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Chlorhexidine Preserves Dentin Bond *in vitro*

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Abstract

Loss of hybrid layer integrity compromises resin-dentin bond stability. Matrix metalloproteinases (MMPs) may be partially responsible for hybrid layer degradation. Since chlorhexidine inhibits MMPs, we hypothesized that chlorhexidine would decelerate the loss of resin-dentin bonds. Class I preparations in extracted third molars were sectioned into two halves. One half was customarily restored (etch-and-rinse adhesive/resin composite), and the other was treated with 2% chlorhexidine after being acid-etched before restoration. Specimens were stored in artificial saliva with/without protease inhibitors. Microtensile bond strengths and failure mode distribution under SEM were analyzed immediately after specimens' preparation and 6 months later. With chlorhexidine, significantly better preservation of bond strength was observed after 6 months; protease inhibitors in the storage medium had no effect. Failure analysis showed significantly less failure in the hybrid layer with chlorhexidine, compared with controls after 6 months. In conclusion, this *in vitro* study suggests that chlorhexidine might be useful for the preservation of dentin bond strength.

Keywords

matrix metalloproteinase; hybrid layer; tooth; microtensile; adhesive

INTRODUCTION

During the last two decades, chemical and technical advances have contributed to increases in resin-dentin bond strength. However, premature loss of bond strength is one of the problems that still affects adhesive restorations (Mjör *et al.*, 2000) and markedly reduces their durability (Carrilho *et al.*, 2005b; De Munck *et al.*, 2005; Frankenberger *et al.*, 2005). The loss of bond strength has been attributed mainly to the degradation of the hybrid layer at the dentin-adhesive interface. Numerous publications have demonstrated the lack of bond stability (Wang and Spencer, 2003, 2005; Yiu *et al.*, 2004; Carrilho *et al.*, 2005a). The notion that deterioration of dentin collagen fibrils contributes to the mechanism responsible for bond degradation was only recently evidenced (Hashimoto *et al.*, 2003; Pashley *et al.*, 2004).

In this context, it has been speculated that a decreasing concentration gradient of resin monomer diffusion within the acid-etched dentin, and a subsequent resin elution from hydrolytically

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unstable polymeric hydrogels within the hybrid layers (Wang and Spencer, 2003) leave the collagen fibrils unprotected and vulnerable to degradation by endogenous metalloproteinases (MMPs). MMPs are a group of 23 mammalian enzymes capable of degrading all extracellular matrix components. Human dentin contains at least collagenase (MMP-8), gelatinases MMP-2 and -9, and enamelysin MMP-20 (Martin-De Las Heras *et al.*, 2000; Sulkala *et al.*, 2002, 2006; Mazzoni *et al.*, 2006). Dentin collagenolytic and gelatinolytic activities (Pashley *et al.*, 2004; Mazzoni *et al.*, unpublished observations) can be suppressed by protease inhibitors (Pashley *et al.*, 2004), indicating that MMP inhibition could be beneficial in the preservation of hybrid layers. This was demonstrated in a recent *in vivo* study, in which the application of chlorhexidine, known to have a broad-spectrum MMP-inhibitory effect (Gendron *et al.*, 1999), significantly improved the integrity of the hybrid layer in a six-month clinical trial (Hebling *et al.*, 2005).

The aim of this *in vitro* study was to evaluate the effect of protease inhibition on resin-dentin bond strength after 6 mos of aging. The test hypothesis, based on the preservation of the hybrid layer *in vivo* (Hebling *et al.*, 2005), was that MMP inhibition by chlorhexidine application prior to formation of the hybrid layer would decelerate the decrease of bond strength frequently seen in the microtensile model after aging (Hashimoto *et al.*, 2003). The secondary hypothesis set that external MMP inhibition, previously seen to suppress the dentin MMP activity (Pashley *et al.*, 2004), would further improve the preservation of the hybrid layer.

MATERIALS & METHODS

Tooth Preparation

Seven unerupted, caries-free third molars were collected after the patients' informed consent had been obtained under a protocol reviewed and approved by the Ethics Committee for Human Studies, Piracicaba School of Dentistry, São Paulo, Brazil. Deep Class I preparations were prepared in the teeth with the use of diamond burs under continuous air/water spray. All preparations' walls were limited by dentin, except for the enamel cavosurface. The tooth preparations were divided buccolingually into 2 halves, which were randomly assigned to one of the 2 bonding groups.

Bonding Procedures

All preparations were etched with 35% phosphoric acid gel (Scotch Etchant, 3M ESPE, St. Paul, MN, USA) for 15 sec, rinsed for 30 sec with tap water, and vigorously dried with oil-/water-free air. The control preparations (n = 7) were re-hydrated with 1.5 μL of distilled water, while the experimental ones (n = 7) were re-hydrated with 1.5 μL of 2 wt% chlorhexidine digluconate solution (chlorhexidine). For both groups, after 60 sec, excess solution was removed with absorbent paper. Two consecutive coats of Single Bond primer/adhesive (3M ESPE, St. Paul, MN, USA) were applied to the entire preparation's surface, and, after solvent evaporation, the preparation was light-cured for 10 sec. Five or 6 increments of resin composite (Z250, 3M ESPE) were obliquely added to the bonded surfaces and individually light-cured for 20 sec, under a halogen light-curing unit with an output of 700 mW/cm². The teeth were stored in distilled water at 37°C for a wk.

Microtensile Bond Testing

Teeth were longitudinally sectioned across the bonded interface in sections perpendicular to the pulpal wall with a diamond saw, to produce a series of 0.9 mm × 0.9 mm × 8 mm beams. From 8 to 10 beams were obtained from each preparation. One-third of those specimens were immediately tested, while the remaining beams were randomly divided and stored at 37°C for 6 mos in artificial saliva (pH 7.1) containing (or lacking) proteolytic enzyme inhibitors. The artificial saliva and protease inhibitors cocktail was prepared as described previously (Pashley

et al., 2004) (Table 1). The storage medium was replaced weekly, since it has been shown to maintain the inhibitors' activity (Pashley *et al.*, 2004). Each specimen was individually fixed to a custom-made testing jig (Geraldeli's device) with a cyanoacrylate glue (Model Repair II Blue, Dentsply-Sakin, Japan), and subjected to tensile load at a crosshead speed of 0.5 mm/min until failure (Instron 4411, Instron Corporation, Canton, MA, USA).

Scanning Electron Microscopy

All fractured specimens were dried at room temperature for 24 hrs in desiccators, and sputter-coated with gold/palladium. Both surfaces of each fracture site were observed under a scanning electron microscope (JEOL-5600 LV, Tokyo, Japan) with 85x magnification at 15 kV. The fracture modes were classified as described previsouly (Hashimoto *et al.*, 2000), except that 5 instead of 4 classes were used: (1) cohesive failure in the composite, (2) cohesive failure in the adhesive resin, (3) failure in the top of the hybrid layer, (4) failure in the bottom of the hybrid layer, and (5) cohesive failure in dentin (Fig. 1). In cases of uncertainty, we used higher magnifications (500–4000x) (Fig. 1) to confirm the nature of fracture. The percentage of each fracture mode was then estimated for each specimen.

Statistical Analysis

We used two-way ANOVA, with Tukey's highly significant difference (HSD) tests, to compare the effects of treatments and storage media on bond strengths, and to compare the distribution of failure modes after 24 hrs and 6 mos of storage within the pretreatments (control *vs.* chlorhexidine). We used Student's *t* test to compare the effects of the treatment modes on the distribution of failure modes. Statistical significance was pre-set at $\alpha = 0.05$.

RESULTS

Microtensile Bond Strengths

Chlorhexidine pre-treatment did not affect *in vitro* bond strength of specimens tested at the immediate testing period (*i.e.*, right after beams' preparation) ($p > 0.05$) (Fig. 2). Six-month storage resulted in significant bond strength reduction of both chlorhexidine and control groups ($p < 0.05$). Storage in artificial saliva without added protease inhibitors reduced bond strength in the control group by 45.3%. In the chlorhexidine group, the reduction was 23.4%. The remaining bond strength was significantly higher in the chlorhexidine group ($p < 0.05$) (Fig. 2). Storage in artificial saliva containing protease inhibitors did not affect the bond strength when compared with that of those stored in artificial saliva without protease inhibitors ($p > 0.05$) (Fig. 2).

Distribution of the Failure Mode

Representative SEM micrographs of the control and chlorhexidine groups are shown in Fig. 1. Of these, 93.5% (129/138) were mixed failures, with practically similar fracture patterns for control and chlorhexidine groups at the immediate testing period (Table 2). After 6 months' storage, a significant increase in the percentage of failures in the bottom of the hybrid layer was observed for the control specimens, but not for chlorhexidine-treated specimens. The failures in the top of the hybrid layer were significantly less prevalent in the chlorhexidine group after 6 months' storage, all for specimens stored with or without protease inhibitors, when compared with immediately tested specimens ($p < 0.05$) (Table 2). Also, after 6 months' storage, significantly less failure occurred in the bottom of the hybrid layer, regardless of the storage medium, and in the top of the hybrid layer in specimens stored with protease inhibitors in the chlorhexidine-treated group when compared with respective controls ($p < 0.05$) (Table 2).

The most prevalent fracture pattern for the control specimens, regardless of the storage time or solution, occurred within the hybrid layer, in either its top or bottom plane. Conversely, after 6 months' storage, the failure in the chlorhexidine group shifted from the hybrid layer predominantly to the adhesive and composite layers ($p < 0.05$ in all cases) (Table 2).

DISCUSSION

Since the application of 2% chlorhexidine on acid-etched dentin resulted in significantly less reduction in bond strength over 6 mos of aging, the primary hypothesis is supported. However, the addition of protease inhibitors to the storage medium did not have any marked effect on bond strength, resulting in the rejection of the secondary hypothesis.

Chlorhexidine has been widely used as an antimicrobial agent, including for disinfection before the placement of restorations. Previous studies have demonstrated that chlorhexidine application prior to acid-etching has no adverse effects on immediate composite-adhesive bonds in dentin (Perdigão *et al.*, 1994; el-Housseiny and Jamjoum, 2000; de Castro *et al.*, 2003), enamel (Filler *et al.*, 1994; el-Housseiny and Jamjoum, 2000), or with resin-reinforced glass-ionomer cements (Cunningham and Meiers, 1997). The antimicrobial efficacy of chlorhexidine used as a preparation disinfectant may, however, be questioned if the surface is subsequently conditioned (Botelho, 2005). Recent studies have examined the use of chlorhexidine after acid-etching, demonstrating initial bond strengths comparable with those of the controls (Pilo *et al.*, 2001; de Castro *et al.*, 2003; Say *et al.*, 2004), as was also observed in this study. While the evidence of chlorhexidine antimicrobial efficacy when used after acid-etching remains to be shown, analysis of the present data apparently indicates its beneficial effects on the preservation of dentin bond strength as an MMP inhibitor, when applied prior to bonding with no further rinsing. When applied in this manner, the naked collagen fibrils were exposed to chlorhexidine that was then sealed into the fibrils by adhesive resins. The reduction of bond strength with the chlorhexidine-treated group (23.4%) is most likely due to the hydrolytic degradation of the adhesive polymer (Carrilho *et al.*, 2005a). The significantly higher cohesive failure rates within the adhesive layers and composite resin in chlorhexidine-treated group (Table 2) support the findings that lower reduction of bond strengths was due to a better preservation of the collagen fibrils.

The lack of effect of protease inhibitors incorporated into the storage solution in preventing reductions in bond strength indicates that the main effect was gained with direct inhibition of dentin-bound MMPs (Pashley *et al.*, 2004). This assumption is supported by recent studies demonstrating that mineral oil used as a storage medium inhibited both the function of dentin-bound MMPs (Pashley *et al.*, 2004) and the hydrolysis of polymerized matrix (Carrilho *et al.*, 2005a), and preserved both the bond strength and the integrity of the hybrid layer (Carrilho *et al.*, 2005b; García-Godoy *et al.*, in press), suggesting the hydrolytic basis of the phenomenon. The possibility of bacteria being responsible for the loss of bond strength in this study is minimal, since the teeth were intact, and they were stored in artificial saliva containing an antimicrobial component (*i.e.*, sodium azide), and no signs of biofilm formation were observed in SEM images. Comparable results with degradation of collagen fibrils in hybrid layers have been demonstrated in the absence of bacteria (Hashimoto *et al.*, 2003; Pashley *et al.*, 2004).

Few studies have examined the failure modes in an entire set of specimens. SEM analysis of the failure mode distribution revealed that 93.5% of the specimens demonstrated mixed failures, with the marked change in the site of failure between control and chlorhexidine groups after 6 months' storage. Quantitative SEM analysis improved the accuracy of the failure mode distribution analysis compared with light microscopy (Hashimoto *et al.*, 2003), and allowed for discrimination between the failures occurring in the top and bottom of the hybrid layer. This is important when one attempts to understand the mechanisms leading to the degradation

of the hybrid layer and the reduction in dentin bond strengths with time. Previously, exposure of the collagen fibrils in the fracture site was demonstrated in aged specimens (Hashimoto *et al.*, 2003), showing the shift of the failure site from the top to the bottom of the hybrid layer. The significantly lower percent failure mode in the hybrid layer, especially in the bottom part, after 6 mos with chlorhexidine treatment indicated that the higher bond strengths observed in this group 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. This may also reflect the better preservation of sub-hybrid layer dentin, in which both progressive demineralization and degradation of dentin collagenous matrix may occur with time (García-Godoy *et al.*, in press).

The present *in vitro* study, as well as a previous *in vivo* study demonstrating preservation of hybrid layers with chlorhexidine treatment after acid-etching (Hebling *et al.*, 2005), was performed with only one adhesive system. Thus, these studies merely provide the proof of concept, and the recommendation of the use of chlorhexidine after acid-etching must at this point be limited to the adhesive system in question. However, other *in vitro* experiments—with various bonding materials demonstrating that chlorhexidine application after acid-etching has no effect on immediate bond strength (Pilo *et al.*, 2001; de Castro *et al.*, 2003; Say *et al.*, 2004)—encourage further experiments to evaluate the preservation of the hybrid layer with different bonding systems.

In conclusion, 2% chlorhexidine application after acid-etching preserves both the durability of the hybrid layer (as seen in the failure mode distribution analysis) and bond strength *in vitro* of aged specimens. The findings correlate well with the recent *in vivo* findings with similar experimental design (Hebling *et al.*, 2005). The most plausible explanation would be the inhibition of dentin matrix-bound MMPs (Gendron *et al.*, 1999), resulting in decreased degradation of hybrid layer and sub-hybrid layer collagen fibrils. While the improvement with chlorhexidine, both in bond strength and in hybrid layer durability in 6 mos, was significant compared with that in the controls, the mean reduction in bond strength was still 23.4% when compared with the initial values. Further *in vitro* and *in vivo* studies are needed to clarify the causes behind the remaining loss of bond strength, to optimize the MMP inhibitory effect (*e.g.*, concentration of chlorhexidine, time of application), and to find the optimal MMP inhibitor that would result in the best time-related preservation of the dentin-adhesive interface.

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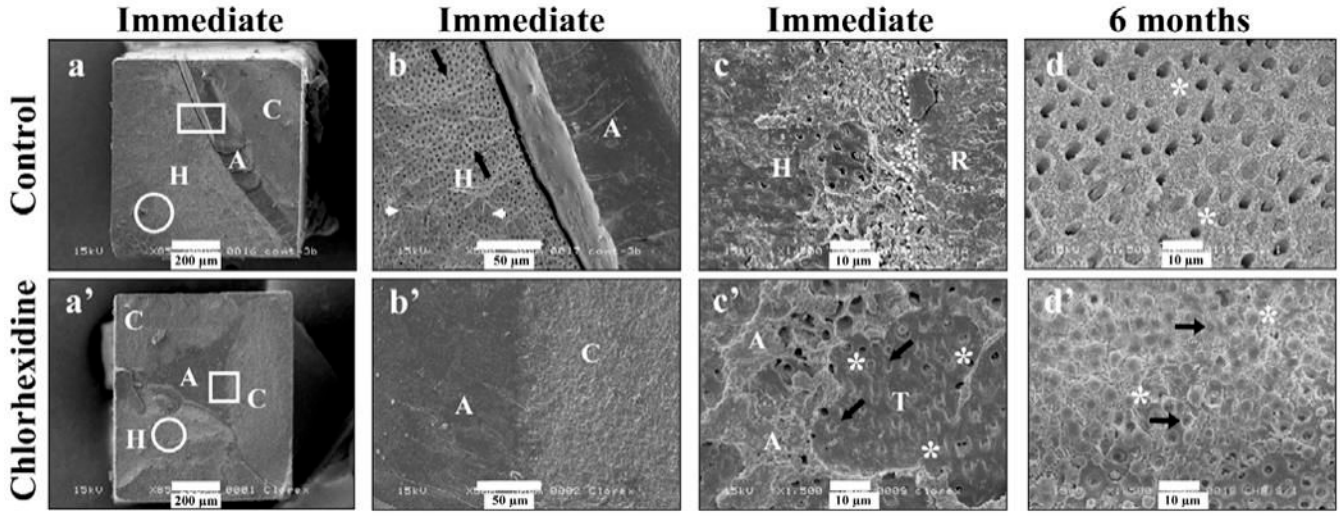

Figure 1.

Representative scanning electron micrographs (SEM) of the dentin side of fractured specimens in different groups. (**a–c**) Immediate testing, control; (**a′–c′**) immediate testing, chlorhexidine-treated; (**d**) six-month control, stored in artificial saliva without protease inhibitors; (**d′**) six-month chlorhexidine-treated, stored in artificial saliva without protease inhibitors. C = resin composite; A = adhesive; H = hybrid layer. (**a**) Low-power magnification demonstrates a mixed failure (partially cohesive in A and C, partially in H). Magnification: 85x. (**b**) Higher magnification of the area limited by a rectangle in (a), showing cohesive failure in the adhesive layer (A), and failure localized at the bottom (between black arrows) or top (between white arrowheads) of the hybrid layer. Magnification: 500x. (**c**) Highest magnification of the area limited by the circle in (a), showing cohesive failure in the resin composite and adhesive (R) and in different depths of the hybrid layer (H) (areas separated with dashed line). Magnification: 1500x. (**a′**) Low-power magnification evidences a mixed failure, partially cohesive in A and C and partially in H. Magnification: 85x. (**b′**) Higher magnification of the area limited by a rectangle in (a′), confirming the partial cohesive failures in the adhesive (A) and composite (C). Magnification: 500x. (**c′**) Highest magnification of the circled area in (a′), showing cohesive failure within the adhesive (A), the rest being located at the top of the hybrid layer (T). Most of the exposed dentinal tubules are filled by resin tags (black arrows), while the intertubular dentin seems to be completely covered by adhesive (asterisk). Magnification: 1500x. (**d**) Highest magnification of control specimen after 6 months' storage, showing a failure localized in the bottom of the hybrid layer, as evidenced by a high density of the dentinal tubules and an uncovered intertubular dentin, with naked collagen fibrils (asterisk). Magnification: 1500x. (**d′**) Highest magnification of chlorhexidine-treated specimen after 6 months' storage shows a cohesive failure localized in the middle of the hybrid layer. Dentinal tubules are completely filled by resin tags (black arrow), and intertubular dentin is covered by adhesive (asterisk). Magnification: 1500x.

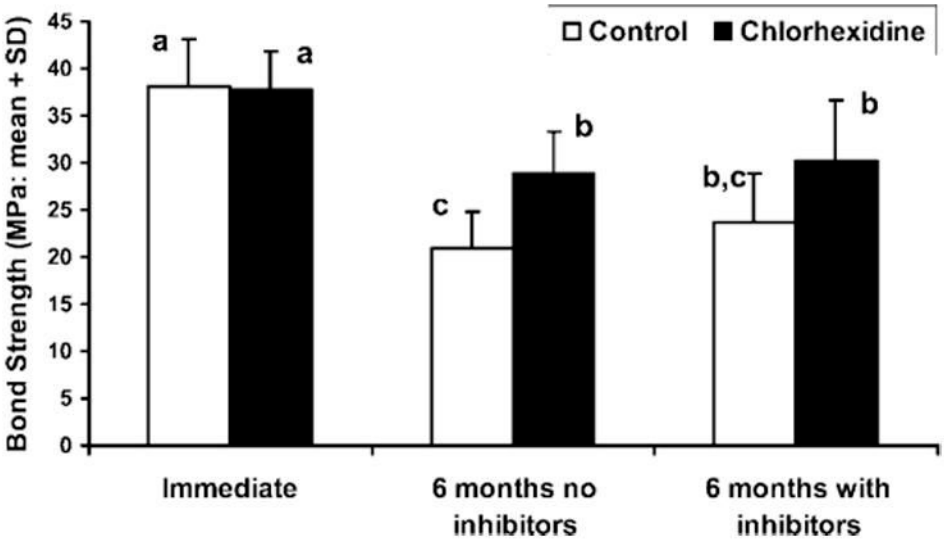

Figure 2.

The bond strengths (mean ± SD) at the immediate testing period (immediate), 6 mos in artificial saliva containing the necessary ions for MMP activation, or in artificial saliva containing additional non-specific protease inhibitors (no inhibitors and with inhibitors, respectively). N = from 19 to 22 in each group. The bars with different letters indicate statistically significant differences (ANOVA with Tukey's test).

Table 1

The Composition of Storage Solutions.

Artificial Saliva[A] **Component**	**Concentration**	**[B] Protease Inhibitor Cocktail** **Component**	**Concentration**
$CaCl_2$	0.70	Tris HCl	65.0
$MgCl_2 \cdot 6H_2O$	0.20	Benzamidine HCl	2.50
KH_2PO_4	4.00	ε-amino-n-caproic acid	50.0
KCl	30.0	N-ethylmaleimide	0.50
NaN_3	0.30	Phenylmethylsulfonyl fluoride	0.30
HEPES buffer (acid)	20.0		

(A) Components of the artificial saliva used for the storage of the specimens (Pashley *et al.*, 2004).

(B) Components of the protease cocktail used in artificial salvia to store half of the six-month specimen. All concentrations are given as mM/L.

Table 2

The Distribution of Failure Modes (in percentage) between the Groups as Observed with SEM

Group	Percentage Failure Modes of Fractured Bonds (%)				
	Cohesive Dentin	Hybrid Layer (bottom)	Hybrid Layer (top)	Cohesive Adhesive	Cohesive Component
Immediate testing					
Control	3.3	14.7^{AB}	34.7	16.7	30.0^{AB}
Chlorhexidine	1.1	16.6	33.4^{AB}	18.4^{AB}	30.5
6 months′ storage, no inhibitors					
Control	5.5	40.2^{aB}	27.5	12.9^{a}	15.2^{a}
Chlorhexidine	0.0	19.1^{B}	22.5	29.1	30.7
6 months′ storage with inhibitors					
Control	6.1	29.5^{a}	36.1^{a}	14.1^{a}	14.1^{a}
Chlorhexidine	0.0	9.3	15.9	37.4	36.5

Values are percentages of the total fractured surface area.

^{a}Control value is statistically significantly different from the chlorhexidine group within the treatment (immediate testing for 6 mos in artificial saliva without or with protease inhibitors, respectively) ($p < 0.05$; Student's *t* test).

^{A}The value is statistically significantly different from the respective value with the same pre-treatment (control or chlorhexidine) in the "6 months′ storage, no inhibitor" group.

^{B}The value is statistically significantly different from the respective value with the same pre-treatment (control or chlorhexidine) in the "6 months′ storage with inhibitor" group ($p < 0.05$; ANOVA with Tukey's HSD test).