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Orientadora: Profa. Dra. Regina Maria Puppin Rontani

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Materiais Dentários da Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas para obtenção do título de Doutora em Materiais Dentários.

Doctorate thesis presented to the Dental Materials Postgraduation Programme of Piracicaba Dental School of the State University of Campinas to obtain the Ph.D. grade in Dental Materials.

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA ANA ROSA COSTA CORRER, E ORIENTADA PELA PROFA. DRA. REGINA MARIA PUPPIN RONTANI.

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Profa. Dra. REGINA MARIA PUPPIN RONTANI

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RESUMO

O propósito deste estudo foi avaliar a influência de diferentes tipos de substrato (Capítulo 1) e tratamento com etanol 100% (Capítulo 2) ou digluconato de clorexidina a 2% (CHX) (Capítulo 3) na resistência e longevidade da união compósito/dentina, após diferentes períodos de armazenagem. Os dentes humanos hígidos foram divididos em 3 grupos, de acordo com o substrato dentinário: (Sd) dentina hígida, (Ci) dentina infectada (manutenção da cárie) e (Ca) dentina afetada (remoção parcial de cárie). As superfícies oclusais de terceiros molares hígidos foram removidas com disco diamantado dupla face sob refrigeração, a fim de expor a superfície plana dentinária hígida, a qual foi submetida ao desenvolvimento da lesão de cárie artificial com S. mutans. Brocas esféricas foram usadas para remover parcialmente o tecido cariado pigmentado, até que permanecesse apenas a dentina levemente corada (Ca). Em seguida, os dentes foram novamente subdivididos, de acordo com o tratamento da superfície: Grupo Controle (Ct) - nenhum tratamento (Capítulo 1); Ct e (Et) – aplicação de etanol 100% (Capítulo 2); e, Ct e (CHX) -aplicação de digluconato de clorexidina a 2% (Capítulo 3). O substrato foi condicionado com ácido fosfórico a 35% por 15 s, lavado por 30 s e seco com leve jato de ar. CHX ou Et foram aplicados após o condicionamento ácido. O sistema adesivo Adper Single Bond 2 (3M ESPE) foi aplicado de acordo com as recomendações do fabricante e fotoativado por 20 s com a fonte de aparelho de luz XL 2500. Sobre a superfície de união foram inseridos incrementos de 2 mm do compósito Z350 (3M ESPE), polimerizados

usando a fonte de luz XL 2500 por 40 s, até confeccionar um bloco com 6 mm de altura. As amostras foram armazenadas em água destilada a 37º C por 24 horas. Após, os dentes foram seccionados perpendicularmente à área de união, de modo a obter palitos com área de secção transversal de 1mm^2. Os palitos foram armazenadas em três períodos: 24 horas, 6 meses e 1 ano. Em seguida, as amostras foram submetidas ao ensaio de resistência de união à microtração usando a máquina de ensaio EZ test (EZS, Shimadzu) a velocidade de 0,5 mm/minuto. Os dados foram submetidos à Análise de Variância e ao teste de Tukey HSD (α=0,05). A união em Sd mostrou valores de resistência de união significativamente maior quando comparado à Ca e Ci. As amostras armazenadas nos períodos de 6 meses e 1 ano resultou na diminuição da resistência de união, independente do tipo de substrato e/ou tratamento. O etanol não foi efetivo em melhorar a resistência de união para os três substratos de dentina avaliados. A clorexidina não influenciou na resistência de união nos períodos de armazenagem avaliados, independente dos substratos.

Palavras-chave: Clorexidina, Etanol, Dentina, Streptococcus mutans, Adesivo Dentinário

ABSTRACT

The aim of this study was to evaluate the effect of 2% chlorhexidine digluconate (CHX) and 100% ethanol wet-bonding (Et) on the degradation of the adhesive system bond strength in sound or artificial caries-affected/infected dentin after different storage times under microtensile test (μTBS). The sound teeth were divided into 3 groups, according to dentin substrates: sound dentin, caries-infected dentin (maintained of caries) and caries-affected dentin (partial removal of caries). Non-carious human third molars had their occlusal enamel removed with a slow speed diamond saw, under copious water-cooling to expose a flat-surfaced sound dentin, which was submitted to the microbiological challenge (S*. mutans*) for the development of artificial caries. Spherical drill was used to remove soft pigmented carious tissue till hard and slightly pigmented dentin remains (Ca). After, the teeth will be assigned into 3 subgroups according to surface treatment: water wet-bonding (Ct) (Chapter 1); Ct and 100% ethanol application (Et) (Chapter 2); and, Ct and chlorhexidine digluconate 2% application (CHX) (Chapter 3). The substrate will was etched with 35% phosphoric acid gel for 15 s, rinsed for 30 s with tap water and dried with oil/water-free air. The CHX or Et was applied for 60 s, just after etching with 35% phosphoric acid gel. The adhesive system Adper Single Bond 2 (3M ESPE) was applied according to the manufacturer's instruction and polymerized for 10 s by a light-curing unit XL 2500. The bonded surfaces were coupled with a composite resin Filtek Z350 (3M ESPE) applied in 2 mm increments and polymerized using a curing unit XL 2500 (3M ESPE) for 40 s for each

increment and to build 6 mm thick block. The restored teeth were stored in distilled water at 37^{o} C for 24 h. After this period, the restored teeth were longitudinally sectioned across the bonded interface to produce a series of 1.0mm^{2} beams. The specimens were then submitted to three storage periods: 24 hours, 6 months, or 1 year. Afterwards, the storage periods, the specimens were submitted to a microtensile bond strength (μTBS) using EZ test machine (EZS, Shimadzu) at a crosshead speed of 0.5 mm/min until failure. Data were submitted to ANOVA and Tukey's HSD test (α=0.05). The bonding with sound dentin showed μTBS values significant higher when compared to caries-affected and caries-infected dentin. The 6 months and 1 year storage periods resulted in decreased bond strengths for all dentin conditions and/or treatment. The ethanol was not effective to improve the μTBS for the three dentin substrates evaluated. The CHX did not affect the 24-hour, 6 and 12 months μTBS, independent of the tested dentin substrates.

Key words: 2% chlorhexidine digluconate, 100% ethanol, dentin, S. *mutans*, adhesive system, microtensile bond strength, s*torage time*

SUMÁRIO

INTRODUÇÃO

A Odontologia minimamente invasiva visa a preservação da maior quantidade possível de estrutura dentária remanescente diante de lesões de cárie. Existe a possibilidade de remover apenas a dentina infectada por bactérias, mantendo, a camada mais interna afetada que teoricamente, seria passível de remineralização (ten Cate JM, 2001) O desenvolvimento de sistemas adesivos e materiais restauradores poliméricos têm inquestionável importância na preservação da estrutura dentária. Procedimentos e produtos que melhorem a qualidade da união, reduzindo a infiltração da água e a degradação do colágeno na camada híbrida são essenciais para manter a interface compósito/dentina mais estável. Diversos estudos de resistência de união têm sido realizados na dentina hígida; entretanto, é bem conhecido que a dentina afetada por cárie é frequentemente encontrada na clínica (Nakajima M *et al*., 1995; 2005; Yoshiyama M *et al*., 2000; 2002).

Dentina infectada e afetada por cárie são condições distintas de um mesmo tecido dental, com diferentes composição química e estrutura morfológica (Fusayama T & Terachima S,1972). Dentina infectada por cárie é caracterizada por uma zona necrótica, superficial, desmineralizada (Ogushi K & Fusayama T, 1975), com fibras colágenas degeneradas e ausência de ligações cruzadas (Kuboki Y *et al*., 1977; Macedo GM *et al*., 2009), além de grande presença de bactérias (Banerjee A *et al*., 2010). Dentina afetada por cárie apresenta pequenas alterações nas ligações cruzadas e nas fibras colágenas (Breschi L *et al*., 2006),

diferindo da dentina hígida pela existência de precipitados mineralizados dentro dos túbulos dentinários (Mobarak EH & El-Badrawy WH, 2012). Remover dentina infectada mantendo fina camada de dentina afetada, circundada por dentina hígida e esmalte nas margens, é uma alternativa proposta com a finalidade de evitar exposições pulpares e preservar estrutura dental, desde que seja possível obter bom selamento marginal e controle da umidade (Banerjee A *et al*., 2010; Fusayama T, 1979)

Diferentes materiais restauradores podem ser empregados para restaurar cavidades que preservem a dentina afetada por cárie, entre estes, sistemas adesivos/compósitos restauradores. Tanto sistemas adesivos quanto compósitos restauradores têm sido estudados e empregados em larga escala na Odontologia, resultando em melhoria nas propriedades e implemento nas indicações clínicas.

Sabe-se que a resistência de união a curto e longo prazo (6 meses) é menor quando estabelecida em regiões de dentina infectada ou afetada pela cárie, mas os valores de união podem ser clinicamente aceitáveis (Yoshiyama M *et al*., 2002; Erhardt MC et al., 2008; Komori PC *et al*., 2009; Macedo GV *et al*., 2009; Li H *et al*., 2011). Alterações existem na união em dentina afetada por cárie devido à deposição de minerais ácido resistentes na região intratubular (Nakajima M *et al*., 2005), tornando o substrato semi-impermeável à água (Yoshiyama M *et al*., 2002), além do fato que mais poros ocorrem na dentina intertubular em função da desmineralização parcial (Huang X *et al*., 2011). A deposição de mineral reduz a permeabilidade dentinária, podendo causar injúria a polpa e impedir a criação de adequada união à dentina (Nakajima M *et al*., 1995; Yoshiyama M *et al*., 2000).

Este fato no procedimento de união é também válido para a dentina infectada por cárie, memso na presença de alterações profundas neste substrato.

O uso de uma camada de agente resinoso hidrófobo (King NM *et al*., 2005), aplicações de múltiplas camadas de adesivo (Pashley EL *et al*., 2002; Hashimoto M *et al*., 2004; 2005), evaporação do solvente (Van Landuyt KL *et al*., 2005), tempo de polimerização prolongado (Cadenaro M *et al*., 2005; 2006; 2009), uso de corrente elétrica para melhorar a impregnação do monômero (Breschi L *et al*., 2009; Pasquantonio G *et al*., 2007), uso de inibidores de metalo-proteinases (Hebling J *et al*., 2005; Brackett WW *et al*., 2007; Carrilho MR *et al*., 2007), *etanol-wet-bonding* (Pashley DH *et a*l., 2007; Cadenaro M *et al*., 2009; Hosaka K *et al*., 2009; Huang X *et al*., 2011) e digluconato de clorexidina (de Castro FL *et al*., 2003; Hebling J *et al*., 2005; Carrilho MR *et al*., 2007; Komori PC *et al*., 2009) são algumas modificações do protocolo clínico padrão que mostraram aumentar a estabilidade da união.

Agentes químicos para prevenir a progressão e recidivas de cárie na dentina podem também ser aplicados. O digluconato de clorexidina é um conhecido agente bacteriano empregado como solução desinfetante de preparos cavitários (de Castro FL *et al*., 2003), sendo também utilizado como *primer* terapêutico para preservar a camada híbrida da dentina do dente humano (Gendron R *et al*., 1999; Hebling J *et al*., 2005),além da função antiproteolítica, inibindo de forma inespecífica a ação de metalo-proteinases (Gendron R *et al*., 1999; Hebling J *et al*., 2005; Pashley DH *et a*l., 2007; Komori PC *et al*., 2009). Entretanto, a causa e a relação do efeito entre os diferentes procedimentos

empregados na técnica convencional e a degradação da dentina na camada híbrida ainda não foram estabelecidas.

A utilização de soluções de etanol em altas concentrações também tem sido propostas para o tratamento da superfície dentinária após o condicionamento ácido (Nishitani Y *et a*l., 2006; Pashley DH *et al*., 2007; Tay FR *et al*., 2007; Sadek FT *et al*., 2008; Cadenaro M *et al*., 2009), mostrando melhoras significativas na resistência de união (Hosaka K *et al*, 2009). Além disso, diminuição do diâmetro da fibrila de colágeno e aumento do espaço interfibrilar nas camadas híbridas foram observados (Hosaka K *et al*, 2009). Assim, o emprego do etanol a 100%, previamente à aplicação do sistema adesivo, funcionaria como retardador da degradação da interface adesiva resina/dentina sadia (Pashley DH *et al*., 2007; Tay FR *et al*., 2007; Sadek FT *et al*., 2008). Embora, este fato pareça promissor, mais evidências se fazem necessárias, principalmente em dentina afetada e infectada por cárie, substratos mais frequentes na clínica.

A excelente efetividade de união dos adesivos dentinários, verificada imediatamente ou em curto prazo, não apresenta durabilidade e estabilidade de união, sendo questionável e multifatorial, pois a degradação na interface dentina/resina composta começa em aproximadamente em 6 meses ou menos (Carrilho MR *et al*., 2005; Costa AR, 2009). A resistência e a estabilidade da união parecem estar associadas à qualidade da camada híbrida. Em outras palavras, esses fatores parecem ser mais dependentes da impregnação adequada ao substrato dentinário pelo sistema adesivo, do que pela espessura ou morfologia da camada híbrida (De Munck J *et al*., 2005). Os co-monômeros usados na maioria

dos adesivos têm características hidrófilas e absorvem de 5 a 12% de água (Yoshiyama M *et al*., 2002; Ito S *et al*., 2005; Malacarne J *et al*., 2006), resultando em plastificação por hidrólise com lixiviação de monômeros não reagidos, o que altera as propriedades mecânicas. Essas camadas tornam-se cada vez mais permeáveis e susceptíveis à hidrólise e a infiltração da resina na dentina condicionada é frequentemente incompleta. Assim, as fibrilas de colágeno expostas podem também ser degradadas pela água circundante presente nesta região (Hashimoto *et al*., 2000; Carrilho *et al*., 2005; Loguercio AD *et al*., 2005) ou pela ativação de MMPs presentes nos tecidos, após o condicionamento ácido da dentina (Pashley DH *et al*., 2004; Mazzoni A *et al*., 2006; Nishitani Y *et al*., 2006; Tay FR *et al*., 2006; Mazzoni A *et al*., 2007).

Assim, melhor infiltração do sistema adesivo reduz sorpção de água e degradação do colágeno, tornando a união resina/dentina mais estável e durável. A comparação entre resistência de união imediata (24 horas), em curto (6 meses) e longo prazo (1 ano) quando estabelecida em dentina hígida, após a manutenção (dentina infectada) ou remoção parcial de cárie (dentina afetada) com ou sem aplicação de digluconato de clorexidina a 2% (CHX) ou etanol a 100% (Et), é escassa na literatura. Embora, técnicas alternativas sejam promissoras na tentativa de melhorar a resistência de união, mais evidências são necessárias, principalmente em dentina afetada ou infectada por cárie, condições mais frequentemente encontradas na prática clínica.

Portanto, o objetivo neste estudo foi:

Capítulo 1

Avaliar *in vitro* ao longo do tempo (24 horas, 6 meses e 1 ano) a interface e a resistência de união produzida por um sistema adesivo convencional de 2 passos (Adper Single Bond 2 – 3M ESPE) à dentina afetada ou infectada por cárie artificial, por meio do ensaio de resistência da união à microtração (μTBS) e da microscopia eletrônica de varredura (MEV). As hipóteses avaliadas foram (1) diferentes substratos dentinários não afetariam a resistência de união do sistema adesivo; (2) os diferentes períodos de armazenagem não afetariam a resistência de união do sistema adesivo aos diferentes substratos dentinários.

Capítulo 2

Avaliar *in vitro* o efeito da aplicação do etanol 100% ao longo do tempo (24 horas, 6 meses e 1 ano) na interface e na resistência de união produzida por um sistema adesivo convencional de 2 passos (Adper Single Bond 2 – 3M ESPE) à dentina afetada ou infectada por cárie artificial, por meio do ensaio de resistência da união à microtração (μTBS) e da microscopia eletrônica de varredura (MEV). As hipóteses avaliadas foram que (1) a aplicação de etanol 100% promoveria aumento na resistência de união nos diferentes substratos dentinários; (2) o tratamento da superfície da dentina com etanol 100% aumentaria a resistência de união aos substratos dentinários, em função dos períodos de armazenagem.

Capítulo 3

Avaliar *in vitro* o efeito da aplicação da clorexidina 2% ao longo do tempo (24 horas, 6 meses e 1 ano) na interface e na resistência de união produzida por um sistema adesivo convencional de 2 passos (Adper Single Bond 2 – 3M ESPE) à dentina afetada ou infectada por cárie artificial, por meio do ensaio de resistência da união à microtração (μTBS) e da microscopia eletrônica de varredura (MEV). A hipótese avaliada foi que o uso da clorexidina a 2% não influencia na resistência de união, em relação aos períodos de armazenagem ou às condições do substrato dentinário.

O presente trabalho é apresentado no formato alternativo de tese de Doutorado de acordo com as normas estabelecidas pela deliberação 002/06 da Comissão Central de Pós-Graduação da Universidade Estadual de Campinas. Os artigos referentes aos Capítulos serão submetidos aos periódicos, *Journal of Dentistry, Operative Dentistry* e *Journal of Adhesive Dentistry,* respectivamente.

CAPÍTULO 1: *In vitro* bond strength of an etch-and-rinse adhesive to sound, caries-affected and caries-infected dentin

ABSTRACT

Objectives: to evaluate the μTBS of an etch-and-rinse adhesive system (Adper Single Bond 2 – SB) to different dentin substrates and water-storage periods.

Methods: 24 dentin surfaces obtained from sound third molars were divided into 3 groups: sound dentin(Sd), caries-affected dentin(Ca), and caries-infected dentin(Ci). Ca and Ci groups were submitted to artificial caries development(broth culture with *S. mutans*). Carious tissue was removed using spherical drills and visual inspection with Caries Detector solution. It was considered as Ci(soft and deeply pigmented dentin) and Ca (hard and slightly pigmented dentin). SB adhesive system was applied and Z350 composite blocks were built. Teeth were stored in deionized water for 24 h at 37º C and sectioned into beams (1.0 mm^2). The specimens were randomized and divided into three water storage periods: 24 hours, 6 months, or 1 year. Specimens were submitted to μTBS using EZ test machine at a crosshead speed of 0.5 mm/min. Failure mode was examined by SEM. Data from μTBS and failure mode were submitted to 2-way ANOVA and Tukey's HSD and Fisher' Exact tests (α=0.05). Bonding interfaces were analyzed by SEM using two additional teeth per group.

Results: The highest μTBS values were verified to Sd and 24h storage. Ci and Ca, 6 months and 1 year presented similar bond strengths. The predominance of mixed and adhesive failures was verified for all groups.

Conclusions: Bonding with Sd showed μTBS values significant higher when compared to Ca or Ci dentin. Storage specimens decreased the μTBS values for all conditions.

Clinical Significance: The bonding to the infected and affected dentin is not suitable procedure compared to sound dentin, due to the poor interaction between adhesive system and altered dentin.

Key-words: sound dentin, caries affected, caries infected, artificial caries, S. *mutans*, adhesive system, microtensile bond strength, storage time.

INTRODUCTION

The current search for aesthetics resulted in an increasing demand from patients for dental treatments using esthetic restorative materials such as composites and ceramics, which are the respective common choice for direct and indirect restorations nowadays. Consequently, the development of dental adhesive systems and the improvement in bonding to tooth tissues have unquestionable importance on this trend.[1,2] Several studies have been performed on bonding to sound non-carious dentin specimens, but it is well-known that the caries-affected dentin is the most frequently substrate found clinically, after cavity preparation.[3-6] The minimally invasive operative management of carious dentin has put into discussion the supposed requirement for complete removal of carious tissues when cleaning and restoring lesions in a tooth.[7,8]

Clinically, caries-infected and caries-affected dentins are very distinct substrates that have different chemical composition and morphological structures.[9]

The caries-infected dentin is a superficial necrotic zone of vastly demineralized substrate,[10] with degenerated collagen fibrils that have lost their cross-linking,[5] besides the presence of bacterial biomass.[11] Conversely, caries-affected dentin is accepted as a variation of reactionary dentin, which is formed in reaction to bland stimuli like caries, presenting small alterations in the cross-linking of its collagen fibrils.[3] Additionally, it contrasts from sound dentin by the existence of mineralized precipitates within the tubules.[4] Investigations established that the caries-affected dentin may be remineralized,[12] making the adhesion to this tissue possible. Therefore, it is recommended to remove only the caries-infected dentin before the bonding procedures involving carious lesions.[11]

The short and long-term bond strengths to caries-infected and caries-affected dentins commonly present lower values for the both substrates, though some of these bond strength values were found to be clinically acceptable.[5,6,13,14] A challenge exists on bonding to caries-affected dentin since the deposition of acid-resistant minerals at the intratubular regions[15] makes this substrate almost impermeable to water,[6] besides the fact that a more porous intertubular dentin become present because of the partial demineralization.[3] While these mineral deposits have the benefit of reducing dentin permeability from harmful agents that can injure the pulp, they may impair the creation of an adequate bonding to dentin.[16] This barrier on bonding procedures is also true for the caries-infected dentin due to the large alterations affecting this substrate. Thereby, concerns still exist regarding the adhesion to these altered dentin substrates in longer periods. Assessments in literature comparing the immediate, short-term (up to 6 months)

and long-term (1 year or more) bond strengths on sound, caries-infected and caries-affected dentins are scarce or inconclusive.[4,17]

The immediate and short-term bonding efficacy of contemporary adhesives has been extensively discussed, but the stability and durability of the resin-dentin interface is dependent upon several factors. High bond strength values can be verified immediately after the bonding procedures. However, these findings are not completely related to the long-term bond stability, since the resin-dentin bonding degradation may initiate in early stages (6 months or less).[18] This might be worsened in bonding to carious substrates after long-term water exposure.[13] The bond strength and durability appear more associated to the hybrid layer quality than other factors.[11]

Procedures and products that improve the quality of bonding, reducing the water infiltration and collagen degradation at the hybrid layer, are of benefit in order to make the resin-dentin interface more stable, even in altered substrates. The removal of the caries-infected dentin layer, leaving the caries-affected dentin lining the cavity with sound enamel margins have been proposed in order to enable good marginal seal and moisture control with the dentin adhesive systems.[6,11] Thus, the association of artificial caries development (broth culture with *S. mutans*) and the application of a water wet-bonding protocol with available adhesive systems on the bond strength to different dentin substrates subjected to storage seem an important topic to be evaluated.

Therefore, the purpose of the present study was to assess the *in vitro* bond strength of an etch-and-rinse 2-step adhesive system to different dentin conditions

(sound, caries-affected and caries-infected dentin) according to storage periods (24 hours, 6 and 1 year). The hypotheses investigated were: 1) the bond strength of the adhesive system will be influenced by the different dentin conditions; 2) the bond strength of the adhesive system to the different dentin conditions will be affected by the storage periods.

MATERIALS AND METHODS

Artificial caries development

Twenty-four sound human third-molars were selected, frozen and used within 3 months following extraction (gathered following informed consent approved by the Committee for Ethics in Research, registration number 041/2010). After, remaining soft tissues were removed using dental hand-scaler and polishing was performed with rubber cup and slurry of pumice. The teeth were mounted in acrylic plates and occlusal enamel was removed using a water-cooled low-speed diamond saw (Isomet 1000, Buehler, Lake Bluff, IL, USA) followed by polishing with 600-grit SiC papers to expose a uniform enamel-free occlusal dentin surface. All surfaces of the teeth submitted to the microbiologic challenge were protected with an acid-resistant varnish layer (Colorama CEIL Ltda., Sao Paulo, SP, Brazil), except for the occlusal dentin surface.

Orthodontic looped wires and silicon hot glue were used to individually fix the teeth to the lid of glass vials immersing them in 40 mL sterile distilled water in order to be sterilized using gamma irradiation (GC-220E, MDS Nordion, Ottawa, Canada) for 32 h at 27ºC with a 14.5 Kgy total radiation dose.[19] The teeth from

groups Ca and Ci were relocated in a sterile second glass vial immersed in 40 mL sterile brain-heart infusion broth (BHI; Difco Laboratories, Detroit, MI, USA) supplemented with yeast extract (Himedia Laboratories, PVT Ltd., Mumbai, India), 0.5% glucose (Synth, LabSynth, Sao Paulo, SP, Brazil), 1.0% sucrose (Synth, LabSynth) and 2.0% *S. mutans* strain (UA159) for development of the artificial carious lesions. The concentration of the bacterial suspension was determined by measuring the absorption at 600 nm (A_{660}) and the adjustment of viable bacteria at A_{660} (colony formation unit – CFU per mL of bacterial suspension) was determined by the optical density of the suspension. Following the inoculation that occurred only in the first day of the experiment, but the culture medium was changed after every 48 h during 14 days.[20] The teeth were divided into three groups (n=8): Sd- sound dentin; Ca- caries affected dentin; and, Ci- caries infected dentin.

Dentin caries differentiation

After the artificial caries development (broth culture with S. mutans), specimens were washed with distilled water and a dye solution (Caries Detector, Kuraray Medical Inc., Okayama, Japan) was used for visual identification of the infected and affected dentin in association to inspection using a sharp explorer.[16] The solution was applied for 10 s over the whole dentin, followed by water rinsing for another 10 s. The dentin was classified in: Sd- sound dentin (hard substrate without dyeing coloration); Ca- caries affected dentin (soft dentin with light-pink coloration); Ci – caries infected dentin (soft dentin with dark-pink coloration). The groups submitted to the microbiologic challenge were assigned to two different

procedures according to the dentin characteristics: Caries infected dentin (Ci) – soft deeply pigmented dentin was remained after microbiology challenge. Caries affected dentin (Ca) - soft pigmented carious tissue was partially removed using spherical carbide burs (n. 8, KG Sorensen, Barueri, SP, Brazil) till hard and slightly pigmented dentin remained.

Previously to bonding procedures, roots were sectioned 1.0 mm bellow the cement-enamel junction using double-sided diamond disc (n. 7020, KG Sorensen) under copious water irrigation. Pulp tissues were removed using excavators and the coronal chambers were etched with 35% phosphoric-acid (Scotchbond Etchant, 3M ESPE, St. Paul, MN, USA) for 30 s, rinsed with water and gently dried with air-spray for 2 s. Adhesive system Adper Single Bond 2 (3M ESPE, St. Paul, MN, USA) was applied according to the manufacturer's instruction and photo activated for 10 s using a light-curing unit XL2500 (3M ESPE, St. Paul, MN, USA) with 700 mW/cm^2. The light power output was checked with digital radiometer Hilux Light Meter (Firt Medica, Greensboro NC, USA). The coronal chamber was filled with composite resin Filtek Z350 (3M ESPE, St. Paul, MN, USA) and photo activated for 40 s using a light-curing unit XL2500 (3M ESPE).

Bonding procedures

For all groups, the dentin surface were etched with 35% phosphoric acid for 15 s (Scotchbond Etchant, 3M ESPE), rinsed with water for 30 s and the water excess was removed with absorbent papers. After, the dentin surface was rehydrated with deionized water using *microbrush* disposable applicators for 60 s,

and the water excess was removed with absorbent papers. The adhesive system Adper Single Bond 2 (3M ESPE) was applied according to the manufacturer's instruction and photo activated for 10 s using a light-curing unit XL2500 (3M ESPE). The adhesive bonded surfaces received composite resin Filtek Z350 (3M ESPE) applied in 2 mm increments and photo activated using a curing unit XL2500 (3M ESPE) for 40 s for each increment to build 6 mm thick block. The restored teeth were stored were stored in distilled water at 37^{o}C for 24 h.

Specimen forming and storage

The specimens were sectioned perpendicular to the bonded area to obtain beams with a transversal bonding area of 1 mm^2 using a water-cooled diamond blade (EXTEC Corporation, Enfield, CT, USA) in a low speed saw machine (Isomet 1000, Buehler, Lake Bluff, IL, USA). Each tooth generated an average of 15 beams, for a total of 120 beams per group. Forty beams per group were designated to three storages time in deionized water at 37ºC for 24 h, 6 months and 1 year. The water was changed every week.

Microtensile bonding test

After storage times, each beam was fixed to the grips of a microtensile device using a cyanoacrylate adhesive (Zapit, Dental Ventures of America Inc., Corona, CA, USA) and the test was conducted in a testing machine (EZ Test, EZS, Shimadzu, Tokyo, Japan) at a crosshead speed of 0.5 mm/min until failure. Bond strength values were calculated in MPa. Data were submitted to two-way ANOVA

and multiple comparisons were performed using the Tukey's post hoc test (SigmaStat, version 3.5.0.54, Systat Software Inc) ($p<0.05$).

Mode of determination failure

The mode of failure the specimen was determined using scanning electron microscopy (LEO 435 VP; LEO Electron Microscopy Ltd., Cambridge, UK) at 100x to 3000x magnifications. The fractured surfaces were classified according to the predominant remaining structure as: Mode 1- adhesive; Mode 2 – cohesive within dentin; Mode 3 – cohesive within composite; and, Mode 4 – mixed, involving bonding agent, composite and/or tooth structure. The results of failure mode classification were submitted to Fisher's Exact test (R, version 2.14.0, The R Foundation for Statistical Computing) ($p<0.05$).

Analysis of the bonding interfaces

Two additional restored teeth (n=2) were vertically sectioned in 2 mm slices (about 8 slices per group), and then embedded in epoxy resin. After each storage times, the slices were wet-polished using 600, 1200 and 2000-grit SiC papers and with decreasingly fine diamond compounds (3 um, 1um, ½ um, ¼ um - Metadi II, Buehler), after each polishing step the specimens were ultrasonically cleaned for 10 min. The specimens were demineralized with 50% H_3PO_4 solution during 5 s, rinsed in distilled water, deproteinezed with 2.5% NaOCl during 10 min. Then, the specimens were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffered solution titrated to pH 7.2 for 72 h and rinsed several times with 0.1 M

sodium cacodylate buffered solution. They were dehydrated in ascending ethanol concentrations (50%, 60%, 70%, 80%, and 90%) for 2 h in each solution and in 100% ethanol for 24 h. The final chemical drying was conducted by immersion in hexamethyldisilazane for 10 min on filter paper inside a covered glass vial with air-drying at room temperature. The specimens were gold-sputtered under high-vacuum environment (MED 010, Balzers Union, Liechtenstein) and analyzed by SEM (LEO 435 VP; LEO Electron Microscopy Ltd., Cambridge, UK) operating on secondary electron mode at 20KV under 1000-5000x magnifications. The profiles of the cross-sections were examined, focusing on the depth of etching, micromechanical entanglement, integrity, homogeneity and continuity along the bonded interface.

RESULTS

Bond strength

The two-way ANOVA showed significant difference for dentin conditions ($p<0.001$) and storage time ($p<0.001$). There was no significant interaction between studied factors ($p=0.875$). The microtensile bond strength (μTBS) and standard deviations for the three dentin conditions and storage time are shown in Table 1.

Table 1 – Mean of μTBS (MPa) and standard deviation (SD) for the different dentin substrates according to the storage time.

Substrate	(0) 24 h	(6) 6 months	(12) 12 months	Mean
(Sd) Sound dentin	48.1 (9.1) ^{A,a}	38.3 (6.9) ^{B,a}	37.3 (8.9) ^{B,a}	**41.2** ^{a}
(Ca) Caries-affected dentin	37.5 (11.3) ^{A,b}	29.0 (9.2) ^{B,b}	30.6 (12.9) ^{B,b}	**32.3** ^{b}
(Ci) Caries-infected dentin	35.6 (12.6) ^{A,b}	24.9 (7.7) ^{B,b}	21.1 (6.9) ^{B,b}	**27.2** ^{b}
Mean	**40.4** ^{A}	**30.7** ^{B}	**29.6** ^{B}	

Means followed by different small letters in column and capital letters in row are significantly different ($p<0.05$).

Regardless of the storage time, the results showed that μTBS (MPa) of Sd was significantly higher in than Ca ($p=0.01$) and Ci ($p<0.001$). No difference was found for Ca and Ci ($p=0.187$). Regardless of the dentin conditions, the storage time 24 hours was significantly higher than that obtained for 6 months ($p=0.004$) and 1 year ($p=0.002$). No difference was found between 6 months and 1 year ($p=0.926$). The highest uTBS were found on sound dentin at 24 hours storage.

Failure mode distribution

The failure mode distribution is shown in Table 2.

Table 2 – Failure mode distribution (%) for the experimental groups.

Experimental Groups		Failure Mode Distribution (%)			
		Adhesive	*Cohesive in dentin*	*Cohesive in composite*	*Mixed*
Sound dentin (Sd) *p=0.001163*	*24 h*	39.5	9.3	2.3	48.9
	6 months	29.8	2.2	25.5	42.5
	1 year	57.8	11.1	11.1	20
Caries-affected dentin (Ca) p=0.01009	*24 h*	40.6	21.7	2.7	35
	6 months	45.7	2.9	22.8	28.6
	1 year	21.8	31.3	3.1	43.8
Caries-infected dentin (Ci) p=0.001118	*24 h*	29.3	19.5	-	51.2
	6 months	40.5	27.1	13.5	18.9
	1 year	35.2	18.9	-	45.9

The predominance of mixed and adhesive failures was verified for all groups. The Fisher's Exact Test for the failure modes within each group showed a significant association between the substrate condition and storage time (two side for all groups, $p<0.01$). The most representative micrographs of failure modes are showed in Figure 1.

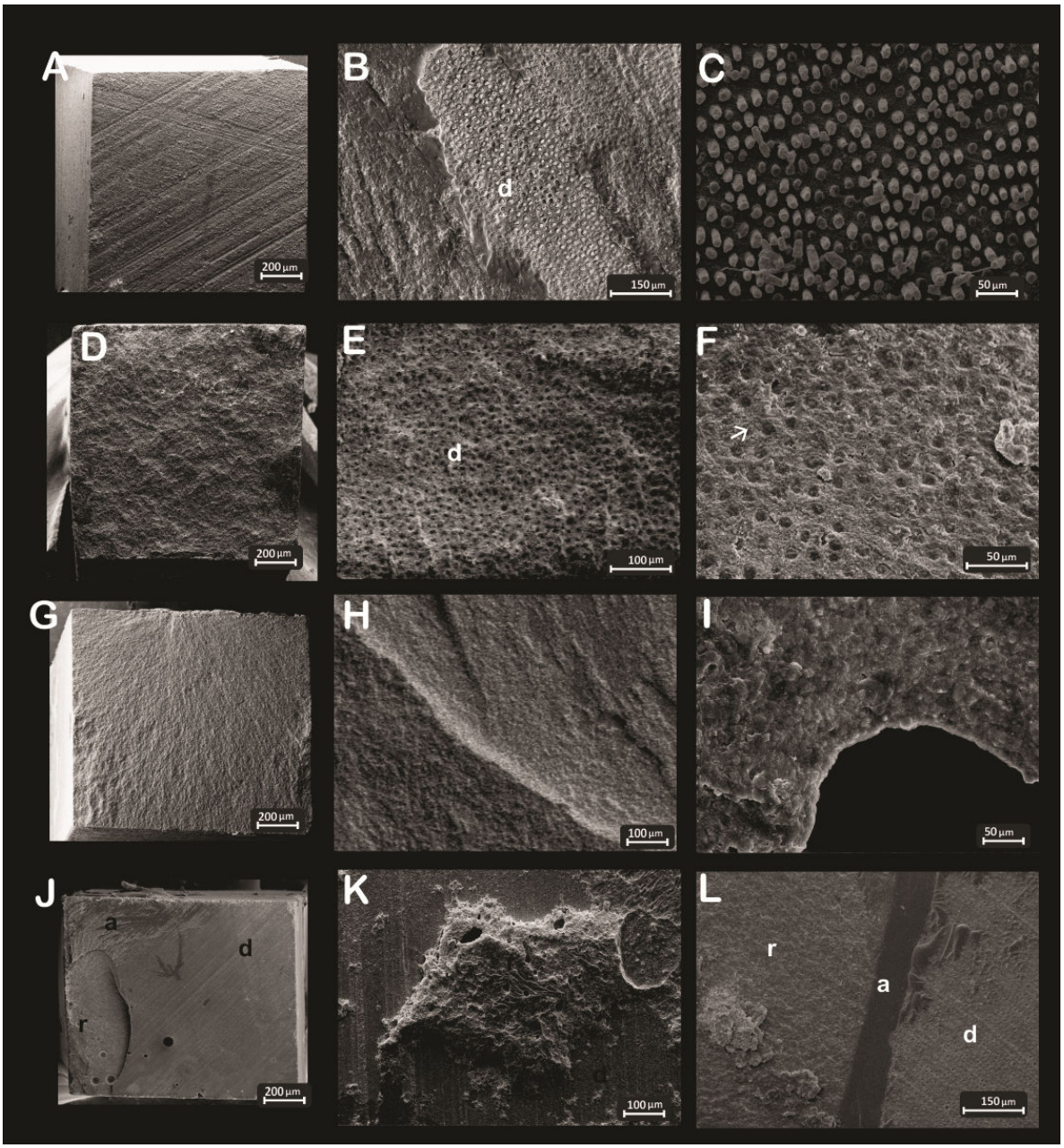

Figure 1: A- Adhesive failure; B – Magnification showing the adhesive detachment from dentin (d); C – Magnification showing the detached resin tags; D – Cohesive failure in dentin; E and F – Higher magnifications showing the dentin fractured surfaces (arrow in F); G – Cohesive failure at the resin-based composite; H – aspect of an internal flaw located at the fractured composite surface; I – aspect of composite fractured surface; J – Mixed failures comprehending the materials of the bonded interfaces showing resin-based composite I, adhesive (a) and dentin (d); K – mixed failure showing the three materials; L – magnification of a mixed failure showing the materials of the bonded interface, resin-based composite I, adhesive (a) and dentin (d) (Magnifications ranged 100X – 3000X).

Bonding interfaces

The SEM characterization of the bonding interfaces for the dentin substrates and storage periods is presented in Figure 2. The water wet-bonding protocol applied for all experimental groups resulted in hybrid layers presenting continuous and homogenous interfaces for the sound dentin (Sd) after 24 h storage (Fig. 2A). After 6 months and 1 year storage observed tag formation and conventional hybrid layer conformation and highly decrease of microtag branches suggesting slightly degradation of the hl were detected (Fig. 2B; 2C). The caries-affected dentin group (Ca) hl presented low resin tags formation after the 24 h storage (Fig. 2D). After 6 months storage hl presenting shorter penetration of tags into dentin were verified (Fig. 2E), and extensive changes were seen after 1 year storage, suggesting increased hl degradation (Fig. 2F). The caries-infected dentin group (Ci) exhibited hl conformation with abnormal tags with gaps at the interface (Fig. 2G). After 6 months storage, hl showing disrupted interface and unusual dentin aspect (Fig. 2H), and after 1 year storage, increased hl degradation showing altered dentin aspect, with some failures at bottom and top of hl and presence of some small and sparse tags were identified (Fig. 2I).

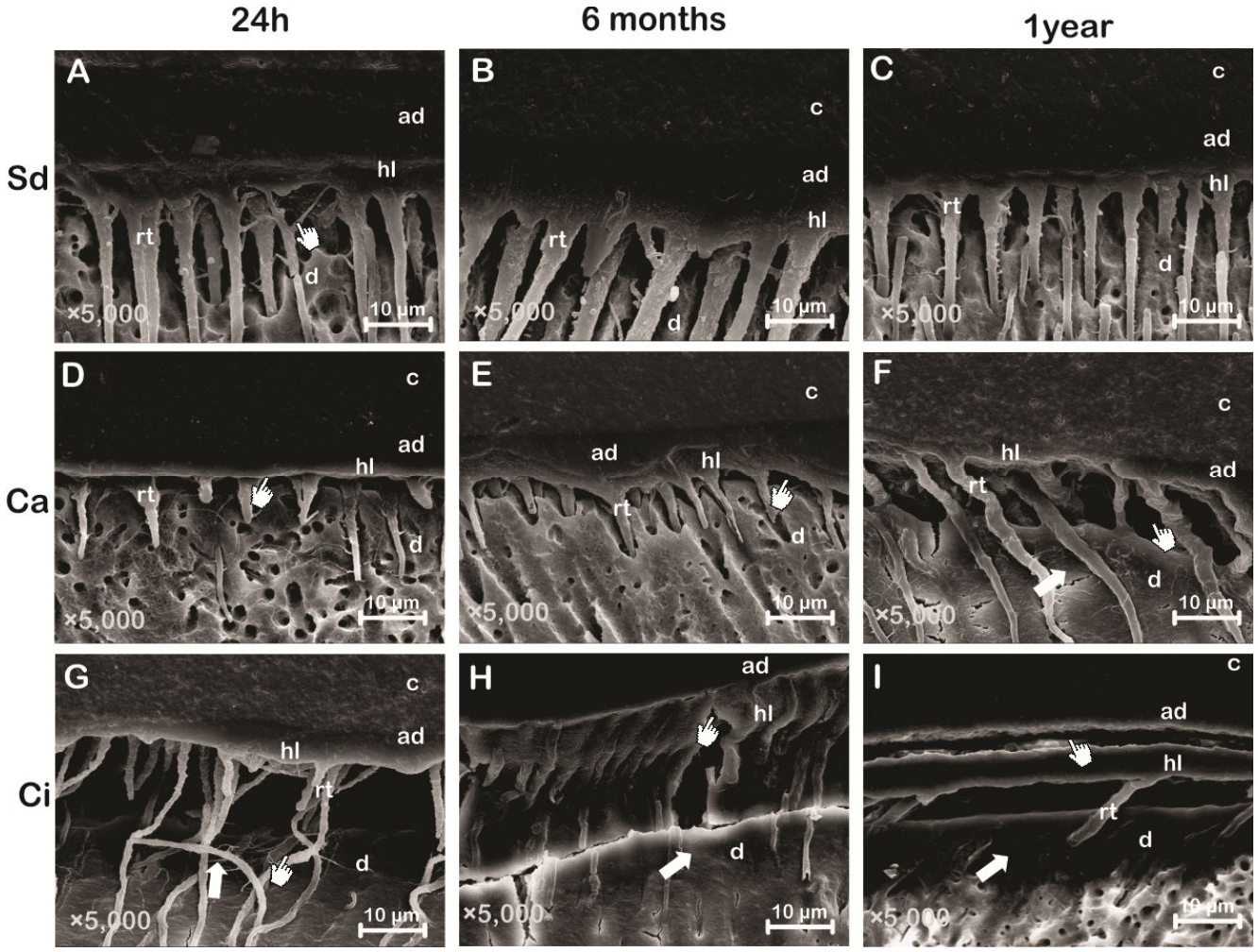

Figure 2: A – (Sd 24 h) Conventional hybrid layer (hl) conformation, presenting continuous and homogenous bonding interface; lateral extensions of microtags branching off at angles from the main resin tags (rt) are visible (pointer); B – (Sd 6 months) tag formation and conventional hl conformation; highly decrease of microtag branches suggesting slightly degradation of the hl over time; C – (Sd 1 year) tag formation and conventional hl conformation; highly decrease of microtag branches suggesting slightly degradation of the hl over time; D – (Ca 24h) hl (pointer) formation and low resin tags formation - unfilled spaces at the bottom of hl ; E – (Ca 6 months) hl formation with tags presenting shorter penetration into dentin; F – (Ca 1 year) increased disruptive areas at the interface (pointer) and a abnormal dentin aspect (arrow); G – (Ci 24 h) gaps at the interface suggesting dentin degradation by microorganism activity during caries producing (arrow) and abnormal tag aspect (pointer); H – (Ci 6 months) hl showing disrupted interface (pointer) and unusual dentin aspect (arrow); I – (Ci 1 year) increased degradation of the hl with some failures at bottom (arrow) and top of hl (pointer) and presence of some small and sparse tags. Arrows also shows altered dentin aspect, suggesting dentin degradation by microorganism activity during caries producing. c: composite; ad: adhesive; hl: hybrid layer; rt: resin tag; and, d: dentin.

DISCUSSION

The first hypothesis that the bond strength of the adhesive system would be influenced by the different to dentin conditions was fully accepted, since the sound dentin group (Sd) presented the highest bond strengths. However, the bond strengths obtained with the same bonding protocol for the caries-affected (Ca) and caries-infected (Ci) groups were statistically similar (Table 1).

The mineral and organic changes that occur in caries-affected dentin make the adhesion more complex than to sound dentin,[17] being associated to lower bond strengths for the contemporary adhesive systems. The present findings are in accordance to other investigations indicating that bonding to caries-affected dentin results in lower bond strengths as related to sound dentin,[11,21,22] even when using etch-and-rinse systems.[6,13,23] These results are attributed to the weaker structure presented by demineralized caries-affected dentin that limits adhesive infiltration due to the tubules filled with acid-resistant mineral deposits,[3,6,15] and to the unusual conformation of the hybrid layer, which is commonly thicker[16]. In addition, the present study showed low resin tags formation with short penetration into demineralized dentin regions (Figs. 2D-2F).[24] Moreover, the increasing water content and the more permeable condition of caries-affected dentin are concerns that may compromise bonding quality/stability over time when using hydrophilic adhesives systems.[13]

Regardless of the adhesive approach,[24] lower bond strengths were already expected for caries-infected dentin,[22] since the hypomineralized dentin is even more porous, with lower mechanical properties and pronounced water content,[25]

besides the presence of bacterial biomass.[26] Caries-infected dentin is also presumed to present almost complete loss of the mineral phase of dentin with a small number of larger apatite crystals and denaturation of the collagen matrix.[6] On this way, bonding can be hampered on this dentin, because fewer larger crystals offer surface area for interacting with the adhesive system. Additionally, it was assumed that the chemical link between the adhesive systems and dentin collagen may contribute for bonding to sound and caries-affected dentin if the last has normal collagen, but it cannot takes place at the denatured matrix of caries-infected dentin.[6] The adhesive infiltration into dentinal tubules may be also complicated by the presence of acid-resistant minerals within dentin tubules. Despite all these facts, no significant differences were verified between the bond strength to the caries-affected or caries-infected dentin. Probably, in this study there was not any difference between Ca and Ci due in vitro challenge, since caries lesions produced in vivo showed acid-resistant mineral deposits.

The second hypothesis that the bond strength of the adhesive system to the different dentin conditions would be affected by the storage periods was also fully accepted. Similar bond strengths were verified for the specimens stored for 6 or 12 months, irrespective of the dentin substrate. However, lower bond strength values were observed for these both storage periods compared to those obtained with the specimens tested immediately (24 h), for all dentin conditions. A great number of adhesive failures were verified for the Sd specimens after 1 year storage period, and the specimens from Ca and Ci groups after 6 months. An increased number of cohesive failures in dentin was observed for the groups Ca

group after 1 year and for the Ci after 6 months. This point may be related to the association of long-term storage and the lower mechanical properties of the dentin altered by caries (Ca and Ci),[26] which probably fractured when the bonding strength has exceeded the cohesive strength of dentin.[6] The adhesive failures observed for the Ca group may also be related to issues with the collagen fibrils partially denatured,[5] and/or to the incomplete infiltration of the adhesive into the demineralized dentin due to the mineral deposits present at the tubules.[3]

The particularly low cohesive strength of the caries-infected dentin due to its low degree of mineralization and disorganization of the collagen-matrix,[16] probably led to the increased cohesive failures observed in dentin. While also resulting in thicker hybrid layers similarly to caries-affected dentin,[22] only superficial monomer infiltration is achieved in this dentin; thus, several dentin tubules remain small and sparces tag formation (Figs. 2G-2I). However, despite the lower bond strengths attained for the caries-affected and caries-infected using an adhesive system with water wet-bonding when compared to sound dentin, the intrinsic weakness of this dentin may not be a problem if enamel and/or sound dentin exist adjacent to the excavated regions of caries altered dentin, as clinical acceptable bond strengths can be delivered with current adhesive systems.[6,11]

The ethanol/water-based adhesives are commonly less sensitive to the different conditions existing in dentin than other etch-and-rinse agents due to the presence of water and the reduced amount of solvents in these systems.[27] In addition, it was shown that the etch-and-rinse 2-step adhesive system used in the present study has polyalkenoic acid copolymers with high molecular weight among

its components, which keep the dimethacrylate monomers at the superficial region of the hybrid layer.[23] Thus, satisfactory bond strengths may be obtained if the components with lower molecular weight such as HEMA can penetrate through all the demineralized dentin and interact with the available collagen fibrils. The bond strengths to caries-affected dentin were shown to become lower when these polyalkenoic acid copolymers are not present in etch-and-rinse bonding agents.[28] Also, the formulation of simplified etch-and-rinse adhesives blends, with solvents and hydrophilic components placed together with hydrophobic monomers in a single bottle yields a hydrophilic polymer after the polymerization step, which behaves similar to a semi-permeable membrane.[29] Consequently, bonding to caries altered dentin which have marked water content becomes even more concerning.

Efficient adhesive systems and polymeric restorative materials have unquestionable significance for the minimally invasive dentistry, which intends to preserve the largest amount of remaining tooth tissues after removing the carious lesions[7] in order to provide better resistance to restored teeth. To assure long-term success to restorations placed on sound dentin or caries altered dentin, the use of adhesive systems with improved marginal sealing capability and stable adhesion to tooth over time is critical.[23] The etch-and-rinse adhesive systems have been shown to attain better bonding to caries-affected and caries-infected dentin as compared to self-etching systems.[6,14,30,31] However, this trend is not absolute when bonding to sound dentin, since bonding to any dentin can be strongly affected by the chemical composition of adhesives. Even though bond strength to dentin altered by

caries is lower than to sound dentin, bonding to both dentins is continuously being implemented and have currently attained relatively high levels.[31]

Although the similar bond strengths verified to caries-affected and caries-infected dentins with the etch-and-rinse agent used, bonding to the last dentin is rarely indicated due to the poor adhesive interaction that occurs. One exception for bonding to caries-infected dentin would occur during periods for adjusting the behavior of uncooperative patients aiming to prevent additional caries progression.[31] A limited number of studies assessing the performance of contemporary adhesive systems on bonding to caries-affected and caries-infected dentins are available at present, and future long-term clinical trials evaluating the restorative parameters studied in the present investigation would be of benefit.

CONCLUSIONS

Within the conditions of this *in vitro* study, the following conclusions can be drawn:

1. The bonding with sound dentin showed μTBS values significant higher when compared to caries-affected and caries-infected dentin.
2. The 6 months and 1 year storage periods resulted in decreased bond strengths for all dentin conditions.

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CAPÍTULO 2: *Long-term evaluation of ethanol wet-bonding to different dentin substrates*

Clinical relevance: The ethanol wet-bonding with 100% ethanol after etching in sound dentin is not a recommended procedure, but in caries-affected dentin can be acceptable, although, degradation may occur over time for all conditions.

ABSTRACT

Objectives: The aim of this study was to evaluate the effect of ethanol wet-bonding on the degradation of the adhesive system to different dentin substrates according to storage time using the microtensile bond strength(μTBS) and analysis of interface bonding(SEM). *Methods & materials:* 48 dentin surfaces obtained from sound third molars were divided into 3 groups: Sound dentin(Sd), Caries-infected dentin(Ci), and Caries-affected dentin(Ca). Ca and Ci groups were submitted to artificial caries development(broth culture with *S. mutans*). Carious tissue was removed using round burs and visual inspection with Caries Detector solution. It was considered as Ci(soft and deeply pigmented dentin) and Ca (hard and slightly pigmented dentin). Teeth were then assigned to 2 subgroups: control – water wet-bonding(Ct), and ethanol – ethanol wet-bonding 100%(Et). The ethanol was applied, just after dentin etching with 35% phosphoric acid gel. After, the dentin surface was bonded with Single Bond adhesive system and Z350 composite blocks were built. Teeth were stored in deionized water for 24 h at 37ºC and longitudinally sectioned across the bonded interface to produce beams (1.0 mm^2). The specimens were randomized into three storage periods:

24 hours, 6 months, and 1 year. Then, they were submitted to μTBS using EZ test machine at a crosshead speed of 0.5 mm/min. The data was submitted to 3-way ANOVA and Tukey's HSD test(α=0.05). *Results:* The Ct showed higher bond strength for Sd and Ci dentin compared to Et, except for Ca dentin, which presented similar values. The specimens stored for 6 months or 1 year showed lower bond strengths and the Et group resulted in preservation of the Ci and Ca hybrid layers after 6 months storage. *Conclusions:* The Et group approach was not effective in producing improved bond strengths to the three dentin substrates evaluated.

Key-words: 100% ethanol, dentin, S. *mutans*, adhesive system, microtensile bond strength, s*torage time*

INTRODUCTION

The minimally invasive approach for restorative dentistry intends to preserve the largest amount of remaining tooth tissues after removing the carious lesions. Hence, the development and use of adhesive systems and polymeric restorative materials have unquestionable importance on this direction.[1,2] Some studies advocated that only the bacteria infected dentin layer (caries-infected dentin) should be removed in carious lesions, since the inner region, the caries-affected dentin, might be remineralized.[3]

It is well established that short and long-term bond strength to caries-infected and –affected dentin result in lower values for the both substrates, although these bond strength values can be clinically acceptable.[4,5] However,

some concerns still remain regarding the adhesion to these dentinal substrates in longer periods. Comparisons between immediate, short-term (6 months) and long-term (1 year) bond strengths in sound dentin, caries infected dentin, and caries affected dentin are still scarce in literature. Additionally, studies evaluating the effects of microbiologic challenges (broth culture with *S. mutans*) and application of ethanol wet-bonding (Et) on the bond strength to these dentinal substrates are also lacking.

Et proposes the surface treatment of the acid etched dentin using solutions with increasing ethanol concentration instead using water,[6-10] and was shown to obtain significant higher bond strength values.[11] The ethanol acts removing the water present at the interfibrillar and intrafibrillar spaces,[12] promoting hydrogel collapse and diminution on the diameter of the collagen fibrils,[7,10,13] what leads to consequent reduction in the matrix volume and expansion of the interfibrillar spaces, thus, allowing better infiltration of the adhesive. This approach can prevent phase separation of adhesive systems containing hydrophobic resin monomers caused by water,[14] since ethanol replaces completely the water prior to the application of the adhesive.[15] The substitution of water by ethanol from the collagen also removes the hydrolytic medium necessary for the functioning of the collagen-bound matrix metalloproteinases (MMPs), responsible for the degradation of exposed collagen fibrils within the hybrid layer.[13] The increased bond strength and durability observed for this technique are also attributed to the better impregnation and sealing of the collagen matrix, minimizing the endogenous collagenolytic

activity.[16] In addition, the ethanol wet-bonding allows using more hydrophobic adhesive systems, since the ethanol-saturated dentin becomes less hydrophilic.[13] Although this technique looks promising,[7,10,13] more evidence is needed, mainly in caries-affected/infected dentin, which are the substrates most frequently found in clinical practice.

The high bond strength values verified immediately after the bonding procedures are not related to the long-term bond stability, since the resin/dentin bonding degradation starts rapidly, namely 6 months or less.[17] The bond strength and its durability seem to be more associated to the hybrid layer quality. In other words, these factors are more dependent of an adequate impregnation of the dentin substrate by the adhesive system than the hybrid layer thickness or its morphology.[18] The co-monomers used in most adhesive systems have hydrophilic characteristics, absorbing up to 5-12% of water,[19] what can lead to matrix plasticizing with lixiviation of unreacted monomers, resulting in decreased mechanical properties. Thus, these layers become even more permeable and consequently more susceptible to hydrolysis, and as the infiltration of the etched dentin by the adhesive is commonly incomplete, the dentin substrate and the adhesive layer may also be degraded by the surrounding water present in this region.[17] Another important degradation mechanism can occur following the acid etching of the dentin, by exposing and activating the metalloproteinase matrix (MMPs) present in this tissue, which may lead to slow cleavage of the collagen peptides.[20,21] Some alternative approaches to improve bond strength\stability have been suggested such as the application of an hydrophobic resin layer

coating,[22] application of multiple adhesive layers,[23] additional solvent evaporation,[24] extended curing,[25] adhesive impregnation using electric current,[26] use of metalloproteinase inhibitors.[27] Although, this technique looks promising,[7,10,13] more evidences are needed, mainly in caries-affected/infected dentin, which are the substrates most frequently found in clinical practice.

On this way, the aim of this study was to evaluate the effect of ethanol wet-bonding using 100% ethanol (Et) on the degradation of the 2-step adhesive system to different dentin substrates (sound, caries-infected and caries-affected) according to storage time (24 hours, 6 and 1 year). The hypothesis investigated in the present study were that (1) the dentin surface treatment with 100% ethanol will improve the bond strength to the three dentin substrate condition; and (2), the dentin surface treatment with ethanol will improve the bond strength to these substrates after storage time.

METHODS & MATERIALS

Sterilization and artificial caries development

Forty-eight sound human molars extracted for clinical purposes were selected, cleansed and stored in 0.1% thymol for 1 month and then stored in distilled water at 4^{o}C until use (gathered following informed consent approved by the Research Ethics Committee, registration number 041/2010). The tooth was mounted in acrylic plates using low-fusion impression compound (Exata, DFL, Jacarepaguá, RJ, Brazil) and the occlusal enamel was sectioned perpendicular to tooth long axis using a water-cooled low-speed diamond saw (Isomet 1000,

Buehler, Lake Bluff, IL, USA) to expose the dentin. Dentin surface was wet ground flat with 180- and 600-grit SiC papers to expose a flat occlusal enamel-free dentin surface. Forty-eight teeth were randomly divided into three groups (n=16): Sd- Sound dentin; Ci- caries-infected dentin; Ca- caries-affected dentin. Then again subdivided into two groups (n=8) according to the surface treatment (100% ethanol application), or no treatment (control). An acid-resistant varnish layer was applied over the entire tooth (Colorama CEIL Ltda., Sao Paulo, SP, Brazil), except at the flat dentin surface, which was posteriorly submitted to the microbiologic challenge for the groups Ci and Ca.

Thirty-two sound molars were individually fixed with orthodontic wire using hot glue on the lid of glass vials containing 40 mL of sterile distilled water and immediately sterilized using gamma irradiation (GC-220E, MDS Nordion, Ottawa, Canada) for 32 h at 27ºC, with a total radiation dose of 14.5 Kgy.[28] After, the teeth were transferred to another sterile glass vial containing 40 mL of sterile brain-heart infusion (BHI) broth (#21152, Becton Dickinson and Company, Spasks, MD, USA) supplemented with yeast extract (Himedia Laboratories, PVT Ltd., Mumbai, India), containing 0.5% glucose (Synth, LabSynth, São Paulo, SP, Brazil), 1.0% sucrose (Synth, LabSynth) and 2.0% *S. mutans* (UA159) for development of the artificial carious lesions. The concentration of the bacterial suspension was determined by measuring the absorption at 600 nm (A660) and the adjustment of the number of viable bacteria to A660, the number of colony-forming units per milliliter of bacterial suspension (cfu/mL) was determined with the use of standard spreading techniques at optical densities. For the

microbiologic challenge, inoculation occurred only in the first day of the experiment, but the broth was renewed every 48 h during 14 days.[29]

Differentiation of the dentin substrates

The detection of the caries-infected and caries-affected dentin was performed using a dye solution (Caries Detector, Kuraray Med. Inc., Okayama, Japan) for identification of those tissues associated to inspection criterion using dental explorers and visual examination.[30] The respective caries-removing endpoints for the experimental groups were: Caries-infected dentin (Ci) – soft deeply pigmented dentin was maintained. Caries-affected dentin (Ca) - soft pigmented carious tissue was removed till hard and slightly pigmented dentin remained. The Ca dentin was removed using a #8 spherical carbide bur (KG Sorensen, Barueri, SP, Brazil) mounted on a slow-speed hand piece. Before the bonding procedures, the root of specimen was sectioned 1.0 mm bellow the cementum-enamel junction using double-face diamond disc (KG Sorensen, Barueri, SP, Brazil) mounted in low-speed handpiece and the pulp tissues were removed using excavators. The coronal chambers were etched with 35% phosphoric-acid (Scotchbond Etchant, 3M ESPE, St. Paul, MN, USA) for 15 s, rinsed with water for 30 s, gently dried with air-spray for 2 s and hybridized using Adper Single Bond 2 (3M ESPE, St. Paulo, MN, USA). The coronal chamber was filled with composite resin Z350 (3M ESPESt. Paul, MN, USA) and photo activated for 40 s by a light-curing unit XL2500 (3M ESPE, St. Paul, MN, USA)

with 700 mW/cm^2, previously checked with digital radiometer Hilux Light Meter (First Medica, Greensboro NC, USA).

Bonding procedures

- Control – water wet-bonding (Ct) groups. The flat dentin surfaces were etched with 35% phosphoric acid for 15 s (Scotchbond Etchant, 3M ESPE), rinsed with water for 30 s and the water excess was removed with absorbent paper. After, the dentin was rehydrated with deionized water using a microbrush for 60 s, and the water excess was removed again with absorbent paper. Then, the adhesive system Adper Single Bond 2 (3M ESPE) was applied according to the manufacturer's instructions and photo activated for 10 s by a light-curing unit XL2500 (3M ESPE).

- Ethanol – wet-bonding with 100% ethanol (Et) groups. The flat dentin surfaces were etched with 35% phosphoric acid for 15 s (Scotchbond Etchant, 3M ESPE), rinsed with water for 30 s and the water excess was removed with absorbent paper. After, the dentin was rehydrated with 1 mL of 100% ethanol using a microbrush for 60 s and the excess was gently air-dried. Then, the adhesive system Adper Single Bond 2 (3M ESPE) was applied according to the manufacturer's instructions and photo activated for 10 s by a light-curing unit XL2500 (3M ESPE).

For both groups, the bonded surfaces were coupled with a composite Filtek Z350 (3M ESPE), applied in 2 mm increments and photo activated using a

curing unit XL 2500 (3M ESPE) for 40 s for each increment and till 6 mm thick block. The samples were stored in distilled water at 37^{o} C for 24 h.

Microtensile bonding strength testing

The specimens were sectioned perpendicular to the bonding interface area to obtain beams with a bonding area of 1 mm^2 using a water-cooled diamond blade (EXTEC Corporation, Enfield, CT, USA) in a low speed saw machine (Isomet 1000, Buehler, Lake Bluff, IL, USA). Each group contained eight teeth, and each tooth generated an average of 15 beams, for a total of 120 beams per group. Forty beams per group were designated to three storages time in deionized water at 37ºC for 24 h, 6 months, and 1 year. After, storage times, each beam was fixed to the grips of a microtensile device using a cyanoacrylate adhesive (Zapit, Dental Ventures of America Inc., Corona, CA, USA) and the microtensile bond test was conducted in a testing machine (EZ Test, EZS, Shimadzu, Tokyo, Japan) at a crosshead speed of 0.5 mm/min until failure.

Bond strength values were calculated in MPa. Data were submitted to three-way ANOVA and multiple comparisons were performed using the Tukey's post hoc test (SigmaStat, version 3.5.0.54, Systat Software Inc) ($p<0.05$).

Failure analysis

The fractured specimens were observed using scanning electron microscopy (SEM) (LEO 435 VP; LEO Electron Microscopy Ltd., Cambridge, UK) at 100x to 3000x magnification. Then, the fractured surfaces on the specimens

were classified according to the predominant remaining structure upon its surface following the described failure mode: adhesive (Mode 1); cohesive within dentin (Mode 2), cohesive within composite (Mode 3); and mixed, involving bonding agent, composite and/or tooth structure (Mode 4). The results of failure mode classification were submitted to Fisher's Exact test (R, version 2.14.0, The R Foundation for Statistical Computing) ($p<0.05$).

Analysis of the bonding interfaces

Two additional restored teeth (n=2) were vertically sectioned in 2 mm slices (about 8 slices per group), were then embedded in epoxy resin (Buehler). After each storage time, the slices were wet-polished using 600, 1200 and 2000-grit SiC papers and with decreasingly fine diamond compounds (3 um, 1um, ½ um, ¼ um - Metadi II, Buehler), after each polishing step the specimens were ultrasonically washed for 10 min. The specimens were demineralized with 50% H_3PO_4 solution during 5 s, rinsed in distilled water, deproteinezed with 2.5% NaOCl during 1 minute. The samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffered solution titrated to pH 7.2 for 72 h and then rinsed several times with 0.1 M sodium cacodylate buffered solution. They were dehydrated in ascending ethanol concentrations (50%, 60%, 70%, 80%, and 90%) for 2 h in each solution and in 100% ethanol for 24 h. The final chemical drying was conducted by immersion in hexamethyldisilazane for 10 min on filter paper inside a covered glass vial and air-drying at room temperature. The specimens were gold-sputtered under high-vacuum ambient (MED 010, Balzers Union, Balzers,

Liechtenstein) and analyzed by SEM (LEO 435 VP) operating on secondary electron mode under 1000-5000X magnification. The cross-section profiles were examined, focusing on the depth of etching, micromechanical entanglement, integrity, homogeneity and continuity along the bonded interface.

RESULTS

Bond strength

The results for the μTBS are shown in Table 1. The triple interaction between factors was not significant (p=0.994). Results of the three-way ANOVA showed significant interaction only for 'Treatment' x 'Substrate' (p=0.046). 'Storage' x Treatment '(p=0.065), 'Storage' x 'Substrate' (p=0.412) did not showed significant interactions between factors. There was significant difference for 'Storage' (p<0.001). The μTBS means (in MPa) after 24 hours was significantly higher (p<0.001) than those obtained for 6 months and 1 year. No difference was found between storage for 6 months and 1 year (p=0.98).

Sound dentin in the control group showed higher μTBS (MPa) values than Ca (p=0.017) and Ci (p<0.001). Within the ethanol group, Ci showed lower μTBS (MPa) values than Ca and Sd. The application of ethanol after acid etching significantly decrease the μTBS for Ci and Sd, but had no significant influence for Ca (p>0.05).

Table 1 - Mean μTBS (MPa) and standard deviation (SD) of dentin substrates according to the storage time.

Substrate	Water wet-bonding (Ct)			Ethanol wet-bonding (Et)			Storage time
Sound dentin (Sd)	41.2 (12.5)	a	A	26.7 (7.8)	a	B	**24h (34.4)**
Caries-affected dentin (Ca)	33.0 (11.4)	b	A	28.4 (6.3)	a	A	**6 months (26.6) ***
Caries-infected dentin (Ci)	26.5 (11.7)	b	A	21.2(8.0)	b	B	**1 year (25.9) ***

Means followed by different small letters in column and capital letters in row are significantly different ($p<0.05$). * Different from 24 h μTBS (MPa), regardless of the other factors.

Failure mode

The failure modes are showed in Table 2 and 3. The predominance of adhesive and mixed failures was verified for all groups. When Ethanol was applied, cohesive failures in dentin decreased. The occurrence of cohesive failures in composite was more evident in stored samples. The Fisher's Exact Test for the failure modes within each group showed a significant association between the 'Substrate' and 'Treatment' related to the 'Storage' (two sided for all groups ($p<0.01$)), using or not ethanol.

Table 2 - Failure mode distribution (%) in water wet-bonding groups (Ct).

Water wet-bonding (Ct)		**Failure Mode Distribution**			
		Adhesive	*Cohesive in dentin*	*Cohesive in composite*	*Mixed*
Sound dentin (Sd)	*24 h*	38.1	7.52	4.76	47.62
	6 months	23.4	2.13	27.66	46.81
p=0.001163	*1 year*	51.12	13.33	13.33	22.22
Caries-infected dentin (Ci)	*24 h*	29.27	24.39	-	46.34
	6 months	37.84	24.33	16.21	21.62
p=0.001118	*1 year*	29.73	18.92	2.7	48.65
Caries-affected dentin (Ca)	*24 h*	40.54	21.62	-	37.84
	6 months	44.11	5.88	23.53	26.48
p=0.01009	*1 year*	19.36	29.03	6.45	45.16

Table 3 - Failure mode distribution (%) in ethanol wet-bonding groups (Et).

Ethanol wet-bonding (Et)		**Failure Mode Distribution**			
		Adhesive	*Cohesive in dentin*	*Cohesive in composite*	*Mixed*
Sound dentin (Sd)	*24 h*	52.78	-	-	47.22
	6 months	55.26	15.79	5.26	23.69
p=0.004257	*1 year*	76.32	5.26	2.63	15.79
Caries-infected dentin (Ci)	*24 h*	34.88	11.64	6.94	46.51
	6 months	44.74	21.05	-	34.21
p=0.0122	*1 year*	60	-	8.57	31.43
Caries-affected dentin (Ca)	*24 h*	38.3	8.51	-	53.19
	6 months	53.5	2.32	4.64	39.54
p=0.01282	*1 year*	45.65	-	6.52	47.83

The most representative micrographs of failure modes are show in Figure 1.

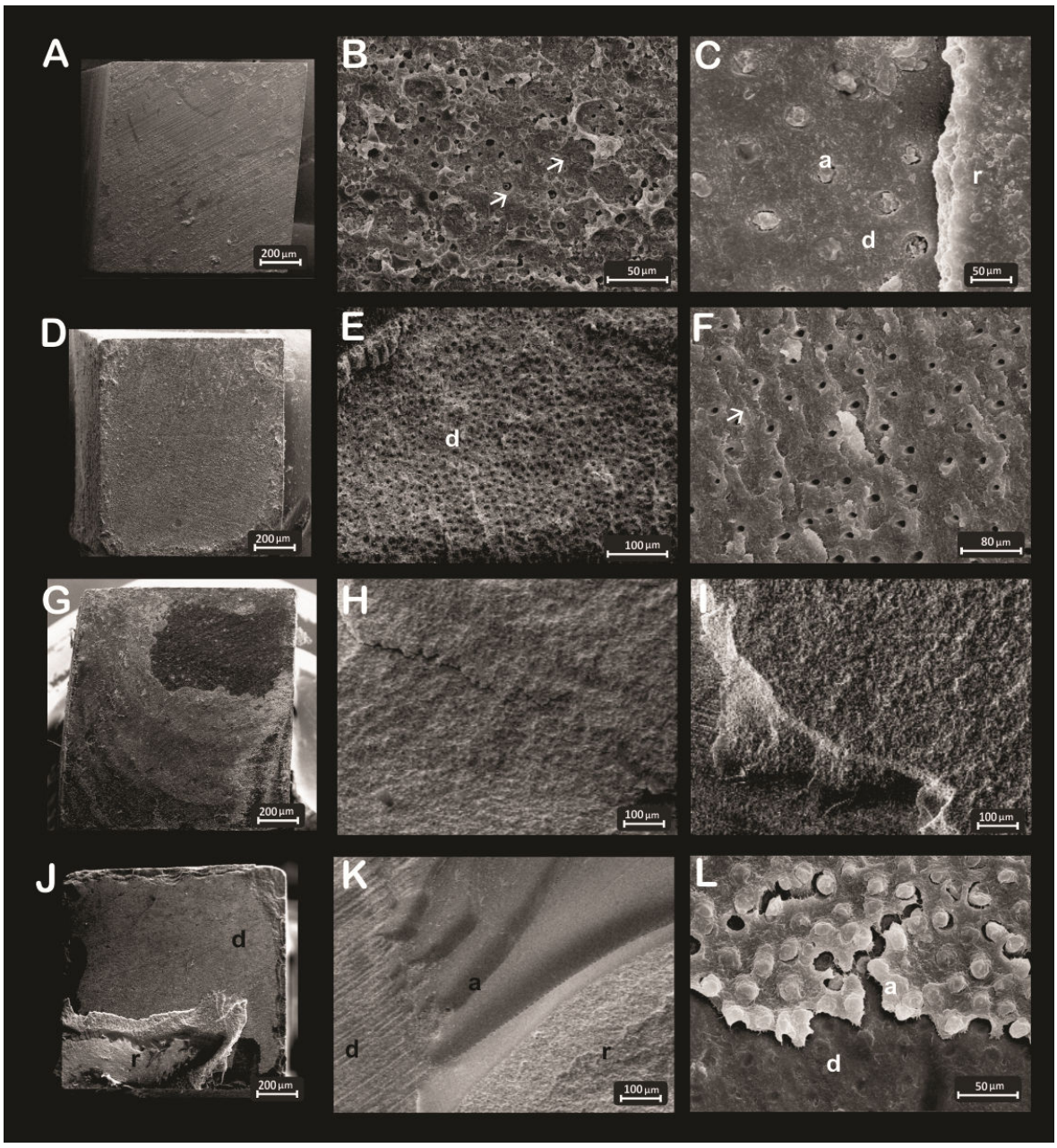

Figure 1: A – Adhesive failure; B – Magnification showing the adhesive detachment from dentin (arrows); C – Magnification showing the dentin (d), dentin tubules filled with adhesive (a), and some areas of detachment from the resin-based composite (r); D – Cohesive failure in dentin; E and F – Higher magnifications showing the dentin fractured surfaces (arrows in F); G – Cohesive failure at the resin-based composite; H – aspect of an internal flaw located at the fractured composite surface; I - aspect of composite fractured surface; J – Mixed failures comprehending the materials of the bonded interfaces; K – Mixed failures showing resin-based composite (r), adhesive system (a) and dentin (d); L – Other magnification of a mixed failure showing the materials of the bonded interface, adhesive system (a) and dentin (d). (Magnifications ranged 100X – 3000X).

Bonding interfaces

The SEM characterization of the bonding interfaces is presented in Figures 2, 3 and 4. For the sound dentin (Sd) the water wet-bonding (Ct) resulted in hybrid layers presenting continuous and homogenous interfaces after 24 h (Fig. 2A). After 6 months storage, some irregularities suggesting beginning of hybrid layer degradation were verified (Fig. 2B), and after 1 year storage, extensive alterations suggesting increased degradation at the dentinal substrate were detected (Fig. 2C). The ethanol wet-bonding (Et) also produced hybrid layers with homogenous and continuous interfaces for the Sd after 24 h (Fig. 2D). Unusual aspect of the resin tags were observed after 6 months storage (Fig. 2E), and disruptive failures on the top of hybrid layer were present after 1 year, suggesting highly degradation of the hybrid layer over time (Fig. 2F).

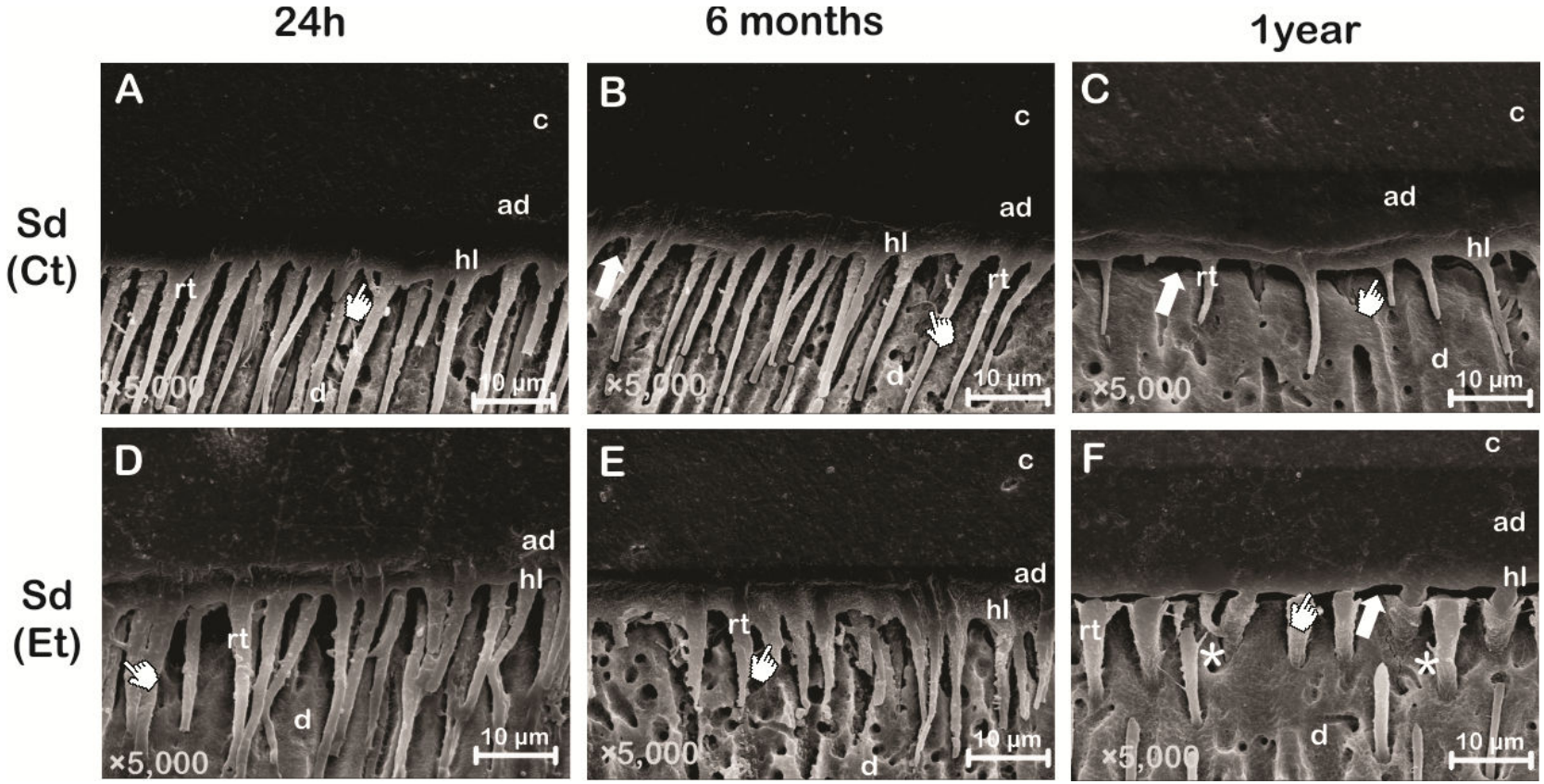

Figure 2: Sound Dentin (Sd) - A (Ct 24 h) – Hybrid layer (hl) after water wet-bonding presenting a continuous and homogenous interface; small lateral extensions of microtags branching off at angles from the main resin tags are visible (pointer); B (Ct 6 months) – slightly irregularities (black areas under hl – arrow) suggesting possible initiation of hl degradation; highly decrease of microtag branches suggesting slightly degradation of the hl over time (pointer); C (Ct 1 year) - extensive alterations suggesting increased degradation at the dentinal substrate (arrows), small and sparse resin tags (pointer); absence of microtag branches suggesting highly degradation of the hl over time; D (Et 24 h) - hl after ethanol wet-bonding presenting a continuous and homogenous interface; small lateral extensions of microtags branching off at angles from the main resin tags are visible (pointer); E (Et 6 months) – unusual aspect of the resin tags (pointer) and shallow penetration; F (Et 1 year) - disruptive failure on the bottom of hl (arrow) and tag constriction (pointer); highly decrease of microtag branches suggesting highly degradation of the hl over time (stars). c: composite; ad: adhesive; hl: hybrid layer; rt: resin tag; and, d: dentin.

The substrate Ci treated with Ct exhibited thick hybrid layers with thin resin tags after 24 h (Fig. 3A). Thick hybrid layers and degradation points were found after 6 months storage (Fig. 3B), and increased hybrid layer degradation with tag separation were identified after 1 year storage (Fig. 3C). The Et group also showed an altered interface after 24 h, with polymeric tags of unusual conformation (Fig. 3D). After 6 months storage, hybrid layers with some empty areas were verified (Fig. 3E). Disruptive failures on the hybrid layer with some areas of dentin cracks were detected after 1 year storage and altered dentin suggesting dentin degradation by microorganism activity during caries producing (Fig. 3F).

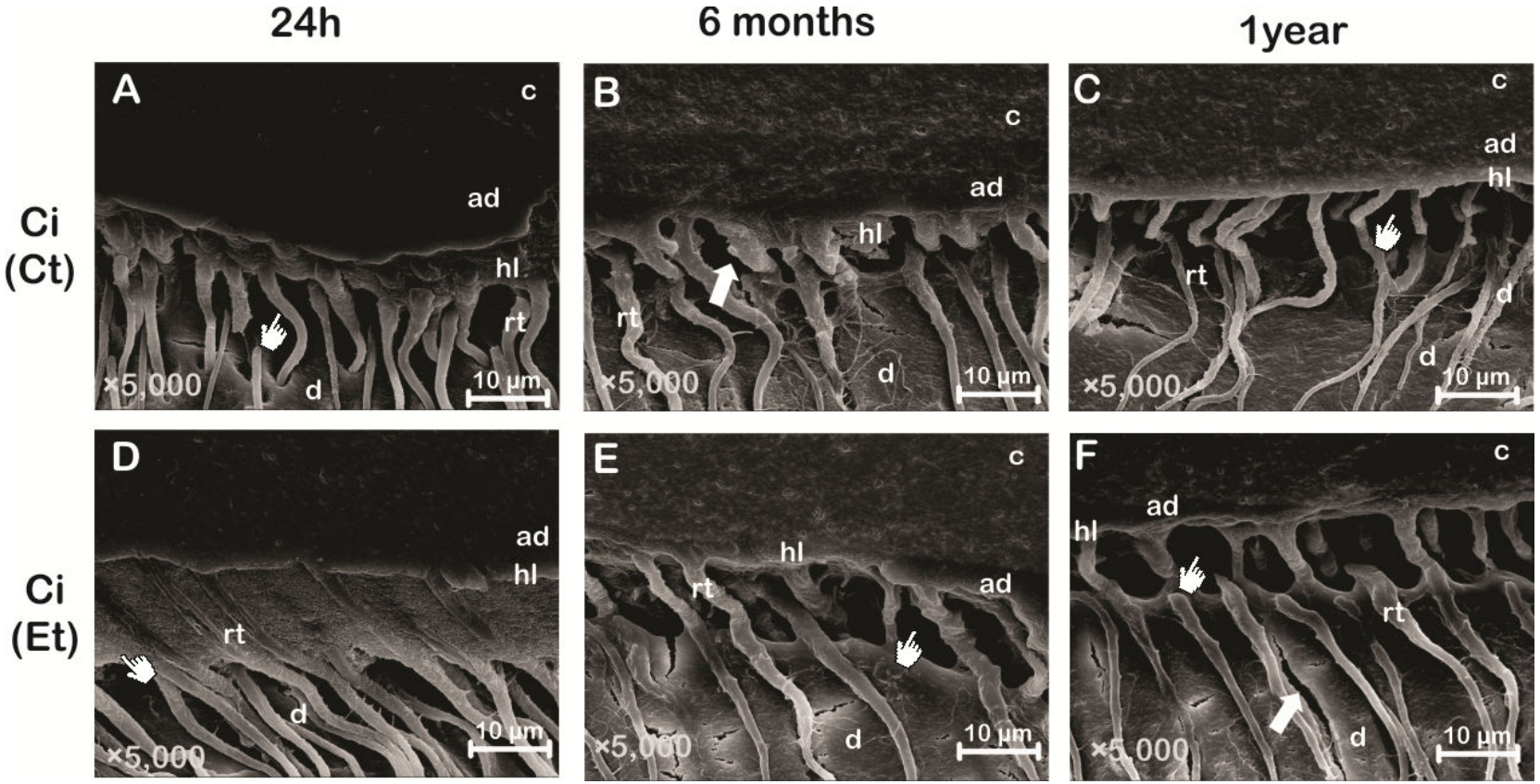

Figure 3: Caries-infected dentin (Ci)- A (Ct 24h) - thick hybrid layer (hl) after water wet-bonding presenting thin resin tags (pointer); B (Ct 6 months) – thick hl and degradation points (black areas - arrow); C (Ct 1 year) – increased degradation (black areas – pointer) of the hl with thin tags separation; D (Et 24h) – hl after ethanol wet-bonding exhibiting an altered interface with polymeric tags of unusual conformation (pointer); E (Et 6 months) - hl with some empty areas (pointer) suggesting highly hl degradation; F (Et 1 year) - disruptive failure of resin tags (pointer) with some areas of dentin cracks (arrow). Arrow also shows altered dentin aspect, suggesting dentin degradation by microorganism activity during caries producing. c: composite; ad: adhesive; hl: hybrid layer; rt: resin tag; and, d: dentin.

Following Ct, the Ca substrate exhibited continuous hybrid layers with polymeric tags after 24 h (Fig. 4A). The 6 months storage showed abnormal aspect of hybrid layers (Fig. 4B), and extensive degradations at the hybrid layer were observed after 1 year storage (Fig. 4C). Ca specimens treated with Et presented thick hybrid layers with continuous and homogenous interface containing polymeric tags of uncommon conformation after 24 h (Fig. 4D). After 6 months storage, hybrid layers showing some resin tag constriction and disruptive regions were verified (Fig. 4E). Unusual hybrid layer with empty areas were identified after 1 year storage, suggesting dentin degradation of demineralized dentin (Fig. 4F).

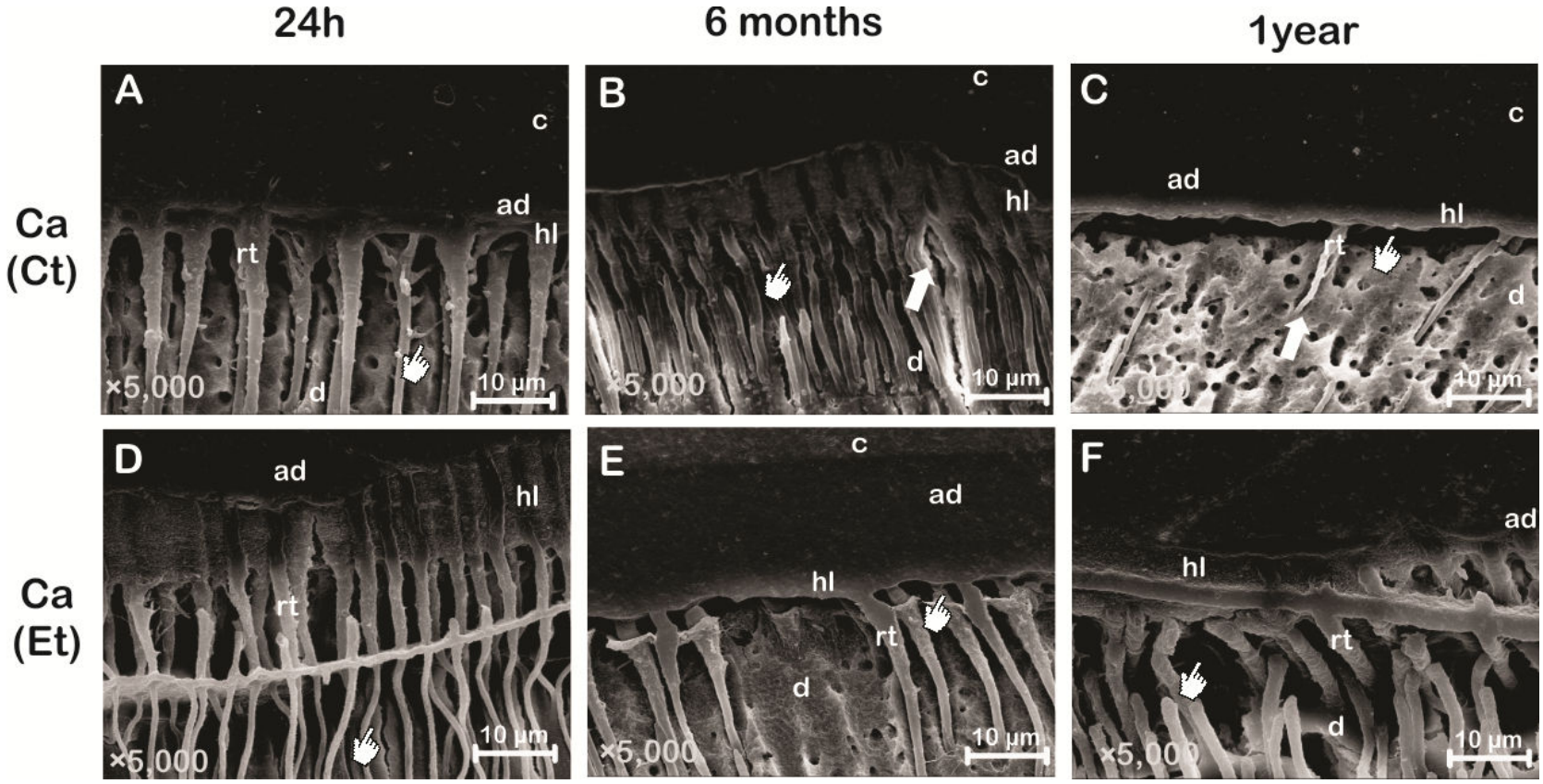

Figure 4: Caries-affected dentin (Ca): A (Ct 24 h) - hybrid layer (hl) after water wet-bonding presenting continuity with polymeric tags; small lateral extensions of microtags branching off at angles from the main resin tags are visible (pointer); B (Ct 6 months) – abnormal aspect of hl (pointer) and some disruptive failures in dentin (arrow); C (Ct 1 year) - extensive hl degradation (pointer) presenting sparse and thin resin tags (arrow); D (Et 24 h) – thick hl after ethanol wet-bonding presenting a continuous and homogenous interface with polymeric tags showing unusual conformation (pointer); E (Et 6 months) - hl showing some resin tag constriction and disruptive regions (pointer); F (Et 1 year) – hl showing unusual conformation and empty areas suggesting dentin degradation of demineralized dentin (pointer). c: composite; ad: adhesive; hl: hybrid layer; rt: resin tag; and, d: dentin.

DISCUSSION

The first hypothesis that the dentin surface treatment with 100% ethanol would improve the bond strength to the three dentin substrates conditions was rejected. The caries-affected dentin was the substrate to present similar bond strengths to the water wet-bonding after the ethanol wet-bonding. The sound dentin and caries-infected dentin showed reduced bond strength values for the specimens treated with ethanol compared to the Ct. In addition to the decreased bond strength, the increased number of adhesive failures and the reduced number of cohesive failures within the dentin substrate for the Sd and Ci ethanol groups suggest difficulties in creating a suitable bonding interface, mainly for the caries-infected substrate. This probably occurred due to the incapability of the adhesive system in correctly infiltrating the dentin and to form a reliable hybrid layer after the ethanol surface treatment.[7,13,16]

The simplified ethanol wet-bonding approach using 100% ethanol for dentin surface treatment instead increasing ethanol concentrations was chosen in order to reduce the technique-sensitiveness and time required for the original protocol.[31] This surface treatment was not effective in producing superior bond strengths for an etch-and-rinse adhesive system to sound and caries-infected dentin. However, the bond strength attained to caries-affected dentin after this ethanol surface treatment was similar to that obtained for the Ct group with water wet-bonding. It is well known that in the ethanol wet-bonding technique, water in dentin is gradually replaced by ethanol with the dentin being saturated with ethanol at the end of the dehydration process.[7,13] When ethanol is applied in larger

volumes, the amount of solvent in the dentin after application of the adhesive system can be excessive if an ethanol-based agent is used, what may impair full solvent volatilization from the substrate.[32] Moreover, the degree of conversion of the polymer may be impacted by the non-volatilized solvent, leading to decreased mechanical properties of the adhesive layer.[33,34] These factors probably interfered with the bonding mechanism of the Sd and Ci groups as revealed by the bond strength results.

The second hypothesis that dentin surface treatment with 100% ethanol would result in better bond strength values to the different dentin substrates after the storage periods evaluated was also rejected. The specimens stored for 6 months or 1 year presented similar bond strength, regardless of the dentin substrate or surface treatment. It has been reported that hybrid layers created using the ethanol wet-bonding technique and hydrophobic adhesive systems resist degradation after long-term storage, regardless of chlorhexidine use.[13] In addition, in other studies the specimens prepared using this protocol also exhibited higher bond strengths after long-term storage compared to the specimens prepared using water wet-bonding and etch-and-rinse adhesive systems.[13] Our findings revealed some degree of hybrid layer degradation for all groups stored for 6 months and 1 year, regardless of the dentin substrate or surface treatment.

The findings of the present study do not fully corroborate with a previous investigation.[13] This fact is possibly related to the differences in the experimental design of the both studies, such as the use of a commercial etch-and-rinse 2-step adhesive system with hydrophilic characteristics instead a hydrophobic resin only,

or the dentin surface treatment with 100% ethanol instead the use of increasing ethanol concentrations. This may be due to the reduced bond strength verified for the groups submitted to 6 months or 1 year storage is the degradation of the hybrid layer, which probably occurred in a large level compared to other studies due to the simplified ethanol wet-bonding approach employed. The dentin surface treatment with 100% ethanol probably promoted incomplete water removal from dentin, impairing complete adhesive infiltration to the substrate, consequently leading to degradation of the collagen matrix and hybrid layer by the mechanisms already mentioned (e.g.: MMPs, phase separation).

The ethanol wet-bonding technique can be a viable approach when surface treatment is performed using increased ethanol concentrations associated to more hydrophobic adhesive systems.[13,16,35] Despite the approach used in our study, the ethanol wet-bonding technique applying 100% ethanol resulted in similar bond strength values between the caries-affected dentin and sound dentin, and in better bond strengths compared to the caries-infected dentin, which is in agreement to previous findings.[31] Moreover, the caries-affected dentin treated with ethanol wet-bonding technique exhibited similar bond strength to the caries-affected dentin treated with water wet-bonding protocol. However, the same behavior was not observed for sound and caries-infected dentin groups. Although satisfactory bond strength results were verified with 100% ethanol surface treatment for caries-affected dentin in the present study and in the literature,[31] some increased levels of nanoleakage were shown to occur in the hybrid layers when caries-affected dentin substrates were bonded using the simplified ethanol

wet-bonding approach, differing from the lower levels of silver impregnation found after the application of a series of increasing concentrations of ethanol.[31]

Due to the mineral and organic changes existing in caries-affected dentin, adhesion to this substrate is more complex than to sound dentin.[36] In fact, μTBS tests showed that the etch-and-rinse adhesive obtained significantly higher bond strength when bonded to sound dentin compared to caries-affected and caries-infected dentin when the water wet-bonding was used. Some investigations also indicated that the caries-affected dentin results in lower bond strengths than that of sound dentin.[5,37] These results are credited to the weakness of the demineralized caries-affected tissue, the limitation of adhesive infiltration, and the hybrid layer conformation caused by tubules filled with mineral deposits.[5,31] Additionally, the more permeable condition and the increasing water content in caries-affected dentin are issues that may influence bonding quality and stability when hydrophilic adhesives are used.[38] The lower bond strengths observed for the caries-infected dentin in either control or ethanol treated groups were already expected, since this substrate is even more porous, with pronounced water content and presence of carious bacteria, which is in agreement to previous results.[5,39]

As seen, in the present results, the simplified ethanol-dehydration protocol achieved significantly lower bond strength values in sound dentin, with better results for the caries-affected dentin substrate. It has been theorized that the acid-resistant mineral deposits present at the tubules of the caries-affected dentin may work as a barrier, reducing water infiltration from the pulp to the dentin surface.[31] Thus the simplified ethanol wet-bonding with 100% ethanol could be effective in

replacing water in this substrate, which means that this approach can be less sensitive in caries-affected dentin than in sound dentin, with potential to assure reliable bond strength. This assumption can be accepted, since the caries-affected dentin specimens treated with the ethanol wet-bonding showed similar bond strengths and similar aspects of the hybrid layers after 6 months storage compared to the caries-affected specimens bonded with the water wet-bonding protocol (Fig. 4). Additionally, the application of less hydrophilic adhesive systems may also produce better bond strength values with the ethanol wet-bonding technique.

The ethanol wet-bonding technique showed encouraging results, but there are some concerns regarding the application of this technique in clinical practice, essentially because of the technique-sensitive and time-consuming protocols. The findings of the present study showed some potential to achieve more stable bonding to caries-affected dentin through ethanol wet-bonding with 100% ethanol. Additional surveys are desired to check the effect of bonding quality and durability on different dentin substrates using the ethanol-wet bonding technique and to make this approach more useful for the clinical practice.

CONCLUSIONS

Within the limitations of the research design of this *in vitro* study, the following conclusions can be drawn:

1 – The ethanol was not effective to improve the μTBS for the three dentin substrates evaluated, regardless of storage time. The application of ethanol, after

acid etching significantly decrease the μTBS for Ci and Sd, but did not influence Ca.

2 – Stored specimens for 6 months and 1 year decreased the μTBS for all conditions.

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CAPÍTULO 3: ***Effect of chlorhexidine digluconate on dentin/resin bond strength at different dentin substrate conditions and storage time***

ABSTRACT

Purpose: to evaluate the effect of 2% chlorhexidine digluconate (CHX) on microtensile bond strength (μTBS) and degradation between an adhesive system and dentin under 3 dentin conditions and storage times. The bonding interface was also analysed using SEM. *Materials and Methods:* Forty-eight dentin surfaces from sound third molar were divided into 3 groups, according to dentin substrates: sound dentin(Sd), caries-infected dentin(Ci) and caries-affected dentin(Ca). Ca and Ci were submitted to development of artificial caries using visual inspection with Caries Detector solution. It was considered as Ci: soft and deeply pigmented dentin and Ca: hard and slightly pigmented dentin. CHX was applied on half of groups, just after etching with 35% phosphoric acid gel. Afterwards, the dentin surfaces were bonded with Adper Single Bond 2(3M ESPE) adhesive system according to manufacturer's instructions. Teeth were longitudinally sectioned across the bonded interface ($1.0mm^2$). The specimens were stored in deionized water at 37°C for 24h, 6 months and 1 year later. Two additional teeth were used to analyze the bonding interfaces by SEM. Data was submitted to ANOVA and Tukey's test($\alpha = 0.05$). *Results:* Ci decreased the bond strength values, when compared to Ca and Sd. Stored samples for 6 months and 1 year decreased the μTBS for all analyzed conditions. *Conclusion:* CHX did not influence bond strength to dentin over time, regardless substrate conditions.

Time is the most important factor in the bond strength degradation. The bonding to caries-infected dentin decreased the bond strength values, when compared to caries-affected and sound dentin.

Key-words: 2% chlorhexidine digluconate, dentin, S. *mutans*, adhesive system, microtensile bond strength, s*torage time*

INTRODUCTION

The 'minimally invasive dentistry' aims to preserve the maximum amount of tooth structure after removal of decayed tissue. The removal of infected dentin only, leaving the innermost layer of dentin, the affected dentin, its a proposed clinical procedure based theoretical in the remineralization ability of the dental hard tissue.[1] Such selective caries excavation should prevent lesion progression, while maintaining the strength and stability of the remaining tooth structure in order to guarantee long-term mechanical resistance to intra-oral forces.[2]

The use of adhesive techniques and polymeric materials also contributes to less tissue removal. It is mandatory to have practical and scientific information background regarding new materials that have been developed and are available to the dental market.[3-6] However, defining the actual endpoint of caries excavation and thus the start-point of restoration is often clinically challenging. In this context, it is known that the short and long term bond strength values decrease when adhesion is performed over affected and infected dentin, although the values obtained may be clinically acceptable.[7,8]

Chemical agents to prevent carious progression or recurrent caries in dentin can be applied. Chlorhexidine (CHX) consists as a well-known

disinfectant/antimicrobial agent,[9] and also has been examined as a therapeutic primer for the preservation of hybrid layers in human dentin.[10-12] While the antimicrobial efficiency of CHX on acid-etched dentin remains unclear, the current data confirm its positive effects on the preservation of bond strength when applied to demineralized normal dentin prior to bonding with no rinsing.[11,13]

According to De Munck et al., 2005,[14] high bond strength values measured immediately does not correlate with bonding longevity, because the resin/dentin bonding is rapidly degraded, i.e., 6 months or less.[13,15] Excellent effectiveness of immediate bond strength does not mean short- and long-term dentin bonding, longevity and stability of bonding interface to dentin. These issues are still very questionable and multifactorial-dependent.[14]

In this context and considering that many questions remains regarding bonding to this type of substrate over longer periods, the current study aimed to compare bond strength of immediate, short- and long-term (6 months and 1 year) on dentin substrate, treated or not with 2% chlorhexidine digluconate (CHX). The null hypothesis was that 2% CHX did not influence on bond strength, irrespective of aging period or dentin substrate condition.

MATERIALS AND METHODS

This study was approved by the Research Ethic Committee (registration number 041/2010). Forty-eight intact recently extracted human molars were selected. Teeth were stored in 0.1% thymol for 1 month and then stored in distilled water at 4ºC until use. The occlusal enamel was sectioned perpendicular

to the tooth long axis with a water-cooled low-speed diamond saw (Isomet; Buehler Ltd., Lake Bluff, IL, USA) to expose the subjacent dentin. Dentin was wet ground flat with 180- and 600-grit silicon carbide paper until a uniform enamel-free dentin surface was obtained.

Artificial caries development

Thirty-two molars were fixed with orthodontic wire on the lids of glass vials containing 40 mL of sterile distilled water and immediately sterilized by gamma irradiation (GC-220E, MDS Nordion, Otawa, Canada) during 32 h, at 27°C, with a total dose of 14.5 KGy.[16] Specimens were then transferred to another sterile glass vial containing 40 mL of sterile brain-heart infusion (BHI) broth (#21152, Becton Dickinson and Company, Sparks, MD, USA) supplemented with yeast extract (Himedia Laboratories, PVT Ltd., Mumbai, India), 0.5% glucose (Synth; LabSynth, São Paulo, SP, Brazil), 1% sucrose (Synth; LabSynth) and 2% *S. mutans* (UA159) for development of the artificial carious lesions. The concentration of this bacterial suspension was determined by measuring absorption at 660 nm (A660). In order to adjust the number of viable bacteria to A660, the number of colony-forming units per milliliter of bacterial suspension (cfu/mL) was determined with the use of standard spreading techniques at carious optical densities. Inoculation occurred only in the first day of the experiment, but the media was renewed every 48 h during 14 days.[17]

Differentiation method: caries-infected and caries–affected dentin

Caries-infected and caries-affected dentin was maintained or partially removed, respectively, after color identification using Caries Detector solution (Kuraray, Med. Inc, Okayama, Japan) associated to inspection criterion using dental explorers and visual examination. The respective caries-removing endpoints for the experimental groups were: Caries-infected dentin (Ci) – soft deeply pigmented dentin was maintained. Caries-affected dentin (Ca) – soft pigmented carious tissue was partially removed till hard and slightly pigmented dentin remained. The caries–affected dentin was removed using a #8 round carbide bur (KG Sorensen, Barueri, SP, Brazil) mounted on a slow-speed hand piece.

Microtensile specimens and bond testing

The root of each specimen was removed above the cementum-enamel junction using a double-face diamond disk (KG Sorensen, Barueri, SP, Brazil) and the pulp chamber was filled with resin composite (Z350, 3M ESPE, St. Paul, MN, USA) light-cured using at 700 mW/cm^2 XL 2500 quartz-tungsten-halogen unit (3M-ESPE, St. Paul, MN, USA), previously conditioned with 35% phosphoric acid for 15 sec (Scotch Bond Etchant, 3M ESPE, St. Paul, MN, USA) and hybridized using Adper Single Bond 2 adhesive (3M ESPE, St. Paul, MN, USA). The sound teeth (n = 48) are randomly divided into three groups (n = 16), according to the dental substrate: sound dentin (n = 16), caries-infected dentin (n = 16), and caries-affected dentin (n = 16). Then, subdivided into two groups (n =

8) according to the surface treatment (2% CHX application), or no treatment (control):

No treatment groups (Control): after conditioning with 35% phosphoric acid for 15 sec (Scotch Bond Etchant, 3M ESPE), dentin was washed for 30 sec and the excess water was removed with tissue paper. After, the dentin was rehydrated with deionized water, using a microbrush for 60 sec and water excess removed with tissue paper. Thereafter, the Adper Single Bond 2 (3M ESPE) adhesive system was applied according to the manufacturer's recommendations.

Treated groups (2% CHX application): after conditioning with 35% phosphoric acid for 15 sec (Scotch Bond Etchant, 3M ESPE), dentin was washed for 30 sec and the water excess was removed with tissue paper. After, the dentin was rehydrated with 2% chlorhexidine digluconate (PRODERMA, Piracicaba, SP, Brazil), using a microbrush for 60 sec and the water excess was removed with tissue paper. Thereafter, the Adper Single Bond 2 (3M ESPE) adhesive system was applied according to the manufacturer's recommendations.

For both groups, the hybridized surfaces were coupled with resin composite (Filtek Z350, 3M-ESPE), applied in 2-mm-thick increments and photo activated using a quartz-tungsten-halogen light-curing unit at 700 mW/cm^2 (XL 2500, 3M-ESPE, St. Paul, MN, USA), the output power was checked with digital radiometer Hilux Light Meter (First Medica, Greensboro NC, USA). Each increment was light-cured for 20 sec. The teeth with composite built-ups were stored in distilled water at 37°C for 24 h. Each specimen was sectioned perpendicular to the bonding interface area to obtain beams with a bonding area

of approximately 1 mm^2 using a water-cooled diamond blade (EXTEC Corporation, Enfield, CT, USA) in a low-speed saw machine (Isomet 1000, Buehler Ltd., Lake Bluff, IL, USA). The cross-sectional area of the bond interface of each beam was measured using a digital caliper (Mitutoyo Corporation, Tokyo, Japan). Bond strength values were calculated and the data supplied in MPa. Beams at specimen peripheries were discarded. Each group had eight teeth, and each tooth generated an average of 15 beans, for a total of 120 beams per group. Microtensile bond-strength data were submitted to three-way ANOVA, and multiple comparisons were performed using the Tukey post hoc test (SigmaStat, version 3.5.0.54, Systat Software Inc) ($p< 0.05$).

Failure mode analysis

The fractured specimens were observed using a SEM (LEO 435 VP; LEO Electron Microscopy Ltd., Cambridge, UK) at 100x to 3000x magnification. The working distances ranged between 22 to 18 mm, according to specimens height. Then, each specimen was classified according to the predominant remaining structure upon its surfaces following the described failure mode: adhesive (Mode 1); cohesive within dentin (Mode 2); cohesive within the composite (Mode 3); and mixed, involving bonding agent, composite and/or tooth structure (Mode 4). The results of failure mode classification were submitted to Fisher's Exact Test (R, version 2.14.0, The R Foundation for Statistical Computing) ($p < 0.05$).

Analysis of bonding interface

Two additional restored teeth (n=2) were vertically sectioned in 2 mm slices (about 8 slices per group), were then embedded in epoxy resin (Buehler, Lake Bluff, IL, USA). After each storage time, the slices were wet-polished using 600, 1200 and 2000-grit SiC papers (Norton SA, São Paulo, Brazil) and with decreasingly fine diamond compounds (3 um, 1um, ½ um, ¼ um - Metadi II, Buehler, Lake Bluff, IL, USA), after each polishing step the specimens were ultrasonically washed for 10 min, demineralized with liquid 50% H_3PO_4 during 5 seconds, rinsed in distilled water, deproteinized with 2.5% NaOCl during 10 minute. The specimens were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffered solution titrated to pH 7.2 for 72 hours and then rinsed several times with 0.1 M sodium cacodylate buffered solution. They were dehydrated in ascending concentrations of ethanol (50%, 60%, 70%, 80%, and 90%) for 2 hours in each solution and in 100% ethanol for 24 h. The final chemical drying was conducted by immersion in hexamethyldisilazane for 10 min on filter paper inside a covered glass vial and air-drying at room temperature.

The specimens were gold coated with sputter coater under high-vacuum ambient (MED 010, Balzers Union, Aktiengeselischaft, Furstentun, Liechtenstein) for 180 seconds at 40 mA and examined using scanning electron microscopy (SEM; LEO 435 VP, Cambridge, England), operated at 20 KV under magnification of 1000-5000x, by the same operator. The cross-section profiles were examined, focusing on the depth of etching, micromechanical

entanglement, integrity, homogeneity and continuity along the bonded interface. The most representative images were selected.

RESULTS

Results for the μTBS are shown in Table 1. Three-way ANOVA showed that significant difference for 'Substrate' ($p<0.001$) and 'Storage' ($p<0.001$) factors, but not for 'Treatment' ($p=0.14$). There was no interaction between factors ($p=0.915$).

Overall, the comparison of μTBS means (in MPa) for sound dentin (40.9) and caries-affected dentin (35.3) groups were significantly higher than caries-infected dentin (28.2). The μTBS (in MPa) after 24 hours (39.3) was significantly higher than storage for 6 months (33.2) and 1 year (31.9). No difference was found between storage for 6 months and 1 year. For treatment, the use of CHX (35.6) did not show significant difference in the μTBS when compared to the control group (34.0).

Table 1. Means (SD) of μTBS (MPa) of dentin substrate exposed to cariogenic challenge.

Substrate	Storage Time						Substrate	Treatment
	24 hours		6 months		1 year			
	Ct	CHX	Ct	CHX	Ct	CHX		
(Sd) Sound dentin	47.8 (10.3)	41.4 (5.0)	39.4 (7.4)	39.9 (8.8)	38.4 (8.9)	38.6 (7.7)	**Sd (40.9) [a]**	**Ct (34.0) ***
(Ca) Caries-affected dentin	38.3 (8.4)	39.2 (10.5)	28.7 (8.5)	40.0 (14.6)	28.6 (9.6)	36.8 (11.4)	**Ca (35.3) [a]**	
(Ci) Caries-infected dentin	37.9 (9.2)	31.3 (7.1)	23.7 (8.2)	27.6 (8.6)	23.1 (5.6)	25.7 (9.0)	**Ci (28.2) [b]**	**CHX (35.6) ***
Storage time	**(39.3) [A]**		**(33.2) [B]**		**(31.9) [B]**			

Means followed by different small letters in column and capital letters in row are significantly different ($p<0.05$).

* Means no difference between Control Group (Ct) and Chlorhexidine treated groups (CHX).

Failure mode

Failure mode results are shown in Tables 2 and 3. Overall, the predominance of the mixed and adhesive failures was detected for all groups. Otherwise, cohesive failures in dentin decreased when chlorhexidine was applied. The occurrence of cohesive failures in composite was more evident in stored samples. The Fisher's Exact Test of the failure modes within each substrate condition showed a significant association between 'substrate condition' and 'storage time' relative to bond strength with in each dentin treatment (using or not chlorhexidine): **Control Group**: p= 0.001163 Sound Dentin, p=0.001118 for Infected-dentin, p=0.01009 for Affected-dentin; **CHX2% Group**: p=0.009804 for sound dentin, p=0.02227 for infected dentin, p=0.004257 for affected dentin. The most representative micrographs of failure modes are shown in Figure 1.

Table 2 – Distribution pattern of failure (%) in control groups.

Control Group		Failure Mode Analysis (%)			
		Adhesive	*Cohesive in dentin*	*Cohesive in composite*	*Mixed*
Sound dentin (Sd) p=0.001163	24 h	35.71	11.90	2.39	50
	6 months	25.53	6.38	25.53	42.56
	1 year	55.56	11.11	15.55	17.78
Caries-infected dentin (Ci) p=0.001118	24 h	26.83	21.95	2.43	48.79
	6 months	40.54	21.62	10.81	27.03
	1 year	32.43	16.20	5.40	45.96
Caries-affected dentin (Ca) p=0.01009	24 h	37.84	24.32	5.40	32.44
	6 months	41.17	20.58	14.72	23.53
	1 year	29.01	22.53	9.68	38.71

Table 3 – Distribution pattern of failure (%) in groups treated with CHX 2%.

Group treated with CHX 2%		Failure Mode Analysis (%)			
		Adhesive	*Cohesive in dentin*	*Cohesive in composite*	*Mixed*
Sound dentin (Sd) p=0.009804	24 h	28.95	7.89	15.79	47.37
	6 months	43.14	3.92	29.41	23.53
	1 year	22.92	6.25	33.33	37.5
Caries-infected dentin (Ci) p=0.02227	24 h	50	6.82	-	43.18
	6 months	40.48	-	11.90	47.62
	1 year	56.41	2.56	5.13	35.9
Caries-affected dentin (Ca) p=0.004257	24 h	66.67	-	4.76	28.57
	6 months	33.33	2.56	12.82	51.29
	1 year	65.79	-	7.89	26.32

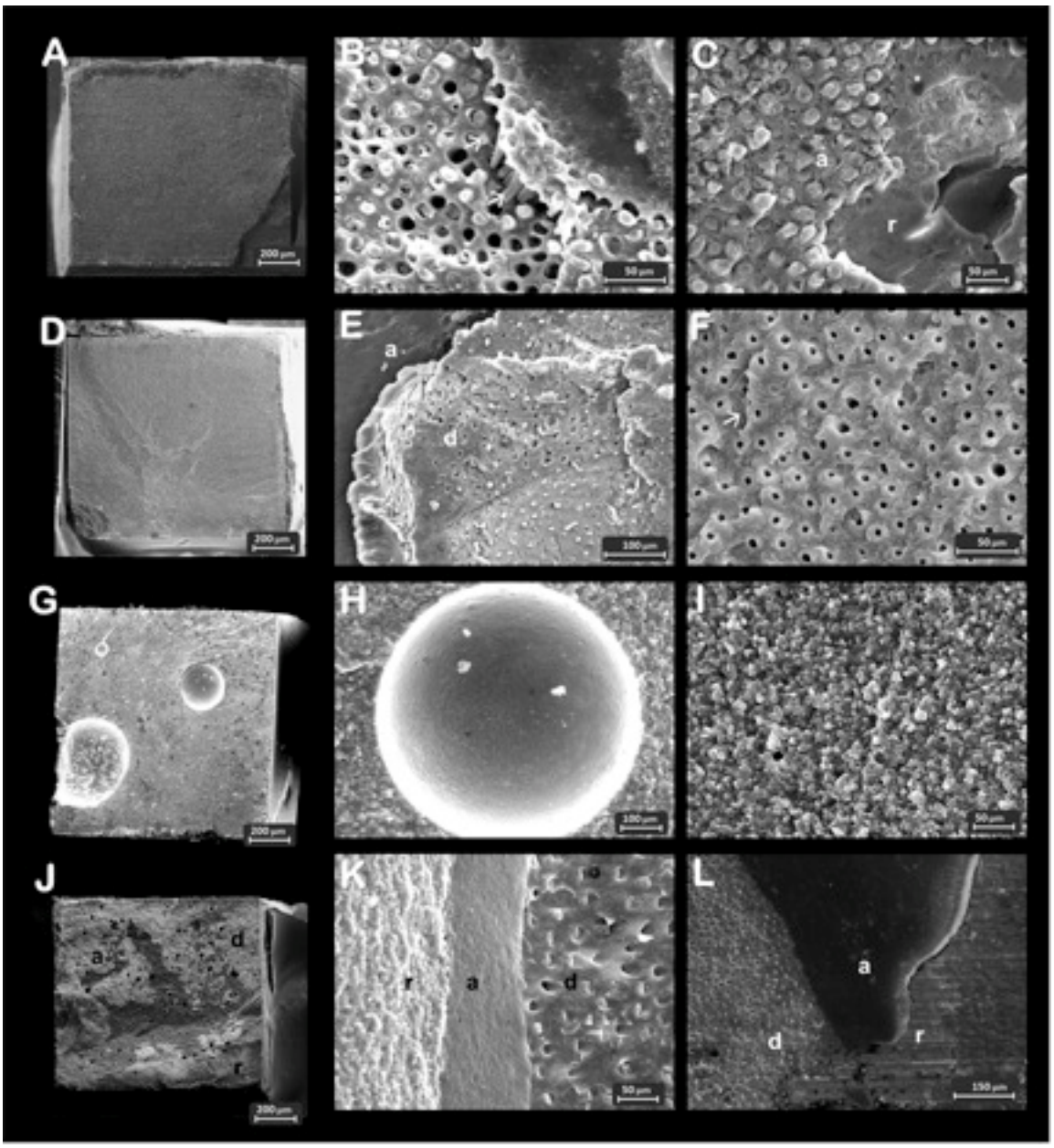

Figure 1- A – Adhesive failure; B – Magnification showing the adhesive detachment from dentin (arrows) suggesting areas the complete detachment of the adhesive agent; C – Magnification showing the adhesive side with the dentinal tubules pattern (a) and some areas of detachment from resin composite (r); D – Cohesive failure within dentin; E and F – Higher magnifications showing the dentin fractured surfaces (d in E and arrow in F); G – Cohesive failure within resin composite; H – aspect of an internal flaw located at the fractured surface; I - aspect of composite fractured surface; J – Mixed failures showing the materials analyzed in the study; K – Mixed failures showing resin composite

SEM characterization – Hybrid layer

The results of SEM characterization is presented and described in details in Figures 2, 3 and 4. Overall, it was observed that all dentin substrate studied showed bonding interface degradation over time. It was observed that regardless substrate conditions, there was bonding interface degradation, although Ci showed the highest bonding interface degradation level. Sd and Ca showed similar aspects of interface degradation, except resin tags conformation.

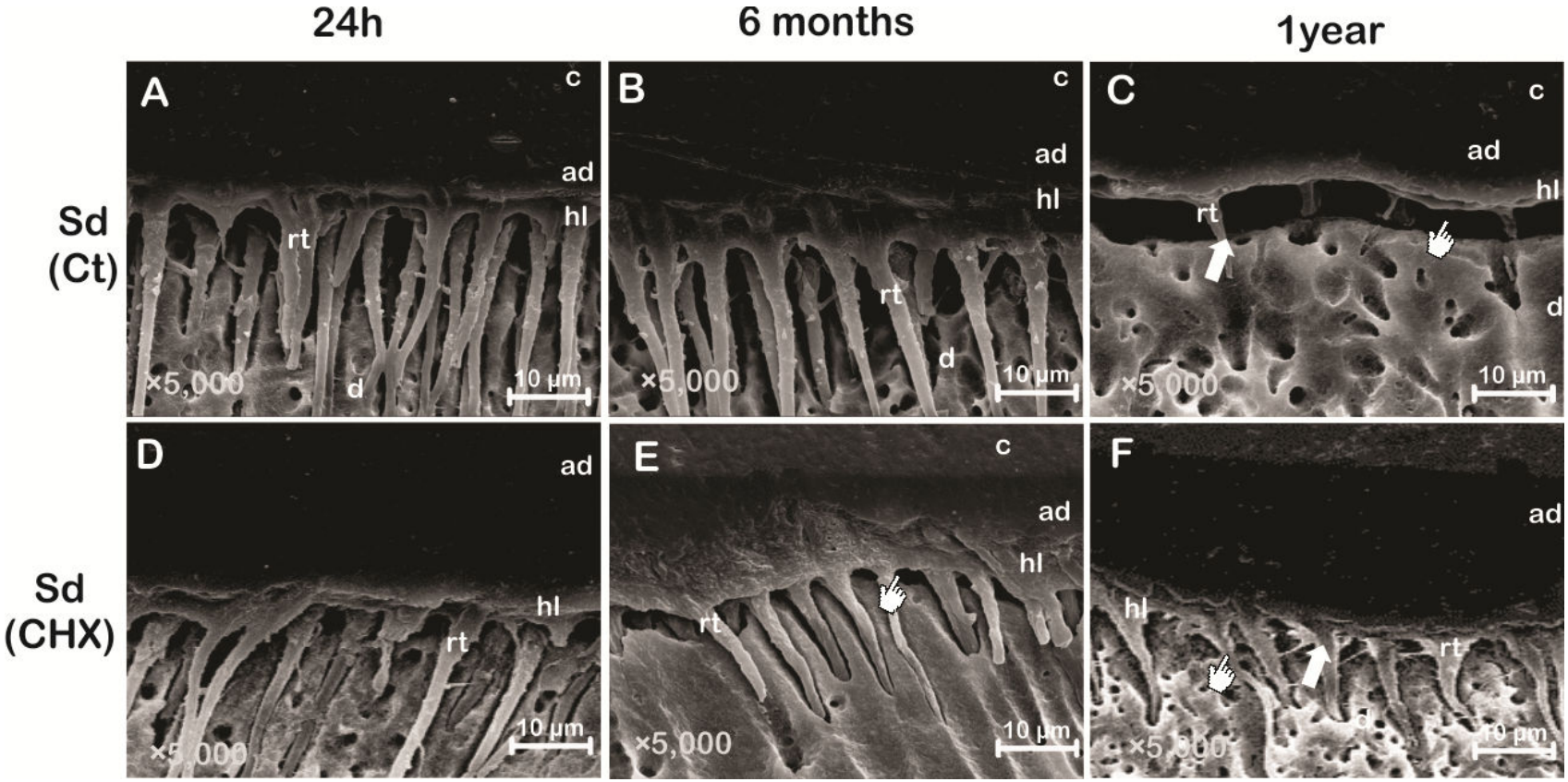

Figure 2 – Sound dentin. A: Ct 24 h– hybrid layer (hl) presenting a continuous and homogenous interface; B – Ct 6 months – hl presenting a continuous and homogenous interface; slightly extensions of microtags branching off at angles from the main resin tags (rt) are visible; C – Ct 1 year – extensive and deep alterations suggesting a higher degree of degradation of dentinal substrate (pointer) and superficial resin tag penetration (arrow); D – CHX 24 h - hl over CHX treated dentin presenting a continuous and homogenous interface; E – CHX 6 months – possible interface flaws seems to originate on bottom of hybrid layer (pointer); F – CHX 1 year – although some flaw and discontinuities are already detected (arrow), the hybridization seems preserved (pointer). c: composite; ad: adhesive; hl: hybrid layer; rt: resin tag; and, d: dentin.

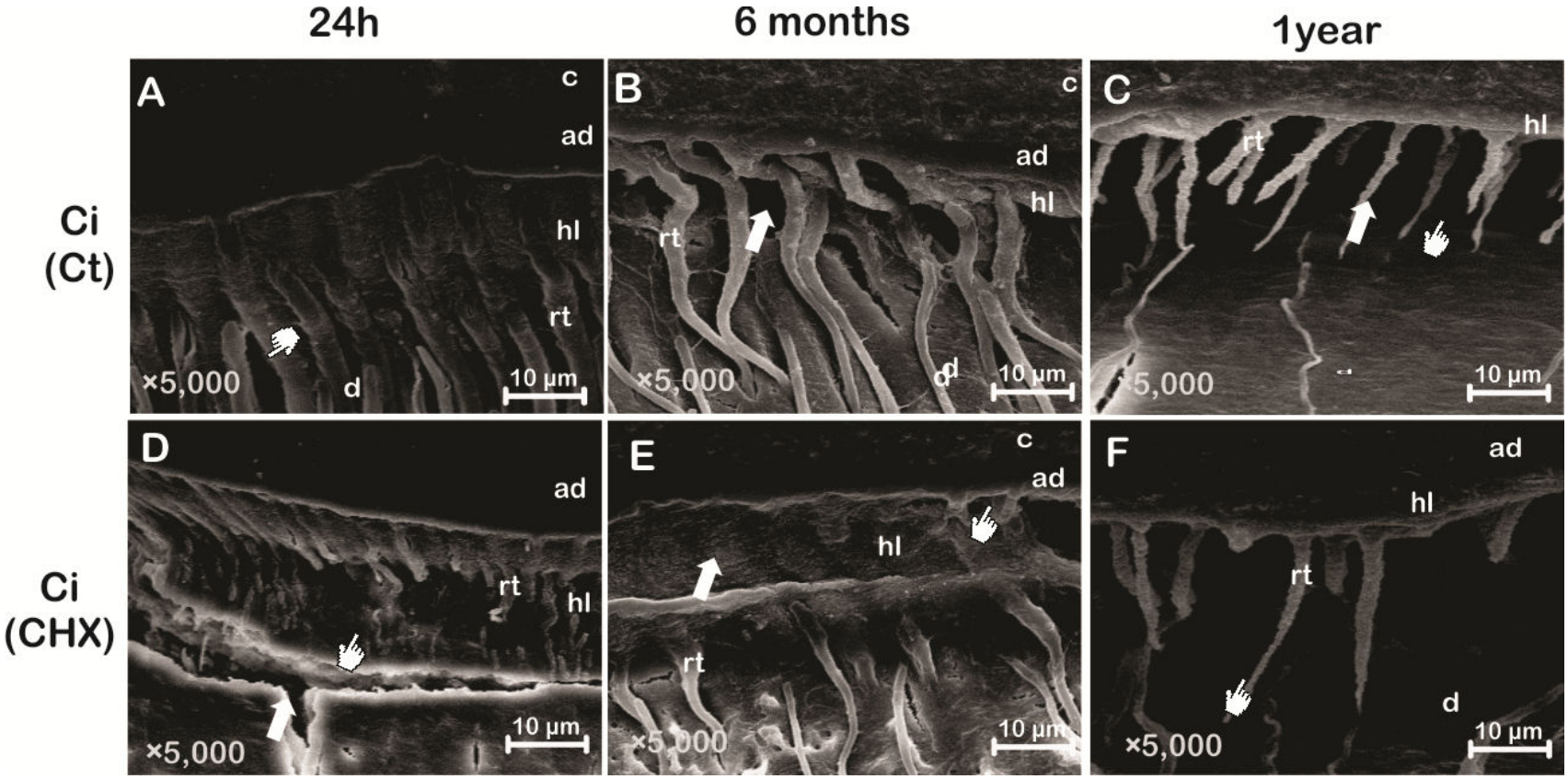

Figure 3 - Caries-infected dentin. A: Ct 24 h – hybrid layer (hl) after water wet-bonding presenting abnormal thin resin tags (pointer); B – Ct 6 months – altered hl and degradation points (black areas - arrow); C – Ct 1 year – extensive and deep alterations (pointer) suggesting a higher degree of degradation of hl; arrow showing thin and abnormal resin tag (rt) aspect; D – CHX 24 h – formation of polymeric tags over abnormal dentin tissue (pointer) and arrow showing some initial dentin flaw; E – CHX 6 months – the interface presents unusual aspect of hl (arrow) and initial flaw detected (pointer); F – CHX 1 year – abnormal resin tag (rt) aspect (pointer). c: composite; ad: adhesive; hl: hybrid layer; rt: resin tag; and, d: dentin.

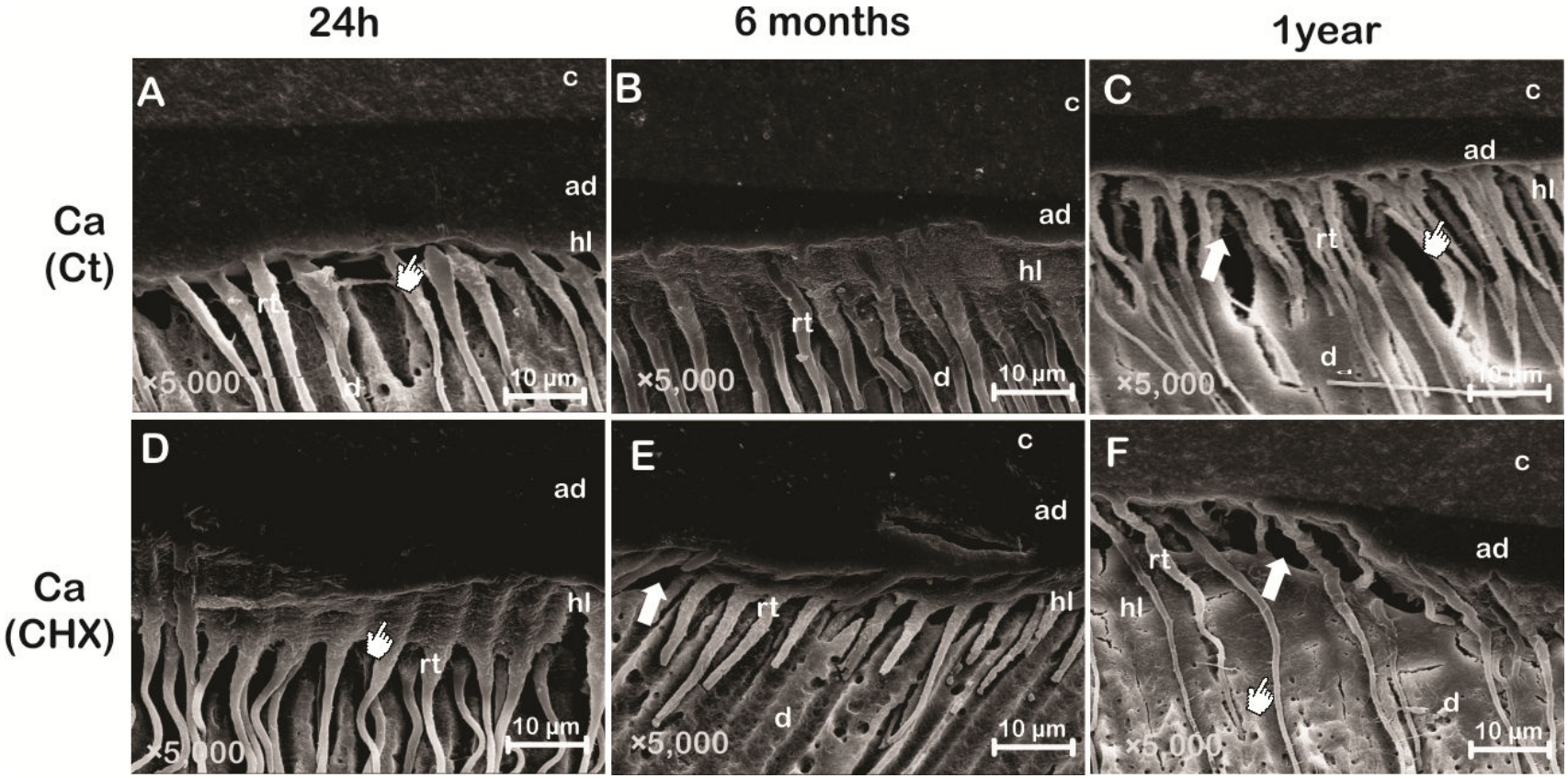

Figure 4 – Caries-affected dentin. A: Ct 24 h– unusual aspect of hybrid layer (hl) presenting some polymeric tags with constriction (pointer); B – Ct 6 months – hl degradation; C – Ct 1 year – extensive alterations suggesting a higher degree of degradation of hl (arrow) and thin and abnormal resin tag aspect (pointer); D – CHX 24 h – pointer showing hybridization of intertubular dentin and unusual aspect of hl; E – CHX 6 months – the interface presents some disruptive failure at bottom of hl showing initial degradation of (arrow); F – CHX 1 year: debonding at the bottom of HL (arrow). Pointer is showing abnormal dentinal tissue aspect suggesting dentin degradation by microorganism activity during caries producing. c: composite; ad: adhesive; hl: hybrid layer; rt: resin tag; and, d: dentin.

DISCUSSION

The null hypothesis was partially accepted. In overall comparison, CHX did not influence bond strength; however, CHX showed similar bond values in sound dentin and caries-affected dentin specimens stored when compared to caries-infected dentin. Therefore, the bonding effectiveness to caries-infected dentin was in general lower than to 'sound' and 'caries-affected' dentin and the different caries-removing techniques resulted in different bonding-receptive dentin substrates. Clinicians frequently must deal with caries-affected and caries-infected dentin, and probably, normal dentin is not the most encountered substrate in clinical situations.[8]

Some studies[11,18] suggested that faster bonding degradation is closely related to increased collagenolytic activity in the caries-affected and caries-infected dentin, when compared to intact normal dentin.[8] Caries-affected dentin usually presents lower dentinal tubule permeability due the presence of mineral crystals blocking fully or partially tubule lumen. Although, this situation could result in lower hydrolytic degradation of hybrid layer due a drastic decrease in the water uptake,[19] this tubule blocking could jeopardize adhesive infiltration towards tubular- and inter-tubular dentin. This corroborates with the finds of the SEM analysis. The formation of small polymeric tags over caries-infected dentin was sometimes detected; in addition, it was observed an unusual hybrid layer conformation thin and abnormal resin tags aspect and altered dentin tissue was the most common find in the SEM characterization, regardless CHX treatment. (Figures 3A-F), The

above cited image conditions may be a possible explanation for the bond strength decrease for Ci group.

In addition to the reduction in bonding strength, the increased number of cohesive failures in caries-infected dentin and caries-affected dentin groups (for the control group) provides further information to corroborate to previous study on changes in dental tissues due to caries activity,[20] which makes these substrates weaker compared to sound dentin. Notwithstanding, the results also indicate that when CHX was applied, the cohesive failures in dentin decreased significantly. In addition to the effectiveness of CHX as an MMP inhibitor or as an anti-microbial agent on bond durability and/or tissue integrity it promoted a decrease in cohesive failures in dentin. Although the current study showed variation in bond strength values among the groups, the means are in a clinical acceptable range. Therefore, a critical evaluation of the failure modes could help in data interpretation and to predict the behavior of bonding over time. It has to be considered that degradation period in this study can be considered a mild challenge and great differences on groups could not be observed, mainly in CHX treatment.

Bonding to caries-affected dentin may rely on the specific chemical composition of dental adhesives.[21] The etch-and-rinse 2-step adhesive (Single Bond 2, 3M ESPE) used contain functional monomers in its composition that can interact with the calcium ions left on the dentin surface, or even with the underlying dentin.[22] It also contains hydrophilic copolymer of the polyalkenoic acid that is believed to form Ca-polyalkenoic acid-base complexes.[23] Assuming that chelating interactions exist between the acidic monomer and mineralized dentin, a reduction

in hydroxyapatite crystallite availability (due to caries demineralization) may have limited its bonding efficacy on caries-infected dentin and even in caries-affected dentin groups. Further studies may evaluate other dental adhesives compositions over the same conditions analyzed in this study.

Degradation of resin-dentin bonds over time was observed in the current study, represented by some interface disruption and discontinuities in samples analyzed by SEM after 6 months and 1 year of water storage, as noted in Figures 2, 3 and 4. The use of metalloproteinase inhibitors as CHX does not negatively influence the immediate bond strength and prevents, or at least decelerates the deterioration of resin-dentin bonds teeth.[7,9,11] Although it was not observed in the current study, chlorhexidine digluconate has potential to minimize the reduction in resin-dentin bond strengths commonly observed after long-term water storage. Auto activation of further pro-MMPs may result in increased enzymatic activity over time[7] and chlorhexidine digluconate could impair this deleterious effect. Moreover, bond durability also depends on both the resistance of adhesive resin and uncovered collagen fibers to degradation over time.[24] This also calls for the need to modify the adhesive monomers or innovation of new versions that are more resistant to hydrolytic degradation and, at the same time, perform well in a clinical common scenario, where microorganism are present.

Under the conditions of this *in vitro* study, it could be concluded that CHX did not influence bond strength to dentin over time, regardless substrate conditions. Time is the most important factor in the bond strength degradation. However, the

bonding with caries-infected dentin decreases the bond strength values, when compared to caries-affected and sound dentin.

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CLINICAL RELEVANCE – The 2% CHX application after etching did not show improved dentin bond strength in the storage time, regardless of the substrates evaluated.

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CONCLUSÃO

Dentro das limitações do estudo pode ser concluído que:

1 – A união em dentina sadia mostrou valores de resistência de união significativamente maior quando comparado às dentinas infectada e afetada por cárie.

2 – O etanol não foi efetivo em melhorar a resistência de união para os três substratos de dentina avaliados.

3 – A união em dentina infectada por cárie diminuiu os valores de resistência de união, quando comparado às dentinas sadia e afetada por cárie para o grupo do etanol. No grupo da técnica úmida, a dentina infectada e afetada por cárie diminuiu a resistência de união em relação à dentina sadia.

4 - A clorexidina não influenciou na resistência de união para os períodos de armazenagem de 24 horas, 6 meses e 1 ano, independente dos substratos de dentina estudados. A união em dentina infectada por cárie diminuiu os valores de resistência de união quando comparado às dentinas sadia e afetada por cárie.

5 – O armazenamento por 6 meses e 1 ano apresentou menor resistência de união para todos os substratos avaliados.

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ANEXO 1

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 **"Estudo in vitro do efeito da clorexidina e do etanol na união dentina/resina em diferentes condições do substrato dentinário e tempos de armazenagem"**, protocolo nº 041/2010, dos pesquisadores Ana Rosa Costa e Regina Maria Puppin Rontani, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 14/05/2010.

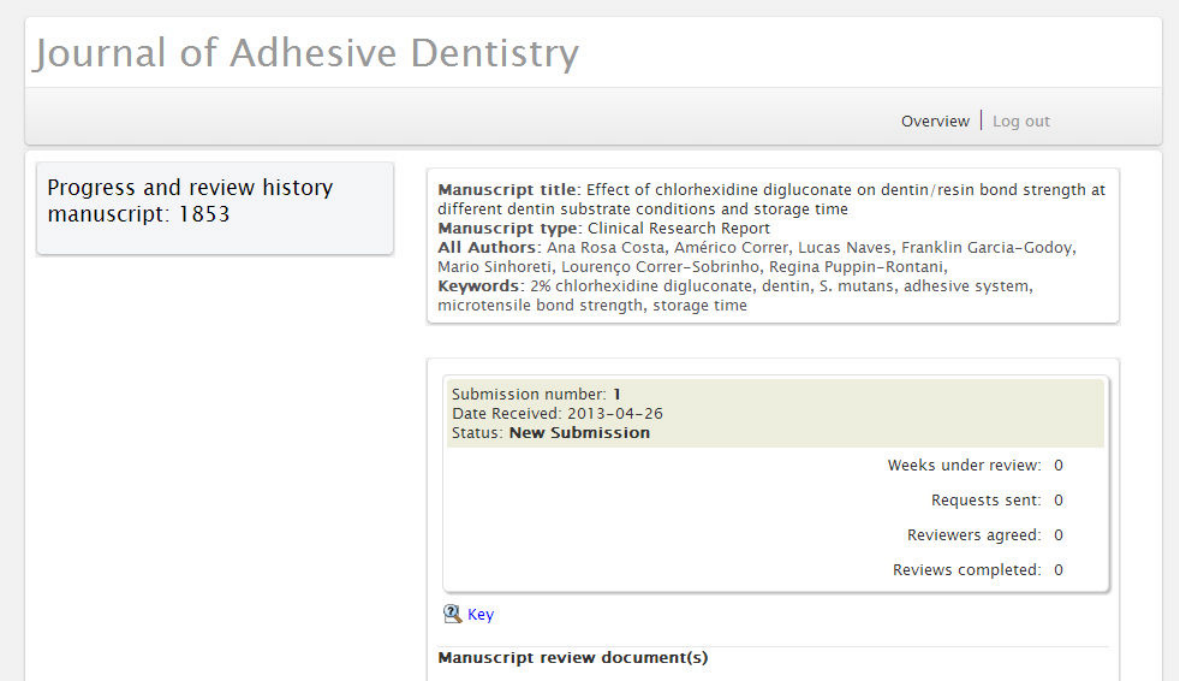
The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Study in vitro the effect of chlorhexidine and ethanol on the bond strength dentin/resin in different conditions of the dentin substrate and storage time"**, register number 041/2010, of Ana Rosa Costa and Regina Maria Puppin Rontani, 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 at 05/14/2010.

Prof. Dr. Pablo Agustin Vargas
Secretário
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
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.

ANEXO 2



Manuscript title: Effect of chlorhexidine digluconate on dentin/resin bond strength at different dentin substrate conditions and storage time.

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