

How erosive drinks and enzyme inhibitors impact bond strength to dentin

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Abstract: Concern has been raised about the bonding of restorative procedures to an erosive lesion, given the change in organic and inorganic composition and structure of this substrate. This *in vitro* study evaluated the effect of erosive drinks and an enzyme inhibitor (2% chlorhexidine digluconate - 2% CHX) on bond strength to dentin. Sixty sound human third molars were selected, and the occlusal enamel was flattened, exposing the dentin surface. The specimens were randomly divided into three groups: AS-Artificial saliva (control group), RC- Regular Cola and ZC- Zero Cola. Twenty specimens were immersed in their respective solution for 1 minute, 3 times a day, over the course of 5 days. After acid etching and before bonding with Adper Single Bond 2, half of the samples of each group (n = 10) were treated with 2% CHX, whereas the other half (n = 10) were not, forming the control group (CONV). All the specimens were restored with Filtek Z250 composite resin filled in Tygon tubes (0.48 mm²), yielding six microcylinders for microshear bond strength testing. Three composite resin microcylinders of each specimen were tested after 1 month, and the remaining microcylinders were tested after 6 months. Failure modes were determined using a stereomicroscope (40x). The data were statistically analyzed by three-way ANOVA and Tukey tests ($\alpha = 0.05$). Overall bonding was reduced after 6 months, regardless of treatment. The 2% CHX enhanced bond strength after 1 month only in the ZC group, and did not enhance bonding performance to demineralized dentin by erosive protocol after 6 months in any group.

Keywords: Chlorhexidine; Dentin-Bonding Agents; Tooth Erosion.

Introduction

The prevalence of erosive lesions has been increasing significantly, prompting an increase in the demand for treatment by patients.^{1,2,3} Erosion is defined as the loss of hard dental tissue resulting from non-bacterial chemical attack usually involving acidic substances.^{1,2,3} It leads to a progressive softening of the tooth surface with subsequent irreversible loss of hard dental tissue.^{3,4} Dental erosion primarily affects the enamel, but can also cause hypersensitivity if it reaches the dentin, or in severe cases of pulp exposure or even tooth fracture.^{5,6} Moreover, it is able to damage restorative materials in the oral cavity, at different levels.^{7,8}

The main etiologic factors for dentin erosion, such as the greater consumption of acidic foods and drinks, together with a more precise



diagnosis of gastro-esophageal disorders, have prompted the scientific literature to heighten the awareness of the so-called tooth-erosion phenomena, and to indicate more appropriate clinical approaches to address this issue.^{9,10,11,12,13} Acidic products, like soft drinks, could increase the risk of dental erosion, depending on the presence of other behavioral and biological risk factors.^{3,4,6,9}

The erosive potential of soft drinks is thought to involve several factors, including pH and buffering capacity, type of acid, frequency of exposure, duration of each episode of erosive exposure, chelating properties and content of calcium and phosphate.⁹

Previous *in situ* studies addressing these issues have found that low-calorie cola is less erosive than regular cola.^{10,11} It has been posited that this lower erosive potential may be related to phenylalanine amino acid, resulting from the hydrolysis of aspartame in the presence of saliva.^{10,11} It is thought that this amino acid may act as a buffer system, increasing the neutralization and buffering of cola drink acids.^{10,11} Since Zero Cola also contains phenylalanine, it could be inferred that Zero Cola is less erosive than regular cola.

These drinks are implicated in compromising the dental substrate to a greater or lesser extent. In many cases, lesions require restorative procedures.^{6,12} The main restorative materials of choice are glass-ionomer cements and composite resins.^{6,12} However, new studies have been focusing on eroded dental tissues as singular bonding substrates for adhesive materials, as pointed out by authors such as Zimerli *et al.*,¹³ 2012, and Francisconi-dos-Rios *et al.*,^{14,15} 2015. All these factors may alter the dental substrate, mostly in dentin. This leads to a deficient hybrid layer formation when restorative bonding materials are used, since they can interfere in the fibril collagen structural features and the dynamics of the organic matrix.^{13,14,15}

The durability of restorations is directly affected by the stability of the hybrid layer.¹⁶ One mechanism for degrading the dentinal organic matrix is through the collagenolytic activity of endogenous matrix metalloproteinase (MMP) enzymes found either in saliva or the organic dentin matrix.^{16,17,18} More recently, evidence has shown that some members of the cysteine

cathepsins (CCs) family may also chemically degrade unprotected collagen fibrils.^{14,15,16,17}

CHX is a potent inhibitor of intrinsic proteolytic enzymes of dentin, and can strongly reduce collagen fiber degradation, thus contributing to long-term stability of the hybrid layer and bond strength.^{19,20,21,22,23} CHX effectiveness on bond durability to various compromised substrates, such as caries-affected dentin, has also been investigated.^{24,25} It seems valuable to evaluate whether using CHX as a protease inhibitor would also favor bond strength durability to eroded dentin. Therefore, this study was designed to evaluate the effect of two types of cola drinks and the use of CHX on bond strength after 1 and 6 months. The null hypotheses are that bond strength to dentin is not affected by any of the following factors: type of erosive solution, use of chlorhexidine or time.

Methodology

Experimental design: This *in vitro* study evaluated three factors: immersion solution on three levels (artificial saliva, Regular Cola and Zero Cola), dentin treatment after acid-etching (conventional-control or associated with 2% CHX) and time (1 and 6 months), both on two respective levels. The response variable used in this comparison was bond strength evaluated by the microshear test.

This study was approved by the Ethic Committee for Human Studies of the Bauru School of Dentistry, *Universidade de São Paulo - USP*, Brazil (Process: 031-2010).

Specimen preparation: Sixty extracted sound human third molars were collected and stored for up to one month in 0.1% sodium azide at room temperature. The roots of the teeth were sectioned 3 mm below the cemento-enamel junction, and the occlusal enamel was removed horizontally (perpendicular to the long axis of the tooth), using a water-cooled diamond-impregnated disc (Extac Corp.; Enfield, 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 60 seconds (Politriz APL-4 Arotec; Cotia, Brazil). The surfaces were examined with a stereomicroscope (Leitz; Wetzlar, Germany) at 40x magnification to ensure presence of enamel only on the peripheral dentin surface. The specimens

were randomly allocated to the experimental groups according to the erosive challenges tested: artificial saliva, Regular Cola or Zero Cola.

Erosive challenge: The carbonated beverages selected to perform the erosion challenges in the present study were Regular Cola and Zero Cola (Coca-Cola, *Cia. Fluminense de Refrigerantes*; Porto Real, Brazil). Artificial saliva was used as a control immersion solution and was prepared by the biochemistry laboratory of the Bauru School of Dentistry at the author's request.

The specimens were randomly divided into three groups ($n = 20$), according to these beverages (Table 1). The Regular Cola and Zero Cola groups were processed according to a pH-cycling model: the teeth were immersed in carbonated beverage (Regular Cola or Zero Cola) for 1 minute, 3 times a day, over five days, and kept in artificial saliva between erosive cycles. The control group was immersed in artificial saliva for the entire experimental period.

Bonding procedures: After specimen demineralization, all the teeth were cleaned with water and dried with absorbent tissue. Half of the specimens were treated conventionally, as follows: acid etching using 35% phosphoric acid (Scotch Etchant, 3M ESPE; St. Paul, USA) for 15 seconds, and rinsing with water for 15 seconds. Excess water was removed by drying with oil-/water-free air, and absorbent paper was applied to yield moist dentin. The remaining samples from each group were treated with 2% CHX (Proderma; Piracicaba, Brazil) right after the conventional treatment, as described previously. After allowing a dwell time of 60 seconds, the dentin surface was gently dried with absorbent paper followed by application of the bonding protocol on

all the specimens: two thin coats of an etch-and-rinse dentin bonding system (Adper Single Bond 2- 3M ESPE; St. Paul, USA) were applied using a disposable microbrush, gently air-dried for 2-5 seconds to allow solvent evaporation, and light-cured for 10 seconds with a LED unit (Radical-SDI; Victoria, Australia). A composite resin (Filtek Z250, 3M ESPE; St. Paul, USA) was inserted into Tygon tubes (Norton Performance Plastics; Granville, USA), with a diameter of 0.79 mm and a height of 1.5 mm. The tubes were then placed on the dentin surface, and the specimens were light-cured for 20 seconds using a LED unit (Radical-SDI; Victoria, Australia) with an intensity of 1000 mW/cm². Six composite resin microcylinders were constructed for each tooth. Pilot tests were performed previously to guarantee that tests would keep from loading or touching other cylinders. The Tygon tubes were carefully removed using a steel blade. The specimens were stored in artificial saliva at 37°C. Three composite resin microcylinders of each specimen were submitted to a microshear bond strength test after 1 month. The remaining microcylinders were stored for up to 6 months at 37°C in weekly renewed artificial saliva, and then submitted to microshear bond strength testing. Each tooth was considered an experimental unit. The average of the three cylinders from each tooth was used to calculate the mean value by tooth at each evaluation time point, as previously recommended.²⁶

Microshear bond strength tests: Specimens were assessed by a microshear test, in a universal testing machine (Emic; São José dos Pinhais, Brazil) operating at a crosshead speed of 0.5 mm/min, using a 500N cell. A thin wire loop (0.2 mm in diameter) was wrapped around the bonded microcylinder assembly, as

Table 1. Comparison of immersion agents.

Immersion agents	Composition*
Artificial saliva**	1.5 mmol/L Ca(NO ₃) ₂ ·2H ₂ O, 0.9 mmol/L Na ₂ HPO ₄ ·2H ₂ O, 150 mmol/L KCl, 0.1 mol/L H ₂ NC(CH ₂ OH) ₃ (TRIS), 0.05 µg/mL F (NaF)
Regular Cola drink	Carbonated water, high fructose syrup, caramel color, phosphoric acid, natural flavors, caffeine content: 23 mg; 8 fl oz. pH = 2.74; tritability = 120 mL (0.1N NaOH)
Zero Cola drink	Carbonated water, caramel color, phosphoric acid, aspartame, potassium benzoate, natural flavors, potassium citrate, acesulfame potassium, phenylalanine, caffeine content: 23 mg/8 fl oz. pH = 3.08; tritability = 91 mL (0.1N NaOH)

* Composition based on manufacturer's information, except for pH and tritability, assessed in the laboratory.

** Prepared in the biochemistry laboratory.

closely as possible to the base of the microcylinder, and aligned with the loading axis of the upper movable component of the testing machine. The shear force at failure was recorded and converted into megapascals (MPa). No cylinders in this test were lost during storage.

Stereomicroscopy analysis: After performing the bonding tests, each specimen was analyzed using a stereomicroscope at 40x magnification (Leitz; Wetzlar, Germany), observing both material and substrate. Failures were categorized as: adhesive failure, mixed failure, cohesive failure in dentin or cohesive failure in resin. The percentage of the failure modes was calculated.

Statistical analysis: The data were calculated and analyzed statistically. The assumptions of equality of variances and normal distribution of errors were checked for all the variables tested. Since the assumptions were satisfied, the data were analyzed by three-way ANOVA and Tukey tests ($p < 0.05$).

Results

The bond strength means and standard deviations are summarized in Table 2.

Immersion solution ($p = 0.134$) and dentin treatment ($p = 0.455$) were not statistically significant factors; however, a significant interaction between the immersion solution and the dentin treatment was detected ($p < 0.000$). Time was the only statistically significant individual variable ($p < 0.000$).

At the 1-month evaluation, no differences between the groups associated with or without chlorhexidine were significant, except for ZC, which showed higher bond strength when associated with CHX (ZC-CHX).

At the 6-month evaluation, no statistical differences were found between the groups associated with or without CHX.

The overall results indicated significant higher bond strength for the immediate evaluation (1 month), as compared with the respective 6-month evaluation, except for ZC-CONV and AS-CHX.

A description of the failure mode distribution is shown in Table 3. All of the interfaces tested (100% of the specimens) were examined and accounted for. Adhesive failures were predominant in all the conditions tested, except for ZC-CONV. No cohesive failures were detected.

Discussion

A greater prevalence of erosive lesions has been reported in the literature, particularly in the initial stage of demineralization, when lesions are limited to the enamel.^{24,25} However, deep lesions involving dentin at different levels have raised concern, since the etiologic factors are not under control.^{3,6}

Based on the results of the present study, we rejected the hypothesis that time is not a significant factor.

The present study demonstrated a significant decrease in dentin bond strength for the tested conditions, except for ZC-CONV and AS-CHX, revealing no stability of bond durability.^{16,17} Bonding to dentin has been considered more difficult and less predictable than bonding to enamel.^{14,15} The main obstacle in dentin bonding is the heterogeneous nature of dentin, with hydroxyapatite deposited on a mesh of collagen fibers.^{13,14,15,22}

Under erosive conditions, this performance may become even more compromised, since mineral loss can

Table 2. Microshear bond strength mean values in MPa.

Time point Challenge	1 month		6 months	
	CONV	CHX	CONV	CHX
AS	12.19/2.21Aa*	9.33/4.92Aa*	7.06/3.42Ba*	5.91/2.96Aa*
RC	13.31/2.49Aa*	12.10/2.08Aab*	6.68/2.67Ba*	7.26/2.79Ba*
ZC	10.01/2.93Aa*	15.74/3.25Aab#	5.96/2.35Aa*	7.77/3.55Ba*

CONV: not treated with chlorhexidine; CHX: treated with chlorhexidine; Control-AS: artificial saliva; RC: demineralized with Regular Cola; ZC: demineralized with Zero Cola.

Different uppercase letters indicate significant differences for the same condition at different time points ($p > 0.05$).

Different lowercase letters indicate significant differences between cola drinks for the same treatment at the same time point ($p > 0.05$).

Different signs indicate significant differences between treatments for the same cola drinks at the same time point ($p > 0.05$).

Table 3. Failure mode distribution according to challenge (%).

Time point	1 month				6 months			
	CONV		CHX		CONV		CHX	
FM CHALLENGE	AD	M	AD	M	AD	M	AD	M
AS	50.00	50.00	100	0.00	86.66	13.33	90.00	10.00
RC	93.33	6.66	100	0.00	73.33	26.66	73.33	26.66
ZC	46.67	53.33	53.33	46.67	80.00	20.00	90.00	10.00

*Adhesive failure (AD), Mixed (M).

expose the organic matrix to degradation.^{13,14,15} Since a small decrease in pH could lead to a significant increase in demineralization, a pH ranging from 3 to 2 could cause different interactions.^{10,11,27} The cola drinks evaluated in the current study had different pH values: Regular Cola had a pH of 2.7, and Zero Cola had a pH of 3.0. Therefore, greater demineralization by Regular Cola would be expected.^{10,11} However, as measured by bond strength, this performance was not confirmed, since all the drinks showed similar performance ($p = 0.134$). The similar performances of these beverages could be explained by their similar buffering capacities. Furthermore, Regular Cola presented a higher concentration of calcium and phosphate, as compared with Zero Cola. This could minimize the influence of its low pH, making its erosive potential similar to that of Zero Cola. Rios *et al.*^{10,11} suggested that Light Cola was less erosive than Regular Cola. They attributed the lower erosive potential of Light Cola to the presence of phenylalanine amino acid, which is produced by the hydrolysis of aspartame in the presence of saliva. The same hypothesis can be inferred for Zero Cola, due to the presence of aspartame. However, a limitation of this interpretation is posed by the use of saliva produced artificially instead of that existing naturally in the oral environment. This may explain the lack of differences between Regular Cola and Zero Cola in the present experiment.

The application of 2% CHX is recommended for use after dentin phosphoric acid etching as a proteolytic inhibitor.^{14,15,19,20,21,22,23} In the present study, 2% CHX did not influence adhesive bond strength over time, except for AS, when comparing AS-CHX after 6 months. The specimens demineralized with Zero Cola and treated with 2% CHX presented higher bond strengths at the 1-month evaluation. However, 2% CHX showed no influence on the bond strength to demineralized specimens previously

treated with any cola drinks after 1 and 6 months ($p > 0.05$). Previous research reported controversial results,^{21,27,28,29,30,31} such as the study by Komori *et al.*,²² 2009. However, it should be stated that these authors tested the effect of CHX on natural carious dentin, which is more complex and more affected than eroded dentin. In carious dentin, a decrease in the diffusion gradient of the resin monomers results in a layer of denuded collagen at the base of the hybrid layer during the bonding procedure, when using an etch-and-rinse adhesive system,²² and leads to the disarrangement of the collagen fiber network. This scenario causes degradation of uncovered collagen by activating host-derived enzymes, such as MMPs^{18,19} and, consequently, degradation of the bond interface.

The use of CHX is supported by evidence of its interaction with these MMPs and CCs¹⁷, minimizing the degradation potential of MMPs along the demineralized layer of collagen. CHX is not reported to be associated with negative effects on bond strength with etch-and-rinse systems.³⁰

Any effort to preserve a hybrid layer is desirable to avoid a reduction in bonding. CHX may prevent collagen degradation, but does not interfere with polymer network stability.³⁰ The polymer continues to be susceptible to water sorption and swelling. Thus, spaces are created in the hybrid layer, and collagen fibers are exposed and can be degraded by MMPs. CHX cannot prevent this biodegradation, only postpone it.^{14,15,29,30} This could partially explain the observation of a continuous reduction in bond strength over time, even in some groups treated with CHX. The current data suggests that 2% CHX was not able to maintain bond strength to normal dentin or dentin previously demineralized with cola drinks. Other strategies, such as ammonium

quaternary molecules with smaller size and effective bind potential, have been studied with successful prospects.²¹ Moreover, other vehicles for CHX, such as gel, could be more effective and should be further investigated.³¹ The association between mechanical and biological strategies may be beneficial in resisting hydrolytic degradation and inactivating the MMPs, thus improving restoration longevity.

Although microtensile bond strength is a reliable *in vitro* gauge to evaluate bond strength,³² the methodology used in this study generates less stress or damage during specimen preparation, compared with the microtensile alternative.³³ Furthermore, restorations made for the microshear bond strength test are small (0.48 mm²), allowing the storage medium to infiltrate the bonding area efficiently. According to the fracture mode evaluation, most of the failures were adhesive in all the conditions tested, except for Z. This is an additional advantage of the microshear test, because

the non-sequential sectioning required to prepare the specimens reduces the occurrence of cohesive substrate fractures.³⁴

Conclusion

Chlorhexidine has been advocated as a potential inhibitor of dentin proteolytic enzymes; however, it does not act positively either on normal/sound or eroded dentin over time.

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