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FACULDADE DE ODONTOLOGIA DE PIRACICABA - FOP

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Avaliação de agentes antibacterianos sobre bactérias cariogênicas, resistência e degradação da união resina/dentina.

Tese de Doutorado apresentada a Faculdade de Odontologia de Piracicaba da UNICAMP, para obtenção do Título de Doutor em Odontologia, Área de Odontopediatria.

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Este exemplar corresponde à versão final da Tese defendida pela aluna, e orientada pelo Prof[a]. Dr[a]. Regina Maria Puppin Rontani.

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" Nada do que vivemos tem sentido, se não tocarmos o coração das pessoas"
(Cora Coralina).

RESUMO

Esta tese apresenta três capítulos. O Capítulo 1 avaliou o efeito dos sistemas adesivos, Clearfil SE Protect (primer com monômero antibacteriano) (CP), Clearfil SE Bond (CB) e Adper Single Bond 2 (SB) e do digluconato de clorexidina 2% (CHX) sobre *Streptococcus mutans*. Bactérias viáveis foram mensuradas depois que, fatias de dentina contaminadas com *S. mutans* foram limpas ou não com CHX e tratadas com os sistemas adesivos, e depois que discos de sistemas adesivos foram incubados em suspensão bacteriana. Para a contagem bacteriana usou-se teste de Friedman, ANOVA-R e teste t (LSD), para o tamanho dos halos de inibição foi utilizado Kruskal-Wallis e teste t ($p<0.05$). CP quando comparado com o CB mostrou maior efeito antibacteriano após 60 min de contato com as bactérias. A CHX não aumentou o efeito antibacteriano dos sistemas adesivos. O CP mostrou efeito inibidor no crescimento de *S. mutans* na superfície do ágar. Conclui-se a CHX não teve efeito no *S. mutans* antes da aplicação dos sistemas adesivos. CP exibiu considerável atividade antibacteriana e pode ser útil para reduzir bactérias cariogênicas. O Capítulo 2 avaliou a atividade antibacteriana do CP e do CB contra *Streptococcus*, *Actinomyces e Lactobacillus*. Discos dos sistemas adesivos foram mantidos em contato com 150 µL de suspensões bacterianas de uma espécie ou misto de bactérias por 15, 30 e 60 minutos. A atividade metabólica foi determinada usando ensaio colorimétrico XTT e a viabilidade bacteriana foi mensurada usando os ensaios MTT e Live/Dead. Os resultados foram submetidos à ANOVA e ao teste f de Scheffe ($p<0.05$). O CP apresentou efeito bactericida na suspensão bacteriana comparado ao CB e ao Controle (suspensão bacteriana sem tratamento) nos três ensaios. Para a suspensão com três espécies bacterianas o CP agiu após 60 minutos de contato. Conclui-se que o CP é bactericida após a polimerização contra bactérias cariogênicas. O Capítulo 3 avaliou o efeito do CP, CB e CHX na resistência da união (RU) e na degradação da interface resina/dentina após ciclagem termomecânica (TM). A RU, degradação adesiva, adaptação marginal e nanoinfiltração foram avaliadas após os espécimes de dentina desmineralizada serem limpos ou não com CHX, tratados com CP ou CB e receberem ou não ciclagem TM (500 ciclos térmicos e 100.000 ciclos mecânicos). Os resultados foram submetidos a ANOVA

três fatores e ao teste de Tukey (5%). A análise do modo de fratura, a porcentagem de fenda marginal e a nanoinfiltração foram analisados de forma descritiva. Não houve diferença estatística para a RU entre os grupos com e sem TM, contudo, a aplicação de CHX aumentou a RU para os espécimes TM e houve predomínio de falha mista. Fenda marginal foi observada em todos os espécimes, mas a porcentagem de fenda foi maior nos espécimes do grupo TM. Todos os grupos mostraram nanoinfiltração na interface adesiva e aumento do depósito de prata foi observado após a ciclagem termomecânica. Conclui-se que o sistema adesivo não afetou a RU. A CHX aumentou a RU e teve um efeito positivo na prevenção da nanoinfiltração após a ciclagem.

PALAVRAS-CHAVE: Sistema adesivo antibacteriano; dentina desmineralizada; digluconato de clorexidina; bactérias cariogênicas; resistência da união; ciclagem termomecânica; nanoinfiltração

ABSTRACT

This Thesis present three chapters. The Chapter 1 evaluated the effect of bonding systems Clearfil SE Protect (primer with antibacterial monomer) (CP), Clearfil SE Bond (CB) e Adper Single Bond 2 (SB) and 2% digluconate chlorhexidine (CHX) on *Streptococcus mutans*. Viable bacteria were verified after that dentine slices contaminated with *S. mutans* were cleaned or not with CHX and treated with adhesive systems, and after that adhesive systems discs were incubated in bacterial suspension. The bacterial counts were analyzed using the Friedman test, ANOVA-R and t test (LSD), and the inhibitory zones were analyzed using the Kruskal-Wallis and t test (LSD) ($p<0.05$). CP when comparing with CB showed higher antibacterial effect after 60 min of bacterial contact. CHX did not improve the antibacterial effect of the bonding systems. Cured CP specimens showed an inhibitory effect on the growth of *S. mutans* on the agar surface. It was concluded that treatment of the dentin surface with CHX did not have an effect on *S. mutans* before applying bonding systems. CP exhibited considerable antibacterial activity and may be useful for reducing cariogenic bacteria. The Chapter 2 investigated the antibacterial effect of CP and CB against *Streptococcus. Actinomyces* and *Lactobacillus.* Discs of adhesive systems were kept in contact with 150 µL of single or mixed bacterial suspension by 15, 30 and 60 minutes. The metabolic activity was determined by XTT assay and the viability bacterial by MTT and Live/Dead assay. Results were submitted to ANOVA and Scheffe's f-test ($p<0.05$). The CP showed bactericidal effect on the bacterial suspension compared with CB and buffer control (bacterial suspension without treatment), in all three assays. To three mixed bacterial suspensions the CP presented effect after 60 contact with bacteria. It was concluded that the CP was bactericidal after cured against cariogenic bacteria. The Chapter 3 evaluated the effect of CP, CB and CHX on the bond strength (BS) and bonding degradation of the resin/dentin interface after thermomechanical cycling (TM). The BS, bonding degradation, marginal adaptation, nanoleakage were analyzed after the specimens demineralized dentin were cleaned or not with CHX, treated with CP or CB and received or not TM (500 cycles thermal/100.000 cycles mechanical). Data were submitted to three-way ANOVA and Tukey's test (5%). The failure mode,

percentage of gap marginal and the nanoleakage were evaluated descripitivelly. There was no difference significance to bond strength among group that received or not TM; however, the CHX maintained the bond strength for specimens of TM group and showed mixed failure for theses groups. Marginal gap was observed on all specimens, but to TM group the percentage of marginal gap was increased. All groups showed nanoleakage at the resin/dentin interfaces and an increase in silver deposition could be notice after thermomechanical cycling. It was concluded that adhesive system did not affect the bond strength. CHX increased the bond strength and had a positive effect to prevent nanoleakage after thermomechanical cycling.

Keywords: Antibacterial adhesive systems; demineralized dentin; chlorhexidine digluconate; cariogenic bacteria; bond strength; thermomechanical cycling; nanoleakage

SUMÁRIO

INTRODUÇÃO GERAL

A lesão de cárie é caracterizada pela desmineralização dos tecidos dentais, causada pela ação de ácidos produzidos por bactérias, principalmente *Streptococcus mutans* e *Lactobacillos* (Loesche, 1986). Esta foi descrita por Fusayama, em 1979, sendo constituída de 2 camadas distintas, uma mais superficial (*outer layer)*, altamente infectada por microrganismos e outra mais interna (*inner layer*), com dentina parcialmente desmineralizada e pouco infectada. O tratamento restaurador conservador, procura remover a camada mais externa (Banerjee *et al.*, 2010), a qual apresenta a estrutura do colágeno irreversivelmente alterada, sem possibilidade de remineralização (Ogushi & Fusayama, 1975) e manter a camada interna, onde as fibrilas colágenas permanecem unidas pelas ligações cruzadas, com possibilidade de remineralização (Ogushi & Fusayama, 1975).

A quantidade de tecido cariado removido durante o preparo cavitário é de relevante importância durante o tratamento restaurador. Uma técnica de preservação da estrutura dentária implica na combinação de quatro princípios: 1- remoção da biomassa central amolecida, para reduzir o grau de infecção da cavidade; 2- aplicação de um material antibacteriano (por exemplo: hidróxido de cálcio), objetivando a indução da aposição de dentina terciária; 3- remineralização da dentina parcialmente desmineralizada e 4- prevenção de reinfecção da dentina por microorganismos da cavidade oral (Wicht *et al.,* 2004).

Clinicamente, a superfície do substrato mais frequentemente encontrada, após a escavação do tecido cariado, consiste de dentina afetada pela cárie (Vaidyanathan *et al*., 2009; Erhardt *et al*., 2008). Essa camada de dentina encontra-se na maior parte ocupada por depósitos minerais ácido resistentes (Nakajima *et al.*, 2005), que frequentemente ocluem os túbulos dentinários, implicando em desafio para a adesão eficiente (Erhardt *et al.*, 2008), uma vez que pode influenciar os parâmetros da dentina desmineralizada, a penetração do sistema adesivo e consequentemente, a adesão dentina/adesivo (Haj-Ali *et al.*, 2006).

Apesar da adesão ao substrato dentinário ser uma questão complexa e difícil, independente desse substrato estar sadio ou afetado pela cárie, as restaurações adesivas são frequentemente realizadas, por requer menor preparo da cavidade, contribuindo para a

preservação da estrutura dental, quando comparadas ao preparo para amálgama (Banerjee *et al*., 2010).

Com o evidente aumento e popularidade do tratamento restaurador conservador (Banerjee *et al.*, 2010), existe maior risco de bactérias residuais permanecerem na cavidade dentária após a remoção da dentina infectada, o que pode resultar em cárie secundária (Weerheijm & Groen, 1999). Sendo assim, soluções desinfetantes têm sido introduzidas como alternativa para reduzir ou eliminar remanescentes bacterianos que possam estar presentes nas paredes da cavidade (Meiers & Kresin, 1996).

Ersin *et al.*, (2006) afirmam que o uso da clorexidina como desinfetante de cavidade reduz o número de bactérias residuais, devido ao amplo espectro de ação apresentado. Geralmente bactérias gram-positivas são mais susceptíveis a ação do digluconato de clorexidina do que as gram-negativas, principalmente *Streptococcus mutans*, o qual mostrou ser mais sensível (Fardal & Turnbull, 1986).

O uso de agentes antimicrobianos como desinfetante de cavidade ou adicionados a sistemas adesivos poderiam reduzir possíveis bactérias residuais presentes na cavidade dentinária e aumentar a taxa de sucesso dos procedimentos restauradores.

Imazato *et al.,* (1994) propôs o emprego de um monômero com propriedades antibacterianas, o brometo de metacriloxidodecilperidíneo (MDPB). Esse monômero é um composto quaternário da amônia, com função antibacteriana, associado a um grupo metacrilato. O agente antibacteriano é co-polimerizado com outros monômeros presentes no sistema adesivo e permanece imobilizado na matriz polimérica, exercendo ação contra bactérias que entram em contato com o monômero (Imazato *et al.*, 1998). Dessa maneira, o monômero MDPB, incorporado ao primer, mostrou ser promissor para inativação de bactérias residuais da cavidade dentária em estudos feitos *in vitro* e *in vivo* (Imazato *et al.*, 1997; 1998; 2001; 2004; Tziafas *et al.*, 2007).

A efetividade da ação antibacteriana do MDPB adicionado ao primer do sistema adesivo autocondicionante de 2 passos, foi demonstrada por Imazato *et al.*, (1994), (1998) com efeito inibitório imediato do primer polimerizado, sem, no entanto, verificar a continuidade deste efeito. Uma vez que este age por contato, e que após a polimerização

ocorre a imobilização do MDPB na rede polimérica, faz-se necessário avaliar o efeito antibacteriano ao longo do tempo.

A busca por um agente de união, capaz de promover o selamento eficaz e duradouro ao substrato dentinário, tem sido um desafio. A diminuição da resistência da união, ao longo do tempo, tem sido atribuída principalmente à degradação da camada híbrida na interface substrato/adesivo (Pashley *et al.*, 2004; Toledano *et al.*, 2007), que pode ocorrer devido a fatores químicos e físico-mecânicos. Os fatores químicos relacionados ao substrato envolvem principalmente ou a degradação das fibrilas colágenas, em decorrência da exposição à agua e enzinas provenientes da dentina e de bactérias; ou relacionados ao polímero, como a degradação hidrolítica (Tay & Pashley, 2003; Pashley *et al.*, 2004). Os fatores físico-mecânicos relacionados aos polímeros, de maneira geral, englobam inúmeros mecanismos que afetam a integridade marginal das restaurações, principalmente: tensão de contração de polimerização dos materiais resinosos; geração de tensões em decorrência da carga mastigatória, as quais podem originar falhas e propagação de fraturas; e mudanças térmicas que ocorrem na cavidade bucal, ocasionando tensões na interface, manisfestadas por contração e expansão do material restaurador (Nikaido *et al*., 2002; de Munck *et al*., 2005; Drummond, 2008).

As metaloproteinases da matriz (MMPs), ou as enzimas capazes de degradar o colágeno da dentina, estão presentes naturalmente na estrutura do complexo dentino pulpar. Essas enzimas podem ser ativadas pela queda de pH, consequente ao tratamento da superfície com primer e adesivo, ou à bioquímica do processo carioso, ocasionando degradação das fibrilas colágenas e consequente nanoinfiltração, as chamadas *water trees* (Pashley *et al*., 2004; Tay *et al*., 2004).

Estudos mostram que a aplicação de clorexidina na limpeza, após preparo cavitário, não apresenta efeito negativo na união da restauração, quanto à resistência ao cisalhamento (el-Housseiny & Jamjoum, 2000; de Castro *et al.*, 2003), pelo contrário, a aplicação de clorexidina pode aumentar a resistência da união (Erdemir *et al.*, 2004), por apresentar efeito inibidor das metaloproteinases (Gendron *et al*., 1999), responsáveis pela degradação da camada híbrida. Isso foi demonstrado em estudo *in vivo*, no qual a aplicação da

clorexidina aumentou significativamente a integridade da camada híbrida em seis meses de avaliação clínica (Hebling *et al*., 2005).

A utilização da clorexidina parece ter um papel favorável na proteção da união sistema adesivo/dentina que, associada ao uso de um adesivo com propriedades antibacterianas, poderia ser promissor na técnica de remoção parcial de tecido cariado e restauração com materiais adesivos, preservando tecido dentário para a restauração e consequentemente diminuindo a possibilidade de exposição pulpar e comprometimento endodôntico.

Sendo a durabilidade da união resina/dentina e a estabilidade do substrato importantes para a longevidade da restauração, torna-se importante avaliar a duração do efeito antibacteriano do agente antimicrobiano MDPB, bem como a interação com o agente antimicrobiano clorexidina, utilizado na limpeza pós-preparo cavitário.

Assim, os objetivos desta TESE[1] composta por 3 capítulos foram: 1- avaliar o efeito bactericida dos sistemas adesivos Clearfil SE Protect (primer com monômero MDPB), Clearfil SE Bond e Adper Single Bond 2 e do desinfetante de cavidade, Digluconato de Clorexidina a 2% na dentina desmineralizada e contaminada; 2 - avaliar a duração da atividade antibacteriana após a polimerização dos sistemas adesivos sobre bactérias cariogênicas; 3 - avaliar a resistência da união, degradação adesiva, adapatação marginal e nanoinfiltração após ciclagem termomecânica.

[1] Este Tese está baseada na resolução da CCPG/002/06, a qual dispõe a respeito do formato das teses de mestrado e doutorado aprovados

CAPÍTULO 1

Effect of 2% chlorhexidine digluconate and dentin bonding systems on demineralized dentin contaminated with *Streptococcus mutans*[2]

Éfani Caroline de Freitas Banzi, DDS,**Cristiane Duque**, DDS, MS, PhD, **Larissa Ferreira Pacheco**, DDS, MS, **Patrícia Almada Sacramento**, DDS, MS, PhD, **Regina Maria Puppin-Rontani***, DDS, MS, PhD, **Franklin Garcia-Godoy**, DDS, MS.

Abstract: Background. The aim this study was to evaluate the inhibitory effect of bonding systems and 2% chlorhexidine digluconate (CHX) on *Streptococcus mutans.*

Methods. Viable bacteria were verified in 1 and 2 assays. After that, dentin slices were cleaned or not with 2% CHX and treated with adhesive systems. Adhesive systems discs were incubated in bacterial suspension, respectively. For assay 3, bacterial inhibition zones around each bonding systems discs were measured and the number of viable bacteria in contact with these discs counted.

Results. Clearfil SE Protect (CP), compared to Clearfil SE Bond showed higher antibacterial effect after 60 min of bacterial contact and lower *S. mutans* counts in dentin than Single Bond. CHX did not improve the antibacterial effect of the bonding systems. Cured CP specimens showed an inhibitory effect on the growth of *S. mutans* on the agar surface.

Conclusion. Dentin surface treatment with CHX did not have an effect on S. mutans before application of bonding systems. CP exhibited considerable antibacterial activity and may be useful for reducing cariogenic bacteria in the remaining dentin.

Clinical Implications. Microorganisms can remain alive after partial caries removal. The application of a dentin bonding system containing MDPB can reduce cariogenic bacteria.

Key Words. Antibacterial bonding systems, dentin, chlorhexidine digluconate

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INTRODUCTION

There has been a increasing interest in dental caries treatments using minimally invasive procedures.[1,2] A procedure that is well-accepted by dentists for avoiding pulp exposure during caries removal includes removal of the infected outer dentin, which is structurally disarranged and highly contaminated, leaving the caries-affected inner dentin prone to remineralization.[3,4] However, since partial caries removal is based on established clinical criteria (darker and harder dentin), it is not possible to confirm the lack of viable cariogenic bacteria in the remaining dentin when using this procedure.[5,6] Therefore, the use of cavity disinfectants and/or liner materials with antimicrobial activity applied on this contaminated tissue, as well as the adequate marginal sealing of the cavity, are essential for eliminating residual microorganisms.[7]

Antibacterial agents have been added to the monomers in adhesive systems. The monomer methacryloyloxydodecylpyridinium bromide (MDPB), developed by Imazato *et al*.[8] is a compound of quaternary ammonium, an antibacterial agent, with a methacryloyl group, with the antibacterial agent covalently bonded to the polymer matrix through copolymerization of MDPB with other monomers during curing.[9] The incorporation of MDPB confers an antibacterial activity to the dentin primer.[9-12] However, this property is reduced after the curing process.[13,14] The MDPB-containing primer has shown inhibitory action mainly against oral streptococci,[6,8,14-16] and penetrates artificially demineralized lesions killing the bacteria in dentin, preventing the progression of root-surface caries.[17,18]

Another way to eliminate remaining bacteria after caries removal is to clean the cavity floor using a disinfectant. Chlorhexidine has been considered the most effective and safe antimicrobial substance for oral use. It has a wide spectrum against Gram positive bacteria (especially mutans streptococci) and Gram negative, aerobic and facultative anaerobic, yeasts and fungi.[19,20] Several formulations of chlorhexidine have been used to control oral biofilm. 2% chlorhexidine solution is an important antimicrobial agent indicated for cavity disinfection before the placement of restorations to reduce or eliminate residual bacteria.[16]

The aim of this study was to evaluate the antibacterial activity of the self-etching dentin adhesive agent Clearfil SE Protect (MDPB containing primer) and 2% chlorhexidine

digluconate, as a disinfecting agent, on demineralized and contaminated dentin. Also, the inhibitory effect of cured Clearfil SE Protect on *S. mutans*, compared to Clearfil SE Bond and Adper Single Bond 2 was evaluated.

MATERIALS AND METHODS

This study was performed under the protocol approved by the Research Ethics Committee of Piracicaba Dental School, (#072/2007), University of Campinas, Brazil. The materials used in this current study are described in Table 1.

Table 1 – Description of composition, manufacturer and batch number, pH value and application technique of the materials used in this study.

Materials	Composition	Manufacturer (Batch number)	pH value *	Application Technique**
Adper Single Bond 2 (SB)	**Etching acid**: Phosphoric acid 37% **Bond**: HEMA; Bis-GMA; dimethacrylates methacrylates; ethanol; water; UDMA, Bisphenol-A glycerolate, polyalkenoic acid copolymer canphorquinone	3M Dental Products, St. Paul, MN, USA (51202)	pH = 0.3 (etching acid) pH = 4.7 (primer + Bond)	a, b, d,f
Clearfil SE Protect (CP)	**Primer**: water, MDP, MDPB, HEMA, Hidrophobic methacrylate **Bond**: MDP, HEMA, Bis-GMA, Hydrophobic dimethacrylate, di-Camphorquinone, N-Diethanol-p-toluidine,silanated colloidal silica	Kuraray Medical Inc, Kurashiki, Tokyo, Japan (Primer: 00047A) (Bond: 00072A)	pH = 1.9 (primer) pH = 2.8 (Bond)	c,d,f
Clearfil SE Bond (CB)	**Primer**: MDP, HEMA, Hydrophobic dimetacrylate, N-Diethanol-p-toluidine, Camphorquinone , water **Bond**: MDP, Bis-GMA, HEMA, silanated coloidal sílica, Hydrophobic dimethacrylate, Camphorquinone , N-Diethanol-p-toluidine	Kuraray Medical Inc, Kurashiki, Tokyo, Japan (Primer: 00830A) (Bond: 01212A)	pH = 2.0 (primer) pH = 2.0 (Bond)	c, d, f
Clearfil AP-X	Silanated barium glass, Silanated colloidal silica, Silanated silica, Bis-GMA, TEGDMA, dl-Camphorquinone	Kuraray Medical Inc, Kurashiki, Tokyo, Japan (# 00985B)	-	e, f

*manufacturer's information **Application technique: a: apply phosphoric acid by 15 seconds; b: rinsed for 15 seconds with sterilized deionized water; c: apply self-etching primer by 20 seconds; d: apply bond; e: 2mm of increment; f: photoactivate. MDP: 10-methacryloyloxydecyl dihydrogen phosphate; MDPB: 12-methacryloyloxydodecylpyridinium bromide; HEMA: 2-hydroxyethyl methacrylate; Bis-GMA: bisphenol-A diglycidil ether dimethacrylate; TEGDMA: Triethyleneglycol dimethacrylate

Assay 1

Preparation of specimens

One hundred and twenty extracted sound human third molars were selected, cleaned with periodontal curettes and pumice slurry with a Robinson brush, and stored in saline solution at 4º C up to 2 months after extraction. Teeth with caries lesions or cracks were excluded. The middle dentin was determined in each individual tooth by the bitewing x-ray technique. The site located half the distance from the enamel-dentin junction to the pulp was determined as the middle dentin and marked with a pen in each tooth. Occlusal portions were sectioned at that point (Isomet, Buehler, Lake Bluff, IL, USA). The roots were cut at the cementoenamel junctions and discarded.

Specimens were painted with an acid-resistant nail polish (Colorama; CEIL Com. Exp. Ind. Ltda., São Paulo, SP, Brazil), except for the flat coronal dentin area (4 x 4x 2 mm), which was further submitted to demineralization.

Dentin demineralization and contamination with S. mutans

The methodology used in the present study was based on the study developed by Imazato *et al*.[17] with some modifications. Demineralized dentin was produced *in vitro* using an acid gel model. Dentin slices were immersed in 5 mL of 6% carboxymethylcellulose acid gel (Proderma Pharmacy, Piracicaba, SP, Brazil) at pH 5.0 and 37º C. The gel contained 0.1 M lactic acid titrated to pH 5.0 with a concentrated KOH solution.[21] The specimens remained in the gel for 24 hours without renewal. This model produced a demineralization depth of about 40 µm, confirmed with polarized light microscopy in a pilot study. After dentin demineralization, the specimens were washed twice in an ultrasound bath with distilled water for 15 min each and dried gently with tissue paper.

Streptococcus mutans UA 159 stock cultures were anaerobically reactivated in Mitis Salivarius Agar (MSA, Difco, Detroit, MI, USA) for 24 h at 37^{o} C. Five colonies were collected from the MSA and cultivated in Brain Heart Infusion (BHI, Difco, Detroit, MI, USA) broth in the same conditions for 18 h. A loopful inoculum of *S. mutans* was transferred to 2 mL of BHI broth, incubated at 37º C for 5 h, and was adjusted to an optical density of 1.0 at 550 nm. Demineralized dentin slices were sterilized in a steam autoclave (Phoenix, SP, Brazil) inside the tubes containing distilled water. The excess water was

removed using sterile tissue paper and the specimens were individually stored in a Petri dish prior to the experiment. A 10 µL aliquot of *Streptococcus mutans* suspension (previously prepared to $3x10^8$ colony forming units (CFU)/ml) was put on the surface of the demineralized specimen and incubated for 1 h and 30 min at 37° C to stimulate bacterial colonization of dentin.

The dentin slices were divided into 20 groups (n=6) according to type of adhesive systems (Clearfil SE Protect – CP; Clearfil SE Bond – CB; Adper Single Bond 2 – SB), surface treated with chlorhexidine (CHX) or not (no CHX), and time of adhesive agent contact with *S. mutans* (15, 30 and 60 min).

Antibacterial activity tests in demineralized dentin specimens

After specimen contamination, for the chlorhexidine groups 5 µL of 2% chlorhexidine digluconate was applied on the demineralized dentin slice surface, left undisturbed for 60 s[22] and removed with sterile tissue paper. The adhesive system was applied as follows: for the self-etching adhesive system (Clearfil SE Protect – CP; Clearfil SE Bond - CB), 5 µL of primer was applied in the dentin surface for 20 s. For Adper Single Bond (SB), the dentin surface was etched with 37% phosphoric acid gel for 15 s and rinsed for 15 s with sterilized deionized water. The entire dentin surfaces were then dried with sterile filter paper and 5 µL of the bonding agent CP, CB and SB was applied over the primer or dentin surface of each group, respectively, and maintained in Petri dishes at 37º C for 15, 30 and 60 min. The dentin/adhesive specimens were then immersed in individual Eppendorf tubes with 1 mL 0.9% NaCl (saline solution) and sonicated (UP400S, Hielscher, Teltow, Germany) for 30 s at a 30% amplitude. The suspension was diluted in decimal series from 10^{-1} to $10^{-3}$and inoculated on BHI agar plates. The plates were incubated anaerobically for 48 h at 37º C and the number of viable bacteria was assessed by counting the colonies formed (CFU/ml).

Assays 2 and 3

Preparation of adhesive system cured disc-shaped specimens

A cylindrical mold (diameter: 6mm, height: 2mm) was filled with the adhesive systems – CP, CB and SB (n=7) and the bottom and top the mold was covered with a glass slide and light cured according to the manufacturers' instructions. The negative control

group was the Clearfil AP-X composite resin (CR), applied using the incremental technique in the mold and light cured for 40 s with Elipar Tri-light unit (ESPE – America Co., Seefeld 82229 - Germany). Light intensity was periodically checked at the beginning of each group (740 mW/cm^2).

Assay 2 - Antibacterial activity of cured disc-shaped specimens

Each cured disc-shaped specimen was immersed in 5 mL sterile distilled water immediately after preparation and agitated for 1 h in a table agitator at 210 rpm to remove the uncured components, and then dried aseptically with tissue paper. A 10 µL aliquot of S. *mutans* suspension of a culture previously adjusted to 3x10^8 CFU/ml was put on the surface of the cured disc-shaped specimens and incubated at 37º C for 1 h. The specimens were then immersed in individual microtubes containing 1 ml of saline solution and sonicated (UP400S, Hielscher, Teltow, Germany) for 30 s at a 30% amplitude. The suspension was diluted in decimal series and inoculated on BHI agar plates. The plates were incubated anaerobically for 48 h at 37º C and the number of viable bacteria was assessed by counting the CFU/ml.

Assay 3 - Inhibitory effect on agar bacterial growth of cured disc-shaped specimen

The bottom surface of the cured specimens of each group (CP, CB, SB, CR) was placed onto a BHI agar plate inoculated with 350 µL of *S. mutans.* Filter disc papers with 5 µL of 2% chlorhexidine digluconate were used as a positive control group. The plates were kept for 1 h at room temperature to allow diffusion of the materials, and then incubated at 37° C for 24 h. Zones of bacterial growth inhibition were recorded in millimeters (mm) using a digital caliper (Mitutoyo, SP, Brazil). Measurements were taken at the greatest distance between two points from the outer limit of the inhibition halo formed around the cured disc-shaped specimen. This measurement was repeated three times and the mean was computed. The specimens that did not produce inhibition zones had the number of viable bacteria in contact with the cured specimens assessed. The cured specimens were immersed in individual microtubes with 1 mL of saline solution and sonicated for 30 s at a 30% amplitude. The suspension was diluted in decimal series and inoculated on BHI agar plates. The plates were incubated anaerobically for 48 h at 37 ºC and the number of viable bacteria was counted (CFU).

Statistical analysis

The bacterial counts (assay 1 and 2) were analyzed using the Friedman test, ANOVA-R and t test (LSD), and the inhibitory zones (assay 3) were analyzed using the Kruskal-Wallis and t test (LSD), at a significance level of 0.05.

RESULTS

Means and standard deviations of bacterial counts were expressed as log values (CFU+1). The constant 1 was added to CFU since many samples showed zero counts. The number of *S. mutans* [log (CFU/ml + 1)] recovered from the demineralized dentin slices is presented in Figure 1. Overall, regardless of treatment the surface with CHX and the time of contact for the adhesive agents with *S. mutans*, CP showed lower *S. mutans* counts in dentin than CB and SB. There was a statistical significant difference between CP and SB ($p<0.0001$), CP and CB ($p<0.0001$) and SB and CB ($p=0.0005$). However, when specimens previously treated or not with CHX were compared, a significant difference was only verified for CB ($p = 0.0013$), and not for CP ($p=0.5622$) or SB ($p= 0.4806$). CHX did not improve the antibacterial effect of bonding systems on contaminated dentin. When considering *S mutans* contact time with the adhesive agents, CP showed better antibacterial effect than CB only after 60 min. When evaluating the 15 and 30 min contact times, *S. mutans* recovery was similar between these groups.

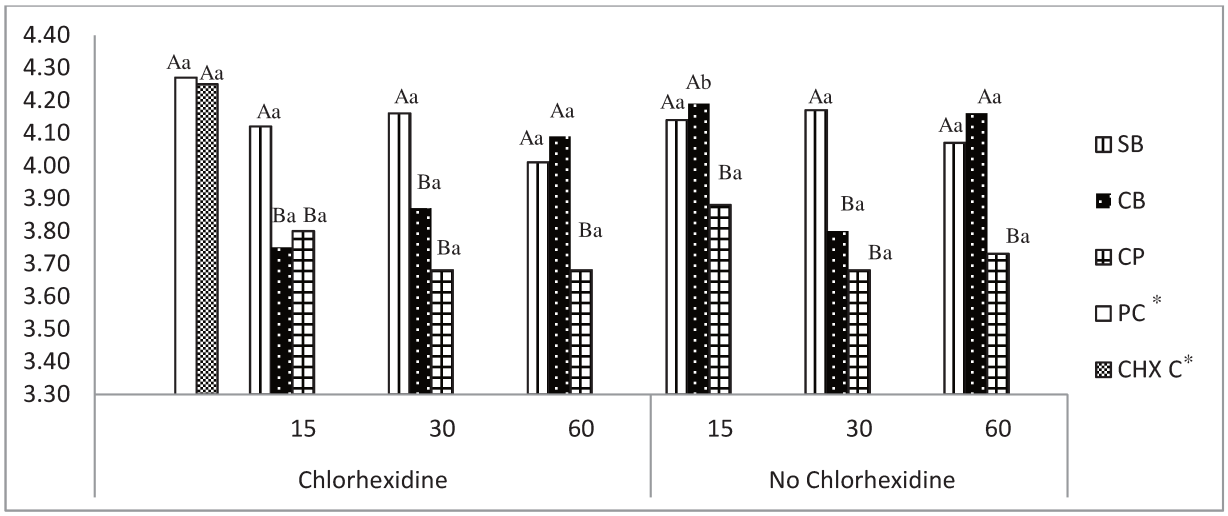

Figure 1 – Number of *S. mutans* [log (CFU/ml + 1)] recovered from the demineralized and contaminated dentin, after treatment or not of the surface with chlorhexidine and application of the adhesive systems.

* PC – positive control (only bacteria); CHX C - 2% chlorhexidine digluconate control.

** Comparing the same material at different times, means followed by the same lower case letter are not statistically different, according to Friedman test, ANOVA-R and t test (LSD) ($p>0.05$)

***Comparing different materials at the same time, means followed by the same upper case letter are not statistically different, according to Friedman test, ANOVA-R and t test (LSD) ($p>0.05$)

Table 2 shows the number of viable *S. mutans* log [log (CFU/ml + 1)] obtained after 1 h of bacterial contact with the cured disc-shaped adhesive system specimens. SB had high counts of *S. mutans*, similar to the negative control group (RC), with no significant difference between those two groups. CP and CB had a bactericidal effect, eliminating bacteria in the specimens. Table 3 presents the values obtained for the inhibition zones against *S. mutans* after contact with the disc-shaped specimens in agar plates. Only the CP and CHX groups presented inhibition zones. However, CP had values significantly lower than CHX. For CB, SB and the negative control group, which did not produce inhibition zones against *S. mutans*, the specimens were removed from the agar plates and the number of bacteria recovered is presented in Table 4. There were no significant differences among the CB, SB and RC groups.

Table 2 - Number of viable *S. mutans* reported as log (CFU/ml + 1) after contact with each cured disc-shaped specimen.

Groups	Mean ± Standard deviation
CP	0 **A***
CB	0 **A**
SB	4.50 ± 4.62 **B**
RC	5.67 ± 6.04 **B**

* Comparing different materials, means followed by the same upper case letter are not statistically different, according to Friedman test, ANOVA-R and t test (LSD) (p>0.05)

Table 3 – Width of inhibition zones (mm) obtained for *S. mutans* according to the groups

Groups	Mean ± Standard deviation
CP	8.19 ± 1.14 **A***
CB	0 **B**
SB	0 **B**
RC	0 **B**
CHX	15.34 ± 1.41 **C**

* Comparing different materials, means followed by the same upper case letter are not statistically different, according to Kruskal Wallis test (LSD) (p>0.05)

Table 4 - Number of viable *S. mutans* reported as log (CFU/ml + 1) under the cured disc-shaped specimen.

Groups	Mean ± Standard deviation
CB	7.34 ± 6.67 **A***
SB	7.65 ± 6.73 **A**
RC	7.53 ± 7.15 **A**

* Comparing different materials, means followed by the same upper case letter are not statistically different, according to Friedman test, ANOVA-R and t test (LSD) (p>0.05)

DISCUSSION

In clinical situations where there is a lack of comprehensive and precise diagnoses of the extent of dental caries, it is difficult to decide how much contaminated dentin must be removed. Although there are some subjective and objective methods to diagnose carious

lesions and differentiate between infected and affected dentin, the clinical criteria do not reflect the existence of cariogenic bacteria or their virulence in the dentin environment.[5] Thus, residual caries following cavity preparation, can potentially lead to secondary caries and failure of the restorative procedure.[23] Therefore, it seems reasonable to suggest that restoration longevity might be improved by using restorative or liner materials with antibacterial properties.

In the present experimental study, dentin surfaces were demineralized and contaminated with *S. mutans*, simulating the characteristics of caries-affected dentin. The specimens were treated or not with 2% chlorhexidine digluconate and three different adhesive systems or both. Two self-etching adhesive systems, which differ in composition by the presence of bactericidal agent MDPB, and one conventional adhesive system were tested. The results of the bacterial recovery in the dentin slices demonstrated that Clearfil SE Protect was more effective in reducing *S. mutans* counts than Clearfil SE Bond after 60min of contact. MDPB incorporation in the primer composition of this adhesive agent likely enhanced the antibacterial effect. This result is consistent with the findings of Imazato *et al*.[17] In their study, primer solution that had been diluted 100 times presented a significant bactericidal effect after 30s of contact with carious dentin samples, showing that, in a hypothetical clinical situation, remaining bacteria inside the tubules may be reduced or eliminated by contact with MDPB. These authors suggested that the potential for arresting the progress of early caries by the application of a bonding system containing MDPB is related to blocking the porous structure of the dentinal lesion and inactivating the bacteria within the lesion.[17] However, depending on the dentin depth, antibacterial components eluted from the adhesives were not able to diffuse and exert their inhibitory effects on cariogenic bacteria.[13]

Schmalz *et al*.[24] and Gondim *et al*.[13] applied adhesive systems, including Clearfil SE Protect and Clearfil SE Bond, on dentin surfaces and evaluated the inhibitory activity of these materials against cariogenic bacteria using agar diffusion methods. The results of these studies were contradictory and those authors demonstrated that the thickness of dentin influenced the diffusion of dental materials. Additionally, those authors indicated that at dentin thicknesses above 400µm, the adhesives do not exert antibacterial actions. Because

of the ability to diffuse into dentin, the use of adhesives in deep cavities must be controlled, since monomers such as TEGDMA (triethylene glycol dimethacrylate), HEMA (2-hydroxyethyl methacrylate), Bis-GMA (Bisphenol A diglycidylmethacrylate), and MDP (10-methacryloyloxydecyl dihydrogen phosphate) are toxic if in contact with pulpal cells.[25,26] Although less cytotoxic than other monomers, such as Bis-GMA, MDPB has a negative influence on odontoblast differentiation and consequently interferes in the process of dentinogenesis.[26]

The utilization of disinfectants of cavities, such as chlorhexidine solutions or varnishes, before application of dentin-bonding systems has been indicated for the prevention of secondary caries in composite restorations.[27,28] Imazato *et al*.[17] indicated the possible advantages of this technique, such as the greater depth of penetration for bonding systems and improved seal of the porous dentin structure. In this current study, cleaning of the dentin surface with chlorhexidine digluconate for 60 s did not result in a significant reduction of viable bacteria. Although chlorhexidine has a considerable antimicrobial effect against caries-associated bacteria,[29] most clinical studies evaluating the effect of chlorhexidine found moderate reductions in microbial samples taken 24 h after gel application.[30,31] The current findings are in accordance with the results of Hauser-Gerspach *et al*.[32] who observed that chlorhexidine gel application for 30s in deep occlusal carious cavities *in vivo* had no significant immediate antimicrobial effects whether the superficial carious dentin layers were removed or not. Van Strijp *et al*.[33] reported in an *in situ* study that 2x daily treatment with 0.2% chlorhexidine for 4 weeks in demineralized dentin did not result in a reduction of the total cultivable microbiota when compared with the control specimens which were treated with water. Mutans streptococci were less sensitive to chlorhexidine when this substance was evaluated in an experimental biofilm and even less in mixed biofilms.[34-36] Furthermore, several tooth components, such as dentin, hydroxyapatite or collagen, have been shown to be able to reduce or inactivate the antimicrobial effect of chlorhexidine.[37] Another factor contributing to the reduction of the antimicrobial effects of chlorhexidine could be its low substantivity in dentin. Substantivity is an intrinsic ability of the chlorhexidine to be retained by oral surfaces and gradually released into oral fluids over many hours.[38]

In this present study, cured disc-shaped specimens were used to evaluate the influence of light curing on the antibacterial effect of the adhesive systems. In the experiment, unpolymerized components were removed from the cured specimens by agitation for 1h. Even with light-activation, Clearfil SE Protect and Clearfil SE Bond presented a bactericidal effect, eliminating bacteria in contact with them. MDPB from Clearfil SE Protect primer co-polymerizes with other monomers, such as Pheny-P (methacryloyloxyethyl phenyl hydrogen phosphate), 5-NMSA (methacryloyl-5-aminosalicylic acid) and HEMA (2-hydroxyethyl methacrylate) when the material is cured. The immobilized agent does not leach out from the material but acts as contact inhibitor against bacteria on surface of the material.[9,11] MDPB could explain the antibacterial activity of Clearfil SE Protect, but not for Clearfil SE Bond, which does not possess this substance in its composition. Primers of self-etching adhesive systems (Clearfil SE Protect or Bond) have low pH (< 2.0) due to the presence of polymerizable acidic monomers, such as MDP, that demineralize and infiltrate the dentin tubules, exerting an inhibitory action against cariogenic bacteria. These monomers are esters originating from the reaction of a bivalent alcohol with methacrylic acid and phosphoric/carboxylic acid.[39] High levels of H^+ ions produce a low pH and may penetrate the bacterial cells and affect their ability to produce ATP and cause damage to molecules and cell structures.[40] Single Bond (SB) presents a pH around 5.0 that is not acidic enough to cause damage to *S. mutans,* when used without phosphoric acid. The absence of antibacterial action of SB observed in this present study is in accordance with Imazato *et al*.[16] and Baseren *et al*.[41]

In agar diffusion tests, only Clearfil SE Protect had an inhibitory effect against *S. mutans.* Several studies have demonstrated that both primers of Clearfil SE Protect and Clearfil SE Bond have antibacterial properties.[9,13,14,16,42-45] In contrast with previous studies, the present study used disc-shaped light cured materials previously cured prior to use in the agar plates. It is likely that the antibacterial activity of Clearfil SE Protect primer was related to the diffusion of non-polymerized monomers on the agar medium, even after light activation, inhibiting the growth of *S. mutans* around the discs. This result confirms the efficacy of MDPB incorporated in the Clearfil SE Protect primer in eliminating cariogenic

bacteria when in contact with this agent that has been immobilized in a polymer network formed after curing the bonding system.

The present study demonstrated the *in vitro* antibacterial action of self-etching adhesive systems and the influence of previous treatment of the dentin surface with chlorhexidine digluconate. The presence of the antibacterial monomer MDPB in Clearfil SE Protect contributed to the inhibitory activity of this material against cariogenic bacteria. The application of 2% chlorhexidine digluconate for 60s prior to the application of bonding agents was not effective in reducing *S. mutans* in demineralized and contaminated dentin. Further clinical trials should be conducted to confirm the *in vitro* inhibitory effect of this antibacterial adhesive system against cariogenic microorganisms.

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CAPÍTULO 2

Metabolic activity and viability of bacteria after different exposure time to the antibacterial adhesive system[3]

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Abstract: Objective: To evaluate the antimicrobial efficacy of Clearfil SE Protect (CP) and Clearfil SE Bond (CB) against six individual oral microorganisms as well as in a mixed bacterial culture. **Methods**: Bacterial culture of single species of *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus gordonii*, *Actinomyces viscosus* and *Lactobacillus lactis*, as well as mixed bacterial culture created were obtained from ATCC. Dentin bonding agent discs were prepared, cured and placed on the bacterial culture of single species or multispecies oral bacteria for 15, 30 and 60 minutes. The antimicrobial property of the two dentin bonding agents was determined by MTT and Live/dead bacterial viability assays, and also by measuring their metabolic activity by XTT assay. All assays were done in triplicates and each experiment repeated at least three times. Significance of results was determined by ANOVA and Scheffe's f-test (5%). **Results**: CB had no significant effect on the viability or metabolic activity of the test microorganisms when compared to the control bacterial culture. The CP was found to be significantly effective against the organisms tested both on single species and on multispecies bacterial culture. Greater than 40% killing was seen within 15 minutes, and the killing progressed with increasing time of incubation. However, longer (60 min) period of incubation was required by CP to achieve similar antimicrobial effective against mixed bacterial culture tested. **Conclusion**: The results demonstrated the antimicrobial efficacy of CP both on single and multispecies bacterial culture. The use of CP may be beneficial in reducing presence of bacterial infections in the cavity preparation. **Keywords**: cariogenic bacteria, antibacterial dentin bonding agent, XTT assay, MTT assay, Live/Dead assay.

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INTRODUCTION

Adhesive restoration is the most common treatment in daily practice in Dentistry and attention has been paid with treatment with minimal intervention. Recent developments in adhesive dentistry allow improved preservation of tooth structure and prevention from pulpal exposition (Ricketts et al., 2006). However, partial caries removal is based on established clinical criteria (darker and harder dentin), and it is not possible to confirm the absence of viable cariogenic bacteria in the remaining dentin when using a minimally invasive procedure. In addition, pulpal inflammation and the recurrent caries beneath restorations can be the results of remaining bacteria (Imazato et al., 1998; Izutani et al., 2011). Many types of bacteria participate in the formation of the dental biofilm (Takahashi & Nyvad, 2011) and consequently of dental caries (Al-Ahmad et al., 2007). Furthermore, coaggregation of streptococci, *Actinomyces*, *Veillonella spp*. and *F. nucleatum* were detected in oral biofilms (Al-Ahmad et al., 2007) and it may reveal interaction of different bacterial species i.e. streptococci and *Actinomyces* (Palmer et al., 2003) decrease the effect of antibacterial agents (Teixiera et al., 2007).

In order to increase the success rate of these restorative procedures, many studies have demonstrated the antibacterial benefits of incorporating antimicrobials into primer of dentin bonding agent against single specie bacteria associated with caries (Imazato et al., 2002; Feuerstein et al., 2007; Imazato et al., 2008; Gondin et al., 2008). Studies with dentin bonding agents have pursued improvements and advancements focusing in the development an antibacterial monomer that kills bacteria (Imazato et al., 1994; 1998; 2008), on higher tolerance (Nishida et al., 2010), increased bond strength (Imazato et al., 2007), providing reliable results (Izutani et al., 2011) and facilitates using.

Therefore, antibacterial agents have been added to the monomers in dentin bonding agent. The monomer methacryloyloxydodecylpyridinium bromide (MDPB), developed by Imazato et al., (1994) is a compound of quaternary ammonium, an antibacterial agent, with a methacryloyl group. This, antibacterial agent covalently is bonded to the polymer matrix through copolymerization of MDPB with other monomers during curing, besides, the antibacterial effects of immediately polymerized dentin bonding are beneficial in the

eradication of residual bacteria in the oral cavity (Imazato et al., 1998). However, this property seems to be reduced after the curing process (Gondim et al., 2008).

Furthermore, previous studies (Imazato et al., 2002; Izutani et al., 2011; Lobo et al., 2005) showed strong antibacterial activity based upon MDPB against *S. mutans* and also against other single bacteria in human dentinal carious lesions as *L. casei* and *A. naeslundii*. Despite of good results for isolated bacteria species, it is not known how the MDPB antibacterial primer could act on bacterial growth and metabolic function of cariogenic bacteria associated, since when they are in association, like biofilm, antibacterial agent show lower antibacterial effect (Teixiera et al., 2007).

The contact time is a preponderant factor when used antimicrobial agents, even though incorporate to polymeric chains. The long-lasting antibacterial activity of polymerized dentin bonding agent may be effective in inactivating bacteria that invade the tooth- dentin bonding interface by microleakage (Feuerstein et al., 2007).

Although antibacterial effect is important when consider antimicrobial agents, viability and citotoxicity are properties that have to be considered. Colorimetric assay (XTT and MTT based) has been used for the non-radioactive quantification of cell proliferation and activation. XTT assay gives information about the metabolic activity of the bacteria based on the reduction of a yellow tetrazolium salt to an orange formazan, while MTT assay determines the viability of bacteria after exposure to the test materials based on the reduction of a yellow tetrazolium salt to a purple formazan. Lately, the method to differentiate live/dead bacteria by staining with fluorescent dyes was introduced (Boulos et al., 1999). The live/dead assay has the advantage of giving results rapidly as it is sensitive for monitoring of bacterial viability and it allow to quantitatively distinguish live and dead bacteria. The purpose of this study was to investigate the effect of contact time of Clearfil SE Protect (MDPB containing primer) and Clearfil SE Bond self-etching dentin bonding agents on five individual oral microorganisms as well as in a mixed bacterial culture on metabolic activity and viability by XTT and MTT assay and Live/Dead assay.

MATERIALS AND METHODS

Bacteria – ATCC strains of *Streptococcus mutans* 31377, *Streptococcus sobrinus* 27351, *Streptococcus gordonii* 10558, *Actinomyces viscosus* 19246 and *Lactobacillus lactis* 12314 were used isolated and in combination of two (*S. mutans* + *S. gordonii*, and *S. mutans* + *A. viscosus*) and three (*S. mutans* + *L. lactis* + *A. viscosus*) bacteria. Inocula from stock cultures were cultivated in 50 mL of Todd Hewitt Broth (THB Difco, Detroit, MI) and incubated at 37° C for 48 h before each experiment. A bacterial culture of each single bacteria and mixed bacteria was adjusted to $1x10^7$ cells/mL.

Preparation of adhesive system discs – Low viscosity polyvinyl siloxane matrix (Aquasil, Dentsply DeTrey, Konstanz, Germany) with a 6 mm diameter and 2 mm high was used to make dentin bonding agents discs. Thus, discs of Clearfil SE Protect (CP) and Clearfil SE Bond (CB) were done on the model and according to the manufacturer's recommendation (Table 1). One drop of self-etching primer was inserted in the matrix, and after 20 seconds one drop of adhesive was inserted over primer and light cured for 20 s (Elipar Tri-light unit, ESPE – America 139 Co., Seefeld 82229 - Germany). The discs were immersed in 0.5 mL of sterile distilled water immediately after preparation and agitated for 1 h to remove the uncured components. The methodology used in the present study was based on the study developed by Imazato *et al*., (1998) with some modifications. Then the samples of discs (in triplicates) were placed into Eppendorf tubes with 150 µL of test bacteria. In order to test the effect of dentin bonding agents on mixed bacterial culture, discs were added to equal amounts of bacteria (75 µL of each for a mixture of two; 50 µL for three mixed bacteria) and incubated for 15 minutes, 30 minutes and 60 minutes. It was considered as control group the bacterial pool (single or mixed bacterial culture). At the end of each incubation period, the antimicrobial effect of the dentin bonding agents was evaluated as described below.

Table 1 – Description of dentin bonding agents, composition, manufacturer and batch number, pH value and application technique of the materials used in this study.

Dentin Bonding Agents	Composition	Manufacturer (Batch number)	pH value *
Clearfil SE Protect (CP)	**Primer**: water, MDP, MDPB, HEMA, Hidrophobic methacrylate, **Bond**: MDP, HEMA, Bis-GMA, Hydrophobic dimethacrylate, di-Camphorquinone, N-Diethanol-p-toluidine, silanated colloidal silica	Kuraray Medical Inc, Kurashiki, Tokyo, Japan (# 61147)	pH = 1.9 (primer) pH = 2.8 (Bond)
Clearfil SE Bond (CB)	**Primer**: MDP, HEMA, Hydrophobic dimethacrylate, N-Diethanol-p-toluidine, Camphorquinone , water **Bond**: MDP, Bis-GMA, HEMA, silanated colloidal silica, Hydrophobic dimethacrylate, Camphorquinone , water, N-Diethanol-p-toluidine	Kuraray Medical Inc, Kurashiki, Tokyo, Japan (# 062071)	pH = 2.0 (primer) pH = 2.0 (Bond)

*manufacturer's information

XTT assay – The bacterial metabolic activity following the exposure to dentin bonding agents discs was measured by the metabolic reduction of XTT (sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate; Roche Applie Science, Indianapolis, IN, USA) to a soluble formazan product by the cells. The bacterial samples following the incubation with test materials were incubated with 50 µL of XTT reagent (final concentration of XTT 0.3 mg/mL). Tubes were incubated at 37° C for 2 hours. The absorbance of the supernatant was measured at 492 nm using an ELISA reader (Spectrostar Nano, BMG Labtech, Allmendgruen, Germany).

MTT assay – The bacterial cell viability was assessed by determining their effects on the ability of the bacterial cells to cleave the tetrazolium salt (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) to a formazan dye, using a kit from Boehringer Mannheim Corp (Indianapolis, IN, U.S.A).

Bacterial culture (150 µL) treated with the test discs were placed in 96-well flat-bottom microtiter plate. Fifteen microliters of the MTT labeling agent was added to each culture well, and the plates were incubated for 4 h at 37° C. After the incubation, 100 µL of solubilizing agent provided by the manufacturer was added and then incubated again overnight. Purple formazan color produced from the MTT by viable cells was read at 560

nm using an automatic enzyme-linked immunosorbent assay reader (ELISA reader, Spectrostar Nano, BMG Labtech, Allmendgruen, Germany).

Bacterial Live/Dead assay – Bacterial toxicity following the exposure to dentin bonding agents discs were measured using the LIVE/DEAD bacterial viability assay kit (Molecular Probes-Invitrogen, Carlsbad, CA, US). Following the treatment of bacteria as described previously, it was incubated with 0.4 μL of fluorescent dye mixture prepared according to the manufacturer's instructions (a mixture of two dyes) for 15 minutes. The combination of two dyes distinguishes live cells from the dead ones based on membrane integrity. The green fluorochrome (SYTO 9) can penetrate intact membranes, while the larger red fluorochrome (propidium iodide) penetrates only compromised cell walls of dead cells, results in red fluorescence by binding to nuclear material of the cells. A standard curve was set up using known ratio of live to dead cells to facilitate the calculation of live and dead cells in the experiment. The florescence emissions was determined at 530 ± 12.5 nm and 645 ± 20 nm using the fluorescent reader (Spectra Max, Molecular Devices, US).

Statistical analysis - All assays were performed using triplicate determinants, and each experiment was performed three times. Data was expressed as mean ± standard deviation and analyzed using a one-way analysis of variance (ANOVA) and Scheffe's F procedure for post hoc comparisons, using StatView® software.

RESULTS

XTT metabolic activity – The table 2 shows the average and the standard deviation of XTT assay by different bacterial culture. The results show that the CB discs did not significantly affect the metabolic activity of bacteria tested either in single bacterial culture or mixed bacterial culture, when compared to the control. Prolonged period contact (60 minutes) with bacteria, CB had no significant effect on the metabolic activity. On the other hand, exposure of test bacteria to the CP discs significantly affected their metabolic activity. CP treatment caused loss of metabolic activity with increasing time of contact with bacteria. In the case of *S. mutans*, about 56% decrease in metabolic activity bacterial was observed in 15 minutes, but after 60 minutes the metabolic activity was reduced to 85% (Table 2). The mixed bacterial cultures were found to be relatively resistant to the

antimicrobial action of CP, but the reduction in metabolic activity was significant ($p < 0.05$) compared to the control (Table 2).

MTT viability assay - Table 3 shows the average and the standard deviation of MTT assay by different bacterial culture. The results of the study show similar findings to that of the XTT assay. Exposure of tested bacteria to CB had no significant effect on their viability compared to the control. The antimicrobial property of CP on both single bacterial culture and mixed culture of bacteria was significant confirming the results obtained with XTT.

Live/Dead staining - The table 4 shows the average and the standard deviation of Live/Dead assay by different bacterial culture. The results show that the CP treatment resulted in significant reduction in % live bacterial cells. Furthermore, the bacterial viability was reduced with increasing incubation times. CP treatment shows statistically significant difference among 15 and 60 minutes of contact with mixed bacterial suspension. Instead, CB had no effect on the viability of bacteria, when compared to the control.

Table 2. Average and standard deviation of XTT assay (metabolic activity) for different dentin bonding agents and different bacterial culture.

	Clearfil SE Bond (CB)			Clearfil SE Protect (CP)			Control(C)
	15 min	30 min	60 min	15 min	30 min	60 min	
S. mutans	0.29±0.09aA$^{¥}$	0.23±0.19aA$^{¥}$	0.28±0.12aA$^{¥}$	0.13±0.04aB^{Ω}	0.08±0.05aB^{Ω}	0.04±0.02aB^{Ω}	0.29±0.13$^{¥}$
S. sobrinus	0.23±0.18aA$^{¥}$	0.21±0.16aA$^{¥}$	0.26±0.15aA$^{¥}$	0.11±0.06aB^{Ω}	0.07±0.06aB^{Ω}	0.04±0.06aB^{Ω}	0.33±0.17$^{¥}$
S. gordonii	0.23±0.06aA$^{¥}$	0.23±0.06aA$^{¥}$	0.24±0.03aA$^{¥}$	0.10±0.04aB^{Ω}	0.10±0.07aB^{Ω}	0.11±0.06aB^{Ω}	0.33±0.14$^{¥}$
A.viscosus	0.32±0.12aA$^{¥}$	0.29±0.13aA$^{¥}$	0.28±0.11aA$^{¥}$	0.11±0.04aB^{Ω}	0.09±0.08aB^{Ω}	0.04±0.08aB^{Ω}	0.30±0.15$^{¥}$
L. lactis	0.28±0.13aA$^{¥}$	0.27±0.09aA$^{¥}$	0.23±0.07aA$^{¥}$	0.14±0.07aB^{Ω}	0.11±0.03aB^{Ω}	0.05±0.04aB^{Ω}	0.34±0.23$^{¥}$
2-species *(S.mutans + S.gordonii)*	0.33±0.12aA$^{¥}$	0.31±0.15aA$^{¥}$	0.31±0.76aA$^{¥}$	0.14±0.08aB^{Ω}	0.12±0.09aB^{Ω}	0.17±0.05aB^{Ω}	0.35±0.16$^{¥}$
2-species *(S.mutans + A.viscosus)*	0.33±0.16aA$^{¥}$	0.32±0.13aA$^{¥}$	0.29±0.13aA$^{¥}$	0.19±0.11aB^{Ω}	0.12±0.08aB^{Ω}	0.15±0.06aB^{Ω}	0.36±0.19$^{¥}$
3-species *(S.mutans + L.Lactis + A. viscosus)*	0.37±0.15aA$^{¥}$	0.37±0.11aA$^{¥}$	0.35±0.16aA$^{¥}$	0.29±0.1aA$^{¥}$	0.24±0.07aA$^{¥}$	0.19±0.09bB^{Ω}	0.37±0.21$^{¥}$

Similar small letters means no statistically significant difference comparing different exposition time, independently by each bacteria and adhesive system. Similar capital letters means no statistically significant difference comparing the same exposition time and adhesive systems, independently by each bacteria. Similar superscript symbol means no statistically significant difference between groups, independently by each bacteria and adhesive system.

Table 3. Average and standard deviation of MTT assay (bacterial viability) for different dentin bonding agents and different bacterial culture.

	Clearfil SE Bond (CB)			Clearfil SE Protect (CP)			Control(C)
	15 min	30 min	60 min	15 min	30 min	60 min	
S. mutans	2.42±0.87aA$^{¥}$	2.26±0.73aA$^{¥}$	2.38±0.91aA$^{¥}$	1.13±0.15aB^{Ω}	0.08±0.02aB^{Ω}	0.02±0.03aB^{Ω}	2.58±0.7$^{¥}$
S. sobrinus	2.33±0.81aA$^{¥}$	2.39±0.81aA$^{¥}$	2.39±0.11aA$^{¥}$	1.03±0.11aB^{Ω}	0.09±0.02aB^{Ω}	0.11±0.05aB^{Ω}	2.29±0.6$^{¥}$
S. gordonii	2.40±0.66aA$^{¥}$	2.24±0.72aA$^{¥}$	2.13±0.62aA$^{¥}$	0.82±0.13aB^{Ω}	0.48±0.02aB^{Ω}	0.27±0.01aB^{Ω}	2.55±0.14$^{¥}$
A.viscosus	2.34±0.41aA$^{¥}$	2.14±0.53aA$^{¥}$	2.28±0.91aA$^{¥}$	1.13±0.12aB^{Ω}	0.07±0.02aB^{Ω}	0.04±0.01aB^{Ω}	2.55±0.6$^{¥}$
L. lactis	2.21±0.26aA$^{¥}$	1.98±0.15aA$^{¥}$	2.06±0.3aA$^{¥}$	1.28±0.14aB^{Ω}	0.36±0.04aB^{Ω}	0.15±0.03aB^{Ω}	3.04±0.41$^{¥}$
2-species *(S.mutans + S.gordonii)*	2.51±0.82aA$^{¥}$	2.38±0.73aA$^{¥}$	2.27±0.81aA$^{¥}$	1.33±0.18aB^{Ω}	1.08±0.02aB^{Ω}	0.58±0.05aB^{Ω}	2.72±0.17$^{¥}$
2-species *(S.mutans + A.viscosus)*	2.34±0.41aA$^{¥}$	2.14±0.53aA$^{¥}$	2.28±0.91aA$^{¥}$	1.63±0.17aB^{Ω}	1.09±0.03aB^{Ω}	0.64±0.03aB^{Ω}	3.15±0.32$^{¥}$
3-species *(S.mutans + L.Lactis + A. viscosus)*	3.54±0.33aA$^{¥}$	3.49±0.36aA$^{¥}$	3.38±0.34aA$^{¥}$	2.98±0.27aA$^{¥}$	2.83±0.08aA$^{¥}$	2.14±0.07bB^{Ω}	3.56±0.39$^{¥}$

Similar small letters means no statistically significant difference comparing different exposition time, independently by each bacteria and adhesive system. Similar capital letters means no statistically significant difference comparing the same exposition time and adhesive systems, independently by each bacteria. Similar superscript symbol means no statistically significant difference between groups, independently by each bacteria and adhesive system.

Table 4 – Percentage of cells Live of Live/Dead assay for different dentin bonding agents and different bacterial culture.

	Clearfil SE Bond (CB)			Clearfil SE Protect (CP)			Control(C)
	15 min	30 min	60 min	15 min	30 min	60 min	
S. mutans	91±8aA$^{¥}$	96±2aA$^{¥}$	92±6aA$^{¥}$	44±13 aB^{Ω}	32±9 abB^{Ω}	23±6 bB^{Ω}	100$^{¥}$
S. sobrinus	93±7aA$^{¥}$	92±6aA$^{¥}$	90±8aA$^{¥}$	40±10 aB^{Ω}	36±7 abB^{Ω}	25±9 bB^{Ω}	100$^{¥}$
S. gordonii	95±4aA$^{¥}$	89±9aA$^{¥}$	84±12aA$^{¥}$	48±11 aB^{Ω}	31±8 abB^{Ω}	20±4 bB^{Ω}	100$^{¥}$
A.viscosus	91±8aA$^{¥}$	96±2aA$^{¥}$	92±6aA$^{¥}$	44±13 aB^{Ω}	32±9 abB	24±6 bB^{Ω}	100$^{¥}$
L. lactis	89±7aA$^{¥}$	84±10aA$^{¥}$	89±6aA$^{¥}$	51±12 aB^{Ω}	44±8 abB^{Ω}	26±7 bB^{Ω}	100$^{¥}$
2-species *(S.mutans + S.gordonii)*	84±6aA$^{¥}$	79±6aA$^{¥}$	77±8aA$^{¥}$	42±7 aB^{Ω}	33±4 abB^{Ω}	22±6 bB^{Ω}	100$^{¥}$
2-species *(S.mutans + A.viscosus)*	93±5aA$^{¥}$	89±4aA$^{¥}$	91±6aA$^{¥}$	48±8 aB^{Ω}	35±5 abB^{Ω}	25±4 bB^{Ω}	100$^{¥}$
3-species *(S.mutans + L.Lactis + A. viscosus)*	100aA$^{¥}$	100aA$^{¥}$	94±4aA$^{¥}$	87±5aA$^{¥}$	84±6aA$^{¥}$	69±6bB^{Ω}	100$^{¥}$

Similar small letters means no statistically significant difference comparing different exposition time, independently by each bacteria and adhesive system. Similar capital letters means no statistically significant difference comparing the same exposition time and adhesive systems, independently by each bacteria. Similar subscribed symbol means no statistically significant difference between groups, independently by each bacteria and adhesive system.

DISCUSSION

Dental caries is still one of the most prevalent infectious diseases affecting humans (Bratthall et al., 2005). *Streptococcus mutans*, and other species of Streptococci are identified as major causative agents of dental caries. *Actinomyces viscous* also a contributor for caries, and considered as an early colonizer of tooth surface (Loesche, 1986; Takahashi & Nyvad, 2011) while *Lactobacillus* is significantly associated with the progressive lesions (Loesche, 1986).

The antimicrobial activity of the two dentin bonding agents (CB and CP) was compared by viability assays, MTT and XTT and also by staining with Live/Dead fluorescent dye. The time periods of exposure of microorganisms to the bonding agents were selected based on our preliminary studies. The bacteria chosen for this study are the microorganisms commonly associated with caries and root canal infections. The antimicrobial activity of the dentin bonding agents was tested on individual microorganisms as well as in mixed bacterial culture condition to simulate their natural occurrence in the caries. The direct contact of dentin bonding agents discs with bacterial suspension provides a quantitative measure of the effect of direct and close contact between microorganisms and the tested material on microbial outgrowth and allows a distinction to be made between bactericidal or bacteriostatic effects when used assay to quantification of viable cells.

Our results showed that the bacterial viability was affected by CP but not by CB both on individual bacteria and also on mixed bacterial cultures. Cured CP discs exhibited antimicrobial activity within 15 minutes of contact and with increasing periods of time up to 60 minutes affected the degree of cell viability (Table 2 and 3). Thus, the dentin bonding agent with monomer MDPB exhibited antibacterial activity against all bacteria tested. Some of the MDPB-contaning primer remains uncured, since there are no photoinitiators in the primer. Then, despite of only little amount of the primer copolymerizes with bond agent and entrapped MDPB in the polymeric network. Therefore, the inhibition of microbial growth when the dentin bonding agent was light-activated may be explained by the fact that MDPB linked to the polymer chain can act controlling the bacteria growth (Imazato et al., 2003).

The antimicrobial activity of CP discs on single bacterial cultures are in accordance with previous studies (Imazato et al., 2002; Turkun et al., 2006; Gondim et al., 2008; Esteves et al., 2010) using agar diffusion method, where a distinct zone of inhibition with isolated bacterial strains was observed. Esteves et al., (2010), evaluated the CP antibacterial activity against five streptococci single species and verified that among the species tested *S oralis* was the most sensitive to self-etching adhesive systems; on the other hand, *S cricetus*, *S mutans* and *S sobrinus* were more resistant. Whereas, Gondim et al., (2008), evaluated the antibacterial activity of CP with or without light-activation, against *S. mutans* and *L. acidophilus* and observed that the non-light-activated material presented significantly greater antibacterial activity against bacteria tested. In the study antimicrobial activity was also observed when the test material was polymerized. The agar diffusion test is generally an acceptable method to test antimicrobial activity of dental materials, since the material to be tested is placed on an agar plate which has been bacteria seeded (Schmalz et al., 2004). For this method, the zones of growth inhibition provided by the material depend on the diffusibility of the material across the culture medium (Turkum et al., 2006). Besides, these studies do not provide quantitative measurement of the antimicrobial activity of the test agents.

The results of our study demonstrated measurable degree of antimicrobial activity of the adhesive systems by all three assay, MTT, XTT and Live/Dead cell staining methods. Based in XTT and MTT assay results of this investigation (Table 2 and 3), the polymerized disc of dentin bonding agent CP showed decreased about 50% of metabolic activity (XTT assay) and viability (MTT assay) of bacteria, it suggests that Clearfil SE Protect agent showed significantly higher antibacterial activity even after the bonding resin had been cured. This finding is agreement with Turkum et al., (2006). In addition, it has to be pointed out that the uncured monomer, in this study, was removed before all assays have been conducted. Tetrazolium salts of MTT and XTT are especially useful for assaying the quantification of viable cells. Thus, based on the results of bacterial metabolic function (XTT assay) and bacterial viability (MTT assay) of this investigation, the primer with MDPB could be bacteriostatic to cariogenic bacteria even after being cured.

The monomer MDPB is synthesized by combining a quaternary ammonium with a methacryloyl group (Imazato et al., 1994). The mechanism of antibacterial effect of quaternary ammonium compounds is believed to be due to the cationic and hydrophobic binding to cell wall components that disturbs membrane function and subsequently induces leakage of cytoplasmic material (Scheie, 1989). As quaternary ammonium compounds have a positive charge, they can interact rapidly with the bacterial cell surface, which is negatively charged (Izutani et al., 2011). MDPB incorporation in the primer has been shown to be an effective method of providing resin-based restorative materials with antibacterial activity (Imazato et al., 1998; Kuramoto et al., 2005; Lobo et al., 2005; Yoshikawa et al., 2007). When the number of viable bacteria was determined after the primer solution was kept in contact with 10^8 CFU of *S. mutans* suspension for 30 seconds, Imazato et al., (1997) found that the primer containing MDPB at 5% was able to kill all the bacteria. Nevertheless, the literature is not reports how long is the antibacterial effect after the bonding resin cured. It is expected that the antibacterial agent is immobilized by copolymerization of MDPB with other monomers contained in the primer would be able to show a long-lasting antibacterial effect after being cured against cariogenic bacteria such as *Streptococcus mutans*, *Streptococcus sobrinus, Actinomyces viscous* and *Lactobacillus* that could remain on caries-affected dentin when using minimally invasive procedures.

Clearfil SE Protect agent exhibited antibacterial effect, from 15 minutes of contact to 60 minutes, time chosen for this study (Table 2, 3 and 4). A slight decrease in tested bacterial viability was seen with increasing periods of contact time. On the other hand, CB had no significant effect on any of the bacteria tested up to 60 minutes of contact. CB failed to show antimicrobial activity even after incubation with bacteria for 24 hours (data not shown). The results of our study corroborated Imazato et al., (2008), evaluating the killing effectiveness of MDPB using a viable cell-staining method, demonstrated antimicrobial activity within 10 minutes of contact with 250 μg/mL of MDPB (Imazato et al., 2008). The effect of CP on the mixed bacterial cultures was more pronounced at 30 minutes and 60 minutes of incubation time (Table 4), unlike the effect seen with single culture bacteria.

Newly developed two-colour live/dead fluorescence staining methods complemented the investigative possibilities by providing a visual differentiation between

living (active) and dead (inactive) bacteria (Al-Ahmad et al., 2008; Boulos et al., 1999). In this study the investigation of Live/Dead assay shows that the dentin bonding agent with antibacterial primer have been killed greater than 45% of bacteria within 15 minutes, and then, killing progressed with increased time of incubation (Table 4).

In clinical situations where there is a lack of comprehensive and precise diagnoses of the extent of dental caries, it is difficult to decide how much contaminated dentin must be removed. Although there are some subjective and objective methods to diagnose carious lesions and differentiate between infected and affected dentin, the clinical criteria do not reflect the existence of cariogenic bacteria or their virulence in the dentin environment (Imazato et al., 2006). There is evidence that *S. mutans* and lactobacilli are the major human dental pathogens (Loesche, 1986), however, several other species of bacteria have been associated with carious lesions (Takahashi & Nyvad, 2011). When a bacterial suspension with *S. mutans* + *L. lactis* + *A. viscosus* in associate was used in this study to simulate the deep caries-affected dentin colonization, the CP treatment showed bactericidal effect after 60 minutes of contact with the bacterial suspension for all three assays (Table 2, 3 and 4). The combination of three testing methods may increase the information value of studies focusing on effects of dentin bonding agent with primer containing antibacterial monomer against microorganisms.

Residual decay following cavity preparation, can potentially lead to caries progression and failure of the restorative procedure (Bergenholtz, 2000). It therefore seems reasonable to suggest that restoration longevity might be improved by using restorative or liner materials with antibacterial properties.

In conclusion, incorporation of MDPB into dentin bonding agent is considered to be effective in providing a post-cured antibacterial effect against cariogenic bacteria isolated or associated. The current dentin bonding agent with MDPB-containing primer can be a promise in a clinical situation on the disinfection of residual-affected dentin in a prepared cavity and can be highly useful for successful restorations.

ACKNOWLEDGEMENTS

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CAPÍTULO 3

Influence of chlorhexidine and adhesive systems on bonding degradation of demineralized dentin/resin interface.[4]

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Abstract: Purpose: To evaluate the effect of chlorhexidine (CHX) and antibacterial monomer (MDPB) on demineralized dentin/resin bonding degradation after thermomechanical cycling.**Methods**: 160 bovine dentin slices were used: one half for push-out bond strength and marginal adaptation, and the others were used for nanoleakage. Eight groups were set according to dentin surface treatment (with CHX or no CHX), thermomechanical cycling (500 cycles 5 - 55^{o} C/100,000 cycles of 50 N loading) (TM) or no thermomechanical cycling (no TM) and adhesive system (Clearfil SE Protect - CP; Clearfil SE Bond - CB), into: CP/CHX/noTM; CP/noTM; CB/CHX/noTM; CB/noTM; CP/CHX/TM; CP/TM; CB/CHX/TM; CB/TM. In the CHX groups, CHX was applied before bonding procedure. Composite resin was used to completing the restorative procedure. After 24 h a half of the specimens (no TM) were submitted to the push-out bond strength and the others to TM before testing. Impression in epoxy resin of each group was obtained to evaluate the marginal adaptation by scanning electron microscopy (SEM). For the nanoleakeage evalution the specimens were prepared as before described and mesio-distal serially sectioned. They were immersed into a silver ammoniacal nitrate solution for 24 h and exposed to photodeveloping solution for 8 h. After polishing, the specimens were observed in backscattered electron beams-SEM. Data were submitted to three-way ANOVA and Tukey's test (5%). Datas of failure mode, marginal adaptation and nanoleakege were used as descriptive analyses. **Results**: There was no significant difference in bonding strength between TM or no TM groups ($p>0.05$); Bond strength values for CHX groups were maintained for specimens of TM group, showing mixed

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failure. Adhesive failure mode was most frequent at no TM group. Marginal gap was observed on all specimens, but to TM group the percentage of marginal gap was increased, regardless adhesive system and CHX treatment. All groups showed nanoleakage at the resin/dentin interfaces and an increase in silver deposition could be notice after TM. Higher percentages of nanoleakage were found in groups without CHX. **Conclusions**: Dentin bond strength was not affected by TM and adhesive system type. CHX increased the bond strength and had a positive effect to decrease nanoleakage and marginal gap after thermomechanical cycling.

Keywords: bond strength; chlorhexidine; adhesive systems; thermomechanical cycling; nanoleakage; caries-affected dentin

INTRODUCTION

The operative dentistry have been shifted from the traditional approach of large extensions and invasiveness in tooth care to procedures more conservative as minimally invasive cavity designs (ten Cate, 2001) in which the bonding surface encountered after caries excavation consists of caries affected dentin and sclerotic dentine (Erhardt et al., 2008).

In contrast to sound dentin, the caries-affected dentin present acid-resistant mineral deposits that frequently occlude the dentin tubule lumem act as barriers that decrease infiltration of resin monomers acid (Nakajima et al., 2005), bacteria and bacterial products (Marshall et al., 2001). It is still a big challenge to seal the resin-dentin interface owing to the heterogeneous character of dentin structure and morphology surface (Yuan et al., 2007). Morphological studies have shown that a perfect hybrid layer is rarely formed in caries-affected dentin due a deeper zone of demineralization, it is more difficult for resin monomers to completely infiltrate the collagen dentin matrix (Wang et al., 2007).

Matrix Metalloproteinases (MMPs) are thought to be responsible for degrading most of the extracellular matrix components, including different types of collagen, in native and denatured forms (Visse & Nagasse, 2003). In addition, the promoting of collagen degradation, it leads to water penetration at the hybrid layer, and an increase in hydrolytic degradation of filling materials, and decreasing the quality of materials that interface with tooth substrate (Pashley et al., 2004). It was shown that the dentinal

collagenolytic activity can be strongly reduced by the use of chlorhexidine (Pashley et al., 2004), a potent MMP inhibitor. Chlorhexidine also prevents or minimizes the auto-degradation of exposed collagen fibrils within incompletely-formed hybrid layers (Carrilho et al., 2007), thereby, contributing to the long-term stability of the hybrid layer and bond strength (de Castro et al., 2003), furthermore the use of chlorhexidine in disinfecting cavity can reduces the number of residual bacteria (Ersin et al., 2006).

The reasons for failure of adhesive restorations are loss of retention and marginal adaptation. Gap on bonding interface allowing that bacterial substrate can come down to restoration and sometimes caries lesion progression would be observed, also bacteria adversely left in cavity preparation could survive for longer than one year (Sharma et al., 2009), which can result in secondary caries. The use of disinfectant solutions is an alternative to reduce or eliminate bacteria from cavity preparations (Ersin et al., 2006). The comprehensive antibacterial adhesive system Clearfil SE Protect, employing an MDPB-containing primer, has been developed (Imazato et al., 1998) and may help to reduce the harmful effects caused by oral bacteria (Izutani et al., 2011).

The durability of the bonding interface between a composite resin and tooth structure affected by caries, since that it constitutes the largest surface area of cavity preparations (Erhardt et al., 2008), and the use a self-etching primer with antibacterial activity or cavity disinfectant are important factors to give more longevity to adhesive restorations. The aim of this study was to analyze the effect of antibacterial monomer MDPB and cavity disinfectant on demineralized dentin/resin bonding strenght, marginal adaptation and nanoleakage after thermomechanical cycling.

MATERIAL AND METHODS

A hundred sixty bovine incisors were selected, 80 for bond strength and marginal adaptation and 80 for interfacial nanoleakage and divided into 8 groups. They were cleaned, and stored in a 0.5% chloramine T solution at 4^{o} C for no more than a week. The roots were sectioned off 1 mm under the cement enamel junction using a double-face diamond saw (K. G. Sorensen, São Paulo, SP, Brazil). The buccal surface was ground flat on a water-cooled mechanical polisher (Metaserv 2000, Buehler, UK Ltd, Lake Bluff, IL 60044-USA) using 80-, 180-, 320-, and 600-grid silicon carbide (SiC) abrasive paper

(Carbimet Disc Set, #305178180, Buehler, UK Ltd, Lake Bluff, IL 60044 - USA) in order to expose a flat dentin area of at least 8 mm^2. These teeth were observed in a stereomicroscope (Zeiss, Manaus, AM, Brazil), at x 25 magnification, to verify whether the enamel has been completely removed.

The dentin surface was fixed in an acrylic base and in the central part of each slice, one conical shape preparation (top diameter of 4 mm, bottom diameter of 3.0 mm and 2 mm in height) was prepared on the flattened surface using a diamond tip (# 3110; K. G. Sorensen, São Paulo, SP, Brazil) mounted in a high-speed handpiece (Kavo, Joinville, SC, Brazil) under constant air water cooling in a standard dental cavity preparation appliance. The diamond tips were replaced after every 10 preparation.

The specimens were randomly divided into 8 groups of 10 specimens each according to chlorhexidine solution treatment, adhesive systems and thermomechanical cycling (TM) or no thermomechanical cycling (no TM) (Table 1). The brand names, main components, manufacturers, batch numbers, pH values and application methods of adhesive systems are shown in Table 2. Clearfil AP-X was used to complete the bonding procedures.

Table 1 –Distribution of groups regarding the adhesive system, CHX application and cycling

No Thermomechanical cycling (no TM)				Thermomechanical cycling (TM)			
Clearfil SE Protect (CP)		Clearfil SE Bond (CB)		Clearfil SE Protect (CP)		Clearfil SE Bond (CB)	
CHX	No CHX	CHX	No CHX	CHX	No CHX	CHX	No CHX

Table 2 – Description of composition, manufacturer and batch number, pH values and application technique of the materials used in this study.

Materials	Composition	Manufacturer (Batch number)	pH value*	Application Technique**
Clearfil SE Protect (CP)	**Primer**: water, MDP, MDPB, HEMA, Hydrophobic methacrylate **Bond**: MDP, HEMA, Bis-GMA, Hydrophobic dimethacrylate, di-Camphorquinone, N-Diethanol-p-toluidine,silanated colloidal silica	Kuraray Medical Inc, Kurashiki, Tokyo, Japan (Primer: 00047A) (Bond: 00072A)	pH = 1.9 (primer) pH = 2.8 (Bond)	a, b, c, e (10s)
Clearfil SE Bond (CB)	**Primer**: MDP, HEMA, Hydrphobic dimetacrylate, N-Diethanol-p-toluidine, Camphorquinone, water **Bond**: MDP, Bis-GMA, HEMA, silanated coloidal sílica, Hydrophobic dimethacrylate, Camphorquinone , N-Diethanol-p-toluidine	Kuraray Medical Inc, Kurashiki, Tokyo, Japan (Primer: 00830A) (Bond: 01212A)	pH = 2.0 (primer) pH = 2.0 (Bond	a, b, c, e (10s)
Clearfil APX	Silanated barium glass, Silanated colloidal silica, Silanated silica, Bis-GMA, TEGDMA, dl-Camphorquinone	Kuraray Medical Inc, Kurashiki, Tokyo, Japan (# 00985B)	-	d, e (40s)

*manufacturer's information **Application technique: a: apply self-etching primer by 20 seconds; b: gently air dry; c: apply bond; d: 2mm of increment; e: photoactivate. MDP: 10-methacryloyloxydecyl dihydrogen phosphate; MDPB: 12-methacryloyloxydodecylpyridinium bromide; HEMA: 2-hydroxyethyl methacrylate; Bis-GMA: bisphenol-A diglycidil ether dimethacrylate; TEGDMA: Triethyleneglycol dimethacrylate

Specimens were coated with a red acid-resistant nail varnish (Colorama; CEIL Com. Exp. Ind. Ltda., São Paulo, SP, Brazil), except 1 mm from the cavity area that would be further submitted to demineralization dentin. Demineralized dentin was produced *in vitro* using an acid gel model. Specimens were immersed in 5 mL of 6% carboxymethylcellulose acid gel (Proderma Pharmacy, Piracicaba, SP, Brazil) at pH 5.0 and 37º C. The gel contained 0.1 M lactic acid titrated to pH 5.0 with a concentrated KOH solution (Van der Veen & ten Bosch, 1996). The specimens remained in the gel for 24 hours without renewal. This model produced a demineralized dentin depth about 40 µm, confirmed in a pilot study of polarized light microscopy. After artificial caries development, specimens were washed two times in an ultrasound bath with distilled water during fifteen minutes each and dried with tissue paper.

Then, at the specimens that received CHX (FGM Joinvile Santa Catarina, Brazil), CHX was applied on the substrate surface as a therapeutic primer and gently blot dried after a dwell time of 60 seconds (Komori et al., 2009). The adhesive systems were then applied according to the manufacturers' instructions, and the composite was inserted in a single increment and light-cured for 40 seconds with an Elipar Tri-light unit (ESPE – America Co., Seefeld 82229 - Germany). Light intensity was periodically measured (740mW/cm^2).

After the light curing procedures, the specimens were stored in distilled water at 37 ^{o}C for 24 h. The restorations were then finished with 600-, 1200- and 2000- grid SiC paper under water and polished with 1 and 0.5 mm diamond paste using a polish cloth under water and ultrasonically cleaning for 30 min between the steps of the finishing procedures.

Thermomechanical cycling procedure (TM) – Specimens from TM group were subjected to 500 cycles at 5 ± 2^{o}C and 55 ± 2^{o}C with a dwell time of 30 seconds, at the same time that a loading device delivered an intermittent axial force of 50 N at 4 Hz totaling 100,000 cycles (Thermal/mechanical cycling, ER 37000, São Paulo, SP, Brasil).

Evaluation of marginal adaptation by SEM - Impressions of each group were taken with a low viscosity polyvinyl siloxane material (Aquasil, Dentsply DeTrey, Konstanz, Germany) and the impressions were poured with epoxy resin (Buehler, Lake Buff, Ill., USA). Afterwards, they were gold-sputter coated (Balzers-SCD 050 Sputter Coater, Liechtenstein) and observed by SEM (JEOL, JSM-5600LV, Scanning Electron Microscope, Japan) for evaluation and classification of the cavity margins. The classification was made with x 200 magnification directly on the microscope screen and observing the entire cavity perimeter according to morphologically defined parameters: 1) Perfect margin: defined as a continuous, gap-free transition between filling and tooth substrate (Figure 1A), and 2) marginal gap: observed as any gap formation and loss of interfacial adhesion (Figure 1B). This classification was defined by Kemp Scholte & Davidson, (1990).

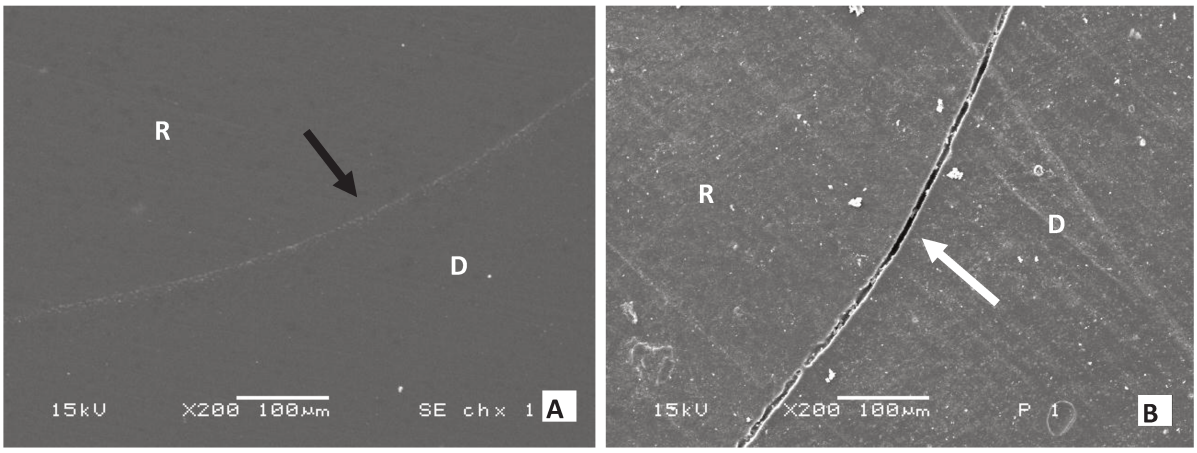


Figure 1 – SEM analysis, x 200 magnification. A. Perfect sealing of the margin (black arrow). B. Marginal gap (white arrow). R – resin composite replica; D –dentin replica

Push-out bond strength test - After 24 h the specimens of no TM group were submitted to the push-out bond strength test and the specimens of TM group after thermomechanical cycling. Thus, an acrylic device with a central hole was attached to the base of a universal testing machine (EMIC, model DL, São José dos Pinhais, PR, Brazil). The central hole was used to control specimen positioning with the restoration bottom side up (smaller diameter of the preparation). A round stainless steel tip (2 mm diameter) attached to the upper grip of the testing machine applied a compressive load at a rate of 0.5 mm/min on the exposed, bottom surface of the restoration and forced the restoration out. The results were recorded in kgF and converted into units of stress (MPa) by dividing by the remaining, conical specimen bonded area. After the test, the fractured specimens were gold-sputter coated (Balzers-SCD 050 Sputter Coater, Liechtenstein) and observed by SEM (JEOL, JSM-5600LV, Scanning Electron Microscope, Japan) and classified in terms of failure: cohesive, adhesive, or mixed failures (Figure 2).

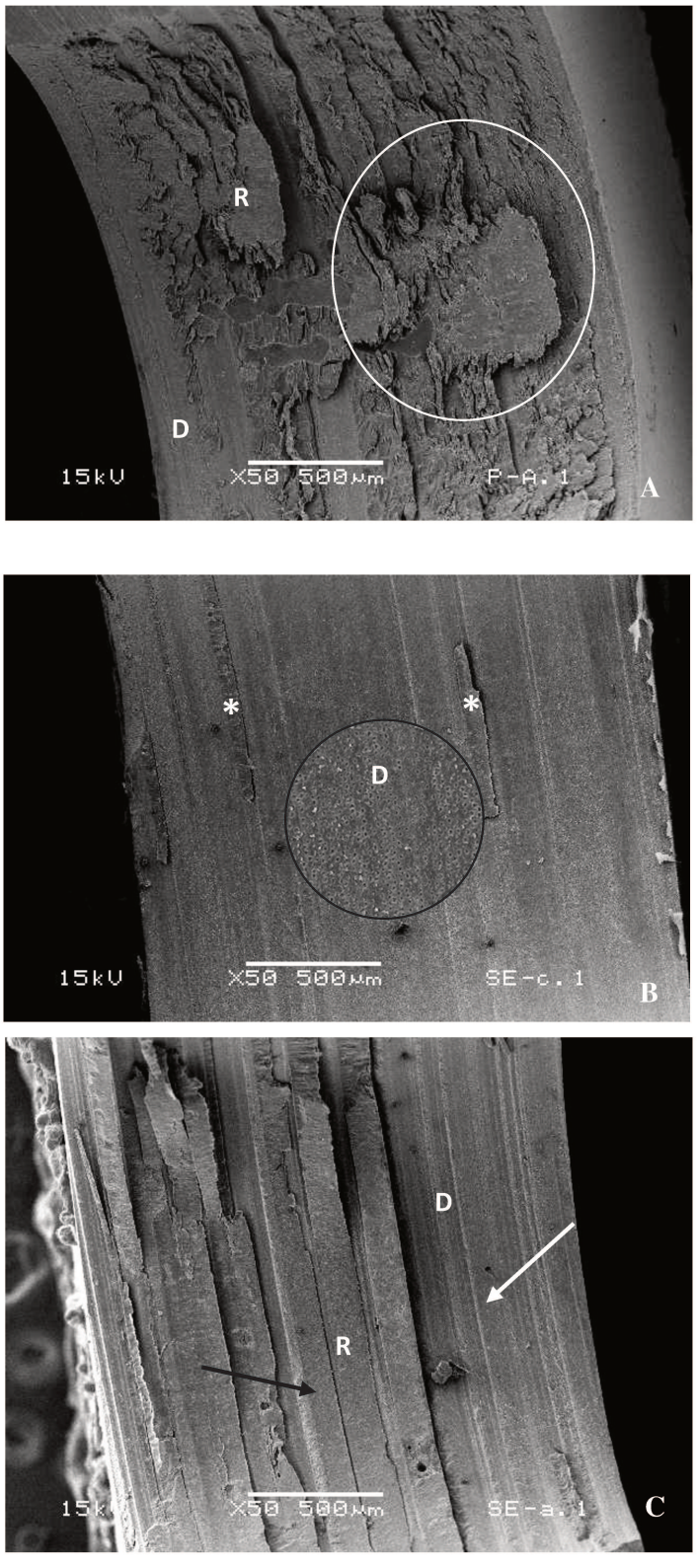
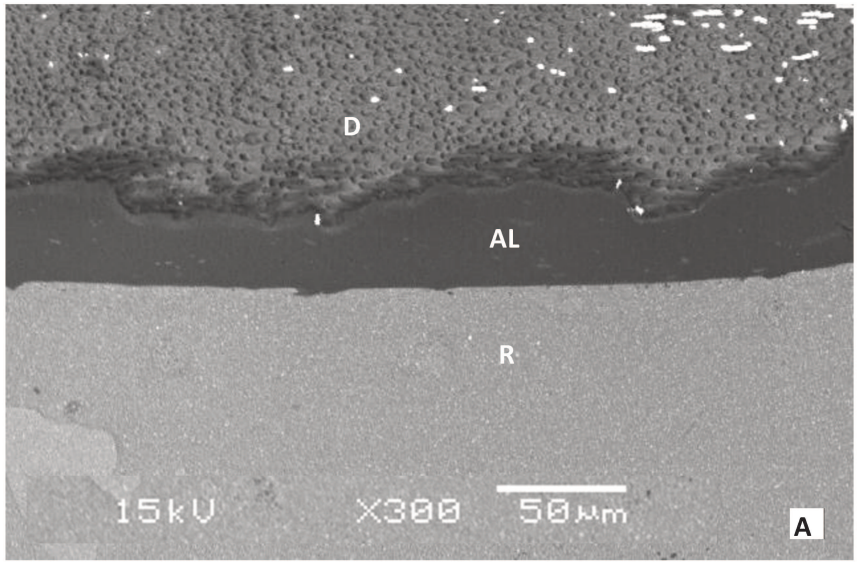


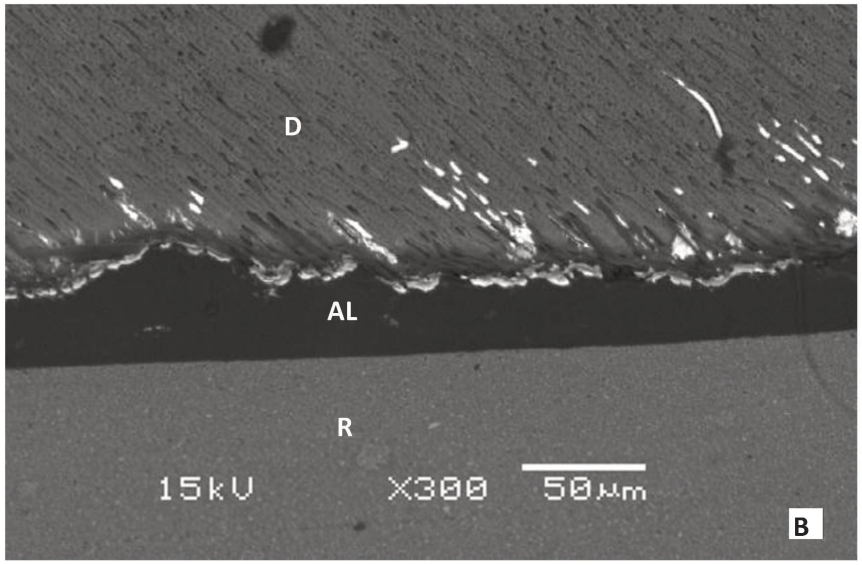
Figure 2 – SEM electronmicrography illustrations used for failure classification (x 50). D - dentin; R – resin composite. (A) **cohesive failure** (resin or dentin), White circle - cohesive failure in resin composite; (B) **adhesive failure**, inside the black circle (x 200 magnification) showing adhesive failure; *adhesive layer; (C) **mixed failure**; black arrow shows cohesive failure in resin composite; white arrow shows adhesive failure in dentin

Interfacial nanoleakage - Eighty bovine incisors were selected for interfacial nanoleakage and all restorative procedures were accomplished as before described and divided into 8 groups following Table 1.

To evaluate the interfacial nanoleakage, each tooth/restoration set was sectioned transversally (Isomet, Buheler, Lake Bluff, IL, USA) under water lubrification producing 0.8 mm thick slices. One slice from each tooth was used in the interfacial nanoleakage assay (n=10).

Specimens were coated with two layers of a red acid-resistant nail varnish (Colorama; CEIL Com. Exp. Ind. Ltda., São Paulo, SP, Brazil) applied up to within 1 mm of the bonded interfaces. To rehydrate specimens, they were immersed in distilled water for 20 min prior to immersion in the tracer solution in total darkness for 24 h. Ammoniacal silver nitrate solution was prepared by the dissolution of 25 g of silver nitrate crystals (Sigma Chemical Co., St. Louis, MO, USA) in 50 mL of distilled water and 50 mL of ammonium hydroxide 28 % (Sigma Chemical Co., St. Louis, MO, USA) yielding a solution of pH = 11.0. Then, the slices were rinsed thoroughly in distilled water and immersed in photodeveloping solution for 8 h under a fluorescent light. Again, the slices were rinsed thoroughly in distilled water and one side of each slice was polished using a water-cooled mechanical grinder (Aropol E, Arotec, São Paulo, SP, Brazil) using 600-, 1200- and 2000-grit silicon carbide abrasive papers (Carbimet Disc Set, # 305178180, Buehler, UK LTD) and with a 3-µm and 1-µm diamond paste with cloth. Specimens were then demineralized with 37 % phosphoric acid for 5 s, washed with distilled water for 30 s, and dried with a tissue paper. Following deproteinization with 10% NaOCl for 5 min, then specimens were washed in an ultrasound bath and dried with tissue paper and left to dry in a desiccator for 24 h at room temperature. Finally, prepared slices were mounted on aluminium stubs, sputter-coated with carbon (Balzers-SCD 050 Sputter Coater, Liechtenstein) and examined with a SEM (JEOL- JSM 5600LV, Tokyo, Japan) under magnifications of 300 x and 800 x. Penetration of silver into the adhesive dentin interface, hybrid layer and adhesive layer were evaluated in a descriptive analyzes and scored as: no leakage, score 0; leakage only in the base of hybrid layer and around resin tags, score 1; leakage inside hybrid layer and/or adhesive, score 2 (Figure 3).





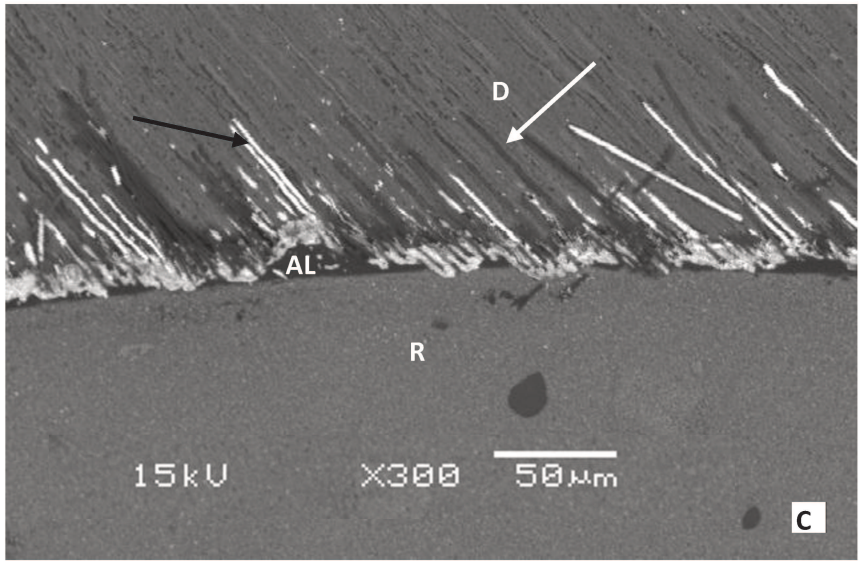


Figure 3 – SEM from resin/dentin interface of x 300 magnification. Nanoleakage scores: R – composite resin; AL – adhesive layer; D – dentine; (A) no leakage, **score 0**; (B) leakage just in the base of hybrid layer and around resin tags, **score 1**; (C) leakage inside hybrid layer and/or adhesive, **score 2**; resin tag free of silver (white arrow) and covered with silver (black arrow).

Statistical analysis

Bond strength data were analyzed with three-way Analysis of Variance and Tukey's HSD post hoc tests ($p<0.05$), after data had been transformed as log. The descriptive analyses were used to datas of marginal adaptation, nanoleakage of resin-dentin interface and failure mode.

RESULTS

The means of bond strenght values and standard deviations, percentage of perfect margin or marginal gap, percentage of scores for the failure mode and nanoleakage score for all the groups are in Table 3.

Table 3 – Means (standard deviation) for bonding strength to dentin, and percentage of perfect margin or marginal gap, percentage of scores for the failure modes and nanoleakage scores regarding the adhesive system, CHX application and TM cycling.

	Group	Bond strenght	Marginal adaptation (%)		Failure modes (%)			Nanoleakage scores **(%)		
		MPa	Perfect	Gap	Adhesive	Mixed	Cohesive	Score 0	Score 1	Score 2
No CYCLING (No TM)	CP/CHX	11.0(6.68)	62.5	37.5	57.14	35.71	7.14	20	80	0
	n		8			14			10	
	CP	12.7(6.54)	62.5	37.5	47.05	35.3	17.64	30	70	0
	n		8			17			10	
	CB/CHX	13.3(4.82)	87.5	12.5	40	40	20	30	70	0
	n		8			15			10	
	CB	14.3(7.08)	75	25	66.67	16.66	16.66	0	100	0
	n		8			12			10	
CYCLING (TM)	CP/CHX	16.1(8.01)	40	60	42.10	52.63	5.26	23	55	22
	n		10			19			9	
	CP	11.0(5.69)	20	80	70	25	5	20	50	30
	n		10			20			10	
	CB/CHX	12.4(5.26)	44.45	55.55	37.5	43.75	18.75	20	65	15
	n		9			16			10	
	CB	8.8 (4.64)	37.5	62.5	72.22	11.1	16.66	0	80	20
	n		8			18			10	

* Similar letters means no statistically significant difference between groups. ** score 0, no leakage; score 1, leakage just in the base of hybrid layer and around resin tags; score 2, leakage inside hybrid layer and/or adhesive,.

Three-way ANOVA showed no significantly interactions among three studied factors: adhesive system, chlorhexidine solution and TM cycling; however, it was observed a significant interaction between chlorhexidine solution use and TM cycling (p=0.047).

The Tukey's HSD test revealed a significantly statistical difference for TM groups treated with CHX, regardless of adhesive system used; the groups treated with CHX solution showed higher bond strength than the groups without treatment (Table 4), and they showed predominance of mixed failure (CP/CHX/TM group and CB/CHX/TM group on the table 3). However, there was no statistical difference among the groups with or without CHX treatment for the specimens of no TM. All of other groups presented predominance of adhesive failure (groups CP/CHX/noTM, CP/noTM, CB/noTM, CP/TM and CB/TM). But, the CB/CHX/noTM group showed similar percentage of adhesive and mixed failure (Table 3).

Table 4 – Bond strength values (MPa) in substrate regards to CHX application

Group	CHX application	
	No	Yes
No TM	13.48 ± 6.64^{Aa}	12.25 ± 5.73^{Aa}
TM	9.87 ± 5.18^{Aa}	14.25 ± 6.86^{Ba}

Similar capital letters means no statistical significant differences between averages in the same row. Similar small letters means no statistical significant differences among averages in the same column.

The evaluation of marginal adaptation in all groups showed gap formation and loss of interfacial adhesion, however, the percentage of marginal gap was higher in the TM group than no TM group. The higher percentage of perfect margin was observed in CB/CHX/noTM group (87.5%). The groups that had CHX treatment showed lower percentage of marginal gap than the others (Table 3).

Also, the results of evaluation, in percentage, of nanoleakage of all the experimental groups are summarized in Table 3. All groups showed nanoleakage at the resin/dentin interfaces. An increase in silver deposition could be notice after thermomechanical cycling. The score 1 was found of indistinct form in all the periods. The score 2 only was found in TM group and was mostly frequent in the groups without previously CHX application.

DISCUSSION

The use of thermomechanical cycling has been sugested as a method of providing *in vitro* simulations of *in vivo* oral conditions. The repetitive contraction/expansion and chewing stresses are simulated by thermal cycling and mechanical cycling, respectively. *In vitro* studies using these types of stresses to evaluate the longevity of restored teeth have been performed (Hakimeh et al., 2000; Nikaido et al., 2002), but few of them have applied these types of stress simultaneously (Nikaido et al., 2002).

Under the conditions of this study, when compared the bond strength among TM group and no TM group there was no significant difference (Table 3), suggesting that in spite of thermomechanical cycling may affect resin adhesion to tooth structure, it was not enough to decreased the bond strength in this study. This findings are according with Paula et al., (2008) that detected that thermal (500 cycles, 5° to 55°C) and mechanical cycling (50,000 cycles, 50N) simultaneously did not have a significant influence on compressive strength. But this result disagree with previous studies (Bedran-de-Castro et al., 2004; Nikaido et al., 2002). In their studies, there was a decrease in microtensile bond strength of a self-etching adhesive system when thermal and mechanical load cycling concomitantly in Class I restorations, using 50 N load force with 100,000 cycles mechanical and 2000 cycles thermal at 5-55^{o}C. The quantity of cycles and the temperatures used on both thermal and mechanical load cycling and the area size seems to be the major difference among different studies (Hakimeh et al., 2000) and can be responsible for the different results. In addition, the difference among the research results can be referred to the type of test. The push-out bond strength test is generally used to evaluate endodontic cements in the radicular canal space (Perdigão et al., 2004). However, in the present study this test was adapted to evaluate strength of restorative composites in a simulated Class I preparation. In bonding strength tests, such as microtensile and microshear tests, the stress is concentrated in small areas. Otherwise, in push-out bond strength test, the applied load is absorbed and dissipated in a greater area, instead of small one as microtensile or microshear bond strength tests.

Regarding the use of CHX, the groups treated with 2% chlorhexidine solution prior to the adhesive systems showed that CHX treatment did not affect the immediate (no TM group) bond strength, regardless of adhesive system (Table 3), suggesting that the use of CHX does not interfere on the formation of hybrid layer. This results are in accordance

with previous studies that used etch & rinse or self-etching adhesive systems in caries-affected dentin (Komori et al., 2009; Mobarak, 2011), and with studies that used sound dentin (de Castro et al., 2003; Breschi et al., 2010; Mobarak, 2011). However, the results are in disaccording with previous studies, but they were conducted using self-etch adhesive systems and sound dentin (Ercan et al., 2009). The authors stated that CHX exerted an adverse effect on shear bond strength when used with the self-etching bonding system.

Nevertheless, after thermomechanical cycling, despite there was no significance difference on bond strength among TM and no TM groups, the CHX treatment increased the bond strength for the specimens of TM group, regardless of adhesive systems (Table 4). This result corroborated partially the findings of previous studies (Chang & Shin, 2010; Campos et al., 2009) that observed that CHX pretreatment did not affect *in vitro* bond strength of the tested specimens during the immediate testing period. However, 10,000 thermo cycles resulted in a significant bond strength reduction in the control group, but lower than that observed in the groups treated with CHX (Chang & Shin, 2010). In addition, Campos et al., (2009) observed that after thermomechanical cycling, 2% CHX applied prior to self-etch adhesive system resulted in higher microtensile bond strength values compared to the control. The difference between the studies can be attributed to the number of cycles. Our study was conducted using thermomechanical cycles concomitantly, i.e, differently from Chang & Shin, (2010) study's. Even in this condition CHX could protect the resin/dentin bonding.

The greater percentagem of mixed failure was observed in the specimens treated with 2% CHX after thermomechanical cycling (Table 3). This findings support that maintenance of bond strength was due to a better preservation of the collagen fibrils, which was referred by Carrilho et al., (2007). The authors observed that 2% CHXapplication preserves both the durability of the hybrid layer and bond strength *in vitro* of aged specimens.

In the current study, when the specimens were submitted to stress from thermomechanical cycling, the percentage of marginal gap formation increased (Table 3), as observed in other studies (Nikaido et al., 2002; Paula et al., 2008). The thermomechanical cycling can cause fissures that propagate through the entire bonding

interface and enable a continuous flow of oral fluids in a process called percolation (Irie & Suzuki, 2001). It could be suggested that this stress of restored teeth results in significant stress between tooth and dental material, thus decreasing the bond strength and increasing the degradation interface. However, the percentage of marginal gap was lower to group that received 2% CHX treatment previously to adhesive systems, this results is according to previous studies that showed that CHX is able to maintain the hybrid layer due to a better preservation of the collagen fibrils (Carrilho et al., 2007).

Even when no gaps can be observed, leakage can occur between the hybrid layer and intact dentin (Sano et al., 1994). This investigation showed nanoleakage at the resin/demineralized dentin interfaces for all groups evaluated (Table 3). Nevertheless, after thermomechanical cycling the percentage of silver deposition was increased, according to Li et al., (2002) which showed that the thermocycled samples (5-55^{o}C for 1500 cycles) showed a slightly more intense silver deposition.

According to the findings of the present study, we may speculate that the increase of silver deposits can be due to degradation of the adhesive interface after thermomechanical cycling; however, this increase was mostly frequent in the groups without previously CHX application. Besides, the frature mode analysis clearly shows adhesive failure for almost all groups, but to groups treated with CHX solution after thermomechanical cycling (Table 3). The percentage of adhesive failure indicates a possible degradation of the adhesive layer and/or hybrid layer.

Similar to incompletely infiltrated sound dentin, the non-infiltrated caries-affected dentin may undergo self-destruction via the release of endogenous matrix metalloproteinases (Pashley et al., 2004). There is also immunohistochemical evidence suggesting that the antigenicities of type I collagen and proteoglycans are masked in caries-affected dentin (Suppa et al., 2006) which may prevent the potential for intrafibrillar remineralization of the partially demineralized collagen matrices in this altered substrate (Erhardt et al., 2008).

Adhesive resins can seal the acid-etched dentin from water, they may preserve their mechanical/chemical properties and, at the same time, protect the collagen against a hydrolytic attack by endogenous MMPs (Komori et al., 2009). Futhermore, CHX applied

on exposed collagen fibrils, may protect the collagen against a dentinal collagenolytic attack by endogenous MMPs (Carrilho et al., 2007). It is an effective synthetic MMP-inhibitor that could directly inhibit the activity of MMP-2, -8 and -9 (Carrilho et al., 2007).

Ours results indicated that regardless of TM cycling and CHX treatment, the adhesive systems CP and CB did not affect the bond strength (Table 3). Except for the presence of the MDPB monomer, the composition of Clearfil SE Bond and Clearfil SE Protect are very similar. It has been suggested that the outstanding hydrolytic stability of MDP and its additional chemical interaction with dentin contributed to the long-term durability of dentin bonding, MDPB contain C=C bonds which are capable of undergoing a free radical polymerization (Ansari et al., 2008), but MDPB would be immobilized within the polymer network and would not induce weakness or degradation in the bonding layer through dissolution and substitution by water (Imazato et al., 2006) such as observed in our study. Furthermore, our results point out that regardless self-etching adhesive system containing or not antimicrobial, 2% CHX using provided an increase on bond strength after thermomechanical cycling, protecting bonding interface. In addition, 2% CHX show an additional advantage due to its antimicrobial activity (Ersin et al., 2006) and can be used in partial removal caries procedures, in a minimally invasive dentistry concept.

CONCLUSION

In conclusion, CHX treatment can maintain the bond strength after thermomechanical cycling regardless self-etching adhesive systems used and it could be able to decrease nanoleakage and marginal gap.

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CONSIDERAÇÕES GERAIS

Agentes desinfetantes, como clorexidina, são utilizados na desinfecção de preparos cavitários (Atac *et al*., 2001; Imazato, 2009) e auxiliam na prevenção à cárie secundária, já que a formação de cárie recorrente ao longo das margens das restaurações é ainda a principal causa de substituição das mesmas (Tyas, 2005). A CHX e o sistema adesivo contendo MDPB, comercialmente denominado Clearfil SE Protect – CP, foram utilizados neste estudo em três experimentos *in vitro* para testar, respectivamente, o efeito dos mesmos na desinfecção de dentina desmineralizada e contaminada por *S. mutans*, na diminuição da viabilidade bacteriana e na degradação e resistência da interface da união à dentina desmineralizada.

O monômero MDPB foi considerado antibacteriano por reduzir o número de bactérias viáveis após a aplicação, porém antes de ser fotoativado (Imazato *et al*., 1997; Imazato *et al*., 1998; Imazato *et al*., 2006). A efetividade desse monômero antes de ser fotoativado foi confirmada nesse estudo quando o primer com monômero antibacteriano foi aplicado sobre a superfície de dentina desmineralizada e contaminada com *S. mutans*. Os resultados mostraram que independente da limpeza da superfície com CHX e do tempo de contato do MDPB com o substrato, o primer com MDPB mostrou ser capaz de diminuir o número de bactérias viáveis. Além disso, ainda no primeiro capítulo deste estudo foi verificada a atividade antibacteriana dos sistemas adesivos fotoativados (CP e Clearfil SE Bond - CB) em contato com *S. mutans* espalhados sobre meio de cultura, teste de difusão em ágar. Notou-se que somente a CHX e o CP apresentaram halos de inibição, no entanto, o MDPB apresentou menor halo de inibição do que a CHX usada como controle positivo.

O teste de difusão em ágar é o ensaio padrão para mensurar a propriedade antibacteriana de um material (Tobias, 1988), pois o componente antibacteriano se difunde através do meio de cultura e a atividade antibacteriana é baseada no tamanho das zonas de inibição (Esteves *et al*., 2010). Sendo assim e diante dos resultados obtidos pode ser sugerido que mesmo após a fotoativação, de acordo com a recomendação do fabricante, o primer com MDPB pode apresentar atividade antibacteriana contra *S. mutans*.

Entretanto, a fotoativação de materiais adesivos causa o aprisionamento dos componentes antibacterianos na matriz polimérica e assim, a liberação desses componentes tende a diminui (Imazato *et al*., 2002). Assim, o efeito antibacteriano do sistema adesivo auto-condicionante pode estar clinicamente restrito a um curto período e a camada superficial de dentina, como verificado por Gondim *et al*., (2008) e Schamlz *et al*., (2004).

Porém, o Clearfil SE Protect pode apresentar um melhor resultado clínico uma vez que depois de fotoativado uma parte das moléculas com estrutura de metacrilol são imobilizadas pela co-polimerização com outros monômeros de metacrilato e a outra parte das moléculas de metacrilol preserva a atividade antibacteriana, mesmo depois de ser imobilizado. Essas características fazem do material contendo MDPB um método potencial de inibição do crescimento bacteriano antes e após a fotoativação (Imazato *et al*., 1994). Para conferir a veracidade dessa informação, discos de sistemas adesivos (CP e CB) fotoativados foram utilizados nos capítulos 1 e 2 e testados por um período de 15, 30 e 60 minutos de contato direto com diferentes suspensões bacterianas.

No Capítulo 1, suspensão de *S. mutans* foi colocada sobre os discos dos sistemas adesivos fotoativados e tanto o sistema adesivo Clearfil SE Protect, contendo MDPB no primer e o Clearfil SE Bond (CB) com a mesma composição, com exceção do MDPB apresentaram o mesmo efeito contra *S. mutans*, ou seja, houve uma diminuição no número de unidades formadoras de colônias (UFC/mL) após uma hora de contato com esses dois sistemas adesivos. No Capítulo 2, os discos fotoativados dos sistemas adesivos CP e CB foram colocados dentro de meio de cultura com diferentes espécies bacterianas associadas à cárie e apenas o sistema adesivo contendo o monômero MDPB foi capaz de diminuir a atividade metabólica e a viabilidade das bactérias.

A diferença entre os resultados dos dois estudos pode ser explicada pela metodologia utilizada. No primeiro estudo, foi colocada sobre os discos de sistemas adesivos menor quantidade (10 µL) de suspensão bacteriana, porém, em maior concentração ($3x10^8$ células/mL) e, em seguida, verificado quantas células ainda eram viáveis pelo crescimento em meio de cultura. Enquanto que no segundo estudo, uma maior quantidade de suspensão bacteriana (150 µL) foi utilizada em menor concentração ($1x10^7$células/mL) e analisado a atividade metabólica e viabilidade das bactérias. Sendo o componente antibacteriano do

sistema adesivo fotoativado capaz de se difundir, e ser mensurado quando presente em caldo de meio de cultura (Giammanco *et al*., 2009), pode explicar a diferença entre os resultados obtidos. Além disso, os primers dos sistemas adesivos auto-condicionantes apresentam um baixo pH (< 2.0) devido a presença de monômeros ácidos polimerizáveis. Assim, pode ser sugerido que acidez dos primers auto-condicionantes tenha agido mais sobre a suspensão bacteriana no primeiro estudo do que no segundo.

De acordo com os resultados obtidos sugere-se que a atividade bactericida do CP apresenta ação duradora mesmo após a polimerização, isto pode ser confirmado com o estudo de Feuerstein *et al*., (2007), que observaram efeito antibacteriano do sistema adesivo Clearfil SE Protect utilizando o teste do contato direto após 7 dias em suspensão de *S. mutans*. Os autores sugerem que nem todos os monômeros de MDPB são co-polimerizados e que aqueles que não são capazes de se ligarem aos polímeros, manifestam as propriedades antimicrobianas. A diminuição da viabilidade bacteriana observada no capítulo 2, após contato com o sistema adesivo fotoativado, pode ser explicada pelo fato de que não ocorre uma completa conversão dos monômeros dentro do polímero e assim, monômeros residuais podem ser liberados para o meio (Imazato *et al*., 2003). Diante disso, foi sugerido que mesmo após a fotoativação, o CP pode apresentar atividade bactericida contra as bactérias cariogênicas presentes nas paredes das cavidades dentárias que estão em contato com esse material.

Contudo, é necessário precaução ao utilizar esse tipo de sistema adesivo, uma vez que estudos anteriores mostraram que a incorporação de agente antibacteriano poderia prejudicar as propriedades mecânicas de adesão ao substrato, e a liberação desse agente, a partir do material, pode resultar em mudanças nas propriedades físicas (Imazato *et al*., 1994; Jedrychowski *et al*., 1983), causando uma diminuição da resistência da união devido a degradação da camada híbrida na interface adesivo/dentina (Pashley *et al*., 2004; Toledano *et al.,* 2007). De tal maneira, o Capítulo 3 desse estudo foi realizado com o objetivo de verificar essa interferência. E encontrou-se que o sistema adesivo com primer contendo o monômero MDPB não alterou a resistência de união, nem a degradação da interface da união. Uma vez que o MDPB ao ser incorporado dentro da cadeia polimérica não enfraquece ou degrada a camada adesiva (Ansari *et al*., 2008).

Também, neste estudo foi analisado o uso da CHX que é um antisséptico utilizado para o controle químico da placa bacteriana e desinfecção de cavidades (Atac *et al*., 2001). A CHX foi utilizada, nos capítulo 1 e 3, como agente desinfetante para a limpeza da superfície dentinária e considerando o encontrado em resultados anteriores de Hebling *et al.,* (2005) com o intuito de preservar a união de materiais resinosos, pois a dentina afetada pela cárie apresenta um aumento da atividade colagenolítica (Dayan *et al*., 1983) devido a um maior número de fibras colágenas expostas (Haj-Ali *et al*., 2006).

A CHX pode preservar essas fibras contra o ataque colagenolítico da dentina (Komori *et al*., 2009), pois é um potente inibidor das MMPs (Gendron *et al*., 1999), responsáveis pela degradação das fibras colágenas e, consequentemente, da camada híbrida (Pashley *et al*., 2004).

Pelos resultados obtidos nos estudos realizados nesta tese, o uso da CHX na limpeza da superfície dentinária desmineralizada e contaminada com *S. mutans* não foi eficaz em eliminar bactérias presentes na superfície de dentina, nem aumentou o efeito antibacteriano dos sistemas adesivos, como visto no capítulo 1. Isto pode ser explicado pelo fato de que alguns componentes dentários, como dentina, hidroxiapatita ou colágeno, mostram reduzir ou inativar o efeito antimicrobiano da CHX (Haapasalo *et al*., 2007). Contudo, a CHX não interferiu na resistência da união, pelo contrário, preservou a resistência da união e a degradação interfacial após desafio de ciclagem termomecânica, no capítulo 3.

A ciclagem termomecânica foi utilizada nesse estudo com o objetivo de simular as tensões de origem térmicas e mecânicas advindas da cavidade oral, decorrentes, respectivamente, da ingestão de alimentos frios e quentes e da carga mastigatória, favorecendo, devido as tensões geradas pela ciclagem termomecânica, à degradação da camada hibrida na interface substrato/adesivo. No entanto, nesse estudo, não foi observado diferença estatística da resistência da união antes e após a utilização da ciclagem termomecânica, apenas observou-se um maior aumento de depósito de nitrato de prata (nanoinfiltração) e de fenda marginal nos grupos que receberam a ciclagem. Tanto a tensão térmica quanto a mecânica podem causar fendas, que se propagam através da interface adesiva e permite uma contínua passagem de fluidos orais, esse processo é conhecido como percolação (Irie & Suzuki, 2001). Diante dos resultados observados, pode ser sugerido que

a ciclagem termomecânica resultou em significante tensão entre o dente e o material restaurador, porém, não foi suficiente para causar perda da resistência da união e deflagar a perda da restauração, mas, se considerado em longo prazo será inevitável. Há que se considerar que diferentemente dos testes mecânicos que utilizam microareas seccionais (microtração e microcisalhamento), o teste utilizado neste estudo empregou uma área maior, com desenho cavitário que oferece tensões de contração de polimerização na interface da união, simulando mais aproximadamente a tensão que ocorre *in vivo*.

Estudos *in vitro* possuem limitações, as quais podem influenciar os resultados. Apesar disso, fica evidente nesse estudo que o sistema adesivo com propriedade antibacteriana, além de auxiliar a eliminação de bactérias residuais, promove um selamento eficaz e é capaz de prevenir a degradação da camada hibrida quando utilizado em conjunto com a CHX. Portanto, a associação da clorexidina com o sistema adesivo Clearfil SE Protect pode tornar-se um procedimento ideal para a prática clínica. Para essa confirmação estudos clínicos randomizados independentes devem ser realizados para demonstrar a evidência científica para a aplicação clínica desses conceitos.

CONCLUSÃO GERAL

Baseando-se nos resultados obtidos deste estudo, concluiu-se que:

1. A incorporação do MDPB no primer do sistema adesivo Clearfil SE Protect contribuiu para atividade antibacteriana desse material contra bactérias cariogênicas.
2. A presença do monômero antibacteriano no sistema adesivo Clearfil SE Protect assegura atividade bactericida antes e após a fotoativação.
3. A CHX não apresentou efeito bactericida quando em contanto com *S.mutans* presentes na superfície de dentina desmineralizada e contaminada.
4. Após 60 minutos de contato com diferentes espécies bacterianas, o Clearfil SE Protect fotoativado colaborou com a diminuição do metabolismo e viabilidade de bactérias cariogênicas.
5. Não houve diferença entre os sistemas adesivos autocondicionantes Clearfil SE Protect e Clearfil SE Bond em relação a resistência da união e a degradação da interface adesivo/dentina desmineralizada.
6. CHX auxiliou a manutenção da resistência da união, bem como preservou a degradação da interface após o desafio da ciclagem termomecânica.

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*De acordo com a norma da UNICAMP/FOP, baseada na norma do International Committee of Medical Journal Editors – Grupo de Vancouver. Abreviatura dos periódiocos em conformidade com o Medline

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APÊNDICES

APÊNDICE 1. Ilustração dos materiais utilizados nos 3 capítulos.

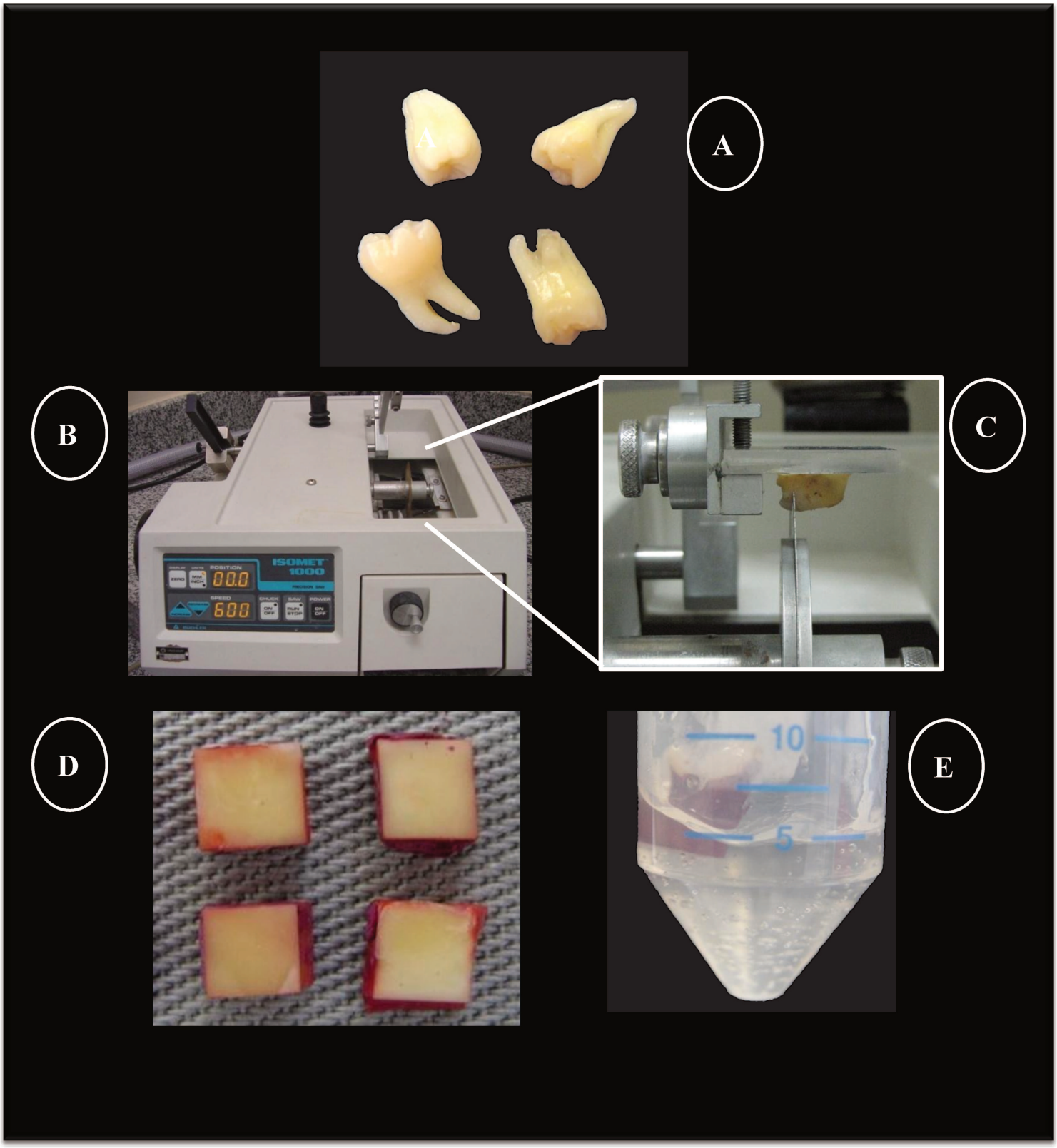
A. Solução de Digluconato de Chorexidina 2% (FGM, Produtos Odontológicos, Joinville, SC, Brasil), utilizada nos capítulos 1 e 3;
B. Sistemas adesivos. B1 – sistema adesivo de 2 passos (*etch& rinse)* Adper Single Bond 2 e ácido fosfórico 37% (3M, Dental Products, St. Paul, MN, EUA), utilizado no cap. 1; B2 – sistema adesivo autocondicionante Clearfil SE Bond (Kuraray Medical Inc, Kurashiki, Tokyo, Japão), utilizado nos cap. 1, 2 e 3; B3 – sistema adesivo autocondicionante Clearfil SE Protect (Kuraray Medical Inc, Kurashiki, Tokyo, Japão), utilizado nos cap. 1, 2 e 3;
C. Resina Composta, Clearfil AP-X (Kuraray Medical Inc, Kurashiki, Tokyo, Japão), utilizada nos cap. 1 e 3.


A
clorhexidina s
B1
3M ESPE
Adper™
Single Bond 2
Adhesive
6g
51202
Scotchbond™
Etchant 3ml 7523
B2
CLEARFIL
SE BOND
BOND
1.25 ml
KURARAY MEDICAL INC.
CLEARFIL
SE BOND
PRIMER
KURARAY MEDICAL INC.
B3
CLEARFIL
PROTECT BOND
PRIMER 6 ml
KURARAY MEDICAL INC.
CLEARFIL
PROTECT BOND
BOND 5 ml
KURARAY MEDICAL INC.
C
A2
CLEARFIL AP-X
2011-02
CLEARFIL AP-X
A2

APÊNDICE 2. Ilustração da metodologia empregada no capítulo 1.

Obtenção e preparo dos espécimes de dentina desmineralizada, ensaio 1

A. Fotografia dos terceiros molares hígidos utilizados;
B. Cortadeira metolográfica (Isomet, Buehler, Lake Bluff, IL, EUA);
C. Visão aproximada: superfície oclusal sendo cortada pelo disco diamantado (Extec, Erios, São Paulo, SP, Brasil);
D. Blocos de dentina com 4x4x2mm;
E. Espécimes de dentina em gel ácido de carboximetilcelulose (Farmácia Proderma, Piracicaba, SP, Brasil).


A
A
B
ISOMET
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POSITION
00.0
SPEED
600
C
D
E
10
5

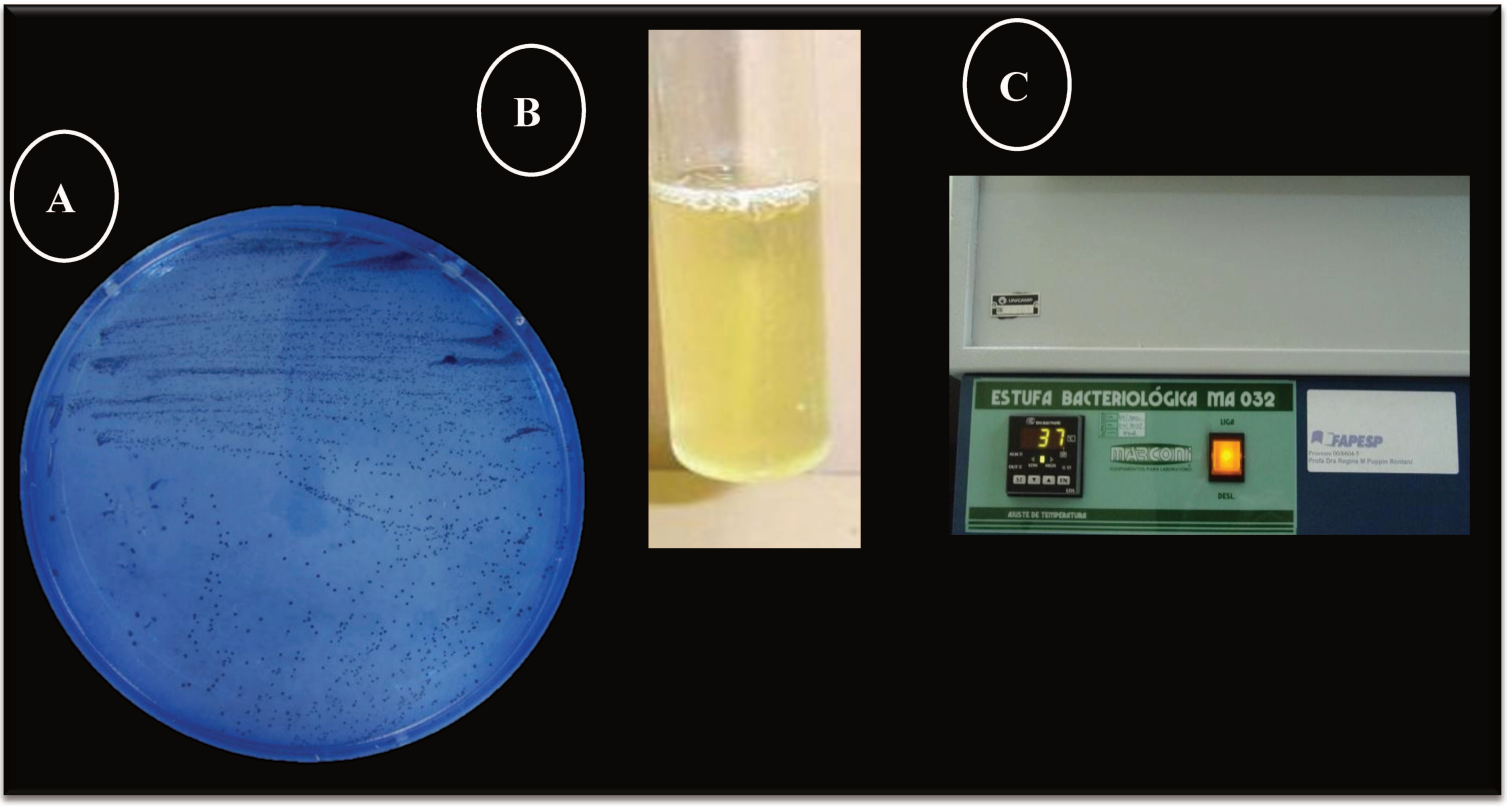
APÊNDICE 3. Ilustração da metodologia empregada no capítulo 1.

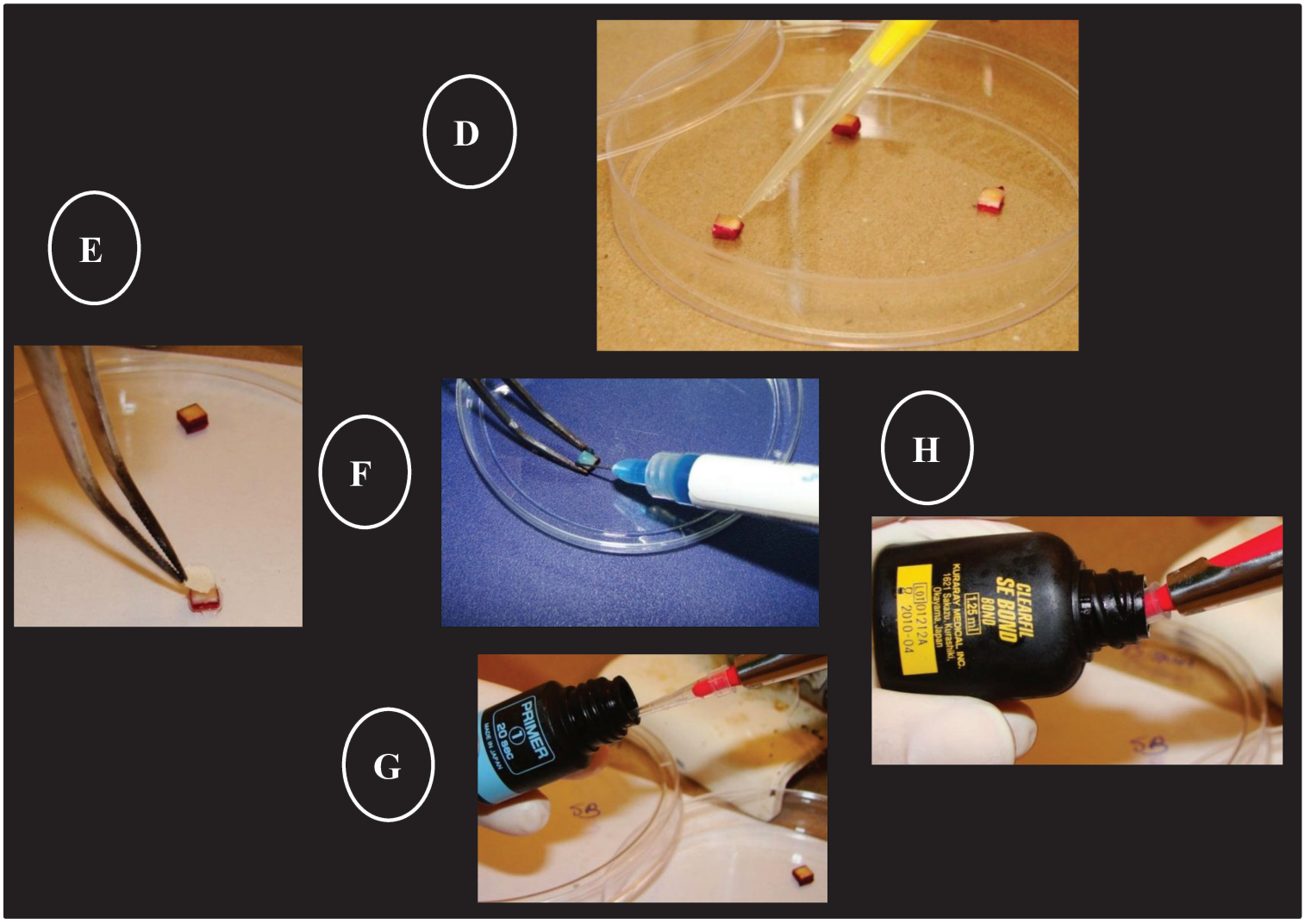
Preparo da suspensão bacteriana utilizada para os 3 ensaios

A. Reativação de *S. mutans* em placa de Agar Mitis Salivarius (MSA, Difco, Detroit, MI, EUA);
B. Suspensão bacteriana de *S. mutans* crescida em caldo de BHI (Brain Heart Infusion, Difco, Detroit, MI, EUA);
C. Estufa bacteriológica (MA032, Marconi, Piracicaba, SP, Brasil)

Preparo dos espécimes de dentina, ensaio1 - cap. 1

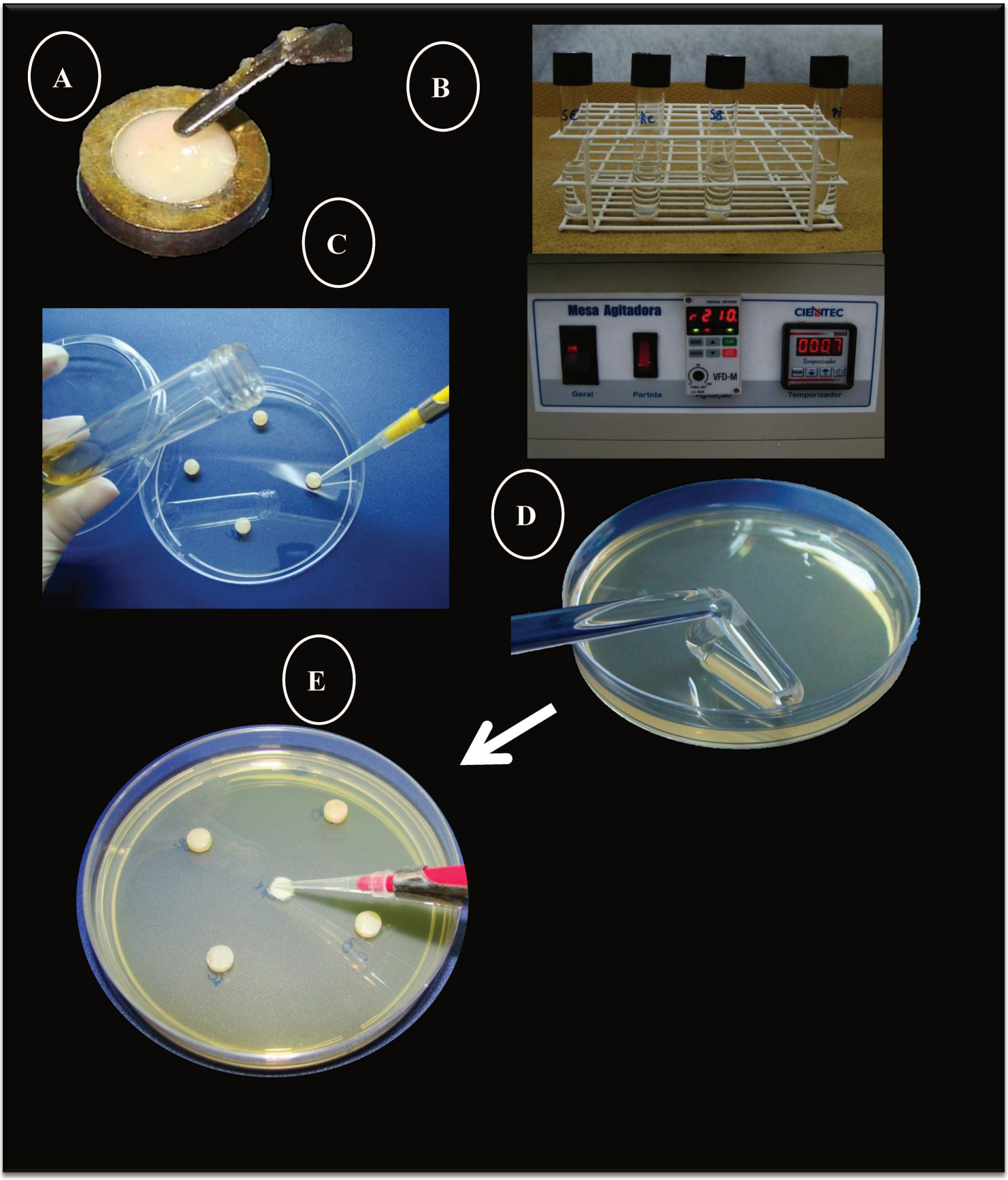
D. Inoculação dos espécimes de dentina desmineralizada com suspensão bacteriana;
E. Aplicação da solução de Clorexidina e secagem com papel absorvente;
F. Condicionamento prévio da dentina com ácido fosfórico 37%;
G. Aplicação do Primer dos sistemas adesivos autocondiconantes;
H. Aplicação do Bond.


A
B
C
ESTUFA BACTERIOLÓGICA MA 032
MARCONI
FAPESP


D
E
F
G
H
PRIMER
20 SEC
CLEARFIL
SE BOND
5 ml
125 ml
KURARAY MEDICAL INC.
1621 Sakazu, Kurashiki,
Okayama, Japan
Lot 01212A
2010-04

APÊNDICE 4. Ilustração da metodologia empregada nos ensaios 2 e 3 do capítulo 1.

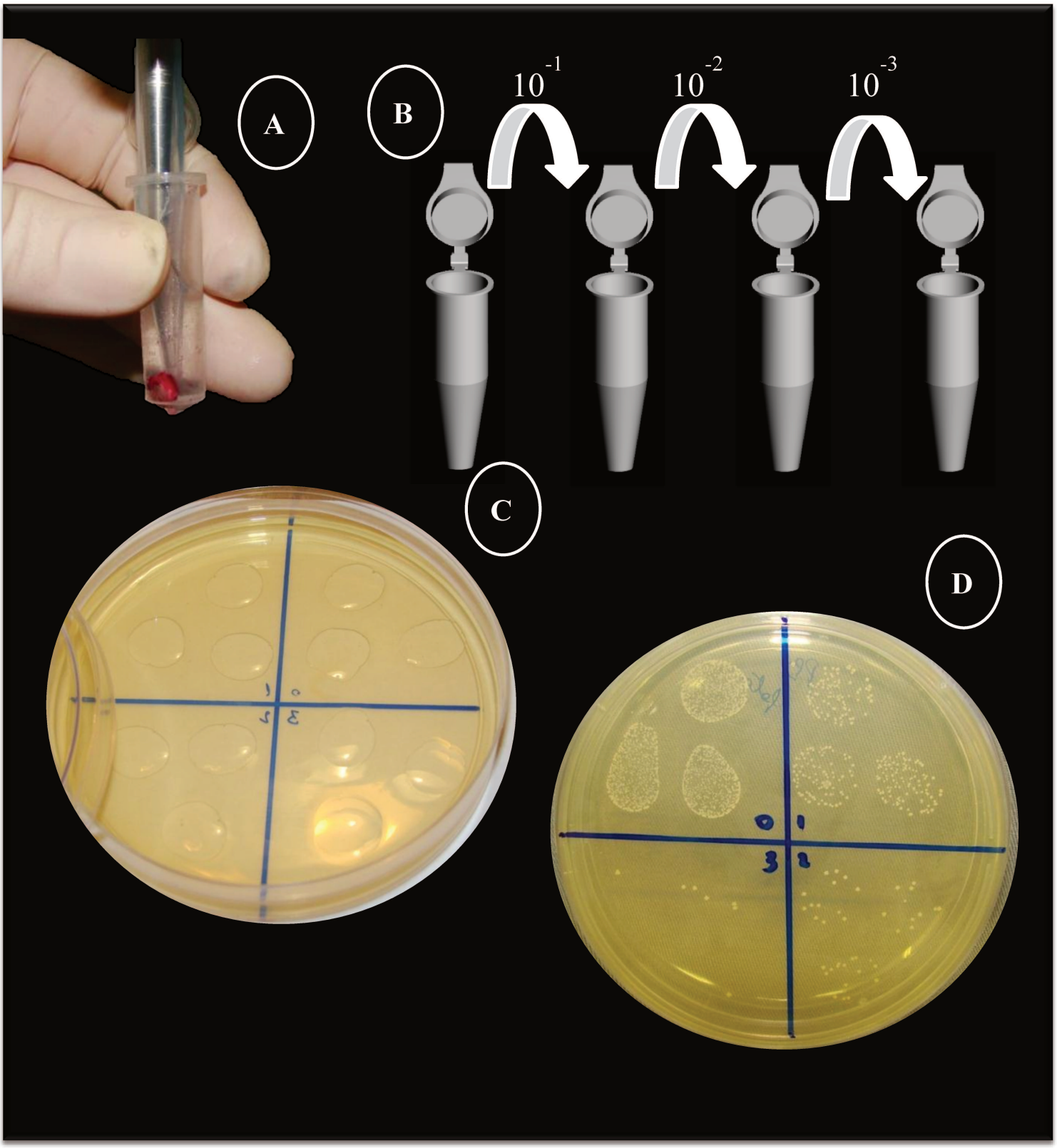
A. Preparo dos espécimes com o auxílio de uma matriz metálica;
B. Mesa agitadora utilizada no processo de eluição dos componentes não polimerizados dos espécimes;
C. Aplicação da suspensão bacteriana sobre os espécimes do ensaio 2;
D. Inoculação de 350µL de suspensão bacteriana sobre placa de BHI, ensaio 3;
E. Colocação dos espécimes sobre o BHI, ensaio 3;


A
B
C
D
E
Mesa Agitadora
CIENTEC
Geral
Partida
VFD-M
Temporizador

APÊNDICE 5. Ilustração da metodologia empregada do capítulo 1.

Mensuração da Atividade Antibacteriana dos 3 ensaios após os respectivos tratamentos.

A. Espécimes dentro de tubos de Eppendorf com solução salina sendo sonicados (UP400S, Hielscher, Teltow, Alemanha);
B. Diluição seriada;
C. Diluições inoculadas em placa de BHI;
D. Crescimento bacteriano após incubação.


A
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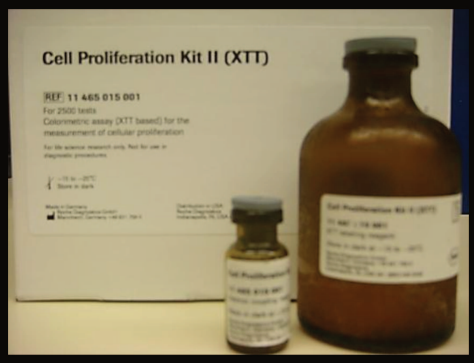
10^{-1}
10^{-2}
10^{-3}
C
D

APÊNDICE 6. Ilustração da metodologia empregada no capítulo 2.

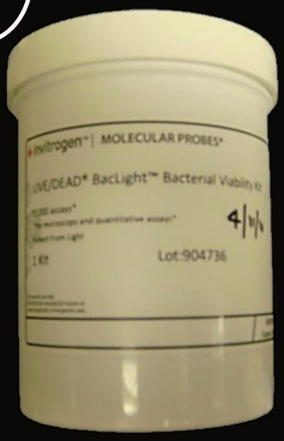
A. Molde de material de moldagem (material leve) a base de silicone por adição (Aquasil, Dentsply DeTrey, Konstanz, Alemanha) utilizado para o preparo dos discos dos sistemas adesivos;
B. Kit XTT - (Roche Applie Science, Indianapolis, IN, EUA);
C. Kit LIVE/DEAD (Molecular Probes-Invitrogen, Carlsbad, CA, EUA);
D. Kit MTT (Roche Applie Science, Indianapolis, IN, EUA);
E. Visão superior da redução do sal brometo de difeniltetrazólio (MTT);
F. Leitor ELISA (Spectrostar Nano, BMG Labtech, Allmendgruen, Alemanha);
G. Leitor Fluorescente (Spectra Max, Molecular Devices, EUA)

A

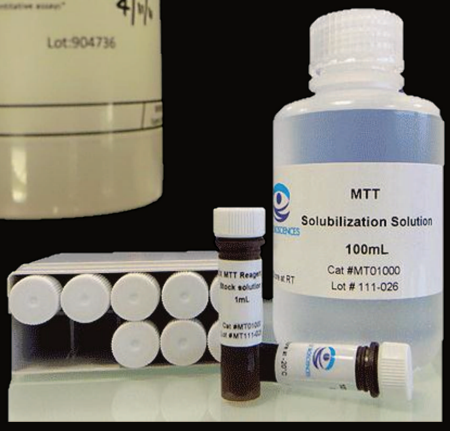
B

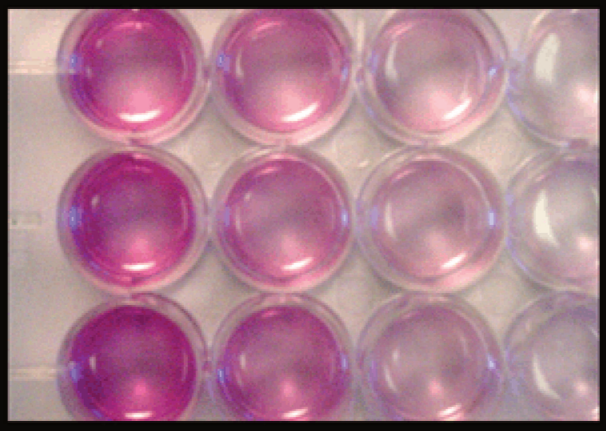

Cell Proliferation Kit II (XTT)
REF 11 465 015 001
For 2500 tests
Colorimetric assay (XTT based) for the measurement of cellular proliferation

C


MOLECULAR PROBES
LIVE/DEAD® BacLight™ Bacterial Viability Kit
Lot:904736

D


MTT
Solubilization Solution
100mL
Cat #MT01000
Lot # 111-026



E

F

G


POWER
MICROPLATE
CUVETTE
SPECTROstar Nano
BMG LABTECH

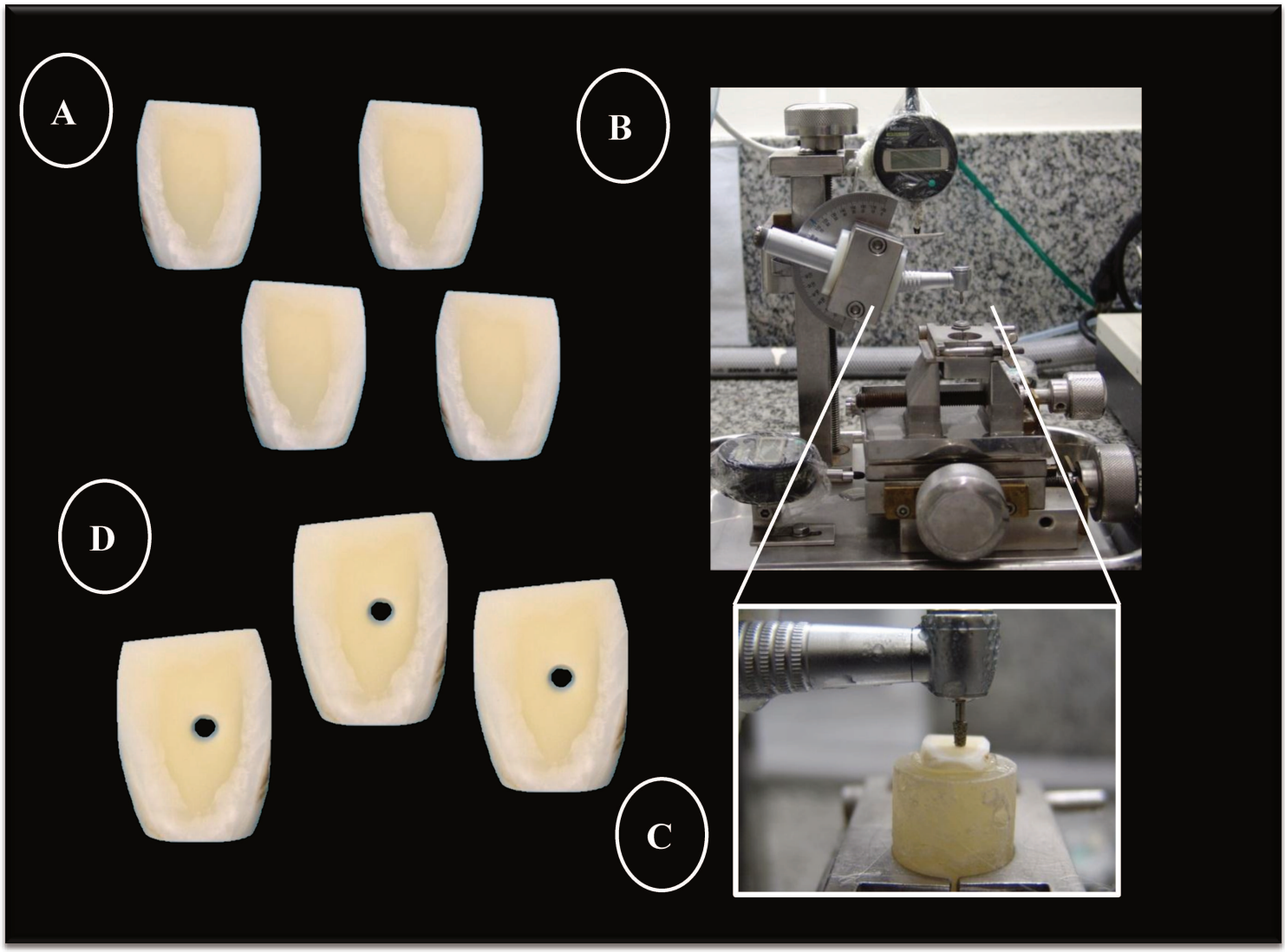
APÊNDICE 7. Ilustração da metodologia empregada no capítulo 3.

Obtenção dos espécimes e dos preparos cavitários

A. Preparo dos dentes bovinos;
B. Máquina padronizadora de preparos cavitários;
C. Visão aproximada da obtenção dos preparos cavitários sobre as fatias de dente bovino;
D. Fatias de dentes bovino após realização dos preparos cavitários cônicos (4mm de diâmetro maior x 3mm de diâmetro menor x 2mm de altura);

Procedimento restaurador

E. Aplicação de Clorexidina por 60s;
F. Aplicação dos sistemas adesivos;
G. Incremento de resina composta;
H. Fotoativação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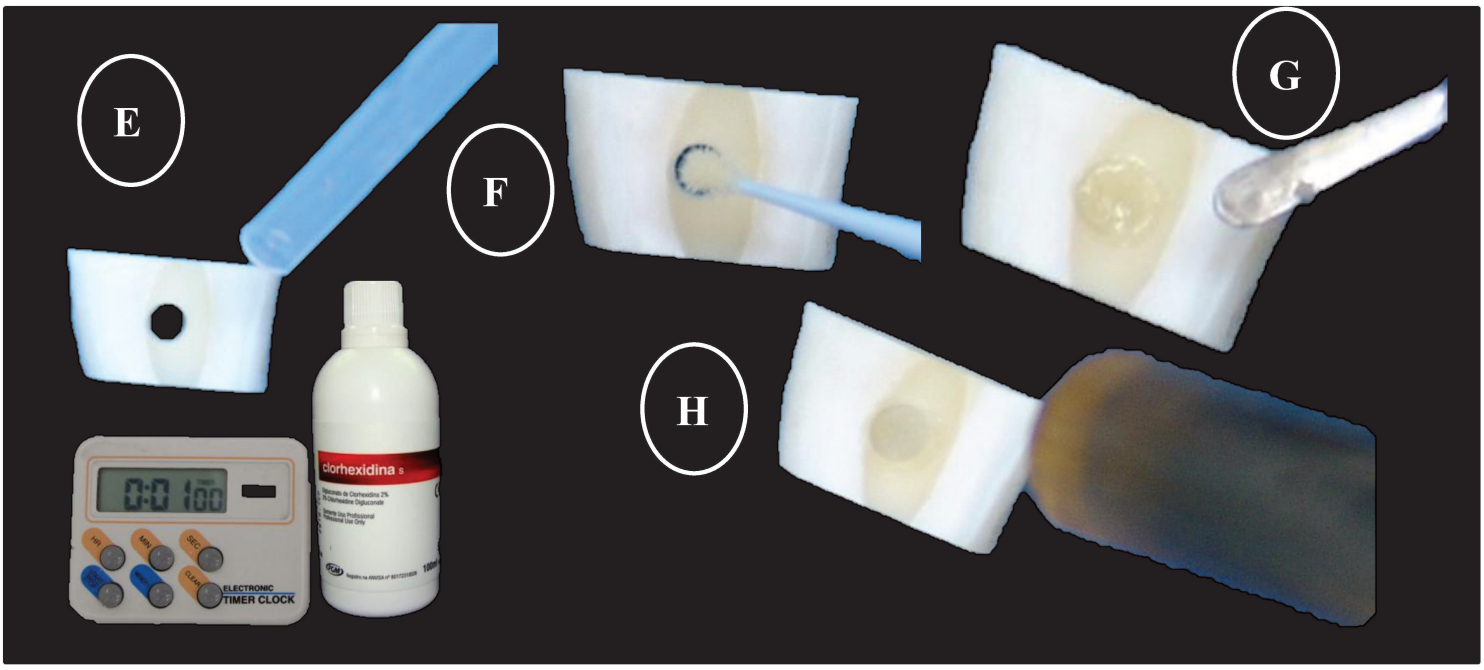
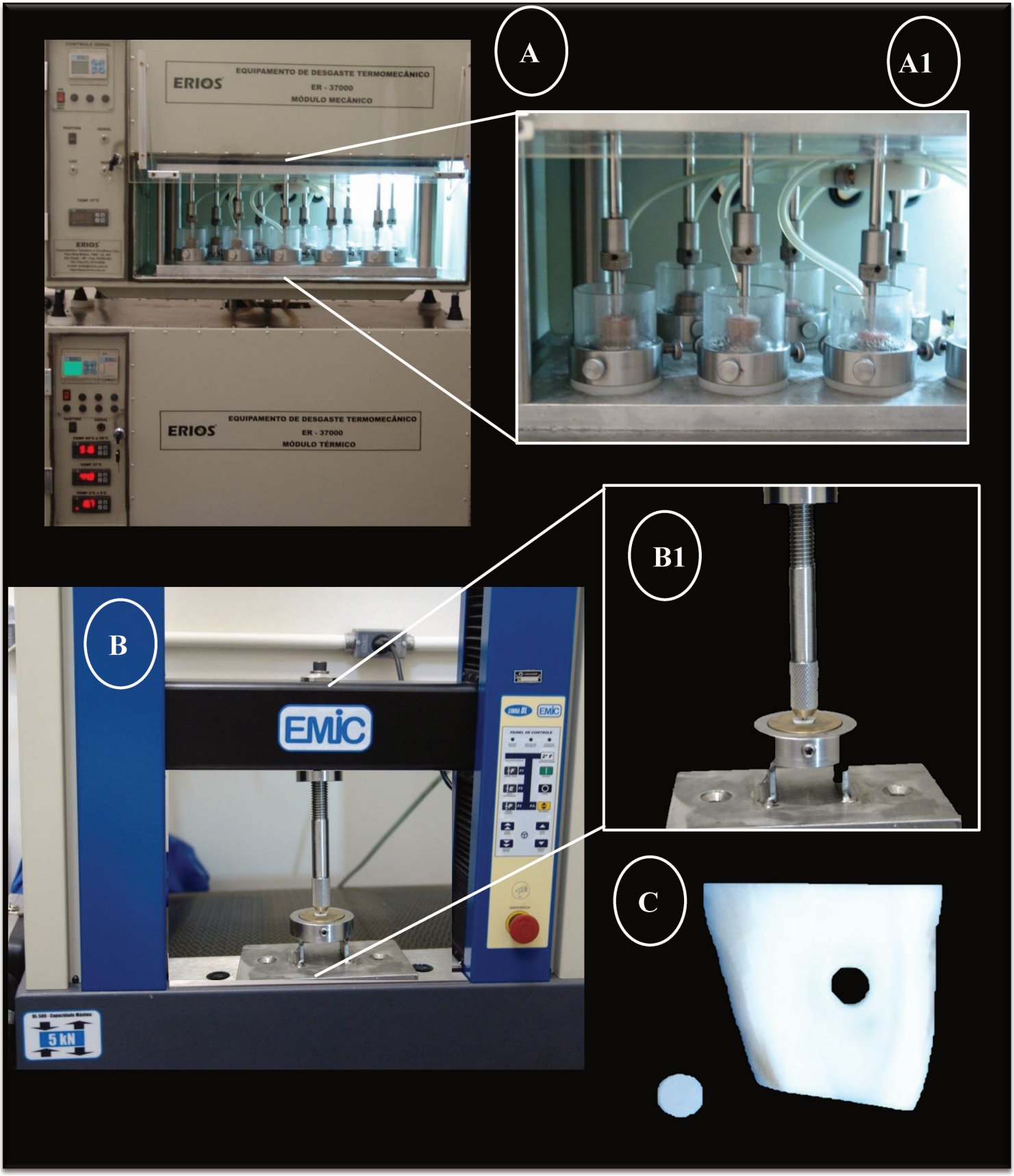

E
F
G
H
clorhexidina
ELECTRONIC
TIMER CLOCK

APÊNDICE 8. Ilustração da metodologia empregada no capítulo 3.

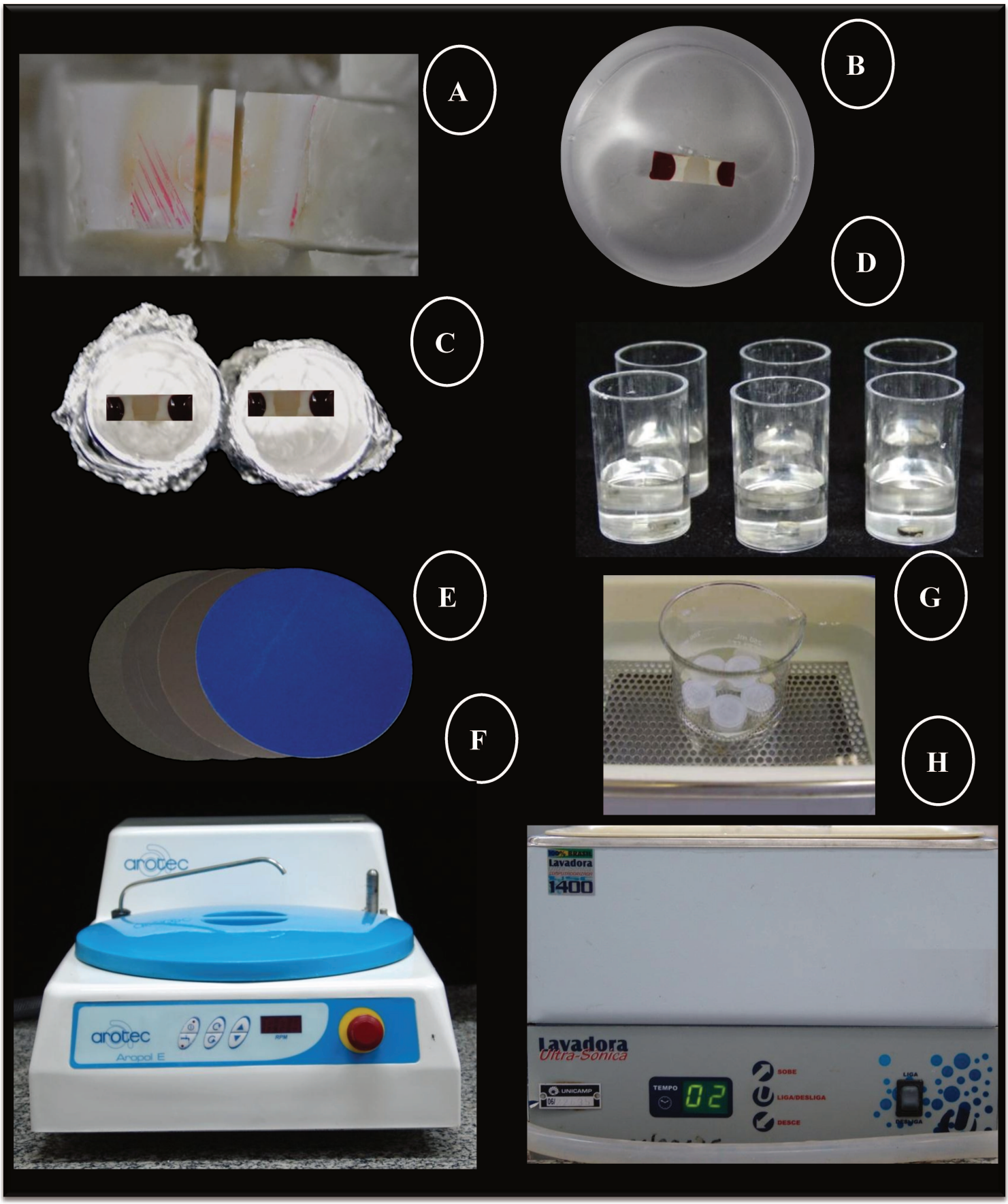
A. Máquina simuladora de ciclagem térmica e mecânica simultânea (ER 37000, Erios, São Paulo, SP, Brasil), A 1- Visão aproximada dos espécimes dentro da máquina de ciclagem;
B. Máquina de ensaio universal (Emic, Linha DL, São José dos Pinhais, PR, Brasil), B 1 - visão aproximada do ensaio *push-out* para mensurar os valores de resistência da união;
C. Fotografia do conjunto dente/restauração após o teste mecânico


A
A1
ERIOS
EQUIPAMENTO DE DESGASTE TERMOMECÂNICO
ER - 37000
MÓDULO MECÂNICO
ERIOS
EQUIPAMENTO DE DESGASTE TERMOMECÂNICO
ER - 37000
MÓDULO TÉRMICO
B
B1
EMIC
5 kN
C

APÊNDICE 9. Ilustração da metodologia empregada no capítulo 3.

Sequência do preparo dos espécimes para análise da nanoinfiltração

A. Fotografia do conjunto dente/restauração seccionado para a obtenção da fatia de espécime com 8 mm de espessura;
B. Visão superior da fatia de dentina/resina/dentina
C. Recipientes de polipropileno utilizados para a imersão individual dos espécimes contendo 5 mL de solução de nitrato de prata;
D. Visão em perfil dos recipientes de polipropileno contendo solução foto-reveladora;
E. Lixas de granulação 800, 1200, 2000 e feltro;
F. Politriz Universal (Aropol E, Arotec, São Paulo, SP, Brasil);
G. Posicionamento do conjunto espécimes/Becker em ultrassom;
H. Aparelho de ultrassom (Ultrasonic Cleaner, Unique, Santo Amaro, SP, Brasil) para lavagem dos espécimes.


A
B
C
D
E
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G
H
arotec
Arapol E
RPM
Lavadora
1400
Lavadora
Ultra-Sonica
UNICAMP
TEMPO
02
SOBE
LIGA/DESLIGA
DESCE
LIGA
DESLIGA

APÊNDICE 10. Ilustração da metodologia empregada no capítulo 3.

Sequência de confecção das réplicas

A. Moldagem dos espécimes utilizando material de moldagem (material leve) a base de silicone por adição (Aquasil, Dentsply DeTrey, Konstanz, Alemanha);
B. Resina epóxica utilizada na proporção 1:5 (Buehler, Lake, Buff, IL, EUA);

Espécimes preparados para observação em Microscopia Eletrônica de Varredura (MEV)

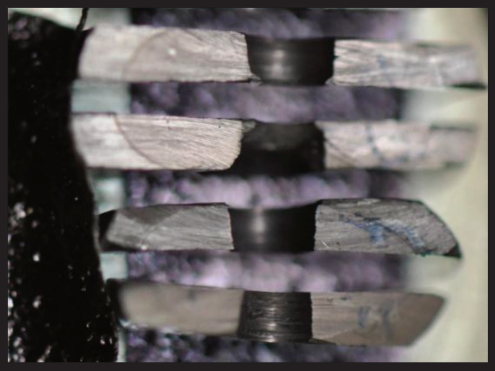
C. Espécimes cortados ao meio após teste mecânico para análise do modo de fratura;
D. Réplica do espécime para avaliar adaptação marginal;
E. Espécimes cobertos com carbono para mensurar a nanoinfiltração;
F. Microscópio Eletrônico de Varredura (JSM 5600LV, JEOL, Tóquio, Japão).

A

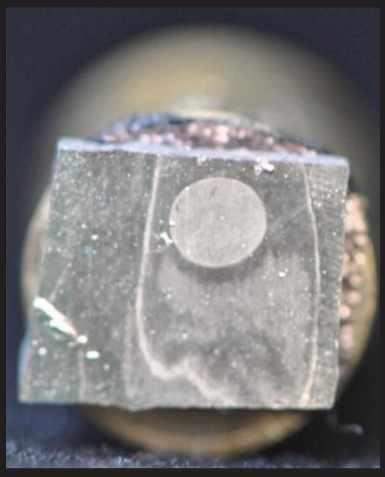
B


No. 20-8130-032
607-500318
1 Quart (0.95l)
BUEHLER®
EPOXICURE™
RESIN
(formerly EPOXIDE)
BUEHLER
USA (847) 295-6500 UK (+44) (0) 24 7658 2158
Deutschland (+49) 0211/974100
website: http://www.buehler.com
20813032-Rev.8

C



D



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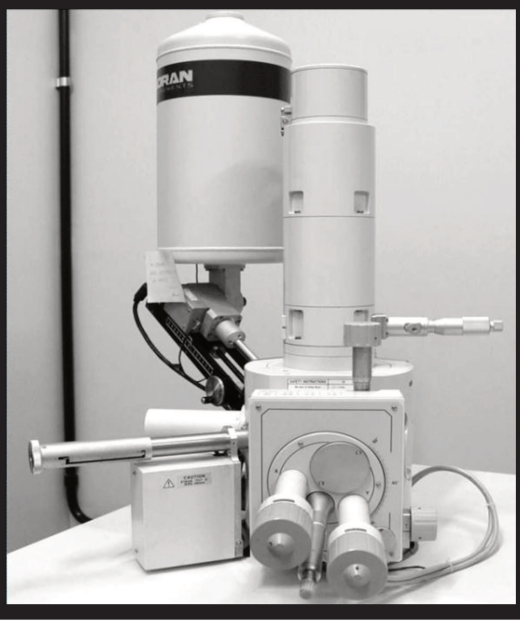

Anexo 1 - Resolução CCPG/002/06 a qual dispõe a respeito do formato das teses de mestrado e doutorado aprovados pela UNICAMP (Parte I)

INFORMAÇÃO CCPG/002/06

Tendo em vista a necessidade de revisão da regulamentação das normas sobre o formato e a impressão das dissertações de mestrado e teses de doutorado e com base no entendimento exarado no Parecer PG nº 1985/96, que trata da possibilidade do formato alternativo ao já estabelecido, a CCPG resolve:

Artigo 1º - O formato padrão das dissertações e teses de mestrado e doutorado da UNICAMP deverão obrigatoriamente conter:

I. Capa com formato único ou em formato alternativo que deverá conter informações relativas ao nível (mestrado ou doutorado) e à Unidade de defesa, fazendo referência à Universidade Estadual de Campinas, sendo o projeto gráfico das capas definido pela PRPG.
II. Primeira folha interna dando visibilidade à Universidade, a Unidade de defesa, ao nome do autor, ao título do trabalho, ao número de volumes (quando houver mais de um), ao nível (mestrado ou doutorado), a área de concentração, ao nome do orientador e co-orientador, ao local (cidade) e ao ano de depósito. No seu verso deve constar a ficha catalográfica.
III. Folha de aprovação, dando visibilidade à Comissão Julgadora com as respectivas assinaturas.
IV. Resumo em português e em inglês (ambos com no máximo 500 palavras).
V. Sumário.
VI. Corpo da dissertação ou tese dividido em tópicos estruturados de modo característico à área de conhecimento.
VII. Referências, formatadas segundo normas de referenciamento definidas pela CPG da Unidade ou por critério do orientador.
VIII. Todas as páginas deverão, obrigatoriamente, ser numeradas, inclusive páginas iniciais, divisões de capítulos, encartes, anexos, etc... As páginas iniciais poderão ser numeradas utilizando-se algarismos romanos em sua forma minúscula.
IX. Todas as páginas com numeração "ímpar" serão impressas como "frente" e todas as páginas com numeração "par" serão impressas como "verso".

§ 1º - A critério do autor e do orientador poderão ser incluídos: dedicatória; agradecimento; epígrafe; lista de: ilustrações, tabelas, abreviaturas e siglas, símbolos; glossário; apêndice; anexos.

§ 2º - A dissertação ou tese deverá ser apresentada na língua portuguesa, com exceção da possibilidade permitida no artigo 2º desta Informação.

§ 3º - As dissertações e teses cujo conteúdo versar sobre pesquisa envolvendo seres humanos, animais ou biossegurança, deverão apresentar anexos os respectivos documentos de aprovação.

Artigo 2º - A critério do orientador e com aprovação da CPG da Unidade, os capítulos e os apêndices poderão conter cópias de artigos de autoria ou de co-autoria do candidato, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, escritos no idioma exigido pelo veículo de divulgação.

Anexo 1 - Resolução CCPG/002/06 a qual dispõe a respeito do formato das teses de mestrado e doutorado aprovados pela UNICAMP (Parte II)

§ único - O orientador e o candidato deverão verificar junto as editoras a possibilidade de inclusão dos artigos na dissertação ou tese, em atendimento à legislação que rege o direito autoral, obtendo, se necessária, a competente autorização, deverão assinar declaração de que não estão infringindo o direito autoral transferido à editora.

Artigo 3º - Dependendo da área do conhecimento, a critério do orientador e com aprovação da CPG da Unidade, a dissertação ou tese poderá ser apresentada em formato alternativo, desde que observados os incisos I, II, III, IV, V e VII do artigo 1º.

Artigo 4º - Para impressão, na gráfica da Unicamp, dos exemplares definitivos de dissertações e teses defendidas, deverão ser adotados os seguintes procedimentos:

§ 1º - A solicitação para impressão dos exemplares de dissertações e teses poderá ser encaminhada à gráfica da Unicamp pelas Unidades, que se responsabilizarão pelo pagamento correspondente.

§ 2º - Um original da dissertação ou tese, em versão definitiva, impresso em folha tamanho carta, em uma só face, deve ser encaminhado à gráfica da Unicamp acompanhado do formulário "Requisição de Serviços Gráficos", onde conste o número de exemplares solicitados.

§ 3º - A gráfica da Unicamp imprimirá os exemplares solicitados com capa padrão. Os exemplares solicitados serão retirados pelas Unidades em no máximo, cinco dias úteis para impressão preto e branco e 10 dias úteis para coloridas.

§ 4º - No formulário "Requisição de Serviços Gráficos" deverão estar indicadas as páginas cuja reprodução deva ser feita no padrão "cores" ou "foto", ficando entendido que as demais páginas devam ser reproduzidas no padrão preto/branco comum.

§ 5º - As dissertações e teses serão reproduzidas no padrão frente e verso, exceção feita às páginas iniciais e divisões de capítulos; dissertações e teses com até 100 páginas serão reproduzidas no padrão apenas frente, exceção feita à página que contém a ficha catalográfica.

§ 6º - As páginas fornecidas para inserção deverão ser impressas em sua forma definitiva, ou seja, apenas frente ou frente/verso.

§ 7º - O custo, em reais, de cada exemplar produzido pela gráfica será definido pela Administração Superior da Universidade.

Artigo 5º - É obrigatória a entrega de dois exemplares para homologação.

Artigo 6º - Esta Informação entrará em vigor na data de sua publicação, ficando revogadas as disposições em contrário, principalmente as Informações CCPG 001 e 002/98 e CCPG/001/00.

Campinas, 13 de setembro de 2006

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 **"Uso de agentes antibacterianos na dentina afetada por cárie. Análises biológica e da morfologia da interface de união"**, protocolo nº 072/2007, dos pesquisadores éfani Caroline de Freitas Banzi 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 10/10/2007.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Use of antibacterial agents in the affected dentine for caries. The bonding interface morphology and biological analysis"**, register number 072/2007, of 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 10/10/2007.

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 3 - Confirmação de submissão do primeiro artigo apresentado nesta Tese para o periódico *The Journal of the American Dental Association*

De: <jadaoffice@ada.org>
Data: quinta-feira, 19 de janeiro de 2012 12:51
Para: <rmpuppin@fop.unicamp.br>
Assunto: JADA - Manuscript ID 036-12

19-Jan-2012

Dear Professor Puppin-Rontani:

Your manuscript entitled "Effect of 2% chlorhexidine digluconate and dentin bonding systems on Streptococcus mutans" has been successfully submitted online and is presently being given full consideration for publication in the The Journal of the American Dental Association.

We will consider your article for publication with the understanding that
- it has not been published previously;
- it has been submitted solely to JADA;
- each author has fully disclosed any financial, economic or other conflicting interests in products or services described in the article.

Before we can put the manuscript into review, we need copies of the Copyright Transfer and Conflict of Interest forms signed by all authors. Please ensure that all authors sign these (you may make copies as necessary) and return them to us by mail or by fax at 1-716-829-6053, (you may disregard this request if you already have submitted the necessary forms with all authors' signatures). If your manuscript is not accepted for publication, all rights will revert to you, and we will destroy the forms and remove your manuscript materials from our active database permanently.

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A decision regarding the disposition of the manuscript will be made after critical evaluation and advisory comment by selected reviewers.

Thank you for submitting your manuscript to The Journal of the American Dental Association.

Respectfully,
The Journal of the American Dental Association Editorial Office