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Two-year Effects of Chlorhexidine-containing Adhesives on the *In Vitro* Durability of Resin-dentin Interfaces and Modeling of Drug Release

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Clinical Relevance

The addition of diacetate chlorhexidine up to 0.2% is a viable method by which to provide a drug release system in adhesive systems to maintain stable resin-dentin adhesive interfaces after two years of water storage.

SUMMARY

Objectives: To evaluate the effects of addition of diacetate chlorhexidine (CHX) at different concentrations into two etch-and-rinse adhesive systems on CHX release, as well as the immediate (IM) and two-year (2-Y) resin-dentin microtensile bond strength (μTBS) and nanoleakage (NL).

Methods: CHX was added to XP Bond (XP) and Ambar (AM) at concentrations of 0.0 wt% (control); 0.01 wt%; 0.05 wt%; and 0.1 to 0.2 wt%. To assess the cumulative CHX release,

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adhesive disks were made in a metallic matrix and after light-curing were stored in water. Ultraviolet spectrophotometric measurements of the storage solution were performed to examine the release kinetics of CHX. For μTBS and NL, the occlusal enamel of molars was removed and the adhesives were applied to the dentin surface after acid etching. After composite resin build-up, specimens were sectioned to obtain μTBS sticks. The specimens were subjected to μTBS and NL at IM and after 2-Y. In addition, specimens underwent examination for CHX using micro–Raman spectroscopy. All data were submitted to statistical analysis (α=0.05).

Results: With regard to CHX release, AM showed a slower and gradual release of CHX while XP released CHX more quickly ($p<0.05$), and CHX was still present in the hybrid layers after 2-Y. Both adhesives showed CHX release at 2-Y water storage. Both CHX-containing adhesives showed higher μTBS values than did the control group ($p<0.05$).

INTRODUCTION

New restorative techniques and materials have become more and more available, allowing increasingly esthetic and conservative procedures. As a result, "Adhesive Dentistry" has gained more attention, with the success of most restorative procedures relying mostly on adhesive systems providing optimal and durable bonding to tooth substrates.

Current adhesive systems are made up of three basic steps: etch, primer, and adhesive (bond). Briefly, an acid is used to demineralize dentin and enamel substrates and to increase their porosity, and the pores are filled with a primer, which is subsequently covered by a hydrophobic agent (bond) to ensure bonding with the resin composite material.[1-3] In the dentin substrate, mineral removal by acid etching exposes the collagen mesh and noncollagenous proteins. It is known that commercially available adhesive systems invariably fail in dentin infiltration[4-8]; thus, many sites of dentin may remain exposed. Thus, matrix metalloproteinases (MMPs) and cysteine cathepsins, which are involved in the degradation of exposed collagen, remain during the bonding procedure via an endogenous proteolytic mechanism, even in the absence of bacteria. These assumptions have been confirmed by *in vitro* and *in vivo* studies.[6,9-14]

Alternatives have been proposed for improving the bonding of adhesives to tooth, such as the use of agents that exhibit enzymatic inhibition.[15] Studies have shown potential inhibition of MMPs in compounds such as epigallocatechin-3-gallate[16,17]; galardin[18]; tetracyclines[19]; benzalkonium chloride[20]; quaternary ammonium salts, such as 12-methacryloyloxydodecylpyridinium bromide[21,22]; and ethylenediamine tetraacetic acid salt,[23,24] thus helping preserve the hybrid layer.

The most commonly and extensively tested compound is chlorhexidine (CHX),[25,26] which is an antimicrobial agent and is one of the first protease inhibitors evaluated in dentistry.[14] However, a majority of the *in vitro* studies applied CHX as an additional primer on acid-etched dentin. This procedure adds an extra step to the bonding protocol and does not fulfill the clinicians' need for simplification.

Sabatini[27] suggested that the inclusion of these protease inhibitor substances into one of the components of the bonding protocol could be an interesting alternative. Recently, the addition of CHX to the acid etchant[28,29] or to the adhesive solution[27,30-34] has been shown to effectively protect the adhesive interface against degradation over time. However, although the addition of CHX to the acid etchant showed promising results, a CHX reservoir is not produced immediately after acid etching. It has been hypothesized[33] that any approach that could provide this controlled release of CHX within the demineralized dentin could improve the durability of the resin/dentin interface by slightly transferring CHX to adjacent collagen. The CHX inclusion into primers and/or adhesives could produce a reservoir for a controlled release. However, no study to date has been found that evaluated the two-year stability of resin/dentin interfaces to CHX-containing adhesive as well as the kinetics of CHX release after two-year storage in water.

Therefore, this *in vitro* study evaluated the effects of CHX diacetate concentration added to two simplified etch-and-rinse (ER) adhesive systems on the release profile of CHX from adhesives as well as the immediate and two-year effects on the micro-tensile bond strength (μTBS) to dentin and nanoleakage at the bonding interface. In addition, micro–Raman spectroscopy was used to detect CHX within the hybrid layer. The following null hypotheses were tested: 1) There is no difference in the CHX release profile for each adhesive system evaluated; 2) The addition of CHX to adhesive composition at different concentrations does not impair immediate μTBS values to dentin and nanoleakage pattern at the bonding interface; and 3) There is no significant difference between immediate and two-year μTBS

Table 1: *Adhesive Systems: Composition and Application Mode*

Adhesive Systems	Composition	Application Mode
XP Bond (DENTSPLY)	TCB-resin, *terc*-butyl alcohol, PENTA, PPD, UDMA, TEGDMA, HEMA, modified carboxylic acid, nanofiller and dimethacrylate	1. Apply phosphoric acid to dentin for 15 s 2. Rinse for 15 s. Dry with absorbent paper. Keep dentin wet. 3. Apply the adhesive for 20 s undisturbed. 4. Gently air for 5 s to evaporate the solvent. 5. Light-cure for 20 s.
Ambar (FGM)	Methacrylate monomers (UDMA and MDP), photoinitiators, co-initiators, stabilizers, inert silica nanoparticles and ethanol	1. Apply phosphoric acid to dentin for 15 s. 2. Rinse for 15 s. Dry with absorbent paper. Keep dentin wet. 3. Apply two coats vigorously by rubbing the adhesive for 20 s (10 s each). 4. Gently air for 10 s to evaporate the solvent. 5. Light-cure for 10 s.

Abbreviations: HEMA, 2-hydroxyethyl methacrylate; PENTA, pentaerythritol pentacrylate monophosphate; PPD, phenylpropanoid; TCB-resin, 1,2,3,4-butane-tetracarboxylic acid ester di2-hydroxyethylmethacrylate; TEGDMA, triethylene glycol dimethacrylate; 10-MDP, 10-methacryloyl oxidecil dihydrogenphosphate; UDMA, urethane dimethacrylate or 1,6-di (methacryloyloxyethylcarbamoil)-3,30,5-trimethylhexano.

values to dentin and nanoleakage patterns at the bonding interface when adhesive systems with CHX are used, regardless of CHX concentration.

METHODS AND MATERIALS

Formulation of the Experimental Adhesives

Four experimental adhesive systems were formulated using the simplified ER adhesive systems XP Bond (XP; Dentsply, York, PA, USA) and Ambar (AM; FGM Produtos Odontológicos Ltda, Joinville, SC, Brazil), according to the addition of different concentrations of CHX (99.9% pure chlorhexidine diacetate; Sigma Chemical Company, St Louis, MO, USA) (wt%): 0.01, 0.05, 0.1, and 0.2. The CHX was added to the adhesive and mechanically mixed by a motorized mixer (stirring). The bonding agents were also tested without any CHX added to their composition (control, commercial material). Therefore, a total of five groups for each adhesive were tested in the present study. Table 1 depicts their detailed composition and application mode.

Release Profile of CHX from Adhesives

Water standard solutions containing 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 μg/mL of CHX were made to obtain an analytical curve with a linear regression between absorbance values and CHX concentrations, using the Genesys 10S UV-Vis Spectrophotometer (Thermo Scientific, Madison, WI, USA). The maximum absorbance of CHX at 260 nm (data not shown) was confirmed. Afterwards, 10 resin discs of each experimental group were produced in a brass mold (5.8 mm diameter, 1.0 mm thick). The unpolymerized adhesive was dispensed to completely fill the mold, and all visible air bubbles trapped in the adhesives were carefully removed. An air stream evaporated the solvent for 40 seconds at a distance of 10 cm. Under a glass cover slip, the adhesive was light-cured for 40 seconds using an LED light source at 600 mW/cm^2 (Radii-cal, SDI, Victoria, Australia). Then, the specimens were removed from the brass mold without undergoing permanent deformation, and each one was individually stored in deionized water.

At appropriate time intervals (one, three, and 12 hours and one, two, three, four, five, six, seven, 10, 12, 14, 21, and 28 days and two years), absorbance values of these storage solutions were obtained at 260 nm and converted into the amount of CHX released based on the linear analytical curve. Thus, the ultraviolet absorbance values at 260 nm of the control groups (0% CHX discs) were subtracted from the values produced from the CHX-containing discs, considering the concomitant monomer release. The cumulative release for the two-year period was represented as the percentage of CHX released and the mass (in milligrams) of CHX release per gram of the adhesive sample.[35,36] MicroMath Scientist TM 2.01 software (Salt Lake City, UT, USA) used mathematical models[37] to build cumulative profiles of CHX released from resin discs and to evaluate the CHX release profile.

Data were fit to first-order, biexponential, zero-order, Weibull, and monolag equations (Table 2). The best fit was chosen considering the correlation coefficient (r), the model selection criteria (MSC), and graphical adjustment. The power law (Korsmeyer-Peppas model: $ft=a\times t^n$) was the semiempirical equation developed to simulate the CHX release mechanism and to describe drug release from polymeric systems.[38] In this equation, ft is the

Table 2: *Mathematical Models Related to Chlorhexidine (CHX) Release Experiments*

Model	Equation[a]
Monoexponential	$\%D = 100(1 - e^{-kt})$
Biexponential	$\%D = 100[1 - (Ae^{-\alpha t} + Be^{-"\beta t})]$
Order zero	$\%D = kt$
Weibull	$\%D = 100[1 - e^{-(t/TD)b}]$
Monolag	$\%D = 100[1 - e^{-k(t-x)}]$

[a] *Where % is the percentage of dissolved drug over time; t, k, α, and "β are the observed dissolution rate constants; A and B represent the initial concentrations of the drug contributing to the two stages of dissolution; TD indicates the time in which 63.2% of the drug is dissolved; and b is the parameter related to structural and geometric characteristics of the resin disc.*

dissolved fraction of CHX at time t; n is the release exponent, indicative of the mechanism of the substance release; and a is the constant incorporating structural and geometric features of the resin disk. After the best model that described the release of CHX from the adhesive systems was determined, the time required to allow 50% release of CHX from each material was calculated, assuming that this equation remained as the dominant release mechanism over time, using MathWorks Matlab TMR 2012a software (Natick, MA, USA).[38]

Tooth Preparation and Bonding Procedures

Twenty-five caries-free extracted human third molars were used. The teeth were collected after the patient's informed consent was obtained. The teeth were disinfected in 0.5% chloramine and stored in distilled water. A flat dentin surface was exposed on each tooth after wet-grinding the occlusal enamel with 180-grit silicon-carbide (SiC) paper. The enamel-free, exposed dentin surfaces were further polished with 600-grit SiC paper for 60 seconds to standardize the smear layer. The adhesives were applied following the manufacturers' instructions (Table 1) and were light-cured using an LED light for 10 seconds at 600 W/cm^2 (Radii-cal, SDI). Resin composite blocks (Opallis, FGM) were incrementally built up on the bonded surfaces (three 1 mm–thick increments), and each resin layer was cured using the same curing light for 40 seconds. A single operator performed all bonding procedures in an environment with controlled temperature and humidity. Five teeth were used for each experimental group (n=5). After storage of the bonded teeth in distilled water at 37°C for 24 hours, they were longitudinally sectioned in both "x" and "y" directions across the bonded interface using a diamond saw in an automated sectioning device (Labcut 1010, Extec Corp, Enfield, CT, USA) under water cooling at 300 rpm to obtain bonded sticks with a cross-sectional area of approximately 0.8 mm^2. The cross-sectional area of each stick was measured using a digital caliper (Absolute Digimatic, Mitutoyo, Tokyo, Japan) to the nearest 0.01 mm and recorded for subsequent calculation of the μTBS. The bonded sticks from the same tooth were randomly divided and assigned to be tested immediately (IM) or after two-year storage [2-Y] in distilled water at 37°C. The storage solution was not changed and its pH was monitored monthly.

μTBS Test

Seven to nine bonded sticks from each tooth at each storage period were attached to a modified device for μTBS testing using cyanoacrylate resin (Super Bonder, Loctite, São Paulo, SP, Brazil) and subjected to a tensile force in a universal testing machine (Kratos, São Paulo, SP, Brazil) at 0.5 mm/min. The failure mode was evaluated at 40× (HMV-2, Shimadzu, Tokyo, Japan) and was classified as cohesive in dentin (failure exclusive within dentin; CD); resin cohesive in resin composite (failure exclusive within resin; CR); adhesive (failure at resin/dentin interface; A), or mixed (failure at resin/dentin interface that included cohesive failure of the neighboring substrates; M).

Nanoleakage (NL) Evaluation

Two bonded sticks from each tooth at each storage period that were not tested for μTBS were subjected to NL evaluation. All sticks were placed in 50 wt% ammoniacal silver nitrate in darkness for 24 hours, thoroughly rinsed 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. Specimens were mounted on aluminum stubs and polished with 1000-grit SiC paper and 6-, 3-, 1-, and 0.25-μm diamond paste (Buehler Ltd, Lake Bluff, IL, USA). Afterwards, they were ultrasonically cleaned, air-dried and gold sputter-coated (MED 010, Balzers Union, Balzers, Liechtenstein) for analysis in a scanning electron microscope (SEM) operated in the backscattered mode and using energy-dispersive X-ray spectrometry (LEO 435 VP, LEO Electron Microscopy Ltd, Cambridge, UK).

Three pictures were taken of each specimen. The first picture was taken in the center of the bonded stick. The other two pictures were taken 0.3 mm to

Table 3: *Chlorhexidine (CHX) Release (in % and mg/g) for All Experimental Conditions After 28 Days and Two Years of Water Storage*[a]

Adhesive System	CHX Concentration, wt%	28 Days		2 Years	
		% of the Original Concentration,	mg/g	% of the Original Concentration,	mg/g
XP Bond	0.01	25.0 ± 1.1 A	0.025 ± 0.013 e	2.0 ± 0.2 B	0.002 ± 0.003 g
	0.05	23.0 ± 1.2 A	0.115 ± 0.002 c	1.6 ± 0.4 B	0.008 ± 0.002 f
	0.1	24.5 ± 1.4 A	0.245 ± 0.018 b	3.0 ± 0.8 B	0.030 ± 0.018 e
	0.2	22.6 ± 1.5 A	0.452 ± 0.047 a	4.2 ± 0.7 B	0.083 ± 0.047 d
Ambar	0.01	10.0 ± 0.9 C	0.010 ± 0.003 k	9.0 ± 0.7 C	0.009 ± 0.003 k
	0.05	10.4 ± 0.7 C	0.052 ± 0.003 j	10.6 ± 0.9 C	0.053 ± 0.004 j
	0.1	13.2 ± 1.1 C	0.132 ± 0.045 i	10.2 ± 0.9 C	0.103 ± 0.045 i
	0.2	12.0 ± 1.2 C	0.240 ± 0.048 h	10.5 ± 1.1 C	0.210 ± 0.048 h

[a] *Comparisons are valid only within adhesive. Analysis per column (n=10 per group). For each adhesive, same capital letters for CHX (%) and lowercase for CHX (mg/g) indicate that there is no statistically significant difference ($p>0.05$).*

the left and right of the first one. As two bonded sticks per tooth were evaluated and a total of five teeth were used for each experimental condition, a total of 30 images were evaluated per group. All images were taken by a technician who was blinded to the experimental conditions. The relative NL percentage within the adhesive and hybrid layer areas was measured in all pictures using image editing software (UTHSCSA ImageTool 3.0 software, University of Texas Health Science Center, San Antonio, TX, USA).

CHX Detection by Micro–Raman Spectroscopy

Two bonded sticks from each tooth at each storage period that were not tested for μTBS were used for CHX detection at the resin/dentin bonding interface. Micro–Raman spectroscopy was performed using Senterra spectroscopy (Bruker Optik; Ettlingen, Germany). The micro–Raman spectrometer was first calibrated for zero and then for coefficient values using a silicon sample. The bonding area was analyzed using the following micro-Raman parameters: 20-mW neon laser with 532-nm wavelength, spatial resolution of ca 3 pm, spectral resolution of ca 5 cm^{-1}, and 100× magnification (Olympus UK, London, UK) to a ca 1-pm beam diameter.[31,39]

Spectra were taken in the middle of the hybrid layer, in an arbitrary area of the intertubular dentin. Care was taken to select an area between two dentin tubules. One site per slice was examined. Accumulation time per spectrum was 30 seconds, and six co-additions were taken per point. Postprocessing of spectra was performed using the dedicated Opus Spectroscopy Software, version 6.5 (Bruker Optik) and consisted of analysis with modeling, which allowed distinguishing spectral components of the adhesive and dentin.

Statistical Analysis

For μTBS and NL, the experimental unit in the current study was the hemi-tooth, since half of the tooth was tested IM and the other half was tested after 2-Y of water storage. The μTBS and NL values of all sticks from the same hemi-tooth were averaged for statistical purposes. The μTBS (MPa) and NL (%) data of each adhesive were subjected to two-way repeated-measures analysis of variance (ANOVA; CHX concentration and storage time). No comparison was made between products. For CHX release, the data (% and mg/g) for each adhesive were analyzed by two-way ANOVA (CHX concentration vs time). A Tukey post hoc test was used for pairwise comparisons ($\alpha=0.05$) using the Statistica for Windows software (StatSoft, Tulsa, OK, USA).

RESULTS

Release Profile of CHX from Adhesive Systems

The analytical curve with linear regression between absorbance values and CHX concentrations had a correlation coefficient of $r = 0.99959$, making it suitable for determining the CHX release (data not shown). The release profiles were fitted to mathematical models and the best model was selected based on the correlation coefficient (r), the MSC (Table 2), and graphic adjustment was conducted using the biexponential equation.[33]

The CHX release values at times 28 days and two years are presented in Table 3. For both adhesives, the cross-product interaction was statistically significant ($p=0.0001$ and $p=0.001$, respectively, for the

Table 4: *Number (%) of Specimens According to Fracture Pattern Mode for All Experimental Conditions Immediately and After Two Years of Water Storage*

CHX Concentration, wt%	Ambar				XP Bond			
	Immediate		2 Years		Immediate		2 Years	
	A/M	C	A/M	C	A/M	C	A/M	C
Control (without CHX)	34 (81.0)	8 (19)	30 (83.4)	6 (16.6)	31 (81.6)	7 (18.4)	27 (77.2)	8 (22.8)
0.01	38 (79.2)	10 (20.8)	32 (84.3)	6 (15.7)	31 (75.7)	10 (24.3)	35 (77.7)	10 (23.3)
0.05	39 (81.3)	9 (18.7)	37 (88.1)	5 (11.9)	34 (81)	8 (19)	29 (78.4)	8 (21.6)
0.1	37 (80.5)	9 (19.5)	33 (86.9)	5 (13.1)	30 (69.8)	13 (30.2)	36 (94.8)	2 (5.2)
0.2	32 (72.7)	12 (27.3)	33 (76.8)	10 (23.2)	37 (74)	13 (26)	32 (82.1)	7 (17.9)

Abbreviations: A/M, adhesive/mixed fracture mode; C, cohesive fracture mode; CHX, chlorhexidine.

percentage of the original concentration and the amount in mg/g). Different CHX concentrations influenced CHX release from the tested adhesive systems. In general, a higher CHX concentration resulted in higher CHX release in both products, and AM showed slower and gradual release when compared to XP, which released CHX at an apparently higher rate. The total amount of released CHX was proportional to the initial concentration of CHX added to the adhesives.

μTBS Test

The fracture patterns of all experimental groups are shown in Table 4. No premature failures were observed in the present study. Cohesive failures were not included in the statistical analysis because of the lower number of specimens with those failure modes. The μTBS means and standard deviations for all groups are shown in Table 5. For both adhesives, the cross-product interaction was statistically significant ($p=0.002$ and $p=0.001$ for AM and XP, respectively). A μTBS drop was noted over time in all XP groups and most AM groups ($p=0.001$ and $p=0.002$, respectively). However, this decrease was much more pronounced in the control group (reduction of 40% to 53%) when compared with the CHX-containing adhesive systems (reduction ranged from 16% to 23% for AM and from 29% to 33% for XP). The only exception was observed when CHX at 0.1 wt% and 0.2 wt% was added to AM, in which case no significant differences were observed between the IM and 2-Y μTBS values.

Nanoleakage

The NL means and standard deviations are shown in Table 6. The cross-product interaction was statistically significant for both adhesives ($p=0.03$ and $p=0.0001$ for AM and XP, respectively). None of the conditions resulted in NL-free interfaces.

For AM and XP, significant differences between immediate and 2-Y groups were seen for all formulations ($p=0.03$ and $p=0.0001$, respectively). However, the highest NL was found in the control group when compared to the NL observed on the bonding interfaces created by CHX-containing adhesives. The 2-Y NL values in the experimental groups were significantly lower than the values observed in the control groups mainly when 0.1 wt% and 0.2 wt% of CHX were added to both adhesives.

Illustrative SEM images of the bonding interfaces created in the control group and experimental group

Table 5: *Means and Standard Deviations of the Microtensile Bond Strength (MPa) for All Experimental Conditions Immediately and After Two Years of Water Storage*[a]

CHX Concentration, wt%	Ambar			XP Bond		
	Immediate	2 Years	% Reduction	Immediate	2 Years	% Reduction
Control (without CHX)	50.3 ± 4.1 A	30.1 ± 4.3 C	40.2	60.2 ± 3.1 a	28.1 ± 3.9 c	53.3
0.01% CHX	53.8 ± 4.0 A	44.8 ± 2.3 B	16.7	63.3 ± 4.7 a	41.9 ± 3.9 b	33.8
0.05% CHX	50.0 ± 4.7 A	41.5 ± 4.0 B	17.0	59.4 ± 4.0 a, b	40.3 ± 3.9 b	32.1
0.1% CHX	52.7 ± 3.5 A	47.4 ± 3.4 A, B	10.0	64.1 ± 4.0 a	45.5 ± 3.9 b	29.1
0.2% CHX	55.6 ± 3.3 A	49.8 ± 3.1A	10.4	63.2 ± 3.1 a	44.7 ± 3.9 b	29.3

Abbreviations: CHX, chlorhexidine.
[a] *Comparisons are valid only within adhesives. For each adhesive, means with the same capital or lowercase letters indicate means statistically significantly different (Tukey test, p>0.05).*

Table 6: *Means and Standard Deviations of the Nanoleakage (%) for All Experimental Conditions After 24 Hours and Two Years of Water Storage*[a]

CHX Concentration, wt%	Ambar		XP Bond	
	Immediate	2 Years	Immediate	2 Years
Control (without CHX)	16.3 ± 4.5 A	24.3 ± 3.1 C	24.4 ± 3.6 a	44.1 ± 4.3 c
0.01	12.5 ± 3.8 A	22.3 ± 3.6 B, C	26.5 ± 3.9 a	36.5 ± 4.7 b
0.05	14.5 ± 4.1 A	21.3 ± 2.6 B, C	27.4 ± 4.6 a	39.7 ± 4.9 b, c
0.1	13.6 ± 4.4 A	17.2 ± 3.3 B	24.5 ± 4.1 a	33.5 ± 3.8 b
0.2	14.3 ± 3.9 A	18.4 ± 4.3 B	26.1 ± 3.3 a	34.5 ± 4.7 b

[a] *Similar uppercase letters indicate means statistically similar (p>0.05) (Tukey test, α=0.05).*

using 0.2 wt% CHX from both adhesive systems at immediate and 2-Y intervals are shown in Figure 1. In the immediate period, NL was nearly restricted to the hybrid layer for both adhesives. Although we did not compare products statistically, XP Bond presented an apparently higher amount of NL, regardless of the CHX concentration, than did Ambar. After two years, all groups showed noticeably more NL, which was present in almost the entire thickness of the hybrid layer and was more pronounced in the control group.

Identification of CHX by Micro–Raman Spectroscopy

Representative Raman spectra performed at the adhesive interface for both adhesive systems are shown in Figures 2 and 3. The relative intensities for CH_2 and CH_3 deformation (1450 cm^{-1}) as well as C-O-C phenyl groups (1113 cm^{-1}) and CH-OH (1260 cm^{-1}) vibrations are associated with methacrylate monomers in the hybrid layer. All these peaks are typically observed when resin/dentin interfaces are evaluated.[39-41]

The peaks associated with CHX digluconate are around 1585 cm^{-1}.[42] This peak was not observed in the control group (Figures 2 and 3). However, it was clearly identified in all CHX-containing adhesives (0.01 wt% and 0.2 wt% CHX groups, Figures 2 and 3). These figures were very similar for the immediate observation and after two years of water storage, indicating that CHX is still present within the hybrid layer even after two years of water storage.

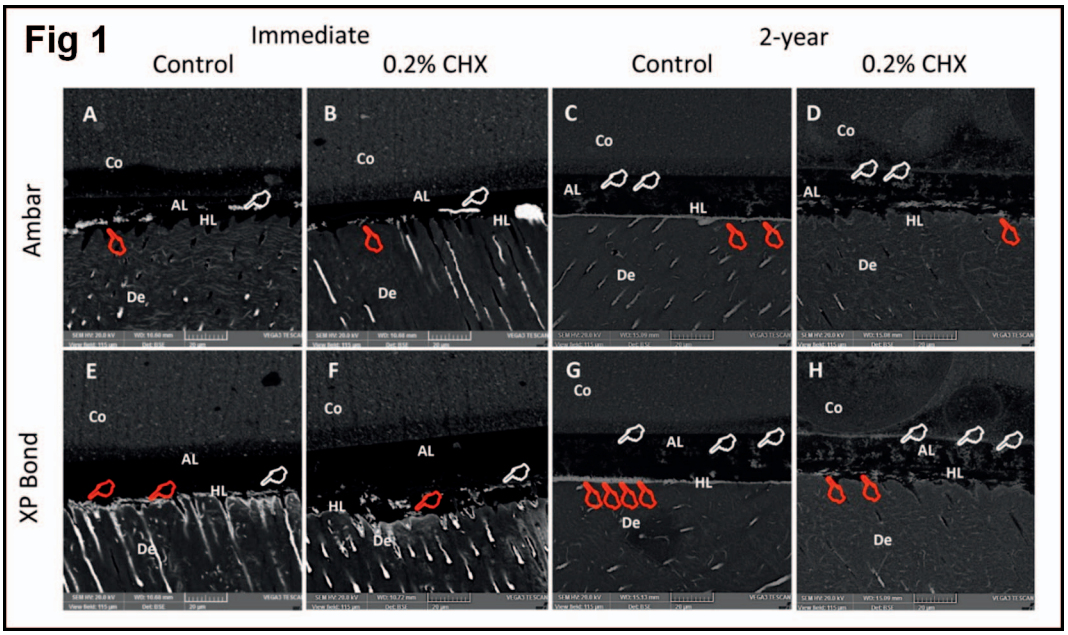

Figure 1. *Scanning electron micrographs of the adhesive interfaces of the experimental groups. Silver nitrate deposits in all groups are mainly within the hybrid layer (red hands), although some deposits were also found within the adhesive layer (white hands). However, after two years of water storage, this deposition was more pronounced in the control group (C and G) when compared to the CHX groups (D and H). As the CHX-containing adhesive showed similar amount and pattern of silver nitrate deposition, we decided to show only one of the groups (Co = composite resin; AL = adhesive layer; HL= hybrid layer; De = dentin).*

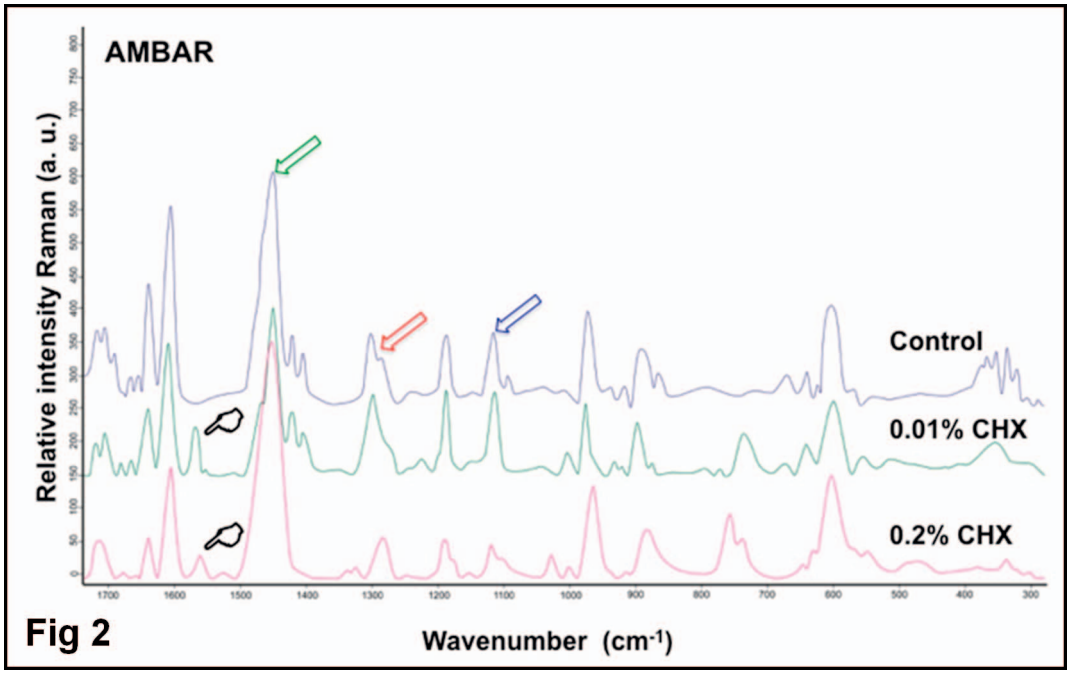

Figure 2. *Raman line-spectra acquired at the adhesive/dentin interface created by Ambar (without CHX [control] and with a 0.01 wt% CHX and 0.2 wt% CHX). The relative intensities for CH2, CH3 deformation (1450 cm^{-1}; green arrow), C-O-C phenyl groups (1113 cm^{-1}; blue arrow), and CH-OH (1260 cm^{-1}; red arrow) vibrations are associated with methacrylate monomers in the hybrid layer; this was observed in all groups. The representative peak of CHX diacetate (1585 cm^{-1}) was only evident in the CHX groups (black hands).*

DISCUSSION

In the current study, a higher concentration of CHX within the bonding agent resulted in a greater amount of released CHX. Moreover, AM showed slower and more gradual release in comparison to that observed in XP. Thus, the first hypothesis was rejected. Differently from the releasing mechanism observed when CHX was added to a primer or to a phosphoric acid, CHX release from bonding agents is quite a bit more complex. When the polymer structure is created after light curing, a porous polymer network composed mostly of hydrophilic monomers and some hydrophobic monomers is formed.[7,8] The amount of hydrophilic monomer within the polymer network determines water sorption and solubility of the bonding agent.[43] Some studies[33,35] have shown a direct positive correlation between these properties and the release profile of drugs added to the bonding agents. In other words, bonding agents with higher water sorption and solubility are capable of releasing more CHX at a higher rate than are bonding agents with lower water sorption and solubility. Differently from AM, XP bond has the highly hydrophilic monomers 2-hydroxyethyl methacrylate (HEMA) and dipentaerythritol pentacrylate monophosphate (PENTA), which were responsible for the higher water sorption and solubility than that observed in AM. As a consequence, faster CHX release from XP Bond (23% of the amount of CHX initially added to the bonding agent) was observed within 28 days when compared to CHX release from AM (11.4% of the amount of CHX initially added to the bonding agent).

After the initial burst release of CHX, both bonding agents showed a continuous but slow release after two years. These results corroborate previous findings[33,35] and may be explained by the water sorption mechanism. After swelling, the mass change in the resin discs due to polymer swelling attains an equilibrium level.[35] After that point, water was not capable of penetrating the polymer network as it did within 28 days[35]; therefore, a small amount of CHX was released from both bonding agents over two years. Indeed, because AM presented lower water sorption and solubility, the slower and continuous water absorption in AM resulted in the apparently higher 2-Y CHX release from AM (an average of 7.8% of the total amount of CHX added to the bonding agent) when compared to that shown by XP (an average of 2.7%).

The addition of CHX to the bonding agents did not impair the immediate μTBS to dentin, nor did it

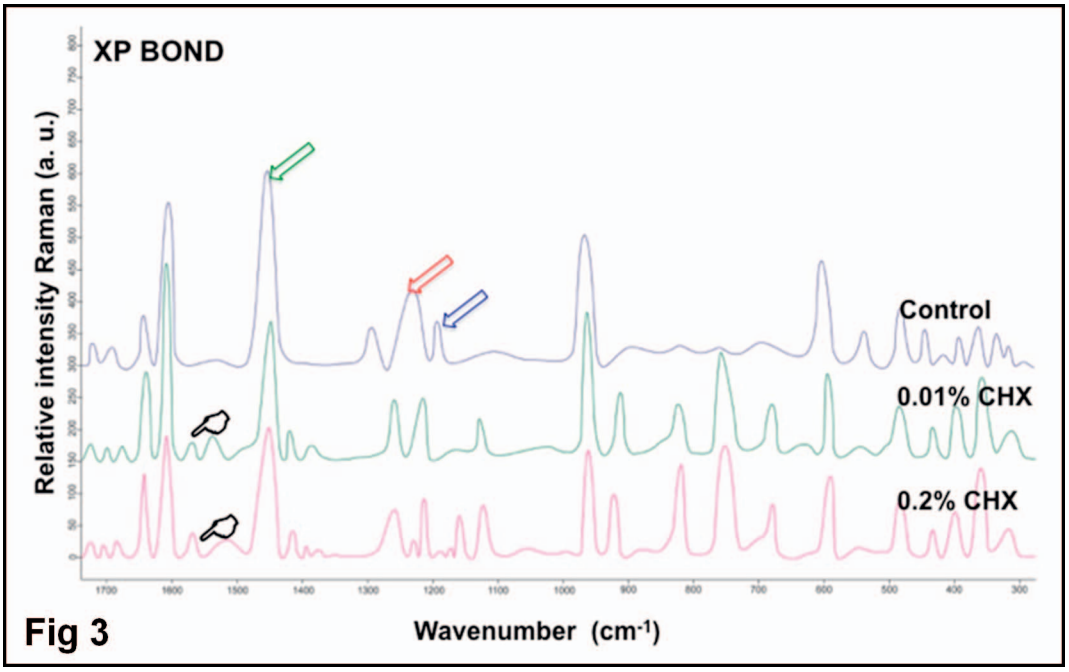

Figure 3. *Raman line-spectra acquired at the adhesive/dentin interface created by XP Bond (without CHX [control] and with a 0.01 wt% CHX and 0.2 wt% CHX). The relative intensities for CH2, CH3 deformation (1450 cm^{-1}; green arrow), C-O-C phenyl groups (1113 cm^{-1}; blue arrow), and CH-OH (1260 cm^{-1}; red arrow) vibrations are associated with methacrylate monomers in the hybrid layer; this was observed in all groups. The representative peak of CHX diacetate (1585 cm^{-1}) was only evident in the CHX groups (black hands).*

impair the NL pattern, so the second hypothesis was accepted. These results corroborate other findings[32,33] and may be mainly attributed to the low CHX content added to the bonding agents. Conversely, previous studies demonstrated that the addition of higher concentrations of CHX (from 1% to 5%) into the bonding agent caused increased resin solubility[35] and decreased modulus of elasticity and degree of conversion of the formed polymer.[44] In addition, CHX diacetate was evaluated in the current study, while other studies[34,45] have incorporated CHX digluconate. The main difference between these compounds is that CHX diacetate is available as a powder, while CHX digluconate is only available as an aqueous solution. In other words, the addition of CHX digluconate to the bonding agents would also add water to the formulation, damaging the adhesive properties through water entrapment.[46,47]

The presence of CHX diacetate did not eliminate hybrid layer degradation within two years in most tested conditions, as lower μTBS values were observed when compared to the immediate values. The only exceptions were noted when AM with CHX at 0.1 wt% and 0.2 wt% were tested, since no significant differences between the immediate and 2-Y μTBS values were recorded. Therefore, the third hypothesis was rejected. It should be noted, however, that all experimental groups showed higher 2-Y μTBS values than did the control groups. Therefore, CHX was apparently capable of attenuating the deleterious effects of MMPs on exposed collagen fibrils, even when a very small amount of CHX was released from the bonding agents. Therefore, it should be noted that CHX has a strong affinity to the dental structure, as it binds to the phosphate groups of mineralized dentin crystallites and to the carboxyl groups of the collagen matrix.[48] This interaction between CHX and dentin matrices is based on electrostatic forces between protonated $NH3^{+}$ in the CHX molecule and negative molecular electrostatic potential of $COOH^{-}$ and OH^{-} in dentin. Therefore, the initial release of CHX may have oversaturated the enzyme binding sites and remained bound to collagen fibrils for later release along with the slow continuing release overtime.[15] For this reason, it is not possible to attribute the CHX protective activity against collagen degradation solely to the small amounts of CHX released from the bonding agents over time. The presence of CHX within the bonding interface over time was confirmed by the Micro-Raman analysis, since the Raman spectra of bonding agents containing CHX still showed the peak referring to the presence of this drug even after two-year storage in water.

Despite the evidence that long-term release of CHX from the bonding agents protected exposed collagen fibrils against collagenolytic attack, most experimental groups still showed a drop in μTBS over time. One could state that most evaluated concentrations were not capable of effectively inhibiting the effects of MMPs to prevent its catalytic activation. However, it should be mentioned that the drop in mechanical strength of the bonding interface after 2-Y is not only related to collagen degradation but also to the degradation of other constituents, such as the composite resin and bonding agent.[18] In this regard, the results indicated that CHX cannot prevent polymer degradation. In other words, water swelling of the bonding agent within the hybrid layer may also result in hygrothermal degradation during aging, such as swelling stresses and the formation of microcracks,[49] which impair the mechanical properties of the plasticized polymer within the hybrid layer. This also helps explain why AM, an adhesive system with lower water sorption and solubility, showed an overall lower percentage of μTBS drop over time than did XP, a bonding agent with more hydrophilic monomer content and higher water sorption and solubility. These results were confirmed by the nanoleakage pattern and SEM analysis, as bonding interfaces created by XP clearly showed more silver deposition than those created by AM. Indeed, the use of bonding agents with lower water sorption and solubility, such as AM, having CHX added at 0.1 wt% or 0.2 wt%, resulted in the most stable bonding interface among all groups. Therefore, the current results demonstrated that the stability of the bonding interface does not rely solely on the CHX dose but also on the mechanical properties of the bonding agent.

In the current study, only two ER adhesive systems were tested, so the results cannot be extrapolated to other adhesive systems, such as self-etching or universal systems. In addition, all analyses were performed on sound, intact third molars. Therefore, different results might be expected when bonding agents containing CHX are applied to caries-affected dentin. Further studies are required to address these issues.

CONCLUSIONS

Within the limitations imposed by this *in vitro* study, the following conclusions were made:

- Although both bonding agents showed an initial CHX burst release profile followed by continuous low release, the general release profile was product-dependent. None of the CHX concentrations impaired the immediate μTBS values and NL patterns, regardless of bonding agent.
- Although most groups showed lower long-term μTBS values when compared to the immediate values, the use of CHX-containing bonding agents resulted in more stable bonding than was associated with the use of regular commercial products.

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Regulatory Statement

This study was conducted in accordance with all the provisions of the local human subjects oversight committee guidelines and policies of the State University of Ponta Grossa. The approval code for this study is 1693/09.

Conflict of Interest

The authors of this manuscript certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

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