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ABSTRACT

Host-derived proteases have been reported to degrade the collagen matrix of incompletely-resin-infiltrated dentin. This study tested the hypothesis that interfacial degradation of resin-dentin bonds may be prevented or delayed by the application of chlorhexidine (CHX), a matrix metalloproteinase inhibitor, to dentin after phosphoric acid-etching. Contralateral pairs of resin-bonded Class I restorations in non-carious third molars were kept under intra-oral function for 14 months. Preservation of resin-dentin bonds was assessed by microtensile bond strength tests and TEM examination. *In vivo* bond strength remained stable in the CHX-treated specimens, while bond strength decreased significantly in control teeth. Resin-infiltrated dentin in CHX-treated specimens exhibited normal structural integrity of the collagen network. Conversely, progressive disintegration of the fibrillar network was identified in control specimens. Auto-degradation of collagen matrices can occur in resin-infiltrated dentin, but may be prevented by the application of a synthetic protease inhibitor, such as chlorhexidine.

KEY WORDS: etch-and-rinse adhesive, *in vivo*, hybrid layer, degradation, chlorhexidine, MMP.

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INTRODUCTION

The susceptibility of contemporary dentin adhesives to water/oral fluid sorption, polymer swelling, and consequent resin leaching has been associated with *in vitro* and *in vivo* manifestations of resin-dentin bond degradation (De Munck *et al.*, 2003; Hashimoto *et al.*, 2003). Apart from these extrinsic factors, intrinsic, host-derived enzymes also appear to be involved in the breakdown of hybrid layers (Hashimoto *et al.*, 2003; Pashley *et al.*, 2004; Tay *et al.*, 2005).

Collagenases are the only proteinases known specifically to cleave native triple-helical collagen at neutral pH (Birkedal-Hansen, 1993). Dentin contains matrix metalloproteinases (MMPs), a group of neutral zinc- and calcium-dependent enzymes that regulate the physiologic and pathologic metabolism of collagen-based tissues (Tjäderhane et. al., 2002; Chaussain-Miller *et al.*, 2006). Evidence of collagenolytic and gelatinolytic activities in partially demineralized dentin treated with etch-and-rinse adhesives (Mazzoni *et al.*, 2006) highlights the potential involvement of these endoproteases in the disruption of incompletely infiltrated collagen fibrils within hybrid layers.

A recent *in vivo* study conducted in primary-school children demonstrated that collagen degradation can occur as early as 6 mos in dentin bonded with a simplified etch-and-rinse adhesive. This rapid degradation may be attributed to the bonding procedures being performed on caries-affected dentin. That study also showed that application of chlorhexidine, a broad-spectrum protease inhibitor, to demineralized dentin prior to bonding preserved collagen integrity for at least 6 mos (Hebling *et al.*, 2005). However, the mechanical correlates of the *in vivo* loss of collagen cross-banding were not measured and require further investigation. Thus, the objective of this *in vivo* study was to evaluate the morphological and mechanical properties of chlorhexidine-pre-treated caries-free dentin bonded with an etch-and-rinse adhesive. The null hypotheses tested were that degradation of the hybrid layer does not take place in clinically intact adhesive restorations, and that chlorhexidine has no detrimental effect on the bond strength to dentin or morphological aspects of hybrid layers.

MATERIALS & METHODS

Clinical Procedures

This study was performed with the approval of the Human Assurance Committee of the University of Guarulhos, São Paulo, Brazil. Twelve individuals with a pair of non-carious third molars in at least partial occlusion, and scheduled for future extraction, were enrolled after their informed consent was received. Class I cavities (3 x 3 x 4 mm) with continuous enamel cavosurface margins were prepared while the individuals were under local anesthesia, and with rubber dam isolation. The control cavities were etched with 35% phosphoric acid (3M ESPE, St. Paul, MN, USA) for 15 sec, bonded with Single Bond (3M ESPE) by the

Table. Bond Strength and Distribution of Failure Modes of *in vivo* Resin-Dentin Bonds

	Bond Strength (MPa)*		Failure Modes (%)**	
	Immediate	14 mos	Immediate	14 mos
Control	29.3 ± 9.2 (14)^{A}	19.0 ± 5.2 (34)^{B}	20% CRB; 15% CHL, 65% M	5% CBR; 30% CHL, 10% CD; 55% M
CHX	32.7 ± 7.6 (17)^{A}	32.2 ± 7.2 (32)^{A}	15% CBR; 30% CHL, 55% M	10% CRB; 30% CRC; 20% CHL, 40% M

* Bond strength values are means ± standard deviations (n = beams/group). Different superscripts indicate statistically significant differences ($p < 0.05$).

** Failure mode abbreviations: CRB, cohesive failure in bonding resin; CHL, cohesive failure in the hybrid layer; CD, cohesive failure in dentin; CRC, cohesive failure in resin composite; M, mixed failure. There were no premature failures of specimens during testing.

moist-bonding technique, and restored with a 1.5-mm-thick layer of a microfilled composite (Clearfil Protect Liner, Kuraray Medical Inc., Osaka, Japan), followed by 2 increments of a hybrid composite (Z250, 3M ESPE). The contralateral experimental cavities received similar treatment, except that the acid-etched dentin were pre-treated with 2 wt% chlorhexidine digluconate (CHX) solution (PRODERMA, Piracicaba, SP, Brazil). Excess CHX was blot-dried after a dwell time of 60 sec, prior to the application of adhesive and resin composite.

The control and experimental teeth (3 pairs) were immediately extracted from three persons who were scheduled to receive orthodontic brackets within the next few days. The other persons were periodically monitored and had their treated teeth extracted (9 pairs) 14 mos later. All restorations were intact, asymptomatic, and had no signs of recurrent caries. All extracted teeth were stored immediately in 0.02% NaN_3-containing phosphate-buffered saline to inhibit microbial growth.

Microtensile Bond Testing

All teeth were sectioned mesio-distally into three 0.9-mm-thick serial sections. One section was further cut into 0.8-mm^2 beams (4 or 5 beams *per* section). Each beam was fixed to a custom-made testing jig (Geraldeli's jig) with cyanoacrylate glue and subjected to microtensile testing at a crosshead speed of 0.5 mm/min until failure (Model 4411, Instron Corporation, Canton, MA, USA). The fractured specimens were sputter-coated with gold/palladium and examined with a scanning electron microscope (JEOL-5600 LV, Tokyo, Japan) at 15 kV. Failure modes were classified as cohesive failures in resin composite (CRC), in bonding resin (CRB), in hybrid layer (CHL), in dentin (CD), or as mixed failures (M). A two-way ANOVA and *post hoc* Tukey's tests were used to analyze the effect of "dentin treatment" (control *vs*. chlorhexidine) and "time of extraction" (immediate *vs*. 14 mos) on bond strength, with $\alpha = 0.05$. The statistical unit was beams, not teeth.

Transmission Electron Microscopy (TEM)

The remaining 2 sections from each tooth were fixed in Karnovsky's fixative for 24 hrs, completely demineralized in 0.5 M ethylenediamine tetra-acetic acid, post-fixed in 1% OsO_4, dehydrated, embedded in epoxy resin, and sectioned by ultramicrotomy according to the TEM protocol described by Pashley *et al.* (2004). Sections stained with 2% uranyl acetate and Reynold's lead citrate were examined with a JEM-1010 TEM (JEOL, Tokyo, Japan) operating at 80 kV.

RESULTS

Both "dentin treatment" and "time of extraction", as well as their interaction, significantly affected the bond strength of *in vivo* resin-dentin bonds ($p < 0.05$). Mean bond strengths and failure mode distribution are summarized in the Table. Chlorhexidine treatment (CHX) did not affect *in vivo* bond strength of the teeth extracted immediately after the restorative procedures ($p > 0.05$). For teeth extracted 14 mos later, *in vivo* bond strength remained stable in the CHX-treated samples ($p > 0.05$), but decreased significantly in the control teeth ($p < 0.05$). Mixed failures were the most common fracture pattern observed, regardless of the experimental condition. After 14 mos of intra-oral functioning, cohesive failure within the hybrid layer was more frequently observed in control samples than in experimental teeth.

The immediately extracted control and experimental specimens, as well as the chlorhexidine-pre-treated experimental specimens retrieved after 14 mos of intra-oral function (Fig. 1A), exhibited intact hybrid layers. In contrast, the 14-month control specimens revealed degraded hybrid layers with 4 progressive zones of disintegration: (1) a "zone of integrity" (Fig. 1B) along the top of the hybrid layer and the periphery of dentinal tubules, with features identical to those observed from similar locations in the experimental specimens; (2) a "zone of partial disintegration" (Fig. 1C), in which sparsely distributed, banded collagen fibrils could still be identified; (3) a "microfibrillar zone" (Fig. 1D), in which the collagen fibrils had completely disintegrated into microfibrillar strands (*i.e.*, gelatin); and (4) a "zone of complete disintegration" (Figs. 1B, 1D) that represented isolated, amorphous regions within the center of the hybrid layers, where the fibrillar characteristics of collagen or gelatin could no longer be seen.

DISCUSSION

Mechanical and ultrastructural disruption of hybrid layers in human dentin in control Class I restorations, under clinical function for 14 mos, supports rejection of the first null hypothesis, that degradation of the hybrid layer does not take place in clinically intact resin-bonded restorations. Conversely, it is surprising that chlorhexidine-treated, acid-etched, resin-bonded dentin exhibited both bond strength and morphological properties after 14 mos in function, resembling the properties of 24-hour specimens. Thus, the second null hypothesis, that chlorhexidine has no detrimental effect on the bond strength and morphological aspects of hybrid layers, must be accepted, and extended to support the adjunctive use of chlorhexidine in acid-etched dentin to delay hybrid layer degradation.

Both control and experimental restorations were completely circumscribed by resin-bonded enamel margins. The percent retention of the restorations was 100%, with no signs or symptoms of clinical deterioration. Hence, the decrease in bond strength and patchy disintegration of the hybrid layers in caries-free control restorations raise concerns that clinically acceptable adhesive restorations may undergo microscopic degradation *in vivo* within 14 mos of function. In the present study, a significant (38%) loss of bond strength was accompanied by severe loss of ultrastructural integrity of the hybrid layer. Other *in vivo* and *in vitro* studies have reported declines in resin-dentin bond strength in time (Hashimoto *et al.*, 2000; De Munck et al, 2003; Koshiro *et al.*, 2004; Donmez *et al.*, 2005). While some of the decline may be due to the plasticizing effects of water (Yiu *et al.*, 2004; Carrilho *et al.*, 2005; Ito *et al.*, 2005; Malacarne *et al.*, 2006), the observed collagen degradation is thought to be the major contributor to the decreased bond strength in the present study.

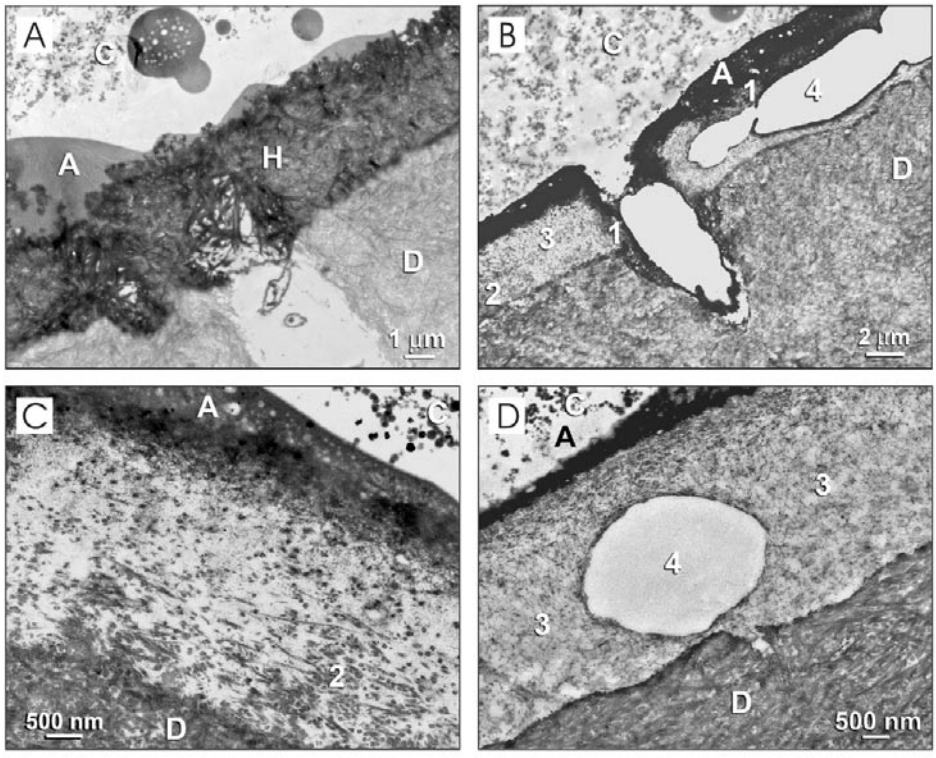

Figure. Transmission electron micrographs of demineralized, stained sections taken from **(A)** a chlorhexidine-pre-treated experimental specimen retrieved after 14 mos of intra-oral function; and **(B,C,D)** multiple control specimens retrieved after 14 mos of intra-oral function, showing the 4 progressive stages of hybrid layer degradation. Captions applicable to all specimens: C, resin composite; A, dentin adhesive; D, intertubular dentin. Caption exclusive to (A): H, hybrid layer. Captions applicable to (B-1D): 1, zone of integrity, where no ultrastructural features of hybrid layer degradation can be observed; 2, zone of partial disintegration, where sparsely distributed, banded collagen fibrils can still be identified; 3, microfibrillar zone, where the multi-stranded collagen fibrils have disintegrated into their microfibrillar components; and 4, zone of complete disintegration, where no fibrillar characteristics can be seen, and regions appear completely amorphous.

The hybrid layers of the control 14-month specimens exhibited partial loss of collagen fibril cross-banding, breakdown of the original multi-stranded fibrils into micro-fibrillar subunits, and multiple micron-sized regions with complete loss of fibrillar characteristics. These ultrastructural events suggest that the breakdown of resin-infiltrated dentin may be governed by host-derived factors, such as the action of endogenous collagenolytic enzymes on partially exposed collagen fibrils. Residual collagenolytic activity was recently confirmed in partially demineralized dentin powder produced from freshly extracted, caries-free teeth, in the absence of exposure to bacterial and salivary MMPs (Pashley *et al.*, 2004; Mazzoni *et al.*, 2006; Nishitani *et al.*, 2006; Tay *et al.*, 2006). While it is theoretically possible for salivary esterases or MMPs to attack resin-dentin bonds (Lin *et al.*, 2005), recent work has shown that incubation of resin-dentin bonds with exogenous collagenase (Toledano *et al.*, 2007) or cholesterol esterase and collagenase (Armstrong *et al.*, 2006) had no additional effect on bond strength over the reductions seen in control groups incubated without exogenous collagenases. These results suggest that salivary enzymes may be too large to penetrate resin-dentin bonds. Penetration of bonds by exogenous collagenase is not necessary if endogenous MMPs are responsible for matrix destruction. The absence of bacteria was confirmed in subsequent TEM examination of the bonded interfaces. Since bonding was performed in caries-free teeth, the observed loss of collagen fibrillar cross-banding in control hybrid layers suggests that the collagenolytic activity is derived from the underlying mineralized dentin. Our thesis is that bonding procedures uncover and activate MMP enzyme activity, while MMPs remain bound to the collagen matrix (Mazzoni *et al.*, 2006; Nishitani *et al.*, 2006). This permits the MMPs to degrade their supporting matrix, causing a partial loss of collagen structure and lower bond strength (Pashley *et al.*, 2004; Hebling *et al.*, 2006; Tay *et al.*, 2006; Carrilho *et al.*, 2007). The ultrastructural features of microfibrillar strands (Zone 3) and multiple amorphous regions (Zone 4) suggest that these are remnant primary and/or secondary by-products of collagen breakdown (Hebling *et al.*, 2005).

The presence of MMP-2 in human dentin has already been identified by a combination of SDS gel electrophoresis and Western blotting, providing indirect evidence for *in vivo* activity of dentin matrix degradation (Martin-de las Heras *et al.*, 2000). That study emphasized the stability of the enzyme when embedded in mineralized matrix. Degradation of type I collagen fibrils was reported in the presence of MMP-2 (gelatinase A), a protease classically known for its gelatinolytic activity, but one that is also an effective collagenase (Aimes and Quigley, 1995). Moreover, a recent study indicated that MMP-8, which is

supposedly the most effective collagenase against type I collagen, is present in both mineralized and non-mineralized compartments of human dentin (Sulkala *et al.*, 2007).

Synthetic MMP inhibitors are being investigated as potential therapeutic agents in the treatment and/or prevention of oral diseases (Sorsa *et al.*, 2004). Chlorhexidine (CHX) has been shown to inhibit MMP-2, -8, and -9 activities directly at extremely low concentrations (*i.e.*, 0.02% for MMP-8, 0.002% for MMP-9, and 0.0001% for MMP-2) (Gendron *et al.*, 1999). These are the same MMPs that have been shown to be present in human dentin (Martin-de las Heras *et al.*, 2000; Sulkala *et al.*, 2007). We speculate that the CHX-saturated demineralized matrix becomes sequestered from interstitial fluids by resin tags that occlude dentinal tubules, by adhesive resin coating collagen fibrils, and by an overlying adhesive layer, which may produce prolonged retention of CHX and inhibition of MMPs. Successful use of chlorhexidine adjunctively with conventional etch-and-rinse adhesives has been previously reported (Hebling *et al.*, 2005; Carrilho *et al.*, 2007). Typical 67-nm cross-banding of collagen fibrils, indicating structural integrity of hybrid layers in the CHX-treated specimens in the current study, confirmed the ultrastructural results reported by Hebling *et al.* (2005). Even more convincing was the observation that the microtensile bond strength of CHX-treated dentin did not change over the 14-month study. In contrast, the bond strength of the control teeth fell 38% over the same time. This is the first report that etch-and-rinse bonds can be stable over the current 14 mos *in vivo*. Previous *in vitro* studies of the ultrastructural (TEM) changes in hybrid layers in normal dentin have required up to 5 yrs (Hashimoto *et al.*, 2000; De Munck *et al.*, 2003) to show the type of degradation that was seen in only 6 mos (Hebling *et al.*, 2005) or 14 mos *in vivo* (the current study). The mechanical stability of resin-dentin bonds may be controlled by the durability of resin-infiltrated collagen fibrils in the matrix. We speculate that chlorhexidine remains bound on collagen, beneath resin that infiltrated interfibrillar spaces. In the CHX-treated cavities, the MMPs would remain inhibited for as long as CHX remained bound to the matrix. Future studies should determine how long CHX remains within the hybrid layer, as well as its optimal concentration for inhibiting MMP activity in dentin, *in vivo*.

The experimental design of the present study does not prove that the effect of CHX on the preservation of the morphological properties of hybrid layers and bond strength was exclusively due to the inhibition of host-derived proteases. Such proof awaits TEM immunolocalization of specific MMPs in dentin, and/or inhibition of matrix degradation by specific monoclonal antibodies to specific MMPs. It is encouraging that CHX treatment conserved both the bond strength and the morphologic features of hybrid layers created *in vivo*. While the present protocol requires additional confirmation, further studies should also test the applicability of other potential, non-toxic, synthetic protease inhibitors, such as tetracycline derivatives, that have been shown to reduce salivary MMP activity *in vitro* (Sulkala *et al.*, 2001). For optimal durability of resin-dentin bonds, preservation of both resin and substrate components (*i.e.*, dentin collagen) should be addressed. These results indicate that the presence of MMPs in the dentin matrix is of more than academic interest. Dentists need to understand the biochemistry of these enzymes and how they may respond to procedures and products used in adhesive dentistry.

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