The aim of this study was to evaluate the effect of cariogenic challenge on the microtensile bond strength values (μTBS) of dentin pre-treated with chlorhexidine digluconate (CHX) or sodium hypochlorite (NaOCl). Thirty-six sound molars were selected and randomly assigned to 3 dentin pre-treatments (distilled water - control, 2% CHX and 10% NaOCl) and 4 aging protocols (24h control, biofilm without cariogenic challenge, biofilm with cariogenic challenge, and 18-month water storage). The same etch-and-rinse adhesive system and composite resin were used for all groups (n=30 beams). For the biofilm groups, dental microcosm biofilms originated from saliva of a healthy donor were grown on the samples with a defined medium enriched with mucin, with or without 10% sucrose, according to the group. After the experimental period, the microtensile test was performed. Data were analyzed with ANOVA followed by Tukey test (p<0.05). The pre-treatment did not influence μTBS for all aging conditions (p=0.188), but the type of aging affected the bond strength (p<0.001). Cariogenic challenge and water storage aging affected the bond stability resulting in a decrease of the μTBS, but the pre-treatments did not influence the μTBS.

**Introduction**

Laboratory tests used to evaluate the mechanical properties of the resin–dentin interface have been referred to as important predictors of the performance of adhesive systems and materials (1). Several methodologies have been proposed trying to better simulate clinical conditions using laboratory tests; however, thermal cycling and water storage are the most common aging protocols used when assessing the resin–dentin interface stability (2,3). The integrity of the tooth–restoration margins may also be influenced by chemical factors, such as pH oscillations, which can simulate the chemical changes that naturally occur in the mouth. In addition, biofilm accumulation and cariogenic challenge are conditions to which the oral environment is daily exposed, and few studies evaluated the effect of those conditions on the bond strength of adhesive systems (4,5).

Endogenous matrix metalloproteinases (MMPs) released by adhesive procedures may degrade collagen in the hybrid layer and compromise the bonding effectiveness of the adhesive system (6). Studies have shown that the bond strength of adhesive systems tends to diminish with time and the interface shows alterations in hybrid layer (3). The degradation process may be caused by inadequate penetration of the resin monomer in the demineralized dentin, inadequate photo-curing of the monomer, water sorption, and resin and dentin degradation activity that contributes to the collagen destruction (3,6). It has been suggested that the suspension of degrading activity of MMPs by protease inhibitors may modify the dentin surface after acid etching (7,8), as an attempt to increase the stability of resin–dentin interface. Studies have shown that chlorhexidine is an effective MMP inhibitor capable to produce a more stable interface (8,9).

Application of substances after dentin etching to promote the removal of collagen fibrils (deproteinization) has also been proposed to avoid early dentin–resin interface degradation on the dentin substrate (10). Removal of collapsed collagen fibrils with sodium hypochlorite (NaOCl) may have the beneficial effect of reducing the sensitivity of the technique and to facilitate diffusion of the adhesive through the demineralized dentin. This procedure alters the dentin morphology, increases the wettability of the substrate, the tubular penetration, the number and length of resin tags and the values of bond strength (11,12).

As secondary caries is one of the most important factors related to restorations’ failure and replacement (13), and considering that there are few studies on the effect of cariogenic challenge on adhesive interface strength, the aim of this study was to evaluate the effect of various types of aging protocols on the microtensile bond strength values (μTBS) of dentin pre-treated with chlorhexidine digluconate (CHX) or sodium hypochlorite (NaOCl).

The tested hypotheses were the following: 1) CHX and NaOCl pre-treatments would promote more stability for the dentin-resin interface in long term, and 2) the aging conditions would affect negatively the adhesive bond strength of restorations.
Material and Methods

The study was approved by the Local Ethics Committee of Research (#210/2011). All materials were used according to the manufacturer’s instructions and the pre-treatment solutions were prepared in a prescription pharmacy (Uso Indicado, Pelotas, RS, Brazil). The same etch-and-rinse adhesive system and composite resin were used for all groups (Table 1).

Preparation of Samples

Thirty-six caries-free human molars were selected and remained stored in chloramine aqueous solution for disinfection until use. The teeth were randomly assigned initially in 3 groups (n=12 teeth), according to the dentin pre-treatments (control - distilled water; pre-treatment with CHX; and pre-treatment with NaOCl). From these 12 teeth per group, 30 resin-dentin beams were randomly selected for each subgroup according to the aging conditions (control - 24 h; biofilm without cariogenic challenge; biofilm with cariogenic challenge; and water storage). The preparation of the samples was done in the same way for all groups. The occlusal surface of the teeth was sectioned transversally to remove occlusal enamel and to expose the dentin. Dentin surfaces were polished with #600 silicon carbide sandpaper in a circular polishing machine (Arotec PL 4, São Paulo, SP, Brazil). The cuts were obtained using precision rotating machine at 200 rpm (Isomet 1000; Buehler, Lake Bluff, IL, USA) with water-cooled diamond saw (Extec Corp., Enfield, CT, USA).

Dentin Pre-treatments

The samples were randomly allocated to 3 types of pre-treatments:

1. Control: immediately after the dentin etching for 15 s with 37% phosphoric acid gel without chlorhexidine, distilled water was applied on the dentin surface for 60 s. After the excess removal with paper towels, the adhesive system was applied to the dentin according the manufacturer's instructions.

2. Chlorhexidine digluconate: after dentin etching for 15 s followed by air/water spray during 30 s and drying with absorbent paper, a 2% chlorhexidine digluconate solution was applied with disposable syringe. After 60 s, the solution excess was removed with paper towels and the adhesive system was applied to the dentin.

3. Sodium hypochlorite (deproteinization): after dentin etching for 15 s followed by air/water spray during 30 s and drying with absorbent paper, a 10% NaOCl solution was applied with disposable syringe. After 60 s, the surface was washed with water for 60 s, the excess water was removed with paper towels, and the adhesive system applied to the dentin.

Restorative Procedures

The restorative procedure was performed immediately after adhesive system application. Each composite resin horizontal increment (3 increments) was light cured for 20 s using a LED light unit (Radii-Call; SDI, Bayswater, VI, Australia) with irradiance of 650 mW/cm². The samples were stored in distilled water at 37 °C for 24 h. One previously trained operator performed the restorative procedures.

Preparation of the Resin-Dentin Beams

The samples were sectioned within each group and 30 stick-shaped beams (cross section of ±1 mm²) of resin-dentin were randomly allocated to each subgroup, according to the aging condition. The beams of the control group (24 h) were immediately submitted to microtensile test. The other beams were exposed to the other aging conditions.

Aging Conditions

1. Control (CO): Beams remained stored in distilled water at 37 °C for 24 h prior to mechanical testing.

Table 1. Description of the tested materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Type</th>
<th>Composition</th>
<th>Application Procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adper Single Bond 2 (3M ESPE)</td>
<td>Etch-and-rinse two-step adhesive system</td>
<td>Bis-GMA, HEMA, dimethacrylates, Polyalkenoic copolymer, ethanol, water, photoinitiator</td>
<td>1. Apply adhesive under pressure for 20 s, 2. Dry with a gentle air stream for 5 s, 3. Photo-cure for 10 s.</td>
</tr>
<tr>
<td>Filtek Z250 (3M ESPE) Shade A2</td>
<td>Hybrid light-cure resin-based composite</td>
<td>Bis-GMA, UDMA and Bis-EMA; 66% filler: zirconium/silica</td>
<td>1. Apply composite 2 mm thick, 2. Photo-cure for 20 s.</td>
</tr>
<tr>
<td>Condact (3M ESPE)</td>
<td>Etching agent (pH 0.7)</td>
<td>35% phosphoric acid gel</td>
<td>1. Apply etchant for 15 s, 2. Rinse for 15 s, 3. Dry with a mild, oil-free air spray.</td>
</tr>
</tbody>
</table>

Bis-GMA: bisphenol glycidyl dimethacrylate; HEMA: 2-hydroxyethyl methacrylate; UDMA: urethane dimethacrylate; Bis-EMA: bisphenol-A ethoxylated dimethacrylate.)
2. Water storage (WS): Beams remained stored in distilled water at 37 °C for 18 months before the mechanical testing, with weekly changes of the storage water.

3. Biofilm without cariogenic challenge (NCC) - no changes of pH: The beams were protected with nail varnish except for the adhesive interface and gamma ray sterilized. In the microcosm model (14), biofilm was formed on beams in cell tissue culture plates with the saliva inoculum obtained from healthy adult volunteer. An aliquot of 0.1 mL of fresh saliva was homogenized and inoculated into each beam. Biofilms were grown for 14 days in triplicate (n=30). The specimens received pure DMM (no sucrose addition) for 24 h and rinsed for 10 s in sterile saline solution and transferred to a new microplate with fresh DMM every 24 h.

4. Biofilm with cariogenic challenge (CC) - with pH change: The same steps used for NCC group were performed, except that for cariogenic challenge the beams received DMM with 1% sucrose (DMM + s) for 6 h, and after that the beams were rinsed for 10 s in sterile saline and transferred to a new 24-well microplate with fresh DMM for 18 h and replaced daily.

### Biofilm Aging

Collection and processing of saliva: fresh stimulated saliva (±20 mL; Parafilm "M", American National Can™, Chicago, IL, USA) was collected by morning from a healthy donor. The donor stopped the oral hygiene for 24 h prior to saliva collection. Saliva was filtered through sterile glass wool, stored in a sterile container and homogenized by vortexing, and divided into two aliquots, one for microbial quantification (data expressed in CFU/mL), and the other was centrifuged (5,000 g at 4 °C for 5 min), the supernatant was removed and the precipitate was frozen for future microbial analyses.

Artificial saliva (DMM), microbial growth and biofilm formation: the defined medium enriched with mucus (DMM) was carried out according to a previously described protocol (15), and contained porcine gastric mucin (2.5 g/L), urea (1.0 mmol/L), salts (in mmol/L CaCl₂ 1.0, MgCl₂ 0.2, KH₂PO₄ 3.5, K₂HPO₄ 1.5, NaCl 10.0, KCl 15.0, NH₄Cl 2.0), mixture of 21 amino acids, 17 vitamins and growth factors. The medium contains the equivalent amino acids in protein/peptide (in mmol/L) at concentrations based on human saliva: alanine (1.95), arginine (1.30), asparagine (1.73), aspartic acid (1.52), cysteine (0.05), glutamic acid (5.41 mmol/L), glutamine (3.03), glycine (1.95), histidine (1.08), isoleucine (2.38) leucine (3.68), serine (3.46) tretonina (1.08), tryptophan (0.43), tyrosine (2.17), valine (2.38) and casein (5.0 g/L). To characterize cariogenic challenge, sucrose was added to the medium for CC aging group at a concentration of 1% (DDM + s).

Saliva was inoculated onto the beams in each well (0.1 mL). After 1 h, the saliva was gently aspirated from the wells and 2 mL of previously prepared DMM was added in each well. The NCC group (without pH changes) did not receive supplemental sucrose in the DMM. However, the samples of CC group (with pH changes) received DMM with 1% sucrose. The medium was daily replaced (14). Biofilms were formed independently on the samples. The micro-wells were incubated in anaerobic condition (80% N₂, 10% CO₂ and 10% H₂), under controlled temperature (37 °C) for 14 days. Proceeding the time of each medium renewal, the plates were thoroughly shaken, and the supernatant was removed at 1st, 4th, 7th and 14th day for microbiological analyses.

Analysis of the biofilm acidity was carried out using pH measurements (Quimis 50w; Quimis Scientific Instruments, Diadema, SP, Brazil) and V621 electrode (Analion, Ribeirão Preto, SP, Brazil) after 1, 4, 7 and 14 days to check the cariogenic potential.

Microbiological collection and analysis: at the same moments, samples of the supernatant of the DMM were collected for microbial analysis. After serial dilution, aliquots were placed in various media for counts: MSB for mutans streptococci, Rogosa for lactobacilli and blood agar for total microorganisms. All plates were incubated in anaerobic conditions and the number of colony-forming units (CFUs) was determined by one trained operator based on colony morphology and cell morphology using both microscope and stereoscopic microscope. The results were expressed as CFU/mg of biofilm (wet weight). After 14 days, the samples were aseptically removed from the wells and non-adherent cells were removed by gently washing in sterile saline solution (2 mL) before the mechanical test.

### Microtensile Bond Strength Test

Prior to the microtensile test, beam cross-sectional area was measured with a digital caliper (Electronic Digital Caliper; King Tools, Mooca, SP, Brazil) to calculate the bond strength in MPa. The beams were fixed with a cyanoacrylate-based glue in a movable jig device, which was fixed to a universal mechanical testing machine (DL 2000; EMIC Equipment and Systems Ltda., São José dos Pinhais, PR, Brazil). The test was carried out with a load cell of 100 kN at 0.5 mm/min cross speed until failure.

### Fracture Analysis

All specimens were examined in stereomicroscopy (Carl Zeiss, Göttingen, Germany) at high magnification in order to evaluate and classify the mode of fractures, as follows: apparently interfacial, cohesive in composite, cohesive in dentin and mixed. A previously trained and blinded operator carried out the preparation for mechanical testing and failure mode evaluation.
Statistical Analysis

Microtensile bond strength data (MPa) were subjected to two-way ANOVA (dentin pre-treatment and aging condition) followed by post-hoc Tukey's test. Additionally, the relationship between the µTBS values and the mode of fracture was analysed with one-way ANOVA. The influence of the dentin pre-treatments and aging conditions on the mode of fracture was assessed by chi-square test. All tests were conducted using SPSS (Statistical Package for Social Sciences, 18.0) and the statistical significance level was set at p<0.05.

Results

The pre-treatment did not influence the microtensile bond strength values for all aging conditions (p=0.188). The aging protocol showed statistically significant results affecting the bond strength of the etch-and-rinse adhesive system to dentin (p<0.001). Table 2 shows the mean and standard deviation for each group. The control group was similar to the biofilm aging group without cariogenic challenge. The control group presented statistically significant higher bond strength values than biofilm aging with cariogenic challenge and water storage (CO=NCC≥CC=WS). The decrease of the microtensile bond strength values was higher for the no pre-treatment group (15.7% for cariogenic challenge aging and 44.6% for water storage), and lower for the CHX (13.4% for cariogenic challenge aging and 20.5% for water storage) and NaOCl (27.4% for cariogenic challenge aging and 25.7% for water storage) pre-treatments groups.

There was no association between the type of fracture and the microtensile bond strength values (p=0.218). The treatment did not influence the fracture mode p=0.205), while the aging protocol statistically influenced the fracture pattern results (p=0.003). The number of apparently interfacial failures increased after aging and the number of mixed failures decreased for the samples aged in water and submitted to cariogenic challenge. This trend was clearly observed for the control dentin pre-treatment (Table 3). No more than five premature failures occurred in each group; these values were excluded from the statistical analysis.

The mean pH of the biofilm for DMM without sucrose was 7.4 pH (±0.05) and for the DMM with sucrose it was 4.7 pH (±0.10). The supernatant pH of the pure DMM in the cariogenic challenge group showed a lower value than the pure DMM without cariogenic challenge. The microbiological distribution was not statistically different for all the dentin pre-treatments (p=0.145) (Fig. 1). Total microorganisms, mutans streptococci and lactobacilli showed a similar distribution in all groups regardless of the treatment. For mutans streptococci there was an increased count until day 7 followed by a decrease in mutans counts (day 14). For total microorganisms, the results showed similar counts during all time periods.

Discussion

The results of this study demonstrated that the new approach for aging strategy induced a remarkable decrease of microtensile bond strength values, comparable to the results obtained after
long-term storage in water, thus the hypothesis that aging conditions would affect negatively the adhesive bond strength of restorations should be accepted. However, the pre-treatments did not influence the bond strength values, rejecting the hypothesis that they would promote more stability for the dentin-resin interface. Several laboratory studies presented significant degradation of resin-dentin bonds after water aging for 6 months (3,16). In contrast, a clinical study showed a suitable longevity of adhesive restorations (13). These conflicting results may somewhat explain why there is an effort to add thermal, mechanical and pH cycling models to simulate clinical conditions (1,4).

The present study used a microcosm biofilm model (14) with sucrose supplementation to induce cariogenic activity at the resin-dentin interface. This is the condition to which the tooth-restoration interface is often exposed in the oral environment. In this study the cariogenic challenge played a significant role in the stability of adhesive interface showing a decrease on microtensile bond strength values with similar results to those found with the water-storage aging. In this study the regimen applied to produce cariogenic challenge may be a suitable method to age adhesive restorations in vitro, with the advantage of mimicking the des-re cariogenic challenge conditions in a more clinically realistic manner than the aging in water only, as it is commonly used in the literature (2,3). Significant decrease of μTBS values were detected in simulated cariogenic challenge after 14 days when compared to the control group where no aging method was performed.

The most vulnerable part of the adhesive restorations to hydrolytic degradation is the resin-dentin interface (16). The destruction of collagen matrix combined with polymer degradation is ascribed as the responsible for the mechanism of bonding degradation (8). This is reported to be the main reason for the considerable decrease of μTBS after water storage aging (3) and may explain the μTBS values of the resin-dentin bonds after 18-month water storage (Table 2). However, it does not clearly elucidate the decreasing in the bond strength values after 14 days of simulated cariogenic challenge period. A previous in vivo study hypothesized that this fact may occur by the activation mechanism of the host-derived MMPs resulting in an intense destruction of the collagen fibrils, responsible for the fast decrease in the resin-dentin bond strength after simulated cariogenic challenge (17).

In cavities formation, bacterial acids are required for demineralization and for the subsequent activation of the host MMPs, since bacteria alone are not able to cause dentin matrix degradation (18). In this study, even using an effective MMP inhibitor, the small quantity of pre-treated dentin was not able to preserve the resin-dentin interface stability. This was shown in the aging with artificial saliva or deionized water (19), even when the CHX is added to the adhesive composition (20). For the CHX dentin pre-treatment, the percentage of reduction of the microtensile bond strength values was smaller for all aging conditions when compared to the control pre-treatment, though it was not statistically significant.

This trend also happened for the deproteinization group (NaOCl). It was reported that dentin deproteinization procedure can increase the adhesive effectiveness, but it may also depend on the applied adhesive system (10). The interaction between the adhesive system and deproteinized dentin results in formation of a hybrid layer different from the conventional procedure. In this condition, the interaction forms a reverse hybrid layer in which the adhesive system is in intimate contact with the chemically altered dentin surface, rich in minerals and similar to the

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Figure 1. Microbiological distribution (log_{10}) of mutans streptococci, total microorganisms and lactobacilli in non-cariogenic (NCC) and cariogenic challenge (CC) groups.
etched enamel (12,21). Adhesive systems seem to interact with the substrate in different ways, and the acetone-based adhesives may be privileged using this technique (12). Nevertheless, the adhesive system used in this study is an ethanol-based adhesive, a fact that could explain the negative effect of dentin pre-treatment on the bond strength values occurred in the aged groups.

The short-term aging under simulated oral conditions with biofilm accumulation and cariogenic challenge increased the occurrence of adhesive failures. Then, a shift from predominance of mixed failures to adhesive failures was detected for the groups aged in water or 14 days in biofilm cariogenic challenge. This result was previously reported in the literature; possibly the effect of aging led to hydrolytic degradation of the adhesive system’s polymer and favored interface fragility (22). In addition, microscopic evaluation showed degradation of the hybrid layer collagen resulting in a weaker adhesive interface (23).

Regarding the microbial composition, the plaque-like biofilms have shown to be affected by sucrose exposure and the biofilm pH, corroborating a previous study (14). Sucrose exposure usually increases the populations of lactobacilli and mutants streptococci in biofilm (24). The results of this study showed the consistency in the biofilms individually formed in the study model, as occurred in previous studies (24,25). There were no statistically significant differences regarding the microbiological distribution for the dentin pre-treatments, which corroborate the idea that all samples were subjected to similar biofilm accumulation conditions, but with different cariogenic situations according to the experimental aging group.

The main limitation of this study is the relatively short exposure time to the biofilm conditions. The biofilm with cariogenic challenge seems to be a suitable short-term methodology to assess the degradation of the resin-dentin interface. However, further long-term investigations regarding its effect on adhesive interface should be evaluated.

In conclusion, within the limitations of the present study, the cariogenic challenge and water storage aging protocols decreased the resin-dentin bond strength values. The biofilm with cariogenic challenge seems to be a suitable short-term methodology to assess the degradation of the resin-dentin interface. The dentin pre-treatments did not influence the stability of resin-dentin bond.

Resumo

O objetivo deste estudo foi avaliar o efeito do desafio cariogênico na resistência de união (RU) da dentina pré-tratada com clorexidina (CRX) ou hipoclorito de sódio (NaOCl). Trinta e seis molares hígidos foram selecionados e randomizados de acordo com 3 pré-tratamentos dentinários (controle, CRX a 2% e NaOCl a 10%) e 4 protocolos de envelhecimento (controle 24h, biofilme sem desafio cariogênico, biofilme com desafio cariogênico, e armazenamento de 18 meses em água). O mesmo sistema adesivo e resina composta foram usados para todos os grupos (n=30).

Biofilme dental de microcosmos originado da saliva de doador saudável foi desenvolvido sobre as amostras com meio enriquecido com mucina adicionada ou não com sacarose a 10%, de acordo com o grupo. Após o período experimental, o teste de microtração foi realizado. Os dados de microtração foram analisados com ANOVA a dois fatores e teste de Tukey (p<0,05). O pré-tratamento não influenciou os valores de RU para todos os protocolos de envelhecimento (p=0,188), porém o tipo de envelhecimento influenciou os valores de RU (p<0,001). O desafio cariogênico e o armazenamento em água afetaram a estabilidade adesiva resultando na diminuição dos valores de RU e o pré-tratamento não influenciou os valores de RU.

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