letters indicate statistically significant difference between DBS in the same condition (pretreatment and time).

Figures

Initial time

Water

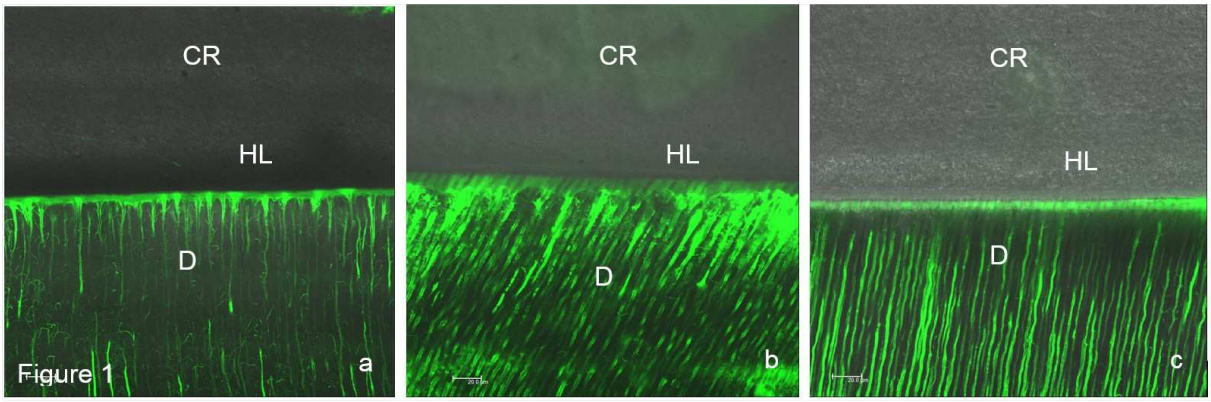


Figure 1

Figure 1- a: SB2-W: Intense fluorescence is observed in the hybrid layer; b: SUER-W: A small proteolytic activity is observed in hybrid layer and a smaller thin layer of adhesive; c: SUSE-W: Notable proteolytic activity is observed in hybrid layer and a larger adhesive layer.

CHX

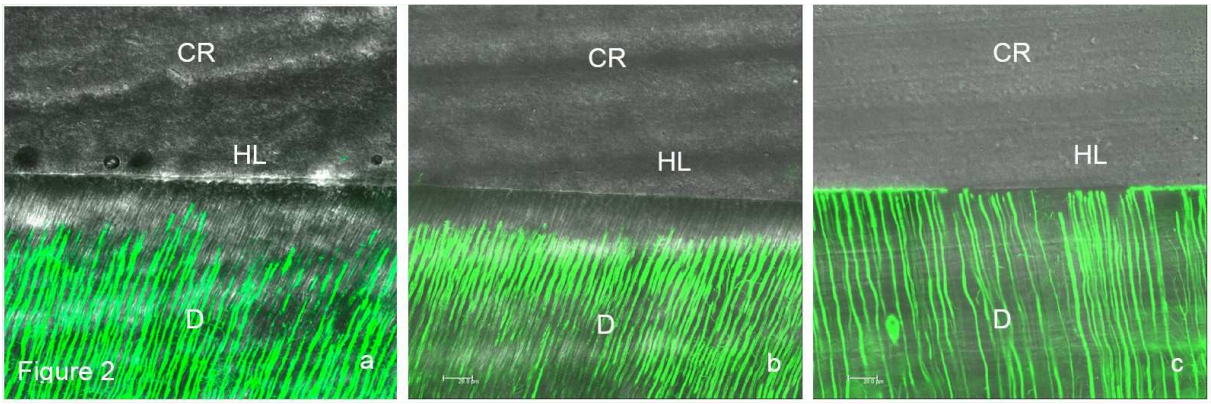


Figure 2

Figure 2- a and b: SB-CHX/SUER-CHX: A non-fluorescence strip is observed below the hybrid layer – A inhibition zone of proteolytic activity promoted by CHX; c: SUSE-CHX: Fluorescence is observed in parts of the hybrid layer indicating proteolytic activity, even after the application of CHX. There is no presence zone of inhibition

6 Months

Water

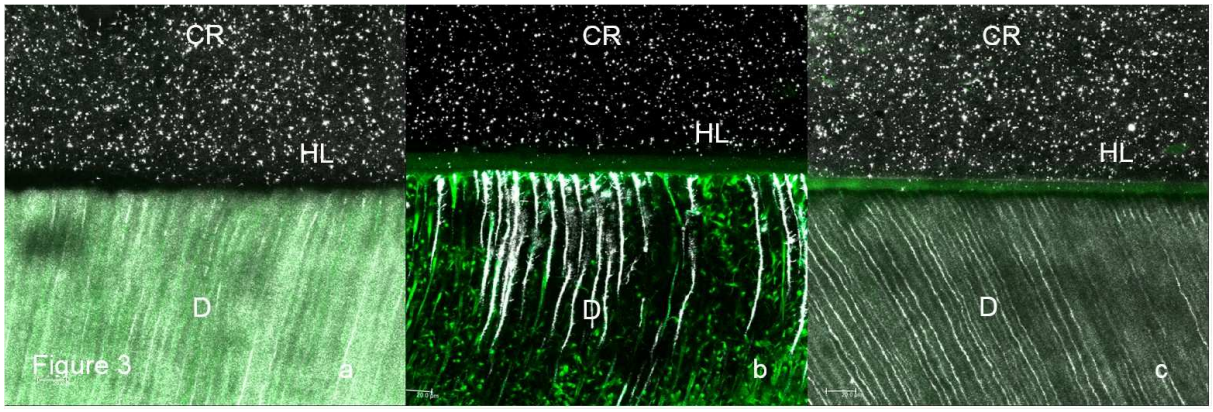


Figure 3-a: SB-W-6m: Fluorescence is observed in all dentin surface, but not in hybrid layer. The adhesive layer is notable and irregular; b: SUER-W-6m: An intensity fluorescence is observed in hybrid layer; c: SUSE-W-6m- Fluorescence is observed all over the dentin, but not in hybrid layer; adhesive layer is more uniform.

CHX

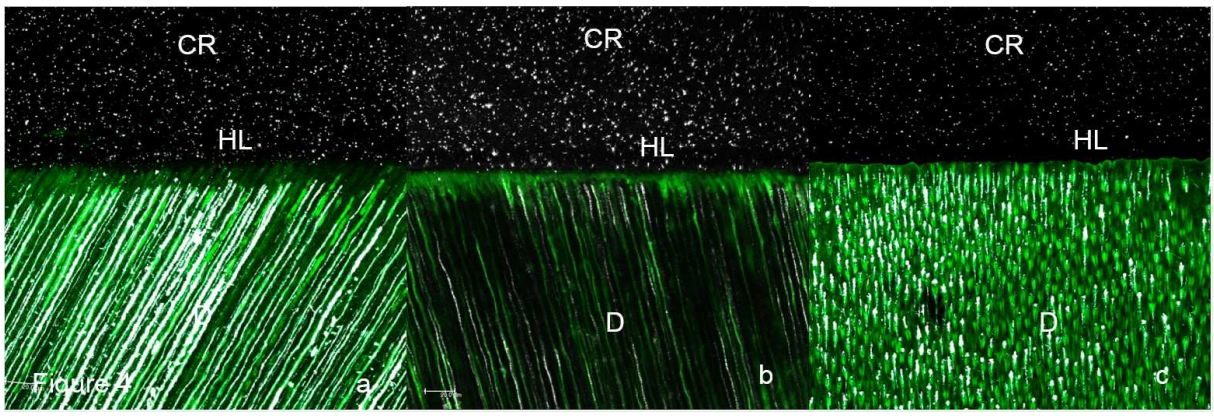


Figure 4-a: SB-CHX-6m: A zone with lower fluorescence is observed below hybrid layer; b: SUER-CHX-6m: Fluorescence is noted in hybrid layer; c: SUSE-CHX-6m: Fluorescence is observed all over dentin surface. In all images, the adhesive layer is regular.

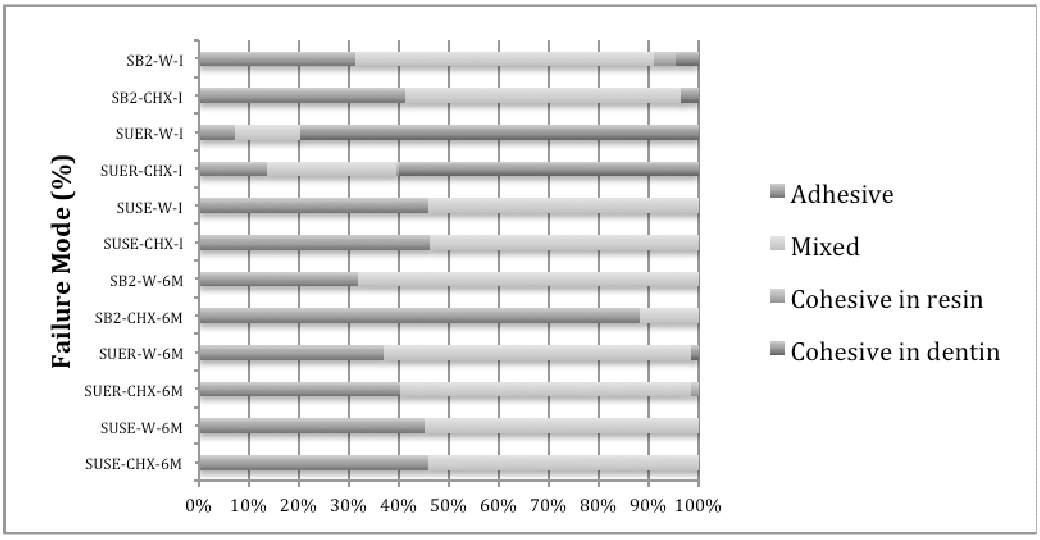


Figure 5 – Failure mode distribution (%) regarding all substrates and pretreatment with inhibitors in immediate, six and eighteen months time.

3 Discussion

3 DISCUSSION

The long lasting of hybrid layer persists as a relevant challenge in adhesive restorative dentistry (Tjadrhane et al., 2013; Breschi et al., 2018) mostly in altered substrate, as carious and eroded dentin (Favetti et al., 2017; Isolan et al., 2018; Siqueira et al., 2018). Demineralized dentin presents a high water concentration (Carvalho et al., 2012) changing its composition, impairing directly in adhesive process (Carvalho et al., 2012). The main difference between carious and eroded dentin consists on the integrity of collagen fibrils (Tjaderhane et al., 2013; Buzalaf et al., 2015; Tjaderhane et al., 2015). Carious dentin presents disorganized mesh of collagen fibrils, loss of mineral content and increase porosity of intertubular dentin (Isolan et al., 2018) leading to an irregular hybrid layer with a thicker adhesive layer (Haj-Ali et al., 2006) and presents low values for microtensile bond strength (Nicoloso et al., 2017; Isolan et al., 2018). However, the collagen cross-linking remains intact, acting like a scaffold to remineralizing dentin (Fusayama et al., 1991; Nakornchai et al., 2004). Gonçalves et al., 2018 proved that with 1.5% STMP solution promotes dentin remineralization after carious lesions. In contrast, eroded dentin presents denude collagen fibrils due to mineral loss, although the collagen structure is maintained (Magalhães et al., 2009; Tjaderhane et al., 2013). These morphological differences could explain that normal and eroded dentin did not show statically difference in article 1 and artificial carious dentin the lower values.

Comparing the profile of MMPs and CCs in sound and normal dentin to carious and eroded dentin (Tjaderhane et al., 1998; Tjaderhane et al., 2013; Buzalaf et al., 2015; Scaffa et al., 2017) and the degradation profile of hybrid layer (Tjaderhane et al., 1998; Pashley et al., 2011), the use of a proteolytic inhibitor seems to be an appropriate strategy to improve the durability of hybrid layer overtime (Carrilho et al., 2007; Carrilho et al., 2010; Tjaderhane et al., 2013), even with some limitations and interactions.

Adper Single Bond Universal (SU) was used in etch-and-rinse mode to expose the highest proteolytic activity (Mazzoni et al., 2006; Apolonio et al., 2017). Chlorhexidine is an antimicrobial and employed in dentistry as cavity cleaner and more recent as proteolytic inhibitor (Gerdron et al., 1999; Hebling et al., 2005; Carrilho et al., 2007) even in lower concentrations. CHX is able to inhibit MMPs but also CCs (Scaffa et al., 2012) and E64 has its specific action in CCs (Tersariol et al.,

2010; Nascimento et al., 2011; Vidal et al., 2014). In article 1, both agents were associated with SU, and CHX showed a negative effect regardless the substrate, leading to a hypothesis of a possible interaction between CHX and 10-MDP (Di Hipólito et al., 2012; Giacomini et al., 2017; da Rosa et al., 2018). Da Rosa et al., 2018 founded that phosphoric acid with chlorhexidine incorporation impaired negatively in SU, which not occurred when SB was used. On the other hand, E64 was only injurious for normal dentin in initial time. An interesting find was observed after 18 months aging, as all groups regardless of the substrate and pretreatment showe no statistical significance difference. CHX is water-soluble and its binding was shown to be a reversible mechanism, and probably due to leaching, the resin–dentin interfaces treated with CHX were shown to be unstable after 18 months (Sadek et al., 2010; Ricci et al., 2010; Frassetto et al., 2016; Breschi et al., 2018). Also, E64 proved not to be efficient in long-term evaluation.

Previous studies suggested this negative relation CHX-10-MDP (Francisconi-dos-Rios et al., 2015a; Francisconi-dos-Rios et al., 2015b; Giacomini et al., 2017). The interaction between 10-MDP and CHX depends on the DBS composition (Montagner et al., 2015; da Rosa et al., 2018) and da Rosa et al., 2018 elucidated a possible bind directly between CHX cations and 10-MDP anions, impairing them functionality of both.

Therefore, a biological assessment was required through *in situ* zymography tests associated with a mechanical test, keeping the sound dentin substrate as control group to evaluate the performance of different categories of DBSs and strategies, adopting the SU in both modes and a two-step MDP-free DBS, Adper Single Bond 2, (SB) providing the article 2.

Mazzoni et al., 2012 used *in situ* zymography to demonstrate the activation of MMPs in the hybrid layer with both etching and adhesive strategies. Generally, the highest level of proteolytic activity of etch-and-rinse DBSs when compared to the self-etching DBSs appears to be correlated with a faster destruction of hybrid layer (Mazzoni et al., 2013; Apolonio et al., 2017). However, this difference may be due to acid etching performed on dentin when using an etch-and-rinse adhesive system, exposing a greater amount of dentin matrix in comparison to the use of self-etching adhesive system (Mazzoni, et al., 2013). In the self-etching adhesives, the exposure of MMPs bound to the matrix was accompanied by an increase in activity, but with activity extension reduced (Mazzoni, et al., 2013).

In article 2, the speculation around the interaction CHX-10-MDP was analyzed, considering if CHX still is effective overtime. It was showed that CHX is able to reduce the proteolytic activity regardless of DBSs up to 6 months aging. Adper Single Bond 2 pretreated with water demonstrated to present more intense activity overtime, while Adper Single Bond Universal pretreated with water showed an increase of proteolytic activity in both mode after 6 months. Mazzoni et al. 2013 described that the acid etching provokes highest level of proteolytic activity due to the large zone of dentin organic matrix exposed, however for self-etching adhesives, proteolytic activity occurs, even activity extension reduced. There are evidences that acid-etching dentin with 37% phosphoric acid, with pH as low as 0.4, can initially keeps MMPs inactivated (Pashley et al., 2004; Mazzoni et al., 2006; De-Vito Moraes et al., 2016), but later when the environment pH gets higher, MMPs would regain their ability to catalyze the degradation of their target tissue components (Tjaderhane et al., 1998; De-Vito Moraes et al., 2016).

Adper Single Bond Universal is considered a “mild” DBS, demineralizing partially the smear layer (Inoue et al., 2001; Yoshida et al., 2004, Rosa et al., 2015) leaving calcium ions to 10-MDP bind. The lower pH around 3 could persist on hybrid, increasing the proteolytic activity overtime. Overall, in article 2, SU in self-etching mode showed the highest values and SU in etch-and-rinse mode was not different from SB for microtensile bond strength.

There is no determination of which mode presents greater results, as it depends on DBS composition (Rosa et al., 2015) and dentin substrate. For eroded dentin, Siqueira et al., 2018 concluded that application mode did not influence for *in vitro* erosion. For Oliveira et al., 2017 self-etching mode is more appropriate as they considered a hypermineralized sclerotic dentin substrate. For carious dentin, Isolan et al., 2018 concluded that etch-and-rinse is the strategy indicated. In relation to CHX, Shadman et al., 2018 showed that CHX did not show any effect in initial bond and Favetti et al., 2017 described that no benefit is found in an evaluation after 36 months on non-carious cervical lesions.

This study highlighted that the successful performance of bonding to dentin is extremely dependent on the dynamic of a complex dental structure, and their alterations and susceptibilities and how it interact with the dentin bonding agents and other materials that are used together. Therefore, even the properties provided by

the functional monomers and chlorhexidine as a proteolytic agent are limited overtime, specially regarding demineralized dentin.

4 Final Considerations

4 FINAL CONSIDERATIONS

The maintenance of integrity and longevity of hybrid layer persist on a challenge for adhesive dentistry. Overall, the bonding procedures are performed above altered dentin, as carious and eroded conditions, however carious dentin is the most challenge substrate. The dentin might be deeply investigated to improve the understanding about how it interact with dental materials.

Chlorhexidine can be used as an enzyme inhibitor, maybe in specific conditions, but it is not able to maintain its activity for a long time. Nevertheless, it did not affect negatively the bond strength overtime and it is able to reduce proteolytic activity up to 6 months.

References

REFERENCES

1. Apolonio FM, Mazzoni A, Angeloni V, Scaffa PM, Santi S, Saboia VP et al. Effect of a one-step self-etch adhesive on endogenous dentin matrix metalloproteinases. Eur J Oral Sci. 2017;125(2):168-172.

2. Breschi L, Maravic T, Cunha SR, Comba A, Cadenaro M, Tjäderhane L et al. Dentin bonding systems: From dentin collagen structure to bond preservation and clinical applications. Dent Mater. 2018;34(1):78-96.

3. Buzalaf MA, Charone S, Tjäderhane L. Role of host-derived proteinases in dentine caries and erosion. Caries Res. 2015;49(1):30-37.

4. Carrilho MR, Carvalho RM, Sousa EN, Nicolau J, Breschi L, Mazzoni A et al. Substantivity of chlorhexidine to human dentin. Dent Mater. 2010;26(8):779-785.

5. Carrilho MR, Geraldeli S, Tay F, de Goes MF, Carvalho RM, Tjäderhane L et al. In vivo preservation of the hybrid layer by chlorhexidine. J Dent Res. 2007;86(6):529-533.

6. Carvalho RM, Tjäderhane L, Manso AP, Carrilho MR, Carvalho CAR. Dentin as a bonding substrate. Endodontic Topics. 2012;21(1):62–88.

7. da Rosa LS, Follak AC, Lenzi TL, Rocha RO, Soares FZM. Phosphoric Acid Containing Chlorhexidine Compromises Bonding of Universal Adhesive. J Adhes Dent. 2018;20(3):243-247.

8. DeVito-Moraes AG, Francci C, Vidal CM, Scaffa PM, Nesadal D, Yamasaki LC et al. Phosphoric acid concentration affects dentinal MMPs activity. J Dent. 2016;53:30-37.

9. Di Hipólito V, Rodrigues FP, Piveta FB, Azevedo Lda C, Bruschi Alonso RC, Silikas N et al. Effectiveness of self-adhesive luting cements in bonding to chlorhexidine-treated dentin. Dent Mater. 2012;28(5):495-501.

10. Favetti M, Schroeder T, Montagner AF, Correa MB, Pereira-Cenci T, Cenci MS. Effectiveness of pre-treatment with chlorhexidine in restoration retention: A 36-month follow-up randomized clinical trial. J Dent. 2017;60:44-49.

11. Francisconi-dos-Rios LF, Calabria MP, Casas-Apayco LC, Honório HM, Carrilho MR, Pereira JC et al. Chlorhexidine does not improve but preserves bond strength to eroded dentin. Am J Dent. 2015a;28(1):28-31.

12. Francisconi-dos-Rios LF, Casas-Apayco LC, Calabria MP, Francisconi PA, Borges AF, Wang L. Role of chlorhexidine in bond strength to artificially eroded dentin over time. J Adhes Dent. 2015b;17(2):133-139.

13. Frassetto A, Breschi L, Turco G, Marchesi G, Di Lenarda R, Tay FR et al. Mechanisms of degradation of the hybrid layer in adhesive dentistry and therapeutic agents to improve bond durability--A literature review. Dent Mater. 2016;32(2):e41-53.

14. Frassetto A, Breschi L, Turco G, Marchesi G, Di Lenarda R, Tay FR et al. Mechanisms of degradation of the hybrid layer in adhesive dentistry and therapeutic agents to improve bond durability--A literature review. Dent Mater. 2016;32(2):e41-53.

15. Fusayama T. Intratubular crystal deposition andremineralization of carious dentin. J Biol Buccale. 1991;19(3):255–262.

16. Gendron R, Grenier D, Sorsa T & Mayrand D. Inhibition of the activities of matrix metalloproteinases 2, 8, and 9 by chlorhexidine. Clin Diagn Lab Immunol. 1999;6(3):437-439.

17. Giacomini MC, Scaffa P, Chaves LP, Vidal C, Machado TN, Honório HM et al. Role of Proteolytic Enzyme Inhibitors on Carious and Eroded Dentin Associated With a Universal Bonding System. Oper Dent. 2017;42(6):E188-E196.

18. Gonçalves RS, Scaffa PMC, Giacomini MC, Vidal CMP, Honório HM, Wang L. Sodium Trimetaphosphate as a Novel Strategy for Matrix Metalloproteinase Inhibition and Dentin Remineralization. Caries Res. 2018;52(3):189-198.

19. Göstemeyer G, Schwendicke F. Inhibition of hybrid layer degradation by cavity pretreatment: Meta- and trial sequential analysis. J Dent. 2016;49:14-21.

20. Haj-Ali R, Walker M, Williams K, Wang Y, Spencer P. Histomorphologic characterization of noncarious and caries-affected dentin/adhesive interfaces. J Prosthodont. 2006;15(2):82-88.

21. Hanabusa M, Mine A, Kuboki T, Momoi Y, Van Ende A, Van Meerbeek B et al. Bonding effectiveness of a new “multi-mode” adhesive to enamel and dentine. J Dent. 2012;40(6):475-484.

22. Hashimoto M, Ohno H, Kaga M, Sano H, Endo K, Oguchi H. Fractured surface characterization: wet versus dry bonding. Dent Mater. 2002;18(2):95-102.

23. Hebling J, Pashley DH, Tjäderhane L, Tay FR. Chlorhexidine arrests subclinical degradation of dentin hybrid layers in vivo. J Dent Res. 2005;84(8):741-746.

24. Huysmans MC, Chew HP, Ellwood RP. Clinical studies of dental erosion and erosive wear. Caries Res. 2011;45(1):60-68.

25. Innes NP, Frencken JE, Bjørndal L, Maltz M, Manton DJ, Ricketts D et al. Managing Carious Lesions: Consensus Recommendations on Terminology. Adv Dent Res. 2016;28(2):49-57.

26. Inoue S, Vargas MA, Abe Y, Yoshida Y, Lambrechts P, Vanherle G et al. Micro-tensile bond strength of eleven modern adhesives to dentin. J Adhes Dent. 2001;3(3):237-246.

27. Isolan CP, Sarkis-Onofre R, Lima GS, Moraes RR. Bonding to sound and caries-affected dentin: a systematic review and meta-analysis. J Adhes Dent. 2018;20(1):7-18

28. Machado CM, Bim Júnior O, Giacomini MC, Graeff MSZ, Garcia FCP, Rios D et al. Effect of ethanol-dissolved rhodamine B marker on mechanical properties of non-simplified adhesives. J Mech Behav Biomed Mater. 2018;84:145-150.

29. Magalhães AC, Wiegand A, Rios D, Honório HM, Buzalaf MA. Insights into preventive measures for dental erosion. J Appl Oral Sci. 2009;17(2):75-86.

30. Marchesi G, Frassetto A, Mazzoni A, Apolonio F, Diolosà M, Cadenaro M et al. Adhesive performance of a multi-mode adhesive system: 1-year in vitro study. J Dent. 2014;42(5):603-612.

31. Mazzoni A, Nascimento FD, Carrilho M, Tersariol I, Papa V, Tjäderhane L et al. MMP activity in the hybrid layer detected with in situ zymography. J Dent Res. 2012;91(5):467-472.

32. Mazzoni A, Pashley DH, Nishitani Y, Breschi L, Mannello F, Tjäderhane L et al. Reactivation of inactivated endogenous proteolytic activities in phosphoric acid-etched dentine by etch-and-rinse adhesives. Biomaterials. 2006;27(25):4470-4476.

33. Mazzoni A, Scaffa P, Carrilho M, Tjäderhane L, Di Lenarda R, Polimeni A et al. Effects of etch-and-rinse and self-etch adhesives on dentin MMP-2 and MMP-9. J Dent Res. 2013;92(1):82-86.

34. Montagner AF, Pereira-Cenci T, Cenci MS. Influence of cariogenic challenge on bond strength stability of dentin. Braz Dent J. 2015;26(2):128-134.

35. Nakabayashi N, Nakamura M, Yasuda N. Hybrid layer as a dentin-bonding mechanism. J Esthet Dent. 1991;3(4):133-138.

36. Nakornchai S, Atsawasuwan P, Kitamura E, Surarit R,Yamauchi M. Partial biochemical characterisation ofcollagen in carious dentin of human primary teeth. ArchOral Biol. 2004;49(4):267–273.

37. Nascimento FD, Minciotti CL, Geraldeli S, Carrilho MR, Pashley DH, Tay FR et al. Cysteine cathepsins in human carious dentin. J Dent Res. 2011;90(4):506-511.

38. Nicoloso GF, Antoniazzi BF, Lenzi TL, Soares FZM, Rocha RO. The Bonding Performance of a Universal Adhesive to Artificially-created Caries-affected Dentin. J Adhes Dent. 2017;19(4):317-321.

39. Nishitani Y, Yoshiyama M, Wadgaonkar B, Breschi L, Mannello F, Mazzoni A et al. Activation of gelatinolytic/collagenolytic activity in dentin by self-etching adhesives. Eur J Oral Sci. 2006;114(2):160-166.

40. Oliveira B, Ulbaldini A, Sato F, Baesso ML, Bento AC, Andrade L et al. Chemical Interaction Analysis of an Adhesive Containing 10-Methacryloyloxydecyl Dihydrogen Phosphate (10-MDP) With the Dentin in Noncarious Cervical Lesions. Oper Dent. 2017;42(4):357-366

41. Pashley DH, Depew DD, Galloway SE. Microleakage channels: scanning electron microscopic observation. Oper Dent. 1989;14(2):68-72.

42. Pashley DH, Tay FR, Breschi L, Tjäderhane L, Carvalho RM, Carrilho M et al. State of the art etch-and-rinse adhesives. Dent Mater. 2011;27(1):1-16.

43. Pashley DH, Tay FR, Yiu C, Hashimoto M, Breschi L, Carvalho RM et al. Collagen degradation by host-derived enzymes during aging. J Dent Res. 2004;83(3):216-221.

44. Ricci HA, Sanabe ME, de Souza Costa CA, Pashley DH, Hebling J. Chlorhexidine increases the longevity of in vivoresin–dentin bonds. Eur J Oral Sci. 2010;118(4):411–416.

45. Rosa WL, Piva E, Silva AF. Bond strength of universal adhesives: A systematic review and meta-analysis. J Dent. 2015;43(7):765-776.

46. Sadek FT, Braga RR, Muench A, Liu Y, Pashley DH, Tay FR. Ethanol wet-bonding challenges current anti-degradationstrategy. J Dent Res. 2010;89(12):1499–1504.

47. Scaffa PM, Breschi L, Mazzoni A, Vidal CM, Curci R, Apolonio F, Gobbi P, Pashley D, Tjäderhane L, Tersariol IL, Nascimento FD, Carrilho MR. Co-distribution of cysteine cathepsins and matrix metalloproteases in human dentin. Arch Oral Biol. 2017;74:101-107.

48. Scaffa PM, Vidal CM, Barros N, Gesteira TF, Carmona AK, Breschi et al. Chlorhexidine inhibits the activity of dental cysteine cathepsins. J Dent Res. 2012;91(4):420-425.

49. Schwendicke F, Frencken JE, Bjørndal L, Maltz M, Manton DJ, Ricketts D et al. Managing Carious Lesions: Consensus Recommendations on Carious Tissue Removal. Adv Dent Res. 2016;28(2):58-67.

50. Shadman N, Farzin-Ebrahimi S, Mortazavi-Lahijani E, Jalali Z. Effect of chlorhexidine on the durability of a new universal adhesive system. J Clin Exp Dent. 2018;10(9):e921-e926.

51. Siqueira FSF, Cardenas AM, Ocampo JB, Hass V, Bandeca MC, Gomes JC et al. Bonding Performance of Universal Adhesives to Eroded Dentin. J Adhes Dent. 2018;20(2):121-132.

52. Tersariol IL, Geraldeli S, Minciotti CL, Nascimento FD, Pääkkönen V, Martins MT et al. Cysteine cathepsins in human dentin-pulp. J Endod. 2010;36(3):475-481.

53. Tjäderhane L, Buzalaf MA, Carrilho M, Chaussain C. Matrix metalloproteinases and other matrix proteinases in relation to cariology: the era of 'dentin degradomics'. Caries Res. 2015;49(3):193-208.

54. Tjäderhane L, Larjava H, Sorsa T, Uitto VJ, Larmas M, Salo T. The activation and function of host matrix metalloproteinases in dentin matrix breakdown in caries lesions. J Dent Res. 1998;77(8):1622-1629.

55. Tjäderhane L, Nascimento FD, Breschi L, Mazzoni A, Tersariol IL, Geraldeli S et al. Optimizing dentin bond durability: Control of collagen degradation by matrix metalloproteinases and cysteine cathepsins. Dent Mater. 2013;29(1):116-135.

56. Van Meerbeek B, Yoshihara K, Yoshida Y, Mine A, De Munck J, Van Landuyt KL. State of the art of self-etch adhesives. Dent Mater. 2011;27(1):17-28.

57. Vidal CM, Tjäderhane L, Scaffa PM, Tersariol IL, Pashley D, Nader HB et al. Abundance of MMPs and cysteine cathepsins in caries-affected dentin. J Dent Res. 2014;93(3):269-274.

58. Wagner A, Wendler M, Petschelt A, Belli R, Lohbauer U. Bonding performance of universal adhesives in different etching modes. J Dent. 2014;42(7):800-807.

59. Wang Y, Spencer P. Analysis of acid-treated dentin smear debris and smear layers using confocal Raman microspectroscopy. J Biomed Mater Res. 2002;60(2):300-308.

60. Yoshida Y, Nagakane K, Fukuda R, Nakayama Y, Okazaki M, Shintani H et al. Comparative study on adhesive performance of functional monomers. J Dent Res. 2004;83(6):454-458.

61. Zarella BL, Cardoso CA, Pelá VT, Kato MT, Tjäderhane L, Buzalaf MA. The role of matrix metalloproteinases and cysteine-cathepsins on the progression of dentine erosion. Arch Oral Biol. 2015;60(9):1340-1345.