UNIVERSIDADE FEDERAL FLUMINENSE

FACULDADE DE ODONTOLOGIA

CARACTERIZAÇÃO E EFEITO ANTIBACTERIANO DE UM SISTEMA ADESIVO EXPERIMENTAL CONTENDO DIFERENTES CONCENTRAÇÕES DE PROANTOCIANIDINA

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Dissertação apresentada à Faculdade de Odontologia da Universidade Federal Fluminense, como parte dos requisitos para obtenção do título de Mestre, pelo Programa de Pós-Graduação em Odontologia.

Área de Concentração: Dentística.

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DEDICATÓRIA

A Deus, por

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RESUMO

Dias PG. Caracterização e efeito antibacteriano de um sistema adesivo experimental contendo diferentes concentrações de proantocianidina [dissertação]. Niterói: Universidade Federal Fluminense, Faculdade de Odontologia;2018.

O objetivo deste estudo foi avaliar o grau de conversão (GC%), a resistência de união (μTBS) à dentina (imediata e após 12 meses de estocagem) e a atividade antibacterina através da contagem do número de células (n°de *S.mutans*) e da atividade metabólica do biofilme (MTT) de um sistema adesivo experimental contendo diferentes concentrações de proantocianidina: 0, 1%, 2%, 4,5% e 6%. Na primeira etapa os adesivos foram aplicados na superfície de dentina oclusal planificada após o condicionamento ácido. Em seguida foram construídos blocos de resina, os quais foram seccionados longitudinalmente para obter espécimes de palitos dentina-resina (1mm^2) os quais foram utilizados para avaliação de μTBS em estocagem imediata e de 12 meses. O GC% dos sistemas adesivos foi medido por espectroscopia de FT-IR. Na segunda etapa foram avaliadas as propriedades antibacterianas pela análise da contagem de células viáveis (Streptococcus mutans) e atividade antibiofilme dos sistemas adesivos através de espectrofotometria. A incorporação de PA não afetou o GC% dos sistemas adesivos. A μTBS imediata foi semelhante para todos os grupos. Após 12 meses de estocagem, PA4,5% apresentou significativa maior μTBS que PA0, enquanto os demais grupos não diferiram de PA0 e PA4,5%. Os grupos PA0 e PA1% tiveram redução significativa da μTBS após 12 meses de estocagem. Nos grupos PA2%, PA4,5% e PA6% a μTBS se manteve após 12 meses de estocagem. Todos os grupos mostraram atividade antibacteriana tanto para contagem do número de células viáveis quanto para atividade antibiofilme, sem diferença estatística. Concluiu-se que PA2%, PA4,5% e PA6% foram capazes de manter a μTBS após 12 meses de estocagem, diferentemente dos grupos PA0 e PA1%. Após 12 meses de estocagem, PA4,5% apresentou significa maior μTBS quando comparado a PA0. A incorporação de PA não afetou o GC% dos sistemas adesivos e todos os sistemas adesivos apresentaram efeito antibacteriano.

Palavras-chave: Adesivos Dentinários, Agentes de ligação cruzada, resistência de união, atividade antibacteriana.

ABSTRACT

Dias PG. Characterization and antibacterial effect of an experimental adhesive system containing different concentrations of proanthocyanidin.[dissertation]. Niterói: Universidade Federal Fluminense, Faculdade de Odontologia; 2018.

The aim of this study was evaluate the degree of conversion (GC%), the dentin bond strength (µTBS) (immediate and after 12 months in water storage) and antimicrobial activity by counting number of cells (number of S. mutans) and activity (MTT) of an experimental system containing different concentrations of proanthocyanidin: 0,1%, 2%, 4.5% and 6%. In the first step the adhesives were applied to flat occlusal dentine surfaces after acid etching. After resin build-ups, specimens were longitudinally sectioned to obtain beam-like resin-dentine specimens (1 mm^2), which were used for evaluation of µTBS and nanoleakage at the immediate and after 12 months storage. The DC% of the adhesive systems was measured by FT-IR spectroscopy. In the second step, antimicrobial properties were evaluated by analyzing the counting of the viable cells (Streptococcus mutans) and the antibiofilm activity of the adhesives systems through spectrophotometry.The incorporation of PA did not affect the DC% of adhesive systems. Immediate µTBS was similar for all groups. After 12 months of storage, PA4.5% presented significant higher µTBS than PA0, while the other groups did not differ from PA0 and PA4.5%. Groups PA0 and PA1% had a significant reduction of µTBS after 12 months of storage. In groups PA2%, PA4.5% and PA6%, µTBS was maintained after 12 months of storage. All groups showed antibacterial activity both counting the number of viable cells and antibiofilm activity, with no statistical difference. It was concluded that PA2%, PA4.5% and PA6% were able to maintain µTBS after 12 months of storage, unlike PA0 and PA1% groups. After 12 months of storage, PA4.5% presented higher mean µTBS when compared to PA0. The incorporation of PA did not affect the GC% of the adhesive systems and all the adhesive systems presented antibacterial effect.

Key words: Dentin-Bonding Agents, Crosslinking Agents, Bond Strength, Antibacterial Activity.

1 – INTRODUÇÃO

A camada híbrida descrita por Nakabayashi *et al.*[1] é essencial para a adesão entre dentina e resina composta. Nesta camada, o colágeno da dentina desmineralizada e a resina do sistema adesivo formam uma rede entrelaçada, a qual é responsável pela longevidade da restauração. O mecanismo responsável pela degradação desta camada ainda não está totalmente claro, mas sabe-se que ocorre uma série de eventos, em três estágios. No primeiro estágio, ocorre a degradação química dos polímeros devido à absorção de água[6], o segundo estágio consiste na eluição dos oligômeros e dos monômeros não reagidos da camada adesiva e da camada híbrida.[7] No terceiro estágio ocorre a depleção das fibrilas colágenas expostas [23] e ocorre o ataque enzimático pelas metaloproteinases (MMPs) presentes na dentina, que degradam as fibrilas colágenas.[8,9] Esta degradação enzimática é crítica para a estabilidade da camada híbrida.[23]

No entanto, as propriedades biomecânicas da dentina podem ser melhoradas com agentes de ligação cruzada, os quais desempenham um importante papel na biomodificação de matrizes de colágeno desmineralizado[37]. Exemplos de agentes de ligação cruzada comumente usados em odontologia são: glutaradeído, genipin, carbodiimida e proantocianidina (PA). [11,16-18]

A PA é um potente agente antioxidante e de ligação cruzada, que apresenta baixa toxicidade. [19] A PA deriva-se de um subgrupo dos compostos flavonóides, que pode ser encontrada no extrato de semente de uva e em uma grande variedade de frutas, vegetais, flores, nozes e sementes.[20] A ação de ligação cruzada no colágeno utilizando PA demonstrou-se segura e eficaz em modelos in vitro e in vivo [7]. O extrato de semente de uva aumenta a tenacidade da dentina desmineralizada[23] e também inibe a progressão da cárie em raízes.[30] Foi comprovado que a PA promove a formação de ligações de hidrogênio no colágeno tipo I, melhorando, assim suas propriedades mecânicas e preservando a matriz colágena da degradação por colagenases.[21,31,37]

Além de atuar como um agente de ligação cruzada e inibidor de MMPs, soluções com PA também podem diminuir a formação de biofilme de *S.mutans,*[28] reduzir a aderência bacteriana e a formação dos polissacarídeos produzidos pelos *S.mutans.*[29] Zhao *et al.* demonstraram no modelo de biofilme de *S. mutans,* o efeito

protetor da PA em lesões de cárie inicial do esmalte, pela diminuição da profundidade da lesão. Esses dados suportaram a hipótese de a PA inibiu a formação e crescimento do biofilme, com a redução da desmineralização de lesões no esmalte.[28]

Para simplificar o uso de agentes de ligações cruzadas, inibidores de MMPs ou agentes antimicrobianos, busca-se incorporar estas substâncias ao sistema adesivo. Visto que as fibras colágenas são parcialmente desnudas pelo condicionamento ácido, a incorporação destes agentes no sistema adesivo permite que eles interajam com as fibras colágenas. [34]

Dessa forma, o objetivo deste trabalho foi caracterizar um sistema adesivo experimental, de condicionamento ácido total e frasco único, contendo PA em diferentes concentrações (0, 1%, 2%, 4,5% e 6%) e avaliar seu efeito na estabilidade da adesão à dentina e seu efeito antimicrobiano. As hipóteses testadas foram:

1. A presença de PA no sistema adesivo poderia aumentar a estabilidade da união à dentina, sem prejudicar a resistência de união imediata e o grau de conversão do sistema adesivo;
2. A presença de PA promoveria um efeito antibacteriano ao sistema adesivo.

2- METODOLOGIA

O pó do extrato de semente de uva contendo 95% de PA[a] foi dissolvido a 30 e 40% (p/p) em acetona. Após agitação magnética[b] por 30 minutos e armazenamento em recipiente fechado por 72h, as soluções foram filtradas (filtro de papel n°6[c]). [23] A solução de acetona/PA a 30% foi utilizada como solvente de sistemas adesivos com PA a 1%; 2% e 4,5%, enquanto a solução de acetona/PA a 40% foi utilizada como solvente do sistema adesivo com PA a 6%. A solução acetona/PA foi adicionada ao sistema adesivo em peso suficiente para atingir a concentração final esperada de PA em relação ao peso total do sistema adesivo. Acetona pura (BIO-GRADE CHEM, 3 More London Riverside, London Bridge,

Londres SE1 2RE, Reino Unido) também foi adicionada aos sistemas adesivos para obter PA a 1%, 2% e 4,5%.

Os sistemas adesivos foram concluídos com os seguintes monômeros (peso%): HEMA (25%), 4-META (30%), TEGDMA (25%) (Essthec, Inc. Essington, PA, USA). Água (4%), canforoquinona (0.5%) e EDMAB (0.5%) (ethyl N, N - dimethyl- 4aminobenzoato – Sigma Aldrich Chemical Company, Inc., Milwaukee, WI, USA) foram também incorporados. A concentração final de acetona nos sistemas adesivos foi de 15% (peso%).

Os components do adesivo foram pesados em uma balança analítica (AUW 220D, Shimadzu, Tokyo, Japan), misturados e homogeneizados em uma centrífuga dupla (150.1 FVZ Speed Mixer DAC, FlackTek Inc., Herrliberg, Germany) em 2400 rpm por 2 minutos.

Desta forma, os sistemas adesivos com diferentes concentrações de PA foram obtidos: 0, 1%, 2%, 4,5% e 6%(p) e os seguintes grupos experimentais respectivamente: PA0, PA1%, PA2%, PA4,5% e PA6%.

2.1 Caracterização dos sistemas adesivos e avaliação da estabilidade da união à dentina

- Avaliação do grau de conversão

O grau de conversão monomérica dos sistemas adesivos (PA0, PA1%, PA2%, PA4,5% e PA6,5%) foi avaliado através de espectroscopia infravermelha com transformada de Fourier (ALPHA-P FT-IR Spectrometer, Bruker Optics, Ettlingen, Alemanha), utilizando a técnica de refletância total atenuada – ATR – (Platinum Single Reflection Diamond Accessory (BrukerOptics, Ettlingen, Alemanha). Quantidades padronizadas dos sistemas adesivos foram depositadas sobre o cristal de ATR, onde foram obtidos espectros no intervalo entre 1800 e 1500 cm^{-1}, para a observação dos sinais em 1608 e 1638 cm^{-1} correspondentes respectivamente as ligações vinílicas aromáticas do bisfenol A e alifáticas do grupamento funcional metacrilato. Os espectros foram obtidos com 40 varreduras e resolução de 4 cm^{-1}. Após, os sistemas adesivos foram fotoativados durante 40 segundos, com irradiância de 650 mW/cm^2 (DEMI, Kerr Corporation, Middleton, WI, EUA) e os espectros obtidos nas mesmas condições. Foram realizadas 5 avaliações para cada sistema adesivo (n=5). A irradiância do fotoativador foi

conferida antes da fotoativação de cada adesivo através de um radiômetro (Modelo 100, Demetron Inc. Danburry, CT, EUA). O grau de conversão (GC%) de cada compósito foi calculado utilizando a razão entre a altura do sinal em 1638 cm^{-1} e em 1608 cm^{-1} dos filmes polimerizados e não polimerizados, de acordo com a seguinte equação:

$GC\% = 100 \times \{1-(R_{\text{filme polimerizado}} / R_{\text{filme não polimerizado}})\}$, onde R = altura da banda em 1638 cm^{-1} / altura da banda em 1608 cm^{-1}

- Avaliação da resistência de união à dentina

Foram utilizados 80 molares humanos hígidos (Aprovação no Comitê de Ética HUAP CAAE: 73679717.9.0000.5243), que foram inicialmente limpos e armazenados durante 7 dias em solução aquosa de cloramina a 0,5% para desinfecção. Após este período, os dentes foram mantidos em água destilada deionizada até o momento da sua utilização. Os dentes foram divididos aleatoriamente em 10 grupos (n=8) em função dos sistemas adesivos avaliados (PA0, PA1%, PA2%, PA4,5% e PA6,5%) e do tempo de armazenagem: imediato e após 1 ano de estocagem. A dentina oclusal dos dentes de cada grupo foi exposta através do corte da superfície oclusal de esmate por uma cortadeira metalográfica (IsoMet 1000, Buëhler, Lake Bluff, IL, EUA) e o esmalte periférico removido com ponta diamantada em alta rotação. A smear layer da superfície oclusal foi padronizada utilizando lixa de SiC #400 e #600 por 1 minuto cada em politriz (DPU 10, Struers, Dinamarca).

Para todos os grupos foi realizado o condicionamento da dentina com ácido fosfórico a 35% por 15 segundos (Condac37, FGM, Joinville, SC, Brasil), lavagem com água por 30 segundos e secagem com papel absorvente. O adesivo experimental com e sem PA foi aplicado em duas camadas consecutivas, seguidas por secagem com ar por 5 segundos a 15cm de distância e fotoativação por 40 segundos com uma irradiância de 650mW/cm² (DEMI, Kerr Corporation, Middleton, WI, EUA).

Tabela 1: Apresentação da composição dos materiais que foram utilizados no estudo.

Material	Nome	Composição (% em peso)	Fabricante
Sistema Adesivo	Experimental	Acetona (15%), HEMA (25%), TEGDMA (25%), 4-META (30%), água (4%), EDMAB (0,5%), canforoquinona (0,5%),	
Compósito	Filtek Z250	Bis-EMA, Bis-GMA, TEGDMA, UDMA, zircônia/sílica com 0,01 a 3,50μm e tamanho médio de 0,6μm e 60% em volume das partículas de carga	3M ESPE, St. Paul, MN, EUA (641480, 631205, 302416,457029, 508764)
Ácido Fosfórico	Condac37%	Ácido fosfórico 37%, espessante, corante e água deionizada	FGM, Joinville, SC, Brasil (080616)
Extrato de Semente de Uva		95% Proantocianidinas	Active Pharmaceutica Palhoça, Santa Catarina,Brasil (GRS201512002)

HEMA:2-Hidroxietil Metacrilato, TEGDMA: trietileno glicol dimetacrilato, 4-META: 4-metacriloxietil trimelitato anidrido, EDMAB: etil 4-dimetil aminobenzoato; Bis-EMA: dimetacrilato glicol A bisfenol etoxilado; Bis-GMA: bisfenol a diglicidil eter dimetacrilato, UDMA: diuretano dimetacrilato.

Um bloco de compósito (Filtek Z250) foi construído sobre as superfícies dentinárias, em incrementos de 1 mm, fotoativados por 40 segundos (DEMI, Kerr Corporation, Middleton, WI, EUA) até 5 mm de altura. Após armazenagem em água destilada a 37˚C por 24 horas, os dentes foram seccionados em dois planos perpendiculares à interface adesiva (IsoMet 1000, Buëhler, Lake Bluff, IL, EUA), produzindo barras de compósito-dentina (palito) com aproximadamente 1 mm^2 de seção transversal. De cada dente foram obtidos de 15 a 23 palitos.

Passado o tempo de estocagem (24 horas ou 12 meses), os palitos tiveram suas áreas de secções transversais mensuradas com um paquímetro digital (MPI/E-101, Mytutoyo, Tóquio, Japão) e fixados com adesivo à base de cianoacrilato (Superbonder Gel, 3M, São Paulo, Brasil) em garras para ensaio de microtração e submetidos a ensaio de resistência de união (DL 2000, EMIC, São

José dos Pinhais, São Paulo, Brasil) com célula de carga de 50 N e velocidade de deslocamento de 1,0 mm/min. Os valores de resistência adesiva (MPa) foram obtidos pela divisão da carga de ruptura (N) pela área de seção transversal dos palitos (mm^2). O valor de resistência de união de cada unidade experimental (dente) foi obtido através do cálculo da média dos valores dos palitos.

O padrão de ruptura dos palitos foi analisado em estereomicroscópio (SZ40, Olympus, Tóquio, Japão – 40x) e classificado em: falha ADESIVA quando a superfície dentinária está livre de compósito remanescente; falha COESIVA quando a ruptura do espécime foi na dentina ou no compósito; e falha MISTA quando a falha adesiva foi associada à perda de dentina ou presença de compósito remanescente. A distribuição das falhas da interface de união foi avaliada pela frequência percentual.

2.2 Efeito antibacteriano dos sistemas adesivos

- Atividade antibacteriana

A cepa padrão de *S. mutans* ATCC 25175 (American Type Culture Collection, Fiocruz, Rio de Janeiro, RJ, BRA) foi utilizada para avaliar a atividade antibacteriana dos adesivos sem (controle) e com diferentes concentrações de PA (PA1%; PA2%; PA4,5% e PA6%). Foram confeccionados discos de adesivo em placas de 96 poços (Corning, NY, USA), de fundo chato estéril. Para isso, 50 µl do adesivo foram aplicados em cada poço, cobrindo por completo o fundo do mesmo e posteriormente foram fotoativados com uma fonte de luz LED (Demi LED Curing Light, Demetron SDS Kerr, Middleton, WI, US) por 40 segundos e irradiância de $1000mW/cm^2$. A irradiância do fotoativador foi aferida por um radiômetro (Demetron, Kerr, CA, US) a cada cinco espécimes confeccionados. A ponta ativa, com diâmetro de 10,5 mm, foi posicionada sobre a entrada do poço durante a fotoativação. Posteriormente, foram aplicados 100 µl de água destilada estéril e mantidos por 7 dias para remoção de monômeros não polimerizados. Após esta etapa, a placa com os adesivos permaneceu por 1 hora sob luz UV para esterilização[48]. Cada poço recebeu 200 µl de uma suspensão celular padronizada de *S. mutans* ($1x10^5$ UFC/ml) em meio BHI (Brain Heart Infusion, Difco, Sparks, USA). A placa foi incubada em baixa tensão de oxigênio à 37°C durante 24 hs.

Após este período, o crescimento celular foi determinado por espectofotometria com comprimento de onda de 550 nm. Utilizou-se a escala de McFarland (Tabela 2) para correlacionar o número de células bacterianas com a densidade óptica encontrada em nm. De acordo com essa escala padrão, quanto maior a turvação do meio líquido maior a concentração de bactérias por ml.

Tabela 2- Escala de McFarland utilizada para correlacionar o número de células bacterianas com a densidade óptica.

Padrão	Concentração bacteriana $x10^6$/ml	Densidade óptica teórica a 550nm
0,5	150	0,125
1	300	0,25
2	600	0,50
3	900	0,75
4	1200	1,00
5	1500	1,25

- Avaliação da atividade metabólica do biofilme de *S. mutans*

Foram confeccionados discos de adesivo em matrizes metálicas de 10 mm de diâmetro por 1 mm de espessura, os quais foram colocados em placas de 24 poços (TPP, 24 ZellkulturFestplatte, SUI) de fundo chato estéril, mantidos em água destilada estéril por 7 dias para remoção de monômeros não-reagidos.

O biofilme foi formado a partir da adição de 2 ml de uma suspensão celular de *S. mutans* ATCC 25175 (American Type Culture Collection, Fiocruz, Rio de Janeiro, RJ, Brasil) em meio de cultura *Brain Heart Infusion* (BHI, Difco, Sparks, EUA) suplementado com 2% de sacarose por poço.

Previamente, a cepa de *S. mutans* ATCC 25175 foi cultivada durante 24 horas em BHI a 37 °C em condições de baixa tensão de oxigênio. O inóculo bacteriano foi ajustado para o ponto correspondente ao 0,5 da escala de McFarland. Posteriormente, a suspensão celular foi diluída 1: 100, e, em seguida, 10 µl do inóculo foi adicionado em cada poço. O volume final de 2 ml por poço foi atingido pela adição de BHI com 2% de sacarose. As placas de cultura de 24 poços

foram incubadas a 37 °C em condições de baixa tensão de oxigênio, durante 48 horas. Durante os 2 dias de formação do biofilme, o meio de cultura foi substituído a cada 24 horas.

A atividade metabólica dos biofilmes de *S. mutans* formados sobre os discos de dentina foi analisada pelo ensaio de redução do brometo de metilotiazolilobrometo de tetrazólio azul (MTT, Sigma Aldrich, St. Louis, EUA). A reação que utiliza o MTT trata-se de um ensaio colorimétrico que mede a redução enzimática de MTT, um tetrazol amarelo, a formazan de coloração púrpura. Para isso, após a lavagem suave com PBS estéril (pH 7,2) dos discos para a remoção das células não aderidas ao biofilme, 1,0 ml de MTT estéril (1 mg / ml em PBS) foi adicionado a cada poço e incubado à 37°C em condições de baixa tensão de oxigênio durante 1 hora. Depois, 1,0 ml de dimetilsulfóxido (DMSO) foi adicionado em cada poço e a placa, mais uma vez, foi incubada durante 20 minutos à temperatura ambiente, protegidas da luz e com agitação suave. Ao final, as suspensões foram submetidas a um leitor de microplacas com comprimento de onda de 540 nm. Uma absorbância maior é indicativo de uma concentração maior de formazan, o que, por sua vez, indica uma maior atividade metabólica do biofilme.

3 - ARTIGO PRODUZIDO

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Characterization and antibacterial effect of an experimental adhesive system containing different concentrations of proanthocyanidin

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Keywords: Dentin-Bonding Agents, Cross-linker, antimicrobial, bond strength.

Characterization and antibacterial effect of an experimental adhesive system containing different concentrations of proanthocyanidin

ABSTRACT

Purpose: The aim of this study was evaluate the degree of conversion (GC%), the dentin bond strength (μTBS) (immediate and after 12 months in water storage) and antibacterial activity by counting number of cells (number of S. mutans) and (MTT) of an experimental adhesive system containing different concentrations of proanthocyanidin: 0,1%, 2%, 4.5% and 6%. **Methods**: In the first step the adhesives were applied to flat occlusal dentine surfaces after acid etching. After resin build-ups, specimens were longitudinally sectioned to obtain beam-like resin-dentine specimens (1 mm^2), which were used for evaluation of μTBS at the immediate and after 12 months storage. The DC% of the adhesive systems was measured by FT-IR spectroscopy. In the second step, antibacterials properties were evaluated by analyzing the bacterial growth (*S. mutans*) and the antibiofilm activity of the adhesives systems through spectrophotometry. **Results:** The incorporation of PA did not affect the DC% of adhesive systems. Immediate μTBS was similar for all groups. After 12 months of storage, PA4.5% presented significant higher μTBS than PA0, while the other groups did not differ from PA0 and PA4.5%. Groups PA0 and PA1% had a significant reduction of μTBS after 12 months of storage. In groups PA2%, PA4.5% and PA6%, μTBS was maintained after 12 months of storage. All groups showed antibacterial activity both counting the number of viable cells and antibiofilm activity, with no statistical difference. It was concluded that PA2%, PA4.5% and PA6% were able to maintain μTBS after 12 months of storage, differently of PA0 and PA1%. After 12 months of storage, PA4.5% presented higher mean μTBS when compared to PA0. The incorporation of PA did not affect the GC% of the adhesive systems and all the adhesive systems presented antibacterial effect.

Clinical Significance: The incorporation of 2%, 4.5% and 6% PA into adhesive systems maintained the dentin μTBS after 12 months storage, without affect DC% of adhesive systems. All adhesive systems presented antibacterial effect when compared to a commercial adhesive system.

Introduction

The hybrid layer described by Nakabayashi et al. [1] is essential for adhesion between dentin and resin. This interlaced network is also responsible for the longevity of the restoration. However, in long-term the degradation of hybrid layer has been related.[2,3,4,5] The mechanism responsible for this degradation is not totally clear, but it is known that a series of events occurs. In the first stage, the chemical degradation of the polymers occurs due to the absorption of water,[6] the second stage consists of the elution of the oligomers and the unreacted monomers of the adhesive layer and the hybrid layer[7], and in the third stage, the collagen fibrils are depleted. In this last stage, the enzymatic attack occurs by the metalloproteinases (MMPs) present in the dentin that degrade the collagen fibrils.[8,9] The enzymatic degradation is critical for the stability of the hybrid layer.[3,4,10]

However, the cross-linkers agents play an important role in the biomodification of demineralized collagen matrices.[11] They can improve strength the collagen fibrils[12,13] and increase the biomechanical properties of dentin[14,15]. Examples of cross-linkers commonly used in dentistry are glutaradehyde, genipin, carbodiimide and proanthocyanidin (PA) .[11,16,17,18]

The PA is a potent antioxidant and cross-linker agent, with low toxicity.[19] It can be found in grape seed extract and in wide variety of fruits, vegetables, flowers, nuts and seeds.[20] PA has shown to promote the formation of hydrogen bonds in type I collagen, thus improving its mechanical properties and preserving the collagen matrix from degradation by exogenous collagenases.[21-25] Grape seed extract is gerally used and has shown to be safe and effectivelly cross-link collagen in both in vitro and in vivo models.[26,27]

In addition to cross-linking and inhibitor of MMPs action, PA solutions may also decrease S. mutans biofilm formation,[28] reduce bacterial adhesion, reduce the formation of the polysaccharides produced by S. mutans,[29] and inhibit the progression of root caries.[30] Zhao et al. demonstrated that in the S. mutans biofilm model the protective effect against PA in caries lesions of the enamel was demonstrated by the decrease of the depth of the lesion and the data of their research supported the hypothesis that the cross-linker inhibited the formation and growth of the biofilm, with the reduction of demineralization in the enamel.[28]

However, the most of studies evaluated the use of solution of PA as pre treatment.[16,17,21,27,30] To simplify the use of crosslinking agents, MMP inhibitors or

antimicrobial agents, they can be incorporated into the adhesive system. Since the collagen fibers are exposed by acid etching, the incorporation of these agents into the adhesive system allows them to interact with the collagen fibers.[15]

Thus, the purpose of the present study was to characterize an experimental etch-and-rinse adhesive system, containing PA (of grape seed extract) in different concentrations and evaluate its effect on stability of dentin adhesion and its antimicrobial effect. The hipothesis tested were: 1) adhesive systems with different concentrations of PA affect the dentin bond strenght (immediate and after 12 months of storage) and the degree of conversion of adhesive systems; 2) adhesive systems with different concentrations of PA affect the viable cell number and antibacterial activity.

Materials and methods

The powdered grape seed extract containing 95% PA[a] was dissolved at 30 and 40% (w/w) in acetone. After magnetic agitation[b] for 30 minutes and 72 hours of storage in closed bottle and, the solutions were filtered (paper filter n∘6)[c].[29] The solution of acetone/PA at 30% was used as solvent of adhesive systems with PA at 1%; 2% and 4.5%, while the solution of acetone/PA at 40% was used as solvent of adhesive system with PA at 6%. The solution acetone/PA was added to adhesive system in weight sufficient to achieve the final concentration expected of PA with respect to the weight total of adhesive system. Pure acetone[d] was also added adhesive systems for obtain PA at 1%, 2% and 4.5%.

The adhesive systems were completed with the following monomers (wt.%): HEMA[e] (25%), 4-META[e] (30%), TEGDMA[e] (25%). Water (4%), Camphorquinone (0.5%) and EDMAB (0.5%) (ethyl N, N -dimethyl- 4aminobenzoato)[f] were also incorporated. The final concentration of acetone in adhesive systems was 15% (wt.%).

The components of the adhesive were weighed in an analytical balance[g], mixed and homogenized in a dual centrifuge[h] at 2400 rpm for 2 minutes.

Thus, adhesive systems with different concentrations of PA were obtained 0, 1%, 2%, 4.5% and 6% (wt), and the following experimental groups respectively: PA0, PA1%, PA2%, PA4.5%, and PA6%.

Caracterization of adhesive systems and evaluation of dentin bonding stability

Degree of conversion (DC%)

Increments of each adhesive system were inserted into a teflon mold (0.785 mm^3) positioned onto an ATR crystal of the FT-IR spectrometer[i] (Alpha-P/Platinum ATR Module, Bruker Optics GmbH, Ettlingen, Germany) and the spectra between 1500 and 1800 cm^{-1} were recorded with the spectrometer operating with 40 scans and at a resolution of 4 cm^{-1}.

Afterwards, the increments were light-cured for 40 seconds with an irradiance of 650mW/cm² [j] (DEMI, Kerr Corporation, Middleton, WI, USA) and the spectra were recorded exactly as performed for the unpolymerized increments. Each adhesive system was evaluated in triplicate (n=3). The DC% was calculated from the ratio between the high sing in 1638 cm^{-1} and 1608 cm^{-1}which were obtained from the polymerized and unpolymerized increments, using the following equation: DC% = 100 x [1 – (Rpolymerized / Runpolymerized)], where R = integrated area at 1638 cm^{-1} / integrated area at 1608 cm^{-1}

Microtensile bond strength (µTBS) measurement

Eighty extracted, caries free, human third molars (Ethical Committee Approval HUAP CAAE:73679717.9.0000.5243) were disinfected in 0.5% chloramine T solution for 7 days, stored in distilled water and used within six months after extraction. The occlusal dentin of the teeth was exposed using a cut machine[k] and the peripheral enamel removed using a diamond bur[l] (#4138). The smear layer of dentin was standardized with #400 e #600-grit SiC papers[m] in politriz[n] for 1 minute. The teeth were divided into 10 groups (n=8) according to the adhesive systems tested (PA0, PA1%, PA2%, PA4.5%, and PA6%) and two times of storage: immediate and after 12 months of storage.

Dentin surfaces were etched with 37% phosphoric acid for 15 seconds[o], rinsed with distilled water for 30 seconds and blot dried with absorbent paper. Two consecutive layers of each adhesive system were applied on active mode, followed by gentle air stream for 5 seconds and light curing for 40 seconds with an irradiance of 650mW/cm² [j]. Five increments of 1 mm tick resin composite[p] (Filtek

Z250) were horizontally added to the bonded surfaces and individually light cured for 40 seconds with an irradiance of $650 mW/cm^2$.

After storage in distilled water at 37°C for 24 h, the teeth were longitudinally sectioned in both the mesio-distal and buccal-lingual directions, across the bonded interfaces[k] producing beams with a crosssectional area of approximately $1 mm^2$. Each tooth provided 15 to 23 beams. After each period of storage, the beams had their adhesive interfaces crosssectional area measured with a digital caliper[q] and were individually fixed to a microtensile device[r] using cyanoacrylate glue[s] and loaded under tension using a universal testing machine[t] at a crosshead speed of 1,0 mm/min until failure occurred. The μTBS (MPa) was obtained by dividing the load at failure (N) by the cross-sectional area of the beam (mm^2). Each failed beam was evaluated with a stereomicroscope[u] at 40x magnification and the failure mode was classified as: adhesive (failures at the adhesive interface), cohesive (failures occurring mainly within dentin or resin composite), or mixed (mixture of adhesive and cohesive failure within the same fractured surface).

Antibacterial effect of adhesive systems

Antibacterial activity

The number of viable S. mutans cells in contact with the adhesives without and at different concentrations of PA (1, 2, 4.5 and 6%) was performed using the ATCC 25175 standard strain. Were made adhesive discs in 96-well plates[t] (Corning, NY, USA) with sterile flat bottom. To do this, 50 μl of the adhesive was applied to each well, completely covering the bottom of the well and then photoactivated with a LED light source[j] for 40 seconds and irradiance of 1000mW / cm^2. The irradiance of the photoactivator was measured by a radiometer[v] for every five specimens made. The active tip, with a diameter of 10.5 mm, was positioned on the entrance of the well during the photoactivation. Subsequently, 100 μl of sterile distilled water was applied and maintained for 7 days to remove unpolymerized monomers. After this step, the plate with adhesives remained for 1 hour under UV light for sterilization. Each well received 200 μl of a standardized cell suspension S. mutans (1x105 CFU / ml) in BHI medium. The plate was incubated at low oxygen tension at 37 ° C for 24hs. After this period, cell growth was determined by spectrophotometry with a wavelength of 550 nm. The Mc Farland

scale was used to correlate the number of bacterial cells with the optical density found in nm. According to this standard scale, the higher the turbidity of the liquid medium the higher the concentration of bacteria per ml.

Biofilm metabolic activity

Adhesive discs were made into metal matrices of 10mm diameter by 1mm thickness, which were placed in sterile flat bottom 24-well plates[w], kept in sterile distilled water for 7 days for removal of unreacted monomers.

Over the sterilized adhesive disks were placed 2.0 mL of Brain Heart Infusion culture medium[x] supplemented with 2 wt% sucrose was added to each well. The strain of S. mutans ATCC 25175[y] used was cultured for 24 hours in BHI at 37.0 ° C under low oxygen tension conditions. The bacterial inoculum was adjusted for optical density (OD) of 0.5 to 550.0 nm in accord with the McFarland scale. The bacterial suspension was diluted to 1: 100, and then 10.0 uL of the inoculum was added to each well. The 24-well culture plates were incubated at 37 ^{o}C under low oxygen tension conditions for 48 hours. Over the 2 days of biofilm formation, the culture medium was replaced each 24 hours.

The cell viability of the S. mutans biofilms on the adhesive disks was analysed by the tetrazolium azide[f] reduction of the methyl tetrazolylbromide bromide assay. The reaction using MTT is a colorimetric assay that measured the enzymatic reduction of MTT, a yellow tetrazole, the purple color formazan. Therefore, 1.0 mL of sterile MTT (1.0 mg / ml in PBS) was added to each well and incubated at 37.0 ^{o}C under low oxygen tension conditions for 1 hour. The discs were then transferred to a new 24-well plate, 1.0 mL of the dimethylsulfoxide (DMSO) was added to each well and the plates were incubated for 20 minutes at room temperature, protected from light and with gentle agitation. The DMSO suspensions were subjected to a microplate reader with wavelength of 540.0 nm. A higher absorbance is indicative of a higher concentration of formazan, which, in turn, indicates a higher metabolic activity of the biofilm.

Statistical Analysis

The data were analyzed using Statgraphics Centurion XVI software[z]. Initially, the normal distribution of errors and the homogeneity of variances were

checked using Shapiro-Wilk´s and Levene´s test, respectively. Based on these preliminary analyses, the DC% data were analyzed by one-way ANOVA. The μTBS data were analyzed by two-way ANOVA and Tukey´s HSD test for multiple comparisons. The antibacterial activity (MTT) data were submitted to one-way ANOVA and Tukey´s HSD test for multiple comparisons. The cell number count data were submitted to one-way ANOVA. The analyses were performed at a significance of α=0.05.

Results

The means and standard deviations of the μTBS test and DC% are summarized in Table 1. For μTBS test two-way ANOVA showed that the independent factor time of storage (p= 0.0000) and the interaction PA concentration vs time of storage (p= 0.0384) were statistically significant. On the other hand, the independent factor PA concentration (p= 0.2106) was not statistically significant. In the immediate time, the μTBS was similar for all groups. After 12 months of storage, the μTBS of PA0 and PA1% decrased significantly, while in the other groups (PA2%, PA4.5% and PA6%) the μTBS did not decrease significantly. When groups were compared after 12 months of storage, the group PA4.5% showed significant larger μTBS than PA0. The other groups were similar to PA0 and PA4.5%.

Table 1. Means and standard deviations of μTBS (MPa) after each period of storage and DC%.

Groups	μTBS		DC%
	Immediate	12 Months	
PA0	29.9 (9.1) Aa	12,4 (3.9) Ab	93.1 (6.3)
PA1%	27.6 (9.8) Aa	13.6 (4.6) ABb	93.2 (4.2)
PA2%	27.9 (8.6) Aa	18.1 (5.4) ABa	94.2 (2.3)
PA4.5%	26.5 (7.4) Aa	26.4 (5.9) Ba	95.0 (1.6)
PA6%	25.2 (6.9) Aa	15.5 (2.1) ABa	93.8 (3.5)

For each column values with the same uppercase letters are statistically similar. For each row, values with the same lowercase letters are statistically similar (Tukey´s HSD, α=0.05). Values of DC% were not statistically different.

With regards DC% data, one-way ANOVA showed no significant difference among groups (p= 0.8471). The incorporation of PA did not affect the DC% of experimental adhesive systems.

The failure mode analysis after µSBS test is presented in Figure 1.The failure mode was predominantly adhesive in all groups.

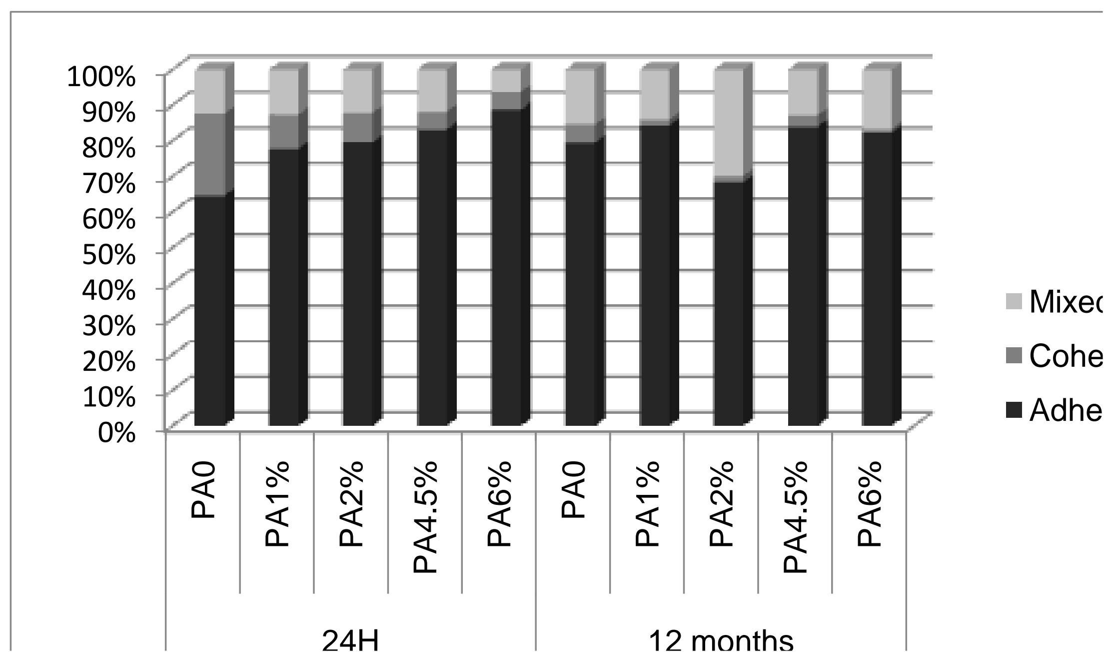

Figure 1. Failure mode (%) of each group after each period of storage in distilled water.

The results of antibacterial activity and biofilm metabolic activity (MTT) of adhesive systems are presented in Table 2. For this analysis, one-way ANOVA showed significant difference among groups (p=0.0000). All groups showed lower cell growth when compared with positive control (BHI+S.*mutans*).

For biofilm metabolic activity (MTT) one-way ANOVA showed no significant difference among groups (p=0.4633). All groups inhibited the production of bacterial biofim. Considering that the group PA0 also caused biofilm inhibition, a comparison was made with commercial adhesive Adper Single Bond 2 (ASB2)[p] All adhesive systems showed significant larger antibacterial activity than ASB2 (p=0.0000).

Table 2- Means and standard deviations of cell growth count and biofilm metabolic activity (density optical - DO).

Groups	Cell growth count (DO)	Biofilm metabolic activity - MTT reduction (DO)
PA0	0.054 (0.006) A	0.062 (0.019) A
PA1%	0.058 (0.002) A	0.048 (0.010) A
PA2%	0.069 (0.003) A	0.081 (0.009) A
PA4.5%	0.053 (0.006) A	0.050 (0.005) A
PA6%	0.051 (0.006) A	0.062 (0.010) A
BHI	0.073 (0.006) A	
BHI+S.*mutans*	0.312 (0.131) B	
ASB2		0.326 (0.031) B

For each column values with the same uppercase letters are statistically similar (Tukey´s HSD, α=0.05). ASB2= Adper Single Bond 2

Discussion

In the present study an experimental adhesive system with known formulation was used.[35]This was done to analyze the real effects of the cross-link and antibacterial agent, since commercially available adhesive systems can present ingredients not described and the exact amount of each component is not reported. Moreover, an experimental adhesive system synthesized with PA could eliminate one step in the restorative procedure.

The PA was choose due to its ability to increase mechanical properties of the dentin,[12] to reduce the biodegradation by host-derived and exogenous proteases,[36] and to be rich in antioxidant polyphenolics that could show antibacterial activity. [37]However, these proprieties of PA never were evaluated when it was inserted into adhesive system.

The first research hypothesis of this study was partially accepted, since the incorporation of PA did not affect the DC% and immediate μTBS of experimental

adhesive systems. However, different concentrations of PA had effect in µTBS after 12 months of storage.

The DC% of adhesive systems was studied because it is an important factor for the dentin bonding durability .[6] Complete polymerization of the adhesive is essential to form a hybrid layer with high quality [15]. A poor polimerization of adhesive system can allow that monomers unreacted/oligomers are leach out of the hybrid layer, exposing collagen fibrils, creating pores in hybrid layer, increasing your permeability, and reducing the sealing ability. [(6-8,38)] In this study, DC% of experimental adhesives range from 93.1 to 95%. All adhesive systems had high DC%, without difference among groups. Therefore, the different concentrations of PA did not interfere with DC% of adhesive systems. This high DC% was similar that found in others previous studies [(39,40).] The presence of TEGDMA, an aliphatic monomer with high flexibility, can increase the reactivity of the adhesive system. Furthermore, HEMA a “solvent-like” monomer may continue reacting with the C=C bonds even after the long chain monomers remain entrapped into the polymer network.[41]

With regard to µTBS, the immediate µTBS was similar for all groups. Thus, the presence of PA in different concentrations did not affect the immediate µTBS. The groups PA0 and PA1% showed significant decrease of µTBS after 12 months of storage. This can indicate that the concentration of 1% of PA was very low, showing not effect on longevity of adhesion. On the other hand, the µTBS after 12 months of storage was maintained for PA2%, PA4.5% and PA6%. This can be explained because capacity cross-linker of the PA[12,17,42]. This can indicate that even the PA inserted into adhesive systems, it was capable to act as cross-linker and MMP-inhibitor. The insertion of PA into dental adhesives can be benefic due the presence of PA in the hybrid layer and their release for a long time. The major structural component of dentin tissue is the type-1 collagen and its inherent strength is derived from the extracellular formation of covalent intermolecular cross-links. A great and consistent increase in the elastic modulus of dentin matrix was observed after the pretreatment with PA-based extracts.[12,15,43] Stiffening and strengthening of dentin collagen has been attributed to increased of interpeptide hydrogen bonding between collagen fibrils.[12] Hydrogen bonds between the protein

amide carbonyl and phenolic hydroxyl groups are considered as the crucial forces for stabilizing PA-treated collagen fibrils.[44]

PAs are known as non-specific MMP-inhibitors, which can also have some role in the prevention resin-dentin bond degradation. The most accepted theory is that MMP inhibitors exert their inhibitory effect through the divalent chelation of metal ions, especially the catalytic zinc atom, so they must have some functional groups for chelating the zinc ion of catalytic domain. In this way prevent MMPs from binding to collagen substrate and its further cleavage. [44] According previous study, [34] the pretreatment of demineralized dentin for 1 min with 5% PA decreased the enzymatic activity up to 64% when compared to the values control group. Besides, pretreatment of dentin for 5 min with 1% PA showed up to 69% of MMP inhibition. [44] A reduction in the amount of released MMP-8, -2 and -9 was observed in the extracts of PA pre-treated demineralized dentin.[34]

An adhesive system similar to Adper Single Bond Plus with 5 wt% PA was also evaluated.[42] Samples treated with PA-incorporated adhesive system, demonstrated the collagen fibrils with intact and normal cross-banding, organization and dimensions.

Another study[43] showed that the insertion of PA in concentrations lower or equal to 2% in the adhesive have any adverse effect on the immediate dentin µTBS, while the adhesive with 3% PA can cause significant damage in µTBS. Differently, in present study none of the concentrations used into adhesive system jeopardized the immediate µTBS. The difference with our study is that none of concentrations used into adhesive system jeopardized the immediate µTBS.

When the results of µTBS after 12 months of storage were evaluated, it can be observed that PA4.5% was the only group with significant greater µTBS than PA0. Then, the concentration of 4.5% of PA can be considered the best for insertion into adhesive system. The 6% concentration may have been beyond ideal because it is a powder that even being filtered may have disrupted adhesion

In immediate and after 12 months of storage evaluation, the adhesive failures were predominant, which can indicate the reliability of the test and demonstrate that the evaluation of the bonding interface occurred.[3]

With regards to antibacterial activity, all adhesive systems showed similar antibacterial activity independent of PA concentration. In previous study was

observed that 3mg/ml crude grape seed PA extract promoted complete inhibition of all bacterial strains tested, the study suggest that polyphenols in PA have bactericidal effects on methicillin-resistant Staphylococcus areus strains due your ability to disrupt cell wall and/or cell membrane and to enlargement cell.[29] The bacterial activity was also tested in root caries, with incubation of S. mutans and Lactobacillus acidophilus. Pretreatment with PA diluted in PBS resulted in the inhibition of root caries lesion compared to the groups 'no treatment' due not only to the decrease in collagen digestibility, but also due to the prevention of dentin demineralization by fixation of collagen on dentin, that may reduce diffusion of calcium and phosphate ions out of the dentin lesion.[37]

Although in our study the biofilm model does not mimic exactly the complex microbial community found in coronal dental plaque, it emphasizes the characteristics of virulence of the biofilm. Furthermore, biofilms using a single organism are advantageous in examining the mechanism of action of therapeutic agents on S. mutans physiology, especially on the glucan-mediated processes involved in the formation of the polysaccharide matrix in biofilm.[45]

Previous study [45] showed that the biofilm development and acidogenicity were significantly affected by topical applications of PA. Activities of GTF B and C (glucosyltransferases) are inhibited significantly by PA. This inhibition has many implications for biofilms development because glucans synthesized by these enzymes are retained on the pellicle promoting accumulation of Streptococcus mutans and other cariogenic bacteria on the tooth surface, and contributing to the formation of the matrix of the biofilms. PA bind to proteins forming protein–polyphenol complexes, which could inhibit the activity of GTFs. Consequently reduced the formation and accumulation of S. mutans biofilms by mostly diminishing the amounts of insoluble glucans in the biofilms matrix also reduced your acidogenicity. Another study [46] determined the influence of the PA application on dental caries development in rats and S. mutans biofilm formation on saliva-coated hydroxyapatite surface. The incidence and severity of smooth-surface caries were significantly reduced in the groups treated with topical applications of PAC significantly reduced the biomass and total amount of extracellular insoluble polysaccharides of S. mutans biofilms (35–40% reduction) compared with the control group .

The results of biofilm metabolic activity corroborated the results of antibacterial activity. All groups showed decreased biofilm metabolic activity, including the control group (PA0). Due to the antibacterial effect showed by PA0, the results of biofilm metabolic activity were compared commercial adhesive system, which showed a high metabolic activity. Therefore, antimicrobial activity was verified in experimental adhesive with or without PA.

A study[47] conducted between adhesive systems with and without antimicrobial showed that all had this capacity, a fact that was attributed to the monomeric HEMA common to all of them, different from what happened in our study, because ASB2 did not present antimicrobial activity, therefore we believe that the 4-META monomer was responsible for the result found .

Consequently , the second research hypothesis was rejected, since the adhesive system without PA also presented antimicrobial activity

Colocar Artigo de 4-meta com efeito antibacteriano.

a Active Pharmaceutica, Palhoça, SC, Brazil

b Quimis®, Diadema, SP, Brazil.

c Whatman, London, UK.

d Bio-Grade Chem, London Bridge, LDN,UK.

e Essthec Inc. Essington, PA, USA.

f Sigma Aldrich Chemical Company, Milwaukee, WI, USA.

g. AUW 220D, Shimadzu, Tokyo, Japan.

h 150.1 FVZ Speed Mixer DAC, Herrliberg, Germany.

i Alpha-P/Platinum ATR Module,Bruker Optics GmbH, Ettlingen, Germany.

j DEMI, Kerr Corporation, Middleton, USA.

k IsoMet 1000Buëhler, Lake Bluff, IL, USA.

l KG Sorensen, Cotia, SP, Brazil.

m Arotec, Cotia, SP, Brazil.

n DPU 10,Struers, Dinamarca.

o Condac37, FGM, Joinville, SC, Brazil.

p 3M Espe, St Paul, MN, USA.

q MPI/E-101, Mytutoyo; Tokyo, Japan.

r ODMT03d, Odeme Biothecnology, Joaçaba, SC, Brazil.

s Superbonder Gel, 3M, São Paulo, Brazil.

t EMIC DL 2000, São José dos Pinhais, SP, Brazil.

u SZ40,Olympus, Tokyo, Japan.

v Demetron Inc. Danburry, CT, EUA.

w TPP, 24 ZellkulturFestplatte, SUI .

x BHI, Difco, Sparks, USA.

y American Type Culture Collection, Fiocruz, Rio de Janeiro, RJ, Brazil.

z STATPOINT Technologies Inc, USA.

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