Universidade Estadual de Campinas
Faculdade de Odontologia de Piracicaba

Eveline Freitas Soares

AVALIAÇÃO DE SISTEMAS ADESIVOS EM DIFERENTES REGIÕES DENTINÁRIAS E DE UMA SOLUÇÃO RE-UMIDIFICANTE COM ANÁLOGOS BIOMIMÉTICOS E ADESIVO COM CÁLCIO-FOSFATO BIOATIVOS EXPERIMENTAIS

EVALUATION OF ADHESIVE SYSTEMS ON DIFFERENT DENTIN REGIONS AND OF AN EXPERIMENTAL RE-MOISTURE SOLUTION WITH BIOMIMETIC ANALOGUES AND ADHESIVE WITH BIOACTIVE CALCIUM-PHOSPHATE

Piracicaba
2017

Eveline Freitas Soares

AVALIAÇÃO DE SISTEMAS ADESIVOS EM DIFERENTES REGIÕES DENTINÁRIAS E DE UMA SOLUÇÃO RE-UMIDIFICANTE COM ANÁLOGOS BIOMIMÉTICOS E ADESIVO COM CÁLCIO-FOSFATO BIOATIVOS EXPERIMENTAIS

EVALUATION OF ADHESIVE SYSTEMS ON DIFFERENT DENTIN REGIONS AND OF AN EXPERIMENTAL RE-MOISTURE SOLUTION WITH BIOMIMETIC ANALOGUES AND ADHESIVE WITH BIOACTIVE CALCIUM-PHOSPHATE

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

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

Orientador: Prof. Dr. Lourenço Correr-Sobrinho

Este exemplar corresponde à versão final da Tese de doutorado defendida pela aluna Eveline Freitas Soares, e orientada pelo Prof. Dr. Lourenço Correr Sobrinho.

Piracicaba
2017

Agência(s) de fomento e nº(s) de processo(s): CAPES

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

Soares, Eveline Freitas, 1987-
So11a Avaliação de sistemas adesivos em diferentes regiões dentinárias e de uma solução re-umidificante com análogos biomiméticos e adesivo com cálcio-fosfato bioativos experimentais / Eveline Freitas Soares. – Piracicaba, SP : [s.n.], 2017.

Orientador: Lourenço Correr Sobrinho.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Remineralização dentária. 2. Biomimetismo. 3. Materiais biomiméticos. 4. Adesivos. 5. Dentina. I. Correr Sobrinho, Lourenço,1960-. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Evaluation of adhesive systems on different dentin regions and of an experimental re-moisture solution with biomimetic analogues and adhesive with bioactive calcium-phosphate
Palavras-chave em inglês:
Tooth remineralization
Biomimetics
Biomimetic materials
Adhesives
Dentin
Área de concentração: Materiais Dentários
Titulação: Doutora em Materiais Dentários
Banca examinadora:
Lourenço Correr Sobrinho [Orientador]
Regina Maria Puppin Rontani
José Augusto Rodrigues
Kamila Rosamilia Kantovitz
Ricardo Danil Guiraldo
Data de defesa: 17-02-2017
Programa de Pós-Graduação: Materiais Dentários

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 17 de Fevereiro de 2017, considerou a candidata EVELINE FREITAS SOARES aprovada.

PROF. DR. LOURENÇO CORRER-SOBRINHO

PROFª. DRª. REGINA MARIA PUPPIN RONTANI

PROF. DR. JOSÉ AUGUSTO RODRIGUES

PROFª. DRª. KAMILA ROSAMILIA KANTOVITZ

PROF. DR. RICARDO DANIL GUIRALDO

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

DEDICATÓRIA

Dedico este trabalho àqueles que acreditaram em mim mais que eu mesma, alimentaram meu viver com oportunidades que nunca imaginei receber e ainda souberam me deixar partir e me guiar para eu voltasse mais forte: meus pais *Sidnei Lopes Soares e Nilse Miranda de Freitas Soares*.

Dedico também aos meus anjos de plantão na Terra: meu irmão *Fábio F. Lopes Soares*, quem arranca minhas maiores gargalhadas mesmo em tempos difíceis; e meu noivo *André Luis Pinho* que, com sua paciência e ternura, me acompanhou pelo meu melhor e pior e ainda assim optou por estar ao meu lado sempre.

Agradeço todos os dias pelo privilégio…
Obrigada por tudo.
Amo vocês!

AGRADECIMENTOS ESPECIAIS

Agradeço em especial ao meu orientador *Lourenço Correr-Sobrinho* por tantos anos de aprendizado, dedicação e paciência. Obrigada por me acolher desde o início, pelo zelo, carinho e amizade!

Agradeço em especial também ao professor *Mário Alexandre Coelho Sinhoreti* pela confiança, prontidão em me ajudar sempre, por me motivar a ser melhor e me proporcionar a oportunidade de uma das melhores experiências da minha vida com a bolsa PDSE, principalmente obrigada pelo convívio com você e sua família tão querida e especial para mim.

Muito obrigada também ao professor *Saulo Geraldeli* por me mostrar como trabalhar com alma, criatividade e amor no que faz. Obrigada à você, professor, e sua família por me receber com tanto carinho e por tantos bons momentos!

Minha desmedida gratidão à vocês!
Muito obrigada!!!

AGRADECIMENTOS

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

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

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

À **Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES** pela concessão da bolsa de doutorado e oportunidade de realizar parte do meu doutorado em uma universidade estrangeira, com a concessão da bolsa de Doutorado PDSE (#99999.005561/2014-00) na **Universidade da Florida (Gainesville, FL, EUA)**.

Ao **Departamento de Odontologia Restauradora e Prótese da Universidade da Florida** (UF) por todo apoio e solicitude desde o primeiro contato, por disponibilizar a estrutura da UF para que a realização deste trabalho fosse possível. Por todo conhecimento compartilhado, convívio afetuoso e preocupação. Exemplo de conduta pessoal e profissional.

Aos **docentes do Curso de Pós-Graduação em Materiais Dentários**, **Profs. Drs. Simonides Consani, Mário Fernando de Goes, Lourenço Correr-Sobrinho, Mário Alexandre Coelho Sinhoreti, Regina Maria Puppin Rontani, Marcelo Gianinni, Américo Bortolazzo Correr, Rafael Xediek Consani, Luis Roberto Marcondes Martins, Fernanda Miori Pascon e Alan Roger** pelos ensinamentos e experiências cotidianas fundamentais para minha formação.

À banca examinadora do exame de obtenção do título de doutorado na área de Materiais Dentários **Profa. Dra. Regina Maria Puppin Rontani, Prof. Dr. José Augusto Rodrigues, Profa. Dra. Kamila Rosamilia Kantovitz e Prof. Dr. Ricardo Danil Guiraldo** pelo aceite do convite, deslocamento, tempo despendido e grande contribuição aos nossos trabalhos acadêmicos.

Às **alunas do curso de pós-doutorado na área de Materiais Dentários Ana Rosa Costa** e **Andréia Bolzan** pela amizade, boa conversa, parceria e carinho.

Aos **técnicos do departamento de Materiais Dentários**, engenheiro **Marcos Blanco Cangiani** e **Selma Segalla** pela disponibilidade e auxílio quando solicitado.

Ao **coordenador do Centro de Microscopia e Imagens da FOP/UNICAMP, Prof. Dr. Pedro Duarte Novaes** e ao apoio nas pessoas queridas de **Adriano Luis Martins e Flávia Sammartino Mariano Rodrigues** que com toda paciência e dedicação nos proporcionaram meios e fins para que nossos projetos fossem realizados.

Ao **Prof. Dr. E.W. Kitajima** e **R.B. Salaroli** do Núcleo de Apoio à Pesquisa em Microscopia Eletrônica, **NAP/MEPA/ESALQ/USP** – Brasil, pelo suporte e uso da microscopia eletrônica de varredura. E ao **LNNano/CNPEM** (Laboratório Nacional de nanotecnologia / Centro Nacional de Pesquisa em Energia e Materiais), pelos ensinamentos e suporte dos **Prof. Drs. J. Bettini, C.A.O. Ramirez e E. Teixeira Neto** na microscopia eletrônica de transmissão e varredura**.**

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

À todos os **amigos da área de Materiais Dentários, amigos de outras áreas e aos meus amigos de vida,** enfrentamos momentos de dificuldades e conquistas. A nossa amizade e troca de experiências foi essencial para meu crescimento pessoal e profissional.

À todos que indiretamente contribuíram para a realização deste trabalho.

Meu caminho foi guardado e abençoado
pela presença de cada um!!!
Obrigada!

RESUMO

Frente ao desafio de conter a degradação da interface compósito/dentina em sua estrutura anisotrópica e complexidade de remineralização, este estudo objetiva avaliar a resistência de união à microtração (μTBS) de adesivos auto-condicionantes e convencionais em diferentes regiões dentinárias (central-DC e proximal-DP) em preparo classe II. Um preparo classe II (mésio-ocluso-distal) foi simulado em 20 terceiros molares humanos extraídos (4x3mm). Adesivos convencionais (Scotchbond Multi Purpose - SBMP e Optibond FL - OPFL) e adesivos auto-condicionantes (Clearfil SE Bond - CSE e Optibond XTR - OPXTR) foram aplicados e a restauração confeccionada. Amostras em palito foram seccionadas (0,9 mm²) e submetidas ao teste μTBS (0.5mm/min). O padrão de fratura foi analisado em estereoscópio (40x) e classificado (coesivo, adesivo, misto/dentina ou misto/resina). Amostras foram observadas em microscopia eletrônica de varredura (n=4). Os dados foram submetidos ao ANOVA 1 fator com parcela subdividida Split-Plot e teste de Tukey (α=0,05). Não houve diferença estatística significante entre todos os grupos testados em DC (p>0,05). Em DP, os valores μTBS para os adesivos convencionais foram significantemente inferiores (p<0,05). O padrão de fratura predominante para adesivos auto-condicionantes foi misto/resina, e para convencionais foi misto/ dentina, com excessão de SBMP (DC) com padrão adesivo. Logo, a localização dentinária em um preparo classe II influenciou μTBS de adesivos convencionais, enquanto o oposto ocorreu para adesivos auto-condicionantes. Com isso, amostras dentinárias foram seccionadas em 4¼ para avaliação do desempenho de um sistema adesivo experimental convencional contendo análogos biomiméticos numa solução re-umidificante e cálcio-fosfato bioativos num adesivo de frasco único. Diferentes protocolos de tratamento foram realizados em cada ¼: solução controle + adesivo controle (G1), solução controle + adesivo experimental (G2), solução experimental + adesivo controle (G3) e solução experimental + adesivo experimental (G4). As amostras foram restauradas e armazenadas (24 horas - 24h e 4 meses - 4m) em solução simuladora de fluído corporal e testadas para μTBS, análise de nanoinfiltração e semi-quantitativa de cálcio-fósforo (Ca/P) por espectroscopia de energia raio-x (EDX) e observadas em microscopia eletrônica de transmissão (MET). Os dados do teste μTBS foram analisados por ANOVA (1 fator, disposição Spit-Plot) e teste de Tukey com comparação pareada (α=0,05). EDX e nanoinfiltração foram

analisados com ANOVA (2 e 1 fatores respectivamente) e teste de Tukey (α=0,05). Não houve diferença estatística entre os grupos em si para 24h quanto μTBS (p>0.05). Em G3 e G4, resultados μTBS permaneceram estáveis em 4m (p>0,05). O padrão de fratura predominante foi misto/dentina, com excessão de G3 (misto/ resina). EDX apontou uma estabilização na proporção Ca/P para G1 (24h e 4m); para G2, G3 e G4 a proporção Ca/P aumentou em 4m. A nanoinfiltração se apresentou reduzida em G4 comparado com G1 e G3. A adição de análogos biomiméticos em uma solução prévia re-umidificante manteve os resultados de resistência de união, estabilizou a proporção Ca/P e reduziu a nanoinfiltração em 4 meses de armazenamento quando aplicada previamente ao adesivo experimental.

Palavras-chave: Remineralização dentária. Mimetismo biológico. Materiais Biomiméticos. Adesivos. Dentina.

ABSTRACT

Facing the challenge to contain degradation of composite/dentin interface in its anisotropic structure and complexity of remineralization, this study aim to evaluate microtensile bond strength (μTBS) of self-etch and etch-and-rinse adhesive systems on different dentin regions (central-CD and proximal-PD) in a class II cavity configuration. A class II (mesial-oclusal-distal) cavity configuration was simulated on 20 extracted human third molars (4x3 mm). Etch-and-rinse adhesives (Scotchbond Multi Purpose - SBMP and Optibond FL - OPFL) and self-etch adhesives (Clearfil SE Bond - CSE and Optibond XTR - OPXTR) were applied and restorations conducted. Beam shape samples were sectioned (0.9 mm²) and submitted to μTBS test (0.5 mm/min). The fracture pattern was analyzed in a stereoscope (40x) and classified (cohesive, adhesive, mixed/dentin or mixed/resin). Samples were observed in scanning electron microscope (n=4). Data was submitted to one way ANOVA with Split-Plot arrangement and Tukey's test (α=0.05). There were no statistically significant differences among all tested groups on CD ($p>0.05$) On PD, μTBS values for etch-and-rinse adhesives were significantly lower ($p<0.05$). The fracture pattern most observed for self-etching adhesives was mixed/resin, and for etch-and-rinse it was mix/dentin, with the exception of SBMP (CD) with adhesive pattern. Therefore, dentin location on a simulated class II cavity configuration influenced μTBS of etch-and-rinse adhesives, while the opposite occurred for self-etching adhesives. From this, dentin samples were sectioned in 4¼ to evaluate the performance of an experimental etch-and-rinse adhesive system containing biomimetic-analogs in a re-moiture solution and bioactive calcium-phosphates in an one-bottle adhesive. Different treatments protocols were performed in each ¼: control solution + control adhesive (G1), control solution + experimental adhesive (G2), experimental solution + control adhesive (G3) and experimental solution + experimental adhesive (G4). Samples were restored and stored (24 hours- 24h and 4 months- 4 m) in simulated body fluid solution and tested μTBS, nanoleakage and semi-quantitative Calcium/ Phosphorus (Ca/P) through energy X-ray spectrometry (EDX) analysis, and observed in transmission electronic microscopy (TEM). The μTBS test data were analyzed using ANOVA (1-way, Split-Plot arragement) and Tukey's test with pairwise comparison (α=0.05). EDX and nanoleakage data were analyzed using ANOVA (2

and 1 way respectively) and Tukey's test ($p<0.05$). There was no statistical difference among groups in 24h for μTBS ($p>0.05$). In G3 and G4, μTBS results remained stable over 4m ($p>0.05$). The fracture pattern most frequently observed was mixed/ dentin, with the exception of G3 (mixed/resin). EDX pointed a stabilization on the proportion Ca/P for G1 (24h and 4m); for G2, G3 and G4, the proportion of Ca/P increased after 4m. Nanoleakage was reduced in G4 compared to G1 and G3. The addition of biomimetic analogues in a prior re-moiture solution maintained bond strength results, stabilized Ca/P proportion and reduced nanoleakage after 4 months storage when applied previously an experimental adhesive.

Keywords: Dental remineralization. Biological mimicry. Biomimetic materials. Adhesive. Dentin.

SUMÁRIO

1 INTRODUÇÃO

O estudo da adesão de compósitos odontológicos em substrato dentinário segue por um processo evolutivo notável. Seu progresso é acompanhado desde estudos que avaliam diretamente a estrutura dentária (Kagayama M *et al.,* 1999, Zaslansky P *et al.,* 2010), bem como novas técnicas para se prolongar a sobrevida de restaurações por meio de uma adesão dentinária eficaz e perdurável (De Munck J *et al.,*2009, Olszta Mj *et al.*, 2003). De maneira desafiadora, a dentina se destaca como um tecido peculiar devido a sua composição e anatomia singulares. Esse tecido, apesar de ser composto majoritariamente por conteúdo mineral (70% em peso de hidroxiapatita), apresenta também considerável quantidade de fluidos (10% em peso) e conteúdo orgânico (20% em peso, de colágeno tipo I, proteínas proteoglicanas, proteínas não colágenas e lipídeos) (Tjäderhane L *et al.,* 2012).

Essa composição variada da dentina se altera conforme o progresso da doença cárie, idade do indivíduo e região específica dentro do substrato dentinário em si. Neste último fator, a disposição dos túbulos dentinários também compõe mais uma importante característica intrínseca dessa estrutura. Quanto mais próximo à junção amelo-dentinária (JAD), menor é a densidade de túbulos dentinários e menores são os seus diâmetros. Enquanto, na região mais próximo à polpa, maior é a densidade e diâmetro dos túbulos dentinários (Marshall GW Jr. *et al.,* 1997, Mjör IA *et al.,* 1996). Além disso, também próximo a região pulpar, há uma maior concentração de dentina peritubular, caracterizada como hipermineralizada, oposto à região próxima à JAD, que apresenta dentina intertubular em maior quantidade. Esta situação é completado com uma grande quantidade de ramificações laterais de túbulos e micro-canais, os quais também são responsáveis pela passagem de líquido e estímulos formando uma rede de anastomose intertubular (Opdam NJ *et al.* 2014, Pashley DH, 1996).

Diante do exposto, diferentes abordagens foram criadas visando o alcance de uma adesão eficaz e duradoura aliada a uma técnica adesiva simples e viável clinicamente. Com isso, mais recentemente, adesivos auto-condicionantes trouxeram simplificação à essa técnica com a novidade de, por

meio de monômeros ácidos (pH≈2), transporem a “smear layer” remanescente dispensando assim o passo do condicionamento com ácido fosfórico. Além disso, essa abordagem ainda propicia união química estável entre hidroxiapatita remanescente e monômeros funcionais, o que manifesta uma adequada e estável resistência de união (Giannini M *et al.*, 2015).

Por outro lado, a técnica de adesão por meio do condicionamento ácido se mantém presente no cotidiano clínico. Com isso, a dificuldade inerente da técnica de manter a quantidade certa de umidade remanescente após o passo de lavagem e secagem relativa da dentina também permanece. Ademais, o condicionamento com ácido fosfórico 37% tende a deixar uma camada de dentina desmineralizada mais espessa comparada à desmineralização através de monômeros ácidos dos adesivos auto-condicionantes (Opdam NJ *et al.* 2014). Consequentemente, uma correspondente infiltração resinosa mais profunda também se faz necessária, processo dificultado pela ascensão de umidade intrínseca do substrato, prejudicando a infiltração resinosa e a polimerização por fotoativação, resultando em uma base da camada híbrida falha e mais sujeita à degradação (Opdam NJ *et al.* 2014, Pashley DH e Carvalho RM, 1997).

Essa degradação, por sua vez, pode ocorrer de diversas maneiras, sendo que dentre elas, a mais frequente tende a se passar justamente na base da camada híbrida não preenchida por resina. Trata-se da degradação pela atividade de enzimas endógenas metaloproteinases (MMPs), além de cisteinas catepsinas que também participam da proteólise intracelular digerindo fibrilas colágenas expostas (Frassetto A *et al.* 2016). Dessa maneira, bloquear a ação dessas enzimas se tornou um objetivo bastante pesquisado no intuito de diminuir a degradação dessa interface. Agentes como clorexidina (CLX), ácido etilenodiamino tetra-acético (EDTA), tetraciclinas, protocianidinas (PA), dentre vários outros compostos são pesquisados por suas propriedades antibióticas, quelantes, agentes de ligação cruzada e inibidores de MMPs (De Munck J et al., 2009,Frassetto A *et al.,* 2016, Tay FR e Pashley DH, 2008). Com isso, seus benefícios englobam o bloqueio de possíveis “sites” disponíveis para ligação com enzimas catalíticas ou a manutenção do arcabouço das fibrilas colágenas

estruturadas através das ligações cruzadas nessa região (De Munck *et al.*, 2009, Tay FR e Pashley DH, 2008).

Outra forma de degradação ocorre por intermédio da água. Com o tempo, espaços vazios resultantes da infiltração incorreta por resina são preenchidos com água remanescente oriunda do próprio tecido dentinário. O resultado dessa propagação de umidade é o enfraquecimento direto da estrutura e organização da camada híbrida, sujeitando a restauração a uma possível falha prematura dependente das condições as quais esse conjunto ficará exposto (Qi YP *et al.,* 2012). Para atenuar essa situação, a diluição de diferentes monômeros visa compatibilizar características químicas diferentes para conferir uma adequada difusão e preenchimento resinoso dessas lacunas. Alguns deles são o HEMA (2-hidroxietil metacrilato), monômero hidrófilo bastante utilizado; bem como o 10-MDP (10-metacriloiloxidecil dihidrogeno fosfato), capaz de se ligar estavelmente ao Ca^{+2} resistindo à degradação por umidade; o MAC-10 (10-ácido metacriloiloxidecametileno malônico), também estável à degradação por umidade; e os monômeros di-metacrilatos, geralmente utilizados na promoção de ligações cruzadas durante a polimerização dos sistemas adesivos, restringindo a propagação da água confinada na estrutura dentinária por entre o material resinoso (Tjäderhane L *et al.*, 2012).

Entretanto, mesmo com o aproveitamento das substâncias inibidoras de MMPs e o avanço da composição dos sistemas adesivos (convencionais e auto-condicionantes), a interface adesiva ainda permanece com sua durabilidade limitada dentro das restaurações (Breschi L *et al.,* 2008). Por isso, uma abordagem diferenciada, com o intuito de agregar diferentes vantagens em um só método vem sendo estudada (Gu LS *et al.*, 2010; Profeta *et al.*, 2013; Abuna *et al.*, 2016) com o objetivo de promover a remineralização do substrato dentinário, preencher lacunas vazias por entre as fibrilas colágenas, fortalecer a estrutura tecidual e evitar a lixiviação por água, além de inibir a atividade de MMPs e consequentemente preservar a interface adesiva ao longo do tempo (Niu LN et al., 2014; Cao CY et al., 2015).

Este processo chama-se remineralização biomimética e consiste, por meio da reprodução simulada de meios naturais, produzir artificialmente

recursos e métodos indutores de mineralização nos tecidos humanos como osso e dente (Wang JM *et al.*, 2013). Para isso, pesquisadores utilizam análogos biomiméticos de proteínas não-colágenas e partículas nano-precursoras de cálcio-fósforo amorfo (CFA) para adentrar por entre as fibrilas colágenas desmineralizadas e assim serem passíveis de atuar na remineralização das mesmas (Wang JM *et al.*, 2013).

Mais especificamente, análogos biomiméticos de proteínas não colágenas como o ácido poliacrílico (PAA), ácido polivinilfosfônico (PVPA) e o trimetafosfato de sódio (STMP), quando em contato com a estrutura colágena, atuam como estabilizadores e agentes sequestradores de partículas CFA. Ou seja, capturam partículas CFA para espaços inter/intrafibrilares formando estruturas padrões para a remineralização por meio de sua capacidade de ligação à matriz colágena. O resultado é a formação de “clusters” de pré-nucleação, os quais se agregam a outras partículas CFA, à estrutura da hidroxiapatita e à própria matriz colágena num processo chamado mineralização “bottom-down” (Liu Y *et al.*, 2011).

Este tipo de remineralização vem sendo estudado através de diferentes abordagens como sua incorporação em dentifrícios (Diamanti I *et al.*, 2011), cimentos remineralizadores de Portland (Gu LS *et al.*, 2010, Liu Y *et al.*, 2011, Sartori N *et al.*, 2016), géis (Guentsch A *et al.*, 2012), pó (Profeta AC *et al.*, 2013), pastas terapêuticas (Cao CY *et al.*, 2013), soluções (Sartori N *et al.*, 2016, Tjaderhane L *et al.*, 2013), cimentos de ionômero de vidro (Watson TF et al., 2014) e sistemas adesivos (Abuna G *et al.*, 2016, Profeta AC *et al.*, 2013). Contudo, apesar dessas investigações, pouca viabilidade clínica é encontrada quando se diz respeito a este tipo de remineralização, especialmente com atuação na base da camada híbrida como principal foco promissor de degradação.

Com isso, um estudo do substrato dentinário em condições laboratoriais específicas se fez pertinente para avaliar o desempenho de sistemas adesivos convencionais frente a auto-condicionantes, testados em diferentes regiões dentinárias por meio da resistência de união à microtração. A partir dele, adaptou-se uma metodologia de distribuição radial no substrato dentinário dos grupos a serem testados para avaliar a capacidade de remineralização

biomimética de um adesivo de frasco único experimental. O qual, foi acompanhado da incorporação de análogos biomiméticos em uma solução re-umidificante de aplicação prévia e da adição de partículas amorfas de cálcio-fosfato bioativos no adesivo experimental. Sua performance foi avaliada por meio do teste de resistência de união à microtração, capacidade de conter a nanoinfiltração, pela proporção cálcio/fósforo presente na camada híbrida e pela análise em microscopia eletrônica de transmissão após 4 meses de armazenamento das amostras.

2 ARTIGOS

2.1 Artigo 1[1]

Title: Microtensile bond strength of adhesive systems in different dentin regions on a class II cavity configuration

[1] Artigo submetido ao periódico Brazilian Dental Journal

Summary

The aim of this study was to evaluate microtensile bond strength (μTBS) of self-etch and etch-and-rinse adhesives systems compared within different dentin regions (central-CD or proximal-PD) in a class II cavity configuration. A class II (mesial-oclusal-distal) cavity configuration was simulated on 20 extracted human third-molars (4mm width/3mm depth). Etch-and-rinse adhesives (Scotchbond Multi Purpose, n=5, SBMP and Optibond FL, n=5, OPFL) and self-etch adhesives (Clearfil SE Bond, n=5, CSE and Optibond XTR, n=5, OPXTR) were applied. Class II restorations were placed using incremental technique and photo-activated (Bluephase/G2). Samples were sectioned on beam shape (1mm² cross-section), placed on Geraldeli's device to μTBS test (0.5mm/min cross-head speed). Fracture pattern were analyzed on stereomicroscope and classified in cohesive-resin, adhesive, mixed/resin or mixed/dentin. Samples (n=4) were prepared for scanning electron microscope observation. Data were submitted to one-way ANOVA with Split-Plot arrangement and Tukey's test (α=0.05). There were no statistically significant differences among SBMP, OPFL, CSE and OPXTR on CD ($p>0.05$). However, on PD for SBMP and OPFL, μTBS values were significantly lower compared to CSE and OPXTR ($p<0.05$). In all groups, mixed failure pattern was more frequently observed, except SBMP/CD (adhesive). In class II type cavity configuration, PD location negatively influenced bond strength of etch-and-rinse adhesive systems. Opposite from self-etching adhesives, which presented higher bond strength values compared to etch-and-rinse adhesives in PD.

Keywords: Dentin, Adhesive, Acid-etching, Composite resin

INTRODUCTION

Modern restorative dentistry highly relies on bonding the restorative materials to tooth hard tissues. Because of that, dentin bonding has been in focus in research field for the last twenty years (1). Since dentin is an anisotropic substrate, bond strength achieved in the adhesive layer connecting restorative materials and dentin structure is affected by dentin tubules orientation, tubules densities and the proportion of intratubular and intertubular dentin (2, 3).

It is known that these tubules are originated from odontoblasts cells tracks from dentin-enamel junction (DEJ) or cementum to pulp chamber (2). Each one of these dentin tubules has a varying radius and an almost straight or slightly wave-like pattern, which penetrates into dentin (4). In its central axis, dentin tubules are approximately parallel in the root part of the tooth, but are obviously radial in the peripheral crown region (4).

Nowadays, this dentin arrangement is still explored through new approaches including different image exams (3), in which it was observed that right beneath enamel (approximately 0.3 mm) an extensive tubule tilting, supposedly because of odontoblasts cells crowding, was detected, which relocates itself in its orthogonal path after that in the same manner as above pulp chamber (3).

Besides tubule orientation, tubules density also plays an important role in bond strength (3). Close to DEJ, tubule density is much lower (occupying just 1% of the total surface area) and with a smaller diameter (0.8μm). While closer to pulp chamber, tubule density is much higher (occupying approximately 22% of the total surface area) and with a larger diameter (2.5μm) (2). Since there are fewer tubules in the periphery of dentin area, there is an enormous variation between peritubular volume percentage from proximal to central dentin area (2). In the outer area, the amount of peritubular dentin is much reduced, while in central dentin, it may predominate in a thicker size (5).

Peritubular dentin, is characterized by a more mineralized and homogeneous substrate compared to intertubular dentin and it is also essentially collagen-free containing mostly apatite crystals (5). While intertubular dentin, as a dominant structure in proximal dentin, separates

tubules and it is composed of a matrix of type I collagen supported by apatite (5). This proportion along with intrinsic characteristics of peritubular and intertubular dentin, added to the already described differences of tubule orientations, often passes unnoticed during experimental projects (6).

On the other side of bonding strength from current adhesive systems, two different approaches are the most frequently used in an attempt to obtain a reliable dentin bonding. The etch-and-rinse technique adhesives systems relies on the removal of the smear layer and exposure of the collagen matrix by acid etching, followed by the application of a primer solution and a bond adhesive or a prime-bonding agent, which combines primer and adhesive resin into one single solution (7). The second approach remains on the use of self-etching primers, in which acid and primer solutions are present in two different bottles or combined in a single one (8). Which has its bonding mechanism based upon the simultaneous etching, priming and bonding to the smear-covered dental tissue, reducing the number of steps for the adhesive procedure, shortening the application time and leading to a lower sensitivity technique (8).

The efficacy of adhesive systems is regularly evaluated *in vitro* by its ability to bond to coronal dentin, usually making use of flat surfaces (6). Which neglects many clinical realities, once higher C-factor increases polymerization contraction stresses over tooth-composite interface. This result from a reduction of the composite property of relaxation after light-curing is carried out introducing plastic deformations that are susceptible to resin degradation. Besides this, it is possible to find dentinal tubules with different orientations on these dentin flat surfaces, due to their radial distribution in relation to the pulp chamber. This structural anisotropy implies that the nature of dentin substrate usually presented for bonding procedure also varies among different locations prompting discrepancies in bond strengths often encountered (2).

The major part of microtensile bond strength (μTBS) studies makes use of flat dentin surfaces where composite blocks are built in increments. In this case, the C-factor (9) can be considered low, differently from what occurs in class I and class II configuration cavities. Thus, this study evaluated the effect of the orientation of dentin tubules in pulpal wall of a class II cavity on μTBS test of two etch-and-rinse and two self-etching adhesives. The null hypotheses

tested were as follows: 1) the different orientations of the dentin tubules would have no effect on μTBS values, and 2) the different types of adhesives would not influence on μTBS.

MATERIALS AND METHODS

Tooth Preparation

Twenty four human third molars extracted for therapeutic reasons were donated under Ethics Committee approval (protocol # IRB 201500060, College of Dentistry, UF). They were certificated as sound molars and the gross debris were removed. The teeth were stored in distilled water at 4ºC for utilization within six months.

Twenty teeth (n=20) had its top occlusal enamel surface and roots from each tooth were sectioned perpendicular to its long axis using a diamond disc (EXTEC Corporation, Enfield, CT, USA) attached to a low-speed cutting machine (Isomet 1000, Buehler Ltda., Lake Bluff, IL, USA) under water cooling. Roots were sectioned 2 mm below the cementoenamel junction and then pulp tissue was removed (Fig. 1A). After that, coronal pulp chamber filling restoration was done using Clearfil SE Bond adhesive system (Kuraray Medical Inc., Tokyo, Japan) and Filtek Z250 flowable composite (3M ESPE, St. Paul, MN, USA) applied according to manufacturer's instructions (Table 1). Each sectioned tooth was glued with a cianocrylate instant adhesive (Loctite Super Glue, Westlake, OH, USA) on a polyvinyl chloride (PVC) stub pre-filled with self-curing acrylic resin (Opti-Cryl, Guarne, Colombia) until bond strength test was performed (Fig. 1B).

A class II cavity configuration (mesial-oclusal-distal, MOD) was simulated in a simplified manner, excluding mesial and distal boxes on each tooth, using a mechanical preparation machine (Elquip, Sao Carlos, SP, Brazil). For that, a high-speed hand piece (Kavo, Joinville, SC, Brazil) with a diamond bur 3097 (KG Sorensen, Cotia, SP, Brazil) was positioned on 90º to tooth long axis and a cavity configuration of 4 mm width by approximately 3 mm depth (Fig. 1C) was performed under water cooling until middle dentin was fully exposed.

Bonding Procedure

Teeth were randomly divided into four groups corresponding to a different type of adhesive system applied according to manufacturers' recommendations (Table 1).

Table 1: Materials, manufacturers, abbreviations, compositions and application procedures

Material	Adhesive system, manufacturer and abbreviation	Composition	Application procedure
Etch-and-rinse 3 steps adhesive systems	Scotchbond MP, 3M-ESPE; SBMP	Primer: HEMA, water, polyalkenoic acid copolymer, water; Bond: BisGMA, HEMA, CQ	Application of etchant (H3PO4) for 15 s, water rinse for 15 s, gently air dry. Application of primer for 15 s, gently air volatilization for 5 s. Application of adhesive, light cure for 10 s.
	Optibond FL, Kerr; OPFL	Primer: HEMA, ethanol, GPDM, MMEP, water, CQ, BHT Bond: TEGDMA, UDMA, GDMA, HEMA, Bis-GMA, filler, CQ, approximately 48wt% filled	Application the etchant (H3PO4) for 15 s, water rinse for 15 s, gently air dry, application of primer actively for 15 s, gently air volatilization for 5 s. Application of adhesive, light cure for 10 s.
Self-etching 2 steps adhesive systems	Clearfil SE, Kuraray; CSE	Primer: 10-MDP, HEMA, hydrophilic dimetachylate, CQ, accelerators, water; Bond: 10-MDP, Bis-GMA, HEMA, hydrophilic dimethaclylate, colloidal silica, CQ, initiators, accelerators	Application of primer for 20 s, gently air volatilization for 5 s, application of adhesive and light cure for 10 s.
	Optibond XTR, Kerr; OPXTR	Primer: GPDM, hydrophilic co-monomers, water, ethanol, acetone Bond: resin monomers, HEMA, inorganic fillers, ethanol	Application of primer actively for 20 s, gently air volatilization for 5 s, application of adhesive for 15 s, light-cure for 10 s.
Microhybrid restorative filler	Filtek Z 250 (A2), 3M-ESPE	Bis-EMA, TEGDMA, UDMA, zirconium, silica	Composite placement through incremental technique with each layer light-cured for 20 s using a LED light curing unit (Bluephase G2, Ivoclar).
Etchant	H_3PO_4	Amorphous silica-thickened 35% phosphoric acid gel	Dentin surface was acid etched using phosphoric acid 35% (3M ESPE) for 15 s, rinsed for 15 s and dried using absorbent pads.

Abbreviations: 10-MDP, 10-methacryloyloxydecyl dihydrogen phosphate; BHT, butylhydroxytoluene; Bis-GMA, bisphenol A diglycidyl methacrylate; CQ, camphorquinone; GDMA, glycerol dimethacrylate; GPDM, glycerol phosphate dimethacrylate; HEMA, 2-hydroxyl methacrylate; MMEP, mono-2-methacryloyloxyethylphthalate; UDMA, diurethane dimethacrylate.

Group SBMP: Dentin (n=5) surface was acid etched using phosphoric acid 35% (3M ESPE) for 15 s, rinsed for 15 s and gently dried with absorbent pads until excess humidity was removed. Scotchbond Multi Purpose adhesive

system, (3M ESPE) was applied. First primer solution was actively spread for 15 s using a disposable brush (Microbrush; Vigodent, Rio de Janeiro, RJ, Brazil), and gently air dried for 5 s, followed by adhesive application.

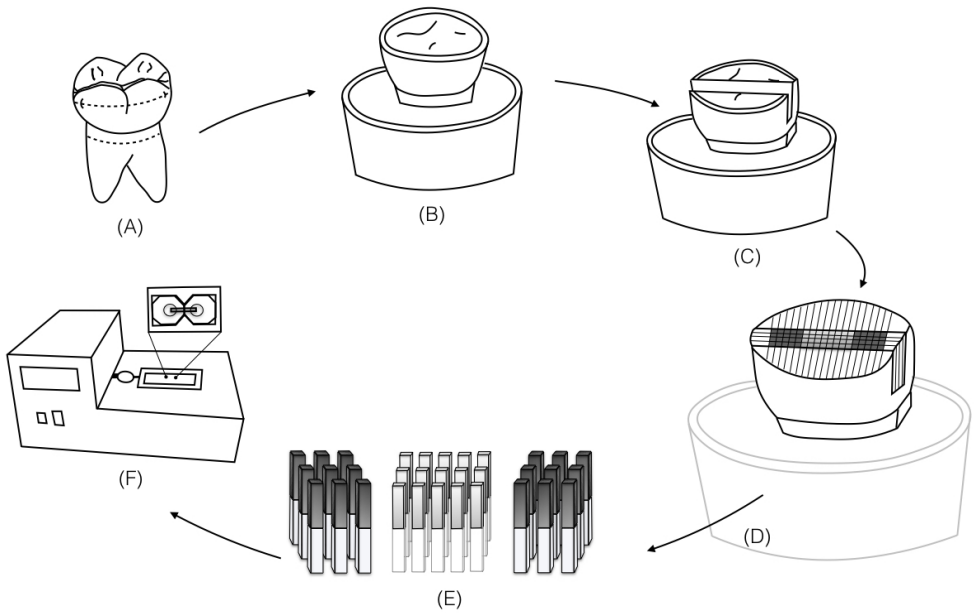
Group OPFL: Dentin (n=5) surface was acid etched as group SBMP, primer solution was spread actively for 15 s and air dried for 5 s, followed by Optibond FL (Kerr Corporation, Orange, CA, USA) adhesive application.

Group CSE: Primer solution was applied for 20 s on dentin surface (n=5) and mild air dried for 5 s before adhesive Clearfil SE Bond (Kuraray) was applied.

Group OPXTR: Primer solution was applied actively for 20 s on dentin surface (n=5) and mild air dried for 5 s before bond adhesive Optibond XTR (Kerr Corporation), be applied for 15 s and air dried for 5 s.

The procedure of light curing was carry out for adhesive layer of each group for 10 s using a LED light curing unit (Bluephase G2, Ivoclar Vivadent, Liechtenstein) in all samples, with 1,392 mW/cm^2 of irradiance. A composite restoration was performed in two horizontal increments, where each layer was light cured for 20 s using the same light curing unit. Samples were stored for 24 h at 37ºC in distilled water.

Figure 1: Schematic illustration of extracted tooth before enamel and root removal (a); Sample fixation on PVC stub pre-filled with acrylic-resin (b); Class II (MOD) 4 mm width x 3 mm depth cavity configuration (c); Composite restoration by incremental technique (d); Beam shape samples acquired from proximal and central dentin location (e); Microtensile bond strength test, μTBS (f).



Microtensile Bond Strength Test *(μTBS)*

Each sample was sectioned in mesial-distal and buccal-lingual directions in perpendicular way to its bonding interface (Fig. 1D) using a diamond disc coupled to a low-speed cutting machine under water cooling at 250 rpm. Samples obtained (Fig. 1E) were beam shaped with at most 1 mm² adhesive area measured with a digital caliper (Mitutoyo Corporation, Tokyo, Japan).

Beams from the same sample were divided into two subgroups according to dentin location from where they were removed. In central dentin group (CD), specimens were located in the middle area of dentin exposed surface, corresponding to the interspace of pulp horns. While in proximal dentin group (PD), specimens were located in distal and mesial area of dentin surface. Enamel specimens or specimens presenting defects as lack of material or irregularities were discarded (Fig. 1D).

After 24 h storage at 37º C in distilled water, specimens were positioned and glued with cyanoacrylate glue (Zapit, Corona, CA, USA) into Geraldeli's jig-2 for μTBS test (Fig. 1F) at 0.5 mm/min speed in a universal testing machine (OMT-100, Odeme Dental Research, Luzerna, SC, Brazil). Final values were express in MPa obtained from the following equation: μTBS = F/A x 0,098; In which, μTBS stands for microtensile bond strength value (MPa), F for microtensile force applied for the test (kgf), A sample bonded area (mm^2)/100 = (cm^2). μTBS results from each area (CD or PD) were pointed as the average of the tested beams for each adhesive system (SBMP, OPFL, CSE and OPXTR) also tested.

Failure mode was classified by observing each fractured beam under stereomicroscope (50 x, Nikon, model SMZ-1B, Tokyo Japan). The mode of failure was classified in adhesive, cohesive, mixed/dentin or mixed/resin type of failure.

Scanning Electronic Microscopy

The same method described above was conducted to restore extra representative samples (n=4) from each adhesive group in central and proximal dentin locations for scanning electronic microscopy (SEM) observation.

Samples were sectioned in 2 mm thick slices with a double sided diamond saw and embedded in epoxy resin (Buehler epoxycure resin and hardener, Agar Scientific Elektron Technology, Stansted, UK). After 24 h curing time, the mounted stubs were finish with silicon carbide sandpaper in ascending granulations (#600-2500, Norton Saint-Gobain, Guarulhos, SP, Brazil) and polished by felt pads with diamond grinding polishing pastes (6μm-0.25μm, Ted-Pella Inc., Redding, CA, USA). Phosphoric acid 50% was applied during 5 s, rinsed and silica-dried for 2 hours. A sputter-coating with gold-palladium for 60 s at 45 mA in a vacuum metalizing chamber (MED 010; Balzers, Liechtenstein) was applied before observation in a SEM (LEO 435 VP; Carl Zeiss, Jena, Germany), operated under 20 kV in different magnifications.

Statistical Analysis

The μTBS mean of the beams in each region of the specimens was calculated. Data was submitted to one-way ANOVA with split-plot arrangement and Tukey´s test for pairwise comparison ($\alpha=0.05$). The factor (parcel) considered was material and as sub-factor (sub-parcel) dentin regions in two levels (central and proximal).

RESULTS

The pre-testing failures (CSE – 1 central and 1 proximal; OPXTR – 1 central and 1 proximal; SBMP – 3 proximal and OPFL – 2 central and 4 proximal) were not included in the statistical analysis. For μTBS test results (Table 2), there were no statistically significant differences among etch-and-rinse adhesives (SBMP - 30.5 MPa and OPFL 29.3 MPa) and self-etching adhesives (CSE - 29.1 MPa and OPXTR - 29.6 MPa) on central dentin location (Table 2, $p>0.05$). Although, when tested on proximal dentin location, etch-and-rinse adhesives (SBMP - 23.2 MPa and OPFL - 22.0 MPa) obtained statistically lower μTBS test values compared do self-etching adhesives (CSE - 27.1 MPa and OPXTR - 28.1 MPa, $p<0.05$). For all groups, mixed type failure pattern was more frequently observed, except for SBMP in central dentin area, which

presented adhesive type of failure more often. Self-etching adhesive systems (CSE and OPXTR) presented generally a mixed/resin type of failure, while etch-and-rinse adhesive systems presented generally a mixed/dentin type of failure (Fig. 2).

SEM observation

SEM images illustrated dentin tubules closer to a parallel position with tooth long axis in central dentin location (Fig. 3A), while in proximal dentin location (Fig. 3B), a tilted angulation was observed. From an occlusal dentin surface perspective, the same pattern could be observed in central dentin (Fig. 3C) and in proximal dentin (Fig. 3D). Lateral diffusion of resin tags (Fig. 3E) and odontoblast processes (Fig. 3F) could be observed in greater magnifications from approximately 7kx.

Table 2: Microtensile bond strength mean values (MPa) for self-etching and etch-and-rinse adhesives for central and proximal dentin locations (p>0.05).

	CENTRAL DENTIN	PROXIMAL DENTIN
CSE	*29.1 ±(5.9) a,A*	*27.1 ±(6.3)a,A*
OPXTR	*29.6 ±(6.1)a,A*	*28.0 ±(4.8)a,A*
SBMP	*30.5 ±(4.7)a,A*	*23.2 ±(5.2)b,B*
OPFL	*29.3 ±(5.5)a,A*	*22.0 ±(6.2)b,B*

Different uppercase letters in the row and lowercase letters in the columns means statistics significance (α=0.05).

Figure 2: Failure modes analysis of debonded specimens (%) after μTBS test.

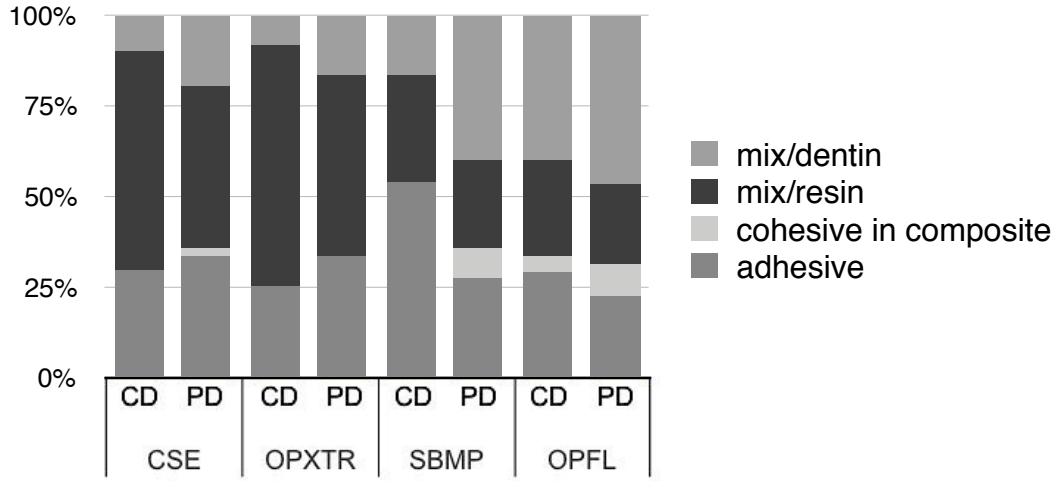
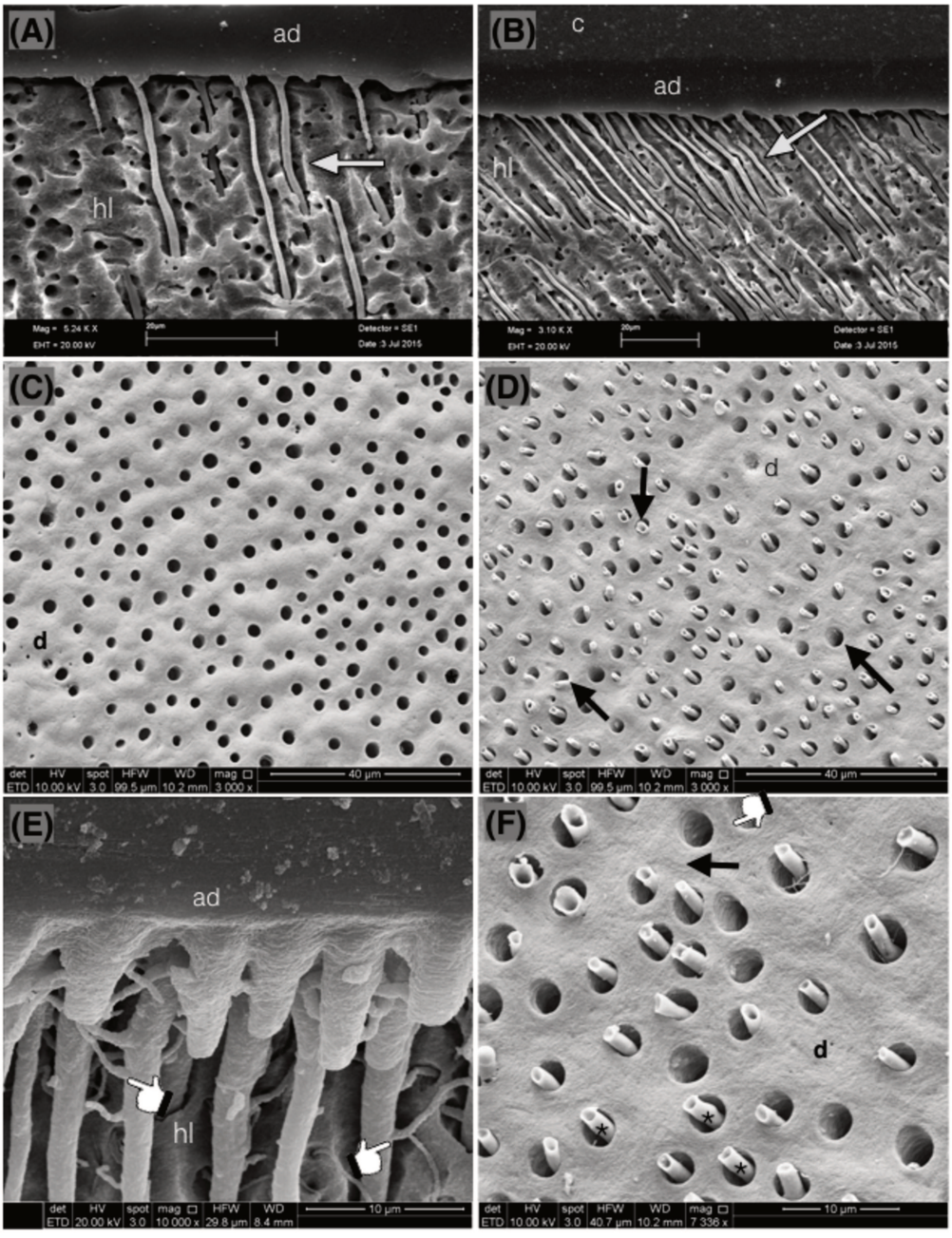


Figure 3: SEM images of dentin aspects regarding hybrid layer formation in different locations. c. Composite; ad. Adhesive; hl. hybrid layer; d. Dentin. (A) and (C) - central dentin location; (B) and (D) - proximal dentin location; (E) shows rich lateral diffusion of resin tags; (F) corresponds to occlusion view of dentin tubules from proximal dentin. Highlighting: “white arrows” pointing dentin tubules filled with resin tags in different angulations; “black arrows” pointing tilted dentin tubules lumen from a occlusal perspective; “Handpoint” showing lateral tags diffusion in proximal dentin; (*) Odontoblast processes.



DISCUSSION

In theory, the bond strength achieved by any dentin bonding agent at any dentin depth is simply correlated with three aspects: the area available for surface adhesion, the area occupied by resin tags and the area of intertubular dentin infiltrated by resin adhesives (10). But in reality, differences in dentin substrate as dentin depth (11, 12), mineral content (2), morphology (3), age (11), disease (4), wetness (2); and contemporaneity in adhesives approaches and compositions (13, 14) gives this subject a more complex perspective. Beyond these matters, in laboratories, flat dentin surfaces are mostly used for μTBS tests. In this case, not only different dentin morphologies are neglected, but a low C-factor is present (9), what contributes to impair a proper reproduction of many clinical conditions.

In this study, μTBS test was conducted comparing central and proximal (mesial and distal) dentin using etch-and-rinse or self-etching bonding agents applied in a class II (mesial-occlusal-distal, C-factor ≅ 2.5) cavity configuration prepared in order to obtain a more pragmatic clinical situation. In this context, smear layer was produced with diamond burs to result in a more dense precipitate towards clinical reality as well. Among the results, proximal and central dentin subtracts were compared, in which self-etching adhesives presented no statistical differences among all tested groups, differently from other studies (13, 15). SBMP and OPFL etch-and-rinse adhesive systems presented lower μTBS values in proximal dentin compared to central dentin. This lead us to a partial acceptation of the first hypothesis*,* since μTBS values were affected by different dentin tubules orientations, but only for etch-and-rinse adhesive systems in proximal dentin area.

This possibly occurred due a non uniform hybrid layer formation once, in this cavity configuration, proximal dentin enclosed a substrate close to DEJ corresponding to tilted angle tubules and tubules from cusps correlative areas due the tubules dentin radius distribution. Under cusps, tubules tend to tilt in a slight “S” shape (4) lightly differing in angle orientation and position from the outer dentin area and the region above pulp chamber (3) as can be seen in dentin microscopy (Fig. 3A, B, C and D). Even though resin tags could be

observed in all groups through SEM confirming a hybrid layer formation, these anisotropy dentin characteristics possibly jeopardized adhesive and substrate intimate contact for SBMP and OPFL, interfering in the arrangement of an uniform hybrid layer (2). This can be the reason for a higher mixed/dentin type of failure (Fig. 2) observed in these groups for proximal dentin (Fig. 3B and D). And may lead to a clinical warning, because of the risk of leaving residual dentin structure unsealed, chancing to cause post-operative sensitivity, bacterial microleakage, marginal staining and/or secondary caries (16).

Besides that, proximal dentin in the class II configuration prepared in this study encompasses a majority of intertubular dentin, a lower dentin tubules density and a higher amount of lateral branches from main dentin tubules (4, 10) pointing these characteristics as important elements for bonding strength in this area. Frequently, higher bond strengths values are presented closer to DEJ in a so called superficial dentin (13, 17). However, in this study, proximal dentin areas presented lower statistical μTBS values for etch-and-rinse adhesives, what rises some interesting points. Intertubular micromechanical resin impregnation may be uncertain from adhesion steps as acid etching demineralization followed by primer and resin diffusion for etch-and-rinse adhesives. This resin tags infiltration via radial diffusion constitutes lateral branches and ramifications from the main lumen contributing substantially to bond strength as can be illustrated in Fig. 3 (E and F). But also, these may compromises μTBS values if the acid etching followed by resin penetration does not succeed evenly, as possibly occurred to etch-and-rinse adhesive systems in proximal dentin.

About dentin substrate at last, in the proximal area there is a reduced intrinsic wetness (5, 18), functioning as a two perspectives situations for bond strength. The first one, is about moist control, having enough wetness to prevent shrinkage of demineralized dentin and consequently keeping the exposed collagen scaffold structured for resin diffusion after acid etching demineralization (19). The second one, is to not exceed the necessary amount of water, avoiding the dilution of some adhesives monomers and phase separation leading to its improper functionality (14, 19). In this case, self-etching

technique has the advantage of a more adaptable approach depending of the intrinsic subtract re-moisture (8, 13, 20).

As part of this behavior, self-etching hydrophilic commercial blends are formulated to alter the smear layer with acidic monomers rather than remove it, creating a permeable membrane through smear layer and smear plugs (20, 8). This contributed to μTBS results in this study, as they did not show statistical differences between each other in central and proximal dentin, and still presented higher μTBS values compared to etch-and-rinse adhesives systems in proximal dentin. This standard behavior through different dentin locations presented by self-etching adhesives contrasted with SBMP and OPFL results, leading to a partial acceptation of the second hypothesis, as different types of adhesives did influence on μTBS values just for proximal dentin region. What highlights recent self-etching adhesives systems as less influenced by different dentin anisotropic characteristics (21, 22).

Both self-etching adhesives applied in this study are considered mild types (pH≈2.0) (14, 23), what contributes to open its path through smear layer in a more balanced manner preventing an overwhelming dentin wetness rise, especially in central dentin area (perpendicular to tubules orientation and closer to pulp chamber). This way, this behavior includes overcoming smear layer and dentin buffering capacity but still maintaining enough monomer concentration to intersect successfully the water (20), what surely reflected in a more even hybrid layer formation through dentin intrinsic differences (Figure 3) and bond strength performance (Table 2).

Furthermore, CSE also demineralize dentin incompletely, leaving remaining hydroxyapatite attached to collagen structure accessible for chemical bonding with 10-MDP (10-methacryloyloxydecyl dihydrogenphosphate) functional monomer (Table 1) by the adhesion/decalcification concept (20) resulting in a low-sensitivity technique (8). While OPXTR has acetone in its chemical composition as its solvent, appealing to a highly hydrophilic volatile component, capable of quickly remove the water and evaporates leaving a further higher concentration of monomers as glycerol phosphate dimethacrylate (GPDM) for resin penetration (24), what also likely contributed to the lack of

statistical differences between self-etching adhesives groups in a various dentin locations.

Within this, adhesive systems approaches and its chemical compositions seem to remains as key elements for the challenges of dentin anisotropy characteristics in agreement with some other studies (21). For these purposes, different distributions of the tested groups can be articulated, as for an example, radial distribution of groups from a middle region on central dentin (25). Further studies are required for a more precise clinical reproduction in reliable laboratory conditions in order to follow dentin anatomy and contribute to resin restorations durability pursuit.

Thus, from the outline of this study, it is concluded that in class II type cavity configuration, dentin location influenced bond strength of etch-and-rinse adhesive systems. In proximal dentin, etch-and-rinse adhesive systems presented lower bond strength results. While self-etching adhesive systems presented homogeneous bond strength values in proximal and central dentin region.

RESUMO

O objetivo deste estudo foi avaliar a resistência de união à microtração (μTBS) de sistemas adesivos auto-condicionantes e convencionais comparados por entre diferentes regiões dentinárias (central-DC ou proximal-DP) em um preparo cavitário classe II. Um preparo cavitário classe II (mesio-ocluso-distal) foi simulado em 20 terceiros molares humanos (4mm largura/3mm profundidade). Adesivos convencionais (Scotchbond Multi Purpose, n=5, SBMP e Optibond FL, n=5, OPFL) e adesivos auto-condicionantes (Clearfil SE Bond, n=5, CSE e Optibond XTR, n=5, OPXTR foram aplicados. Restaurações classe II foram realizadas usando a técnica incremental e fotoativadas (Bluephase/ G2). As amostras foram seccionadas em forma de palito (1mm^2 secção transversal), posicionadas no dispositivo de Geraldeli para o teste μTBS (velocidade transversal de 0,5mm/min). Padrão de fratura foi analisado em estereoscópio e classificado em coesivo-resina, adesivo, misto/resina, misto/ dentina. Amostras (n=4) foram preparadas para observação em microscópio

eletrônico de varredura. Os dados foram submetidos ao ANOVA um fator e teste de Turkey (α=0,05). Não houve diferença estatística significante entre SBMP, OPFL, CSE, e OPXTR em DC (p>0,05). Entretanto, para SBMP e OPFL em DP, valores μTBS foram significativamente menores comparados com CSE e OPXTR (p<0,05). Em todos os grupos, o padrão de fratura misto foi o mais frequentemente observado, exceto em SBMP/CD (adesivo). Em um preparo classe II, a localidade da DP influenciou negativamente a resistência de união de sistemas adesivos convencionais. Oposto aos adesivos auto-condicionantes, os quais apresentaram valores de resistência de união maiores comparados com adesivos convencionais em DP.

ACKNOWLEDGMENT

The authors are thankful to CAPES scholarship support, EW Kitajima and RB Salaroli for SEM equipment support in the “NAP/MEPA/ESALQ/ USP” (Piracicaba, SP, Brazil), to J Bettini, E Teixeira Neto and CAO Ramirez for FEI Quanta 650 FEG support in the Brazilian Nanotechnology National Laboratory (LNNano, CNPEM, Campinas, SP, Brazil).

REFERENCES

1. Amaral CM, Correa D de S, Miragaya LM, Silva EM. Influence of Organic Acids from the Oral Biofilm on the Bond Strength of Self-Etch Adhesives to Dentin. Braz Dent J. 2015 Oct;26(5):497-502.
2. Marshall GW Jr., Marshall SJ, Kinney JH, Balooch M. The dentin substrate: structure and properties related to bonding. J Dent 1997;25:441-458.
3. Zaslansky P, Zabler S, Fratzl P. 3D variations in human crown dentin tubule orientation: A phase-contrast microtomography study. Dent Mater 2010; 26 e1–e10.
4. Nanci A. Ten cate’s oral histology: development, structure and function. 6th ed. St. Louis: Mosby; 2003.

5. Tjaderhane L, CARRILHO MR, BRESCHI L, Tay FR & PASHLEY DH. Dentin basic structure and composition - an overview. Endodontic Topics 2012; 20:3–29.
6. Carneiro KK, Meier MM, Santos CC, Maciel AP, Carvalho CN, Bauer J. Adhesives doped with bioactive niobophosphate micro-filler: degree of conversion and microtensile bond strength. Braz Dent J. 2016; 27:705-711.
7. Gan J, Liu S, Zhou L, Wang Y, Guo J, Huang C. Effect of Nd:YAG Laser Irradiation Pretreatment on the Long-Term Bond Strength of Etch-and-Rinse Adhesive to Dentin.. Oper Dent. 2017 Jan/Feb;42(1):62-72.
8. Giannini M, Makishi P, Ayres AP, Vermelho PM, Fronza BM, Nikaido T *et al*. Self-etch adhesive systems: a literature review. Braz Dent J. 2015;26:3-10.
9. Feilzer AJ, De Gee AJ, Davidson CL. Setting stress in composite resin in relation to configuration of the restoration. J Dent Res 1987;66:1636-1639.
10. Toledano M, Osorio R, Ceballos L, Fuentes MV, Fernandes CA, Tay FR *et al*. Microtensile bond strength of several adhesive systems to different dentin depths. Am J Dent 2003;16:292-298.
11. Burrow MF, Takakura H, Nakajima M, Inai N, Tagami J, Takatsu T. The influence of age and depth of dentin on bonding. Dent Mater 1994;10:241-246.
12. Lopes MB, Sinhoreti MA, Gonini Júnior A, Consani S, McCabe JF.Comparative study of tubular diameter and quantity for human and bovine dentin at different depths. Braz Dent J 2009;20:279-283.
13. Pegado REF, Amaral FLB, Flório FM, Basting RT. Effect of different bonding strategies on adhesion to deep and superficial permanent dentin. Eur Journ 2010;4:110-117.
14. Van Landuyt KL, Snauwaert J, De Munck J, Peumans J, Yoshida Y, Poitevin A, *et al*. Systematic review of the chemical composition of contemporary dental adhesives. Biomaterials 2007;28;3757–3785.
15. De Munck J, Mine A, Cardoso MV, Neves AA, Van Landuyt KL, Poitevin A *et al*. Effect of dentin location and long-term water storage on bonding effectiveness of dentin adhesives. Dent Mater 2011;30: 7–13.

16. Fernández EM, Martin JA, Angel PA, Mjör IA, Gordan VV, Moncada GA. Survival rate of sealed, refurbished and repaired defective restorations: 4-year follow-up. Braz Dent J. 2011;22:134-139.
17. Foscaldo T, Dos Santos GB, Miragaya LM, Garcia M, Hass V, da Silva EM.Effect of HEMA Phosphate as an Alternative to Phosphoric Acid for Dentin Treatment Prior to Hybridization with Etch-and-Rinse Adhesive Systems. J Adhes Dent. 2016;18(5):425-434
18. Yoshikawa T, Sano H, Burrow MF, Tagamil J, and Pashley DH. Effects of dentin depth and cavity configuration on bond strength. Dent Res 1999; 78:898-905.
19. Pashley DH, Ciucchi B, Sano H and Horner JA. Permeability of dentine to adhesive agents. Quintessence Int 1992; 24: 618-633.
20. 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;27:17-28.
21. Marchesi G, Frassetto A, Visintini E, Diolosa M, Turco G, Salgarello S, Di Lenarda R, Cadenaro M, Breschi L. Influence of ageing on self-etch adhesives: one-step vs. two-step systems. Eur J Oral Sci 2013; 121:43–49.
22. Szesz A, Parreiras S, Reis A, Loguercio A. Selective enamel etching in cervical lesions for self-etch adhesives: A systematic review and meta-analysis. J Dent. 2016 Oct;53:1-11.
23. Schiltz-Taing M, Wang Y, Brown BSD, Chen L. Effect of tubular orientation on the dentin bond strength of acidic self-etch adhesives. Oper Dent 2011; 36:86-91.
24. Walter R, Swift EJ, BOUSHELL LW, BRASWELL K. Enamel and dentin bond strengths of a new self-etch adhesive system. J Esthet Restor Dent 2011;23:390–398.
25. Geraldeli S, Perdigão J, Larson WK. A novel micro-tensile dentin bond strength method using paired dentin quarters. J Dent Res 2002;80:abstract 3373.

2.2 Artigo 2

Title: Experimental re-moiture solution with biomimetic analogues and acid-etching adhesive system and calcium-phosphates bonding performance

Abstract

The aim in this *in vitro* study was to evaluate bonding performance of an experimental etch-and-rinse adhesive system containing biomimetic analogs (BA) and bioactive calcium-phosphates (BAP). A control (C) blend of a re-moiture solution (90-wt% distilled water and 10-wt% HEMA) and a single-bottle adhesive (10-wt% HEMA, 10-wt% UDMA, 20-wt% TEGDMA, 15-wt% BisEMA, 10-wt% BisGMA, 32-wt% ethanol and 3-wt% photoinitiator system) were formulated. In experimental groups, BA (5-wt% polyacrylic acid and 10-wt% polyvinylphosphonic acid) were added in the re-moiture solution and BCP (ß tricalcium-phosphate and monocalcium phosphate mono-hydrated) were added in the adhesive. Thirty extracted human molars were sectioned in 4¼, for experimental treatments: C solution + C adhesive (G1), C solution + experimental adhesive (G2), experimental solution + C adhesive (G3) and experimental solution + experimental adhesive (G4). Restorations were performed and stored for 24h and 4 months (4m) previously to microtensile bond strength (μTBS) test. Representative samples were directed to nanoleakage, energy dispersive X-ray spectrometry (EDX) and transmission electronic microscopy (TEM) analysis. The μTBS test data were analyzed using ANOVA (1-way, Split-Plot arragement) and Tukey's test with pairwise comparison ($\alpha=0.05$). EDX and nanoleakage data were analyzed using ANOVA (2 and 1 way respectively) and Tukey's test ($p<0.05$). There was no statistical difference among groups in 24h for μTBS ($p>0.05$). In G3 and G4, μTBS results remained stable over 4m ($p>0.05$). The predominant fracture pattern was mixed/dentin, except G3 (mixed/resin). EDX pointed a stabilization on the proportion Ca/P for G1 (24h and 4m); for G2, G3 and G4, the proportion of Ca/P increased after 4m. Nanoleakage was reduced in G4 compared to G1 and G3. The addition of BA in a prior re-moiture solution maintained bond strength results, increased Ca/P proportion and reduced nanoleakage after 4 months storage when applied previously an experimental adhesive.

Keywords: Biomimetic remineralization. Bond strength. Adhesive. Collagen. Acid-etching.

1 Introduction

Dentin/composite interface vulnerability remains challenging dentistry research to overcome degradation in a functional, accessible and practical protocol. While etch-and-rinse technique requires a prior phosphoric acid etching of dentin and enamel simultaneously, which demands a great attention from clinicians in order to achieve the proper amount of moisture remained before hybridization is completed (25). If over dried, this process can be jeopardized by collagen fibrils collapse, hampering resin diffusion through demineralized dentin structure (3). To overcome that, re-moisture solutions are available to be used after acid-etching procedure and before single-bottles adhesive application, offering an option to control humidity in dentin subtract (3).

Despite this, long term durability of composite restorations are often questionable, once bonding degradation takes place as soon as the adhesive interface is photo-activated (7). Different types of bonding degradation occurs inward adhesive interface. The first and more current one, endures in the bottom part of hybrid layer through endogenous enzymatic degradation of collagen, mediated by metalloproteinase matrix MMP's and cathepsines enzymes. Still, this degradation can be temporary inhibited by some dentinal conditioners, as chlorhexidine, tetracyclines, proanthocyanidins, EDTA, among others (16, 35).

A second type of degradation consists occurs when after dentin etching (by phosphoric acid or acidic monomers) and sequent adhesive penetration, intrinsic water held in dentin demineralized collagen matrix arises from intrafibrillar and extrafibrilar portions (4). This water arisen may fill each empty space poorly infiltrated by resin monomers during hybridization process or improperly polymerized throughout bond interface (6). Hence, this structural rearrangement and a weaken of the integrity of this interface leads this structure into a more susceptible form of premature clinic failures depending on whatever conditions this restoration will oppose during its clinical life-time (2, 4).

The last type of degradation, apart from enzymatic and water ascendency mediated degradation, is the questionable stability of silica-coating

over substrate surface to induce stable and durable covalent bonding between ≡Si-O-Si≡ elements within dentin restorative materials (19). This form of degradation may be prevented by the improvement of chemical bonding between these agents with new silane monomers to promote hydrolytic stability and durable bondings (20).

Still after all, the problem seems to remain, once it is impossible for these monomers to reach such inner dentin parts as collagen intrafibrillar spaces, even because their chemical chain sizes are often bigger than the actual space for them to over through inward collagen fibrils (2). Additionally, these manners pointed above to prevent adhesive interface degradation tends to solve punctual problems, instead of come with a single approach to prevent different types of degradation.

Because of that, the perception of biomineralization, as a non-classical particle-based crystallization concept (23, 36) that uses intrinsic alive organisms to excrete inorganic minerals, draws attention to be explored aiming to overcame these degradation issues towards an integrated way. This biomineralization process often occurs for example in sea-shells, bones and teeth in a natural and a highly spacial organized and hierarchical growth by the deposition of biominerals such as calcium carbonate, amorphous silica and calcium phosphate salts (23, 36). In human tissues, this mechanism is regulated by non-collagenous proteins, which mediates the calcification of collagen matrix through the deposition of carbonated apatite inorganic phases (23, 36). Specifically in teeth structure, along with non-collagenous proteins, some MMPs and enzymes previously secreted by odontoblasts participates of biomineralization transferring carboxylic acid and phosphate functional groups to be linked with Ca/P available sites for the beginning of nucleation and consecutive apatite crystallization development (23, 29).

For its laboratory reproduction in a therapeutic way aiming to overcome degradation in dentin structure, the utilization of native non-collagenous proteins turns to be still economically unfeasible and highly time-consuming (11, 23). This way, the utilization of polyelectrolyte and poly(acid) macromolecules to mimic functional domains of these natural intrinsic proteins is investigated in an

biomimetic mineralization approach (1, 10, 12, 17, 22, 27, 32). Which based on nature mimecry of mineralization rationale, biomimetic analogues of non-collagenous proteins and amorphous calcium/phosphate (ACP) nano-precursors particles are applied to fill in demineralized collagen network (5, 15, 23).

To pursuit this in a partially or fully empty space caused by acid dissolution of interfibrillar and intrafibrillar mineral content within dentin collagen fibril, biomimetic analogues such as polyacrylic acid (PAA), polyvinylphosphonic acid (PVPA) or sodium trimetaphosphate (STMP) are employed (1, 13, 27). The first one, PAA, acts as a sequestration analogue, binding itself into specific dentin matrix protein 1 (DMP1) sites stabilizing ACP nano-particles from aggregating it-selves in the outer edge of interfibrillar and intrafibrillar collagen fibrils (13, 27). On the other hand, PVPA and STMP are described as template analogues by its linkage capacity to collagen matrix and attraction capability for ACP nano-particles (13). The combination of different biomimetic analogues allows biomimetic mineralization to take place through a stable entrance of these components within collagen fibrils followed by the entrance of calcium-phosphate ions to arrange pre-nucleation clusters. What consists of ACP nano-precursors and apatite structure aggregation in highly organized templates throughout intra/interfibrillar spaces and between different collagen fibrils in a bottom-down mineralization mechanism (18).

Despite all the advances in biomimetic remineralization field, little information is available concerning the direct addition of biomimetic analogues and ACP particles in etch-and-rinse experimental adhesives (1, 27). The target is to develop a clinical practicable approach together with an economical viable set formulated to reach the bottom part of hybrid layer, including inner areas in collagen fibrils after acid-etching demineralization. Particularly, this circumstance reproduces a challenging environment, once, this sensitive-technique, even if well performed during adhesion procedure, still persists with a considerable amount of remained water and less mineral content to be remineralized (31). Besides this, etch-and-rinse technique continues to be clinical performed routinely in many countries, what makes this concept still

demanding to follow ongoing approaches in dentin adhesion field and its innovations.

Within this, the aim of this in *vitro* study was to evaluate the re-mineralizing potential of a combination of an experimental re-moiture solution containing biomimetic analogs and an experimental etch-and-rinse adhesive system (single-bottle) containing bioactive calcium-phosphates, after 4 months storage in simulated body fluid (SBF). The hypothesis were: (i) The association of the experimental re-moiture solution and adhesive system, at least, would maintain μTBS values after 4 months storage in SBF; (ii) The application of re-moiture solution containing biomimetic analogs and an experimental adhesive system containing bioactive calcium-phosphates would aid in the deposition of apatite within and around of the exposed collagen in the hybrid layer.

2. Materials and Methods

2.1. Experimental Adhesive Formulation

An experimental 3-step acid-etching adhesive system was formulated, which was composed of a prior re-moiture solution and a single-bottle adhesive. Control prior re-moiture solution was a blend composed of (% by weight): 90% distilled water and 10% 2-hydroxyethyl methacrylate (HEMA). This prior re-moiture solution was used for control groups and did not have the addition of biomimetic analogues. Experimental prior re-moiture solution composition was equal as described above, but with the addition of 5% polyacrylic acid (PAA) and 10% polyvinyl phosphonic acid (PVPA) as biomimetic analogues, applied on experimental groups for re-moiture solution.

A matrix blend of a single-bottle adhesive was formulated with the following composition (wt%): 10% of HEMA; 10% urethane dimethacrylate (UDMA); 20% triethylene glycol dimethacrylate (TEGDMA); 15% bisphenol-A-ethoxylate dimethacrylate glycidil (Bis-EMA); 10% bisphenol-A-dimethacrylate glycidil (Bis-GMA); 32% ethanol; 1% of dimethylamino-ethyl benzoate (EDAB); 0.5% camphorquinone (CQ); and 1.5% diphenyl iodonium hexafluorophosphate

(DPIHP) and applied on control group. For experimental groups, in this adhesive blend, 20% of solid phase bioactive calcium/phosphorus components were added as follows: 40 mol% of mono-calcium phosphate monohydrate (MCFM), 40 mol% of beta-tricalcium phosphate (β-TCP) and 20 mol% of calcium hydroxide (CaOH) (Table 1).

All solutions manufactured in this study were blended in a laboratory environment with control conditions as temperature, humidity and light source. Stirring occurred during 30 min for re-moiture solution and one hour for adhesive blend, until it both were completely homogeneous.

2.2 Dentin Bonding Procedure

Thirty extracted human third molars free of caries or intrinsic fractures were collected under ethical committee approved protocol #063/2014 (FOP/ UNICAMP), stored in 5% chloramine-T solution at 4°C and used within three months following extraction. Teeth had its gross debris removed with curettage instruments. Correspondingly to figure 1 (1), roots were sectioned perpendicular to its long axis 2 mm below cement-enamel junction, with a diamond saw (EXTEC Corporation, Enfield, CT, USA) linked into a laboratory sawing machine (Isomet 1000, Buehler Ltda., Lake Bluff, IL, USA). Occlusal enamel was removed in the same manner (Figure 1) and dentin exposed surface was double-checked for enamel remains, being worn in case of remained enamel with #600 sandpaper attached into a low-speed methalographic polisher machine (Arotec APL-4, Cotia, SP, Brazil).

Each sample was then glued to a phenolic tube pre-filled with acrylic resin (Figure 1.a). This set was mounted into a sawing machine and positioned for dentin substrate sectioning performed by a 0.15 thick diamond disk (Buehler, Lake Bluff, IL, USA). Dentin substrate was rotated inside a precision cutter accessory (Isomet, Buehler, Lake Bluff, IL, USA) after each sectioning in order to obtain 90º angled sections among four dentin quarters, accordingly with figure 1.a schematic illustration. Dentin surface went through a standard smear layer simulation through 600-grit silicon carbide paper grinding under running

water (21) and each quarter was randomly assigned for one of the following groups (n=30): (G1) control solution + control adhesive, (G2) - control solution + experimental adhesive, (G3) experimental solution + control adhesive and (G4) experimental solution + experimental adhesive, likewise figure 1.b.

For restoration procedure (Table 1, Figure1), all dentin quarters not treated were maintained protected by a thick insulation tape (3M ESPE Rubber tape, St. Paul, USA) until treatment was conducted. First, dentin surface etching procedure was carried out with 35% phosphoric acid (Ultradent, South Jordan, USA) during 15 s, rinsed for 15 s and blot dried. The experimental re-moiture solution was applied actively during 10 s and gently air dried for 5 s. The adhesive coat was spread actively for 15 s using new disposable microbrushs followed by air volatilization for 5 s and light curing for 10 s with a light-emitting diode (LED) Bluephase G2 (Ivoclar Vivadent, Schaan, Liechtenstein) with 1,392 mW/cm^2 of irradiance. Resin-based composite (Filtek Z250, 3M ESPE, St. Paul, USA) was layered over the adhesive surface accordantly with incremental technique restoration and light cured separately for a total 40 s. Each dentin quarter received a different combination of re-moiture solution and adhesive with or without bioactive components as mentioned above (G1, G2 G3 and G4; table 1, figure 1.b). Dentin quarters were color tagged with permanent markers for posterior group identification and stored individually in identified hermetic selling multiwell plates (Eppendorf AG, Enfield, Connecticut, USA).

Table 1: Groups abbreviations, corresponded materials tested, its compositions and application method utilized.

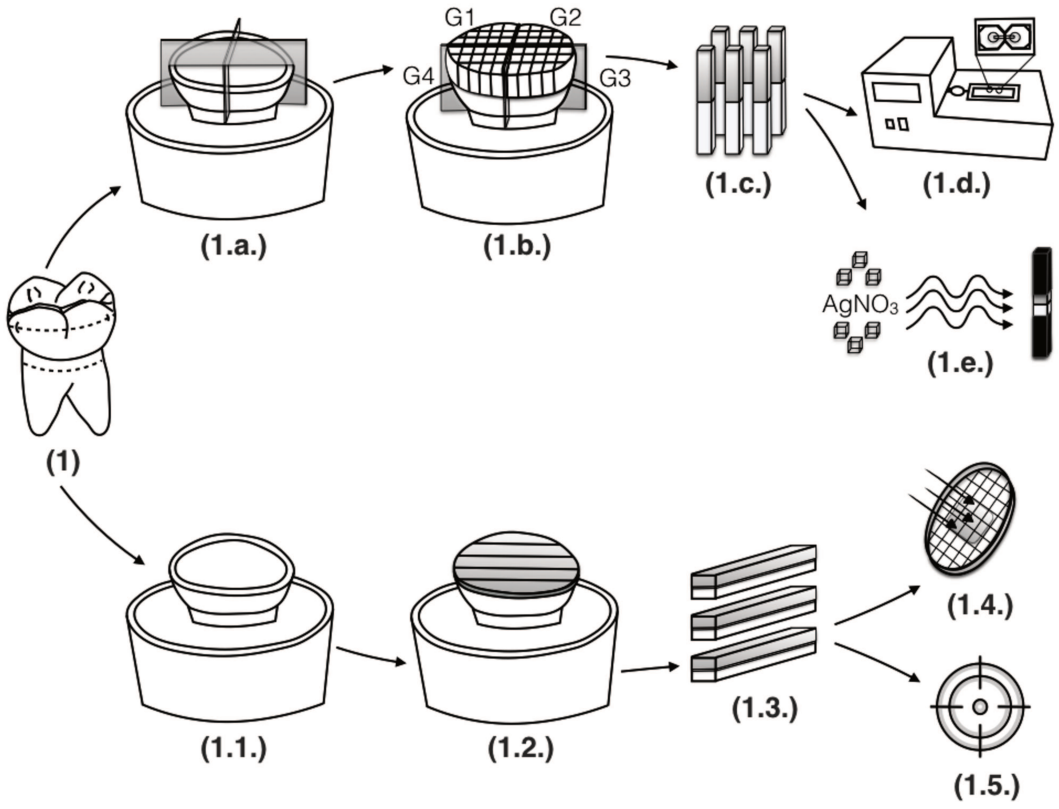
Group abbreviation	Material	Composition	Application method
G1	Control re-moiture solution	HEMA, distilled water	Re-moiture solution with/without analogues: After phosphoric acid etching (15 s) rinsing(15 s) and drying, solution is applied actively (15 s) and air dried (5 s).
	Control adhesive	HEMA, UDMA, TEGDMA, BisEMA, BisGma, ethanol, photoiniciators	
G2	Control re-moiture solution	HEMA, distilled water	
	bioactive calcium-phosphates adhesive	HEMA, UDMA, TEGDMA, BisEMA, BisGMA, ethanol, photoiniciators + βTCP, MCPM, CaOH	
G3	Biomimetic analogues re-moiture solution	HEMA, distilled water + PAA and PVPA	Adhesive with/without bioactive calcium-phosphates: Actively application of adhesive (15 s), air volatilization (5 s) and light curing (10 s).
	Control adhesive	HEMA, UDMA, TEGDMA, BisEMA, BisGma, ethanol, photoiniciators	
G4	Biomimetic analogues re-moiture solution	HEMA, distilled water + PAA and PVPA	
	Bioactive calcium-phosphates adhesive	HEMA, UDMA, TEGDMA, BisEMA, BisGMA, ethanol, photoiniciators + βTCaP, MCaPM, CaOH	

Abbreviations: hydroxyethyl methacrylate (HEMA), urethane dimethacrylate (UDMA), triethylene glycol dimethacrylate (TEGDMA), ethoxylated bisphenol-A dimethacrylate (BisEMA), bisphenylglycidyl dimethacrylate (BisGMA), beta-tri-calciumphosphate (βTCP), monocalciumphosphate monohydrated (MCPM), calcium hydroxide (CaOH), polyacrylic acid (PAA), polyvinyl phosphonic acid (PVPA).

2.3. Storage Medium

Each individual hermetic sealing multiwell plate was fulfilled with 1.5 ml of a remineralizing medium of simulated body fluid (SBF) solution weekly changed during 4 months storage. SBF solution was previously prepared by dissolving the following components: 205.2 mM NaCl, 6.3 mM $NaHCO_3$, 4.5 mM KCl, 1.5 mM $KHPO_4 \cdot 3H_2O$, 2.25 mM $MgCl_2 \cdot 6H_2O$, 3.75 mM $CaCl_2$ and 0.75 mM Na_2SO_4 (pH=7.3 at 37ºC).

Fig.1: Schematic illustration of methods design. (1) Extracted tooth sectioning location; (1.a.) Dentin sectioning in quarters and matrix strip positioning before restoration; (1.b.) Dentin quarters restorations with different treatments accordingly with G1, G2, G3 and G4 protocols; sectioning for microtensile bond strength test (μTBS). (1.c.) μTBS samples in beam shape of ± 0.9 mm^2 adhesive area stored for 24 h and 4 m in SBF (simulated body fluid) solution. (1.d.) μTBS test in Geraldeli's jig-2 testing device. (1.e.) nanoleakage analysis of adhesive area after 4 months storage. (1.1.) and (1.2.) Dentin exposition, restoration and sectioning for TEM and EDX analysis. (1.3.) Beams were obtained with adhesive area parallel to its long axis. (1.4.) Ultrathin adhesive area sections on farmvar grips were observed through TEM and (1.5.) representative samples of each groups were analyzed in EDX-SEM.



2.4. Microtensile Bond Strength Test (μTBS)

Restored specimens were sectioned in X and Y directions creating composite-dentin beams of approximately 0.9 mm² adhesive interface (figure 1.c). Half of the obtained beams from each group were tested immediately (24 h) and other half kept stored in SBF solution at 37ºC for 4 months (4 m) before μTBS test. For μTBS test, beams were actively gripped onto a Geraldeli's jig-2 (Figure 1.d) with cyanoacrylate glue and tested in a universal testing machine (OM100, Odeme Dental Research, Luzerna, SC, Brazil) at 0.5 mm/min. Cross-sectional area was measured with a digital caliper (Mitutoyo Corporation, Tokyo, Japan) after fracture. Final values were expressed in MPa calculated from the following equation: μTBS = F/A x 0,098; In which, μTBS stands for microtensile bond strength value (MPa), F for microtensile force applied for the test (kgf), A sample bonded area (mm^2)/100 = (cm^2). For μTBS test, each quarter was defined as the average of the tested beams (n=30). Failure mode was determined by evaluating each beam with stereomicroscope (50x, Nikon, model SMZ-1B, Japan). The mode of failure was classified in adhesive (A), cohesive in composite (C), mix/resin (MR) or mix/dentin (MD).

2.5. Nanoleakage analysis

After sectioning dentin quarters to obtain a set of beams samples, one beam located on middle dentin region was collected from each dentin quarter corresponded to tested group of 4 m storage for posterior nanoleakage analysis (Figure 1.e). Beams were immersed in 50 wt% ammoniacal silver nitrate ($AgNO_3$) aqueous solution in total darkness for 24 h. Afterwards, beams were rinsed abundantly with distilled water to dismiss the excess of $AgNO_3$ and immersed in photo-developing solution (Kodak Developer D-76; Kodak Brazil, Sao Jose dos Campos, SP, Brazil) for 8 h under fluorescent light in order to reduce silver ions into metallic silver grains. Silver-impregnated beams were embedded in epoxy resin and abraded using SiC papers with crescent abrasion of #600-4000 grids (Norton Saint-Gobain Abrasives, Worcester, MA, USA). Polishing was performed using diamond pastes (Buehler, Lake Buff, IL, USA) in the sequence of 6, 3, 1 and 0.25 μm particles sizes. Between each one of

abrasive and polishing steps, stubs were submitted to an ultrasonic cleanup bath for 20 minutes.

Samples were air-dried, dehydrated in silica-gel overnight and sputter-coated with 20 nm layer of carbon for 60 s at 45 mA in a vacuum metallizing chamber (MED 010; Balzers, Liechtenstein) and subjected to a scanning electron microscopy (JEOL JSM-IT300LV, Tokyo, Japan) analysis operated under 15 kV in backscattered electron mode. All images are representative areas with a 1000 x magnification (working distance between 11.9 – 16.3 mm) with 10 µm scale bars. SEM images were attached to Image J software in 8 bit format, adhesive interface was selected and submitted to pixels counting accordingly with the actual distance given by SEM scale bar information.

2.6. Transmission electron microscopy (TEM) analysis

Four extracted human teeth were sectioned removing occlusal enamel and below pull chamber structure in order to obtain a middle dentin slab of 1.5 mm thickness. A representative restoration from each group was performed following the same restoration procedure as described above, with the exception of 1 mm height flow-resin composite restoration (A2, Filtek Supreme Ultra Flowable Restorative, 3M-ESPE, St. Paul, USA). Alike figure 1.2, four sections (1 mm width) on mesial-distal directions were performed to obtain three beams for each group with its adhesive layer parallel to its long axis (Figure 1.3). Each set of three beams were stored in SBF solution in the same manner as described above for 4 months (4 m).

After storage period, specimens were immersed in a fixative Karnovsky solution (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M sodium phosphate buffer [pH 7.4]) for 24 h and rinsed 3×10 min with 0.1M sodium phosphate buffer solution. For post- fixation step, samples were immersed in 1% osmium tetroxide solution for 1 h and rinsed for 3×10 min with 0.1M sodium phosphate buffer solution. Ascending grades of ethanol dehydration (30-95%) were kept for 1 h each and 3 h for 100% ethanol. Then, samples were gradually infiltrated with epoxy resin (50-100%) and cured for 48 h in pure epoxy resin at 60ºC (33).

For TEM observation (Figure 1.4), approximately 70 nm ultrathin sections of each specimen were collected on a 75 mesh formvar coated copper grid (Ted Pella Inc., Redding, CA, USA) obtained from an ultra-microtome sectioning (UC6, Leica Microsystems, Heerbrugg, Switzerland) with a diamond knife (Histo Diatome, Biel, Switzerland). Samples were observed in a TEM (Hitachi 7600, Hitachi Ltd., Tokyo, Japan) at 80 kV and x50k and x100k magnification.

2.7 Energy Dispersive X-ray Spectrometry (EDX)

Three extra representative samples from each tested group (24 h and 4 m) were collected after TEM ultra-thin sections were obtained for EDX analysis. Sections were analyzed perpendicularly to dentin adhesive layer surface (figure 1.3). Samples were sectioned and embedded in epoxy resin as TEM description above, and positioned on an acrylic stub for silica-dehydration during 24 h and sputter-coated with a 20-nm layer of carbon (MED 010 Baltec, Balzers, Liechtenstein) for SEM observation and EDX analysis (JEOL, JSM-5600LV, Tokyo, Japan). An energy dispersive X-ray (EDX, Vantage, NORAN Instruments, Middleton, WI, USA) automatic chain and 50 mm beam diameter apparatus was attached to SEM to identify elemental inquiry on treated dentine surface at 15 kV in backscattering mode. The spectrometer was coupled to a computer for data collection and processing (Vantage, NORAN Instruments, Middleton, WI, USA).

A semi-quantitative analysis was conducted to track calcium (Ca) and phosphorus (P) elements on hybrid layer area for each treated group. Ten random areas on hybrid layer were selected with a resolution of 7 μm and each spectrum was acquired during 100 s (voltage 15 kV, working distance 20 mm). Data were calculated using a Ca and P ratio as parameters from X-ray emitted.

2.8 Statistical analysis

For μTBS test, data were submitted to one-way ANOVA with Split-Plot arrangement and Tukey´s test for pairwise comparison (α=0.05). The factor (parcel) considered was material (G1, G2, G3, G4) and as sub-factor (sub-parcel) storage time in two levels (24h and 4 months). Failure mode was determined by frequency percentage of failure pattern observed. In

nanoleakage analysis, one-way ANOVA and Tukey´s test (α=0.05) were performed. For EDX, Ca and P radio were submitted to two-way ANOVA and Tukey´s multiple comparisons test (α=0.05).

3. Results

3.1. Microtensile bond strength (μTBS)

Statistical analysis of μTBS results pointed significant interaction between tested factors ($p<0.05$). At immediate testing (24 h), there was no statistical significant difference among all tested groups (table 2). While after 4 months storage degradation, re-moiture solution with analogues and experimental adhesive with bioactive calcium-phosphates (G4) presented statistical significant increased μTBS values compared to all others group (Table 2).

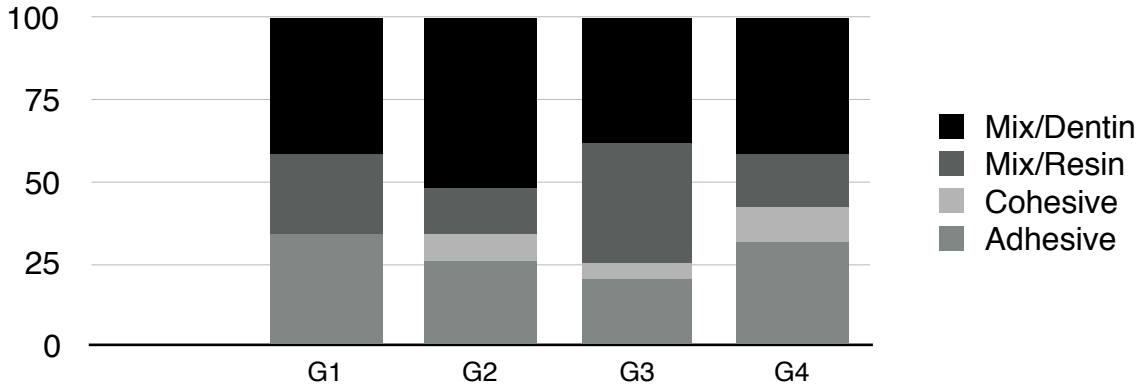
Control group (G1) and the group containing only bioactive calcium-phosphates in adhesive (G2) presented statistical significant reduction on theirs μTBS results after 4 m storage. While groups containing biomimetic analogues in re-moiture solution (G3 and G4) did not present statistical difference from 24 h to 4 m storage (Table 2). Failure pattern mode distribution showed a predominance of mix/dentin type of failure for all groups, except for G3, which presented a mix/resin type of failure more often observed (Figure 2).

Table 2: Statistical results obtained from μTBS test, EDX analysis from 24 hours and 4 months storage time; and nanoleakage statistical analysis (%) of 4 months storage samples.

	***μ*TBS (MPa)**		**EDX (Ca/P ratio)**		**nanoleakage (%)**
	24 h (mean ± s.d.)	**4 months (mean ± s.d.)**	**24 h (mean ± s.d.)**	**4 months (mean ± s.d.)**	**4 months (mean ± s.d.)**
G1	26.12±3,30 a,A	19.68±4.43 b,B	2.18±0.16 a,A	2.36±0.15 b,A	15.00±3.01 ab
G2	29.71±3.43 a,A	20.15±4.48 b,B	2.13±0.10 a,B	2.88±0.51 a,A	13.43±2.71 bc
G3	24.90±4.98 a,A	20.52±4.52 b,A	2.01±0.21 a,B	2.33±0.18 b,A	18.22±1.07 a
G4	27.13±5.20 a,A	28.75±5.36 a,A	2.27±0.16 a,B	2.44±0.09 ab,A	11.73±2.94 c

Different uppercase letters in the row and lowercase letters in the columns means statistics significance (α=0.05)

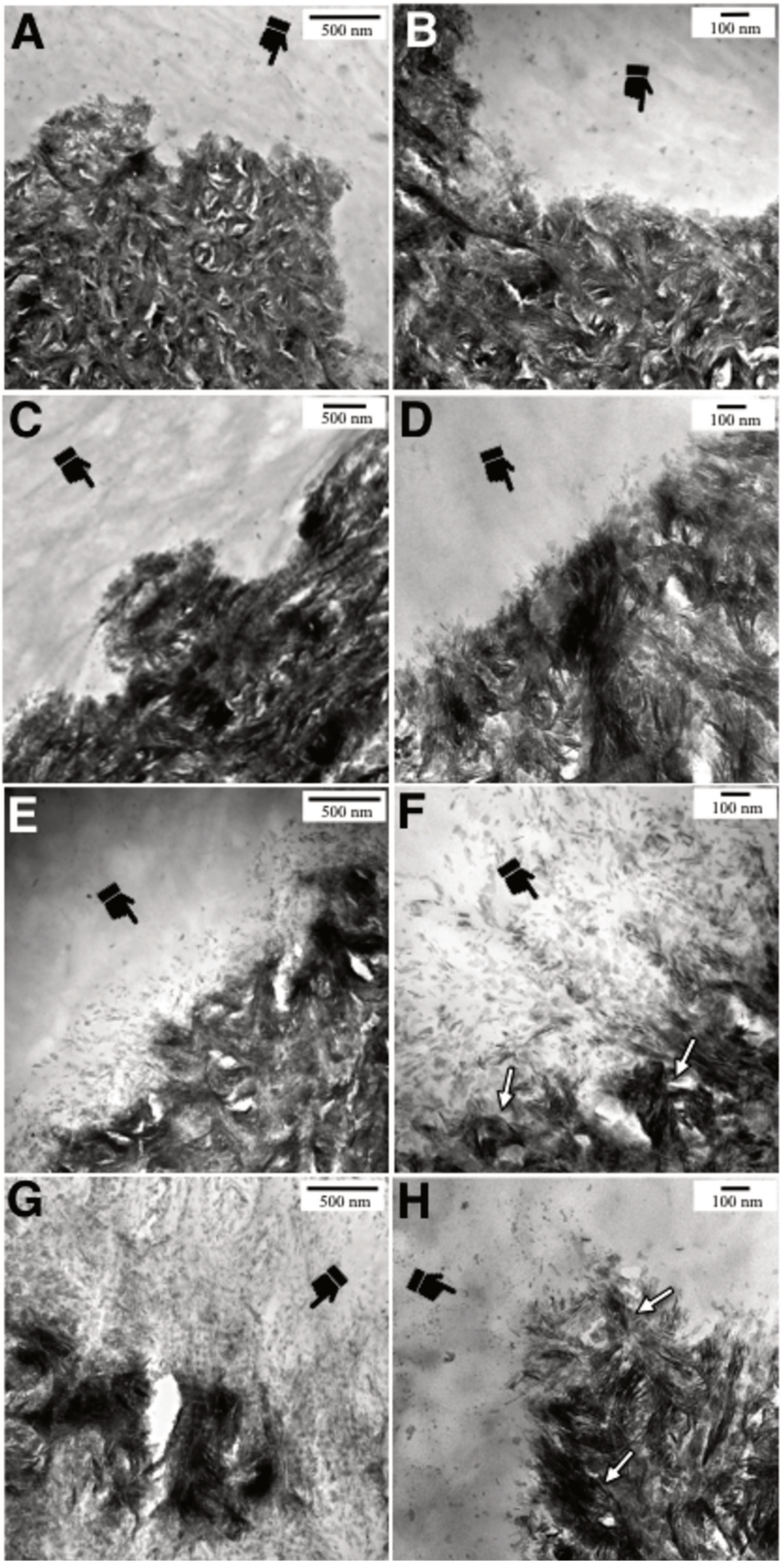
Fig.2: Failure pattern mode distribution of μTBS tested samples.



3.2 Dentin Remineralization - TEM analysis

The observations from TEM ultra structure analysis illustrates the results from representative samples of each tested group after 4 months storage in SBF solution (figure 3). Figure 3: E, F, G and H exposes biomimetic remineralization present in hybrid layer area as apatite deposition when experimental re-moiture solution was used with or without the application of the experimental adhesive (G3 and G4). It also points indications of biomimetic remineralization within collagen fibrils. In other side, figure 3 (A, B) as control group (G1) and figure 3 (C,D) as control solution and experimental adhesive group (G2) maintained a hybrid layer with the absence of the biomimetic remineralization pattern throughout dentin periphery.

Fig.3: Unstained TEM ultra structure observation with 50-100 kx magnification (80kv) illustrating each group´s dentin collagen and hybrid layer area after 4 months storage in SBF solution. A, B: control group (G1), no indications of apatite deposition in collagen fibers neither in dentin periphery (hand-pointed); C and D: control re-moiture solution and experimental adhesive (G2), as G1, there are no indications of biomineralization, dentin periphery is hand-pointed; E,F: experimental re-moiture solution with control adhesive (G3) with indications of apatite deposition over dentin periphery (hand-pointed) and indications of biomineralization within dentin structure (white arrows); G,H: experimental re-moiture solution and experimental adhesive (G4), as G3, there are indications of apatite deposition throwout dentin periphery (hand-pointed) and within dentin structure (white arrows).


A
500 nm
B
100 nm
C
500 nm
D
100 nm
E
500 nm
F
100 nm
G
500 nm
H
100 nm

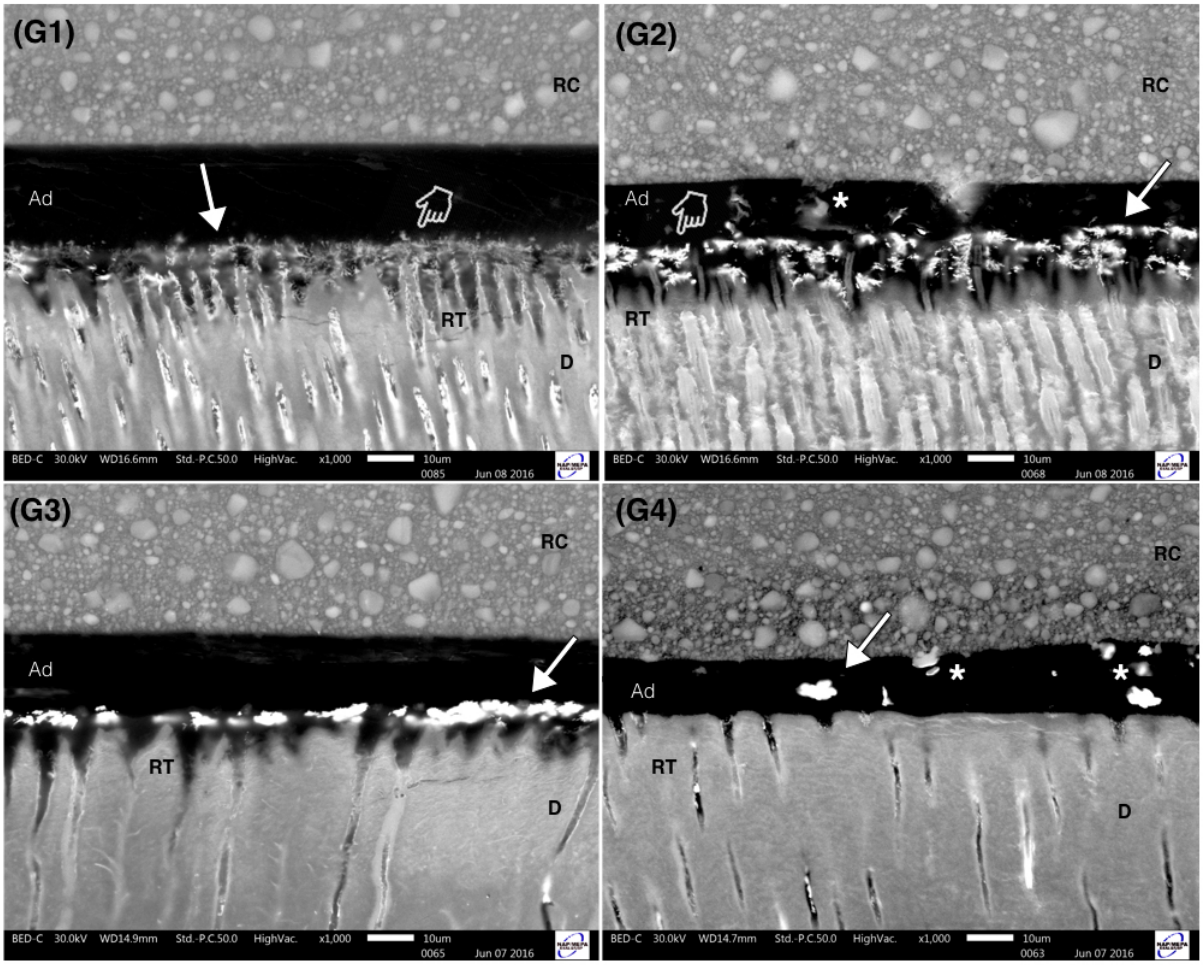
3.3. Dentin Remineralization - EDX analysis

Different Ca/P ratios were detected by EDX spectrometry analysis in different regions of hybrid layer within 24 h and 4 m SBF storage. At immediate EDX analysis (24 h), no statistical significant differences were found on Ca/P ratios among all tested groups in hybrid layer area. After 4 m storage, all groups that received biomimetic analogues and/or bioactive calcium-phosphates (G2, G3 and G4) presented statistical significant higher Ca/P ratios (table 2). G1, as control group, maintained Ca/P ratio after 4 m storage.

3.4. Nanoleakage

Nanoleakage statistical analysis performed in percentage comparison after 4 m storage in SBF solution is represented in table 2. It showed that G4 presented reduced percentage nanoleakage area compared to G1 and G3 groups ($p<0.05$) and did not present statistical significant difference with control solution and bioactive calcium-phosphates group (G2) ($p>0.05$). This numerical reduced pattern of silver nitrate deposits can be observed on SEM micrographs on figure 4, image G4, where scarcely few silver nitrate deposits may be noticed throughout top hybrid layer area. The group with analogues in re-moiture solution and control adhesive (G3) presented the greater numerical percentage of nanoleakage (Fig. 4, G3) after 4 m storage, not differing statistically from control group (Fig. 4, G1).

Fig. 4: Nanoleakage SEM representative micrographs of dentin treated with tested control and experimental adhesive systems at 1000x magnification on backscattering mode (30 kV, wd= 14.9-16.6). (G1) represents control group; (G2) control solution/bioactive calcium-phosphates adhesive; (G3) analogues solution/control adhesive; (G4) analogues solution/bioactive calcium-phosphates adhesive, which presented lower statistical percentage of nanoleakage with reduced silver nitrate deposits after 4 m SBF storage. Water trees can be observed in images (G1) and (G2). Abbreviations are: RC, resin composite; Ad, adhesive interface; RT, resin tags; D, dentin surface. White arrows highlight silver nitrate particles infiltration, (*) shows bioactive calcium-phosphates particles.



4. Discussion

This *in vitro* study demonstrated that, in the presence of SBF solution storage, the inclusion of biomimetic analogues in a prior re-moiture solution of an experimental adhesive system preserved μTBS results over 4 months, decreased its nanoleakage compared to control group and increased calcium/ phosphorus ratio in hybrid layer area. Thus, first hypothesis was accepted, once this association did maintain μTBS values for 4 months. The use of biomimetic analogues and bioactive calcium-phosphates may be a rich environment to back-fill apatite deficiencies over time and current demands as local chemical alterations. Besides μTBS test, nanoleakage analysis, TEM observations and EDX data integrates this scenario also providing constructive miscellaneous information for this debate.

The presented μTBS methodology embraces μTBS test to be conducted over dentin quarters instead of each treatment be performed on a whole tooth (dentin surface). This singularity has the advantage of observing this treatment over a greater number of dentin surfaces and its particularities, hence enriching this investigation. From that point, it was observed that whenever PAA and PVPA biomimetic analogues of dentin phosphoproteins were applied through a re-moiture solution, with or without the subsequent application of bioactive calcium-phosphates into adhesive, μTBS values were preserved after 4 months storage. This displays biomimetic analogues as PAA and PVPA as key-elements behind biomimetic remineralization rationale (Table 2).

Indeed, as suggested by some authors (12, 13, 17, 18), these biomimetic analogues seems to diffuse into and along collagen fibers being able to link into some specific locations of collagen structure. This goes on before amorphous calcium phosphate nanoprecursors are attracted inside intrafibrillar spaces, where their deposition conjectures apatite formation though pre-nucleation clusters. These pre-nucleation clusters eventually aggregate each other building up higher structured organized liquid-like ACP nanoparticles. After this, a mineralization self-assembly template is developed constructing a crystallographic alignment structure, which, when combined, encircles

intrafibrillar and interfibrillar collagen spaces and current apatite seed crystallites in a so-called biomimetic remineralization (8, 9, 23, 24).

This principle known as bottom-up approach has being recently investigated (1, 22, 27). Although substantial data is available regarding this subject, the incorporation of biomimetic analogues and bioactive calcium-phosphates in adhesives is quite innovative. The addition of different bioactive compounds (sodium–calcium–aluminum–magnesium silicate hydroxide, HOPC; aluminum–magnesium–carbonate hydroxide hydrates, HCPMM; and titanium oxide) in Portland cement presented a reduction of hybrid layer degradation (27). While a self-etching adhesive system synthesized containing PAA and STMP biomimetic analogues also presented signs of propitious remineralization (1). Still this last work, when PAA was added separately in primer solution, a reduction on μTBS values were observed after 6 months storage in SBF, oppositely than when both analogues were used together (1). These both works brightened this research´s objective to incorporate biomimetic analogues in therapeutic materials. Thus, in agreement with Cao et al., 2015 (5), the combination of different biomimetic analogues may be favorable to guide, stabilize and template ACP nano-precursors at the same time, what probably lead the outcomes of this research.

Besides PAA characteristics to stabilize ACP nano-precursors and prolong its life, the addition of a different biomimetic analogue as PVPA gives in the ability to recruit ACP nano-precursors to template and also to inhibit matrix metalloproteinase (MMPs) activity (23, 34). What upgrades this approach, once endogenous MMPs and some cysteine cathepsins becomes entrapped in these circumstances being disabled to act and surrounded by apatite replacement, what has been called “fossilized” state (34). This fact helps supports collagen structure to maintain its properties during this long process of biomimetic remineralization, what probably also corroborated to maintain μTBS values over 4 months, avoiding different degradation´s entraps at the same time as mentioned earlier.

Another way to investigate degradation behavior is through nanoleakage analysis (26). The groups with addition of biomimetic analogues and bioactive

calcium-phosphates together in adhesive system (G4) obtained the less percentage of nanoleakage (table 2), in accordance with EDX data and with μTBS results obtained after 4 months storage (table 2). The addition of analogues only (G3) jeopardized the integrity of hybrid layer surface presenting the highest numerical nanoleagake percentage (table 2). This may be due intrinsic characteristics of the adhesive itself (30). Single-bottle adhesive systems presents in its composition hydrophilic and hydrophobic monomers in the same vial, while the re-moiture solution applied in this study had plenty water and hydrophilic content (table 1). After acid-etching procedure, dentin surface presents a low energetic state making a hydrophilic re-moiture solution welcome to get in and still reestablish collagen fibrils framework (25). After that, resin should penetrate opened path and fulfill collagen empty spaces before light-curing. But from another perspective, these single-bottle adhesives tends to present monomers separation during dentin penetration, which probably was emphasized by the water content inserted before by re-moiture solution in this environment. What might jeopardize light-curing performance and leave willing resin area more available for degradation.

In the same situation, control group (G1) and groups with the addition of biomimetic analogues or bioactive calcium-phosphates only (G2, G3) presented higher content of silver nitrate deposits along hybrid surface (Figure 4). However, experimental adhesive with biomimetic analogues and bioactive calcium-phosphates (G4) appeared less affected by nanoleakage compared to control group and G3 (table 2). This emphasizes the concern of adding biomimetic analogues preferentially along with a direct source of bioactive calcium-phosphates properly available, in order to create an auspicious setting for biomimetic remineralization (5).

In addition to that, TEM ultrastructural observation and EDX spectra counting were carried out for a more punctual examination of stored sample´s hybrid layer area. Both TEM images and EDX data statistical results pointed compelling indications of biomimetic remineralization of hybrid layer area for G3 and G4 groups after 4 months storage. Likewise, TEM ultrastructural analysis of G3 and G4 groups (Figure 3) denotes a consistent pattern of apatite deposition over dentin periphery within hybrid layer. Also, denotes some intrafibrillar and

extrafibrillar apatite deposition within collagen fibrils (figure 3.F and 3.H), what lead second tested hypothesis as accepted. The tested experimental adhesive system with biomimetic analogues and bioactive calcium-phosphates assisted mineral precipitation within and around exposed collagen fibers in some regions of hybrid layer after 4 m storage in SBF solution.

Differently than control group (G1) results obtained from μTBS test, nanoleakage analysis and TEM ultrastructural observation, these remineralization characteristics in G4 group inferences bottom-up biomimetic remineralization taking place in hybrid layer area. Which likely propitiated the stabilization of μTBS results, reduction of $AgNO_3$ deposits during nanoleakage test and an increase in Ca/P ratio pointed by EDX analysis after 4 m SBF storage in G4 group. This overcome supports dentin-adhesive interface to resist with its structural integrity throughout chemical and biological demands. As a consequence from “smart” materials research field, this may indicate a promising approach for long-lasting restorations. Therefore, within the limitations of this *in vitro* study, further investigations are required to submit biomimetic remineralization approach to new challenges as caries affected dentin substrate and prolonged periods of evaluation before clinical trials.

5. Conclusion

The addition on biomimetic analogues in a prior re-moiture solution of an experimental adhesive maintained μTBS results over 4 months STB storage, reduced nanoleakage compared do control group and increased hybrid layer Ca/P proportion when applied along with control adhesive or bioactive calcium-phosphates adhesive.

Acknowledgments

The authors are thankful to Capes/Brazil for financial support (#99999.005561/2014-00), to Dr. P.D. Novaes and A.L. Martins for SEM equipment support in Image and Microscopy Center (CMI-FOP/UNICAMP), to Dr. E.W. Kitajima and R.B. Salaroli for SEM equipment support in the NAP/

MEPA/ESALQ/USP, to Dr. J. Bettini, Dr. E Teixeira-Neto, Dr. C.A.O. Ramirez for TEM training in Brazilian Nanotechnology National Laboratory (LNNano, CNPEM, SP, Brazil) and to Interdisciplinary Center for Biotechnology Research (University of Florida) for TEM equipment support.

Conflict of interest

All authors declare no financial and personal conflict of interest.

References

1. Abuna G, Feitosa VP, Correr AB, Cama G, Giannini M, Sinhoreti MAC, Pashley DH, Sauro S. Bonding performance of experimental bioactive/ biomimetic self-etch adhesives doped with calcium-phosphate fillers and biomimetic analogs of phosphoproteins. J. Dent. 2016; 52: 79-86.
2. Bertassoni LE, Orgel JP, Antipova O, Swain MV. The dentin organic matrix – limitations of restorative dentistry hidden on the nanometer scale. Acta. Biomaterialia 2012; 8: 2419–33.
3. Brackett MG, Li N, Brackett WW, Sword RJ, Qi YP, Niu LN, Pucci CR, Dib A, D.H. Pashley DH, Tay FR The critical barrier to progress in dentine bonding with the etch-and-rinse technique. J. Dent. 2011; 39: 238-248.
4. Breschi L, Mazzoni A, Ruggeri A, Cadenaro M, Di Lenarda R, De Stefano DE. Dental adhesion review: aging and stability of the bonded interface. Dent. Mater. 2008; 24: 90–101.
5. Cao CY, Mei ML, Li Q, Lo ECM, Chu CH. Methods for Biomimetic Remineralization of Human Dentine: A Systematic Review. Int. J. Mol. Sci. 2015; 16: 4615-4627.
6. Carvalho RM, Manso AP, Geraldeli S, Tay FR, Pashley DH. Durability of bonds and clinical success of adhesive restorations. Dent. Mater. 2012; 28: 72–86.
7. De Munck J, Meerbeek VB, Yoshida Y, Inoue S, Vargas M, Suzuki K, Lambrechts P, Vanherle G. Four-year Water Degradation of Total-etch Adhesives Bonded to Dentin. J. Dent. Res. 2003; 82: 136-40.
8. Dey A, Bomans PHH, Müller FA, Will J, Frederik PM, de With G & Sommerdijk NAJM. The role of prenucleation clusters in surface-induced calcium phosphate crystallization. Nat. Mater. 2010; 9:1010–1014.
9. Du LW, Bian S, Gou BD, Jiang Y, Huang J, Gao YX, et al. Structure of clusters and formation of amorphous calcium phosphate and hydroxyapatite: from the perspective of coordination chemistry. Crystal Growth and Design 2013, http://dx.doi.org/10.1021/cg400498j.
10. Gandolfi MG, Taddei P, Siboni F, Modena E, de Stefano ED, Prati C. Biomimetic remineralization of human dentin using promising innovative calcium-silicate hybrid “smart” materials. Dent. Mater. 2011; 27: 1055–1069.

11. Gower LB. Biomimetic model systems for investigating the amorphous precursor pathway and its role in biomineralization. Chem. Review 2008;108: 4551–627.
12. Gu LS, Kim J, Kim YK, Liu Y, Dickens SH, Pashley DH, Ling JQ, Tay FR. A chemical phosphorylation-inspired design for type I collagen biomimetic remineralization. Dent. Mater. 2010; 26: 1077–1089.
13. Gu LS, Kim YK, Liu Y, Takahashi K, Arun S, Wimmer CE, Osorio R, Ling JQ, Looney SW, Pashley DH. Immobilization of a phosphonated analog of matrix phosphoproteins within cross-linked collagen as a templating mechanism for biomimetic mineralization. Acta. Biomater. 2011; 7: 268–277.
14. Gu LS, Kim YK, Liu Y, Ryou H, Wimmer CE, Dai L, Arola DD, Looney SW, Pashley DH, Tay FR. Biomimetic analogs for collagen biomineralization. J Dent. Res. 2011; 90: 82–87.
15. Imazato S, Ma S, Chen J, Xu HHK Therapeutic polymers for dental adhesives: Loading resins with bio-active components. Dent. Mater. 2014; 30: 97–104.
16. Longhi M, Cerroni L, Condò SG, Ariano V, Pasquantonio G. The effects of host derived metalloproteinases on dentin bond and the role of MMPs inhibitors on dentin matrix degradation. Oral & Implantology. 2014; 7: 71-79.
17. Liu Y, Li N, Qi YP, Niu LN, Elshafiy S, Mao J, Braschi L, Pashley DH, Tay FR. The use of sodium trimetaphosphate as a biomimetic analog of matrix phosphoproteins for remineralization of artificial caries-like dentin. Dent. Mater. 2011; 27: 465–477.
18. Liu Y, Mai S, Li N, Yiu CKY, Mao J, Pashley DH, Tay FR. Differences between top-down and bottom-up approaches in mineralizing thick, partially demineralized collagen scaffolds. Acta. Biomater. 2011; 7: 1742–1751.
19. Lung CYK and Matinlinna JP. Aspects of silane coupling agents and surface conditioning in dentistry: An overview. Dent. Mater. 2012; 28: 467–477.
20. May LG, Passos SP, Capelli DB, Özcan M, Bottino MA,Valandro LF. Effect of silica coating combined to a MDP-based primer on the resin bond to Y-TZP ceramic. J. Biomed. Mater. Res. 2010; 95B: 69–74.
21. McInnes PM, Wendt Jr. SL, Retief DH, Weinberg R. Effect of dentin surface roughness on shear bond strength. Dent. Mater. 1990; 6: 204-207.

22. Niu L, Jee SE, Jiao K, Tonggu L, Li M, Wang L, Yang Y, Bian J, Breschi L, Jang SS, Chen J, Pashley DH & Tay FR. Collagen intrafibrillar mineralization as a result of the balance between osmotic equilibrium and electroneutrality. Nat. Mater. (2016): doi:10.1038/nmat4789.
23. Niu LN, Zhang W, Pashley DH, Breschi L, Mao J, Chen JH, Tay FR. Biomimetic remineralization of dentin. Dent. Mater. 2014; 30: 77–96.
24. Olszta MJ, Odom DJ, Douglas EP, Gower LB. A new paradigm for biomineral formation: mineralization via an amorphous liquid-phase precursor. Connect. Tissue Res. 2003; 44 :326–34.
25. Pashley DH , Tay FR, Breschi L, Tjäderhane L, Carvalho RM, Carrilho M, Tezvergil-Mutluay A. State of the art etch-and-rinse adhesives. Dent. Mater .2011; 27: 1–16.
26. Pioch T, Staehle HJ, Duschner H, García-Godoy F. Nanoleakage at the composite-dentin interface: a review. Am. J. Dent. 2001;14: 252-8.
27. Profeta AC, Mannocci F, Foxton R, Watson TF, Feitosa VP, De Carlo B, Mongiorgi R, Valdré G, S. Sauro S. Experimental etch-and-rinse adhesives doped with bioactive calcium silicate-based micro-fillers to generate therapeutic resin–dentin interfaces. Dent. Mater. 2013; 29: 729:741.
28. Qi YP, Li N, Niu LN, Primus CM, Ling JQ, Pashley DH, Tay FR. Remineralization of artificial dentinal caries lesions by biomimetically modified mineral trioxide aggregate. Acts. Biomater. 2012; 8: 836–842.
29. Qin C, D'Souza R, Feng JQ. Dentin matrix protein 1 (DMP1): new and important roles for biomineralization and phosphate homeostasis. J. Dent. Res. 2007; 86: 1134–41.
30. Tay FR, Frankenberger R, Krejci I, Bouillaguet S, Pashley DH, Carvalho RM, Lai CNS. Single-bottle adhesives behave as permeable membranes after polymerization. I. In vivo evidence J. Dent. 2004; 32: 611–621.
31. Tay FR and Pashley DH Biomimetic remineralization of resin-bonded acid-etched dentin J Dent Res 2009 88: 719-724.
32. Tay FR and Pashley DH. Guided tissue remineralisation of partially demineralised human dentine. Biomaterials 2008; 29: 1127–1137.
33. Tay FR, Moulding KM, Ashley DH. Distribution of nanofillers from a simplied-step adhesive in acid-conditioned dentin. J. Adhes. Dent. 1999; 1:103-117.

34. Tezvergil-Mutluay A, Agee KA, Hoshika T, Tay FR, Pashley DH. The inhibitory effect of polyvinylphosphonic acid on functional matrix metalloproteinase activities in human demineralized dentin. Acta. Biomat. 2010;6: 4136–42.
35. Tjäderhane L, Nascimento FD, Breschi L, Mazzoni A, Tersariol IL, Geraldeli S, Tezvergil-Mutluay A, Carrilho M, Carvalho RM, Tay FR, Pashley DH. Strategies to prevent hydrolytic degradation of the hybrid layer-A review. Dent. Mater. 2013; 29: 999-1011.
36. Xu A, Ma Y, Cölfen H. Biomimetic mineralization. J. Mater. Chem. 2007; 17: 415–4

3 DISCUSSÃO

Os testes de resistência de união dentinária *in vitro* com adesivos comerciais ou experimentais denotam a capacidade de sobrevida desses materiais em diferentes desafios simulados, com o intuito de conhecer sua resposta e anteceder seu padrão de comportamento. Com isso, dois sistemas adesivos convencionais (SBMP e OPFL) e dois sistemas adesivos auto-condicionantes (CSE e OPXTR) foram testados em dentina central e proximal em um preparo tipo classe II (MOD) simulado quanto sua resistência de união à microtração (μTBS).

Os sistemas adesivos auto-condicionantes apresentaram um desempenho mais homogêneo por entre as diferentes regiões dentinárias. Enquanto os adesivos testados que requerem condicionamento ácido prévio apresentaram redução significativa nos valores μTBS quando aplicados em dentina proximal. Esse padrão de comportamento foi correlacionado com algumas características conhecidas do substrato dentinário também ilustradas em microscopia eletrônica de varredura (artigo 1, figura 3).

Dentre elas, a angulação natural dos túbulos dentinários em regiões periféricas da dentina. O que parece não ter exercido efeito direto na desmineralização por ácido fosfórico em si desse substrato. Até mesmo porque em dentina central, com alto conteúdo de dentina peritubular (hipermineralizada), esse comportamento também não se mostrou afetado dentre os resultados μTBS. Entretanto, pode ter prejudicado a difusão resinosa subsequente por entre essa região de menor densidade de túbulos dentinários e maior quantidade de dentina intertubular. Assim, tem-se um substrato com poucos túbulos e muitas ramificações laterais, o que possivelmente dificultou a difusão dos monômeros resinosos por entre essa malha tubular, diminuindo a resistência de união dos adesivos convencionais em dentina proximal.

Com esse resultado e uma frente de adesivos convencionais experimentais a serem testados, uma metodologia diferenciada para teste μTBS foi aplicada com o intuito de minimizar essa diferença de resultados μTBS nessas condições (Geraldeli *et al.*, 2002). Para isso, a divisão da superfície de dentina a ser testada foi realizada com secções equivalentes de 4 quartetos distribuídos radialmente (artigo 2, figura 1.b, apêndice 2). Dessa maneira, duas vantagens foram acolhidas: (i) uma maior variedade de dentina foi incluída no teste, o que não ocorreria caso cada grupo

fosse testado individualmente por dente; (ii) uma diluição da variação região dentinária testada dentre um maior número de dentes incluídos entre os quartetos testados, trazendo maior confiabilidade dos dados obtidos.

Dentre esses dados, os resultados do teste μTBS realizado para avaliar o comportamento de um sistema adesivo experimental convencional de frasco único, aplicado após a utilização de uma solução re-umidificante também experimental. Esta solução foi criada com base em opções comerciais disponíveis (Aqua-prep F, Bisco, Schaumburg, IL, EUA) ou mesmo experimentais, como clorexidina ou tetraciclina (Sartori N *et al.*, 2013, Oliveira HL *et al.*, 2016). As quais funcionam como uma alternativa para contraposição de erros cometidos após o condicionamento ácido, como a secagem demasiada da superfície dentinária depois do passo de lavagem.

Neste tipo de solução, análogos biomiméticos (PAA e PVPA) foram adicionados para aplicação prévia a um adesivo experimental contendo partículas bioativas de cálcio-fosfato. O que permitiu a viabilidade da lógica do estudo, uma vez que assim os análogos ficaram passíveis de exercerem sua função antecedendo o caminho das partículas de cálcio-fosfato bioativas. Estas por sua vez, apesar de não serem o fator-chave do processo de remineralização biomimética, mostram-se com atuação direta sobre ele (Kim J et al., 2010). Uma vez que sua propriedade bioativa intrínseca confere a esses materiais uma propensão à ligações químicas em tecidos biológicos adjacentes. E assim proporciona um meio propício para os análogos biomiméticos iniciarem a segmentação estruturada de remineralização por entre as cadeias de fibrilas colágenas do tecido dentinário.

Desta maneira, a resistência de união à microtração se manteve estável depois de 4 meses de armazenamento para o grupo com adição de análogos biomiméticos na solução (G3) e o grupo experimental (G4), enquanto houve redução nos valores μTBS para os grupos controle (G1) e somente com fosfato bioativos no adesivo (G2) (artigo 2, tabela 2). Ademais, os análogos biomiméticos possuem a capacidade de se difundirem por entre e dentro de espaços vazios das fibrilas colágenas, numa dimensão tão reduzida, que muitas vezes, nem mesmo as cadeias poliméricas são capazes de atravessarem (Niu LN *et al.*, 2014). Enquanto, partículas amorfas de cálcio-fosfato atuam como nanoprecursores à formação de “clusters” de pré-nucleação que se agregam entre si e com estruturas vizinhas, se organizando

em um padrão cristalográfico de alinhamento altamente organizado. Quando unidos às fibrilas colágenas da região, circundam a porção intrafibrilar e interfibrilar destas, formando pequenos cristais de hidroxiapatita no processo de remineralização biomimética (Dey A *et al.*, 2010, Du LW *et al.*, 2013, Niu LN *et al.*,2014), o que provavelmente colaborou na redução da porcentagem de nanoinfiltração observada para o grupo experimental G4 (artigo 2, tabela 2, figura 4).

Por fim, a concentração cálcio-fósforo da dentina aumentou no período de 4 meses de armazenamento para os grupos G2, G3 e G4 (artigo 2, tabela 2). Bem como a análise por meio do microscópio eletrônico de transmissão demonstrou um padrão uniforme e localizado de deposição de cristais de apatita por toda a região adjacente a dentina tratada com análogos biomiméticos (G3 e G4), oposto ao que se observou sem a utilização destes (G1 e G2) (artigo 2, figura 3). Tais resultados refletem as considerações acima acerca de remineralização biomimética somente quando interligados entre si. Uma vez que após quatro meses de armazenamento, a resistência de união foi conservada, a proporção de cálcio-fosfato se mostrou aumentada, a degradação da interface adesiva amenizada e foi observado a deposição uniforme de hidroxiapatita adjacente região dentinária, indicadoras fundamentais e plausíveis de um substrato mineralizado.

4 CONCLUSÃO

Baseado nos resultados desse estudo, pode-se concluir que a região da dentina é capaz de afetar os resultados de resistência de união para sistemas adesivos com condicionamento ácido prévio. A adição de análogos biomiméticos em uma solução experimental re-umidificante e de cálcio-fosfato bioativos em um adesivo experimental proporcionou resultados estáveis quanto à resistência de união à microtração. Além disso, reduziu o padrão de nanoinfiltração e aumentou a concentração de cálcio-fósforo depois de 4 meses de armazenamento em solução SBF com indícios de remineralização biomimética também dentro e ao redor de fibrilas colágenas da região de camada híbrida dentinária observadas em TEM.

REFERÊNCIAS*

- Abuna G, Feitosa VP, Correr AB, Cama G, Giannini M, Sinhoreti MAC, Pashley DH, Sauro S. Bonding performance of experimental bioactive/biomimetic self-etch adhesives doped with calcium-phosphate fillers and biomimetic analogs of phosphoproteins. Journal of Dentistry 2016(52):79-86.
- Breschi L, Mazzoni A, Ruggeri A, Cadenaro M, Di Lenarda R, De Stefano DE. Dental adhesion review: aging and stability of the bonded interface. Dental Materials 2008(24):90–101.
- Cao CY, Mei ML, Xu J, Lo ECM, Li Q, Chu CH. Biomimetic mineralisation of phosphorylated dentine by CPP-ACP. Journal of Dentistry 2013(41):818-825.
- De Munck J, Van den Steen PE, Mine A, Van Landuyt KL, Poitevin A, Opdenakker G, Van Meerbeek B. Inhibition of enzymatic degradation of adhesive-dentin interfaces. Journal of Dental Research. 2009;88(12):1101-6.
- Dey A, Bomans PHH, Müller FA, Will J, Frederik PM, de With G & Sommerdijk NAJM. The role of prenucleation clusters in surface-induced calcium phosphate crystallization. Nature Materials 2010(9):1010–1014.
- Diamanti I, Koletsi-Kounari H, Mamai-Homata E, Vougiouklakis G. In vitro evaluation of fluoride and calcium sodium phosphosilicate toothpastes, on root dentine caries lesions. Journal of Dentistry 2011(39):619–628.
- Du LW, Bian S, Gou BD, Jiang Y, Huang J, Gao YX, et al. Structure of clusters and formation of amorphous calcium phosphate and hydroxyapatite: from the perspective of coordination chemistry. Crystal Growth and Design 2013, http://dx.doi.org/10.1021/cg400498j.
- Frassetto A, Breschi L, Turco G, Marchesi G, Di Lenarda R, Tay FR, Pashley DH, Cadenaro M. Mechanisms of degradation of the hybrid layer in adhesive dentistry and therapeutic agents to improve bond durability-A literature review. Dental Materials 2016;32(2):e41-53.
- Geraldeli S, Perdigão J, Larson WK. A novel micro-tensile dentin bond strength method using paired dentin quarters. Journal of Dental Research 2002; 80:abstract 3373.

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

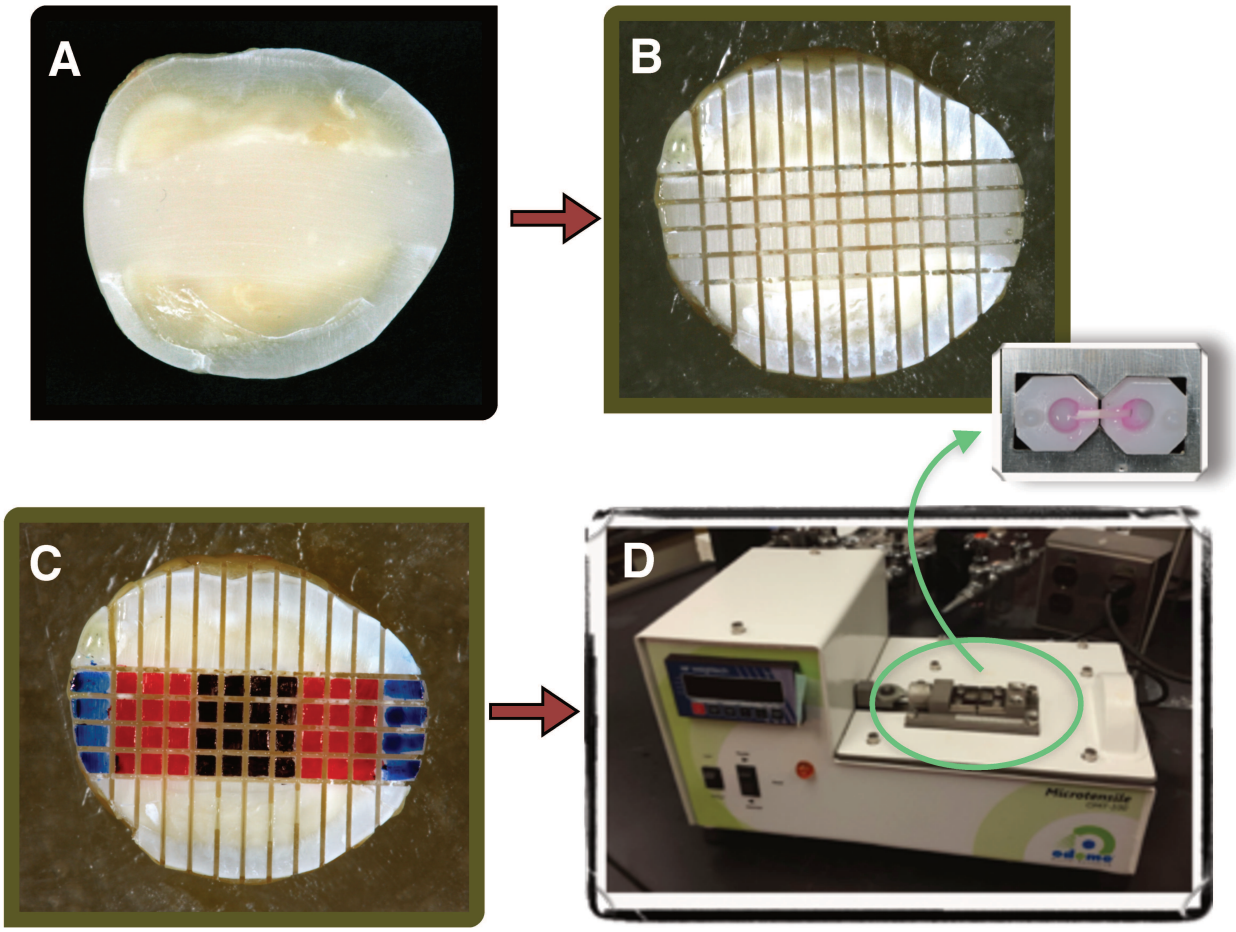
- Giannini M, Makishi P, Ayres AP, Vermelho PM, Fronza BM, Nikaido T, Tagami J. Self-etch adhesive systems: a literature review. Brazilian Dental Journal. 2015; 26(1):3-10.
- Gu LS, Kim J, Kim YK, Liu Y, Dickens SH, Pashley DH, Ling JQ, Tay FR. A chemical phosphorylation-inspired design for type I collagen biomimetic remineralization. Dental Materials 2010(26):1077–1089.
- Guentsch A, Seidler K, Nietzsche S, Hefti AF, Preshaw PM, Watts DC, Jandt KD, Sigusch BW. Biomimetic mineralization: Long-term observations in patients with dentin sensitivity. Dental Materials 2012(28):457–464.
- Kagayama M, Sasano Y, Sato H, Kamakura S, Motegi K, Mizoguchi I. Confocal microscopy of dentinal tubules in human tooth stained with alizarin red. Anatomy and Embryology 1999(199):233–238.
- Kim J, Arola DD, Gu L, Kim YK, Mai S, Liu Y, Pashley DH, Tay FR. Functional biomimetic analogs help remineralize apatite-depleted demineralized resin-infiltrated dentin via a bottom–up approach. Acta Biomaterialia 2010;6(7):2740-50. 2740–2750.
- Liu Y, Li N, Qi YP, Niu LN, Elshafiy S, Mao J, Braschi L, Pashley DH, Tay FR. The use of sodium trimetaphosphate as a biomimetic analog of matrix phosphoproteins for remineralization of artificial caries-like dentin. Dental Materials 2011(27):465–477.
- Liu Y, Mai S, Li N, Yiu CKY, Mao J, Pashley DH, Tay FR. Differences between top-down and bottom-up approaches in mineralizing thick, partially demineralized collagen scaffolds. Acta Biomaterialia 2011(7): 1742–1751.
- Marshall GW Jr., Marshall SJ, Kinney JH, Balooch M. The dentin substrate: structure and properties related to bonding. Journal of Dentistry 1997;25:441-458.
- Mjör IA, Nordahl I. The density and branching of dentinal tubules in human teeth. Archives of Oral Biology 1996(41):401–412.
- Niu LN, Zhang W, Pashley DH, Breschi L, Mao J, Chen JH, Tay FR. Biomimetic remineralization of dentin. Dental Materials 2014(30):77–96.
- Oliveira Hde L, Tedesco TK, Rodrigues-Filho LE, Soares FZ, Rocha Rde O. Doxycycline as a matrix metalloproteinase inhibitor to prevent bond degradation: the effect of acid and neutral solutions on dentin bond strength. General Dentistry. 2016;64(2):14-7.

- Olszta MJ, Odom DJ, Douglas EP, Gower LB. A new paradigm for biomineral formation: mineralization via an amorphous liquid-phase precursor. Connective Tissue Research 2003(44):326–34.
- Opdam NJ, van de Sande FH, Bronkhorst E, Cenci MS, Bottenberg P, Pallesen U, Gaengler P, Lindberg A, Huysmans MC, van Dijken JW. Longevity of posterior composite restorations: a systematic review and meta-analysis. Journal of Dentistry Research 2014;93(10):943-9.
- Pashley DH, Carvalho RM. Dentine permeability and dentine adhesion. Journal of Dentistry 1997;25(5):355-72.
- Pashley DH. Dynamics of the pulpo–dentin complex. Critical reviews in oral biology and medicine 1996(7):104–133.
- Profeta AC, Mannocci F, Foxton R, Watson TF, Feitosa VP, De Carlo B, Mongiorgi R, Valdré G, S. Sauro S. Experimental etch-and-rinse adhesives doped with bioactive calcium silicate-based micro-fillers to generate therapeutic resin–dentin interfaces. Dental Materials 2013(29):729:741.
- Qi YP, Li N, Niu LN, Primus CM, Ling JQ, Pashley DH, Tay FR. Remineralization of artificial dentinal caries lesions by biomimetically modified mineral trioxide aggregate. Acts Biomaterialia 2012(8):836–842.
- Sartori N, Peruchi LD, Phark JH, Duarte S Jr. The influence of intrinsic water permeation on different dentin bonded interfaces formation. Journal of Dentistry 2016(48):46-54.
- Sartori N, Stolf SC, Silva SB, Lopes GC, Carrilho M. Influence of chlorhexidine digluconate on the clinical performance of adhesive restorations: a 3-year follow-up. Journal of Dentistry 2013;41(12):1188-95.
- Tay FR and Pashley DH. Guided tissue remineralisation of partially demineralised human dentine. Biomaterials 2008;(29):1127–1137.
- Tjäderhane L, CARRILHO MR, BRESCHI L, Tay FR & PASHLEY DH. Dentin Basic structure and composition - an overview. Endodontic Topics 2012;(20)3–29.
- Tjäderhane L, Nascimento FD, Breschi L, Mazzoni A, Tersariol IL, Geraldeli S, Tezvergil-Mutluay A, Carrilho M, Carvalho RM, Tay FR, Pashley DH. Strategies to prevent hydrolytic degradation of the hybrid layer-A review. Dental Materials 2013;29(10):999-1011.

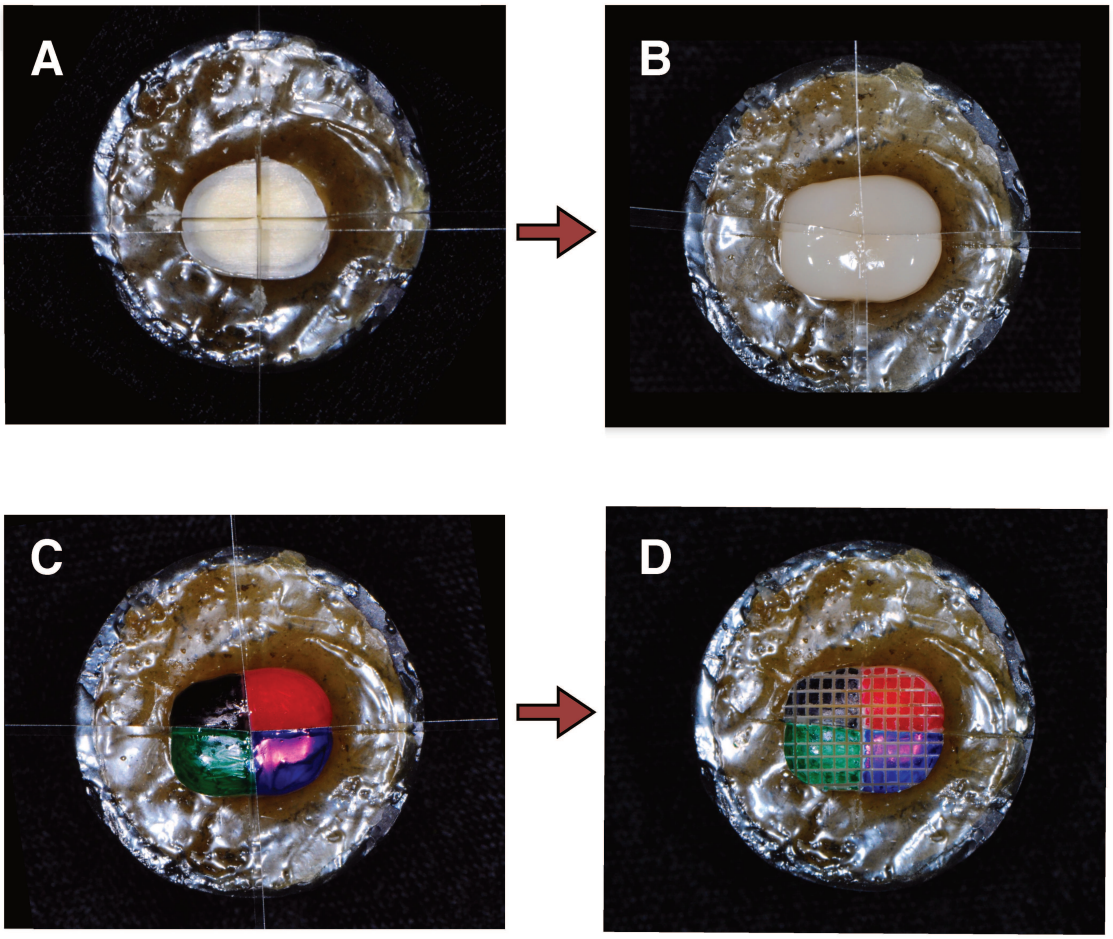
- Van Landuyt KL, Snauwaert J, De Munck J, Peumans J, Yoshida Y, Poitevin A, Coutinho E, Suzuki K, Lambrechts P, Van Meerbeek B. Systematic review of the chemical composition of contemporary dental adhesives. Biomaterials 2007(28): 3757–3785.
- Wang JM, Chen Y, Li L, Sun J, Gu XH, Xu XR, Pan HH, Tang RK. Remineralization of dentin collagen by meta-stabilized amorphous calcium phosphate. CrystEngComm 2013(15):6151–6158.
- Watson TF, Atmeh AR, Sajini S, Cook RJ, Festy F. Present and future of glass-ionomers and calcium-silicate cements as bioactive materials in dentistry: Biophotonics-based interfacial analyses in health and disease. Dental Materials 2014(30):50–61.
- Zaslansky P, Zabler S, Fratzl P. 3D variations in human crown dentin tubule orientation: A phase-contrast microtomography study. Dental Materials 2010(26) e1–e10.

APÊNDICE

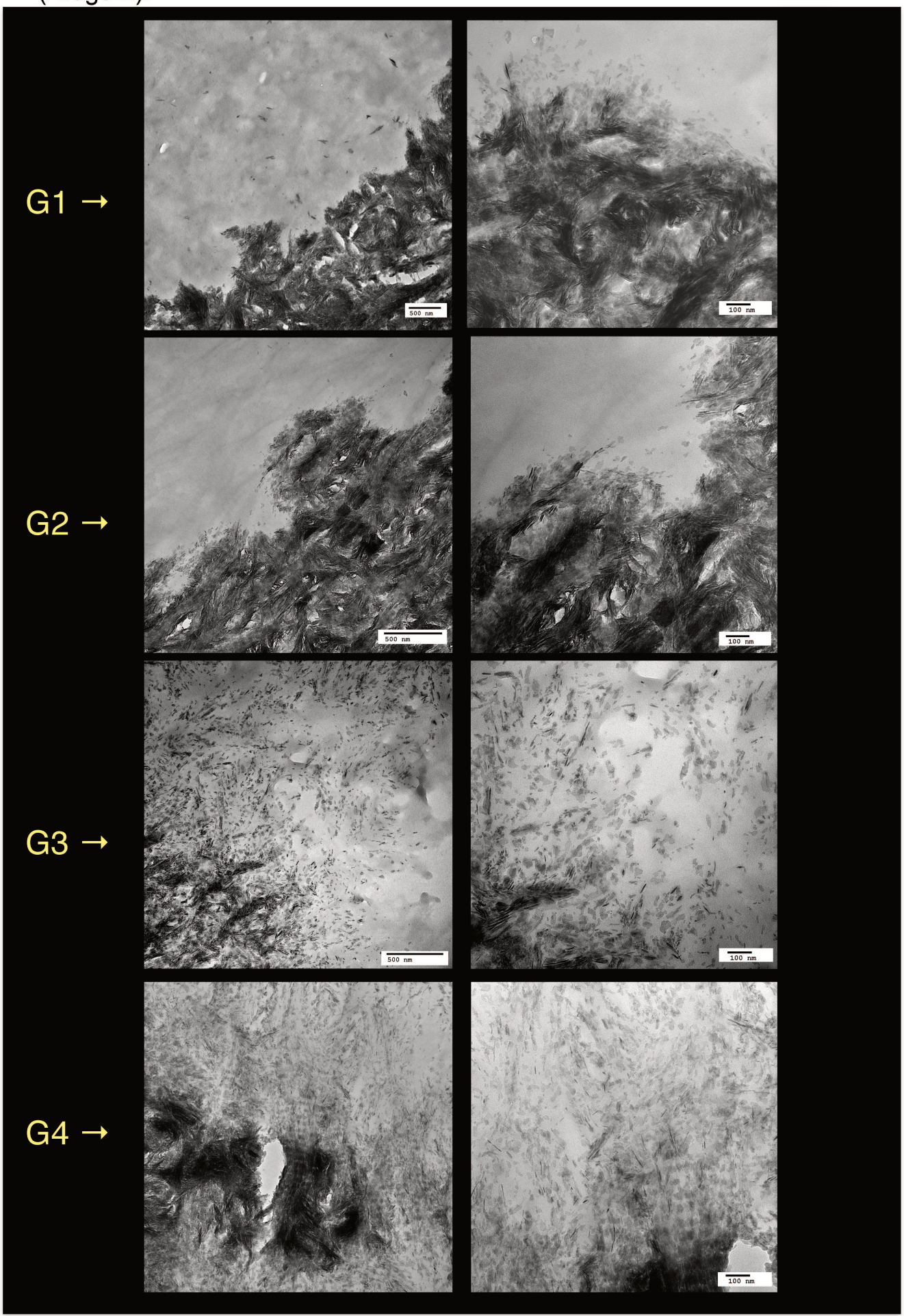
Apêndice 1: Metodologia ilustrada do teste μTBS (Artigo 1). (A) restauração com compósito (Z250) pela técnica incremental em preparo classe II (MOD) simplificado; (B) secções transversais no sentido mésio-distal e vestibulo-lingual para obtenção dos espécimes em palito; (C) divisão dos grupos testados, azul corresponde ao esmalte excluído do teste, vermelho à dentina proximal e preto à dentina central; (D) máquina de ensaio μTBS com dispositivo de Geraldeli 2.



Apêndice 2: Metodologia ilustrada do teste μTBS (Artigo 2). (A) seccionamento da dentina em 4¼ com proteção lateral usando tiras de poliéster pré-cortadas; (B) restauração com compósito (Z250) pela técnica incremental de acordo com o protocolo de tratamento de cada grupo testado; (C) identificação da amostra de acordo com protocolos de tratamento testados; (D) seccionamento e divisão dos espécimes em G1, G2, G3 e G4.



Apêndice 3: Imagens extras de microscopia eletrônica de transmissão (Artigo 2).



Anexo

Anexo 1

- **Certificado do comitê de ética em pesquisa**

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Avaliação do potencial remineralizador de uma solução reumidificadora contendo análogos biomiméticos associada a um sistema adesivo experimental com fosfatos de cálcio bioativos "**, protocolo nº 063/2014, dos pesquisadores Eveline Freitas Soares e Lourenço Correr-sobrinho, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 10/09/2014.

The Ethics Committee in Research of the Piracicaba Dental School - University of Campinas, certify that the project **"Evaluation of the remineralizing potential of a remoisture solution containing biomimetic analogs associated with an experimental adhesive system with bioactive calcium phosphate "**, register number 063/2014, of Eveline Freitas Soares and Lourenço Correr-sobrinho, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee on Sep 10, 2014.

Prof. Dr. Felippe Bevilacqua Prado
Secretário
CEP/FOP/UNICAMP

Profa. Dra. Lívia Maria Andaló Tenuta
Coordenadora
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.