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Análise química, microbiológica e da resistência de união à dentina do sistema adesivo contendo monômero antibacteriano e íons fluoreto

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas para obtenção do Título de Doutor em Materiais Dentários.

Orientadora: Prof. Dra. Regina Maria Puppin Rontani

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A Comissão Julgadora dos trabalhos de Defesa de Tese de DOUTORADO, em sessão pública realizada em 20 de Janeiro de 2009, considerou a candidata FABÍOLA GALBIATTI DE CARVALHO aprovada.

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Dedico este trabalho

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RESUMO

O monômero antibacteriano 12-metacriloxidodecilpiridinio de brometo (MDPB) foi incorporado, juntamente com o flúor, ao sistema adesivo Clearfil Protect Bond (CPB) para auxiliar na prevenção de cárie secundária. Desta forma, o objetivo deste estudo foi avaliar em três experimentos *in vitro* o efeito desses componentes na desinfecção de cavidade, durabilidade da união à dentina afetada por cárie (DAC) e prevenção de cárie secundária. No Capítulo 1, o efeito antibacteriano do MDPB foi avaliado em biofilme de *S. mutans* presentes na superfície de dentina a partir de quatro tempos de aplicação do primer contendo MDPB (20s, 90s, 300s e 590s). A viabilidade celular foi avaliada utilizando microscópio confocal de varredura a laser (CLSM) e contagem de unidades formadoras de colônias. Somente a partir de 300s de aplicação do primer foi verificado o efeito bactericida do MDPB contra biofilme de *S. mutans* na dentina. No Capítulo 2, a degradação à DAC artificial foi avaliada utilizando teste de microtração (μTB) após a aplicação dos sistemas adesivos CPB e Adper Scotchbond Multi-Purpose (SBM) e armazenamento em cultura de *S. mutans* por três dias e água deionizada por três meses. A presença do flúor e MDPB no sistema adesivo CPB não preveniu a degradação da união à DAC, pois não houve diferença nos valores de μTB entre CPB e SBM nos dois métodos de degradação. Assim, o meio de armazenamento dos espécimes com *S. mutans* por 3 dias foi tão eficaz na degradação à DAC quanto o armazenamento em água por 3 meses. No Capítulo 3, a inibição da cárie secundária por CPB e SBM foi avaliada após 5 dias de desafio cariogênico com métodos químico (gel ácido) e biológico (meio com *S. mutans*). A formação da zona de inibição à cárie (IZ) pelos sistemas adesivos foi avaliada por CSLM e a distribuição de cálcio e fósforo para investigar a perda mineral na dentina ao longo da interface dentina/restauração foi avaliada por análise de fluorescência por raios-x (μ-EDX). O sistema adesivo CPB produziu IZ mais espessa e menor perda mineral quando submetido ao desafio químico, mas produziu a menor espessura de IZ e a maior perda mineral quando submetido ao desafio biológico. O sistema

adesivo SBM induziu valores intermediários de perda mineral e IZ frente aos dois desafios. Desta forma, o método de desenvolvimento de cárie secundária artificial e a camada híbrida criada por CPB e SBM influenciaram na inibição de cárie secundária por sistemas adesivos. Dentro das limitações deste trabalho pode-se concluir que o monômero MDPB presente no sistema adesivo CPB possuiu efeito bactericida contra biolfime de *S. mutans* na superfície de dentina após 300 s de sua aplicação. Além disso, este monômero não preveniu a degradação da união após armazenamento em cultura de *S. mutans* e água deionizada, bem como a desmineralização da dentina na interface dente/restauração após desafio cariogênico químico e biológico.

Palavras-chave: **MDPB, sistemas adesivos, *S. mutans*, resistência de união, degradação, cárie secundária, microscopia confocal de varredura a laser.**

ABSTRACT

The antibacterial monomer 12-methacryloyloxydodecylpyridinium bromide (MDPB) was incorporated with fluoride component in Clearfil Protect Bond adhesive system to help in secondary caries prevention. Thus, the present study conducted three *in vitro* experiments with the aim to evaluate the fluoride and MDPB effects in cavity disinfection, bonding durability on caries-affected dentin (CAD) and secondary caries prevention. In Chapter 1, the MDPB antibacterial effect was evaluate against *S. mutans* biofilm on dentin surface after four application times of MDPB primer (20s, 90s, 300s e 590s). The cellular viability was evaluated by confocal laser scanning microcopy (CLSM) and viable count test. MDPB had bactericidal effect against *S. mutans* biofilm on dentin after 300s of primer application. In Chapter 2, the artificial CAD bonding degradation was evaluated using microtensile bonding strength test (μTB) after CPB and Adper Scotchbond Multi-Purpose (SBM) adhesive systems application and storage in *S. mutans* culture for three days and deionized water for three months. The fluoride and MDPB in CPB adhesive system did not prevent the CAD bonding degradation because there was no statistical difference between μTB values of CPB and SBM in both degradation methods. Therefore, *S. mutans* culture was an effective method to produce degradation *in vitro* in a short period of time as 3 months of water storage. In Chapter 3, the secondary caries inhibition by CPB and SBM was evaluated after chemical (acidic gel) and biological (*S. mutans* culture) challenges during 5 days. The inhibition zone formation (IZ) by adhesive systems used was evaluated by CLSM. The calcium and phosphorus distribution was evaluate by X-Ray fluorescence analysis (μ-EDX) to investigate the mineral loss on dentin along dentin/restoration interface. CPB adhesive system produced the thickest IZ and lowest mineral loss when it was submitted to chemical challenge; however the same adhesive system produced the lowest value of IZ thickness and the highest mineral loss in the biological challenge. SBM adhesive system showed intermediate mineral loss and IZ thickness values in both cariogenic challenges. Thus, the artificial caries method

and the hybrid layer quality of CPB and SBM affected the secondary caries inhibition by adhesive systems. Based on the findings of this study, and within the limitation of this investigation, could be concluded that the MDPB monomer in CPB adhesive system had bactericidal effect after 300 s of its application. Furthermore, this monomer did not prevent the bonding degradation after *S. mutans* culture and water storage, as well as the dentin demineralization at tooth/restoration interface after chemical and biological cariogenic challenges.

Keywords: **MDPB, adhesive systems, bond strength, degradation, secondary caries, confocal laser scanning microscopy.**

SUMÁRIO

INTRODUÇÃO

A longevidade das restaurações adesivas está relacionada com fatores químicos e físico-mecânicos de degradação da união dente/restauração. Os fatores químicos ocasionam principalmente a degradação da resina adesiva e fibrilas de colágeno em decorrência da exposição à água e enzimas provenientes da dentina e de bactérias (Tay and Pashley, 2003; Pashley *et al.*, 2004). Os fatores físico-mecânicos englobam inúmeros mecanismos que afetam a integridade marginal das restaurações, principalmente: tensão de contração de polimerização dos materiais resinosos; concentrações de tensões em decorrência da carga mastigatória, as quais podem originar falhas e propagação de fraturas; e mudanças de temperatura que ocorrem na cavidade bucal ocasionando tensões na interface devido à diferença dos coeficientes de contração e expansão do material restaurador e das estruturas dentárias (de Munck *et al.*, 2005; Drummond 2008). Embora todos estes fatores estejam envolvidos na adaptação marginal das restaurações, a principal causa de substituições das mesmas é ainda a formação de cárie ao longo das margens (Fontana *et al*., 2000; Tyas *et al*., 2005). Assim, componentes como o flúor e o monômero 12 - metacriloxidodecilpiridinio de brometo (MDPB) foram incorporados aos sistemas adesivos para auxiliar na prevenção da cárie recorrente e minimizar a degradação da união (Imazato *et al*., 1998; Itota *et al*., 2002; Nakajima *et al*., 2003).

O monômero MDPB é um composto de amônia quaternária, com função antibacteriana, associado a um grupo metacrilato e foi desenvolvido com os objetivos de eliminar bactérias presentes na dentina, após a remoção da cárie, e minimizar a invasão bacteriana na interface dente/restauração (Imazato *et al*., 1997). O agente antibacteriano é co-polimerizado com outros monômeros presentes no sistema adesivo e permanece imobilizado na matriz polimérica, exercendo ação somente contra bactérias que entram em contato com o polímero (Imazato *et al*., 1998). Os íons fluoretos presentes em sistemas adesivos concentram-se na base da camada híbrida (Ferracane *et al*., 1998), e podem, com

o tempo, difundir-se para a dentina subjacente e aumentar a resistência à cárie (Itota et al., 2002). O efeito do flúor presente em sistemas adesivos na inibição de cárie recorrente foi avaliado por estudos *in vitro* após desafio cariogênico com métodos químicos de formação de cárie, como gel e soluções ácidas ou ciclagem de pH (Han *et al*., 2002; Savarino *et al*., 2004). Alguns estudos relataram que os íons fluoreto formam uma zona de inibição à cárie na interface dentina/restauração (Han *et al*., 2002; Dionysopoulos *et al*., 2003; Savarino *et al*., 2004; Shinohara *et al*., 2006). O efeito do monômero MDPB na inibição da cárie recorrente em dentina foi avaliado concomitantemente com o flúor, por meio de desafios químicos. Entretanto, a presença de bactérias no desafio cariogênico se torna indispensável para a avaliação do efeito antibacteriano do MDPB na inibição da cárie.

O efeito do flúor presente em sistemas adesivos também foi avaliado na durabilidade da união à dentina hígida (Nakajima *et al.*, 2003; Peris *et al*., 2007). Porém, clinicamente, pode ser encontrada a dentina afetada por cárie após a remoção de cárie. A resistência de união imediata a este substrato foi menor comparada à dentina hígida (Nakajima *et al*., 2000; Yoshiyama *et al*., 2002; Yoshiyama *et al*., 2003; Yazici *et al*., 2004), no entanto dados sobre a qualidade e a durabilidade da união à dentina afetada por cárie são escassos. Apenas Erhardt *et al*. (2008) investigaram a durabilidade da união de sistemas adesivos à dentina afetada por cárie, a qual foi menor quando comparada à dentina hígida devido à presença de zona rica em colágeno mais espessa, resultante da incompleta infiltração do adesivo (Nakajima *et al*., 1999) e a maior quantidade de água presente (Ito *et al*., 2005), e também devido à presença de depósitos minerais no interior dos túbulos (Nakajima *et al*., 1999). O principal método utilizado para avaliação da durabilidade de união de sistemas adesivos à dentina é o armazenamento dos espécimes em água, por um período de tempo, para posterior teste de resistência de união (de Munck *et al*., 2005). Contudo, Peris *et al*. (2007) utilizaram outro método de armazenamento dos espécimes, a ciclagem de pH. O objetivo de utilizar este método de armazenagem foi para investigar o

efeito do flúor presente em sistemas adesivos na resistência e durabilidade de união à dentina hígida após um desafio cariogênico, já que sistemas adesivos fluoretados minimizaram a degradação da interface adesiva após armazenamento em água (Nakajima *et al*., 2003). A presença do MDPB poderia também auxiliar na durabilidade da união, devido ao seu efeito antibacteriano, mas para este fim uma solução de armazenamento dos espécimes contendo bactérias cariogênicas precisaria ser utilizada. Entretanto, não existem estudos na literatura que avaliaram a durabilidade de sistemas de união com flúor e MDPB à dentina, hígida ou afetada por cárie, após armazenamento em meio contendo bactérias.

O monômero MDPB possui efeito contra *Streptococcus mutans* e bactérias presentes em lesões de cárie (Imazato *et al*., 2001), porém este efeito foi principalmente investigado contra bactérias planctônicas ou por meio do teste de difusão em agar (Imazato *et al*., 1997; Imazato et al., 2001; Ozer *et al*., 2005; Yoshikawa *et al*., 2007; Imazato *et al*., 2008; Gondim *et al*., 2008). Como as bactérias que permanecem no substrato dentinário, após a remoção do tecido cariado, ficam aderidas na superfície e nos túbulos dentinários (Kidd *et al*., 1993), o efeito antibacteriano do MDPB nestas bactérias poderia diferir daquele testado em bactérias planctônicas. Porém, ainda não existem investigações da ação desinfetante do MDPB contra bactérias aderidas na dentina.

Deste modo, o presente trabalho foi dividido em três capítulos com o objetivo de investigar: (1) o efeito bactericida do primer contendo MDPB contra biofilme de *S. mutans* na dentina por microscopia confocal de varredura a laser, em tempo real; (2) a resistência de união de sistemas adesivos com e sem MDPB/flúor à dentina afetada por cárie artificial quando submetidos ao armazenamento em água e cultura de *S. mutans*; (3) a inibição de cárie recorrente utilizando sistemas adesivos com e sem MDPB/flúor após desafio cariogênico químico e biológico.

CAPÍTULO 1

Real-time bactericidal effect of MDPB on *S. mutans* biofilm

ABSTRACT

The antibacterial monomer MDPB (12-methacryloyloxydodecylpyridinium bromide) has never been investigated against biofilm. This study hypothesized that there are differences in bactericidal effect against *S. mutans* biofilm between primers with (Clearfil Protect Bond - CPB) and without MDPB (Clearfil SE Bond - CSE) by confocal microscopy (CLSM) and viable bacteria counting (CFU). Bovine dentin surfaces were submitted to *S. mutans* biofilm formation for 18 h. Live/Dead stain and primers were applied on samples to CLSM analysis during 590 sec. The primers and saline (control group) were also applied on other samples during 20, 90, 300, and 590 sec and CFU were assessed by counting colonies. CLSM images showed no bactericidal effect of CSE, while CPB showed gradual increase of dead bacteria. CFU decreased significantly after 300 and 590 sec of CPB application, but no difference was found to CSE. MDPB primer had bactericidal effect against *S. mutans* biofilm after 300 sec.

KEY WORDS: MDPB, biofilm, *S. mutans*, adhesive system, viability staining

INTRODUCTION

The use of cavity disinfectants before restoration is an important clinical step because bacteria remain in dentin after removal of caries (Kidd *et al.*, 1993). The antibacterial monomer 12-methacryloyloxydodecylpyridinium bromide (MDPB) is a compound of the antibacterial agent quaternary ammonium and a methacryloyl group, which is covalently bound to polymer matrix by its copolymerization with other monomers (Imazato *et al.*, 1998). MDPB incorporated into the self-etching adhesive system would inactivate the residual bacteria and also prevent bacterial invasion through the gap tooth/restoration (Imazato *et al.*, 1997). It has shown antimicrobial effect against oral streptococci, lactobacilli, and microorganisms clinically isolated from root caries (Imazato *et al.*, 2001; Yoshikawa *et al.*, 2007). Studies evaluating the MDPB antibacterial effect were usually conducted in bacterial suspension (Imazato *et al.*, 1997, 1999, 2001, 2008). However, bacteria in dentin are present inside tubules and adhered on dentin surface after removal of caries. This fact could interfere in the MDBP effect, since bacteria adherent on surfaces, forming biofilms, are less susceptible to antimicrobials than planktonic microorganisms (Chambless *et al.*, 2006).

Confocal laser scanning microscopy (CLSM) has been used to evaluate bacterial viability on dentin biofilms (Roland *et al.*, 2006), using fluorescence dye to differentiate between live and dead bacteria (Imazato *et al.*, 2008). Also, CLSM can demonstrate the real-time death of microorganisms and has never been used to test the real-time viability of *Streptococcus mutans* biofilm after MDPB application. The hypothesis tested was that there is a difference in the MDPB bactericidal effect against *S. mutans* biofilm over 590 sec by CLSM analysis in real-time and count of viable bacteria test.

MATERIALS AND METHODS

Adhesive systems

Two self-etching/priming solutions were tested: Clearfil Protect Bond – CPB, containing MDPB, MDP (10-methacryloxydecyldihydrogen phosphate), HEMA (2-hydroxyethylmethacrylate) and water; and Clearfil SE Bond – CSE, containing MDP, HEMA, dl-camphorquinone, N,N-diethanol-p-toluidine and water.

Samples preparation

One hundred seventeen bovine incisors were obtained and stored in 0.1% thymol solution. The buccal portion of enamel was wet ground (Arotec, São Paulo, SP, Brazil) using 400 and 600 grits silicon carbide paper (Saint-Gobain, São Paulo, SP, Brazil). Dentin samples with 4 x 4 mm were obtained of each tooth using a low-speed diamond saw (Isomet, Buehler, Lake Bluff, IL, USA) and ground with 240 grit silicon carbide paper to reach 1.5 mm thickness. All samples were sterilized by steam autoclave (Phoenix, Araraquara, SP, Brazil) at 121°C for 15 min (Amaecha *et al.*, 1998).

Bacterial colonization on dentin

The dentin surface of each sample was exposed under static conditions to 20 µL aliquot of *S. mutans* UA159 suspension in brain heart infusion broth (BHI) (Becton Dickinson, Sparks, MD 21152, USA) (approximately 1 x 10^9 CFU) adjusted to an optical density of 0.6 at 600 nm to bacterial adherence. After 2 hr at room temperature, the non-adhering cells were removed by washing three times with saline solution (Montanaro *et al.*, 2004). A sample was placed in each well of a 24-well multi-dish polystyrene plate (Nunclon, Thomas Scientific, Swedesboro, NJ, USA). Two mL of BHI broth supplemented of 1% (w/v) sucrose (LabSynth, Diadema, SP, Brazil) were then added to the wells (Kreth *et al.*, 2004) to simulate bacterial colonization. The plates were incubated for 18 hr at 37ºC in 5% supplemented CO_2 environment. The lack of contamination in the media of each well was verified using Gram staining and by plating samples. The 18 hr incubation

period was established by a previous study, which verified that it was sufficient to develop a thin *S. mutans* biofilm without producing demineralization of dentin specimens, since the demineralization process could increase the dentin autofluorescence (van der Veen and ten Bosch 1996) and harm the biofilm visualization by CLSM.

Assessment of bactericidal effects by CLSM

Live:Dead Baclight bacterial viability stain (L13152) (Molecular Probes, Eugene, OR, USA) was used in this study. It consists of a two nucleic acid-binding stains mixture: Syto 9 and propidium iodide. Syto 9 stains all viable bacteria in green, while propidium iodide stains in red the bacteria whose membranes were damaged (non-viable bacteria). Three samples were used for each primer or solution application: CPB, CSE and saline (control group). After 18 hr bacterial colonization, the non-adhering cells were removed by washing three times with saline solution (Montanaro *et al.*, 2004). Live:Dead was mixed according to the manufacturer's instructions and one drop was applied directly to surface of each sample (Roland *et al.*, 2006). After 15 min in dark incubation (Boulos *et al.*, 1999), the stain surplus was removed by absorbent paper and 10 µL of each solution was applied on the samples' surface, according to each group. Immediately after, samples were analyzed by CLSM (LSM 510 META, Zeiss, Jena, Germany). A preliminary sample, after each primer application, was used to standardize the gain and offset values. An excitation wavelength of 488 nm was used, and all light emitted between 500 – 550 nm and over 560 nm was collected by different filters. The scan mode *time series* was used to take a series of time-lapse scans (*xyt*) at intervals of 10 sec during 590 sec using continuous scanning with 10X objective lens. Scans were taken in 8 bits at a resolution of 512 by 512 pixels. The region of interest (ROI) of each sample was defined by *ROI mean* tool selecting all extension of image, which plotted a fluorescence intensity of viable and non-viable bacteria versus time chart.

Assessment of bactericidal effects by viable bacteria count

Four primer application times were tested based on CLSM results (20 sec, 90 sec, 300 sec, 590 sec). One-hundred eight dentin samples were used and twelve groups were obtained according to the application time of each primer or saline (n=3): (G1) saline 20 sec; (G2) saline 90 sec; (G3) saline 300 sec; (G4) saline 590 sec; (G5) CPB 20 sec; (G6) CPB 90 sec; (G7) CPB 300 sec; (G8) CPB 590 sec; (G9) CSE 20 sec; (G10) CSE 90 sec; (G11) CSE 300 sec; (G12) CSE 590 sec. Groups 1, 2, 3, and 4 were considered control groups of each time. The experiment was conducted in triplicate.

After 18 hr of bacterial colonization as described before, 10 µL of each primer or saline solution was applied to the surface of samples. The solutions were kept on contact with *S. mutans* biofilm, without disturbing, according to each group. After each experimental time, the excess of solutions was removed by sterile absorbent paper. Samples were immersed in individual eppendorfs with 1 mL saline solution and sonicated for 30 sec at 30% amplitude (UP400S, Hielscher, Teltow, Germany). The suspension was diluted in decimal series and inoculated on BHI agar plates containing bacitracin (2U/mL). The plates were incubated for 48 hr at 37ºC in 5% supplemented CO_2 environment and the number of viable bacteria (CFU) were assessed. The data of CFU were submitted to Kruskal-Wallis and Dunn's Multiple Comparisons tests at a significance level of $p<0.05$.

RESULTS

CLSM images showed *S. mutans* biofilm on dentin surface with viable and non-viable colonies (green and yellow/red, respectively) and non-stained (black) bubble-like structures within the biofilm architecture. Some dead bacteria were observed at time zero (Figs. 1B, E, H). After CSE application no bactericidal effect was observed during all 590 sec; indicating *S. mutans* were viable during the

period evaluated (Fig. 1A). Figure 2B showed a constant line of non-viable bacteria during 590 sec, indicating that there was no increase of dead bacteria after CSE application. The intensity of viable bacteria increased over 300 sec and then declined. The period of 0 to 90 sec did not show change between viable and non-viable bacteria after CPB application. After 90 sec, a slight color change to a yellowish or orange was seen for some colonies, indicating the death of bacteria after CPB application (Fig. 1D). At 590 sec, yellowish and red colonies were predominant (Fig. 1F). Figure 2C showed an inverse relation between viable and non-viable bacteria fluorescence intensity by the time. There was a decreasing of viable bacteria fluorescence intensity and an increasing of non-viable bacteria fluorescence intensity. The control group demonstrated viable bacteria during 590 sec (Fig.1G). A constant line of fluorescence intensity of viable and non-viable bacteria was observed (Fig. 2A).

Table shows the quantitative analysis of the number of viable bacteria (CFU) of *S. mutans* biofilm on dentin after CPB and CSE application. There was no statistical difference among CFU counting after 20, 90, 300, and 590 sec of CSE application. The CFU decreased significantly after 300 and 590 sec of CPB application, but there was no statistical difference between 20 and 90 sec. The comparison among control, CSE and CPB groups in each time showed that there was no statistical difference among groups at 20 sec of saline or primers application. At 90 sec CPB showed significant CFU decreasing compared to control group; while at 300 and 590 sec, both primers showed CFU decreasing with no difference between them.

DISCUSSION

The present study demonstrated that MDPB can be considered a bactericidal agent against *S. mutans* present in biofilm even at high cell density (10^9CFU/mL), since it affects the bacteria membrane that could be detectable by fluorescent indicators. Antimicrobials compounds that do not directly affect the cell membrane,

such as those that interfere with protein or nucleic acid synthesis, are normally considered bacteriostatic and are not suitable agents to be studied by viability staining method (Hope and Wilson, 2004). As MDPB is a derivate of quaternary ammonium, it has high affinity for negatively charged bacterial cells by a nitrogen atom on pyridinium ring, which binds to cell surface. The cell membrane loses its electrical balance, creating a disruption in the cytoplasmic membrane and the cell dies (Scheie 1989).

The MDPB real-time effect against *S. mutans* biofilm by CLSM evaluation had been never tested and showed similar results with the CFU test among times of each primer action. CLSM images of the CSE group remained green in all period tested (Fig. 1A) and no statistical difference was found among CFU up to 590 sec (Table). Likewise, the increase of red staining in CLSM images (Fig. 1D) corroborated the CFU decrease after 90, 300, and 590 sec after CPB application (Table). These results confirmed previous studies that MDPB-containing primer has an antibacterial effect against *S. mutans* colonized in dentin (Kitasako *et al.*, 2004; Imazato *et al.*, 2006; Turkun *et al.*, 2006). The acidic monomer (MDP) present in both primers also can have antibacterial activity because of its low pH (1.9) (Gondim *et al*., 2008). However, dentin can act as buffer, because the acidic dissolution of dentin apatite may be neutralized by bonding between dentin ions and H+ ions of monomer (Gondim *et al*., 2008). In the present study, dentin apatite may have been initially dissolved due to the acid production of biofilm. As the pH value was additionally decreased after primer application, the dentin ions may not have neutralized the high concentration of H^+ ions, maintaining a low pH. Therefore, when CFU results were compared to the control group, there was a CFU reduction after both applications of primer. However, viable bacteria were observed after CSE application by CLSM. In this method the stain used was diluted in saline (pH ~ 7.0), according to manufacturer's instructions. It is possible that the pH of the stain may have increased the biofilm pH. Consequently, the pH of MDP may have been neutralized by the buffering action of dentin, leading to the

visualization of viable bacteria by CLSM. CFU results of the CPB group may also have been influenced by MDP; however, the MDPB bactericidal effect was shown by the viability stain test. Further investigations are needed to clarify this fact.

Bacterial biofilms associated with surfaces are complex three-dimensional structures in which bacteria are embedded in a matrix mainly made of exopolysaccharides (EPS) (ten Cate 2006). EPS functions as an ion-exchange matrix and hinders the penetration of positively-charged antimicrobial agents (Stewart 2003). The activation of an adaptive stress response and changes in gene and protein expression of bacteria are also factors that makes biofilms 10 to 1000 times less sensitive to antimicrobial agents than planktonic bacteria (Mah and O'Toole 2001, Hope and Wilson 2004). The current results indicate that the reduced biofilm susceptibility to antimicrobial agents with live bacteria was found after 590 sec, using even higher MDPB concentration than Imazato *et al.*, who carried out a study against bacterial suspension in the same time and methodology as the current study.

Some studies reported decline *S. mutans* counting in small pieces or chips of dentin and bacterial suspension after 20 sec application of CPB primer (Imazato *et al.*, 1997, 1999, 2001, 2006; Turkun *et al.,* 2006). However, the present study showed a latter bactericidal MDPB effect (300 sec). The EPS among biofilm channels may have impaired the penetration of MDPB and consequently its action, because the mechanism of action for MDPB is based on the contact of immobilized antibacterial molecules with bacteria (Imazato *et al.*, 1998).

Some characteristics of *in vivo* biofilm are visualized in Fig. 1. The bubble-like structures that were voids were possibly filled with biological substances, such as EPS and glycoproteins, which are not stainable by the stain used (Wood *et al.*, 2000); or dead bacteria that were initially present, that might have been located in deeper regions of the biofilm and had reduced access to nutrients from the exposed medium (Hope *et al.*, 2002). Additionally, the inherent variability between

and within biofilms (Roland *et al.*, 2006) caused the difference found among fluorescence intensity for the control group, CSE and CPB (Fig. 2). Furthermore, the laser beam may have excited a region with a higher number of bacteria and stain concentration causing this difference, which did not influence the results.

As the viability staining method provided a rapid and sensitive way to test the MDPB bactericidal effect against a *S. mutans* biofilm, further investigations need to be conducted against other cariogenic microorganisms in biofilm or multispecies biofilm. Some attempts were made in this present study to test the MDPB effect after bond application and cure, but the bond autofluorescence interfered with the bacteria visualization using CLSM. In conclusion, MDPB had bactericidal effect against *S. mutans* biofilm after 300 sec, as evidenced by real-time CLSM and CFU counting evaluations.

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Table. Number of viable *S. mutans* (CFU) recovered after Clearfil Protect Bond (CPB) and Clearfil SE Bond (CSE) primer application at different times. Values are expressed in median (minimum/maximum).

Groups	20s *	90s	300s	590s
Control	2.5×10^{9} **A,a** (10^{4} /10^{9})	1.0×10^{9} **A,a** (10^{8}/10^{9})	5.3×10^{9} **A,a** (10^{8}/10^{9})	2.5×10^{9} **A,a** (10^{8}/10^{9})
CSE	9.0×10^{4} **A,a** (10^{4} /10^{6})	3.6×10^{3} **A,ab** (10^{3}/10^{4})	8.8×10^{2} **A,b** (10^{2}/10^{3})	9.6×10^{2} **A,b** (10^{2}/10^{3})
CPB	1.0×10^{5} **A,a** (10^{4}/10^{5})	1.0×10^{3} **AB,b** (10^{2} /10^{3})	4.8×10^{2} **B,b** (10/10^{2})	5.6×10^{2} **B,b** (10^{2} /10^{3})

* For each row: values with identical uppercase letters indicate no statistically significant difference (P>0.05). For each column: values with identical lowercase letters indicate no statistically significant difference (P>0.05) according to Dunn's Multiple Comparisons test; n=9 for each time of each primer/saline application.

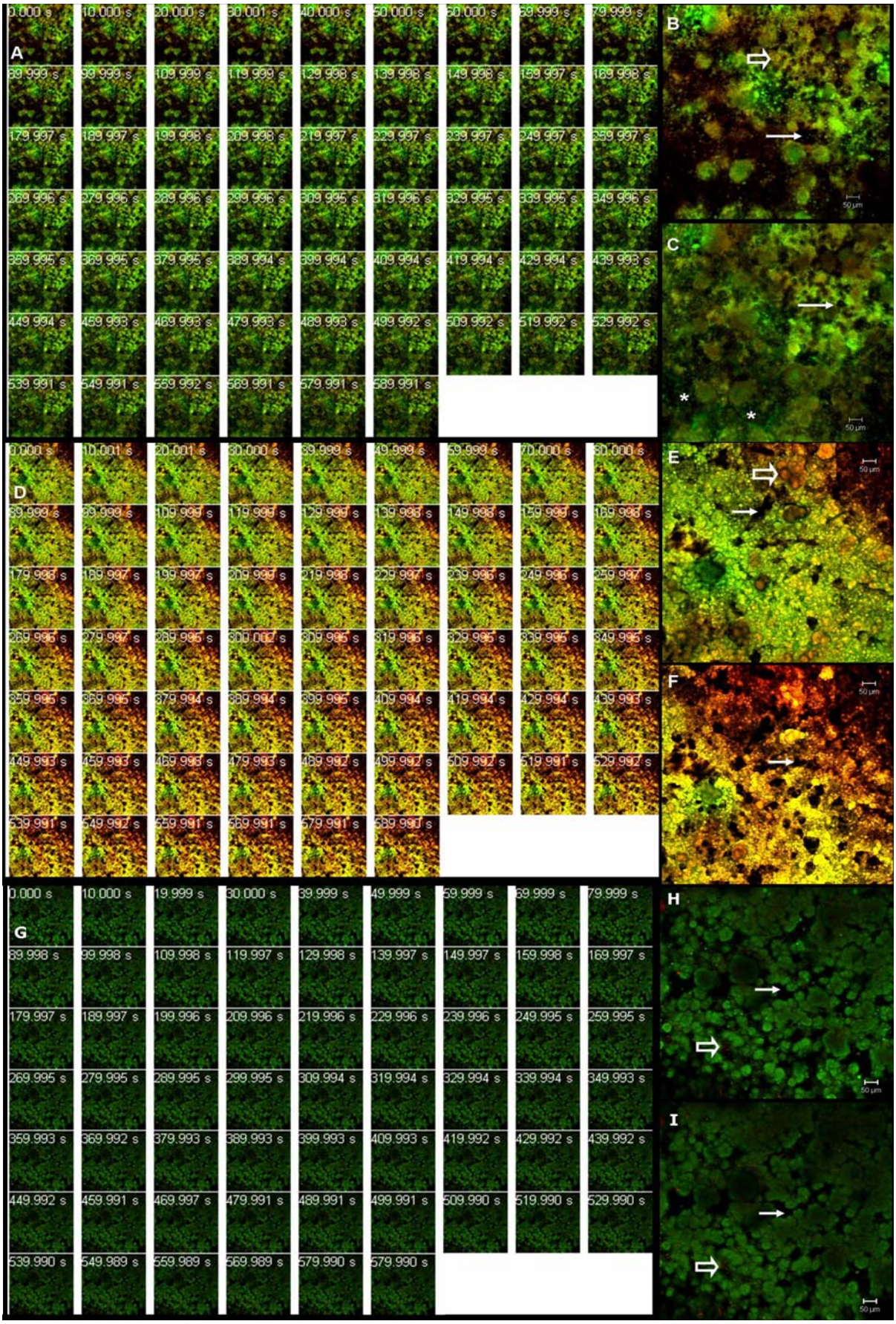
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Figure 1. *S. mutans* biofilm on dentin surface after Clearfil SE Bond (CSE), Clearfil Protect Bond (CPB) and saline application visualized by CLSM. Images were recorded in real-time over 590 sec. Viable bacteria are stained green and non-viable bacteria are stained yellowish/red color. Figs. A, D, G shows image series of time-lapse scans according to the time (upper-left side of each scan). No bactericidal effect was observed after CSE (Fig. A) and saline application (Fig G) because all colonies were stained green over 590 sec. After CPB application was observed a gradual increase of non-viable bacteria (yellowish/red colonies) during 590 sec (Fig. D), indicating the bactericidal effect of MDPB. Non-viable bacteria were predominant at 590 sec (Fig. F). Figs. B, E and H are higher magnification of image at zero time, showing the presence of dead bacteria (open arrow), which are also visualized at 590 sec (Figs. C, F, I). The non-stained (black) bubble-like structures within the biofilm architecture (arrow) are voids filled with exopolysaccharides (EPS) (Figs. B, C, E, F, H, I). *Low-intensity noise that may be attributed to the fluorescence of primer.

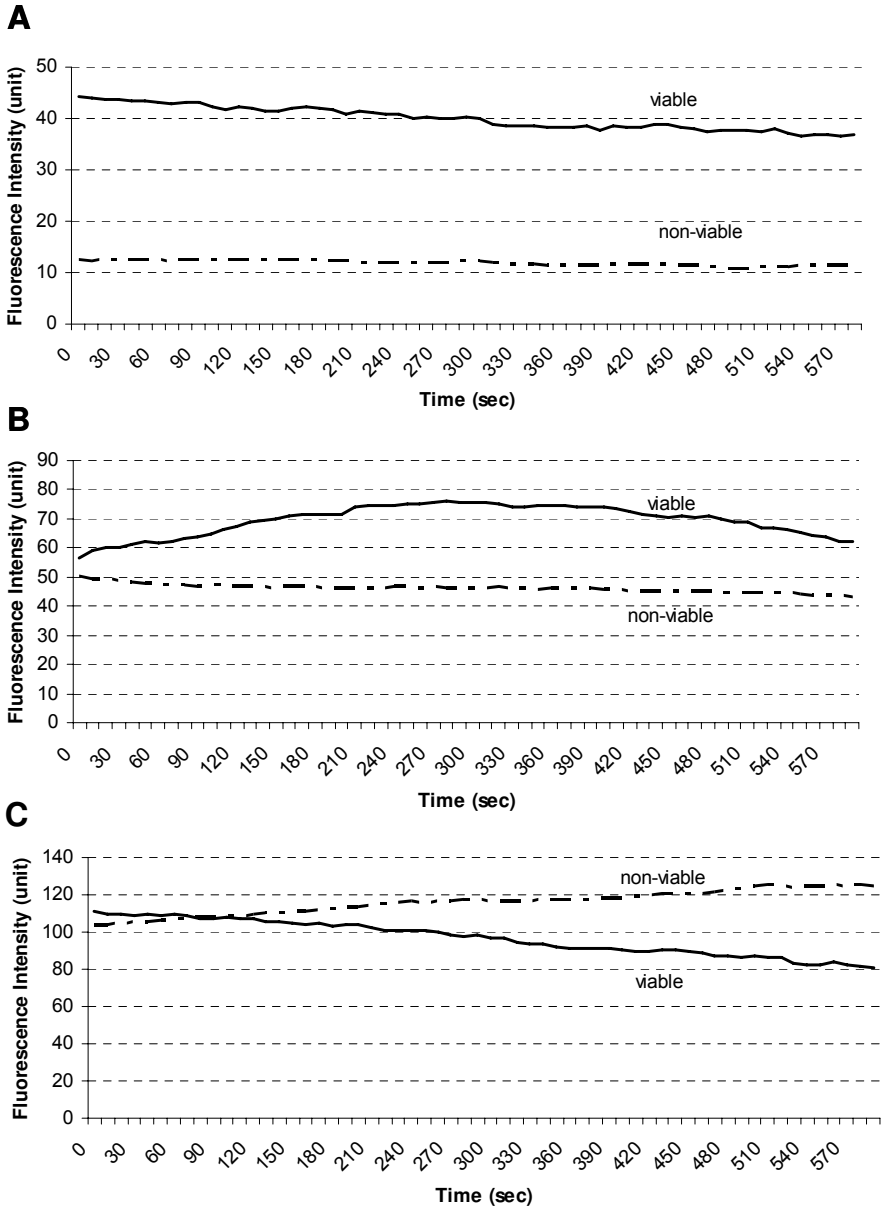

Figure 2. Fluorescence intensity of viable and non-viable bacteria versus time. (**A**) Constant line of viable and non-viable bacteria during 590s after saline application (control group). (**B**) Constant line of non-viable bacteria during 590 sec, indicating that there was no increase of dead bacteria after Clearfil SE Bond application. The intensity of viable bacteria increased until 300 sec and after declined. (**C**) Decreasing of viable bacteria intensity and increasing of non-viable bacteria intensity after Clearfil Protect Bond application.

CAPÍTULO 2

Biodegradation of artificial caries-affected dentin bonding interface of fluoride and MDPB-containing adhesive system

Abstract

The aim of this study was to evaluate the effect of a fluoride/MDPB-containing adhesive system on the durability of bonding to permanent artificial caries-affected dentin (CAD) exposed to *S. mutans* culture and water storage. Twelve third molars were selected. Flat surfaces of dentin were submitted to artificial caries development in *S. mutans* and BHI broth. Caries-infected dentin was removed with burs according to clinical criteria and CAD cavities were restored with Adper Scotchbond Multi-Purpose (SBM) and Clearfil Protect Bond (CPB) (n=6). Non-trimmed resin-dentin bonded interfaces (1mm^2) were stored in *S.mutans* + BHI for 3 days or in deionized water for 3 months and after that subjected to microtensile bond strength (μTBS) test. The control group was not submitted to storage and immediate μTBS testing was performed. Fractographic analysis was performed after μTBS testing. Four molars were restored as described, and morphological analysis of hybrid layer (HL) was evaluated by scanning electron microscopy (SEM). Two-way ANOVA with split-plot design and Tukey's tests were performed. No difference was found between μTBS values of SBM and CPB irrespective of the groups. A significant decrease in μTBS values after *S. mutans* culture and water storage was observed, but no difference was found between them. SBM and CPB showed similar hybrid layer thickness and resin tag formations. The fluoride/MDPB-containing adhesive system did not prevent the degradation of CAD bond strength in both degradation methods.

Keywords: caries-affected dentin, degradation, adhesive, hybrid layer, water storage, *Streptococcus mutans*

1. Introduction

Bonding to carious dentin has been investigated because in clinical situations some areas along the pulpal floor are composed of caries-affected dentin (CAD) after cavity preparation. CAD bond strength has shown significantly lower values than those to normal dentin with self-etching [1,2] and etch-and-rinse adhesive systems [3,4,5]. The intratubular deposition of calcium phosphate crystals by the caries process, which can interfere in adhesive infiltration into the tubules and prevent resin tags formation, as well as the higher degree of porosity of intertubular dentin contribute to the lower CAD bond strength [2,4,6].

The limited and irregular size and shape of naturally-formed CAD may harm the standardization of the substrate for bonding analysis [7,8], thus artificial models have been used to simulate CAD [7,8,9]. Biological models have been used in few studies [10,11] and their demineralization mechanism seems to be more analogous to the in vivo situation than that of chemical methods [12]. Especially in secondary caries formation, since the cariostatic effects of restorative materials are obtained by means of acids and enzymes produced by bacteria [10,12,13], approaching the features of the oral environment.

As secondary caries formation is still the main cause of restoration replacements [14], components such as fluoride and antibacterial monomer, 12-methacryloyloxydodecylpyridinium bromide (MDPB), have been incorporated into adhesive systems to aid in caries prevention and the longevity of restorations. Fluoride ions are concentrated at the base of hybrid layer and can penetrate into dentin [15], increase dentin resistance to caries attack [10] and minimize the bonding degradation [16]. MDPB is a compound of antibacterial agent quaternary ammonium and a methacryloyl group, which is covalently bound to the polymer

matrix by its copolymerization with other monomers [17] and it has shown bactericidal activity against *Streptococcus mutans* and other oral bacteria [18,19].

The quality and durability of bonds between adhesive resins and dentin are important data for understanding restoration longevity. CAD bonding durability has been little investigated [5] and showed higher susceptibility to water degradation over the time than sound dentin [5]. Water storage of specimens submitted to the microtensile bond strength is the main method used to evaluate the durability of resin material bonding [5,16,20]. Recently, Peris et al. [21] stored specimens in pH cycling solutions to evaluate the influence of adhesive systems containing fluoride on the bond strength to sound dentin. As MDPB has an antibacterial effect, it would be interesting to evaluate the effect of MDPB on bond strength durability when storage solution containing bacteria was used. Furthermore, the collagenolytic and gelatinolytic activities of *S. mutans* [22] might interfere in bonding degradation. To date, no study has reported the effect of fluoride ions and MDPB on CAD bond strength when subjected to the *S. mutans* culture degradation method.

The aim of this study was to investigate the effect of a fluoride/MDPB-containing adhesive system on the bonding durability to artificial CAD exposed to *S. mutans* culture and water storage. The hypotheses tested were (1) there is no difference between *S. mutans* culture and water storage on CAD microtensile bond strength (μTBS); (2) there is no difference in CAD μTBS between etch-and-rinse without fluoride/MDPB and self-etching fluoride/MDPB-containing adhesive systems irrespective of the degradation method.

2. Materials and methods

2.1. Preparation of dentin samples

This study was performed under the protocol approved by the Ethics Committee of Piracicaba Dental School - University of Campinas. Sixteen sound third molars were selected for this study. Teeth were stored in 0.1% thymol at 4ºC and used within 1 month after extraction. The occlusal enamel was sectioned

perpendicular to the long axis of the teeth using a low-speed diamond saw under water cooling (Isomet, Buehler Ltd, Lake Bluff, IL, USA) to expose adjacent dentin. The dentin surface was wet ground flat with 180 and 400 grits silicon carbide abrasive paper (Saint-Gobain Ltda, SP, Brazil) until all enamel had been removed. The roots were sectioned and discarded. The pulp chamber was filled with composite resin (TPH Spectrum, Dentsply Caulk, Milford, DE, USA). Samples were covered with acid-resistant nail varnish (Colorama, CEIL Ltda., SP, Brazil) leaving the coronal dentin uncoated for artificial caries development.

2.2. Artificial caries development and restoration

Samples were fixed in the lids of glass containers with orthodontic wire and were sterilized in a gamma irradiation chamber (Gammacell 220 Excel, GC-220E; MDS Nordion, Ottowa, Canada) [23]. After this, samples were transferred to another glass container containing 40 mL of sterile brain heart infusion broth (BHI) (Becton Dickinson and Company, Sparks, MD 21152, USA), supplemented with yeast extract (Himedia Laboratories PVT Ltd., Mumbai, India), 0.5% glucose (Synth, LabSynth, Diadema, SP, Brazil), 1% sucrose (Synth, LabSynth, Diadema, SP, Brazil) and 2% *S. mutans* [9]. *Streptococcus mutans* UA159 was used in this study. This strain was selected for genomic sequencing [24]. To prepare the inoculum, this microorganism was obtained from the culture collection of the Department of Microbiology and Immunology, Dental School of Piracicaba, and grown on Mitis salivarius agar plates at 37ºC for 48 h in a 5% supplemented CO_2 environment. Subsequently, single colonies were inoculated into 5 mL of BHI broth and incubated at 37ºC for 24 h. An overnight culture was adjusted to 1 x 10^6 (colony forming units (CFU)/mL) and an optical density of 0.6 at 600 nm. Then 800 µL of bacterial suspension was inoculated into the culture medium only on the first day, but the medium was renewed every 48 h for 14 days [9]. Every 48 h, the lack of contamination and purity of the medium were verified using Gram staining and plating methods.

The soft and infected carious tissue was removed with #8 round tungsten carbide burs (KG Sorensen, Barueri, São Paulo, Brazil) in a slow-speed handpiece. The tactile sensation criterion with dental explorers and visual examination were used to identify CAD [25]. Twelve permanent teeth were randomly assigned into two groups for μTBS testing, according to the adhesive system used (n=6) (Table 1): etch-and-rinse adhesive system Adper Scotchbond Multi-Purpose (SBM) (3M ESPE, St Paul, MN, USA) and self-etching adhesive system Clearfil Protect Bond (CPB) (Kuraray, Okayama, Japan).

Dentin surfaces were restored using the adhesive systems according to their manufacturers' instructions (Table 1). The bonded surfaces were coupled with a hybrid resin composite (TPH Spectrum, Dentsply Caulk, Milford, DE, USA, Lot No. 317309) incrementally built up to form 4-mm-thick cores. Each increment was light cured for 20s and the restored teeth were stored in distilled water at 37°C for 24 h.

Each specimen was cross-sectioned perpendicular to the resin-dentin interface with a slow-speed saw (Isomet, Buehler Ltd, Lake Bluff, IL, USA) under water cooling, yielding square sticks of approximately 1 mm^2, in accordance with the non-trimming version of the microtensile bond test reported by Shono et al. [26]. A total of 18-20 sticks per teeth were obtained. No premature debonding occurred during sample preparation.

2.3. Storage solutions for bond strength degradation

The sticks were coated with an acid-resistant nail varnish except for a window of dentin measuring 8.0 mm^2 around the bonded interface. This area was exposed to storage solutions. The sticks obtained from each tooth were equally and randomly divided into three subgroups according to the storage solutions: 1- no solution – immediate μTBS (control group); 2- BHI supplemented with 1% sucrose with *S. mutans* (3 days) [9]; 3- deionized water for 3 months. Samples were individually placed in eppendorf tubes and sterilized in a gamma irradiation

chamber at 100% relative humidity (Gammacell 220 Excel, GC-220E; MDS Nordion, Ottawa, Canada) [23].

A pilot study was conducted to test the influence of gamma irradiation on μTBS, fluoride release and the antibacterial effect of MDPB. The fluoride release tests were conducted with samples of CPB bond using fluoride-specific electrode, the agar diffusion test was used to test the antibacterial effect of MDPB against *S. mutans* before and after gamma irradiation. No difference was found between irradiated and non-irradiated groups with respect to μTBS values, fluoride release and antibacterial effect against *S. mutans.*

For storage in the *S. mutans* culture, each sample was placed into an eppendorf tube containing 1 mL BHI supplemented with 1% sucrose [10]. Next 20 μL *S. mutans* was inoculated into each eppendorf tube, as described before, only on the first day and the culture media were renewed daily. The numbers of viable bacteria were 2 x 10^8 CFU/mL in each change of medium. The degradation area was 8.0 mm^2/mL of *S. mutans* culture. Samples were kept in *S. mutans* culture for 3 days. This protocol was based on results from previous pilot studies, with the aim of establishing a period for keeping samples immersed in *S. mutans* culture without causing high demineralization rates in dentin, which would make it impossible to perform the microtensile bond strength testing due to high incidence of cohesive failures in dentin. The storage period of 3 days was enough to cause a uniform demineralization at CAD dentin/adhesive interface with very low percentage of cohesive failures in dentin. Samples of subgroup 3 were stored in individual eppendorf tubes containing 1 mL of deionized water for 3 months. The water was renewed every 7 days [5]. The degradation area was 8.0 mm^2/mL of deionized water.

2.4. Microtensile bond strength

The exact dimensions of all samples were measured with a digital caliper (Starret 727-6/150; Starret, Itu, SP, Brazil) to calculate bond strength before bond-

testing. Samples were stressed to failure under tension in a Bencor Multi-T device (Danville Engineering, San Ramon, CA, USA) in a universal testing machine (Instron model 4411; Canton, MA, USA) at crosshead speed of 0.5 mm/min. Means and standard deviations were calculated and expressed in MPa. All fractured samples were evaluated under an optical microscope (Leica-DM 5000B, Leica, Germany) at 40X magnification and failures were designated as adhesive, mixed or cohesive failures in either dentin or resin/adhesive. Representative sticks of each group were mounted on aluminum stubs, sputter-coated with gold (Balzers-SCD 050 Sputter Coater, Liechtenstein) and observed by scanning electron microscopy - SEM (JEOL- JSM 5600LV, Tokyo, Japan) at 90X and 1000X magnification. The percentage of each failure mode was calculated.

2.5. SEM Evaluation of dentin/resin interface

Four third molars not used for μTB testing were used to evaluate the morphology of the CAD dentin-resin interface and resin tags formation. Samples were submitted to artificial caries development and restoration as described before. Samples were sectioned perpendicularly to the bonded surface and polished on a water-cooled mechanical grinder (Arotec, Cotia, SP, Brazil) using 400, 600, 1200 and 2000 grit silicon carbide abrasive paper and a 1-μm diamond paste. After this, samples were gently decalcified (50% phosphoric acid for 3 s), deproteinized (2% NaOCl solution for 10 min) and maintained in a desiccator at 37ºC for 1h. They were mounted on stubs, sputter-coated with gold (Balzers-SCD 050 Sputter Coater, Liechtenstein) and observed under SEM (JEOL- JSM 5600LV, Tokyo, Japan) at 2500X magnification.

2.6. Statistical analysis

The bond strength data were submitted to two-way ANOVA with split-plot design and Tukey's test at a 5% level of confidence. The plots were represented by the factor adhesive system (SBM and CPB) and sub-plots were represented by the factor treatment (immediate μTBS (control), *S. mutans* culture and water storage).

3. Results

Two-way ANOVA with split-plot design revealed that there were significant differences for the factor storage solution (p = 0.0001). However, no differences were detected for the factor adhesive system (p = 0.5490) or for the interaction between the two factors (p = 0.4167). Tukey's test showed significant decrease in μTBS values after *S. mutans* and water storage ($p < 0.05$), but no difference was found between μTBS values obtained from *S. mutans* culture and water storage, irrespective of the adhesive system tested. The means and standard deviations are summarized in Table 2.

The distribution of fracture mode pattern for the groups evaluated is shown in Fig. 1. A high prevalence of mixed fracture was observed for all groups, irrespective the treatment and adhesive system. *S. mutans* culture storage showed cohesive fracture in dentin on the edges (Figs. 2A and B). SBM showed fractures located at the bottom of hybrid layer with open tubules without peritubular dentin in both storage media (Figs. 3A and B). The hybrid layer and tags inside tubules were found in some regions (Figs. 3A and B). CPB fracture patterns after *S. mutans* and water storage showed fractures located at the top of hybrid layer, with peritubular dentin and adhesive around the tubules (Figs. 4 and 5).

SEM interface micrographs showed similar hybrid layer thickness and resin tags formation between SBM and CPB adhesive systems. No funnel-shaped tags with lateral branches were observed (Fig. 6).

4. Discussion

The artificial caries development with *S. mutans* used in this study was a dynamic model because the broth was renewed every 48 h and a gradual decrease in pH occurred after the broth was changed [10]. According to Tjaderhane et al. [27] carious lesion formation involves dentin demineralization

followed by degradation of the exposed organic matrix. Although *in vitro* evaluations cannot completely reproduce all the factors involved in the natural caries process, the artificial caries model containing *S. mutans* can simulate dentin demineralization by acid production as well as the degradation of the organic matrix due to the collagenolytic and gelatinolytic activities of *S. mutans* [22]. Concerning to the bonding strength, this study showed that the initial μTBS values in artificial CAD developed by *S. mutans* were similar to those of other studies tested on natural CAD for both adhesive systems used [4,25].

S. mutans culture storage has never been investigated before to evaluate the antibacterial effect of MDPB, as well as the effect of fluoride on CAD μTBS. *S. mutans* culture and water storage decreased the μTBS values, which was also reflected by the increase in the percentage of adhesive failures after storage in the two solutions (Fig. 1). Bonding degradation occurred mainly due the presence of water. Similar to incompletely infiltrated sound dentin, the non-infiltrated CAD may undergo self-destruction via the release of endogenous metalloproteinases present in dentin matrix [28]. As these enzymes are hydrolases, they require water to break peptide bonds in the collagen molecules, causing hydrolytic degradation of collagen [28]. In addition, the polymer networks may be “plasticized” by water sorption from environment, decreasing their mechanical properties [20]. Erhardt et al. [5] also showed that there was a decrease in CAD bond strength after water exposure when a fluoride/MDPB-containing adhesive system was used, corroborating our results. The present study showed that *S. mutans* culture is as effective a method for producing degradation *in vitro* as 3 months of water storage, but in a short period of time (3 days). *S. mutans* have collagenolytic and gelatinolytic activities [22] that could accelerate the degradation process. In addition, the acid production by *S. mutans* decreased the pH of the medium and caused dentin demineralization at the CAD dentin/adhesive interface. Probably, this demineralization reduced the strength of the CAD/adhesive interface at the edges (Fig. 2B), irrespective of the presence of fluoride and MDPB in the adhesive

systems used. The lack of difference in μTBS values between the storage solutions provided indirect evidence that the hydrolytic degradation of dentin collagen may proceed in the absence of bacterial colonization, as proposed by Pashley et al. [28]. Nevertheless, the collagenolytic and gelatinolytic activities of *S. mutans* and other cariogenic microorganisms in the degradation of the collagen fibrils in the CAD hybrid layers need to be better clarified, since the lower durability of CAD bond strength in comparison with that of sound dentin might also be attributed to the collagenolytic activity of bacteria present in CAD, because residual bacteria are found in dentin after caries removal [29].

Although the adhesion mechanism differs between self-etching and etch-and-rinse adhesive systems [30], in the present study there was no difference between the μTBS values of CPB and SBM adhesives systems, as previously reported by other studies [4,5]. The SEM interface micrographs (Fig. 6) also showed no difference in hybrid layer thickness and resin tags formation between the adhesives systems, confirming the μTBS results. Probably, the demineralization of substrate due to the artificial CAD development eliminated the difference in resin tags morphology created by self-etching and etch-and-rinse adhesive systems. Etch-and-rinse adhesive systems create funnel-shaped tags in sound dentin due to the demineralization of peritubular dentin after etching with phosphoric acid [31,32]. The micrographs of fractured specimens showed a more aggressive pattern in the SBM group after degradation by *S. mutans* culture or water exposure with open tubules devoid of peritubular dentin (Fig. 3). Whereas, fractured specimens of the CPB group showed peritubular dentin around tubules (Figs. 4 and 5).

It has been reported that fluoride inhibits a number of enzymes including esterases [33] and could prevent the dentin degradation [16]. As fluoride is released within the hybrid layer [15], it might inhibit the enzymes released from the mineralized matrix (metalloproteases) from attacking the components of the hybrid layer or prevent the release of these enzymes due to its remineralization action

[16]. However, in the present study there was no difference in μTBS values between adhesive systems with or without fluoride, suggesting that neither fluoride nor MDPB have an influence on μTBS values 24 h after restoration and exposure to *S. mutans* culture or water. A possible explanation for this fact is that fluoride ions could have been surrounded by resin matrix and/or hybrid layer and its contact with water was restricted, because its movement might be limited by the matrix and dentin [34]. Moreover, the small amount of adhesive system used during the bonding procedure may also have had an influence. According to the manufacturer's technical information, CPB has less than 1% of surface-treated sodium fluoride that is sodium fluoride with a unique, semi-permeable polymer capsule, which remains in the bonding layer after fluoride ions are released. This capsule is very important because it helps to maintain the initial mechanical properties of the material, avoiding the formation of voids in the resin matrix by fluoride leaching [35]. However, it might be an additional barrier to fluoride release. Peris et al. [21] reported that Des/Re solutions used to store specimens for μTBS testing presented very low fluoride ion release (lower than 0.03 ppm) by adhesive systems bonding to dentin.

Likewise fluoride, the MDPB monomer did not influence the bond strength durability, mainly in *S. mutans* culture storage. MDPB is an antibacterial monomer that copolymerizes with other monomers and the antibacterial agent is covalently bonded to the polymer network [17]. The immobilized antibacterial agent does not leach out from the material, thus the MDPB action mechanism is based on the contact of immobilized antibacterial molecules with the bacteria [17]. In the present study, the MDPB monomers were probably intermixed in the polymer network and restricted to a small portion of the restoration exposed to *S. mutans* culture, i.e adhesive layer and hybrid layer. Consequently, the antibacterial effect of MDPB was decreased due to the lower contact area with *S. mutans.* Imazato et al. [36] suggested that after cure, MDPB can suffer the same phenomenon as the pyridinium-type polymer, N-benzyl-4-vinylpyridinum bromide (BVP). BVP showed no bactericidal effect after cure, because the cross linking formation unable the

antibacterial component to penetrate the cell wall of bacteria, therefore the interaction between the surface of bacterial cells and polymer halt at the adsorption stage [37].

The present study showed that the fluoride/MDPB-containing adhesive system did not prevent the degradation of CAD bond strength irrespective the degradation method. The hypotheses were accepted. The fluoride release mechanism of adhesives systems in dentin, as well as the effect of MDPB, needs to be evaluated in further studies to test their behavior under intra-oral conditions and to clarify the role of these components in CAD and sound dentin bonding durability.

5. Conclusions

Based on the results of this study it can be concluded that the fluoride/MDPB-containing adhesive system did not prevent the degradation of CAD bond strength after *S. mutans* culture or water storage. Three days of *S. mutans* culture is as effective a method for producing degradation *in vitro* as three months of water storage.

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Table 1. Main components and application mode of adhesive systems used in the study

Materials	Composition (batch number)	Application mode
Adper Scotchbond Multi-Purpose (3M-ESPE, St Paul, MN, USA)	*Acid Conditioner:* 37% Phosphoric acid (7523 5FB) *Primer:* water, ethanol, HEMA, copolymer of polyalkanoic acid (3008) *Bond:* Bis-GMA, HEMA, dimethacrylate, initiators (7583)	Apply *acid conditioner* for 15s, rise for 15s, dry gently for 2s. Leave moist. Apply *primer*, dry gently for 5s. Apply *bond*, light cure for 10s.
Clearfil Protect Bond (Kuraray, Okayama Japan)	*Primer:* 10-MDP, MDPB, HEMA, water (00017A) *Bond:* 10-MDP, Bis-GMA, HEMA, hydrophobic methacrylate, dl-Camphorquinone, N,N-Diethanol-p-toluidine, silanated colloidal silica, surface-treated sodium fluoride (00027A)	Apply *primer* for 20s. Mild air stream. Apply *bond*. Gentle air stream. Light cure for 10s.

10-MDP: 10-methacryloxydecyl dihydrogen phosphate; HEMA: 2-hydroxyethyl methacrylate; MDPB: 12-methacryloyloxydodecylpyridinium; Bis-GMA: bis-phenol A diglycidylmethacrylate.

Table 2. Microtensile bond strengths values of adhesive systems in each degradation method.

	Adhesive systems	
Degradation methods	Adper Scotchbond MP*	Clearfil Protect Bond
Control (n=6)	26.8 ± 3.4 $^{A, a}$ (37)	23.1 ± 8.4 $^{A, a}$ (34)
Strep. mutans culture (n=6)	17.0 ± 4.4 $^{B, a}$ (37)	17.5 ± 4.6 $^{B, a}$ (34)
3 months water (n=6)	18.5 ± 2.4 $^{B, a}$ (35)	17.1 ± 5.7 $^{B, a}$ (35)

*Values are mean ± standard deviation bond strengths in MPa. The number of sticks tested for each group is described inside brackets. For each column: values with identical uppercase letters indicate no statistically significant difference (P>0.05). For each row: values with identical lowercase letters indicate no statistically significant difference (P>0.05) according to Tukey's test.

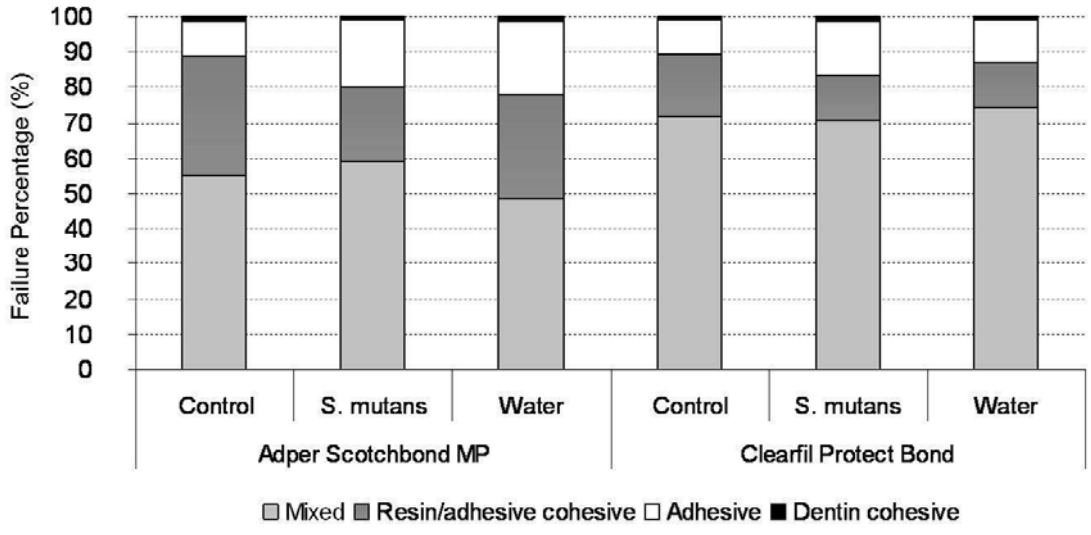

Figure 1. Distribution of failure mode patterns for each group (%).

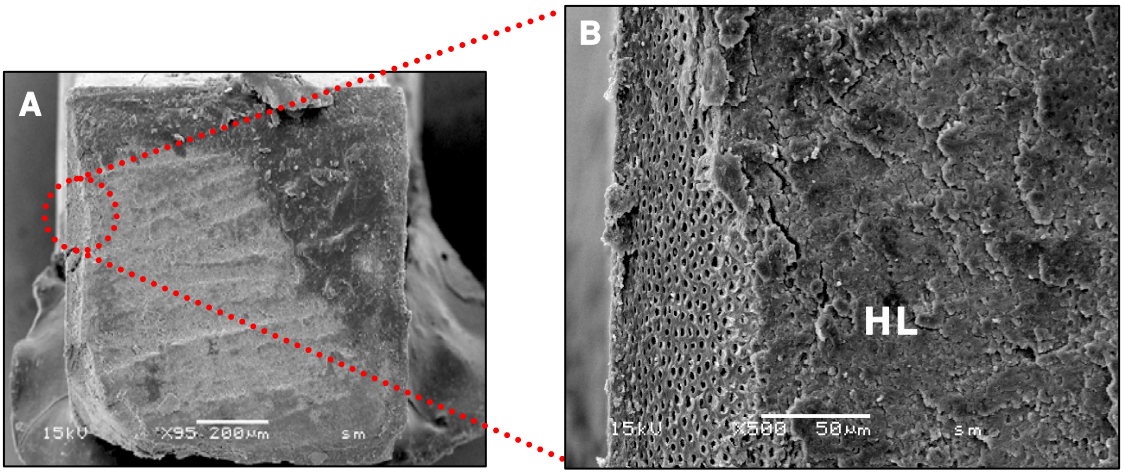

Figure 2. SEM image of the fractured dentin surface of specimen bonded with SBM stored in *S. mutans* culture for 3 days. (A) Mixed failure (95X). (B) Higher magnification view (1000X) of mixed failure, dentin cohesive fractures occurred on the edges of the specimen due to demineralization acid by *S. mutans*. HL: hybrid layer.

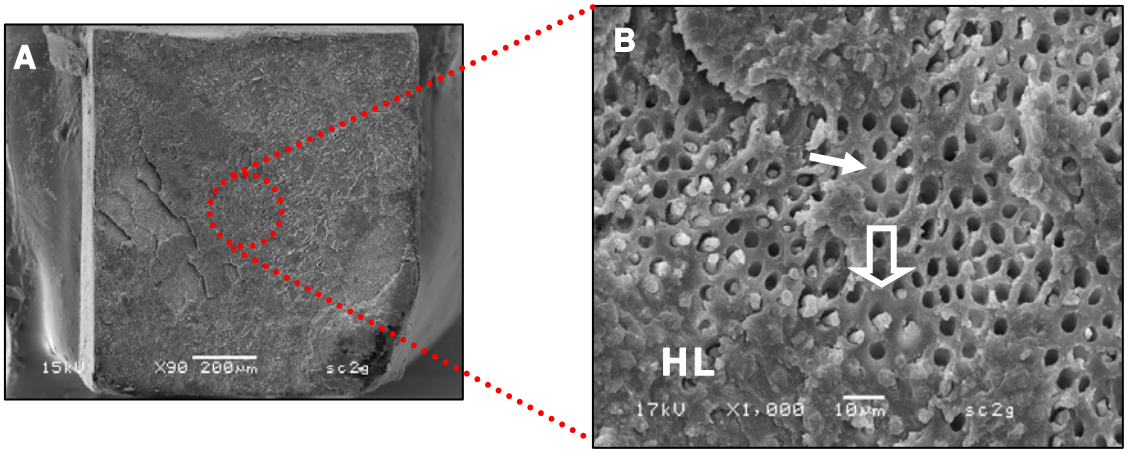

Figure 3. SEM image of the fractured dentin surface of specimen bonded with SBM stored in deionized water for 3 months. (A) Mixed failure (90X). (B) Higher magnification view (1000X) of mixed failure located at the bottom of hybrid layer (HL), with open tubules without peritubular dentin (arrow). Presence of resin tags inside tubules (open arrow).

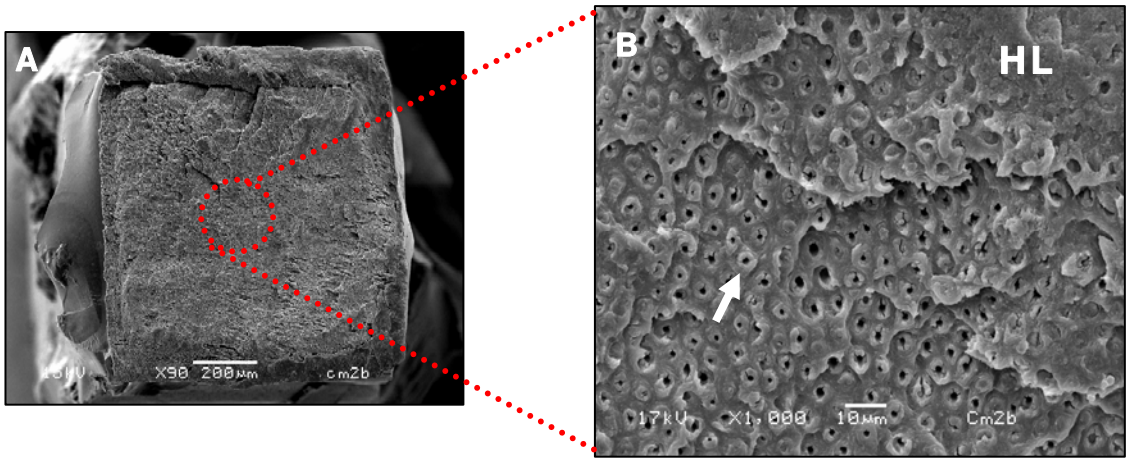

Figure 4. SEM image of the fractured dentin surface of specimen bonded with CPB stored in *S. mutans* culture for 3 days. (A) Mixed failure, dentin cohesive fractures occurred in the edge of the specimen due to demineralization acid by *S. mutans* (90X) (B) Higher magnification view (1000X) of mixed failure located at the top of hybrid layer (HL), with peritubular dentin and adhesive around the tubules (arrow).

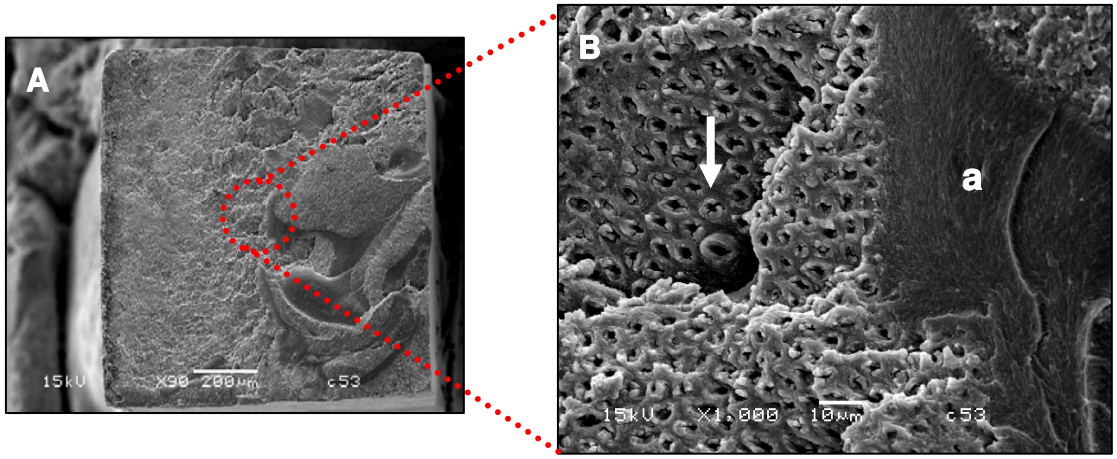

Figure 5. SEM image of the fractured dentin surface of specimen bonded with CPB stored in deionized water for 3 months. (A) Mixed failure (90X). (B) Higher magnification (1000X) showing the presence of peritubular dentin and adhesive around tubules (arrow). a: adhesive.

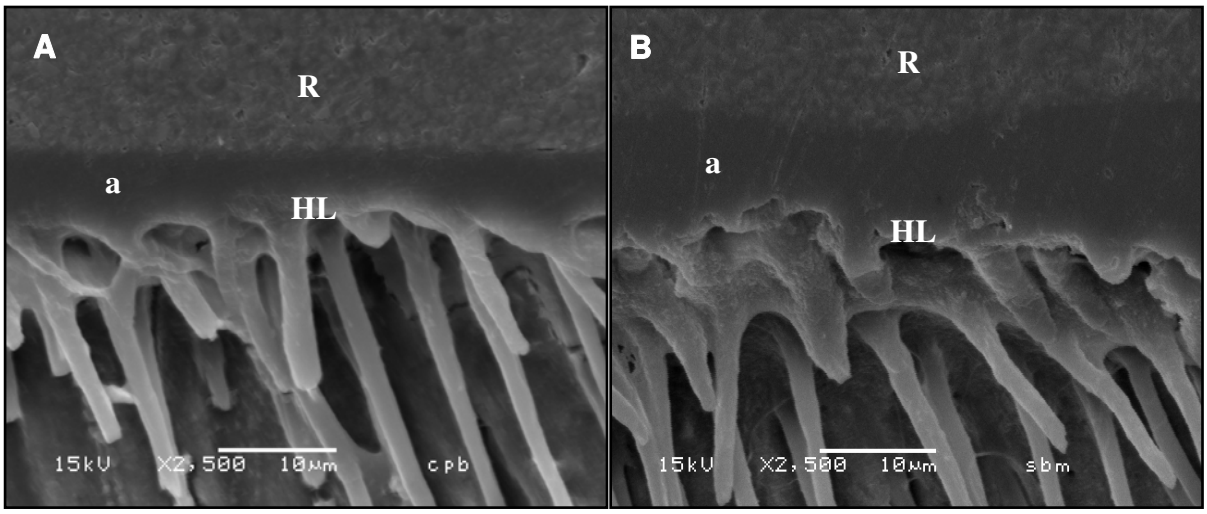

Figure 6. SEM interface micrographs (A) CPB interface (B) SBM interface. Similar hybrid layer thickness (HL) and resin tags formation were observed between SBM and CPB adhesive systems. R: resin; a: adhesive layer.

CAPÍTULO 3 *

Mineral distribution and CLSM analysis of secondary caries inhibition by fluoride/MDPB-containing adhesive system after cariogenic challenges

Abstract

Objective: To evaluate the inhibition zone formation (IZ) and mineral distribution along the interface of adhesive systems either containing fluoride or not, and antibacterial monomer (MDPB) after chemical and biological artificial caries challenges.

Methods: Forty eight third molars were used. Artificial caries was developed with *S. mutans* in a 4 x 4 mm area of occlusal dentin surface. Carious dentin was removed and cavities were restored with Adper Scotchbond Multi-Purpose (SBM) and Clearfil Protect Bond (CPB) (n=24). Samples were submitted to secondary caries development by chemical (C) (acidic gel) or biological (B) (*S. mutans* culture) methods for 5 days. Four groups were tested (n=12): (1) SBM+C; (2) SBM+B; (3) CPB+C; (4) CPB+B. The IZ and outer lesion (OL) formations were analyzed by confocal laser scanning microscopy (CLSM). The distribution of calcium (Ca) and phosphorus (P) content along the interface was analyzed by micro X-ray fluorescence spectrometer by energy-dispersive (μEDX).

Results: The frequency of IZ formation and mean values of IZ thickness differed among the groups. The CPB+C group presented the lowest OL depth. μEDX analysis showed that CPB had the highest mineral loss by the biological method, but the lowest mineral loss by the chemical method. SBM+C and SBM+B groups showed intermediate values of mineral loss.

*** Trabalho submetido para o periódico *Journal of Dentistry***

Conclusion: The mineral loss along the dentin/restoration interface was affected by the artificial caries method, and hybrid layer formation by adhesive systems used. The fluoride and antibacterial monomer of adhesive system did not prevent secondary caries formation.

Key-words: fluoride, MDPB, adhesive system, inhibition caries, confocal microscopy

Introduction

The fluoride incorporated in adhesive systems can contribute to the inhibition of secondary caries formation,[1-4] since fluoride ions are released at the base of hybrid layer and can penetrate into dentin.[5] Fluoride resistance to secondary caries is more related to inhibiting the demineralization process and optimizing remineralization than its antibacterial activity.[1,4,6] Thus, the presence of an antibacterial monomer in adhesive systems, such as 12-methacryloyloxydodecylpyridinium bromide (MDPB), may have an antibacterial effect and help the fluoride ions to prevent caries formation, by avoiding the bacterial invasion through the tooth/restoration gap. MDPB is a compound of the antibacterial agent of quaternary ammonium and a methacryloyl group, which is covalently bound to polymer matrix by its copolymerization with other monomers.[7] This monomer has shown antibacterial effect against oral streptococci, lactobacilli and microorganisms clinically isolated from root caries.[8,9]

Caries inhibition by restorative materials has been evaluated by the formation of an inhibition zone or acid-base resistant zone (IZ).[1,10,11] The inhibition zone is created along the interface, between adhesive and dentin, by fluoride ions released from adhesive systems after the cariogenic challenge,[1,3,11,12] and all studies have used sound dentin in this evaluation. Microscopically, this zone showed similar birefringence and radiopacity to that of sound dentin.[10,13] Tsuchiya

et al.[11] described the formation of an acid-base resistant zone after acid-base challenge in the same area as the inhibition zone. This zone is created adjacent to the adhesive layer by adhesive systems with or without fluoride, and it is partly related to the amount of fluoride released from fluoride-containing adhesives, but mainly to the quality of the hybrid layer.[11] Therefore, IZ formation demonstrates an attempt to inhibit caries formation induced by materials, although it is not able to state the amount of mineral loss, which has not been extensively investigated.[14] It is important to establish the degree and site of mineral loss in dentin along the margins of restorations after cariogenic challenge, in order to evaluate the potential of each adhesive system to prevent marginal dentin demineralization, by a quantitative or semi-quantitative method..

Fluoride/MDPB-containing adhesive systems have shown IZ formation after chemical challenge with acidic solutions.[2,4] However, due to the antibacterial effect of MDPB, the biological caries method would be more suitable to investigate the inhibition of secondary caries formation by fluoride/MDPB-containing adhesive systems.

Confocal laser scanning microscopy (CLSM) is a non-destructive method to evaluate IZ formation and the samples can be morphologically observed after a single cutting and polishing, thus avoiding technical artifacts.[13] The X-ray fluorescence spectrometer by energy-dispersive is also a non-destructive analytical technique to analyze tooth mineral content. The fluorescent radiation emitted by elements due to X-ray radiation is proportional to their content in a tissue.[15]

The aim of this study was to evaluate (1) the depth of outer lesion and IZ formation by a self-etching fluoride/MDPB-containing adhesive system and a total-etch adhesive system without fluoride/MDPB after chemical and biological artificial caries methods using CLSM; (2) the distribution of calcium (Ca) and phosphorus (P) in the inhibition zone by X-Ray fluorescence analysis. The null hypothesis

tested was that the IZ formation and distribution of Ca and P in this zone do not differ between the two adhesive systems and artificial caries methods.

Materials and Methods

2.1. Specimens preparation

This study was conducted under the protocol approved by the Ethics Committee of Piracicaba Dental School - University of Campinas. Forty eight sound third molars were selected for this study. Teeth were stored in 0.1% thymol at 4ºC and used within 1 month after extraction. The occlusal enamel was sectioned perpendicular to the long axis of the tooth using a low-speed diamond saw under water cooling (Isomet, Buehler Ltd, Lake Bluff, IL, USA) to expose adjacent dentin. The dentin surface was wet ground flat with 180 and 400 grit silicon carbide abrasive paper (Saint-Gobain Ltda, São Paulo, SP, Brazil) until all enamel had been removed. Samples were covered with acid-resistant nail varnish (Colorama, CEIL Ltda., São Paulo, SP, Brazil) leaving an uncoated window measuring 4 x 4 mm in the center of the dentin surface for caries development.

2.2. Artificial caries development and restoration

Samples were fixed in the lids of glass container, using orthodontic wire, and sterilized in a gamma irradiation chamber (Gammacell 220 Excel, GC-220E; MDS Nordion, Ottowa, Canada).[16] After this, they were transferred to another glass container containing 40 mL of brain heart infusion broth (BHI) (Becton Dickinson and Company, Sparks, MD 21152, USA), supplemented with yeast extract (Himedia Laboratories PVT Ltd., Mumbai, India), 0.5% glucose (Synth, LabSynth, Diadema, SP, Brazil), 1% sucrose (Synth, LabSynth, Diadema, SP, Brazil) and 2% *Streptococcus mutans*.[17] *S. mutans* UA159 was used in this study.[18] To prepare the inoculum, this microorganism strain was obtained from the culture collection of the Department of Microbiology and Immunology, Dental School of Piracicaba, and

grown on Mitis salivarius agar plates at 37ºC for 48 h in a 5% supplemented CO_2 environment. Subsequently, single colonies were inoculated into 5 mL of BHI broth and incubated at 37ºC for 24 h. An overnight culture was adjusted to 1 x 10^6 (colony forming units (CFU)/mL) with an optical density of 0.6 at 600 nm. Then 800 µL of bacterial suspension was inoculated into the culture medium only on the first day, but the medium was renewed every 48 h for 14 days.[17] Every 48 h, the lack of contamination and purity of the medium were verified using Gram staining and plating methods.

The carious tissue was removed with cylindrical tungsten carbide burs (#57 KG Sorensen, Barueri, SP, Brazil, lot 01/0105) in a slow-speed handpiece. The depth of each cavity was standardized at 2.0 mm. A new bur was used for every five cavity preparations. The prepared samples were randomly divided into two groups according to the adhesive system used (n=24): (1) Adper Scotchbond Multi-Purpose etch-and-rinse adhesive system (SBM) (3M-ESPE, St Paul, MN, USA) and (2) Clearfil Protect Bond self-etching adhesive system (CPB) (Kuraray, Okayama, Japan). The adhesive systems were applied according to the manufacturers' instructions (Table 1) and a non-fluoridated composite resin was inserted in a single increment (TPH Spectrum, Dentsply Caulk, Milford, DE, USA Lot No 317309) and light cure for 20s with a quartz-tungsten-halogen light-curing unit at 550 mW/cm^2 (Tri-Light, 3M/ESPE, St Paul, MN, USA). Samples were stored in a humid environment at 37ºC for 24 h.[19] Subsequently, the restorations were polished with aluminum-oxide abrasive disks of medium, fine, and superfine- grit (Sof-Lex, 3M/ESPE, St Paul, MN, USA, Lot No. 2380B). Each sample was analyzed using a stereomicroscope at 40X magnification to ensure that there was no excess material overlying the restoration/tooth interface. Two layers of acid-resistant nail varnish were applied 1 mm away from the restoration margins, leaving a total area of 36.0 mm^2 of the restorations margins exposed to the caries challenges. All teeth were stored in 100% relative humidity and sterilized in a

gamma irradiation chamber (Gammacell 220 Excel, GC-220E; MDS Nordion, Ottowa, Canada).[16]

2.3. Biological and Chemical Caries challenges

The groups were randomly divided according to the caries challenge and adhesive systems used (n=12): (1) SBM and chemical challenge (SC); (2) SBM and biological challenge (SB); (3) CPB and chemical challenge (CC); (4) CPB and biological challenge (CB).

2.3.1. Chemical caries challenge

Samples fixed in the lids of glass container with orthodontic wire were immersed in 5 mL of sodium carboxymethylcellulose acid gel made up with 0.1M lactic acid titrated with a concentrated KOH solution at pH 4.0 at 37ºC without renewal[20] for 5 days.

2.3.2. Biological caries challenge

Samples fixed in the lids of glass container with orthodontic wire were immersed in 40 mL of BHI broth (Becton Dickinson and Company, Sparks, MD 21152, USA) with 1% sucrose.[20] Then 800 µL of *S. mutans* suspension, prepared as describe before, was inoculated into the culture medium only on the first day, but the medium was renewed every 24 h for 5 days. Every 24 h, the lack of contamination and purity of the medium were verified using Gram staining and plating methods.

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After the caries challenges, samples were rinsed thoroughly with water. The roots were sectioned and discarded and the pulp chamber was filled with composite resin (TPH Spectrum, Dentsply Caulk, Milford, DE, USA). Samples were embedded in epoxy resin (Buehler, Buehler Ltd, Lake Bluff, IL, USA) and a cut was made through the center of each restoration, parallel to the long axis of the tooth, using a diamond sectioning saw (Buehler, Buehler Ltd, Lake Bluff, IL, USA). The

cut surfaces of the samples were serially polished with 400, 600, 1200 and 2000 grit silicon carbide abrasive paper and with a 1 µm diamond paste (Metadi Buehler, Buehler Ltd, Lake Bluff, IL, USA). After this, 0.5 mm thick slices of polished surface were obtained, using a diamond sectioning saw, for CLSM and X-Ray fluorescence analyses.

2.4. CLSM analysis

The IZ formation and outer lesion depth were analyzed by CLSM (LSM510 META, Zeiss, Germany). The Argon gas laser source was used with an excitation band at 488 nm wavelength and a 505 nm long pass filter to detect the autofluorescence of sound and carious dentin.[17] Histotomographic images (parallel to the occlusal surface) were recorded on the sample surface in scan mode plane using 40X objective lenses. The resulting sets of confocal optical sections were collected by LSM image browser software as stacks of images and 3-D images were obtained. Scans were taken in 8 bits at a resolution of 512 by 512 pixels. Standard settings for contrast, brightness and laser power were used for all images. When the IZ was present, its thickness was measured using LSM image browser software. The depth of the outer lesion was measured at a distance of 100 µm from the restoration margin[21] and the results were analyzed by two-way ANOVA and Tukey's test at a 5% level of confidence.

2.5. X-Ray Fluorescence Analysis

Semi-quantitative elemental analyses of calcium (Ca) and phosphorus (P) were carried out by a micro X-ray fluorescence spectrometer by energy-dispersive (µ-EDX 1300, Shimadzu, Kyoto, Japan), equipped with a rhodium X-ray tube and a Si (Li) detector cooled by liquid nitrogen (N_2). The equipment was coupled to a computer system for data processing. The energy range of scans was from 0.0 to

40.0 eV. The voltage in the tube was set at 50 kV, with automatic current adjustment. The analyses of Ca and P characteristic emissions were taken longitudinally on the sample surfaces, with incident beam diameter of 50 μm. The line mapping was taken using 40 x 1 points with a step of 10 μm along the dentin/restoration interface of each sample. Consequently, the analysis was performed in a line 400 μm long and 50 μm thick. The scans were performed with a count rate of 50 s per point (live time) and a dead time of 25%.

The equipment was adjusted using a certified commercial reagent of stoichiometric hydroxyapatite (Aldrich, synthetic $Ca_{10}(PO_4)_6(OH)_2$, grade 99.999%, lot 10818HA) as reference. The measurements were collected using the fundamental parameters of characteristic X-ray emission of the elements Ca and P. The elements O and H were used as chemical balance. The reference was also used as a point in the intensity curve calibration. The energy calibration was performed using internal standards for light elements.

The Ca and P content profiles were expressed in % weight (wt) as a function of depth (μm) for each group. The measured points of Ca and P profiles located along the interface were linear fitted (from 50 to 300 μm). The fitting equation gives the angular coefficient, β of each sample profile, enabling the mineral loss among the groups to be compared. The angular coefficient is determined by the line slope to depth axis. The higher is the line sloping, the higher is the angular coefficient β. Consequently, the higher β is, the shorter is the interval length of mineral loss at the interface. The interval of points was selected using the region where the depletion of Ca and P content effectively occurred. The points of Ca and P content located from 0 to 50 μm were not included in the statistical analysis because their values were close to zero. The points of Ca and P content from 320 to 380 μm were considered in sound dentin due to the minimal variation of mineral content. The Ca/P rate was performed using mineral stoichiometry and the one-way ANOVA test at a 95% confidence level. Tukey-Kramer Multiple Comparisons were

used to evaluate the significance of the mineral content variability of the substrate among groups.

Results

The outer lesion depths are shown in Table 2. There were statistically significant differences between CC and SB, CC and CB groups. Among the other groups there were no statistical differences. The CC group showed the lowest outer lesion depth (224.8 ± 1.5 μm) (Table 2).

Figure 1 shows the inhibition zone and outer lesion formations by CLSM. Only for SB group the IZ formation was unclear. The outer lesions of secondary caries in Figure 1A, B and D were removed when the samples were cut. The frequency (%) of IZ formation for each group and the mean values of IZ thickness are shown in Table 2.

The Ca and P content profiles (% wt) as a function of depth (μm) for each group are shown in Figure 2. The Ca and P contents of all samples were strongly affected by the treatments. The biological caries method showed changes in the angular coefficient for the Ca demineralization profile (Figure 2A), irrespective of the adhesive system used. These changes were slighter for P (Figure 2B) than for the Ca demineralization profile. Figures 2C and D show the linear fitting of the Ca and P profiles at the interface to demonstrate the mineral loss among all groups. CC group showed the highest angular coefficient for both Ca and P content (β = 113.54 and 49.41, respectively) and, thus, the lowest mineral loss. Whereas, the CB group showed the lowest angular coefficient for both Ca and P content (β = 101.67 and 44.55, respectively) and therefore, the highest mineral loss. SM and SG groups showed intermediate values of angular coefficient for Ca (β = 104.77 and 108.02, respectively) and P (β = 47.28 and 46.79, respectively).

The Ca/P rate in sound dentin was shown in Figure 3. There were no differences in Ca/P molar rate among the groups, indicating that the degree of mineral content of sound regions was similar for all groups.

Discussion

In this study initial caries lesions were developed in order to simulate the operative procedure of caries removal that happens in the clinical situation, since the restorations are often placed not in sound but in affected dentin. In addition, the area (10.000 μm^2) to evaluate the mineral distribution along the interface by μ-EDX analysis was selected because the fluoride ions released from adhesive systems can diffuse into dentin up to 30 µm from the interface,[2] the IZ thickness created by fluoride adhesive systems ranges from 1 to 17 µm thickness[1,3,4] and the monomer MDPB does not leach out from the material,[22] thus its antibacterial effect can be restricted to the interface.

The results of the present study showed that the IZ formation and its mineral distribution were influenced by artificial caries methods and adhesive systems. As there was no difference of Ca/P content in sound dentin among groups (Figure 3), the dentin substrate was standardized to compare the effects of fluoride and MDPB on caries inhibition. Although the CLSM showed IZ formation by the fluoride/MDPB-containing adhesive system in both caries methods (Figures 1A and B), the μ-EDX analysis showed that this adhesive system had the highest mineral loss when submitted to the biological caries method and the lowest mineral loss when submitted to the chemical caries method (Figures 2C and D). These results were probably due to the gel not being renewed. The non-renewal of gel method was previously used by others studies for secondary caries formation.[20,23] Thus, close to dentin/restoration surface, the gel may have been saturated with minerals released from dentin. In addition the fluoride ions released by adhesive system may have reacted with Ca ions released from dentin, forming calcium fluoride

(CaF_2), which may have been deposited on the dentin surface[24] and led to protection against demineralization. This fact would confirm the lower outer lesion depth (Table 2), lowest mineral loss (Figures 2C and D) and IZ formation by CLSM analysis (Figure 1A) for CC group. Otherwise, the biological method is dynamic due to the exchange of bacterial medium every 24 h for 5 days. The medium exchange removed the fluoride and Ca/P ions released; this may have created an unsaturated environment surrounding the dentin/restoration, and the fluoride ions released were not sufficient to produce any protective effect.[24] Consequently, the mineral loss was higher and IZ was thinner than that found in chemical caries formation method (Figures 1A, 1B, 2C and 2D).

The low pH of the medium in both caries methods caused dentin demineralization. However, in the biological method the *S. mutans* metabolism may have helped the caries to develop with a more aggressive pattern than in the chemical method due to several physiological factors of *S. mutans*. Exopolysaccharides synthesis increases the porosity and cariogenic potential of biofilm on the dentin/restoration surface[25] and the collagenolytic and gelatinolytic activities of *S. mutans* can contribute to caries progression by collagen degradation.[26] In addition to the change of medium, these factors could be related to the higher mineral loss of CB group (Figures 2C and D). Possibly, the biological caries method caused high demineralization and degradation of dentin and the fluoride ions and MDPB monomer did not exert their cariogenic effects. Thus, the micromechanical attachment of adhesive to dentin had more influence on the formation of IZ and the mineral loss than the effects of fluoride and MDPB.

IZ formation by non-fluoridated adhesive systems has previously been reported by other studies.[1,4,6,12,21] These zones were associated with the hybrid layer created by the adhesive system, since the hybrid layer is acid resistant.[4,27] Furthermore, the monomer penetrating deeper than hybrid layer helped the formation of the acid-base resistant zone.[4,11] The acid resistance of the hybrid layer may be associated with the quality of this layer,[11] which involves several factors

related to the hydrolytic degradation of resin-dentin bonding.[28-30] de Munck et al.[31] reported that three-step ethanol-water-based etch-and-rinse adhesives, such as SBM, showed the formation of separate coupling resin-layer and had lower hydrophilicity compared with two-step etch-and-rinse and self-etching adhesive systems that had an influence on lower bonding degradation.

In this study the effect of MDPB could not be investigated alone, because fluoride is also a component of this adhesive system. However, the antibacterial effect of fluoride has not been much related to its cariostatic effect, because the fluoride concentrations released from restorations in vivo are not high enough to affect the metabolism of caries-associated bacteria.[32,33] The MDPB action mechanism is based on the contact of immobilized antibacterial molecules with the bacteria, because MDPB copolymerizes with other monomers after curing and the antibacterial agent is covalently bonded to the polymer network, thus MDPB is not leached out from the material.[22] Because of this action mechanism, the antibacterial effect of MDPB on preventing caries formation is restricted to the margin of restorations involving the first micrometers of dentin along the interface.

Although the highest mineral loss of the CB group may have been related to the dynamic mechanism of the biological caries method as regards fluoride action, the antibacterial effect of MDPB was not enough against *S. mutans* to prevent demineralization along the interface, even with IZ formation in this group, because μ-EDX analysis showed values of Ca and P content close zero up to 50 μm depth along the interface (Figures 2A and B). This fact occurred to all groups of this study, regardless of adhesive system or caries method used. This occurred in all the groups of this study, irrespective of adhesive system or caries method used. Although some protection occurred along the interface, such as IZ formation, the 50 μm deep demineralization site created a path for the invasion of fluids and bacteria, increasing the risk of restoration failure. Therefore, the evaluation of mineral distribution analysis in the area of IZ formation provides additional information about the caries inhibition potential of restorative materials.

In addition to the results of mineral content degree, μ-EDX analysis was also important, because the open V-shaped notches at the margins of the restorations, as shown in the SB group by CLSM (Figure 1D), can lead to inaccurate evaluation of IZ formation.[21] These notches were also demonstrated in previous studies[1,21,23] and were caused by dissolution of mineral crystallites and shrinkage of the residual collagen matrix by unavoidable dehydration of the artificial lesions during the sample preparation procedures.[21,23]

The adhesive systems used in the present study, with or without fluoride and MDPB, were not able to inhibit the development of secondary caries on dentin surfaces under the conditions investigated. The fluoride-containing adhesive systems associated with fluoridated composite resin or compomer have shown better results in the inhibition of secondary caries formation,[12,14,21] because the IZ formation occurs according to amount of fluoride released by restorative materials.[1,3,12,21] *In situ* or *in vivo* investigations are necessary to evaluate the effect of fluoride/MDPB-containing adhesive systems against secondary caries formation, because the intra-oral condition differs from the artificial caries methods used by studies, and these methods can influence the results. Based on the results, the null hypothesis was rejected because there were differences in the IZ formation and mineral distribution along dentin/restoration interface between the two adhesive systems and artificial caries methods used.

Conclusions

Dentin demineralization along the dentin/restoration interface was affected by the artificial caries method and adhesive systems used. The fluoride and antibacterial monomer of adhesive system did not prevent secondary caries formation.

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Table 1. Main components and application mode of adhesive systems used in the study

Materials	Composition (batch number)	Application mode
Adper Scotchbond Multi-Purpose (3M-ESPE, St Paul, MN, USA)	*Acid Conditioner:* 37% Phosphoric acid (7523 5FB) *Primer:* water, ethanol, HEMA, copolymer of polyalkanoic acid (3008) *Bond:* Bis-GMA, HEMA, dimethacrylate, initiators (7583)	Apply *acid conditioner* for 15s, rise for 15s, dry gently for 2s. Leave moist. Apply *primer*, dry gently for 5s. Apply *bond*, light cure for 10s.
Clearfil Protect Bond (Kuraray, Okayama Japan)	*Primer:* 10-MDP, MDPB, HEMA, water (00017A) *Bond:* 10-MDP, Bis-GMA, HEMA, hydrophobic methacrylate, dl-Camphorquinone, N,N-Diethanol-p-toluidine, silanated colloidal silica, surface-treated sodium fluoride (00027A)	Apply *primer* for 20s. Mild air stream. Apply *bond*. Gentle air stream. Light cure for 10s.

10-MDP: 10-methacryloxydecyl dihydrogen phosphate; HEMA: 2-hydroxyethyl methacrylate; MDPB: 12-methacryloyloxydodecylpyridinium; Bis-GMA: bis-phenol A diglycidylmethacrylate.

Table 2. The depth of outer lesion, thickness and frequency of IZ formation for all groups.

Groups	Outer lesion (μm)*	IZ thickness (μm)	IZ formation (%)
CB	253.8 ± 3.9 ^{A}	4.8 ± 2.3	50
SB	255.4 ± 2.7 ^{A}	5.6 ± 2.2	16.7
CC	224.8 ± 1.5 ^{B}	7.7 ± 1.3	75
SC	243.3 ± 0.7 ^{A,B}	6.0 ± 1.8	50

* mean ± standard deviation. Means followed by the same upper cases did not showed statistically significant difference according to Tukey's test (p>0.05).

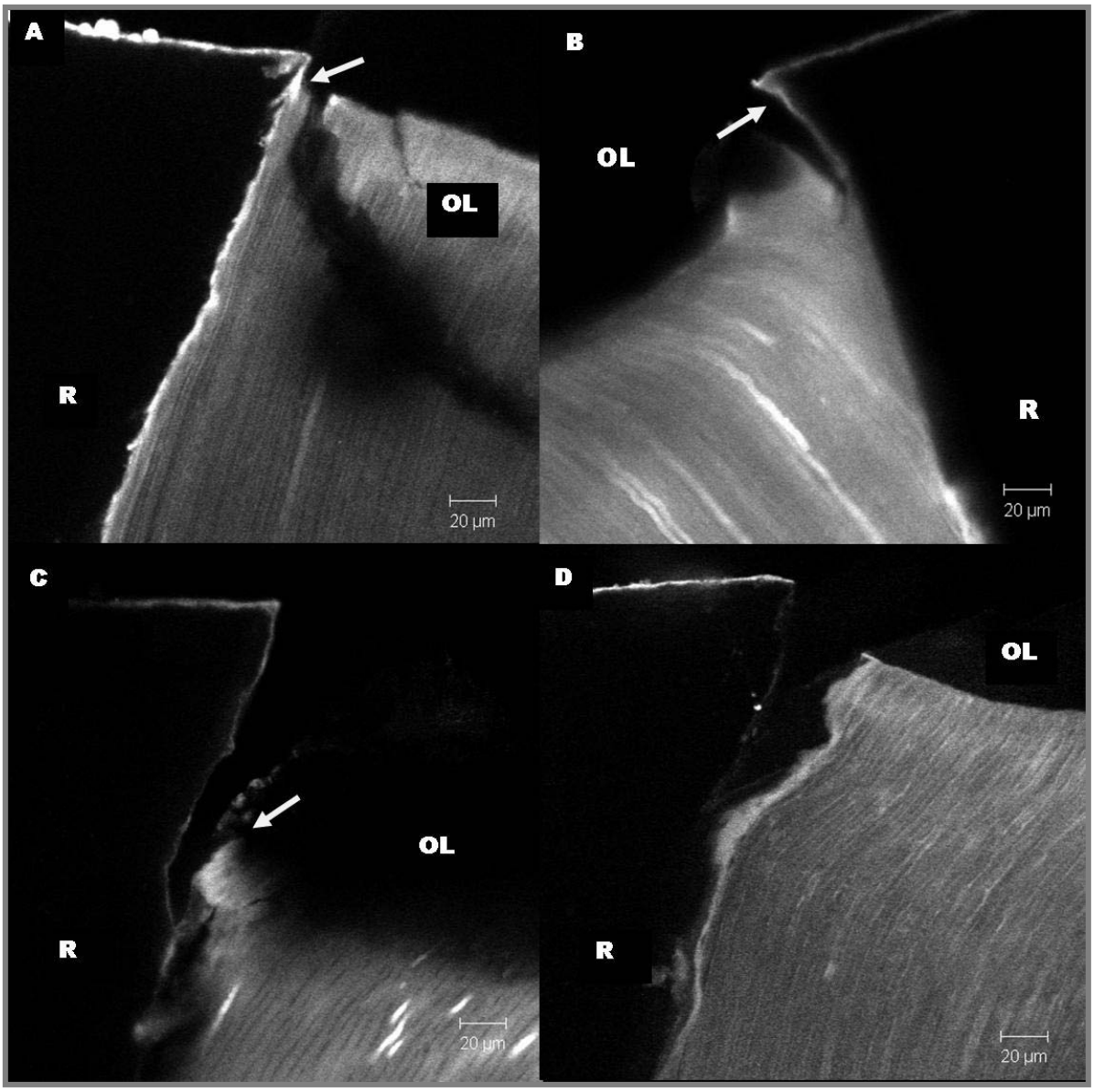

Figure 1. CLSM images of inhibition/acid-base zone (IZ) formation by adhesive systems after biological or chemical artificial challenge. (A) CC group; (B) CB group; (C) SC group; (D) SB group. The IZ formation was observed in CC, CB and SC groups, but it is unclear for SB group. OL: outer lesion; R: restoration; → indicate the IZ formation. (40X magnification).

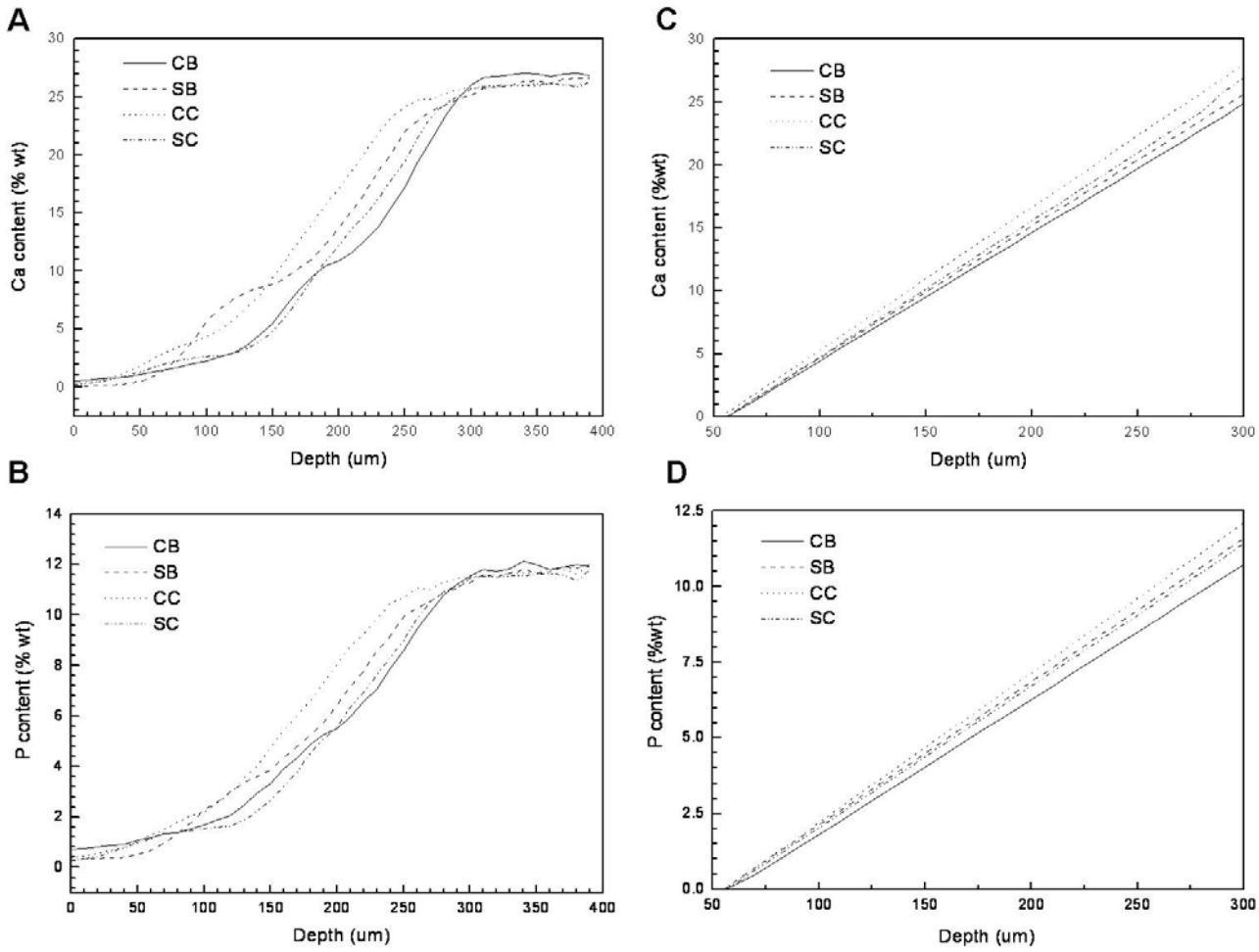

Figure 2. (A) Ca content profile in all groups (% wt as a function of depth in μm). **(B)** P content profile in all groups (% wt as a function of depth in μm). **(C)** Ca content (% wt) linear fitting as a function of depth (μm). **(D)** P content (% wt) linear fitting as a function of depth (μm). **(C,D)** The mineral loss was represented by angular coefficient, *β*, which is linked to the line sloping. CC showed the lower and CB the highest mineral loss. SC and SB showed intermediate mineral loss.

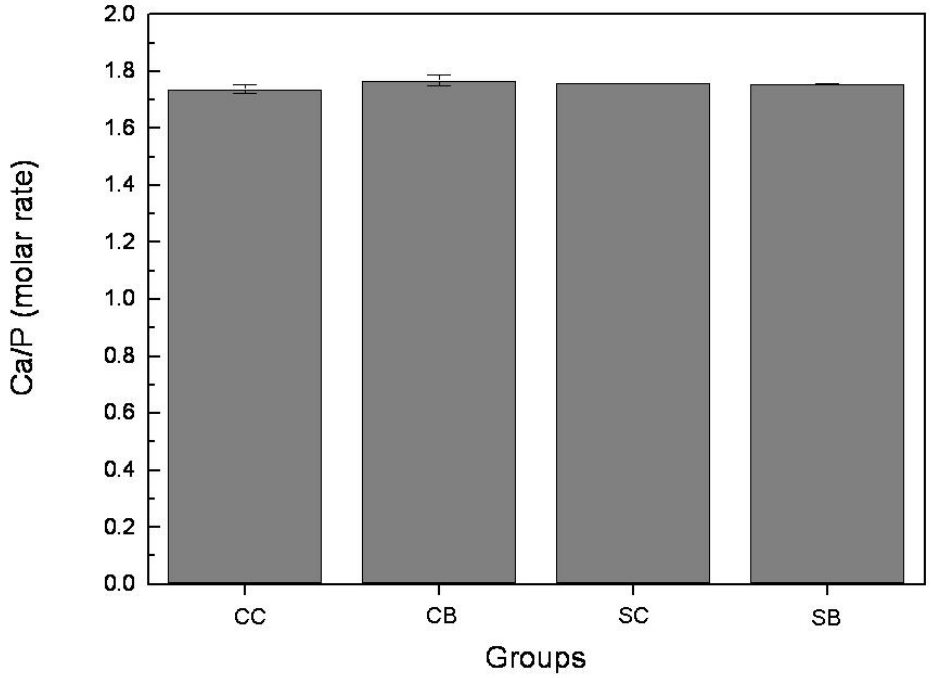

Figure 3. Graph of Ca/P molar rate in sound dentin from 320 to 380 μm depth. There was no statistical difference among the groups by Tukey's test (p>0.05).

CONSIDERAÇÕES GERAIS

Componentes com propriedades cariostáticas, como flúor e monômero antibacteriano (MDPB), foram incorporados aos sistemas adesivos para auxiliar na prevenção à cárie, já que a formação de cárie secundária ao longo das margens das restaurações é ainda a principal causa de substituições das mesmas (Fontana *et al.*, 2000; Tyas *et al*., 2005). O sistema adesivo contendo flúor e MDPB, comercialmente denominado Clearfil Protect Bond - CPB (Kuraray, Okayama, Japão), foi utilizado neste estudo em três experimentos *in vitro* para testar o efeito desses componentes na durabilidade da união à dentina afetada por cárie, prevenção de cárie secundária e desinfecção de cavidade.

O monômero MDPB foi considerado antibacteriano por reduzir o número de bactérias viáveis após a sua aplicação (Imazato *et al.,* 1997; Imazato *et al.,* 1998; Imazato *et al.*, 2006). Porém, Imazato *et al.* (2008) verificaram que o MDPB possuiu efeito bactericida em células planctônicas utilizando método de viabilidade bacteriana com indicadores fluorescentes. Do mesmo modo, os resultados do Capítulo 1 demonstraram que o MDPB possuiu atividade bactericida quando aplicado sobre biofilme de *S. mutans* na dentina. O efeito bactericida do monômero causou a ruptura da membrana das células bacterianas, a qual foi visualizada em CLSM pelo corante Live:Dead Baclight (Molecular Probes, OR, USA). Entretanto, o efeito bactericida do MDPB em biofilme de *S. mutans* sobre a dentina foi tardio (após 300s de sua aplicação) comparado ao tempo de aplicação do primer recomendado pelo fabricante (20s). A presença de polissacarídeos extracelulares no biofilme pode ter influenciado no tempo de ação do monômero. Este capítulo demonstrou que o primer contendo MDPB, sem posterior aplicação do adesivo, diminuiu o número de bactérias viáveis aderidas na dentina, mesmo em alta densidade bacteriana, podendo ser utilizado como desinfetante de cavidade. Porém, como o tempo de aplicação de 20s foi testado principalmente em bactérias planctônicas (Imazato *et al.*, 1997; Imazato *et al.*, 2001; Ozer *et al.*, 2005), estudos ainda são necessários para avaliar o tempo de aplicação do

primer, viável clinicamente, para que o MDPB tenha efeito bactericida em bactérias presentes na dentina.

Como o monômero MDPB é bactericida, a presença de bactérias torna-se indispensável nos experimentos *in vitro* para testar este efeito. Até o presente momento, estudos na literatura não tinham avaliado o efeito bactericida do MDPB na degradação da união à dentina, bem como na inibição de cárie secundária em meios contendo bactérias. Para este fim, o meio cariogênico com *S. mutans* foi utilizado nos Capítulos 2 e 3. No Capítulo 2, o período de 3 dias de armazenamento dos espécimes em meio contendo *S. mutans* demonstrou ser tão efetivo na degradação da união à dentina afetada por cárie quanto o armazenamento em água deionizada por 3 meses. No Capítulo 3, foi sugerido que o meio com *S. mutans* (método biológico) seria mais "agressivo" que o gel ácido (método químico) para o desenvolvimento de cárie secundária, porque o método biológico foi dinâmico, com trocas periódicas do meio, e possuiu a presença de bactérias que auxiliaram na formação de cárie. O mesmo período de formação de cárie artificial (5 dias) foi utilizado nos dois métodos, simulando um alto desafio de cárie, para comparação dos efeitos do flúor e MDPB na proteção da desmineralização da dentina. De acordo com os resultados obtidos, foi sugerido que o alto potencial do método biológico em desmineralizar a dentina limitou o efeito cariostático, principalmente, do MDPB na proteção à cárie. Desta forma, poderia ser questionado se o MDPB presente no sistema adesivo não possuiu efeito somente porque foi submetido a um alto desafio de cárie. Entretanto no Capítulo 2, a presença de desmineralização da dentina nos espécimes de microtração submetidos ao método de degradação com *S. mutans,* em um período de desafio menor que o do Capítulo 3 (3 dias), foi uma evidência indireta da ausência de efeito cariostático do MDPB, como também do flúor.

Embora o capítulo 1 deste trabalho tenha demonstrado que o MDPB possuiu efeito bactericida quando o primer foi utilizado, nos capítulos 2 e 3 este efeito, bem como o efeito cariostático do flúor, não influenciaram diretamente na

durabilidade da resistência de união à dentina afetada por cárie e na inibição de cárie secundária. De maneira geral, o mecanismo de ação antibacteriano do MDPB e a forma de incorporação do flúor ao sistema adesivo e seu mecanismo de liberação foram os fatores relacionados aos resultados obtidos nestes dois capítulos. O mecanismo de ação do MDPB ocorre por contato do grupo piridínio (agente antibacteriano) com as bactérias (Imazato *et al*., 1995; Imazato *et al*., 1998), entretanto este agente antibacteriano é imobilizado e covalentemente unido à cadeia polimérica após a polimerização (Imazato *et al*., 1998). Assim, o efeito antibacteriano do MDPB é prejudicado após a fotoativação do adesivo, como demonstrado nos estudos de Imazato *et al*. (1995) e Imazato *et al.* (1998), pois o contato do grupo piridínio com a membrana da célula bacteriana fica restrito após a formação da rede de polímeros. Com relação ao flúor, segundo o fabricante, este elemento é incorporado no adesivo na forma de fluoreto de sódio envolvido por uma cápsula semipermeável de polímero. Esta cápsula permanece na matriz resinosa após liberação dos íons fluoreto, impedindo a formação de "porosidades ou falhas" no material, mas poderia ser uma barreira adicional para a liberação dos íons, como sugerido no Capítulo 2. Além disso, os íons fluoretos também poderiam ficar entremeados na matriz resinosa, após a fotoativação, dificultando sua liberação (Toba *et al*., 2003). Em estudo piloto realizado, a liberação de íons fluoreto de amostras de adesivo do CPB foi de 0,287 ppm F^-. Entretanto essa quantidade de fluoretos liberada não pode ser considerada relevante, porque menor quantidade de adesivo é aplicada à dentina durante o procedimento clínico, e uma área menor do adesivo é exposta ao ambiente externo, justamente devido a sua interação com a dentina. Assim, provavelmente menor quantidade de íons fluoreto seja liberada. Por esta razão, Peris *et al*. (2007) reportaram que a liberação de íons fluoreto do sistema adesivo CPB foi menor que 0,03 ppm F^-, após união com a dentina.

Além dos fatores citados anteriormente, os resultados do Capítulo 3 foram principalmente influenciados pelo método artificial de desenvolvimento de cárie e

camada híbrida formada pelos sistemas adesivos. Neste capítulo, o flúor presente no sistema adesivo, após desafio cariogênico com método químico, proporcionou menor perda mineral na dentina ao longo da interface, pois mesmo que tenha sido liberado em pequena quantidade, provavelmente este íon ficou disponível no gel, ao redor da restauração, e pode ter reagido com os íons Ca^{+2} liberados da dentina, formando fluoreto de cálcio (CaF_2) na superfície, como sugerido por Itota *et al.* (2003). Desta forma, menor desmineralização da dentina ocorreu. Diferentemente, a pequena quantidade de flúor liberada pelo sistema adesivo foi removida com as trocas periódicas do meio no método biológico.

A utilização adicional de um método quantitativo ou semi-quantitativo para analisar o potencial de cada adesivo em prevenir a perda mineral foi sugerida neste capítulo. O método mais utilizado neste tipo de estudo é a avaliação da formação da zona de inibição ou ácido-base resistente (IZ) (Itota *et al.,* 2002; Hara *et al.,* 2005; Shinohara *et al.*, 2006), embora a espessura desta zona seja relacionada com maior ou menor proteção à cárie, este é um método de avaliação qualitativo e nenhuma informação é fornecida quanto ao grau e ao sítio da perda mineral. A análise por µ-EDX mostrou que houve diferença na perda mineral entre os materiais, de acordo com os métodos de desenvolvimento de cárie a que foram submetidos. Entretanto, em todos os grupos ocorreu a formação de cárie secundária, sendo mais uma evidência da redução do efeito antibacteriano do MDPB após a fotoativação e da quantidade de flúor presente no sistema adesivo não ser suficiente para impedir a formação de cárie. Embora, estudos relataram que sistemas adesivos fluoretados associados a resinas compostas contendo flúor ou compômeros produziram maior espessura de IZ e menor perda mineral, estes compostos também não inibiram a formação da lesão de cárie (Savarino *et al.*, 2004; Hara *et al.*, 2005).

Clinicamente, mesmo que o sistema adesivo proteja a dentina na interface por meio da formação da IZ e menor perda mineral, a formação da lesão de cárie adjacente às margens da restauração pode ser uma indicação para a sua

substituição. Mesmo que a presença da IZ ou maior conteúdo mineral ao longo das margens das restaurações pudessem protegê-las da desmineralização adicional, foi demonstrado no capítulo 3, que todos os grupos apresentaram completa desmineralização da dentina da superfície externa até 50 μm de profundidade ao longo da interface. Esta área desmineralizada funcionaria como um caminho do meio externo para a interface da restauração, por onde bactérias e fluidos se infiltrariam prejudicando a longevidade da mesma. Adicionalmente, o efeito positivo da liberação de íons fluoreto do sistema adesivo seria encoberto pela infiltração de água necessária para ao processo de dissociação do fluoreto de sódio, influenciando na degradação da união do material/dente com o tempo (Itthagarun *et al.*, 2004).

Experimentos *in vitro* possuem limitações, as quais podem influenciar os resultados, como observado no capítulo 3. Desta forma, estudos *in vivo* ou *in situ* precisam ser realizados para testar o efeito do flúor e MDPB na durabilidade da união, na inibição à cárie e na desinfecção de cavidades.

CONCLUSÃO

Dentro das limitações do presente estudo, as seguintes conclusões foram obtidas:

1. O primer contendo MDPB apresentou efeito bactericida contra biofilme de *S. mutans* na dentina após 300 segundos de aplicação;

2. O flúor e o monômero MDPB presentes no sistema adesivo não preveniram a degradação da união à dentina afetada por cárie após armazenamento dos espécimes em meio contendo *S. mutans* por 3 dias e água deionizada por 3 meses;

3. O período de 3 dias de armazenamento dos espécimes no meio contendo *S. mutans* foi tão efetivo na degradação da união à dentina afetada por cárie quanto o período de 3 meses de armazenamento em água.

4. O método de desenvolvimento de cárie secundária artificial influenciou no efeito do flúor e MDPB, presentes no sistema adesivo, em prevenir a desmineralização da dentina ao longo da interface;

5. Sistemas adesivos com e sem flúor e MDPB não preveniram o desenvolvimento da cárie secundária artificial nas margens das restaurações;

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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 periódicos em conformidade com o Medline.

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ANEXO

1. Declaração do Comitê de Ética e Pesquisa

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

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Desempenho de sistemas de união à dentina decídua e permanente afetada por cárie quando submetidos ao desafio cariogênico. Análise mecânica e micromorfológica**", protocolo nº **111/2005**, dos pesquisadores **FABIOLA GALBIATTI DE CARVALHO 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 28/10/2005.

The Research Ethics Committee of the School of Dentistry of Piracicaba - State University of Campinas, certify that project "**Performance of adhesive systems in primary and permanent affected caries dentin submitted to caries challenge. Mechanical and micro-morphological analysis**", register number **111/2005**, of **FABIOLA GALBIATTI DE CARVALHO and REGINA MARIA PUPPIN RONTANI**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for researching in human subjects and was approved by this committee at 28/10/2005.

Cinthia Pereira Machado Tabchoury
Secretária
CEP/FOP/UNICAMP

Jacks Jorge Júnior
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.

2. Materiais e Métodos - Capítulo 1

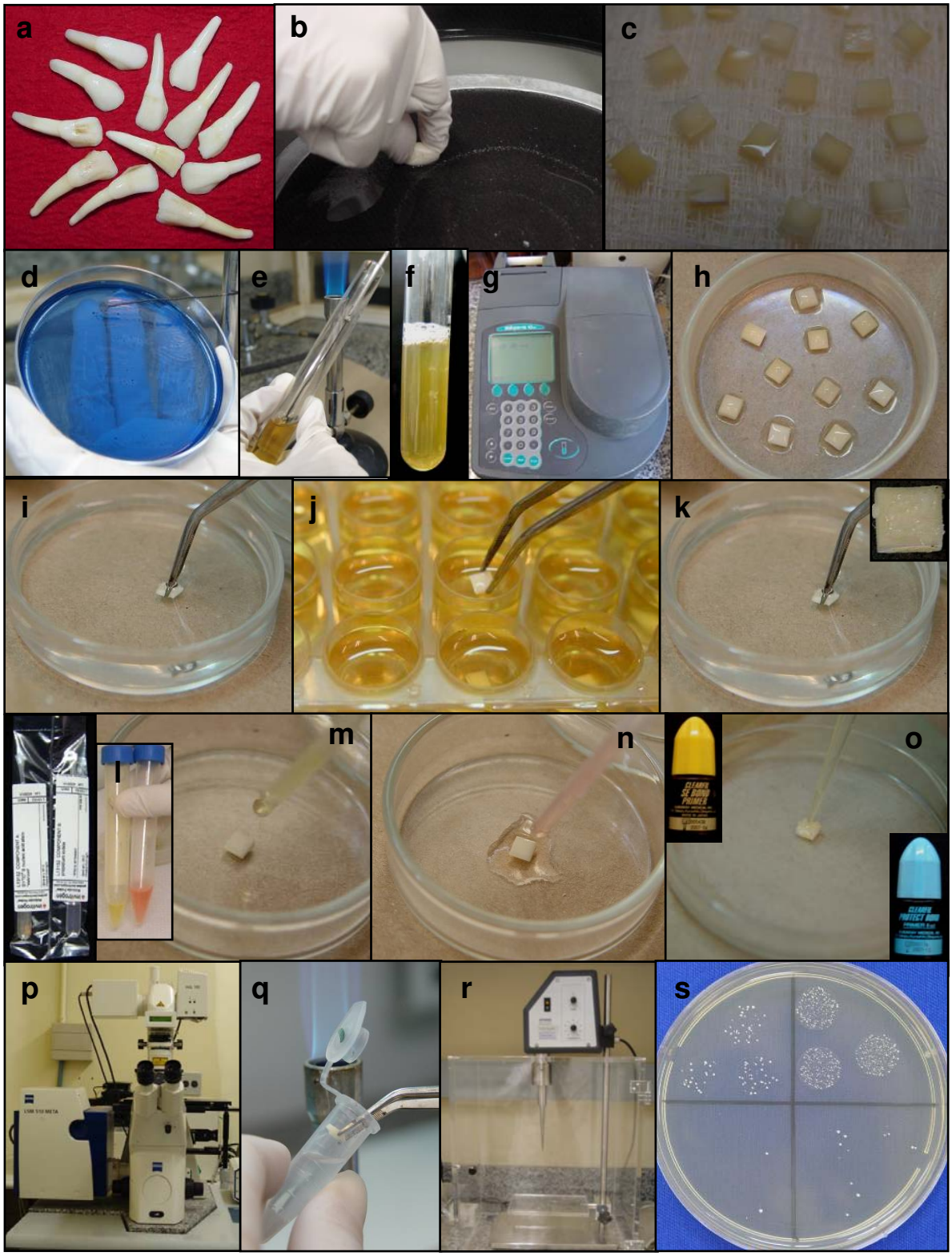

Figura 1. Metodologia utilizada no Capítulo 1. **a.** incisivos bovinos utilizados no estudo; **b.** remoção do esmalte e exposição da dentina subjacente; **c.** obtenção de blocos de dentina de cada dente medindo 4 x 4 x 1,5 mm, os quais foram posteriormente autoclavados; **d, e, f.** preparo do inóculo de *S. mutans* UA 159; **d.** microorganismo semeado em meio de cultura específico (MSA) por 48h; **e.** colônias inoculadas em 5 mL BHI por 24h; **f.** inóculo de *S. mutans*; **g.** análise da densidade óptica (do) em espectrofotômetro (do=0.6 em 600 nm de comprimento de onda); **h.** 20 µL do inóculo depositado sobre a superfície de cada amostra para a aderência bacteriana por 2 h; **i.** remoção das bactérias não aderidas em solução salina; **j.** desenvolvimento do biofilme por meio de imersão de cada amostra em poços contendo 2 mL de BHI com 1% de sacarose durante 18 h; **k.** remoção das bactérias não aderidas em solução salina; biofilme formado na superfície de dentina; **l.** corante de viabilidade celular utilizado (Live/Dead Baclight), preparo do corante segundo o fabricante; **m.** aplicação do corante Syto 9; **n.** aplicação do corante Iodeto de propídeo; **o.** aplicação de 10 µL de solução salina (grupo controle) ou primers dos sistemas adesivos Clearfil SE Bond e Clearfil Protect Bond; **p.** análise em microscópio confocal de varredura a laser (CLSM); **q.** amostras imersas em 1 mL de salina presentes em eppendorfs (após os procedimentos realizados nos itens **a – k**) para realização do teste de contagem de unidade formadoras de colônias (CFU); **r.** sonicador (UP400S) utilizado para deslocar as bactérias aderidas, para posterior realização da diluição seriada e plaqueamento; **s.** placa de BHI após 48h em estufa de 5% CO_2; crescimento das colônias de *S. mutans*.

3. Materiais e Métodos - Capítulo 2

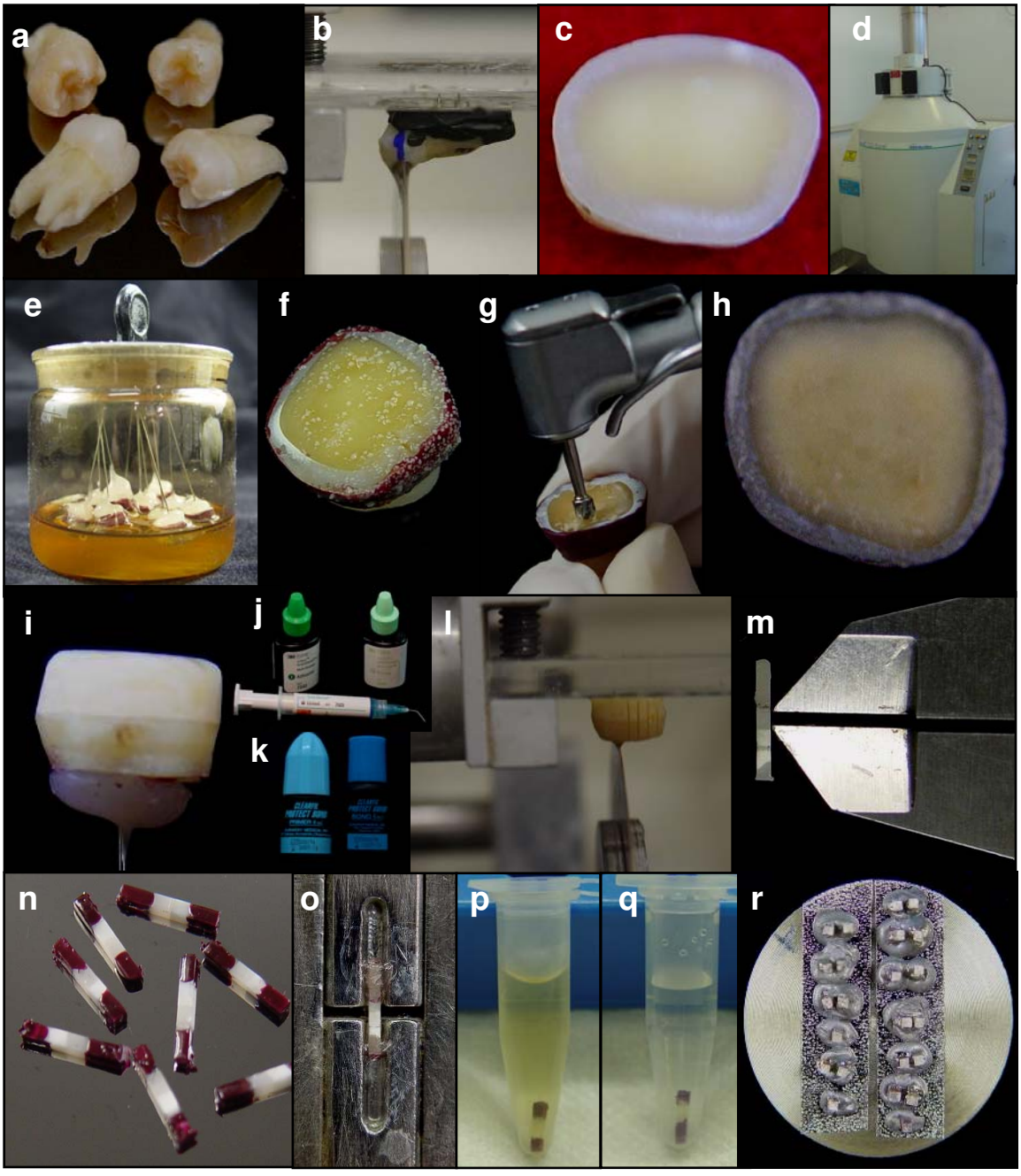

Figura 2. Metodologia utilizada no Capítulo 2. **a.** molares permanentes utilizados no estudo; **b.** remoção do esmalte com disco diamantado e exposição da dentina subjacente; **c.** isolamento da amostra com verniz ácido-

resistente exceto na dentina exposta; **d.** esterilização das amostras por raios-gama; **e.** amostras suspensas em frasco para desenvolvimento da cárie artificial com *S. mutans* UA 159 por 14 dias; **f.** cárie desenvolvida na superfície e biofilme formado; **g.** remoção da dentina infectada artificial com broca esférica carbide em baixa rotação; **h.** dentina afetada por cárie artificial; **i.** bloco de resina composta restaurada na superfície de dentina com os sistemas adesivos Clearfil Protect Bond ou Adper Scothbond Multi-Purpose e resina composta TPH; **j, k.** sistemas adesivos utilizados para restauração; **l.** obtenção dos palitos de 1 mm^2 para o teste de microtração; **m.** área delimitada dos palitos com verniz ácido-resistente para o armazenamento das amostras; **n.** palitos isolados com verniz ácido-resistente a 1 mm da interface; **o, p**, **q.** grupos do estudo segundo o tipo de armazenamento; **o.** grupo controle – microtração imediata; **p.** armazenamento em cultura de *S. mutans* por 3 dias; **q.** armazenamento em água deionizada por 3 meses; **r.** análise do padrão de fratura em microscópio eletrônico de varredura para após o teste de microtração.

4. Materiais e Métodos - Capítulo 3

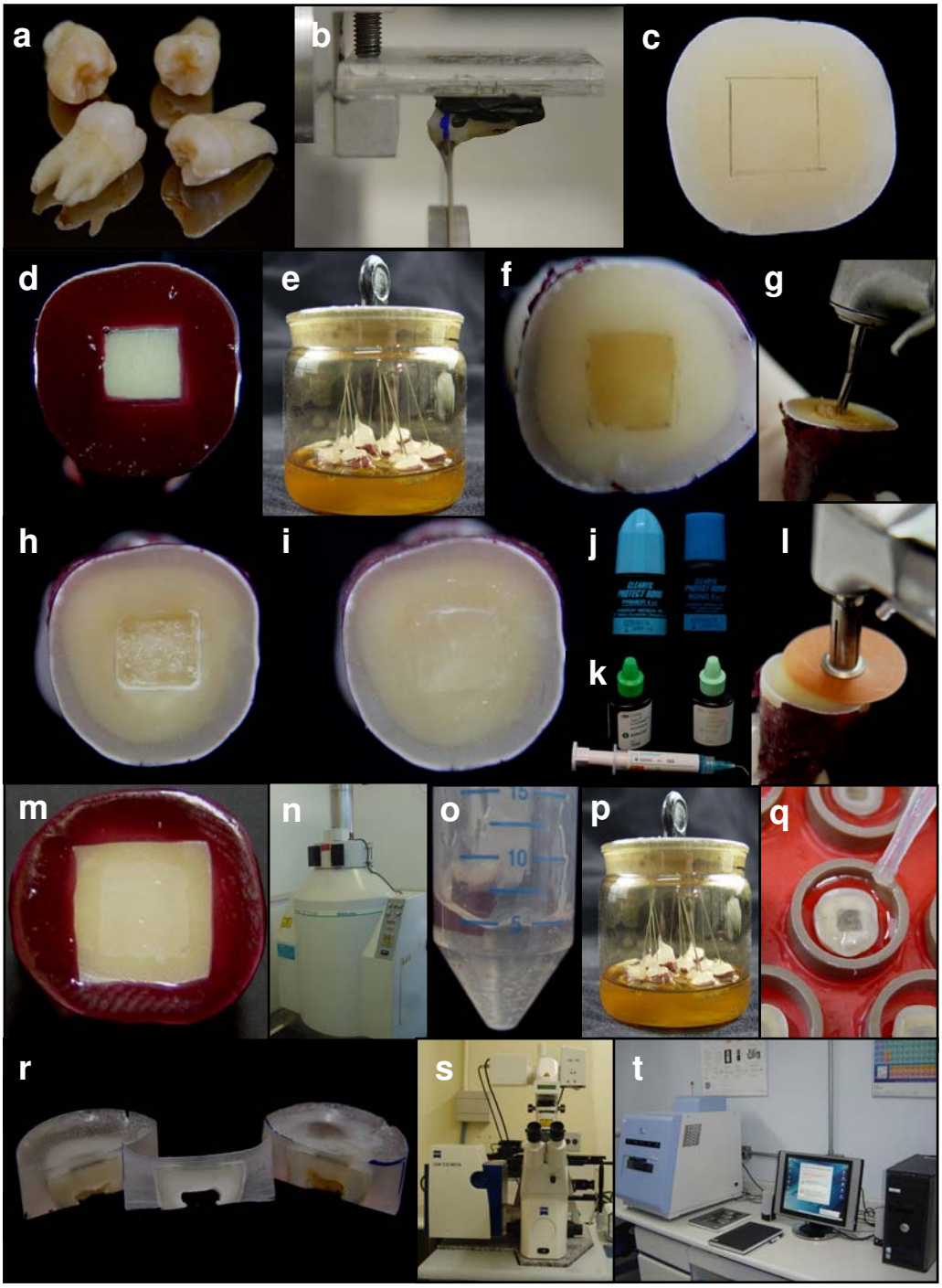

Figura 3. Metodologia utilizada no Capítulo 3. **a.** molares permanentes utilizados no estudo; **b.** remoção do esmalte com disco diamantado e

exposição da dentina subjacente; **c.** delimitação da área de desenvolvimento da cárie artificial na superfície de dentina; **d.** amostras isoladas com verniz ácido-resistente exceto na região delimitada para posterior esterilização por raios-gama; **e.** amostras suspensas em frasco para desenvolvimento da cárie artificial com *S. mutans* UA 159 por 14 dias; **f.** dentina cariada na superfície delimitada; **g.** remoção da dentina infectada artificial com broca carbide em baixa rotação; **h.** cavidade com profundidade em torno de 2 mm; **i.** restauração com os sistemas adesivos Clearfil Protect Bond ou Adper Scothbond Multi-Purpose e resina composta TPH; **j, k.** sistemas adesivos utilizados para restauração; **l.** polimento da restauração com discos Sof-lex; **m.** área delimitada com verniz ácido-resistente a 1 mm da interface para desenvolvimento dos desafios cariogênicos; **n.** esterilização das amostras restauradas por raios-gama; **o.** desafio cariogênico com gel ácido; **p.** desafio cariogênico com *S. mutans*; **q.** embutimento das amostras em resina epóxica; **r.** obtenção de fatia de 0,5 mm da área restaurada e submetida aos desafios cariogênicos; **s.** análise em microscópio confocal de varredura a laser (CLSM); **t.** análise de fluorescência de raios-x (μEDX).

4. Comprovante de envio de trabalho – Artigo do Capítulo 3

Submission Confirmation for Journal of Dentistry

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Kind regards,

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