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Role of Proteolytic Enzyme Inhibitors on Carious and Eroded Dentin Associated With a Universal Bonding System

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Clinical Relevance

Carious dentin is a challenging substrate with which to establish an effective bond. Using an etch-and-rinse mode with universal adhesive system, stable resin-dentin bond strength over time can be achieved for those substrates. However, the use of E-64 associated with it presented better performance compared with CHX. Thus, it appears to be an appropriate bonding strategy for the maintenance of bond strength to eroded and carious dentin over time.

SUMMARY

Objectives: The aim of this study was to evaluate the effect of proteolytic inhibitors on the bond strength of a universal adhesive system (etch-and-rinse mode) applied to artificial carious and eroded dentin. Methods: Ninety molars were prepared and randomly divided into three groups according to the substrate: N, no challenges; ACD, artificial carious dentin simulation and ERO, artificial erosion simulation

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with orange juice. All groups were redivided into three subgroups according to the dentin pretreatment: W, water; CHX, 2% digluconate chlorhexidine; and E-64 (trans-epoxysuccinyl-L-leucylamido-[4-guanidino] butane), 5 μM E-64 inhibitor. They constituted a total of nine groups (n=10): N-W, N-CHX, N-E64, ACD-W, ACD-CHX, ACD-E64, ERO-W, ERO-CHX, and ERO-E64. All specimens were restored with Adper Single Bond Universal/Filtek Z250. Beams (0.64 mm^2) were obtained and subjected to the microtensile test (μTBS) in a universal testing machine at 0.5 mm/min. The failure mode of the interfaces was determined by optical microscopy (40× magnification). Data were statistically analyzed by three-way analysis of variance and Tukey tests ($p<0.05$). Results: All individual factors ($p<0.0001$) and the interaction between substrate and treatment ($p=0.0011$) and between substrate and time ($p=0.0003$) were statistically significant. The caries substrate contributed negatively to bond strength. Chlorhexidine reduced bond strength for normal and eroded conditions. Only the normal substrate was negatively affected by time despite the pretreatment. Conclusions: The universal bonding system appears to be a promising bonding strategy for the maintenance of bond strength to affected dentin. E-64 did not affect bonding to the dentin in contrast to the use of chlorhexidine, which, when associated with the universal system, did affect the microtensile bond strength for artificial carious dentin.

INTRODUCTION

Dental caries and erosion are the most clinically significant demineralizing events, provoking changes in dental hard tissues, depending on the etiologic agent and its intensity and frequency. In dentin, dental caries result in a mineral loss of the organic matrix and disorganization of the collagen fibrils.[1] Dental erosion is an increasingly common multifactorial event[1,2] associated with dietary habits or gastric disorders that favor the presence of acid components in the oral cavity.[2] However, different from caries, microorganisms are not involved in the erosion, which determines distinct clinical consequences.

Based on an improved understanding of the dental remineralizing mechanism and the use of advanced materials and technique, affected dentin can be maintained.[3,4] During the bonding process, a hybrid layer involves the interlocking of the bonding agent in a demineralized organic matrix based on collagen (90%) and noncollagen proteins (10%).[5] Therefore, in both carious and eroded clinical conditions, this layer might be more susceptible to degradation by intrinsic collagenolytic enzymes, such as matrix metalloproteinases (MMPs) and cysteine cathepsins (CCs), which are present in saliva and in dental hard tissues.[1,5-8] It has been shown that MMPs and CCs, exposed by the loss of minerals, can be activated in acidic conditions and act synergistically in the degradation of the adhesive interface through the hydrolytic degradation of collagen fibrils.[5,6] Therefore, strategies to paralyze or minimize their activity might be beneficial to dentin protection.

Several studies have demonstrated the inhibitory potential of several synthetic and natural inhibitors of MMP and CC activity.[1,5,7] The role of chlorhexidine in this process has been investigated the most.[1,5,7,9] Chlorhexidine is a widely employed proteolytic inhibitor, mainly due to its nonspecific action, practical use, substantivity, and low cost.[10,11]

E-64 (trans-epoxysuccinyl-L-leucylamido-[4-guanidino] butane) is a well-known specific inhibitor of CCs.[12] It is synthetically prepared, and its inhibition role is irreversible (covalent type) since there is a connection to the active site between the inhibitor and CCs. In 2010, Tersariol and others[13,14] introduced E-64 into dentistry at a concentration of 5 μM. In 2011, Nascimento and others[6] evaluated its proteolytic activity on CCs with satisfactory results. As it is soluble in water, it can be indicated for clinical use as a solution, following a similar protocol as digluconate chlorhexidine (CHX). Until now, its potential use in dental tissues has been limited to its effect on the progression of erosion,[15] and its effect on dentin bond strength has never been tested.

Bonding to tooth substrates is researched primarily using *in vitro* models with healthy dentin as a substrate to evaluate the impact of the mechanisms of interaction of different restorative materials under various adhesive protocols.[11,15] However, clinically, it is commonly tested on caries- or erosion-modified dentin that render the tissue biochemically, structurally, and mechanically different from that used in major laboratory studies. Thus, studies using naturally altered substrates or simulating demineralization conditions aim to reproduce situations in the laboratory in a more clinically relevant way.[16-21]

Simultaneously, universal restorative systems have been proposed to allow versatility in clinical

work, as they can be used in etch-and-rise and self-etching modes and in dry and wet conditions.[22-26] However, previous studies have shown controversial results. While one study demonstrated promising longevity results,[26] more susceptibility to degradation was observed when universal adhesives were applied on acid-etched dentin,[22,23] or no differences were found between etch-and-rinse and self-etch modes.[24,25] In this study, a universal adhesive system was used in etch-and-rise mode since an acidic condition activates enzymatic action, allowing for the better evaluation of the role of the inhibitors. As such, more studies are needed to better understand the degradation of the dentin organic matrix by host-derived enzymes when this new adhesive approach is used in distinct substrate conditions.

This study aimed to investigate the use of enzyme inhibitors associated with a new adhesive technology in clinically relevant conditions. The objective was to evaluate the performance of a universal adhesive system (etch-and-rinse mode) on bond strength to dentin in different conditions (artificial carious dentin or eroded dentin) after pretreatment with proteolytic inhibitors over time. The null hypotheses tested were as follows: 1) there is no difference in dentin bond strength to normal, carious, and eroded dentin; 2) there is no difference in dentin bond strength after treating the affected dentin with CHX or E-64; and 3) there is no difference on bond strength over time (six months) regardless of the substrate and pretreatment.

METHODS AND MATERIALS

Ninety extracted caries-free third molars were collected and stored for no more than one month in 0.1% sodium azide at room temperature. The roots were sectioned 3 mm below the cement–enamel junction. The occlusal enamel was removed horizontally (perpendicular to the long axis of the tooth) using a water-cooled diamond disc (Extec Corp, Enfield, CT, USA) to expose a flat dentin surface. The dentin surface was ground flat, and a smear layer was standardized using 600-grit SiC paper under running water for 30 seconds (Politriz APL-4 AROTEC, Cotia, São Paulo, Brazil). The specimens were divided according to the pretreatment of the dentin before the bonding procedures as follows: normal dentin (control [N]), artificial carious dentin (ACD) or erosion (ERO). The specimens in the control group were maintained in artificial saliva (1.5 mM $Ca[NO_3]_2{\cdot}4H_2O$, 0.9 mM $NaH_2PO_4{\cdot}2H_2O$, 150 mM KCl, 0.1 mol/L Tris, 0.03 ppmF, pH 7.0) at 37°C for seven days. Artificial carious dentin lesions were created by cycles of six hours of demineralization (2 mM $Ca[NO_3]_2{\cdot}4H_2O$, 2 mM $NaH_2PO_4{\cdot}2H_2O$, 0.075 mM acetate buffer, 0.02 ppm F, pH 4.0), followed by 18 hours of incubation in artificial saliva with remineralizing action. Daily renewal cycles were performed for five days followed by 48 hours of incubation in remineralizing solution.[27]

The erosive challenge consisted of immersion in industrialized orange juice with a pH of 3.2 (Suco Del Valle do Brasil, Coca-Cola, Americana, São Paulo, Brazil), composed of water, sugar, orange juice concentrate, natural flavor, citric acid, and antioxidant ascorbic acid, for five minutes, three times per day, for five days. As the consumption of this beverage is increasing, laboratory protocols have investigated its impact.[28] Between the erosive challenges and after the completion of the erosion, the specimens were stored in daily renewed artificial saliva.

Both substrates were assessed by transverse microradiography, after all cycles were completed, to validate the creation of artificial carious dentin and eroded dentin. For the artificial carious dentin substrate, we observed a demineralized subsurface layer with the preservation of the outer enamel surface, an aspect of carious lesions. For the artificially eroded substrate, a thin, worn superficial layer was evident.

All specimens were etched with 37% phosphoric acid (Dentsply, Catanduva, São Paulo, Brazil) for 15 seconds. Specimens from each dentin substrate were subdivided into three pretreatment groups (n=10), including application of distilled water (W), 2% chlorhexidine digluconate aqueous solution pH 5.8 (CHX, Pharmácia Específica, Bauru, São Paulo, Brazil), or 5 μM E-64 aqueous solution (E-64, Sigma-Aldrich, St Louis, MO, USA) pH 5.5 (E-64). After passive application for 60 seconds, excess was removed with absorbent paper. Then the adhesive system (Adper Single Bond Universal, 3M ESPE, St Paul, MN, USA) was applied according to the manufacturer's instructions and light cured using a 1000-mW/cm^2 LED unit (Radical, SDI, Bayswater, VIC, Australia). Two increments of 2-mm layers of the resin-based composite (Filtek Z350 Universal Restorative, 3M ESPE) were layered and light cured for 20 seconds each. The specimens were immersed in artificial saliva for 24 hours at 37°C and then longitudinally sectioned, perpendicularly to the bonding interface, using an Isomet 1000 digital saw (Buehler, Lake Bluff, IL, USA) to obtain beams of ≅ 0.64-mm^2 area (0.8×0.8 mm) beams. Each beam was measured at the dentin–adhesive interface using a

Table 1: *Mean Values (MPa) and Standard Deviations of the Test Conditions*[a]

	W		CHX		E-64	
	Immediate	**6 mo**	**Immediate**	**6 mo**	**Immediate**	**6 mo**
N	35.32 (5.30) AaΔ	27.45 (5.33) Aa†	28.36 (5.88) AbΔ	16.50 (3.89) Ab†	28.33 (5.42) ABbcΔ	20.80 (3.71) ABbc†
ERO	29.85 (4.77) AaΔ	26.07 (4.96) AaΔ	22.53 (4.76) ABbΔ	20.13 (4.62) ABbΔ	30.23 (6.51) AaΔ	27.70 (5.32) AaΔ
ACD	23.42 (4.95) BabΔ	20.28 (3.55) BabΔ	18.31 (3.50) BbΔ	16.50 (3.90) BbΔ	24.51 (4.41) BaΔ	20.80 (3.71) BaΔ

Abbreviations: ACD artificial carious dentin; CHX, digluconate chlorhexidine; ERO, artificial erosion simulation;E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane; N, normal dentin; W, water.

[a] *Different uppercase letters indicate differences between substrates in each treatment and time (columns) ($p<0.05$). Different lowercase letters indicate differences between treatments in each substrate and time (rows) ($p<0.05$). Different symbols (Δ,†)indicate differences between times (immediate vs 6 mo) in each substrate and treatment ($p<0.05$).*

digital caliper (Mitutoyo America, Aurora, IL, USA) to obtain the exact surface area of the interface, which was fixed to the Bencor Multi-T testing apparatus (Danville Engineering Co, Danville, CA, USA) with cyanoacrylate resin (Super Bonder Flex Gel-Loctite, Henckel Ltda, Itapevi, São Paulo, Brazil) and tested in tension in a universal testing machine (Instron 3342, Instron Co., Canton, MA, USA) at a 0.5-mm/min crosshead speed and with a 500 N load cell. Each tooth was considered the experimental unit. From each specimen (tooth), an average of eight to 10 beams was obtained for each time (after 24 hours and six months). During the six-month aging period, all beams were stored in a weekly renewed artificial saliva at 37°C.

The microtensile bond strength (μTBS) was expressed in MPa by dividing the maximum load (kgf) by the specimen cross-sectional area (mm^2). Each fractured surface was analyzed with a handheld digital microscope (DINO-LITE^{plus} digital microscope, AnMo Electronics Corp, Hsinchu, China) at 40× magnification, and failure was classified in the adhesive (failure in the adhesive layer), cohesive in dentin, cohesive in composite resin, or mixed. For the statistical analysis, as the tooth was the experimental unit, an average of all beams per tooth was performed to determine the tooth μTBS. Data were calculated and statistically analyzed with Statistica software (Statsoft, Tulsa, OK, USA). Assumptions of a normal distribution and equality of variance were tested for all the variables using the Kolmogorov-Smirnov and the Levene test, respectively. As the assumptions were satisfied, the data were subjected to three-way analysis of variance ($p\leq0.05$), followed by the Tukey test ($p<0.05$) for individual comparisons.

RESULTS

All tested factors (substrate, pretreatment, and time) were statistically significant ($p<0.0001$). Significant interactions between substrate and treatment ($p=0.0011$) and between substrate and time ($p=0.0003$) were also detected. Table 1 shows the mean and standard deviation values (MPa) of dentin bond strength and comparisons of all substrates and pretreatment methods.

Overall, the results indicate that bond strength was compromised related to three conditions: artificial carious dentin condition, chlorhexidine as a pretreatment, and six months of aging. Bond strength trended lower in the artificial carious dentin condition, even with chlorhexidine as a pretreatment, at six months, and an analysis of interactions was performed.

Regarding the substrate, artificial carious dentin consistently demonstrated the lowest bond strength values regardless of pretreatment and time. Overall eroded dentin also had lower bond strength immediately and after six months of aging compared to normal dentin, except when E-64 was used. However, no statistical significance was observed. Bond strengths to dentin decreased during aging. For all groups based on normal dentin, a significant difference between the immediate and the six-month groups was observed, regardless of pretreatment. In the ACD and eroded dentin groups, a reduction of dentin bond strength after aging was noted, although it was not statistically significant in all pretreatment groups.

The CHX groups had the lowest bond strength values regardless of the substrate and time. E-64 significantly reduced bond strength of normal dentin, both immediately and after six months of aging. However, the same trend was not observed for artificial carious and eroded dentin. In ACD, CHX bond strengths were significantly lower than in E-64-treated samples immediately and after six months. Interestingly, the use of E-64 preserved the bond strength overtime (after 6 months) for both ACD and ERO, and a significant reduction after 6 months was observed only for normal dentin.

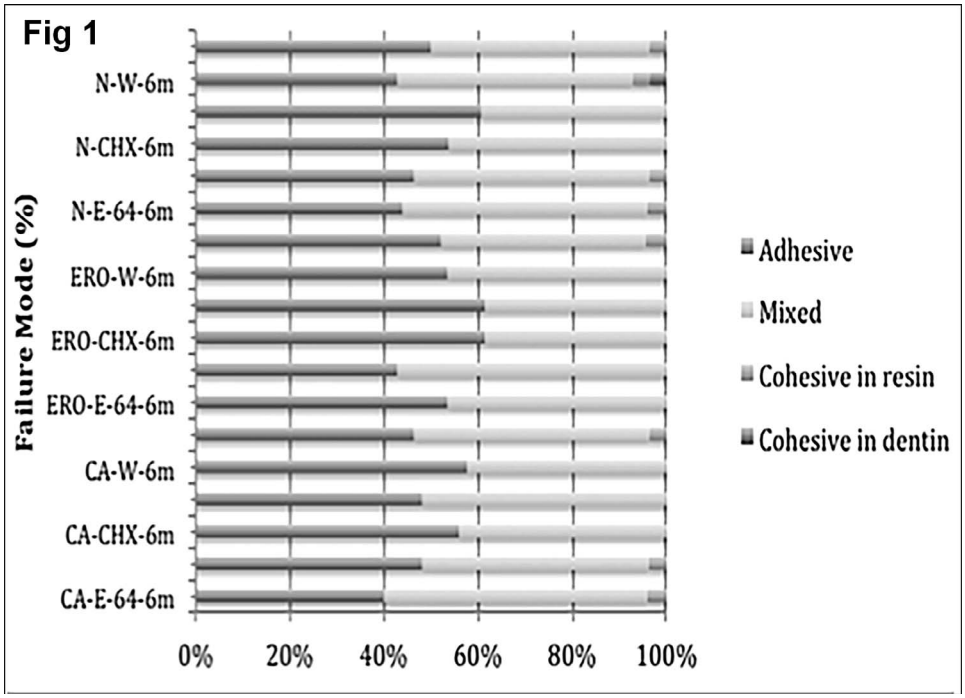

Figure 1. *Failure mode distribution (%) for all substrates and pretreatment with inhibitors immediately and at six months.*

The distribution of failure mode analysis is presented in Figure 1, revealing that adhesive and mixed failures were predominant in all groups, independent of the substrate condition, treatment, and aging.

DISCUSSION

Most studies use healthy substrates to understand the mechanism involved in the hydrolytic degradation of the hybrid layer. In this study, artificial simulation of carious and eroded dentin was performed to obtain the most common substrates seen in clinical practice, beyond normal dentin. These different substrates were associated with the use of enzyme inhibitors in bonding protocols of a universal dentin bonding system.

The results demonstrated significant differences in adhesion according to the dentin substrate condition. The normal substrate showed the highest value, and the carious dentin showed the lowest bond strength value, while the eroded substrate did not show a significant difference compared to normal dentin. For this reason, the first null hypothesis was rejected. This outcome was supported by previous studies showing that, in general, hybrid layers that are poorly infiltrated by adhesives,[17] such as carious and eroded substrates, have changed adhesion.[29,30] It suggests clinical relevance since most techniques for carious dentin removal leave caries-affected and even caries-infected dentin to serve as the bonding substrate,[30,31] and the removal method may also affect immediate dentin bond strength.[31]

For the eroded substrate, previous studies also reported the impact of erosion on reducing bond strength to dentin.[19,20] It is likely that the presence of the collapsed demineralized fibrils and the high water content may prevent the proper polymerization of adhesives. In the eroded dentin, the thicker layer of collagen exposed by erosion cannot be adequately infiltrated by resin monomers.

Orange juice was used in this study to promote eroded substrate, as it is a relevant source of vitamin C. This vitamin has been thought to play a potent antioxidant role, and thus its use has been investigated as an adjunctive strategy to improve resistance against hybrid layer degradation.[32,33] According to Gotti and others,[33] vitamin C acts by reducing the potential degradation effects of free radicals and hydrolysis. They found that antioxidant-doped adhesives appear to have a positive effect on the durability of the adhesive interface since their bond strength after 24 hours of water storage was maintained or increased over time. Therefore, this mechanism can explain the similar performance of normal and eroded substrates.

In regard to pretreatment with enzyme inhibitors, CHX and E-64 reduced bond strength for normal but not for caries- or erosion-affected dentin immediately and after six months of aging, resulting in the rejection of the second null hypothesis.

Previous studies have reported that MMPs and CCs typically coexist in normal dentin.[1,9,13,34] In carious lesions, they contribute to the degradation of the dentin organic matrix.[1] In the dental bonding process, these enzymes participate in the degradation of the hybrid layer.[8,35] Evidence also suggests their role in eroding dentin.[20] Strong evidence also indicates that there is an increase of CC in carious dentin with increasing depth toward the pulp, suggesting that MMPs and CCs have synergistic and dependent activities.[7] Based on all this evidence, the pretreatment of dentin substrates before bonding could improve the biological conditions for adequate bonding over time.

The significant reduction in the immediate dentin bond strength with both inhibitors was an unexpected finding. Chlorhexidine is the most commonly used nonspecific proteolysis inhibitor in bond strength durability studies and the only one used clinically.[15] In general, it has demonstrated satisfactory performance, as it did not affect the immediate dentin bonding and postpones the reduction in bond strength.[8,18-20,36,37]

A reasonable speculation about this performance might be the possibility of an interaction between CHX and 10-methacryloyloxydecyl-dihydrogen phosphate (MDP), present in the adhesive system. Adper Single Bond Universal presents MDP as a relevant ingredient that is a phosphate and bifunctional monomer that is able to bind chemically to dental substrate.[22-26,38] CHX binds to both the mineral and the organic components of dentin,[39] so it can be speculated that it may have affected the competitive MDP bonding to calcium, thus contributing to the reduction in bond strength.

On the other hand, the lower dentin bond strength in normal dentin may be related to the pH of the applied inhibitors. Even though only mildly acidic (pH 5.8 and 5.5 for CHX and E-64, respectively), 60-second applications may have increased demineralization. The mild acid pH CHX solutions may have provoked an erosive effect. This, in turn, would have resulted in demineralized collagen that is too thick, leading to poor penetration of adhesives and thus a reduction of the immediate bond strength.[40]

Nevertheless, a recent study demonstrated that 2% CHX did not have a significant effect on the immediate or 12-month bond strength of Adper Single Bond Universal Adhesive.[41] However, similar to most CHX bonding studies, the pH of the solution was not reported. The effect of pH on the CHX effect on the dentin bond remains to be evaluated.

While bond strength to normal dentin was reduced significantly during aging, nonsignificant changes were seen in the affected substrates regardless of the pretreatment. In eroded dentin, CHX again showed lower bond strength compared to the control in contrast to E-64, and in artificial carious dentin, bond strength in the CHX-treated group was significantly lower than that of the E-64 group at both time points. This result suggests that, in caries- and erosion-modified substrates, factors other than pH may exert an effect on bonding.

The third hypothesis was also rejected. It is interesting to note that while bond strength showed a significant time-related reduction in normal dentin, the loss of bond strength was markedly lower in eroded or artificial carious dentin regardless of the treatment.

Both enzyme-inhibiting solutions, CHX and E-64, promoted significantly lower bond strengths after six months when compared to the immediate values in normal dentin, especially with CHX, which is supposed to inhibit both MMPs and CCs. As it also occurred even though the immediate bond strength was already significantly reduced, a hypothesis of an interaction between MDP and CHX may be considered.

For the artificial carious and eroded dentin, all groups had reduced bond strength. Mechanisms that may explain this result include the mode of inhibitor application or drying, the effect of the acidity of CHX and E-64 on the matrix collagen, or the fact that SB Universal is not susceptible to inhibitors.

This is in accordance with previous findings[18] and has been proposed to be caused by the inferior quality of the hybrid layer in caries-affected dentin.[30] Logically, this should lead to an increased rate of hydrolytic degradation of the hybrid layer components because, in caries-affected layers, the integrity of collagen is seriously affected[1,42] and the levels of MMPs and CCs are increased.[7,9] It would be expected that bond strength would be lost faster relative to intact dentin.

However, in the poorly structured hybrid layer of artificial carious and eroded dentin, the bond based on the micromechanical interlocking of collagen is not as imperative as in the "normal" hybrid layer; thus, the loss of collagen may have a lesser effect.[1] The remaining low bond strength may be due to the weaker physical forces (eg, the van der Waals forces), which may be less susceptible to structural changes in the adhesive–dentin interface. As stated by Marshall and others,[43] van der Waals forces occur

at every interface; however, they are often supplemented by significant contributions from strong bonds that may be present. Whatever the explanation, the difference should perhaps be considered in future research. First, dentin-bonding studies using only normal intact dentin give overly optimistic estimations of dentin bond strength and should perhaps be replaced or complemented by studies using caries-affected dentin. Second, since the lower bond strength of caries-affected dentin is widely acknowledged,[29,30] perhaps more emphasis should be placed on attempts to improve immediate bond strength in carious dentin rather than the preservation of bond strength in intact dentin, as is currently being done. If the immediate bond strength of carious dentin can be increased or reach levels similar to intact dentin, then it will be interesting to see if bond strength reduction remains nonsignificant. That kind of adhesive would be of an actual clinical benefit, as dentin bonding is almost always performed on caries- or erosion-modified substrates.

Moreover, it is important to note the relationship between proteolytic enzyme inhibitors and the universal adhesive system, especially CHX, which seems to present an interaction with MDP. On the other hand, E-64 resulted in a lower decline of bond strength over time, mainly in erosive and carious substrates. For aging, the use of CHX and E-64 demonstrated no difference at baseline or at six months, and thus it is possible that proteolytic enzyme inhibitors have an interaction with altered substrates.

CONCLUSIONS

In conclusion, the present study confirms the previous findings of significantly lowered bond strength in artificial carious dentin compared to normal intact dentin. Simulated erosion also impaired bond strength to dentin. Proteolytic enzyme inhibition did not improve the durability of bond strength associated with the universal bonding system. However, E-64 preserved bond strength overtime in carious and eroded dentin so this treatment may be a promising strategy for hybrid layer longevity. Research should focus more on identifying methods to improve the initial bond strength in eroded and caries-affected dentin rather than trying to eliminate the degradation of the hybrid layer components created on intact dentin.

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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 Ethic Committee for Human Studies of Bauru School of Dentistry, University of São Paulo, Brazil. The approval code for this study is 16558913.2.0000.5417.

Conflict of Interest

The authors of this article 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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