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Effect of 2% Chlorhexidine Digluconate on the Bond Strength to Normal Versus Caries-affected Dentin

PCP Komori • DH Pashley • L Tjäderhane • L Breschi
A Mazzoni • MF de Goes • L Wang • MR Carrilho

Clinical Relevance

Attempts to use CHX adjunctively with conventional etch-and-rinse adhesives may play a clinically relevant role in the preservation of resin-dentin bonds.

Paula CP Komori, DDS, MS, PhD student, Department of Restorative Dentistry, Dental Materials, Piracicaba School of Dentistry, University of Campinas, Brazil

David H Pashley, DMD, PhD, regents' professor, Department of Oral Biology, School of Dentistry, Medical College of Georgia, Augusta, GA, USA

Leo Tjäderhane, DDS, PhD, professor, Institute of Dentistry, University of Oulu and Oulu University Hospital, Oulu, Finland

Lorenzo Breschi, DDS, PhD, professor, Department of Biomedicine, Unit of Dental Sciences and Biomaterials, University of Trieste, Trieste, Italy and IGM-CNR, Unit of Bologna c/o IOR, Bologna, Italy

Annalisa Mazzoni, DDS, PhD student, Department of SAU&FAL, University of Bologna, Bologna, Italy

Mario Fernando de Goes, DDS, PhD, professor, Department of Restorative Dentistry, Dental Materials, Piracicaba School of Dentistry, University of Campinas, Piracicaba, SP, Brazil

Linda Wang, DDS, PhD, professor, Department of Operative Dentistry, Dental Materials and Endodontics, University of São Paulo, Bauru School of Dentistry, Bauru, SP, Brazil

*Marcela R Carrilho, DDS, PhD, professor, Department of Restorative Dentistry, Dental Materials Area, Piracicaba School of Dentistry, University of Campinas and GEO/Bandeirante University of São Paulo, São Paulo, SP, Brazil

*Reprint request: Rua Alagoas, 475 ap 13B, CEP 01242-001, São Paulo, SP, Brazil; e-mail: marcelacarrilho@gmail.com

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SUMMARY

This study evaluated the effect of 2% chlorhexidine digluconate (CHX) used as a therapeutic primer on the long-term bond strengths of two etch-and-rinse adhesives to normal (ND) and caries-affected (CAD) dentin. Forty extracted human molars with coronal carious lesions, surrounded by normal dentin, were selected for this study. The flat surfaces of two types of dentin (ND and CAD) were prepared with a water-cooled high-speed diamond disc, then acid-etched, rinsed and air-dried. In the control groups, the dentin was re-hydrated with distilled water, blot-dried and bonded with a three-step (Scotchbond Multi-Purpose-MP) or two-step (Single Bond 2-SB) etch-and-rinse adhesive. In the experimental groups, the dentin was re-hydrated with 2% CHX (60 seconds), blot-dried and bonded with the same adhesives. Resin composite build-ups were made. The specimens were prepared for microtensile bond testing in accor-

dance with the non-trimming technique, then tested either immediately or after six-months storage in artificial saliva. The data were analyzed by ANOVA/Bonferroni tests (α=0.05). CHX did not affect the immediate bond strength to ND or CAD (*p*>0.05). CHX treatment significantly lowered the loss of bond strength after six months as seen in the control bonds for ND (*p*<0.05), but it did not alter the bond strength of CAD (*p*>0.05). The application of MP on CHX-treated ND or CAD produced bonds that did not change over six months of storage.

INTRODUCTION

The restorative treatment of carious teeth has undergone profound changes since the outer, non-contaminated layer of carious dentinal lesions was found to be suitable for remineralization.[1] Operative procedures have shifted from the removal of large extensions of the affected tooth to the completion of more conservative, minimally invasive cavity designs[2-3] in which the affected, non-contaminated dentin can be deliberately retained in the preparation.

Contemporary etch-and-rinse and self-etch adhesives play a significant role in the coupling of resin composites to caries-affected dentin by providing reasonable initial bond strength.[4-5] However, morphological studies have shown that a perfect hybrid layer is rarely formed in caries-affected dentin.[6-7] This hypomineralized, porous substrate allows for deeper penetration of the etchant, leading to a deeper demineralization of the dentin.[4,8-9] With a deeper zone of demineralization, it is more difficult for resin monomers to completely infiltrate the collagen dentin matrix.[6-7] Accordingly, the prevalence of exposed, unprotected collagen fibrils within the hybrid layers formed in caries-affected dentin may be greater when compared to hybrid layers in non-carious dentin.[6]

The durability of resin-dentin bonds made to normal,[10] sclerotic[11] or caries-affected dentin[12] (normal or caries-affected), using contemporary, simplified adhesives has been shown to be poor, as these adhesives do not perfectly seal the exposed dentin.[13-14] Moreover, increasing the concentration of ionic and polar comonomers in simplified adhesives to make them more hydrophilic results in increased water sorption,[15-17] which ultimately lowers their mechanical properties by polymer plasticization.[15-16,18] Thus, the use of hydrophilic, simplified etch-and-rinse adhesives may never achieve the goal of creating a durable coupling between the resin composites and resin-bonded dentin.

During caries progression, dissolution of some of the mineral phase of dentin leads to exposure of some of the organic matrix that can then be degraded by bacterial enzymes[19] and host-derived collagen-degrading proteases,[20-22] such as the matrix metalloproteinases (MMP) found either in saliva[21,23] or dentin organic matrix.[24-26] MMPs are thought to be responsible for degrading most of the extracellular matrix components, including different types of collagen, in native and denatured forms.[27-28] It has been suspected that the activity of host-derived MMPs may also be involved in the degradation of unprotected collagen within incompletely resin-infiltrated acid-etched dentin,[29-32] which could explain the progressive thinning of the hybrid layers seen in numerous *in vivo*[30,32-33] and *in vitro*[10,31] aging studies.

Persistent collagenolytic activity exhibited by unbonded, partially demineralized human dentin substrates, in the absence of bacteria, was recently associated with a morphological disintegration of dentinal collagen fibrils.[29] Nevertheless, it was also shown that the dentinal collagenolytic activity can be strongly reduced by the use of chlorhexidine,[29] a potent MMP inhibitor. Chlorhexidine also prevents or minimizes the auto-degradation of exposed collagen fibrils within incompletely-formed hybrid layers,[30,32] thereby, contributing to the long-term stability of the hybrid layer and bond strength. However, to date, the research has been limited to a single two-step etch-and-rinse adhesive (Single Bond, 3M/ESPE, St Paul, MN, USA).

The current study evaluated the effect of a 2% chlorhexidine digluconate primer (CHX) on the durability of resin-dentin bonds in normal *versus* caries-affected dentin, using three-step or two-step etch-and-rinse adhesives. The tested hypotheses were that: 1) CHX does not cause a detrimental effect on the immediate bond strength to either normal or caries-affected dentin; 2) Preservation of bond strength to both normal and caries-affected dentin over time is not adhesive- or CHX-dependent.

METHODS AND MATERIALS

Tooth Preparation

Forty extracted human molars with coronal caries lesions were collected after the patients' informed consent had been obtained under a protocol reviewed and approved by the Ethic Committee for Human Studies, Piracicaba School of Dentistry/UNICAMP, Brazil. Selected teeth were stored for one month or less in 0.5% chloramine T solution at 4°C. Occlusal enamel and dentin were removed horizontally (perpendicular to the long axis of the tooth) through the carious lesion mesio-distally, using a water-cooled diamond-impregnated disc (Buehler Ltd, Lake Bluff, IL, USA) to expose a flat dentin surface, wherein the carious lesion was surrounded by normal, sound dentin. Exposed dentin surfaces were stained with Caries Detector solution (Kuraray Medical Inc, Tokyo, Japan) for 10 seconds and rinsed with water. The carious lesion was then manually excavated until there was no more bright-red-staining dentin. Caries-affected dentin was defined as dentin that was colorless to light pink, firm and opaque. The entire dentin surface was then ground flat with 600-grit SiC paper under running water.

Bonding Procedures

All dentin surfaces were etched with 35% phosphoric acid gel (Scotch Etchant, 3M ESPE) for 15 seconds, rinsed for 30 seconds with tap water and vigorously dried with oil/water-free air. Teeth assigned to the control groups were re-hydrated with 15 µL of distilled water, gently blot-dried and bonded with one of the etch-and-rinse adhesives: the three-step Scotchbond Multi-Purpose (MP, 3M ESPE) or the two-step Single Bond 2 (SB, 3M ESPE) according to the manufacturer's instructions. In the experimental groups, dentin was re-hydrated with 15 µL of 2% CHX (PRODERMA, Piracicaba, SP, Brazil). After a dwell time of 60 seconds, the dentin surface was gently blot-dried and bonded with one of the two adhesives. Three 1.5-mm thick layers of a resin composite (Filtek Z250, 3M ESPE) were incrementally placed over the bonded dentin surfaces and individually light-cured for 20 seconds using a quartz-tungsten-halogen curing-light unit (XL 3000, 3M ESPE) with an output of 700 mW/cm^2. The teeth were stored in 0.02% NaN_3-containing phosphate-buffered saline (PBS, pH 7.4) at 37°C for a week.

Microtensile Bond Testing

The teeth were longitudinally sectioned across the bonded interface using a water-cooled diamond saw in order to produce a series of 0.9-mm thick slabs. Each slab was then cut into multiple 0.9 mm x 0.9 mm x 8 mm beams. Twelve to 14 beams were obtained from each bonded tooth. Half of those specimens were immediately tested, while the remaining specimens were stored at 37°C for six months in NaN_3-artificial saliva (pH 7.1) (Table 1) to inhibit microbial growth. The storage medium was replaced weekly. Each beam specimen was individually fixed to a custom-made testing jig (Geraldeli's jig) with a cyanoacrylate glue (Model Repair II Blue, Dentsply-Sakin, Japan) and subjected to tensile load at a crosshead speed of 0.5 mm/minute until failure (Instron 4411, Instron Corporation, Canton, MA, USA). All fractured surfaces were sputter-coated with gold/palladium and had their failure mode examined under scanning electron microscopy (JEOL-5600 LV, Tokyo, Japan) at 85x magnification at 15 kV. The fracture modes were classified as: 1) cohesive failure in resin (CR), if the fracture occurred exclusively within the resin composite, adhesive or both; 2) cohesive failure in dentin (CD); 3) interfacial failure, if the fracture site was located entirely between the adhesive and dentin (I); 4) mixed failure (M), if the fracture site continued from the adhesive into either the resin composite or dentin. In case of uncertainty, higher magnifications (500-4000x) were used to confirm the nature of the fracture. The number of prematurely debonded specimens per each tested group was also recorded but not included in the statistical analysis to determine the final mean bond strength.

Table 1: *Components of the Artificial Saliva Used for Storage of the Specimens*

Component	Concentration (mmol/L)
$CaCl_2$	0.70
$MgCl_2 \cdot 6H_2O$	0.20
KH_2PO_4	4.00
KCl	30.0
NaN_3	0.30
HEPES buffer (acid)	20.0

Based on Pashley and others (2004) Journal of Dental Research ***83*** *216-221.*

Statistical Analyses

The bond strength results of all actual tested specimens were analyzed by multiple ANOVA. In principle, normal (ND) and caries-affected (CAD) dentin substrates were separately analyzed by two three-way ANOVA analyses, having as main factors the dentin pretreatment (control *vs* 2%CHX), adhesives (MP *vs* SB) and testing time (immediately vs six months after aging). Comparisons between the bond strength of ND *vs* CAD substrates at the baseline (immediately tested) or after six months of aging were then individually performed by two three-way ANOVA analysis, having the following main factors: dentin condition (ND *vs* CAD), dentin pretreatment (control *vs* 2% CHX) and adhesives (MP *vs* SB). Bonferroni's adjustment tests were used to examine *post hoc* differences among the groups. ANOVA and Tukey's tests were also used to compare the effect of the pretreatment (control *vs* 2% CHX) on the distribution of failure modes either in normal or in caries-affected dentin. Statistical significance was preset at α=0.05.

RESULTS

Results of the bond strengths reported as the mean and standard deviation are summarized in Figures 1 and 2. CHX pretreatment did not affect the *in vitro* immediate bond strength of specimens tested right after the beams preparation to serve as baseline results ($p>0.05$), regardless of the dentin condition (normal or caries-affected dentin) or the tested adhesive (MP or SB) (Figure 1). In general, six-month storage in artificial saliva resulted in a significant reduction if bond strength for the control and the CHX-pretreated groups were bonded with the simplified tested adhesive, the two-step etch-and-rinse system Single Bond 2 ($p<0.05$) (Figure 1). The only exception was specimens of caries-affected dentin pretreated with CHX for which the bond strength of SB was not altered

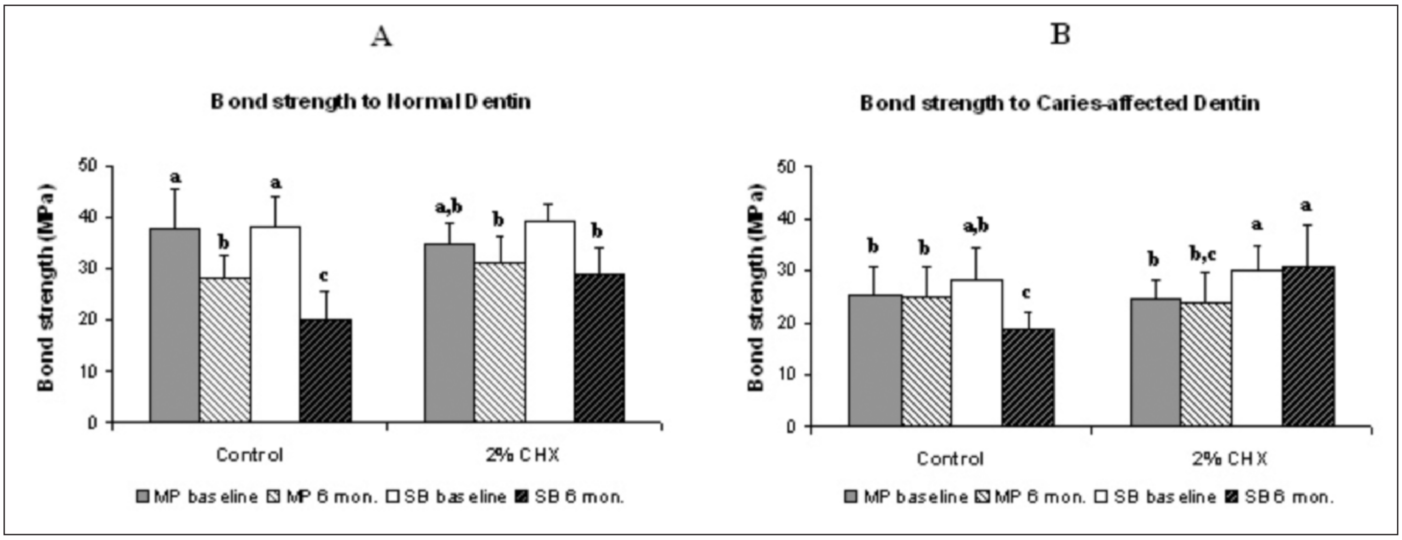

Figure 1: *Bond strengths (MPa) to normal (**Figure 1A**) and caries-affected (**Figure 1B**) dentin in either control (**Control**) or CHX-pretreated (**2% CHX**) specimens that were tested immediately (**MP baseline** and **SB baseline**) or after six months of aging in artificial saliva (**MP six months and SB six months**). **MP**= Scotchbond Multi-purpose adhesive; **SB**= Single Bond 2 adhesive. The height of the bars is the bond strength mean; half-brackets indicate plus one standard deviation. Groups identified with different letters were statistically different (p<0.05).*

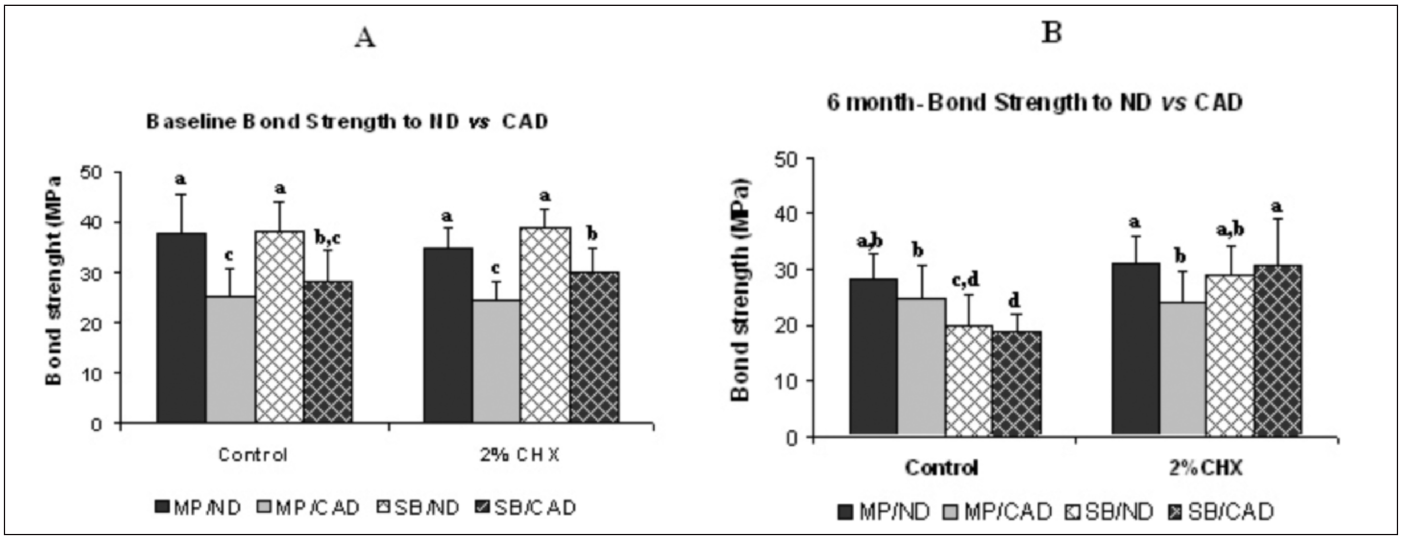

Figure 2: *Bond strengths (MPa) to normal (**ND**) versus caries-affected (**CAD**) dentin for control (**Control**) and CHX-pretreated (**2% CHX**) specimens that were tested immediately (**Figure 2A**) or after six months of aging in artificial saliva (**Figure 2B**). **MP**= Scotchbond Multi-purpose adhesive; **SB**= Single Bond 2 adhesive. The height of the bars is the bond strength mean; half-brackets indicate plus one standard deviation. Groups identified with different letters were statistically different (p<0.05).*

over the six months of storage in artificial saliva (p>0.05) (Figure 1B). In contrast, the bond strength of specimens bonded with the three-step MP system did not vary significantly over the six months of storage (p>0.05) (Figure 1), except for the control specimens of normal dentin that exhibited a significant decrease in bond strength at this testing period (p<0.05) (Figure 1A).

The immediate bond strengths (baseline results) of both tested adhesives to normal dentin were significantly higher than to caries-affected dentin (p<0.05) (Figure 2A). At this period, differences in bond strength resulting from using SB or MP were observed for caries-affected dentin pre-treated with CHX, with SB giving higher bond strength than MP (p<0.05) (Figure 2A). After six-months storage, differences between the bond strength to normal and caries-affected dentin were not significant (p>0.05), irrespective of the dentin treatment and tested adhesive (Figure 2B). At this period, control specimens bonded with MP exhibited higher bond strength when compared with control specimens bonded with SB (p<0.05) (Figure

Table 2: *Distribution of Failure Modes (in percentage) Between Groups, As Observed with SEM*

Group	Pretreat	Test	Fract/Deb	Percentage of Failure Mode of Fractured Bonds (%) CR	CD	I	M
MP/ND	C	IM	36/0	$25.0^{b,A}$	$0.0^{c,B}$	$25.0^{b,A}$	$50.0^{a,C}$
	CHX		30/0	$0.0^{c,D}$	$0.0^{c,B}$	$25.0^{b,A}$	$75.0^{a,A}$
	C	6 months	48/2	$6.6^{c,C}$	$3.3^{d,A}$	$16.6^{b,B}$	$66.6^{a,B}$
	CHX		40/1	$11.1^{c,B}$	$0.0^{d,B}$	$22.2^{b,A}$	$66.6^{a,B}$
MP/CAD	C	IM	20/1	0.0^{d}	$10.0^{c,B}$	$20.0^{b,B}$	$70.0^{a,B}$
	CHX		19/1	0.0^{c}	$11.0^{b,B}$	$10.8^{b,C}$	$78.2^{a,A}$
	C	6 months	31/9	0.0^{d}	$20.8^{b,A}$	$7.0^{c,D}$	$68.7^{a,B}$
	CHX		28/5	0.0^{d}	$20.0^{b,A}$	$12.2^{c,A}$	$67.8^{a,B}$
SB/ND	C	IM	29/0	$10.3^{c,B}$	$3.4^{d,B}$	$17.2^{b,A}$	$69.1^{a,B}$
	CHX		32/0	$14.4^{b,A}$	$6.5^{d,A}$	$10.4^{c,B}$	$68.7^{a,B}$
	C	6 months	43/1	$4.6^{c,C}$	$0.0^{d,C}$	$16.3^{b,A}$	$79.0^{a,A}$
	CHX		48/1	$16.6^{b,A}$	$0.0^{f,C}$	$12.5^{c,B}$	$70.8^{a,B}$
SB/CAD	C	IM	22/2	0.0^{d}	$9.0^{c,B}$	$24.2^{b,A}$	$66.6^{a,B}$
	CHX		25/1	0.0^{d}	$11.8^{c,B}$	$17.6^{b,C}$	$70.6^{a,A}$
	C	6 months	33/9	0.0^{c}	$20.6^{b,A}$	$19.7^{b,BC}$	$59.1^{a,C}$
	CHX		34/6	0.0^{c}	$17.0^{c,A}$	$22.0^{b,AB}$	$61.0^{a,C}$

***MP**: Scotchbond Multi-purpose system; **SB**: Single Bond 2 system; **ND**: normal dentin; **CAD**: caries-affected dentin; **C**: control dentin pretreatment; **CHX**: chlorhexidine dentin pretreatment. **IM**: values obtained at the immediate testing period; **6 months**: values obtained after six months of aging; **Fract/Deb**: total number of fractured/debonded specimens (that is, failed before the test). **CR**: cohesive failure in resin; **CD**: cohesive failure in dentin; I: interfacial failure; **M**: mixed failure. Values are the percentages of failure modes in fractured specimens under the same type of dentin and adhesive (grouped and identified with the same shading background). Analyses comparing different types of dentin and/or adhesives were not performed. Different superscript lower case letters (analysis in row) and different superscript upper case letters (in column) indicate statistically significant differences ($p<0.05$) (ANOVA and Tukey's tests).*

2B). For all the tested groups and conditions, the most prevalent fracture pattern observed was the mixed failure, followed by interfacial failure (Table 2). Cohesive failures in resin were more prevalent for specimens of normal dentin, while the prevalence of cohesive failure in dentin was greater for caries-affected specimens (Table 2 and Figure 3).

DISCUSSION

Since the application of 2% CHX did not affect the immediate bond strength of the tested etch-and-rinse adhesives to normal or caries-affected dentin, the primary tested hypothesis is supported. Nevertheless, the results showed that the preservation of bond strength to normal dentin over the six months of storage in artificial saliva was significantly dependent on the adhesive and the application of 2% CHX, resulting in the partial rejection of the secondary tested hypothesis. This finding supports the concept that the use of chlorhexidine as a therapeutic primer may produce hybrid layers within normal dentin that, at least at middle-term (six months), would be mechanically more stable than untreated caries-affected dentin. Since all tested specimens were stored in the same artificial saliva containing the antimicrobial agent (sodium azide) that was replaced weekly, the possibility of bacteria being responsible for the loss of bond strength in this study is minimal. The artificial saliva always remained very clear, indicating that no microbial growth occurred.

In addition to its well-known disinfectant/antimicrobial benefits, CHX has been examined as a therapeutic primer for the preservation of hybrid layers in human dentin using etch-and-rinse adhesives.[30-32] While the antimicrobial efficiency of CHX on acid-etched dentin remains unclear, the current data confirms its positive effects on the preservation of bond strength when applied to demineralized normal dentin prior to bonding with no rinsing. CHX applied on exposed collagen fibrils, then sealed in place with adhesive resin/resin composites, may protect the collagen against a dentinal collagenolytic attack, thereby delaying the typical degradation seen when using contemporary, simplified dental adhesives.[30-32]

CHX is an effective synthetic MMP-inhibitor that, even in low concentrations (0.02 –0.0001%), could directly inhibit the activity of MMP-2, -8 and -9.[34] These are the same MMPs that have recently been found in human dentin.[24-26] At low concentrations, the inhibitory effect of CHX on MMPs is thought to be related to a cation-chelating mechanism, wherein the sequestration of metal ions, such as calcium and zinc, would hamper the activation of the catalytic domains within MMPs.[35-36] Nevertheless, the MMP inhibitory effect of CHX seems to be dose-dependent[34] and, at high concentrations, it may likely inactivate MMPs by enzyme denaturation[37] rather than by chelation of cations.

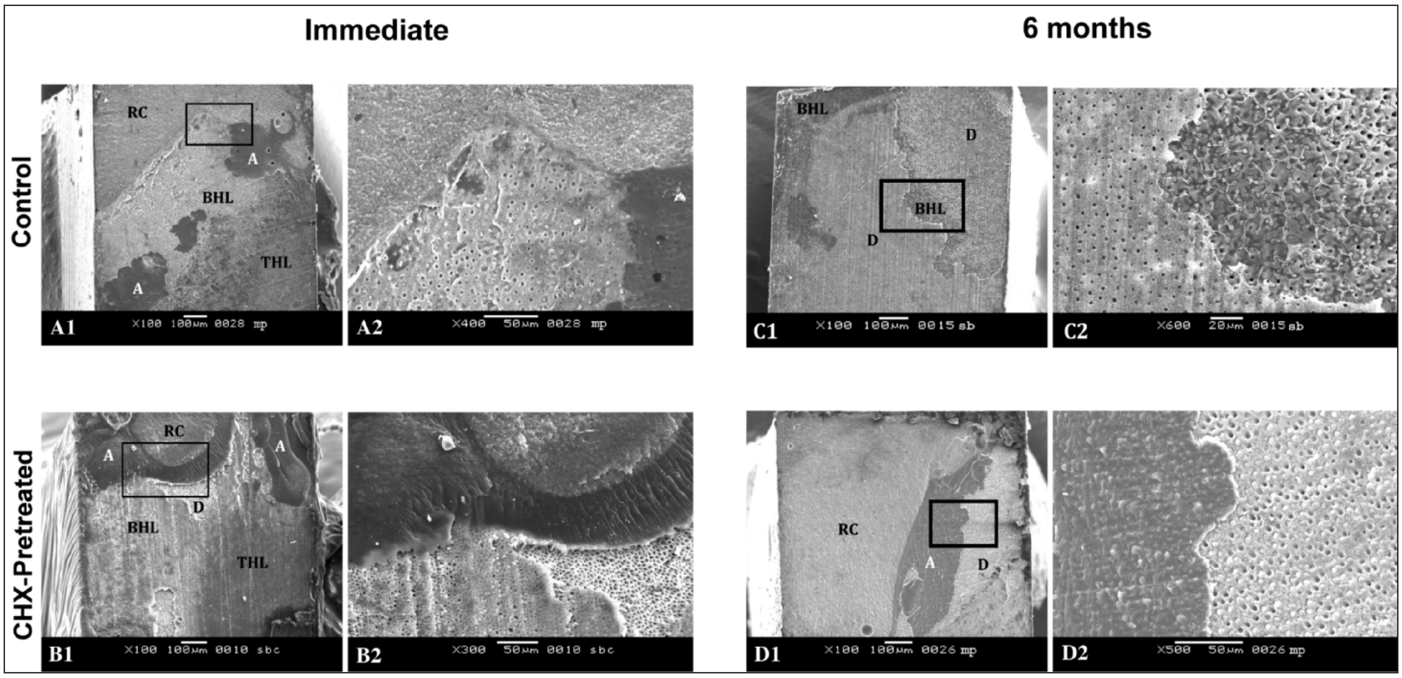

Figure 3: *Representative scanning electron micrographs (SEM) of fractured specimens composed of caries-affected dentin that was pretreated or not with chlorhexidine (**Control** or **CHX**) and submitted to tension immediately (**Immediate**) or after six months of storage in artificial saliva (**six months**). A1) Low-power magnification (100x) demonstrates a mixed failure of a control specimen immediately tested; A2) Higher magnification (400x) of the area limited by a rectangle in A1, showing cohesive failures in the adhesive (A), resin composite (RC) and the bottom of the hybrid layer (BHL). B1) Low-power magnification (100x) demonstrates a mixed failure of a CHX-pretreated specimen immediately tested; B2) Higher magnification (300x) of the area limited by a rectangle in B1, showing cohesive failures in the adhesive (A), resin composite (RC), bottom of the hybrid layer (BHL) and dentin (D). C1) Low-power magnification (100x) demonstrates a failure almost exclusively in dentin (D) for a control specimen tested after six months of storage in artificial saliva; C2) Higher magnification (600x) of the area limited by a rectangle in C1, showing a large cohesive failure in the dentin (D) and a restricted cohesive failure at the bottom of the hybrid layer (BHL). D1) Low-power magnification (100x) demonstrates a mixed failure of a CHX-pretreated specimen tested after six months of storage in artificial saliva; D2) Higher magnification (500x) of the area limited by a rectangle in D1, showing cohesive failures in the adhesive (A) and dentin (D). **RC**= resin composite; **A**= adhesive; **D**= dentin; **BHL**= bottom of hybrid layer; **THL**= top of hybrid layer.*

In this context, the reduction of bond strength in CHX-treated dentin would be more dependent on the degradation of hydrophilic polymerized adhesives. In fact, the current results also showed that, regardless of the dentin condition (normal or caries-affected), reductions in bond strength over six months were significantly greater for specimens bonded with the simplified two-step etch-and-rinse system—Single Bond 2—when compared to that observed for specimens bonded with the conventional three-step etch-and-rinse system—Scotchbond Multi Purpose (Figure 1). Although none of the current adhesive systems appear to guarantee perfect-sealed, leakage-free restorations for a significant period of time,[11] good clinical performance of the three-step etch-and-rinse adhesives over the simplified etch-and-rinse adhesives is notable.[38-39] The presence of a relatively more hydrophobic, non-solvated resin over hybridized dentin, when using three-step etch-and-rinse adhesives, may improve the quality of the dentinal sealing by decreasing the permeability of the resin-bonded interface.[14,40-42]

If adhesive resins can seal the acid-etched dentin from water, they may preserve their mechanical/chemical properties and, at the same time, protect the collagen against a hydrolytic attack by endogenous MMPs. MMPs are enzymes that require water to hydrolyze peptide bonds in the collagen molecules.[29] Thus, besides being important to guaranteeing the integrity of the resin components of hybrid layers,[43] the lower permeability of hydrophobic adhesives may have an additional effect on the durability of the resin-dentin interfaces by preventing water sorption into the dentinal matrix, thereby limiting collagen hydrolysis by dentinal MMPs.

Since carious dentin has been reported to exhibit increased collagenolytic activity when compared with intact normal dentin,[44] it was previously considered that the degradation of caries-affected dentin-bonded interfaces by dentinal collagenases may occur much more rapidly than in resin-bonded interfaces made in normal, non-carious dentin.[30] Interestingly, most of the currently tested caries-affected dentin-bonded specimens did not exhibit a significant reduction in bond strength over the six months of storage in artificial saliva, except for the control group bonded with Single Bond 2 (Figure 1B). The authors of the current study speculate that this result may be because the concentration of collagenases bound to collagen fibrils in caries-affected dentin may be relatively low. Nevertheless, the significant increase in the rate of cohesive failure in bonded caries-affected dentin accompanied by a higher number of premature debonding failures (Table 2) indicate that the mechanical stability of caries-affected resin-dentin bonds must be carefully interpreted.

As reported in previous studies,[4-5,45] the caries-affected/adhesive interfaces exhibited significantly lower bond strength values than normal dentin/adhesive interfaces, regardless of the adhesive (MP or SB) and dentin treatment (control or CHX) (Figure 2). Additionally, compared to non-carious dentin, the caries-affected dentin forms resin-bonded interfaces that are more complex, being composed of multiple zones (resin-infiltrated dentin, poorly-infiltrated dentin, exposed dentin and partially demineralized dentin).[6] The presence of these different zones, each with different stiffness/flexibility, raises concern whether this variable structure will be able to act as a strong foundation to dissipate stress from resin polymerization shrinkage, occlusal loading and thermal dimensional changes.[46] The softer the caries-affected dentin, the lower its ultimate tensile or cohesive strength.[9] It is this lower cohesive strength that might be responsible for the higher incidence of cohesive failures in caries-affected versus normal dentin (Table 2).

CONCLUSIONS

For optimal durability of resin-dentin bonds, preservation of resin and dentin matrix components should be equally considered. Although the current results do not prove that CHX can directly inhibit the collagenolytic activity of dentin, it is encouraging that CHX-treatment has once more demonstrated its therapeutic action on the preservation of bond strength of etch-and-rinse adhesives to normal dentin. While the current protocol requires additional confirmation, further studies should also test the applicability of CHX and other synthetic MMPs inhibitors as therapeutic primers combined with the use of hydrophobic dental adhesives applied to acid-etched dentin saturated with ethanol instead of water.[47]

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