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MARIA ELISA MARTINS MOURA

DESENVOLVIMENTO E AVALIAÇÃO DE UM NOVO ADESIVO ODONTOLÓGICO PARA REMINERALIZAÇÃO BIOMIMÉTICA DE INTERFACES ADESIVAS

FORTALEZA

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Odontologia da Faculdade de Farmácia, Odontologia e Enfermagem da Universidade Federal do Ceará, como requisito parcial para a obtenção do Título de Mestre em Odontologia.

Área de Concentração: Clínica Odontológica.

Orientador: Prof. Dr. Victor Pinheiro Feitosa.

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RESUMO

Uma recente alternativa para promover uma maior durabilidade às restaurações é a remineralização biomimética. Ela utiliza dois análogos de fosfoproteínas da dentinogênese e poderia ser realizada utilizando um adesivo autocondicionante de dois passos contendo fosfatos de cálcio bioativos no adesivo e um novo análogo biomimético (EDTF, etilenodiamino tetrametileno-fosfonato) no *primer*, agindo como os dois diferentes tipos de análogos já conhecidos. Assim, um adesivo experimental foi preparado e aplicado com mistura prévia com fosfato de mono-cálcio mono-hidratado e beta fosfato tri-cálcio no *bond*. Além disso, foram desenvolvidos *primers* de acordo com o delineamento experimental. Um *primer* sem análogos e sem partículas no *bond* (controle negativo, CN), um contendo os análogos mais utilizados na literatura trimetafosfato (TMP) de sódio e ácido poliacrílico (APA) (TMP/APA - controle positivo, CP), e outros contendo EDTF/TMP, EDTF/APA ou somente EDTF foram preparados. Um grupo somente com partículas no *bond* e sem os análogos no *primer* também foi usado. Estes sistemas adesivos foram aplicados em dentina afetada por cárie (DAC) simulada artificialmente e em dentina hígida de molares humanos extraídos. Após períodos de 24h e 6 meses de armazenamento, eles foram avaliados por teste de microtração, nanoinfiltração e espectroscopia Micro-Raman da interface (camada híbrida) e da dentina subjacente. Os dados de microtração foram analisados estatisticamente com ANOVA dois fatores e Teste de Tukey (α=5%). Na microtração dos hígidos só houve diferença estatística ($p<0,05$) nos grupos EDTF/TMP apresentando um aumento na resistência de união após 6 meses e no grupo EDTF/APA que teve um declínio. Em DAC, houve uma queda nos grupos CN, EDTF/TMP e EDTF/APA ($p<0,05$). Na análise de nanoinfiltração em DAC, o CN revelou a presença de fendas e degradação na região de dentina subjacente de forma imediata, e na camada de adesivo após o envelhecimento, o que não foi observado nos demais grupos. Na espectroscopia Micro-Raman foi possível observar a presença de mineralização mais acentuada nos grupos CP, EDTF, EDTF/TMP e somente partículas, porém isso não foi observado nos grupos CN e EDTF/APA. Conclui-se que os melhores resultados para a adesão e a remineralização da dentina afetada por cárie são obtidos com o uso dos análogos tradicionais (TMP/APA) ou com EDTF sozinho e associado ao TMP, não afetando a adesão à dentina hígida.

Palavras-chave: remineralização, adesivo dentinários, fosfato de cálcio.

ABSTRACT

A recent alternative to promote higher durability for restorations is biomimetic remineralization. It utilizes two analogues of phosphoproteins from dentinogenesis and could be undertaken by using two-step self-etch adhesives containing bioactive calcium phosphates in adhesive and a new biomimetic analog (EDTMP, ethylene-diamine-tetramethylene-phosphonic acid) in primer, acting as both known different analogues. Thus, an experimental adhesive was prepared and applied with previous mixture of monocalcium phosphate monohydrate and beta-tricalcium phosphate fillers in adhesive resin. Primers without analogues and bond without fillers (negative control, NC), one containing most used analogues in literature sodium trimetaphosphate (TMP) and polyacrylic acid (PAA) (positive control, PC – TMP/PAA) and others containing TMP/EDTMP, EDTMP/PAA or only EDTMP were prepared. A group (Fillers) containing CaP fillers in adhesive and no analogues in primer was also used. Such adhesives were applied on caries-affected dentin (CAD) simulated artificially and on sound dentin of extracted human molars. After 24h or 6 months water storage, specimens were surveyed by microtensile bond strength (μTBS), nanoleakage and Micro-Raman spectroscopy of interface (hybrid layer) and underlying dentin. Data was statistically analyzed by two-way ANOVA and Tukey's test (α=5%). Microtensile test on sound dentin showed statistical difference ($p<0.05$) only for EDTMP/TMP with increase in μTBS after aging and for EDTMP/PAA with decrease. In CAD, there was reduction of μTBS in NC, EDTMP/TMP and EDTMP/PAA ($p<0.05$). Nanoleakage in CAD revealed presence of gaps and degradation of underlying dentin immediately in NC specimens, and in adhesive layer after aging; this was not observed in further groups. In Micro-Raman spectroscopy it was possible to note presence of more intense mineralization in PC, EDTMP, EDTMP/TMP and only Filler, but it was not observed in NC and EDTMP/PAA groups. In conclusion, overall best outcomes of adhesion and remineralization on caries-affected dentin were attained by using the two traditional analogues (TMP/PAA) or with EDTMP alone and associated with TMP, without affecting bond strength to sound dentin.

Keywords: remineralization, dental adhesive, calcium phosphate.

SUMÁRIO

Introdução Geral

1 INTRODUÇÃO GERAL

As restaurações em resina composta compreendem atualmente a grande maioria das restaurações diretas realizadas, devido à diminuição do uso do amálgama (RUEGGEBERG, 2011). O advento da adequada fotopolimerização (RUEGGEBERG, 2011), juntamente das notáveis melhorias nas fases monoméricas e inorgânicas (FERRACANE, 2011) fizeram com que esses materiais evoluíssem significantemente (MITRA et al., 2003).

Em geral, os compósitos atuais possuem propriedades mecânicas adequadas (FERRACANE, 2011). Entretanto, a degradação das interfaces adesivas ainda é um problema muito significativo que leva a substituição de um grande número de restaurações. O elo mais fraco nas restaurações em resina composta hoje é certamente a união com a dentina (SPENCER et al., 2010), pois a estabilidade da união com o esmalte é muito mais duradoura (LOGUERCIO et al., 2008).

Com o advento da odontologia minimamente invasiva, foi proposto um tratamento de lesões mais profundas, adotando-se o Tratamento Restaurador Atraumático (ART) (1990) com o objetivo de remover de forma seletiva a cárie, ou seja, removendo totalmente o tecido cariado infectado e deixando a dentina afetada pela cárie, pois esta é passível de ser remineralizada (YAMAGA et al., 1972). Os conceitos de ART combinados com o advento da última geração de sistemas adesivos têm influenciado radicalmente o tratamento restaurador (SONODA et al., 2005; SAURO et al., 2012). Em geral, a presença de dentina cariada resulta em camadas híbridas e adesões menos resistentes (NAKAJIMA et al., 1995, NAKAJIMA et al.,1999, YOSHIYAMA et al., 2002, YOSHIYAMA et al., 2003). A menor eficácia de adesão à dentina afetada que a dentina sadia está relacionada às alterações que ocorrem neste substrato como consequência da progressão da cárie. Em primeiro lugar, a redução aleatória no conteúdo mineral e a perda de cristalinidade da fase mineral remanescente, associado às alterações na estrutura secundária do colágeno resultam em um substrato de dentina com menores dureza e módulo de elasticidade que a dentina sadia, apresentando uma menor resistência mecânica (WANG, SPENCER, WALKER, 2007, OGAWA et al., 1983, MARSHALL et al., 2001).

Além disso, os sistemas adesivos aplicados na dentina possuem um grande problema ao longo do tempo que é a degradação da sua interface de união. A

degradação da união obtida com a utilização dos sistemas adesivos ocorre normalmente de três maneiras (VAN LANDUYT et al., 2010, BRACKETT et al., 2011). Logo após a polimerização do adesivo, regiões desmineralizadas e pobremente infiltradas por resina assim como as regiões de solvente não evaporado na camada de adesivo são observadas através de ensaios de nanoinfiltração (TAY et al., 2002). Essas regiões de colágeno exposto são comumente encontradas e são as zonas mais suscetíveis a degradação inicial (CARRILHO et al., 2007). A degradação do colágeno desprotegido é acelerada por enzimas conhecidas como metaloproteinases (MMPs) (CARRILHO et al., 2007) e catepsinas (NASCIMENTO et al., 2012), essas podem ser inibidas por diversas substâncias já conhecidas (BRESCHI et al., 2008, SCAFFA et al., 2012). Essas substâncias já foram inclusive incorporadas aos sistemas adesivos com sucesso (ALMAHDY et al., 2012, YIU et al., 2012).

A segunda principal forma de degradação é a hidrólise do polímero (ITO et al., 2010), gerada pelo contato direto com a água do tecido dentinário, com a saliva e com a água presente no adesivo e não evaporada antes da polimerização. A hidrólise do polímero e do colágeno presentes na camada híbrida são reduzidas ou elevadas de acordo com a hidrofobia dos monômeros do sistema adesivo (BRESCHI et al., 2008), a qual diminui ou aumenta a sorção de água na região de união (ITO et al., 2010). A hidrólise do polímero é mais lenta que os outros tipos de degradação; no entanto, pode ser também acelerada por enzimas conhecidas como esterases (KOSTORYZ et al., 2009). Novos monômeros têm sido desenvolvidos com o intuito de diminuir a ação das esterases e a degradação do polímero (PARK et al., 2009). A terceira e mais recentemente conhecida frente de degradação é o desprendimento das partículas de carga e o desprendimento das mesmas da matriz de resina do adesivo (VAN LANDUYT et al., 2010, BRACKETT et al., 2011). O espaço deixado pelas partículas de carga do adesivo na matriz polimérica é subsequentemente preenchido por água, o que acelera as duas degradações supracitadas (VAN LANDUYT et al., 2010, BRACKETT et al., 2011).

Dessa forma, é observado que a maior barreira para a grande durabilidade da união das restaurações é a degradação promovida pela água (CARRILHO et al., 2005, BRACKETT et al., 2011, FEITOSA et al., 2012) que pode ou não ser acelerada por enzimas (FRASSETTO et al., 2016). A estratégia mais inovadora para diminuição da degradação e promoção de maior durabilidade das restaurações é remineralização

biomimética (SAURO E PASHLEY 2016) que consiste na formação de cristais de apatita nas regiões de colágeno exposto e nos espaços internos da camada de adesivo deixados pelas partículas de carga ou formados pela hidrólise do polímero (BRACKETT et al., 2011). A técnica de remineralização biomimética da dentina vem sendo amplamente divulgada nos últimos anos (GANDOLFI et al., 2011, BRACKETT et al., 2011, CAO et al., 2015, PADOVANO et al., 2015) desde sua introdução por Tay e Pashley em 2008 (TAY; PASHLEY, 2008). Ela mostrou ser mais eficiente que o uso de inibidores de colagenases (BRACKETT et al., 2011) e em preservar a integridade da interface resina-dentina ao longo prazo (KIM et al., 2010). A remineralização biomimética é uma metodologia que imita o processo natural de mineralização e representa uma abordagem diferente, pois esta técnica preenche o colágeno da dentina desmineralizada de forma interfibrilar e intrafibrilar (CAO et al., 2015).

A substância fornecedora de minerais mais utilizada atualmente para a remineralização biomimética é o cimento Portland (silicato de cálcio) bioativo, o qual mostrou ótimos resultados em relação à remineralização da dentina e interface de união quando aplicado em laboratório (KIM et al., 2010, PROFETA et al., 2012). Entretanto, apesar do cimento Portland ser uma ótima fonte de íons e minerais, existem indícios de que quando ocorre sua completa dissolução há formação de espaços e estes, então, são preenchidos por água que levam ao processo de degradação (PROFETA et al., 2012). Uma nova alternativa é a bruxita (fosfato de di-cálcio di-hidratado) que é um predecessor estável da hidroxiapatita em soluções levemente ácidas (JOHNSSON; NANCOLLAS,1992). Sua transformação em hidroxiapatita ao longo do tempo já foi demonstrada em condições fisiológicas após dissolução desse fosfato de cálcio (STULAJTEROVA; MEDVECKY, 2008). A bruxita pode ser um substituto viável ao cimento Portland na remineralização biomimética, pois pode induzir a formação de novos cristais de hidroxiapatita. Além disso, a bruxita pode ser formada através da mistura equimolar de beta-tri-cálcio fosfato (β-TCP) e mono-cálcio fosfato mono-hidratado (MCFM) com grande captação de água (CAMA et al., 2009), inclusive quando misturado em composições resinosas odontológicas experimentais (MEHDAWI et al., 2009).

A biomineralização da dentina é modulada pelas proteínas não colagenosas da dentina que possuem alta afinidade por íons cálcio e pelas fibrilas colágenas. A proteína de matriz dentinária (DMP1) e a fosforina dentinária (DMP2), por exemplo,

são responsáveis por regular a nucleação e o crescimento de minerais, contudo, são muito difíceis de se extrair e purificar de forma natural. A busca por substâncias com uma ação semelhantes a essas proteínas levou ao desenvolvimento de análogos biomiméticos (HE, GEORGE, 2004, WIESMANN, et al., 2004, TAY, PASHLEY, 2008).

Um dos análogos é o análogo estabilizador da nucleação de nano-precursores de apatita que é um agente quelante como o EDTA, o ácido poliaspártico ou o ácido poliacrílico (APA) (KIM et al., 2010, BRACKETT et al., 2011). O APA é o mais usado nos artigos já publicados. O segundo análogo é um análogo guia que se liga nas fibrilas colágenas expostas e funciona como um modelo para a mineralização coordenada dos nano-precursores estabilizados (KIM et al., 2010, BRACKETT et al., 2011). Os análogos guia mais encontrados são tri- e poli-metafosfato hidrolisados (TMP) e o ácido poli-vinil-fosfônico. A ligação entre os poli-fosfonatos provenientes do TMP e o colágeno é muito eficiente e certamente outros reagentes baseados em poli-fosfonatos podem ser utilizados para o mesmo propósito. Um tipo de EDTA fosfonatado chamado ácido etileno-diamino-tetra (metileno-fosfônico) que será chamado de “EDTF” é também um quelante eficiente (HAN et al., 2008, ZENG et al., 2010) e também se liga de forma adequada com proteínas como o colágeno (LIU et al., 2013). Assim, o EDTF poderia funcionar como os dois diferentes análogos biomiméticos facilitando e deixando menos oneroso o processo de remineralização biomimética. Este estudo avalia se o EDTF é capaz de atuar como ambas as proteínas de matriz não colagenosas, guia e estabilizadora, na remineralização biomimética induzida por um sistema adesivo autocondicionante misturado a fosfatos de cálcio.

Proposição

2 PROPOSIÇÃO

2.1 Objetivo Geral

Formular e avaliar os efeitos sobre a degradação da interface adesiva de adesivos autocondicionantes experimentais com fosfatos de cálcio bioativos e com *primers* contendo um novo análogo biomimético que promova a remineralização em dentina hígida e afetada por cárie *in vitro*.

2.2 Objetivos Específicos

- Formular adesivos autocondicionantes de dois passos, os quais terão essencialmente como *bond* monômeros hidrofóbicos sem solvente misturados com diferentes fosfatos de cálcio (MCFM e β-TCP), como *Primers* os análogos biomiméticos e avaliar a adesão à dentina.

- Avaliar o desempenho do EDTF como análogo guia e análogo estabilizador;

- Avaliar o desempenho do EDTF no sistema adesivo autocondicionante sobre a resistência de união em dentina hígida e em dentina afetada por cárie artificialmente.

- Avaliar a influência dos diferentes sistemas de adesivos autocondicionantes sobre nanoinfiltração com nitrato de prata na interface de união formada pelo adesivo e a dentina hígida ou afetada por cárie em microscopia eletrônica de varredura.

- Avaliar a presença do fosfato na camada híbrida da dentina hígida e afetada por cárie através da espectroscopia Micro-Raman.

Capítulo

3 CAPÍTULO

Esta dissertação está baseada no Artigo 46 do Regimento Interno do Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará que regulamenta o formato alternativo para dissertações de Mestrado e teses de Doutorado, e permite a inserção de artigos científicos de autoria ou coautoria do candidato. Por se tratar de estudos envolvendo seres humanos, ou parte deles, o projeto de pesquisa foi submetido à apreciação do Comitê de Ética em Pesquisa da Universidade Federal do Ceará, tendo sido aprovado. Assim sendo, esta dissertação é composta de um artigo científico que será submetido ao periódico *Journal of Dental Research (JDR)*, conforme descrito abaixo:

INVETIGATION OF NEW BIOMIMETIC ANALOG FOR SELF-ETCH DENTIN BONDING AND REMINERALIZATION

MOURA MEM, DE PAULA DM, LEMOS MVS, YOSHIHARA K, SAURO S, RODRIGUES LK, FEITOSA VP

ABSTRACT

Dentin biomimetic remineralization represents today a feasible strategy to improve durability of resin-dentin bonds. It commonly uses two biomimetic analogues of phosphoproteins to stabilize (chelant) and guide (polyphosphonate) intrafibrillar mineralization of collagen. Herein, we proposed a new reagent (chelant polyphosphonate) to be tested as both analoges added to a self-etching primer with an adhesive containing calcium phosphate fillers. EDTMP (ethylene-diamine-tetramethylene-phosphonic acid) was the new analog and prepared primers were: without analogues and adhesive without fillers (negative control, NC), one containing main analogues sodium trimetaphosphate (TMP) and polyacrylic acid (PAA) (positive control, PC) and others containing TMP/EDTMP, EDTMP/PAA or only EDTMP. Fillers group possessed CaP-fillers in adhesive and no analogues in primer. Adhesives were applied on caries-affected dentin (CAD) or on sound dentin of extracted human molars. After 24h or 6 months water storage, specimens were assessed by microtensile bond strength (μTBS), nanoleakage and Micro-Raman spectroscopy of interface (hybrid layer and underlying dentin). Data was statistically analyzed by two-way ANOVA and Tukey's test (α=5%). Microtensile test of sound dentin showed statistical difference ($p<0.05$) only for EDTMP/TMP with μTBS increase after aging and for EDTMP/PAA with decrease. In CAD, there was μTBS reduction in NC, TMP/EDTMP and EDTMP/PAA ($p<0.05$). Nanoleakage revealed presence of gaps and degradation of adhesive layer and underlying dentin in NC specimens after aging; this was not observed in further groups. Micro-Raman spectroscopy depicted presence of more intense mineralization in PC, EDTMP, TMP/EDTMP and only Fillers, but no remineralization in NC and EDTMP/PAA groups. In conclusion, best outcomes of adhesion and remineralization on caries-affected dentin were attained using two traditional analoges (TMP/PAA) or with EDTMP alone, proving the effectiveness of this new agent.

Keywords: remineralization, dental adhesive, calcium phosphate.

INTRODUCTION

The concept of minimally invasive dentistry is applied for caries treatment intending to selectively remove carious dentin, thereby preserving caries-affected dentin which is prone to remineralization (Sauro and Pashley 2016). The backgrounds of conservative dentistry and recent improvements on dental adhesives have enhanced the handling of remaining carious dentin (Sonoda et al., 2005, Sauro et al., 2012). Conversely, the adhesion of bonding agents to caries-affected dentin represents a new challenge for restorative dentistry (Lynch et al., 2012; Jant, Sigusch, 2009).

Dentin remineralization is more complex and less effective than that of enamel (Shen et al., 2010; Xu et al., 2011). Main differences are the few residual apatite crystals and extensive exposure of organic matrix (principally type 1 collagen) in dentin (Marshall et al. 1997). Therefore, organized dentin remineralization may not be expected in such substrate by means of only ion-releasing materials (Niu et al., 2014). Mineralization of dentin collagen may occur as interfibrillar or intrafibrillar, the latter is attained when precipitation of calcium phosphates is accomplished in interstitial spaces within collagen fibrils. Such approach warrants successful bottom-up (organized) mineralization instead of classic top-down (disorganized) mineralization achieved with mineral precipitation only surrounding the fibrils (Silverman, Boskey, 2004). These minerals are readily dissolved by pH variations and are not able to fully recover dentin's mechanical properties (Kinney et al., 2003; Bertassoni et al., 2011). Contrariwise, intrafibrillar (bottom-up) mineralization oriented by biomimetic remineralization (Colfen et al., 2005) fulfils all internal spaces of collagen fibrils and further gaps, providing the complete recovering of dentin stiffness and flexural strength (Niu et al., 2014).

Biomimetic remineralization mimics natural process of dentin mineralization with the aim of stabilizing and guiding amouphous calcium phosphate nano-precursors (Shen, Zhang, Anusavice, 2010; Xu et al., 2011) modulated by analogs of non-colagenous proteins (Cao et al., 2013; Boukpessi et al., 2008). The extraction or chemical synthesis of such natural proteins is difficult and very expensive; thus, recent investigations focused on the development and study of synthetic analogues to play this role (Tay and Pashley, 2008, Sauro and Pashley 2016). Some artificial analogues employed are the chelating agents like polyacrylic acid (PAA) and polyaspartic acid which stabilize the nucleation of mineral as nano-precursors (Kim et al., 2010, Brackett et al., 2011, Sauro and Pashley 2016). Further analogues bind the collagen fibril and provide the template to embed the stabilized nano-calcium phosphates, thereby guiding organized intrafibrillar mineralization (Kim et al., 2010, Brackett et al., 2011). Most used template analogues are trimetaphosphate (TMP) and polyvinyl-phosphonic acid.

A molecule similar to EDTA called ethylene-diamine-tetra (methylene-phosphonic) acid (EDTMP) is an efficient chelating agent (Han et al., 2008, Zeng et al., 2010) and also binds adequately to several proteins including type I collagen (Liu et al., 2013). Therefore, EDTMP has the potential to act as both biomimetic analogues (chelating agent with polyphosphonates that bind to collagen). Moreover, EDTMP is already applied in medicine associated with Samrium-153 (Patricio et al., 2014) for treatment of bone metastasis caused by cancer (Kalef-Ezra, Valakis, Pallada, 2015). However, EDTMP has never been tested for dentin biomimetic remineralization so far. Therefore, the objective of this investigation is to evaluate if EDTMP is able to act alone (play the roles of both template and stabilizing biomimetic analogues) on bond strength, nanoleakage, and remineralization of sound and caries-affected dentin used

in a self-etch primer associated with ion-releasing calcium phosphates in adhesive. Study hypothesis is that EDTMP can induce adequate dentin bond strength and remineralization after aging.

MATERIALS AND METHODS

Experimental adhesives

An experimental resin-based primer was prepared mildly acidic by mixing 20 wt% glycerol-dimethacrylate-phosphate (GDMA-P), 8.5 wt% hydroxyethyl-methacrylate, 10 wt% urethane-dimethacrylate (UDMA), 20 wt% de-ionized water, 40 wt% absolute ethanol, 1 wt% ethyl 4-dimethyl-amine benzoate and 0.5 wt% camphoroquinone. Using this control primer, four additional experimental primers were formulated by adding the biomimetic analogues EDTMP, PAA and/or TMP: i) Positive Control 5 wt% polyacrylic acid and 5 wt% sodium tri-metaphosphate (PAA/TMP, Mw 1800); ii) EDTMP - 10 wt% ethylenediamine tetramethylene phosphonic acid (EDTMP) iii) 5 wt% EDTMP + 5 wt% PAA (EDTMP/PAA); iv) 5 wt% EDTMP + 5 wt% TMP (EDTMP/TMP). The pHs of primers were all adjusted to 2.1 using NaOH when necessary.

A further experimental co-monomer blend was prepared resin by mixing 30 wt% UDMA, 30 wt% ethoxylated bisphenol- A-diglycidyl-dimethacrylate (BisEMA), 38.5 wt% triethylene-glycol-dimethacrylate (TEGDMA), 1 wt% ethyl 4-dimethyl- amine benzoate and 0.5 wt% camphoroquinone. The control was then used to formulate the experimental ion-releasing adhesive. This latter was created by adding 20 wt% Ca/P micro-filler (10–50 μm particle size) constituted of a mixture of monocalcium phosphate monohydrate (MCPM) (45 mol%), beta-tricalcium phosphate (β-TCP) (45 mol%) and

$Ca(OH)_2$ (10 mol%) (Abuna et al. 2016). All resin blends created were stirred in darkness for 1h under continuous sonication in order to obtain homogenous solutions. All reagents were donated by Esstech Inc. (Essington, USA), except TMP, PAA, MCPM and β-TCP that were bought in Sigma Aldrich (St. Louis, USA), and EDTMP which was purchased from Santa Cruz Biotechnology (Dallas, USA).

Preparation of artificial caries-affected dentin

Sixty dentin specimens (n = 10) were prepared from extracted human third molars obtained under approval of institutional Ethics Committee and stored in 0.1% thymol solution at 4 °C for no longer than four months. Each tooth was horizontally cut to expose a flat middle dentin surface using a slow-speed water-cooled diamond saw (Isomet; Buehler, Lake Bluff, USA) in order to remove occlusal enamel crown and the roots. Exposed dentin surfaces were grounded using 320-grit SiC abrasive papers under water irrigation for 30s to create a standardized smear-layer.

Half of the specimens were subjected to pH cycling to create artificial caries-affected dentin. The dentin surface was polished with 1200-grit SiC papers to create a smooth surface. All further surfaces were protected with acid-resistance nail varnish. A layer of partially demineralized dentin with approximately 200μm-thickness was created according to Qi et al. 2012 on the uncoated surface by pH cycling using the demineralizing solution containing 1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 50 mM acetic acid and 5 mM NaN_3 adjusted to pH 4.8. The remineralizing solution was consisted of 1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4, 0.13 M KCl and 5 mM NaN_3 buffered to pH 7.0 with HEPES buffer. Each specimen was immersed in 10mL demineralizing solution for 8h followed by immersion in 10mL of remineralizing solution for 16h, with fresh solutions

used in each cycle. This procedure was realized during 14 days at ambient temperature (Qi et al., 2012).

Dentin bonding procedures

The specimens (n = 5) were divided into six groups of sound dentin and six of caries-affected dentin: 1) Negative control (primer without analogues + bond without fillers); 2) Positive control (PAA/TMP in primer) + Bond with MCPM/βTCP; 3) Fillers - Primer without analogues + Bond with MCPM/βTCP fillers; 4) Primer with only EDTMP + Bond with MCPM/βTCP; 5) EDTMP/TMP in the primer + Bond with MCPM/βTCP; 6) EDTMP/PAA + Bond with MCPM/βTCP. On each dentin specimen, the self-etching primers were actively applied for 20s, gently air-dried for 3 s and the adhesive resin applied vigorously for 20s with final light-curing for 20s using a LED unit (DB-85, Dabi Atlante, Ribeirao Preto, Brazil), followed by incremental placement of two 2mm-thick horizontal layers of resin composite (Filtek Z350XT, 3M-ESPE, St. Paul, USA), light-cured for 40s each.

Microtensile bond strength (μTBS)

Resin-bonded specimens were sectioned in resin-dentin sticks (0.9 mm x 0.9 mm) for microtensile bond strength testing. Sticks from the most peripheral area presenting residual enamel were excluded. After this procedure, half of the sticks per tooth was tested after 24h in deionized water whereas the other half was submitted to water immersion (aging) for 6 months. The sticks were attached to a jig with a cyanoacrylate cement (Super Bonder gel, Loctite, Henkel Corp., Rocky Hill, USA) and tested to tensile failure in a universal testing machine (EMIC; Sao Jose do Rio Preto, Brazil) with a 500-N load cell and 0.5 mm/min cross-head speed. The exact cross-sectional area of each tested stick was measured with a digital caliper. The μTBS

results were calculated and expressed in MPa. The μTBS values obtained from the sticks of the same resin-bonded tooth were averaged and the mean bond strength of each individual tooth was used as one unit for statistical analysis. Five resin bonded teeth (n = 5) were evaluated for each sub-group. The μTBS data were statistically analyzed by two-way ANOVA (bonding agents and aging period) and Tukey's HSD test at 5% significance level. Subsequent to the μTBS testing, the mode of failure of each fractured stick was determined using a stereomicroscope (Olympus SZ 40-50; Tokyo, Japan) at 100X magnification. The fractures were classified as adhesive, mixed, cohesive in composite or cohesive in dentin.

Nanoleakage analysis

Two resin-dentin sticks were selected from each bonded tooth and storage condition during the cutting procedure (n=10). These sticks were immersed in 50wt% ammoniacal silver nitrate ($AgNO_3$ (aq)) solution in total darkness for 24h (Tay et al., 2002). Subsequently, the specimens were rinsed with distilled water to remove the excess of silver nitrate and immersed in photo-developing solution for 8h under light to reduce silver ions into metallic silver grains. The silver-impregnated sticks were embedded in epoxy resin and polished using 600-, 1200-, 2000-grit SiC papers and diamond pastes (Buehler, Lake Bluff, IL, USA) with 3, 1, and 0.25 μm particle sizes, and ultrasonically cleaned of 15min after each abrasive/polishing step. Specimens were finally air-dried, dehydrated overnight in silica gel under vacuum, coated with carbon and analyzed using SEM (Inspect 50, FEI, Amsterdam, Netherlands) and observed in backscattered electron mode at 20 kV.

Micro-Raman Spectroscopy

For resin-dentin interface remineralization survey, one bonded stick per tooth in each storage condition (n=5) was selected. Micro-Raman spectra of hybrid layer and underlying dentin were obtained in Xplora Raman microscope (Horiba, Paris, France). HeNe laser in 633nm wavelength and 3.2mW power was used as excitation source. Equipment was calibrated with silicon standard sample supplied by the manufacturer. Laser was focused using a 100X magnification lens (Olympus) to determine ~1µm diameter focal point and acquisition was obtained for 10s with 3 accumulations in 900-1000 cm^{-1} range. Two scannings were undertaken in each specimen, first at hybrid layer and then at underlying dentin (5µm below hybrid layer).

RESULTS

For sound dentin, no statistical difference was observed between immediate and aged results of bond strength for positive control, negative control, Fillers, EDTMP ($p>0.05$) (Figure 1). However, EDTMP/PAA group presented significant reduction on µTBS and EDTMP/TMP increased bond strength after aging ($p=0.001$). In caries-affected dentin (Figure 2), positive control, Fillers and EDTMP groups showed no difference on µTBS between 24h and 6 months ($p>0.05$), but negative control, EDTMP/TMP and EDTMP/PAA presented significantly diminished µTBS after aging ($p<0.05$) (table 2). The predominant fracture mode was adhesive for all groups.

Nanoleakage images are presented in Figure 3. Increase in interface degradation was noted after aging for caries-affected dentin bonded with negative control adhesive (Fig. 3A and 3B). Positive control achieved noteworthy remineralization of underlying (caries-affected) dentin (Figs. 3C and 3D). Such

remineralization occurred in Fillers group (no analogues in primer), but with different conformation (Fig. 3F). EDTMP presented noteworthy mineralization after 6 months as observed in Fig. 3H. Both in EDTMP/TMP and EDTMP/PAA groups, slight adhesive degradation and no remineralization of underlying dentin were observed after aging (Figs. 3J and 3M).

On remineralization analysis by Micro-Raman spectroscopy (Figure 4), peaks at 960cm^{-1} (apatite phosphate vibration), 970cm^{-1} (β-TCP phosphate vibration) and 980cm^{-1} (brushite phosphate vibration) were assessed. All spectra of sound dentin specimens were performed at hybrid layer and those in CAD were at underlying dentin. No mineral formation was detected in immediate and aged samples of negative control (Fig. 4A1) whereas positive control demonstrated increase of 980cm^{-1} peak (brushite precipitation) either in 24h or after 6 months, and some residual β-TCP (Fig. 4B1). Fillers group showed no formation of mineral at hybrid layer in 24h, and deposition of brushite (980cm^{-1}) and apatite (960cm^{-1}) after aging (Fig. 4C1). EDTMP alone achieved apatite precipitation after aging (Fig. 4D1), whilst EDTMP/TMP and EDTMP/PAA depicted formation of brushite after aging (Figs. 4E1 and F1).

Raman spectra obtained in caries-affected dentin showed Negative control with absence of minerals (Fig. 4A2). Positive control and EDTMP/TMP depicted the presence of β-TCP and brushite in 24h and appearance of apatite after aging (Figs. 4B2 and E2). Group with only fillers showed brushite at 24h and β-TCP after 6 months (Fig. 4C2). Only EDTMP demonstrated peaks of β-TCP and brushite after aging (Fig. 4D2), whereas EDTMP/PAA at 24h depicted β-TCP, but with no clear signs of mineral precipitation after aging (Fig. 4F2).

DISCUSSION

Based on the outcomes, study hypothesis, that EDTMP is able to induce adequate bond strength and remineralization after aging, needs to be accepted. Yet, its association with sodium trimetaphosphate (TMP) was successful in such tasks, thereby reducing degradation even on caries-affected dentin.

Many strategies have been proposed to remineralize partially-dentin, for example the use of fluoride, amourphous calcium phosphate or bioglass (Shen, Zhang, Anusavice, 2010; Xu et al., 2011; Profeta et al., 2013, Luo et al 2016). Nevertheless, most processes are focused on rapid and intense mineral deposition which causes only interfibrillar remineralization. (Kim et al., 2010). With this classic method of remineralization, mineral deposition does not reach the region affected by caries' acids which is intrafibrillar dentin collagen (Kinney et al., 2003; Tay and Pashley, 2008). Intrafibrillar mineralization not only increases mechanical properties, but also protects dentin structure from bacterial acids and endogenous enzymes (Balooch et al., 2008; Kinney et al., 2003).

Biomimetic remineralization is a different approach to solve this issue, because it tries to fulfil also intrafibrillar collagen by the incorporation of stabilized nanoprecursors of amourphous calcium phosphate inside fibrils (Tay and Pashley, 2008). This process is modulated by analogs of non-collagenous phosphoproteins of dentin (Tay and Pashley, 2008; Cao et al., 2015, Niu et al., 2014). Biomimetic remineralization occurs only in presence of analogs, once they are accountable for the maintenance of calcium phosphates in very small (nanometer) size sufficient to penetrate demineralized collagen fibril, this role is performed by stabilizing analog which should be a potent chelant (Tay and Pashley, 2008).

Moreover, further analogues possess affinity with collagen fibril in order to guide the crystallization of nano-calcium phosphates (template analogue) (Tay and Pashley, 2008). Most used analogues in literature are polyacrylic acid, polyaspartic acid, polyvinyl-phosphonic acid and trimetaphosphate (Cao et al., 2015). As majority of analogues are acidic, their addition to dental adhesives would be more suitable in self-etch adhesives. Incorporation of some analogues in self-etch adhesives was only recently reported (Abuna et al., 2016). However, the solubility of analogues in primers and adjustment of solutions' pH containing acidic functional monomers are some shortcomings still present in preparation of adhesives.

EDTMP is an efficient chelator (Han et al., 2008, Zeng et al., 2010) with the ability to bind its polyphosphonates to phosphate and amine functionalities of collagen (Liu et al., 2013). This compound is widely used in medicine associated with Samarium for the treatment of bond tumours (Patricio et al., 2014; Kalef-Ezra, Valakis, Pallada, 2015), but has never been investigation in Dentistry to our knowledge. Chemical structure of EDTMP molecule is very similar to EDTA (ethylenediamine tetraacetic acid), a substance very established in dental clinical practice, except for the replacement of four acetic functionalities by four phosphonic groups. Indeed, EDTMP might work as the two different biomimetic analogs, turning the process easier (to prepare materials and dissolve main compounds) and with lower cost for a future perspective of clinical applicability of biomimetic remineralization.

Microtensile outcomes from sound dentin specimens did not present many statistical differences (Figure 1). This was expected because, in intact dentin, most collagen fibrils are preserved and highly mineralized as well as mild self-etch adhesives expose less collagen than etch-and-rinse ones (De Munck et al., 2005). For EDTMP/TMP primer used in sound dentin, there was an increase ($p<0.05$) on bond

strength after 6-month aging, what did not happen in positive control (TMP/PAA). One may speculate that EDTMP is a stabilizing (chelator) analogue more efficient than polyacrylic acid, thereby affording rapid remineralization of resin-dentin interface. Conversely, primer containing EDTMP/PAA presented bond strength reduction. As EDTMP and PAA are potent chelators, they may react preferably with most calcium, jeopardizing the chemical interaction of the acidic functional monomer. Different from functional monomer, these analogues do not possess polymerizable functionality; thus, such interaction (EDTMP-Ca and PAA-Ca) in excess has no contribution to the true dentin adhesion. Less chemical bonding of functional monomer has demonstrated to impair the durability of self-etch adhesives (Feitosa et al. 2014), what could explain the decrease of bond strength. Furthermore, the over-demineralization promoted by three agents (primer containing acidic monomer, EDTMP and PAA) might expose more collagen, creating spots of degradation and more activity of proteases (Brackett et al., 2011).

Caries-affected dentin represents a substrate with high clinical relevance, once the modern cavity preparations and caries removal tend to leave often CAD which is prone to remineralization (Sauro et al., 2015). Positive Control, EDTMP and Fillers groups depicted stable bond strength after aging, but reductions were found in negative control, EDTMP/TMP and EDTMP/PAA. Nanoleakage micrographs of CAD specimens from Negative Control group presented gaps, failures and silver deposition at underlying dentin (Fig. 3A) after 24h, whilst after aging all these factors were observed along with degradation (silver deposition) in adhesive layer (Fig. 3B). This sort of degradation after aging was not found in further groups.

All groups except negative control showed presence of calcium phosphate fillers in adhesive layer (Fig. 3). Although these fillers were partially dissolved to supply ions

for remineralization, the MCPM/βTCP system used did not promoted gaps in adhesive layer (Abuna et al., 2016), proving the mineral recharge based on inorganic reaction with water to form brushite (dicalcium phosphate dihydrate). In positive control (TMP/PAA), degradation of hybrid layer and underlying dentin was reduced (Fig. 3D) with very sparse silver deposits, thereby confirming previous investigations (Tay and Pashley, 2008, Niu et al., 2014, Abuna et al., 2016). However, some fractures were observed (arrows in Fig. 3D) in these specimens. This was likely cause by partial degradation of collagen, as no MMPs inhibitors or collagen cross-linkers were applied to avoid initial degradation of partially demineralized collagen of CAD.

Mineralization of underlying dentin was also noted in underlying dentin of Fillers (no analogues in primer) specimens, but with different morphology (Fig. 3F) than positive control specimens. Dentin remineralization, mediated by an ion-releasing adhesive in absence of biomimetic analogues, is disorganized and random. It was noted that the distance between tubules apertures in remineralized dentin (arrows in Fig. 3F) was different from intact dentin observed at the bottom of the micrographs and some silver deposits were present, indicating incomplete remineralization. Such remineralization represents the classic (top-down) remineralization only in interfibrillar spaces (Kinney et al., 2003). EDTMP presented hybrid layer and underlying dentine (re-)mineralization with characteristics of inter- and intra-fibrillar deposits (Fig. 3H). In EDTMP/TMP specimens, nanoleakage survey showed that adequate dentin remineralization was not afforded as well as in EDTMP/PAA group which depicted increase of silver impregnation both at hybrid layer and at underlying dentin.

Micro-Raman spectroscopy of negative control showed no formation of mineral in sound dentin. Nevertheless, precipitation of brushite and beta-tricalcium phosphate were observed initially and after 6 months. Indeed, apatite formation did not occur due

to the low pH (Cao et al. 2015) highly acidic primer containing analogues. In Fillers group, rapid apatite formation was observed (Fig. 4C1), likely due to the absence of analogues and easier increase of pH. In sound dentin, primer containing only EDTMP achieved the formation of brushite at hybrid layer, which was not found in EDTMP/TMP and EDTMP/PAA groups. In caries-affected dentin, negative control did not afford mineral detection, but positive control showed βTCP and brushite initially, whereas these minerals transformed into apatite with some residual brushite. These findings corroborate with previous studies depicting mineralization with apatite modulated by analogues over time (Abuna et al, 2016; Sauro et al., 2015).

In Fillers group, the higher pH does not favour formation of brushite, which is precipitated in 3-5 pH range (Feitosa et al., 2013); thus, the brushite observed after 24h may have been transformed into βTCP after aging (Fig. 4C2), or it could be residual βTCP. Due to its strong chelating nature, primer with only EDTMP uptakes lots of calcium ions, impairing the initial formation of brushite. However, after aging such mineral was detected in caries-affected dentin. Outcomes of EDTMP-only primer presented consistent remineralization, but slower than further groups (Fillers and positive control). Indeed, this was caused by the higher concentration of EDTMP in comparison with other analogues, thereby attaining more “sequestration” of calcium and reduced speed of inorganic reaction. Group EDTMP/PAA (combination of chelators) also showed ineffective mineral formation at caries-affected dentin (Fig. 4E2) likely due to the low supply of calcium. Future studies should focus on the reduction of biomimetic analogues concentration in order to accomplish optimal organized (bottom-up) remineralization with maximum reaction rate. In summary, it may be concluded that stable dentin bonds with adequate remineralization is provided

by primers containing TMP/PAA (positive control), EDTMP and EDTMP associated with TMP, proving the ability of EDTMP as template analogue and stabilizing analogue.

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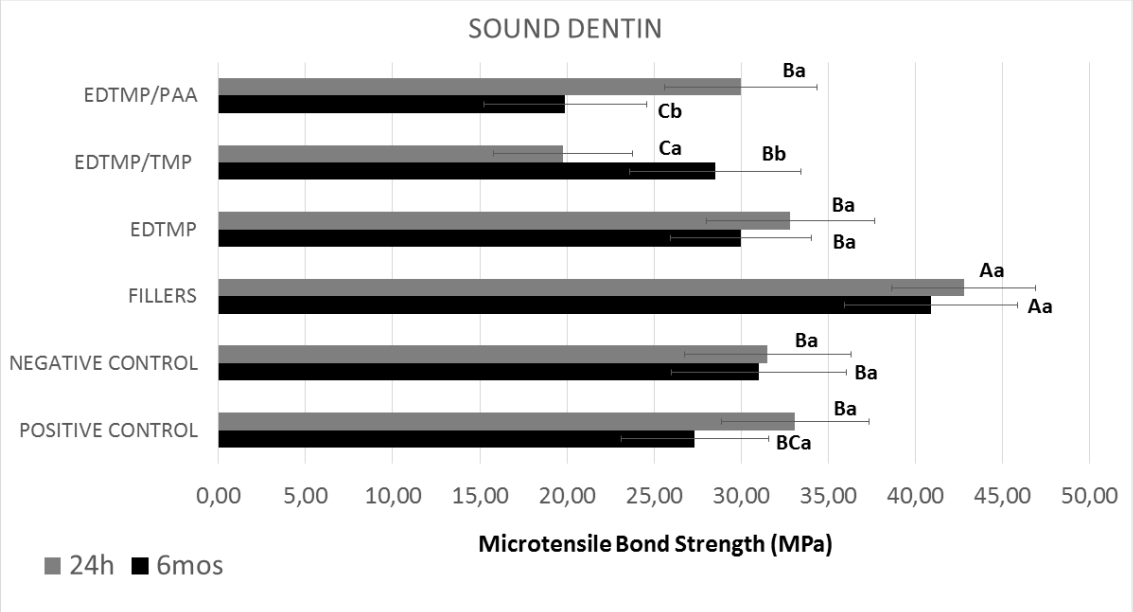

Figure 1. Graph depicting the microtensile bond strength results of sound dentin specimens. Different capital letters indicate statistically significant differences ($p<0.05$) among different primers in same period. Different lowercase letters indicate significant differences ($p<0.05$) between 24h and 6 months.

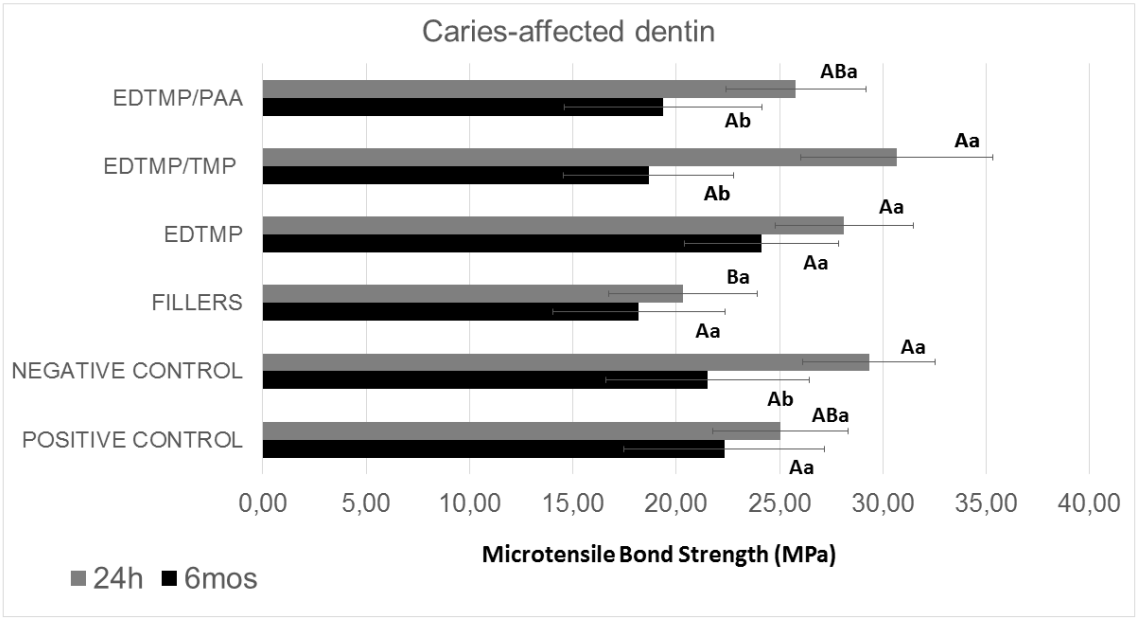

Figure 2. Graph depicting the microtensile bond strength results of caries-affected dentin (CAD). Different capital letters indicate statistically significant differences ($p<0.05$) among different primers in same period. Different lowercase letters indicate significant differences ($p<0.05$) between 24h and 6 months.

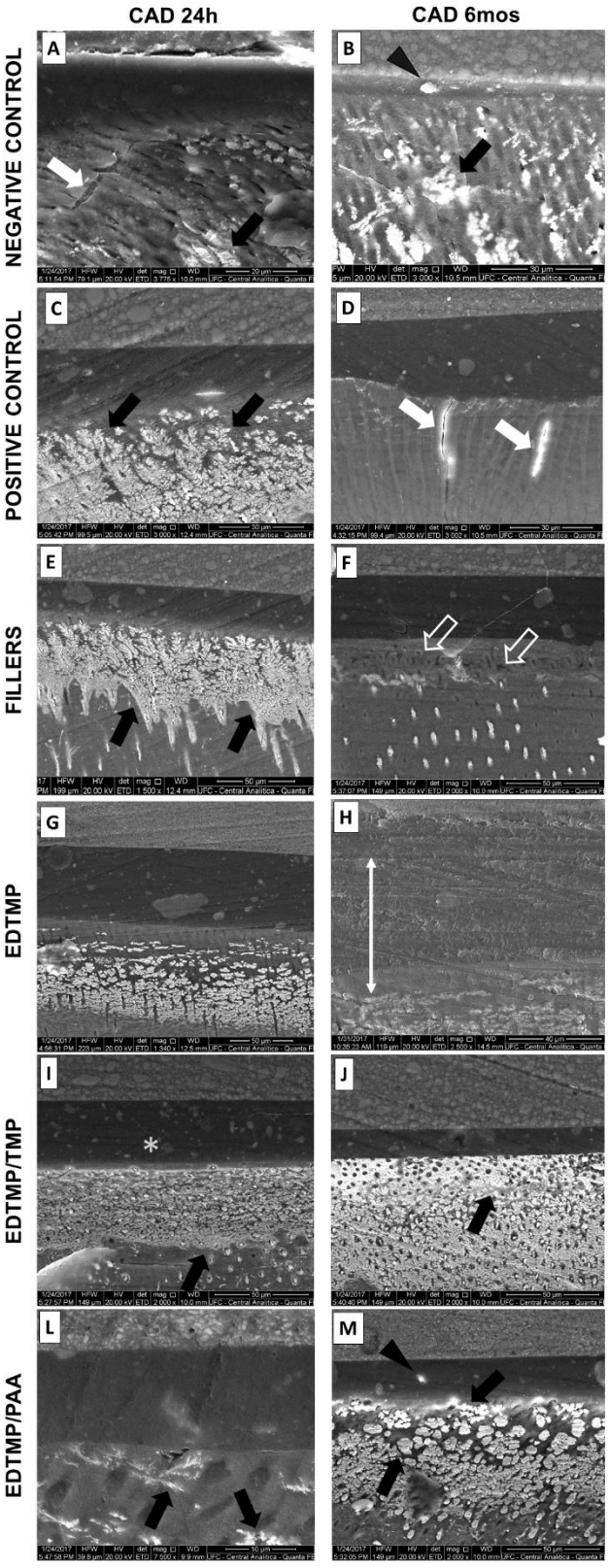

Figure 3. SEM micrographs of resin-dentin interfaces illustrating the most common nanoleakage characteristics observed after silver uptake. The open arrows indicate deposits of silver and black

arrows indicate silver deposits in caries-affected dentin, and white arrows indicate gaps likely due to collagen partial cleavage. Black arrowheads indicate degradation inside the adhesive layer. Open white arrows indicate alteration in dentin tubule morphology and organization after remineralization.

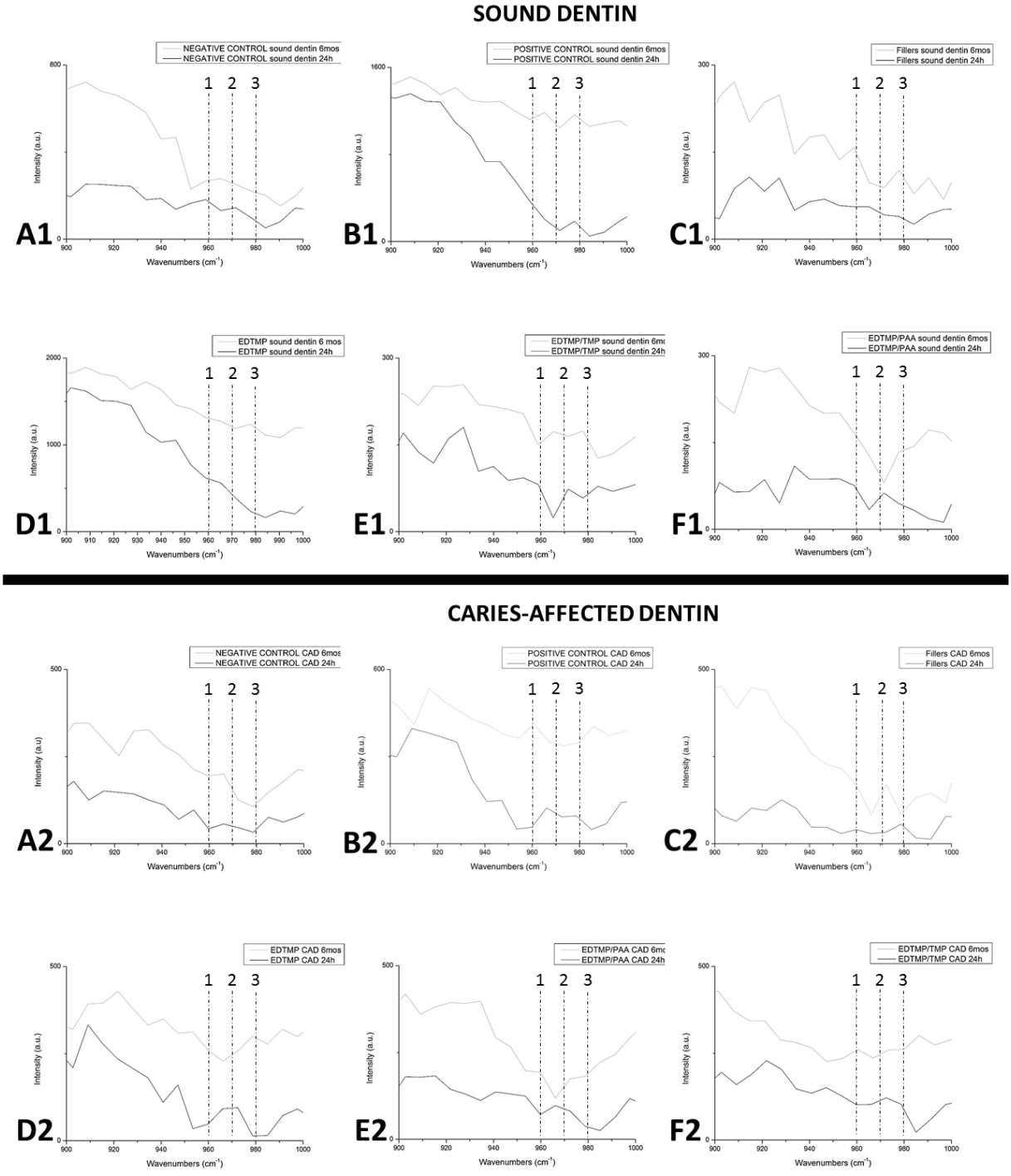

Figure 4. Micro-Raman spectra in hybrid layer of sound dentin specimens (upper panel) and in caries-affected dentin (bottom panel).

Conclusão

4 CONCLUSÃO

Em dentina hígida, os adesivos controle positivo, EDTF e EDTF/TMP obtêm adesão e maior capacidade de remineralização após envelhecimento. Em dentina afetada por cárie, o controle positivo e o EDTF sozinho foram mais eficazes em mineralizar a dentina e preservar a adesão à dentina ao longo do tempo. O EDTF pode ser usado como análogo biomimético em primers autocondicionantes promovendo efetiva remineralização da dentina afetada por cárie.

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UNIVERSIDADE FEDERAL DO CEARÁ/ PROPESQ

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: AVALIAÇÃO DO DESENVOLVIMENTO DE UM NOVO ADESIVO ODONTOLÓGICO PARA REMINERALIZAÇÃO BIOMIMÉTICA DE INTERFACES ADESIVAS

Pesquisador: Maria Elisa Martins Moura

Área Temática:

Versão: 2

CAAE: 55285616.3.0000.5054

Instituição Proponente: Departamento de Clínica Odontológica

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.610.783

Apresentação do Projeto:

Projeto de pesquisa da mestranda Maria Elisa Martins Moura sobre as falhas de adesão entre dente/restauração em curto espaço de tempo. É objetivo avaliar uma restauração terapêutica baseada na remineralização biomimética utilizando um adesivo autocondicionante fotopolimerizável contendo fosfatos de cálcio bioativos e um novo análogo biomimético (EDTF,etilenodiamino tetrametileno-fosfonato). Serão utilizados uma incorporação de análogos controles, um adesivo experimental autocondicionante de dois passos e primers autocondicionantes específicos. Estes sistemas adesivos serão aplicados em dentina afetada por cárie simulada artificialmente e em dentina sadia de dentes humanos extraídos. Após períodos de 24h e 6 meses de armazenamento,serão avaliados por teste de microtração, nanoinfiltração e microdureza da interface e da dentina adjacente.O teste de Kolmogorov-Smirnov será utilizado para avaliar a distribuição normal e os valores serão submetidos à análise estatística com ANOVA dois fatores (primer experimental e tempo de armazenagem) e Teste de Tukey ($p<0,05$).

Objetivo da Pesquisa:

Formular e avaliar adesivos experimentais fotopolimerizáveis com fosfato de cálcio bioativos com primers contendo um novo análogo biomimético que promova a remineralização satisfatória em

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dentina afetada por cárie in vitro.

Avaliação dos Riscos e Benefícios:

A pesquisa é de baixo risco para o operador visto que se trata de uma pesquisa realizada integralmente em laboratório, pondo o pesquisador em contato com material biológico fixado.

Quanto aos benefícios destaca-se a possibilidade de se empregar nos serviços de saúde um material restaurador de baixo custo com capacidade de repor o tecido dentário destruído e de promover uma maior longevidade da restauração.

Comentários e Considerações sobre a Pesquisa:

Trata-se de uma pesquisa de caráter investigativo e de mérito científico.

Considerações sobre os Termos de apresentação obrigatória:

A pesquisadora apresentou a este comitê: projeto, folha de rosto, declaração de concordância, autorização do local de execução, carta de encaminhamento, currículo do pesquisador, solicitação de dispensa de TCLE e Termo de doação de dentes por fiel depositário.

Recomendações:

Não se aplica.

Conclusões ou Pendências e Lista de Inadequações:

Não se aplica.

Considerações Finais a critério do CEP:

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_663864.pdf	06/06/2016 10:54:35		Aceito
Folha de Rosto	folha.pdf	06/06/2016 10:54:03	Maria Elisa Martins Moura	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	dispensa.pdf	18/04/2016 11:33:19	Maria Elisa Martins Moura	Aceito
Projeto Detalhado / Brochura Investigador	Projeto.docx	18/04/2016 11:18:18	Maria Elisa Martins Moura	Aceito

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Continuação do Parecer: 1.610.783

Cronograma	Cronograma.docx	18/04/2016 11:18:01	Maria Elisa Martins Moura	Aceito
Declaração de Instituição e Infraestrutura	Instituicao.pdf	03/04/2016 20:10:01	Maria Elisa Martins Moura	Aceito
Outros	CUSTEIO.pdf	17/03/2016 09:38:50	Maria Elisa Martins Moura	Aceito
Outros	doacao.pdf	17/03/2016 09:38:01	Maria Elisa Martins Moura	Aceito
Outros	COMEPE.pdf	17/03/2016 09:37:34	Maria Elisa Martins Moura	Aceito
Declaração de Pesquisadores	pesquisadores.pdf	17/03/2016 09:35:28	Maria Elisa Martins Moura	Aceito
Orçamento	Orcamento.docx	17/03/2016 09:35:07	Maria Elisa Martins Moura	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

FORTALEZA, 28 de Junho de 2016

Assinado por:
FERNANDO ANTONIO FROTA BEZERRA
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