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Influence of Chlorhexidine and/or Ethanol Treatment on Bond Strength of an Etch-and-rinse Adhesive to Dentin: An *In Vitro* and *In Situ* Study

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Clinical Relevance

Bond strength suffered degradation over time and was not influenced by dentin treatments with chlorhexidine and/or ethanol. Adhesive bond degradation was less affected under *in situ* conditions than *in vitro*.

SUMMARY

The aim of this study was to evaluate the effect of a chlorhexidine and/or ethanol application on the bond strength of an etch-and-rinse, hydrophobic adhesive system either under *in vitro* aging or *in situ* cariogenic challenge. The dentin surface of 36 human third molars were flattened and allocated into four groups to be treated with chlorhexidine, ethanol, or chlorhexidine + ethanol or left unexposed to any solution (control) (n=9). Then, a resin composite restoration was made on the dentin surface and longitudinal sticks were obtained. Sticks from each tooth were assigned to three test conditions: stored in water *in vitro* for 24 hours, stored in water *in vitro* for 6 months, or worn *in situ* for 14 days. During *in situ* wear time, a high-cariogenic challenge condition was simulated. Specimens were tested for microtensile bond strength (μTBS). Multivariate analysis of variance and Tukey's test showed that chlorhexidine, ethanol, or chlorhexidine + ethanol did not affect the μTBS. The *in vitro* μTBS values were significantly lower for the specimens stored for 6 months than for those stored for 24 hours. Intermediate μTBS values were shown by the specimens worn *in situ*. Thus, use of chlorhexidine and/or ethanol was incapable of containing the degradation

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at the bond interface in the *in vitro* model. The *in situ* model was capable of reducing bond strength similarly to the *in vitro*/6 months model. Despite this, the *in situ* bond strength was still similar to that of the *in vitro*/24-hour model.

INTRODUCTION

Enamel and dentin are tissues that differ in composition, structure, and water concentration.[1] Although a lasting seal occurs between adhesive systems and enamel, the same has not been observed in dentin, in which bond strength decreases over time.[2-4]

Extrinsic and intrinsic factors are related to the integrity of the bond interface, such as the chemical and physical stability of the composite and dentin, the adhesive system itself, and the collagen fibers involved in the hybridization process.[5] The intrinsic water content of dentin and water coming from the oral cavity negatively affect the bond stability over time.[6]

Additionally, in an attempt to simplify the protocols of adhesive application, many adhesive systems have become increasingly hydrophilic, which can result in a trend toward hydrolytic degradation. In view of this knowledge, the use of hydrophobic dimethacrylates is expected to reduce water sorption.[7] However, during a conventional wet-bonding technique, the remaining water can potentially promote phase separation of the hydrophobic monomer, which has less solubility.[8] Studies[8,9] have demonstrated that use of anhydrous solvents, such as ethanol (ETH), on demineralized dentin keeps the demineralized collagen matrix expanded and ready for receiving the adhesive system. Also, the tissue would be less hydrophilic, which would prevent the phase separation of the hydrophobic monomer and result in a more stable bond over time.

Nevertheless, it has been observed that there is degradation of the hybrid layer even in well-sealed restorations. It has been demonstrated that the decrease in bond strength is not solely related to the resin material.[10] Another factor seems to play an important role in this process: collagen proteolysis.[11]

Dentin contains enzymes from the metalloproteinase (MMP) family, which exhibit a collagenolytic and gelatinolytic action capable of degrading collagen fibrils not encapsulated by the adhesive system.[3,11] It has been observed that chlorhexidine gluconate is an MMP inhibitor capable of maintaining hybrid layer integrity by preventing collagen from degrading.[10,12-15]

Studies have evaluated the application of ethanol with the aim of favoring the use of either hydrophobic monomers[9,16-19] or chlorhexidine as an MMP inhibitor.[11,15,20,21] However, it is still unclear whether the association of these two substances would act additively or synergistically.

One of the most commonly used methods to simulate bond degradation is the storage of restorations in water.[22] Such models, however, do not properly simulate the processes taking place in an intraoral situation. One of these processes involves the pH fluctuations caused by cariogenic challenges, which can reduce bond strength.[23]

In addition, *in situ* models are appropriate for evaluating bond degradation because host-derived MMPs can originate from saliva and from dentin.[24] Recently, Reinke and others[23] reported that the *in situ* model seems to be a suitable short-term methodology to investigate the degradation of resin-dentin bonds under a more realistic condition.

Therefore, the aim of this study was to compare the bond strength of a three-step adhesive system at time intervals of 24 hours and 6 months, and in an *in situ* cariogenic challenge. The following null hypotheses were tested: 1) Neither chlorhexidine nor ethanol, used alone or in combination, influences bond strength values; 2) time has no influence on bond degradation; and 3) *in situ* cariogenic challenge does not influence bond strength.

METHODS AND MATERIALS

Experimental Design

The experimental design of this study followed a randomized complete block design with repeated measures, in a 4x3 factorial scheme. The following factors were under study:

- Treatment at four levels: control, chlorhexidine, ethanol, and chlorhexidine + ethanol;
- Test condition at three levels: after *in vitro* storage for 24 hours or 6 months and *in situ*.

The response variable was microtensile bond strength, measured in MPa. In this study each tooth was considered an experimental unit. Therefore, beams taken from each tooth (*in vitro*/24 hours, *in vitro*/6 months, and *in situ*) were not independent. Due to this dependent relationship, multivariate analysis of variance (MANOVA) was applied.

Selection and Preparation of Teeth

Thirty-six sound, recently extracted human third molars were randomly selected from a pool of extracted teeth (stored in a 0.1% thymol solution)

Table 1: *Materials, Manufacturers, Composition, Description, and Mode of Application of Materials*

Material	Manufacturer	Lot	Description
All Bond 3	(Bisco Inc)	Lot 1000004752	Conventional three-step application adhesive system: acid, part A (primer), and part B (light-activated adhesive).
Filtek Z100	(3M ESPE)	Lot N142512BR	Microhybrid resin composite
Chlorhexidine (FGM)		Lot 301110	Solution
100% Ethanol	(Chemco Ltda, Brazil)	Lot: 24631	Solution

Abbreviations: MgNTG-GMA, tolylglycine glycidyl methacrylate, definition; Bis-GMA, *bisphenol A glycidylmethacrylate; BPDN, biphenyl dimethacrylate; HEMA, 2-hydroxyethyl methacrylate; TEGDMA, triethylene glycol dimethacrylate.*

from the São Leopoldo Mandic School of Dentistry, with appropriate ethical approval from the local ethics committee (protocol 2010/0294).

Teeth were sectioned perpendicular to the long axis at the amelodentinal junction. Two-thirds of the most apical root portion was also removed, exposing the pulp chamber, so dentin thickness could be standardized to 2 mm. Pulp chambers were filled with an adhesive and resin composite (Single Bond and Z100 shade A2, 3M/ESPE, St Paul, MN, USA). The exposed occlusal dentin was flattened and polished using a metallographic grinder (Politriz Aropol 2V, Arotec, Cotia, São Paulo, Brazil) with 600-grit silicon carbide abrasive paper. Samples were randomly divided into four experimental groups (n=9) to be treated with chlorhexidine, ethanol, or chlorhexidine + ethanol, or left untreated. At this time, all specimens were submitted to ethylene oxide sterilization.[25,26]

Restorative Procedures

Table 1 describes the materials used, manufacturers, lots, manufacturers' instructions, and dentinal treatments. Specimens were prepared (Table 2) according to the experimental group to which they were assigned. After the adhesive procedures, the resin composite (Z100, 3M/ESPE) was inserted in three increments of 2 mm and individually light polymerized for 40 seconds between each increment, using a halogen light unit at 450 mW/cm^2 (Demetron LC, Kerr, Danbury, CT, USA). Irradiance was monitored with a radiometer (RD-7, Ecel Ind. e Com. Ltda, Ribeirão Preto/São Paulo, Brazil).

Specimen Preparation for Microtensile Test and Storage

Samples were individually fixed on acrylic plates. This appliance was duly fixed to a precision cutter (Isomet 1000, Buehler, Lake Bluff, IL, USA) used to serially section the samples from the resin composite, parallel to their long axis in the mesiodistal and vestibular-lingual directions, with a distance of 1 mm between the cuts, thereby obtaining around 12 sticks per tooth. One-third of the sticks were randomly assigned to be stored in distilled water at 37°C for 24 hours. Another third was kept in distilled water at 37°C for 6 months, and the remaining sticks were assigned to be worn *in situ*.

In Situ Test

After signing an informed consent form, nine volunteers of both genders, aged 20 to 50 years,

Table 2: *Description of the Experimental Groups and Treatments*

Groups	Treatments
1	Acid etching for 15 seconds, washing with water, removing excess water, and adhesive system application
2	Acid etching for 15 seconds, washing with water, removing excess water, chlorhexidine application for 30 seconds, removing excess chlorhexidine, and adhesive system application.
3	Acid etching for 15 seconds, washing with water, removing excess water, 100% ethanol application for 1 minute, drying, and adhesive system application
4	Acid etching for 15 seconds, washing with water, removing excess water, 100% ethanol application for 1 minute, drying, chlorhexidine application for 30 seconds, removing excess chlorhexidine, and adhesive system application

Table 1: *Materials, Manufacturers, Composition, Description, and Mode of Application of Materials (ext.)*	
Composition (Main Components)	**Application Mode**
Acid: 32% phosphoric acid with benzalkonium chloride	Acid etching of dentin for 15 seconds, washing, removing excess humidity, application of 2 coats of primer, drying for 5 seconds, primer polymerization for 10 seconds, adhesive application, and light polymerization for 20 seconds
Part A: Ethanol, MgNTG-GMA	
Part B: Bis-GMA, BPDN, HEMA, photoinitiator, and stabilizer	
Bis-GMA and TEGDMA	2-mm layers
2% Chlorhexidine digluconate	Acid etching for 15 seconds, washing with water, removing excess water, chlorhexidine application for 30 seconds, removing excess chlorhexidine, and adhesive system application.
100% Ethanol	Acid etching for 15 seconds, washing with water, removing excess water, 100% ethanol application for 1 minute, drying, and adhesive system application.

were enrolled. The inclusion criteria were as follows: stimulated salivary flow >0.7 mL/min, available to follow the schedule established for the experiment, and no active caries lesions. The exclusion criteria were as follows: taking medication; pregnant or lactating, having a periodontal disease; wearing removable dentures, orthodontic appliances (fixed or removable), or occlusal plates; or presenting systemic diseases.

The sticks assigned to the *in situ* test were mounted in an intraoral appliance, which was fabricated of self-polymerizing acrylic resin made from the volunteers' casts. The appliances contained four niches, and one stick from each of the four experimental groups was mounted. The sticks were fixed into their respective niches using a gauze and wax. Palatal appliances were then inserted into each participants' mouth.

The *in situ* phase volunteers were instructed in how to use the dentifrice (Sorriso Colgate Palmolive Company, São Bernardo do Campo, São Paulo, Brazil) and toothbrush (Colgate Palmolive Company, São Bernardo do Campo, São Paulo, Brazil) provided by the researcher. Seven days later, the appliance was inserted and worn for 14 days.[27] Cariogenic challenges were started on the second day, as the first day served to allow saliva pellicle formation. The volunteers were instructed to remove the palatal appliance and drip a 20% sucrose solution into each niche four times a day (8:00 AM, 11:00 AM, 3:30 PM, and 7:00 PM). After 5 minutes of dripping the solution, the appliance was reinserted into the oral cavity.[27] During the experimental period, the volunteers were instructed to brush their teeth after the main meals (7:30 AM, 12:30 PM, and 8:00 PM) and to use the device continually, removing it only for oral hygiene and during meals.

Microtensile Test

Sticks were attached to an acrylic testing device using a cyanoacrylate adhesive (Super Bonder Gel, Henkel Ltda, São Paulo, Brazil) and subjected to tensile stress in a universal testing machine (MEM-2.000 model, EMIC, São José dos Pinhais, Paraná, Brazil) at a crosshead speed of 0.5 mm/min and a 20N load cell until fracture. After testing, fractured surfaces were observed under a stereomicroscope to determine the mode of failure according to one of the four criteria: 1) adhesive fracture, 2) part adhesive fracture and part cohesive in resin, 3) cohesive fracture in resin, and 4) cohesive fracture in dentin.

Statistical Analysis

Data were submitted to MANOVA and Tukey's test. The significance level was set at 5%.

RESULTS

Table 3 summarizes the mean values and standard deviations found for each group.

Table 3: *Means (MPa) (Standard Deviation) of Bond Strength Values Per Treatment and Test Condition*			
Treatment	**Test Condition**		
	***In Vitro* 24 h**	***In Situ***	***In Vitro* 6 mo**
Control	28.77	21.38	19.37
	(11.02)	(7.27)	(5.74)
Chlorhexidine	24.43	19.12	17.47
	(8.01)	(8.87)	(6.62)
Ethanol	20.86	19.69	19.28
	(7.09)	(12.05)	(9.68)
Chlorhexidine + ethanol	27.01	26.86	21.22
	(17.86)	(18.78)	(9.97)

Table 4: *Mean Values (Standard Deviation), According to Test Condition, Regardless of the Dentin Treatment*

Test Condition	Bond Strength (MPa)
In vitro 24 h	25.27 (11.66) a
In situ	21.76 (12.39) ab
In vitro 6 mo	19.33 (7.97) b

Distinct letters indicate statistical differences between groups ($p<0.05$)

MANOVA revealed no significant interaction between treatment and test condition ($p=0.7657$). No differences in microtensile bond strength were caused by the treatments applied on the dentin ($p=0.5410$). Test Condition had a significant effect on microtensile bond strength ($p=0.0174$). Tukey's test showed that, regardless of the treatment applied to the dentin, under *in vitro* conditions significantly lower bond strength values were observed when the sticks were stored for 6 months (Table 4). Bond strength values of the sticks submitted to the *in situ* condition did not differ from those observed in the *in vitro* conditions (24 hours and 6 months).

With regard to the failure mode, for all test conditions, cohesive failures were the most common, followed by mixed failures and adhesive failures, regardless of the treatment received by the dentin (Table 5).

DISCUSSION

The results of this study demonstrated that water storage had a significant influence on the adhesive interface degradation, regardless of the dentin treatment. Thus, null hypothesis 1 was rejected and null hypothesis 2 was accepted. The loss of bond strength along the hybrid layer may occur by degradation of the resin material, collagen fibrils, or both.[2,28]

Ideally, adhesive systems should have formulations based on hydrophobic monomers, as they are more stable both chemically and mechanically.[16] However, hydrophobicity is incompatible with dentinal humidity. Therefore, in order to use hydrophobic adhesive systems it is necessary to alter the dentinal substrate. Because of this aspect, the use of ethanol (ethanol wet-bonding) has supported the rationale that a less hydrophilic substrate would favor the encapsulation of unprotected collagen by hydrophobic monomers.[16-18] In fact, many studies have shown promising results for this technique,[16-18,29,30] even though most of them used increasing concentrations of ethanol. However, clinically, this appears to be unfeasible, seeing that it excessively increases the clinical working time. Therefore, the technique used in this study was dehydration with 100% ethanol for 1 minute.[31] Considering that null hypothesis 1 was rejected because all dentin treatments (including application of ethanol) yielded no differences in bond strength, it may be suggested that this simplified technique may not have been sufficient to dehydrate the dentin in order to improve the penetration of the adhesive system used and thereby prevent degradation of the hybrid layer.[32]

Moreover, it is to be expected that a hydrophobic material, such as the adhesive system used in the present study, would maintain bond integrity for a longer time. Nevertheless, it was observed that this did not occur, as there was a decrease in bond strength over the 6-month period of water storage. Perhaps acid etching for 15 seconds demineralized the dentin to a depth that was not completely achieved by the adhesive monomer,[33] thus leaving the unprotected collagen more vulnerable to proteolytic degradation. Ethanol in small concentrations is known to favor the mechanism of polymerization and a degree of conversion of adhesive systems. Nevertheless, these properties may be jeopardized when the ethanol concentration appears to be above 30%.[6] The All Bond 3 system contains approximately 49% ethanol after the mixture of primers A and B (per the manufacturer). This concentration of ethanol in an

Table 5: *Failure Mode According to Treatment and Test Conditions*

Failure Mode	*In Vitro* 24 h				*In Situ*			
	Control	Chlorhexidine	Ethanol	Chlorhexidine + Ethanol	Control	Chlorhexidine	Ethanol	Chlorhexidine + Ethanol
Adhesive	10%	20%	35%	23%	19%	29%	6%	24%
Cohesive in resin	27%	29%	22%	27%	33%	33%	18%	24%
Cohesive in dentin	27%	31%	26%	27%	29%	24%	47%	24%
Mixed	36%	20%	17%	23%	19%	14%	29%	28%

adhesive added to the high-concentrated ethanol applied on the dentin lead us to think that the dentin was supersaturated with ethanol. This may explain the results seen in the groups in which the ethanol was applied. This hypothesis may be confirmed by the fracture patterns. After 6 months there was a large quantity of fractures of the cohesive type in the resin and adhesive, showing adverse effects of ethanol on the resin material polymerization and on bond stability.

However, it is known that control of dentinal humidity alone does not prevent the degradation of collagen fibers that are incompletely infiltrated by the monomer. This degradation may also occur through the action of MMPs.[11,14,31] When the dentin is etched during adhesive procedures, the MMPs are activated and may slowly degrade the collagen fibrils. It is known that MMPs may be inhibited by protease inhibitors, such as chlorhexidine. Consequently, it has been demonstrated that the use of chlorhexidine can possibly decrease the hybrid layer degradation, even at low concentrations.[12]

Although a reduction in immediate bond strength has been demonstrated when chlorhexidine is used,[34] this was not observed in the present study (null hypothesis 1 was rejected), and it has not been corroborated by other authors.[10,15,21,35,36] Nevertheless, the results of the present study showed no significant effect of chlorhexidine on bond strength stability, results that were also found in the literature.[37] These differences may be attributed to the diverse forms of dentin surface preparation, different ages of dentin, diverse measurement techniques used (such as the mode of force application), and material properties (such as modulus of elasticity and size of samples tested).[38] Moreover, the chlorhexidine may have lost its capacity of substantivity. Considering that the small extensions of the sticks represent a form of accelerated aging, so that water could easily diffuse from the surface toward the center, thus, the water diffusion could lead to dilution or displacement of the chlorhexidine and loss of substantivity.[15]

Table 5: *Failure Mode According to Treatment and Test Conditions (ext.)*

In Vitro 6 mo			
Control	**Chlorhexidine**	**Ethanol**	**Chlorhexidine + Ethanol**
4%	12%	33%	15%
28%	27%	33%	11%
28%	37%	10%	41%
40%	24%	24%	33%

In vitro methods provide important information about the fundamental factors involved in the degradation at the resin/dentin interface. However, *in vitro* methods fail to consider the complexity of the intraoral medium (eg, bacterial plaque, enzymes, acidity of foods, chemical agents).[28] Therefore, in this study an *in situ* model was used, which is usually adopted to exacerbate the cariogenic challenge,[27] as no other methodology appropriate for evaluating degradation at the bond interface in an *in situ* model has been found in the literature. The results showed that the *in situ* condition was statistically similar at 24 hours and at 6 months, demonstrating degradation at the bond interface to a limited extent, so null hypothesis 3 was partially accepted. Nevertheless, the interference of the model was not as exacerbated as that which occurred during *in vitro* storage for 180 days. This fact shows that perhaps the deleterious effect of time is more important in bond degradation than the immediate oral conditions. In view of these results, further research needs to be conducted to develop an *in situ* methodology that simulates adhesive interface degradation.

Thus, knowledge of the importance of bonding with reference to the clinical longevity of restorations combined with the knowledge of the limitations of contemporary adhesive systems, explain why the use of chlorhexidine and ethanol associated with a hydrophobic adhesive system was not capable of containing the degradation of the bond interface in the *in vitro* model after 180 days of storage.

CONCLUSION

The use of chlorhexidine and/or ethanol could not contain the degradation at the bond interface in the *in vitro* model. The *in situ* model was capable of reducing bond strength similarly to the *in vitro*/6-month model. Despite this, *in situ* bond strength was still similar to the *in vitro*/24-hour model.

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