Chlorhexidine-containing Acid Conditioner Preserves the Longevity of Resin-dentin Bonds

R Stanislawczuk • RC Amaral • C Zander-Grande
D Gagler • A Reis • AD Loguercio

Clinical Relevance

The addition of CHX digluconate in the acidic conditioner may be an excellent tool to increase the stability of collagen fibrils within the hybrid layer against host-derived metaloproteinases without the need for additional steps for the bonding protocol.

SUMMARY

The current study evaluated the effect of 2% chlorhexidine digluconate (CHX) on the immedi- ate and six-month resin-dentin bond strength (BS) and nanoleakage pattern (NL) of etch-and-rinse adhesives when applied in aqueous or associated to the phosphoric acid conditioner. The occlusal enamel of 42 caries-free extracted molars was removed in order to expose a flat dentin surface. In groups 1 and 2 (control–C), the surfaces were acid etched with conventional phosphoric acid, and the adhesives Prime&Bond NT (PB) and Adper Single Bond 2 (SB) were applied after rinsing, drying and rewetting with water. In groups 3 and 4 (Ac/CHX), the adhesives were applied in a similar manner, however, a 2% CHX-containing acid was previously applied. In groups 5 and 6 (CHX), the adhesives were applied according to the control group; however, the rewetting procedure was performed with an aqueous solution of 2% CHX for 60 seconds. Composite buildups (Opallis, FGM) were constructed incrementally, and the specimens were longitudinally sectioned in the "x" and "y" directions to obtain bonded sticks (0.8 mm^2) to be tested in tension at 0.5 mm/minute immediately or after six months of water storage. For NL, two bonded sticks from each tooth were coated with nail varnish, placed in silver nitrate and polished down with SiC paper. Resin-dentin interfaces

Rodrigo Stanislawczuk, DDS, MS, graduate student, Department of Restorative Dentistry, Universidade Estadual de Ponta Grossa, Ponta Grossa, Brazil

Roberto César do Amaral, DDS, MS, professor, Department of Dental Materials and Restorative Dentistry, Universidade do Oeste de Santa Catarina, Joaçaba, Brazil

Christiana Zander-Grande, DDS, MS, graduate student, Department of Restorative Dentistry, Universidade Estadual de Ponta Grossa, Ponta Grossa, Brazil

Diego Gagler, engineer, undergraduate student, Department of Mechanical Enginner, Universidade do Oeste de Santa Catarina, Joaçaba, Brazil

Alessandra Reis, DDS, PhD, adjunctive professor, Department of Restorative Dentistry, Universidade Estadual de Ponta Grossa, Ponta Grossa, Brazil

*Alessandro Dourado Loguercio, DDS, MS, PhD, adjunctive professor, Department of Restorative Dentistry, Universidade Estadual de Ponta Grossa, Ponta Grossa, Brazil

*Reprint request: Rua Carlos Cavalcanti, 4748, Bloco M, Sala 64A–Uvaranas, Ponta Grossa, PR, 84030-900, Brazil; e-mail: aloguercio@hotmail.com

were analyzed by EDX-SEM. The BS and NL data from each adhesive was submitted to two-way repeated measures ANOVA and Tukey's test (α=0.05). After six months of water storage, significant reductions in BS were observed for both adhesives in the control group (p<0.05). When Ac/CHX or CHX was used, no significant reductions in BS were observed for both systems. Nanoleakage was more evident in the control group than in the experimental groups (p<0.05), even after six months. The use of CHX in an aqueous solution or associated with the acid conditioner was effective for reducing degradation of resin-dentin bonds after six months of water storage.

INTRODUCTION

There is a general consensus that resin-dentin bonds created by contemporary hydrophilic dentin adhesives deteriorate over time.[1-2] The premature loss of bond strength is one of the problems that still affects adhesive restorations[3] and markedly reduces their durability.[4-5] The loss of bond strength has primarily been attributed to degradation of the hybrid layer at the dentin-adhesive interface.[6-8]

For etch-and-rinse simplified adhesives, a decreasing gradient of resin monomer diffusion within acid-etched dentin[6] results in incompletely infiltrated zones along the bottom of hybrid layers that contain denuded collagen fibrils.[7] These zones are micromorphologically seen as different modes of nanoleakage within the hybrid layers;[8] they are sites that are susceptible to degradation.[9-10] It has been speculated that this decreasing gradient of resin monomer diffusion within acid-etched dentin and the resin elution from hydrolytically unstable polymeric hydrogels within the hybrid layers[10] leave collagen fibrils unprotected and also vulnerable to degradation by endogenous metaloproteinases (MMPs) in a way that is similar to what occurs in caries progression[11-13] and periodontal disease.[14]

MMPS are a group of 23 mammalian enzymes capable of degrading all extracellular matrix components. Human dentin contains collagenase (MMP-8) and gelatinases (MMP-2 and –9), among others.[12,15-16] This dentin collagenolytic and gelatinolytic activity can be suppressed by protease inhibitors,[17] indicating that MMP inhibition could be beneficial in preservation of the hybrid layers. Therefore, one might consider that preventing the degradation of incomplete resin-infiltrated collagen fibrils by MMPs is an important issue to be investigated, since this could be the key to increasing the durability of restorations that involve bonding to dentin substrate. Several *in vitro* and *in vivo* studies suggested that the application of chlorhexidine (CHX) solution to acid-etched dentin for 60 seconds could maintain the stability of adhesion to dentin over time,

while bond strength significantly decreases in teeth without CHX application and a progressive disintegration of the fibrillar network can be identified.[18-21]

Despite the advantages of using 2% CHX, using it for 60 seconds after acid etching means that another bonding step needs to be accomplished during the restorative procedure, and this is in contrast to the clinician's need for simplification.[22] CHX is a safe, widely used compound in dentistry and can be delivered in different modes and in high and low concentrations for considerable periods of time to reduce the number of microorganisms in plaque and saliva.[23]

Recent studies have examined the use of CHX before, during or after acid-etching, demonstrating initial bond strengths comparable to those obtained without using CHX.[20,24-26] The ideal would be the application of CHX as one part of the bonding component, since it would not add an extra step to the bonding protocol. Therefore, the purpose of the current study was to evaluate the effect of 2% CHX digluconate on immediate and six-month resin-dentin bond strength (BS) and the nanoleakage pattern (NL) of two etch-and-rinse adhesives when applied in aqueous or associated to the phosphoric acid conditioner.

The null hypothesis to be tested is that no significant difference between immediate and six-month bond strengths and nanoleakage will be observed whether or not CHX (in aqueous solution or associated with 37% phosphoric acid) is used prior to adhesive application.

METHODS AND MATERIALS

Teeth Selection and Preparation

Forty-two extracted, caries-free human third molars were used. The teeth were collected after the patients' informed consent. The University of Ponta Grossa Review Board approved this study. The teeth were disinfected in 0.5% chloramine, stored in distilled water and used within six months of extraction. A flat and superficial dentin surface was exposed on each tooth after wet grinding the occlusal enamel on #180-grit SiC paper. The enamel-free, exposed dentin surfaces were further polished on wet #600-grit silicon-carbide paper for 60 seconds to standardize the smear layer.

Restorative Procedure

Two different solvent-based, etch-and-rinse adhesive systems were tested: Adper Single Bond 2 ([SB] 3M ESPE, St Paul, MN, USA), an ethanol-/water-based system and Prime&Bond NT ([PB] Dentsply De Trey, Konztanz, Germany), an acetone-based system (Table 1). Forty two teeth were divided into six groups according to the following description: in groups 1 and 2 (control [C]), the surfaces were acid etched with conventional phosphoric acid and the adhesives PB and SB were applied after rinsing, drying and rewetting with

Table 1: *Adhesive Systems: Composition, Groups and Application Mode*				
Adhesive Systems	**Composition**	**Groups Characteristics**	**Groups**	**Application Mode(*)**
Prime&Bond NT (PB-Dentsply De Trey, Konstanz, Germany)	1 Adhesive–UDMA, Bis-GMA, PENTA, butylated hydroxitoluene, 4-ethyl dimethyl aminobenzoate, cetilamine hydrofluoride, initiator and acetone	Conditioning with 37% phosphoric acid followed by wetting with water	Control	a1, b, c, d1, e, f, g
		Conditioning with 37% phosphoric acid followed by rewetting with 2% aqueous CHX	CHX	a1, b, c, d2, e, f, g
		Conditioning with 37% phosphoric acid containing 2% CHX followed by rewetting with water	Ac/CHX	a2, b, c, d1, e, f, g
Adper Single Bond 2 (SB-3M ESPE, St Paul, MN, USA)	1. Adhesive–Bis-GMA, HEMA, dimethacrylates, silica nanofiller (5 nm), polyalquenoic acid copolymer, initiators, water and ethanol	Conditioning with conventional 37% phosphoric acid followed by rewetting with water	Control	a1, b, c, d1, e, f, g
		Conditioning with 37% conventional acid containing 2% aqueous CHX	CHX	a1, b, c, d2, e, f, g
		Conditioning with 37% phosphoric acid containing 2% CHX followed by rewetting with water	Ac/CHX	a2, b, c, d1, e, f, g
(*) **a1**: *conventional 37% phosphoric acid conditioning (15 seconds);* **a2**: *conditioning with 37% phosphoric acid containing 2% CHX (15 seconds);* b: *rinsing (15 seconds);* c: *air-drying (30 seconds);* **d1**: *– dentin rewetted with water for 60 seconds;* **d2**: *dentin rewetted with 2% CHX for 60 seconds;* **e**: *two coats of adhesive;* **f**: *air-drying for 10 seconds at 20 cm;* **g**: *light-curing (10 seconds–600 mW/cm².)*				
UDMA – urethane dimethacrylate; Bis-GMA: bisphenol A diglycidyl methacrylate; PENTA–dipentaerythritol pentacrylate monophosphate; HEMA: 2-hydroxyethyl methacrylate				

water, according to the directions in Table 1. In groups 3 and 4, the adhesives were applied in a similar manner; however, a 2% chlorhexidine digluconate containing acid (Ac/CHX) was previously applied. In groups 5 and 6 (CHX), the adhesives were applied according to the control group; however, the rewetting procedure was performed with an aqueous solution of 2% CHX for 60 seconds.

The adhesives were light cured with a halogen light set at 600 mW/cm² for 10 seconds (VIP, BISCO, Schaumburg, IL, USA). Resin composite buildups (Opallis, FGM, Joinville, SC, Brazil) were placed on the bonded surfaces (three increments of 1.5 mm each), which were individually light activated for 40 seconds each. All the bonding procedures were carried out by a single operator at 24°C and 50% relative humidity. Seven teeth were used for each experimental group.

Microtensile Testing

After storing the bonded teeth in distilled water at 37°C for 24 hours, they were longitudinally sectioned in both the "x" and "y" directions across the bonded interface using a diamond saw in a Labcut 1010 machine (Extec Corp, Enfield, CT, USA) under water cooling at 300 rpm to obtain bonded sticks with a cross-sectional area approximately 0.8 mm². The number of premature debonded sticks (D) per tooth during specimen preparation was recorded. The cross-sectional area of each stick was measured with the digital caliper to the near-

est 0.01 mm and recorded for subsequent calculation of the microtensile bond strength (Absolute Digimatic, Mitutoyo, Tokyo, Japan). The bonded sticks that originated from the same teeth were randomly divided and assigned to testing immediately or after six months of storage in distilled water at 37°C. The storage solution was not changed and its pH was monitored monthly.

At each storage time period, each bonded stick was attached to a modified device[27] for microtensile testing with cyanoacrylate resin (Zapit, Dental Ventures of North America, Corona, CA, USA) and subjected to a tensile force of 0.5 mm/minute in a universal machine (Emic, São José dos Pinhais, PR, Brazil). The failure modes were evaluated at 400x (HMV-2, Shimadzu, Tokyo, Japan) and classified as cohesive (failure exclusive within dentin or composite, C), adhesive (failure at the resin/dentin interface, A) or adhesive/mixed (failure at the resin/dentin interface that included cohesive failure of the neighboring substrates, A/M).

Nanoleakage Test

Two bonded sticks from each tooth prepared for microtensile testing were coated with two layers of nail varnish applied up to 1 mm of the bonded interfaces. The specimens were rehydrated in distilled water for 10 minutes prior to immersion in the tracer solution for 24 hours. Ammoniacal silver nitrate was prepared according to the protocol previously described by Tay and others.[8] The sticks were placed in ammoniacal sil-

ver nitrate in darkness for 24 hours, rinsed thoroughly in distilled water and immersed in photo developing solution for eight hours under a fluorescent light to reduce silver ions into metallic silver grains within voids along the bonded interface.

All the sticks were wet-polished with 600 SiC paper to remove the nail varnish. The specimens were then placed inside an acrylic ring, which was attached to a double-sided adhesive tape, they were then embedded in epoxy resin. After the epoxy resin had set, the thickness of the embedded specimens was reduced to approximately half by grinding with silicon carbide papers under running water. The specimens were polished with 1000-grit SiC paper and 6, 3, 1 and 0.25 μm diamond paste (Buehler Ltd, Lake Bluff, IL, USA) using a polishing cloth. The specimens were ultrasonically cleaned, air dried, mounted on stubs and gold coated (MED 010, Balzers Union, Balzers, Liechtenstein). Resin-dentin interfaces were analyzed in a field-emission scanning electron microscope operated in the backscattered electron mode using energy dispersive x-ray spectrometry (EDX) (LEO 435 VP, LEO Electron Microscopy Ltd, Cambridge, UK). The amount of silver nitrate within the adhesive layer, hybrid layer and resin tags in each stick was measured with EDX in three regions (5 x 5 μm) of the bonded stick (left, center and right) (Figure 1).[28] The intensity of the backscattered electrons generated by electron bombardment by x-rays can be correlated to the atomic number of the element within the sampling volume. Hence, qualitative elemental information can be revealed. The characteristic x-rays emitted from the sample serve as fingerprints and give elemental information of the samples, including quantitative analysis. The percentage of silver nitrate deposition was calculated based on the amount of Ag presented in the pre-selected areas. The total length of the bonding interface (adhesive layer,
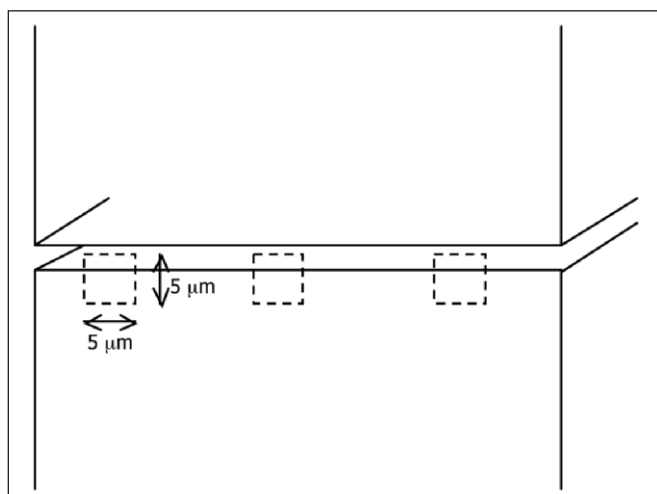

Figure 1. *Schematic drawing of a resin-dentin interface showing the areas where the silver nitrate uptake was measured by SEM-EDX.*

hybrid layer and resin tags) scanned for silver nitrate uptake measurement was approximately 75 μm². The silver nitrate uptake was expressed as the area amount of silver nitrate uptake divided by the total area measured. This value was multiplied by 100, and the mean value for each region was expressed as a percentage of the total area evaluated. The mean of three measures of each stick was averaged for statistical purposes.

Statistical Analysis

The experimental unit in the current study was the hemi-tooth, since half of the sample was tested immediately and the other half was tested only after six months. The mean of the microtensile bond strength values and percentage of nanoleakage of all sticks from the same tooth were averaged for statistical purposes. The prematurely debonded specimens were included in the hemi-tooth mean for microtensile bond strength. The average value attributed to specimens that failed prematurely during preparation was arbitrary and corresponded to approximately half of the minimum bond strength value that could be measured in the current study (ca 9.8 MPa). The specimens with cohesive failure were excluded from the data analysis. The microtensile bond strength and nanoleakage means for every testing group was expressed as the average of the seven hemi-teeth used per group and expressed in MPa.

The microtensile bond strength (MPa) and percentage of silver nitrate penetration (nanoleakage) data for each adhesive were subjected to two-way repeated measures analysis of variance and a Tukey's post-hoc test for group-wise comparisons (α=0.05). The repeated measure was the hemi-tooth. The correlation between the bond strength measurements and nanoleakage of each adhesive system in each experimental condition was analyzed by simple linear regression analysis. The strength of the association between these two variables (bond strength measurements versus nanoleakage uptake) was estimated with the Pearson product–moment correlation statistics (α=0.05).

RESULTS

The mean cross-sectional area ranged from 0.74 to 0.92 mm² and no difference among groups was detected (*p*>0.05). The percentage of specimens with premature debonding and the frequency of each fracture pattern mode is shown in Tables 2 and 3. SB had a lower overall pretest failure rate than PB. No cohesive failure in composite occurred in the immediate time. A low percentage of cohesive failure in dentin occurred for both adhesives after six months.

Microtensile Bond Strength Test (BS)

The overall BS values for SB and PB are shown in Table 4. The cross-product Treatment vs Storage Time was statistically significant for both adhesive systems (*p*<0.05). For both adhesives, no significant reductions

Table 2: *Number and Percentage of Specimens (%) and Total of Sticks Tested According to Fracture Pattern Mode and Premature Debonded Specimens From Prime & Bond NT (*)*

Adhesive/Storage Time	Prime & Bond NT							
	Immediate				6 Months			
Pattern Fracture	A/M	C	Debonded	Total	A/M	C	Debonded	Total
Control	30 (67)	0 (0)	10 (33)	40	22 (75.8)	1 (4.5)	6 (20.6)	29
CHX	39 (75)	1 (2.5)	9 (22.5)	49	28 (96.5)	0 (0)	1 (3.5)	29
Ac/CHX	58 (83)	0 (0)	9 (17)	67	29 (72.4)	0 (0)	8 (27.6)	37

(*) A/M–adhesive/mixed fracture mode; C–cohesive fracture mode; Debonded–premature debonded specimens.

Table 3: *Number and Percentage of Specimens (%) and Total of Sticks Tested According to Fracture Pattern Mode and the Premature Debonded Specimens From Adper Single Bond 2 (*)*

Adhesive/Storage Time	Adper Single Bond 2							
	Immediate				6 Months			
Pattern Fracture	A/M	C	Debonded	Total	A/M	C	Debonded	Total
Control	38 (85)	2 (5)	4 (10)	44	27 (64.3)	0/0	15 (35.7)	42
CHX	52 (96.3)	0 (0)	2 (4.8)	54	30 (100)	0/0	0/0	30
Ac/CHX	58 (87.6)	2 (3.4)	6 (10)	66	30 (76.9)	0/0	9 (23.1)	39

(*) A/M–adhesive/mixed fracture mode; C–cohesive fracture mode; Debonded–premature debonded specimens.

Table 4: *Overall Microtensile Bond Strength Values and the Respective Standard Deviations (MPa) Obtained in Each Experimental Condition and Statistical Significance (*)*

Conditions	Prime&Bond NT		Adper Single Bond 2	
	Immediate		6 Months	
Control	22.0 ± 9.7 B	14.6 ± 3.1 C	27.2 ± 6.1 a	20.4 ± 2.1 b
CHX	21.9 ± 4.7 B	23.4 ± 2.1 B	31.1 ± 3.1 a	31.1 ± 2.6 a
Ac/CHX	30.6 ± 9.0 A	25.7 ± 2.5 A,B	28.4 ± 4.4 a	27.1 ± 1.4 a

(*) Comparisons are only valid within each adhesive system. For each adhesive, means of each group with the same capital or lower case are not significantly different (Tukey's test, $p>0.05$).

Table 5: *Overall Percentage of Nanoleakage Uptake (%) and Respective Standard Deviations (obtained in each experimental condition) and Statistical Significance (*)*

Conditions	Prime&Bond NT		Adper Single Bond 2	
	Immediate		6 Months	
Control	28.1 ± 4.9 C	52.8 ± 6.6 D	26.0 ± 6.0 b	39.9 ± 5.6 c
CHX	13.8 ± 3.4 A	38.1 ± 12.4 C	17.6 ± 4.7 a	28.7 ± 16.7 b
Ac/CHX	13.7 ± 6.6 A	29.5 ± 3.5 C	17.8 ± 4.7 a	20.8 ± 6.4 a

(*) Comparisons are only valid within each adhesive system. For each adhesive, means with the same capital or lower case are not significantly different (Tukey's test, $p>0.05$).

in bond strength value were observed when CHX was used either in an aqueous solution (CHX) or in the phosphoric acid conditioner (Ac/CHX) ($p>0.05$). On the other hand, significant BS reductions were observed in the control groups ($p=0.0001$).

Nanoleakage Test

The overall nanoleakage uptake (NL) for SB and PB are shown in Table 5. The cross-product Treatment vs Storage Time was statistically significant for both adhesive systems ($p<0.05$). A lower percentage of silver nitrate deposition was observed for PB in CHX or Ac/CHX when compared to the control group in the immediate time ($p=0.01$). After six months of water storage, a significantly higher silver nitrate uptake was observed for both adhesives under the experimental conditions, however, this uptake was far less pronounced for the CHX and Ac/CHX groups compared to the controls ($p=0.001$).

Representative SEM images at the resin-dentin interfaces for the experimental conditions are depicted in
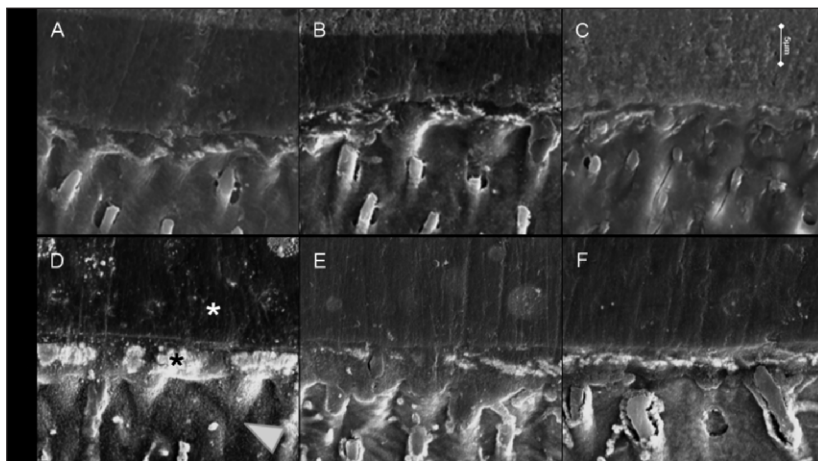
Figure 2. *Representative backscattered SEM images of the resin-dentin interfaces bonded with Prime & Bond NT in the immediate (A to C) and six-month periods (D to F). The control groups are represented by Figures 2A and 2D; CHX groups by Figures 2B and 2E and CHX/Ac groups by Figures 2C and 2F. The amount of silver penetration in Figures 2A, B and C (immediate) was lower and practically occurred at the hybrid layer. Only a few dentin tubules were infiltrated by silver nitrate. After six-months (Figures 2D, E and F), most silver nitrate deposition occurred in the control group (Figure 2D), with silver deposition occurring almost throughout the entire thickness of the hybrid layer. It can be seen that the amount of silver penetration in Figures 2E and 2F was lower than in Figure 2D. However, when all six-month groups (Figures 2D, E and F) were compared to their respective groups in the immediate period (Figures 2A, B and C), significantly higher silver nitrate impregnation occurred after six months. (White asterisk = adhesive layer; black asterisk = hybrid layer and white triangle = dentin)*
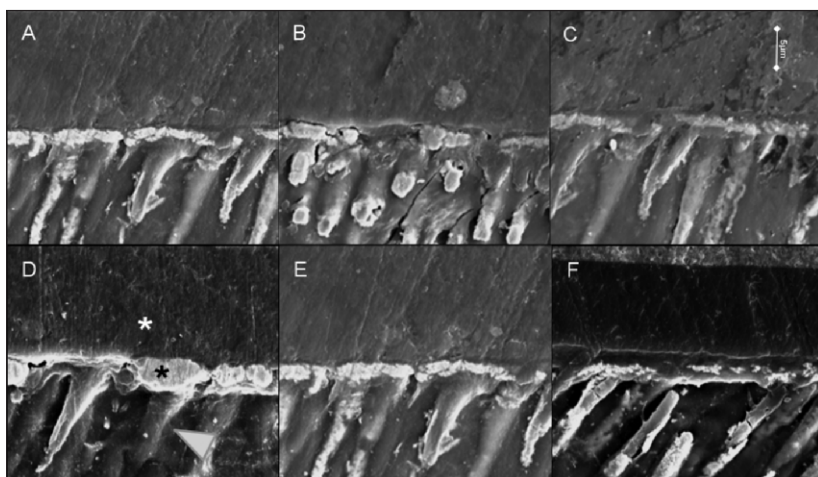

Figure 3. *Representative backscattered SEM images of the resin-dentin interfaces bonded with Adper Single Bond 2 in the immediate (Figures 3A to 3C) and six-month periods (Figures 3D to 3F). The control groups are represented by Figures 3A and 3D; the CHX groups by Figures 3B and 3E and the CHX/Ac groups by Figures 3C and 3F. The amount of silver penetration in Figures 3A, B and C (immediate) was lower and practically occurred at the hybrid layer. After six months (Figures 3D, E and F), a higher amount of silver nitrate deposition occurred in all groups, usually at the hybrid layer. This was more pronounced for the control group (Figure 3D), in which an intense penetration of silver nitrate can almost be seen throughout the entire thickness of the hybrid layer. (White asterisk = adhesive layer; black asterisk = hybrid layer and white triangle = dentin)*

Figures 2 and 3. All groups bonded with PB showed a lower amount of silver nitrate penetration, which practically occurred at the base of the hybrid layer (Figures 2A, 2B and 2C). Only a few dentin tubules were infiltrated by silver nitrate in immediate time for the control and CHX groups (Figures 2A and 2B, respectively). After six months, the control group showed the highest amount of silver nitrate uptake (Figure 2D), which nearly occurred throughout the entire thickness of the hybrid layer. Regarding SB, very little silver nitrate deposition was seen in the immediate groups (Figure 3A, 3B and 3C). However, after six-months, an intense penetration of silver nitrate could nearly be seen throughout the entire thickness of the hybrid layer in the control group (Figure 3D). In Figures 3E and 3F, few dentin tubules were infiltrated by silver nitrate.

Regression analysis revealed a linear and negative relationship between the mean bond strength and mean silver nitrate uptake for PB and SB (Figure 4) (R^2=0.87, p=0.001).

DISCUSSION

CHX is a widely used antimicrobial agent that possesses a broad spectrum of activity against oral bacteria and has low toxicity.[29] This is why several studies have proposed the use of CHX for cavity disinfection before placement of restorations. The ideal moment for CHX application has been questioned, leading some authors to use it to treat cavity preparation before or after acid etching.[24-27]

Authors who apply CHX after acid etching believe that this procedure can increase the wetting of dentin for primers, as CHX solution produces some debris on the surface and within the tubules of etched dentin.[24,30] Certain CHX properties, including strong positive ionic charge; ready binding to phosphate groups; strong affinity to the tooth surface, which is increased by acid etching and, finally, an increase in surface-free energy of enamel and perhaps dentin, are the likely reasons responsible for the good resin-dentin bond strengths obtained when CHX is applied after acid etching.[23,30] Fortunately, according to the results of the current study compared to those published in the literature, the application of CHX produces no detrimental effect on dentin adhesion in immediate time.[24-27]

Recently, Pashley and others[17] proposed that the currently accepted technique of applying a CHX disinfecting solution to acid etched dentin prior to the use of etch-and-rinse simplified adhesives systems may have additional potential merits in preventing the degradation of collagen fib-

rils in dentin hybrid layers, apart from its widely known antimicrobial property, as mentioned in the introduction. Several *in vitro* and *in vivo* studies of the same working group validated the concept that CHX may prevent exposed collagen within dentin bonds from degradation, thereby improving its longevity.[18-21]

The results of the current study corroborated with such findings. When a 2% CHX solution was used to rewet dentin instead of using water, which was used in the control group, no significant degradation of resin-dentin bonds in terms of bond strength values and a nanoleakage pattern was observed. However, contrary findings were observed in the control groups, which demonstrated reductions of approximately 33.6% and 25% for PB and SB, respectively, in terms of bond strength values after six months of water storage.

The significantly lower percentage of silver deposition in the hybrid layer, especially in the bottom part after six months in the CHX groups, indicates that the higher microtensile bond strengths values observed in these groups reflected the preservation of hybrid layer collagenous matrix, especially in the bottom zone, where partially exposed collagen fibrils are most prone to initial enzymatic degradation. The strong and negative correlation between bond strength values and silver nitrate penetration observed in the current investigation also attests to this finding. This may also reflect better preservation of the sub-hybrid layer dentin in which both progressive demineralization and degradation of the dentin collagenous matrix may occur with time.[6-7,9-10]

Some studies have attempted to correlate the data from nanoleakage with bond strength values in the same specimens.[31-34] However, none of these studies has succeeded in obtaining a significant correlation between these two properties. In the current study, a negative and linear correlation was observed between these two properties. The main difference observed in the current study and the aforementioned ones is that the bonded interfaces were evaluated in the short- and long-term period. This allowed time for the degradation process to take place and promote significant changes in the resin–dentin adhesive interface. One could speculate that the highly significant correlation between decreases in bond strength and increases in silver nitrate uptake reflect degradation at the resin–dentin interfaces. Reis and others[28] also observed an inverse relationship between bond strength and silver nitrate uptake for the etch-and-rinse adhesive system. However, this matter still deserves further attention, since another study that also evaluated the durability of bonded interfaces by means of bond strength measurements and silver nitrate uptake has not observed any correlation between these two properties.[35]
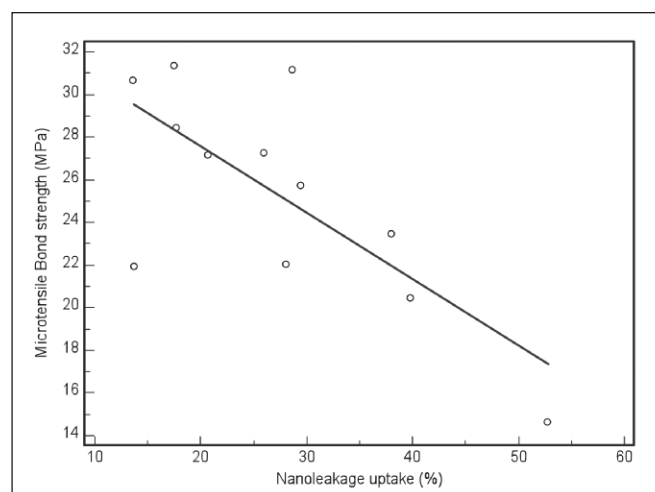

Figure 4. *Relation between the means of microtensile bond strength (MPa) and nanoleakage uptake (%) for each group.*

The degradation of incompletely infiltrated zones within hybridized dentin by host-derived MMPs (MMP-2; MMP-8 and MMP-9) within the dentin matrix may be prevented by the use of MMP inhibitors, such as CHX. Gendron and others[36] showed that CHX demonstrates beneficial antiproteolytic properties, and they proposed two different mechanisms of action involved in MMPs inhibition: a chelating mechanism for inhibition of MMP-2 and -9 and, in the case of MMP-8, CHX may interact with the essential sulfhydryl groups and/or cysteine present in its active site.

However, of interest were the results observed in the Ac/CHX groups. No negative effect both on immediate bond strength values[26] and nanoleakage pattern was observed when this acid was employed; but most importantly, no significant degradation of the resin-dentin bonds was detected when CHX containing phosphoric acid was used prior to the adhesive application. Contrary to the application of 2% chlorhexidine solution (CHX) for 60 seconds, 2% chlorhexidine-containing phosphoric acid (Ac/CHX) is completely washed out after 15 seconds of application, reducing the period of time that CHX is in contact with the dentin substrate and its concentration by the time the adhesive is applied.

However, it seems that the CHX concentration employed so far to prevent host-derived MMPs from degrading exposed collagen fibrils is higher than what is likely needed. Some studies have shown that CHX can inhibit proteases at extremely low concentrations.[36-37] For instance, Gendron and others[36] demonstrated that MMP-2, -8 and –9 activities can be inhibited by CHX at concentrations of 0.0001%, 0.02% and 0.002%, respectively. As most of the studies employ CHX at higher concentrations,[18-21] MMP-2 and MMP-9 are probably inactivated by protein denaturation[37] rather than by the chelation of cations.[36]

Therefore, one might envision that even small concentrations of CHX, such as what remains after acid rinsing in the Ac/CHX group, can lead to the same inhibition effects produced by the employment of an aqueous solution for 60 seconds, one that remains on the dentin surface prior to adhesive application. It is worth stressing that only acid-etching is able to reduce the collagenolytic activity of dentin.[17] Conditioning dentin with 37% phosphoric acid for 15 seconds can reduce the collagenolytic activity by 65% compared to no dentin demineralization.[17] It is likely that the association with CHX can increase this potential.

Another explanation that can be given is that CHX binds to exposed collagen sites at such a fast rate that even as short a period as 15 seconds could be enough to prevent resin-dentin degradation. This has been described in the literature as substantivity, even though it is far more correlated to the residual antibacterial activity of CHX in human dentin,[38-39] and its inhibitory effect on MMPs activity was described in the literature as substantivity (residual antibacterial activity) of the CHX in human dentin.[38-39] This is why CHX is one of the most used antimicrobial agents, that is, it is an agent that can have a therapeutic effect for a prolonged period of time. This property is only possible because of the release of positively charged molecules from CHX-treated dentin and its ability to adsorb to the surfaces of the oral cavity.[39-40] This ability to adsorb to the surfaces of the oral cavity can also be the same for collagen fibrils, which probably preserves degradation of the hybrid layer after long-term water exposure.

Antimicrobial substantivity of CHX in contact with root dentin has been reported to vary from 48 hours to 21 days.[38,41-42] However, other investigators reported that this period of time can be extended for up to 12 weeks.[39] Obviously, there is a considerable debate in the literature regarding the time for dentin treatment to induce substantivity, but future studies should be encouraged to investigate these hypotheses.

Another concern raised from the results of the current investigation resulted from the stability of CHX in low pH solution, such as 37% phosphoric gel. Unfortunately, the substantivity of CHX is decreased in lower pH,[43-45] although the significance of this observation is not entirely understood. The gel employed in the current investigation was a commercial gel and was not manipulated by the authors. However, even considering that CHX possesses lower substantivity in low pH, this group (Ac/CHX) behaved in a similar way to that of using an aqueous solution of CHX (CHX), which probably indicates that CHX was somewhat active inside the acidic gel. An increased denaturation of protein, such as MMPs, at the lowest pH could theoretically counter-balance the reduced binding.[45] However, it was already demonstrated that 0.1% CHX can still preserve its function in low pH (around 2).[43] Future

studies should be encouraged to investigate MMPs inhibitory effect of CHX in low pH environments and/or solutions.

Unfortunately, there is no evidence to document the antimicrobial efficacy of CHX when used prior to or after acid-etching. In the case of phosphoric acid, previously reported observations claim that CHX achieves its higher inhibitory effect at higher (pH 6-10) pH values than at lower ones.[44]

Regardless of the reasons that led the Ac/CHX groups to achieving stable bond strength and less permeable adhesive interfaces over the six-month period, this is an excellent option for clinicians. The inclusion of CHX in the acidic conditioner does not add an extra step in the bonding protocol and can significantly improve stability of the bonds over time.

CONCLUSIONS

In conclusion, 2% CHX application, either in an aqueous solution or associated with the acid conditioner, preserves durability of the resin-dentin interface. Further *in vivo* studies are needed to clarify whether the use of a 2% CHX-containing acid preserves resin-dentin interface after long-term function.

References

1. De Munck J, Van Landuyt K, Peumans M, Poitevin A, Lambrechts P, Braem M & Van Meerbeek B (2005) A critical review of the durability of adhesion to tooth tissue: Methods and results *Journal of Dental Research* **84(2)** 118-132.

2. Breschi L, Mazzoni A, Ruggeri A, Cadenaro M, Di Lenarda R & De Stefano Dorigo E (2008) Dental adhesion review: Aging and stability of the bonded interface *Dental Materials* **24(1)** 90-101.

3. Mjör IA, Moorhead JE & Dahl JE (2000) Reasons for replacement of restoration in permanent teeth in general dental practice *International Dental Journal* **50(6)** 361-366.

4. Carrilho MR, Carvalho RM, Tay FR, Yiu C & Pashley DH (2005) Durability of resin-dentin bonds related to water and oil storage *American Journal of Dentistry* **18(6)** 315-319.

5. Reis A, Grandi V, Carlotto L, Bortoli G, Patzlaff R, Rodrigues Accorinte Mde L & Dourado Loguercio A (2005) Effect of smear layer thickness and acidity of self-etching solutions on early and long-term bond strength to dentin *Journal of Dentistry* **33(7)** 549-559.

6. Spencer P & Wang Y (2002) Adhesive phase separation at the dentin interface under wet bonding conditions *Journal of Biomedical Materials Research* **62(3)** 447-456.

7. Hashimoto M, Ohno H, Kaga M, Sano H, Endo K & Oguchi H (2002) The extent to which resin can infiltrate dentin by acetone-based adhesives *Journal of Dental Research* **81(1)** 74-78.

8. Tay FR, Pashley DH & Yoshiyama M (2002) Two modes of nanoleakage expression in single-step adhesives *Journal of Dental Research* **81(7)** 472-476.

9. Hashimoto M, Ohno H, Sano H, Kaga M & Oguchi H (2003) *In vitro* degradation of resin-dentin bonds analyzed by microtensile bond test, scanning and transmission electron microscopy *Biomaterials* **24(21)** 3795-3803.

10. Wang Y & Spencer P (2003) Hybridization efficiency of the adhesive-dentin interface with wet bonding *Journal of Dental Research* **82(2)** 141-145.

11. Tjäderhane L, Larjava H, Sorsa T, Uitto VJ, Larmas M & Salo T (1998) The activation and function of host matrix metalloproteinases in dentin matrix breakdown in caries lesions *Journal of Dental Research* **77(8)** 1622-1629.

12. Sulkala M, Larmas M, Sorsa T, Salo T & Tjäderhane L (2002) The localization of matrix metalloproteinase-20 (MMP-20, enamelysin) in mature human teeth *Journal of Dental Research* **81(9)** 603-607.

13. van Strijp AJ, Jansen DC, DeGroot J, Ten Cate JM & Everts V (2003) Host-derived proteinases and degradation of dentine collagen *in situ* *Caries Research* **37(1)** 58-65.

14. Lee W, Aitken S, Sodek J & McCulloch CA (1995) Evidence of a direct relationship between neutrophil collagenase activity and periodontal tissue destruction *in vivo*: Role of active enzyme in human periodontitis *Journal of Periodontal Research* **30(1)** 23-33.

15. Martin-De-Las Heras S, Valenzuela A & Overall CM (2000) The matrix metalloproteinase gelatinase A in human dentine *Archives of Oral Biology* **45(9)** 757-765.

16. Mazzoni A, Mannello F, Tay FR, Tonti GA, Papa S, Mazzotti G, Di Lenarda R, Pashley DH & Breschi L (2007) Zymographic analysis and characterization of MMP-2 and -9 forms in human sound dentin *Journal of Dental Research* **86(5)** 436-440.

17. Pashley DH, Tay FR, Yiu C, Hashimoto M, Breschi L, Carvalho RM & Ito S (2004) Collagen degradation by host-derived enzymes during aging *Journal of Dental Research* **83(3)** 216-221.

18. Hebling J, Pashley DH, Tjäderhane L & Tay FR (2005) Chlorhexidine arrests subclinical degradation of dentin hybrid layers *in vivo* *Journal of Dental Research* **84(8)** 741-746.

19. Brackett WW, Tay FR, Brackett MG, Dib A, Sword RJ & Pashley DH (2007) The effect of chlorhexidine on dentin hybrid layers *in vivo* *Operative Dentistry* **32(2)** 107-111.

20. Carrilho MR, Carvalho RM, de Goes MF, di Hipólito V, Geraldeli S, Tay FR, Pashley DH & Tjäderhane L (2007) Chlorhexidine preserves dentin bond *in vitro* *Journal of Dental Research* **86(1)** 90-94.

21. Carrilho MR, Geraldeli S, Tay F, de Goes MF, Carvalho RM, Tjäderhane L, Reis AF, Helding J, Mazzoni A, Breschi L & Pashley D (2007) *In vivo* preservation of the hybrid layer by chlorhexidine *Journal of Dental Research* **86(6)** 529-533.

22. Tay FR & Pashley DH (2003) Have dentin adhesives become too hydrophilic? *Journal of the Canadian Dental Association* **69(11)** 726-731.

23. Fardal O & Turnbull RS (1986) A review of the literature on use of chlorhexidine in dentistry *Journal of the American Dental Association* **112(6)** 863-869.

24. Pilo R, Cardash HS, Oz-Ari B & Ben-Amar A (2001) Effect of preliminary treatment of the dentin surface on the shear bond strength of resin composite to dentin *Operative Dentistry* **26(6)** 569-575.

25. de Castro FL, de Andrade MF, Duarte Junior SL, Vaz LG & Ahid FJ (2003) Effect of 2% chlorhexidine on microtensile bond strength of composite to dentin *Journal of Adhesive Dentistry* **5(2)** 129-138.

26. Soares CJ, Pereira CA, Pereira JC, Santana FR & do Prado CJ (2008) Effect of chlorhexidine application on microtensile bond strength to dentin *Operative Dentistry* **33(2)** 183-188.

27. Perdigão J, Geraldeli S, Carmo AR & Dutra HR (2002) *In vivo* influence of residual moisture on microtensile bond strengths of one-bottle adhesives *Journal of Esthetic and Restorative Dentistry* **14(1)** 31-38.

28. Reis A, Grande RHM, Oliveira GMS, Lopes GC & Loguercio A (2007) A 2-year evaluation of moisture on microtensile bond strength and nanoleakage *Dental Materials* **23(7)** 862-870.

29. Johnson BT (1995) Uses of chlorhexidine in dentistry *General Dentistry* **43(2)** 126-140.

30. Perdigão J, Denehy GE & Swift EJ (1994) Effects of chlorhexidine on dentin surfaces and shear bond strengths *American Journal of Dentistry* **7(2)** 81-84.

31. Guzman-Armstrong S, Armstrong SR & Qian F (2003) Relationship between nanoleakage and microtensile bond strength test at the resin–dentin interface *Operative Dentistry* **28(1)** 60-66.

32. Cenci M, Demarco F & de Carvalho RM (2005) Class II composite resin restorations with two polymerization techniques: Relationship between microtensile bond strength and marginal leakage *Journal of Dentistry* **33(7)** 603-610.

33. Loguercio AD, Reis A & Ballester RY (2004) Polymerization shrinkage: Effects of constraint and filling technique in composite restorations *Dental Materials* **20(3)** 236-243.

34. Ding PG, Wolff D, Pioch T, Staehle HJ & Dannewitz B (2009) Relationship between microtensile bond strength and nanoleakage at the composite-dentin interface *Dental Materials* **25(1)** 135-141.

35. Okuda M, Pereira PN, Nakajima M & Tagami J (2001) Relationship between nanoleakage and long-term durability of dentin bonds *Operative Dentistry* **26(5)** 482-490.

36. Gendron R, Greiner D, Sorsa T & Mayrand D (1999) Inhibition of the activities of matrix metalloproteinases 2, 8 and 9 by chlorhexidine *Clinical and Diagnostic Laboratory Immunology* **6(3)** 437-439.

37. Grenier D (1993) Reduction of proteolytic degradation by chlorhexidine *Journal of Dental Research* **72(3)** 630-633.

38. Basrani B, Santos JM, Tjäderhane L, Grad H, Gorduysus O, Huang J, Lawrence HP & Friedman S (2002) Substantive antimicrobial activity in chlorhexidine-treated human root dentin *Oral Surgery Oral Medicine Oral Pathology Oral Radiology & Endodontics* **94(2)** 240-245.

39. Rosenthal S, Spångberg L & Safavi K (2004) Chlorhexidine substantivity in root canal dentin *Oral Surgery Oral Medicine Oral Pathology Oral Radiology & Endodontics* **98(4)** 488-492.

40. Gjermo P, Bonesvoll P & Rölla G (1974) Relationship between plaque inhibitory effect and retention of chlorhexidine in the human oral cavity *Archives of Oral Biology* **19(11)** 1031-1034.

41. White RR, Hays GL & Janer LR (1997) Residual antimicrobial activity after canal irrigation with chlorhexidine *Journal of Endodontics* **23(4)** 229-231.

42. Leonardo MR, Tanomaru Filho M, Silva LA, Nelson Filho P, Bonifácio KC & Ito IY (1999) *In vivo* antimicrobial activity of 2% chlorhexidine used as a root canal irrigating solution *Journal of Endodontics* **25(3)** 167-171.

43. Hjeljord LG, Rolla G & Bonesvoll P (1973) Chlorhexidine-protein interactions *Journal of Periodontal Research* **8(Supplement 12)** 11–16.

44. Turesky S, Glickman I, Warner VD & Mirth DB (1973) *In vitro* plaque inhibition by chlorhexidine gluconate and homologues-effect of pH and pretreatment *Journal of Periodontology* **44(11)** 709-711.

45. Gjermo P, Bonesvoll P, Hjeljord LG & Rölla G (1975) Influence of variation of pH on chlorexidine mouth rinses on oral retention and plaque-inhibiting effect *Caries Research* **9(1)** 74-82.