Adhesive systems and secondary caries formation: 
Assessment of dentin bond strength, caries lesions depth and fluoride release

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Article info
Article history:
Received 31 March 2005
Received in revised form 29 November 2005
Accepted 10 January 2006

Keywords:
Fluoride releasing materials
Microtensile bond strength
In vitro secondary caries
Depth of caries formation

Abstract
Objectives. The present study evaluated the microtensile bond strength and caries formation on adhesive/dentin interfaces before and after dynamic chemical formation of secondary caries.
Methods. Restorations were prepared on the dentin surface of 80 bovine incisors using four adhesive systems: two fluoride-free (Single Bond and Clearfil SE Bond) and two fluoride containing (Optibond Solo Plus and Clearfil Protect Bond). The restored teeth were then sectioned into multiple slabs that were further trimmed at the bonded interface to a cross-sectional area of 1 mm². Half of the slabs were subjected to secondary caries formation using a pH cycling model (treated groups); while the other half was used as the control group (no pH cycling). The specimens designated for bond strength evaluation were subjected to microtensile bond strength test (µTBS). Caries lesions formation was assessed by polarized light microscopy at different depths from the adhesive–dentin bonded interface. The fluoride ion concentration was evaluated using the de/remineralization solutions (De/Re).
Results. No differences in µTBS were observed among the adhesive systems in both the control and treated conditions. Secondary caries significantly reduced the values of µTBS for all adhesives (p < 0.05). Optibond Solo Plus presented the lowest caries formation at 5 µm depth. Fluoride concentrations present in the De/Re were less than 0.03 ppm, regardless of the adhesive system tested.
Significance. Bond strength values significantly decreased after in vitro secondary caries formation. Fluoride present in adhesive systems is not capable of inhibiting secondary caries or maintaining bond strength values following caries formation.

1. Introduction
Recent developments in adhesive systems have resulted in a greater application of these agents on daily dental practice. Contemporary adhesives systems present satisfactory bonding to enamel and dentin [1]. However, replacement of the restorations due to secondary caries formation is still a major problem and of great concern in dentistry [2].
Caries development is a dynamic process between demineralization and remineralization of dental hard tissues that eventually results in cavitation [3]. Caries development depends upon acids and enzymes produced by bacteria present in dental biofilm [4,5]. The products of dental biofilm include lactic, acetic and citric acids, which reduce tooth surface pH causing hard tissue demineralization [5]. Fluoride plays a significant role in the inhibition of this process, through mineral deposition [6–7]. Based on this property, many restorative materials that release fluoride have been used with the purpose of contributing to secondary caries prevention [7–9]. Therefore, adhesive systems containing fluoride have been developed and introduced in the dental market. The fluoride in these materials induces an anti-cariogenic activity by increasing dentin resistance to acids present in the oral cavity [10,11]. These adhesives also inhibit secondary caries development by bonding to dental tissues [10]. According to Ferracane et al. (1998) [12] fluoride ions in these systems are released directly to the cavity wall and easily penetrate the dentin. Featherstone et al. (1986) [13] reported that these ions increase mineralization and reduce demineralization of dentin, thus offering resistance against secondary caries. However, to date, no study has reported the effect of fluoride ions on the bond strength of composite restorations subjected to artificial secondary caries formation.

Thus, this in vitro study aimed to evaluate the influence of adhesive systems with and without fluoride on the microtensile bond strength of composite restorations to dentin, subjected or not to dynamic chemical secondary caries formation using a pH cycling model. Further aims were to assess the depth of dentin carious lesions and quantify fluoride release from the adhesive systems.

2. Materials and methods

2.1. Teeth preparation and restoration

Eighty bovine mandibular incisors were obtained and stored in 0.1% thymol solution. The buccal portion of enamel was removed using silicon carbide paper (grits—240, 400 and 650/Carborundum, Saint-Gobain Abrasivos LTDA, Guarulhos, SP, Brazil) fixed in a mechanical grinder (MaxiGrind Soflemest, São Paulo, SP, Brazil), under constant water irrigation. Dentin surfaces were exposed and inspected to ensure no remnants of enamel were left. Fig. 1 describes the methodology used in the present study.

Teeth were randomly assigned into four groups (n=20), according to the adhesive system used (Fig. 1B):

- G1: total-etch fluoride-free adhesive system Single Bond—SB (3M ESPE Dental Products Division, St. Paul, MN, USA);
- G2: total-etch fluoride containing adhesive system OptiBond Solo Plus—OS (Kerr Corporation, Orange, CA, USA);
- G3: Self-etching primer fluoride-free Clearfil SE Bond—SE (Kuraray Co., Ltd., Umeda, Osaka, Japan);
- G4: self-etching primer fluoride containing Clearfil Protect Bond—PB (Kuraray Co., Ltd., Umeda, Osaka, Japan).

Dentin surfaces were restored using the adhesive systems that were applied according to the manufacturers instructions. Then a 3 mm × 3 mm × 8 mm (height, width and length) resin “crown” (composite resin Filtek Z250—3M ESPE) was build up incrementally over the dentin. The specimens were stored in moist environment, over wet cotton, at 37°C for 24 h. All four groups were then randomly allocated into two subgroups (n=10), according to the chemical treatment: treated – subjected to chemical induction of secondary caries (treated groups) and control – no pH cycling.

Restorations were sectioned perpendicularly to the bonded interface into approximately 1.0 mm-thick slabs. A total of six slabs per teeth were obtained (Fig. 1C) and the adhesive/dentin interface was further trimmed using a fine diamond bur (#105FH—KG Sorensen Ind. e Com. LTDA, Barueri, SP, Brazil) under copious water spray to produce a cross-sectional surface area of approximately 1.0 mm². No premature debonding occurred during sample preparation.

2.2. Demineralization-remineralization cycling (pH cycling)

The slabs from the treated group were coated with an acid-resistant nail varnish except for a 4.0 mm²-window of dentin around the bonded interface (Fig. 1D). The specimens were subjected to four demineralisation-remineralization cycles at 37°C. Each cycle was consisted of a 4 h immersion in demineralization solution followed by a 20 h immersion in remineralization solution. The demineralizing solution [14] was composed of 2.0 mM Ca, 2.0 mM P in a buffer solution of 74.0 mM of acetate at pH 4.3 (it was used 6.25 mL of solution/mm² of exposed dentin) [15]. The remineralizing solution [14] was composed of 1.5 mM Ca, 0.9 mM P in a buffer solution of 20.0 mM Tris (hydroxymethyl)-ammoniomer at pH 7.0 (it was used 3.125 mL/mm² of exposed dental area) [15].

This cycling model was based on results from previous pilot studies, which aimed to establish a standardized protocol for the proposed methodology. The authors were looking for a non-aggressive caries model that would allow uniform demineralization at the dentin/adhesive interface. It was observed during the pilot study that several protocol used resulted in high demineralization rates that unable the microtensile bond strength testing due to high incidence of cohesive failures in dentin during the testing. Thus, the model used in this study (four cycles, 4 h-immersion in demineralization solution and 20 h-immersion in remineralization solution), allowed the formation of secondary caries and evaluation of the interface bond strength values with very low percentage of cohesive in dentin.

2.3. Microtensile bond strength

Four slabs of each tooth/restoration were selected for the microtensile bond testing and the other two were prepared for polarized light microscopy, for both treated and control groups.

Specimens were positioned in a microtensile testing device attached to a Universal Testing Machine (EMIC LTDA, São José...
Fig. 1 – Experimental design. (A) Bovine incisors. (B) Composite specimen prepared on dentin with the different adhesive systems used in the study. (C) Six slabs of specimen obtained after transversal sectioning. (D) Half of the samples were prepared for the chemical induction of caries (treated group), with remaining 4 mm² of dentin exposed around the adhesive interface. (E) Control group. (F) Model of pH cycling. (G) Four slices of each tooth were submitted to microtensile testing and the remaining two were evaluated in polarized light microscopy. (H) Evaluation of fluoride concentration on DE/RE solutions used on pH cycling.

dos Pinhais, PR, Brazil) at a crosshead speed of 0.5 mm/min until failure. Specimens were then carefully removed from the grips with a scalp knife and the cross-sectional area at the site of fracture was measured (digital calliper—Carl Mahr, GmbH, Esslingen, Germany) once more to ensure an accurate cross-sectional area value. A mean bond strength value was obtained for each tooth. Means and standard deviations were calculated and expressed in MPa. The bond strength data was statistically analyzed using two-way analysis of variance ANOVA and Tukey’s test at a 5% level of confidence.

In order to evaluate fracture patterns, debonded specimens were fixed in 10% neutral formalin for at least 8 h prior to SEM examination. All fractured specimens were placed on SEM stubs and allowed to air dry. Specimens were then gold sputter-coated and observed with scanning electron microscopy (SEM 1600 LV, Joel, Tókio Japan) so that micro-
scopic fracture patterns and debonded interfaces morphology could be assessed. The patterns of fracture were designated as interface (hybrid layer), cohesive in adhesive resin, cohesive in dentin and mixed failure (association of two or more failures) [16]. The percentage of each failure mode was calculated from the frequency observed in each experimental group.

2.4. Specimen preparation for polarized light microscopy

Two slabs of each tooth from both control and treated groups were randomly selected and prepared for the evaluation of demineralization by polarized light microscopy. Slabs were embedded in epoxy resin (Epoxicure Resin, Buehler, USA) and polished using silicon carbide paper (grit #600) in a mechanical grinder, under constant water irrigation. Then the specimens were manually polished using carbide paper grit 600 and 1200 until a final thickness of 0.10 ± 0.02 mm was obtained [9]. Special care was taken so that the surface remained free from the embedding material.

Polished tooth sections were immersed in deionized water and photographed under polarized light microscopy (Leica, MLST). Standard 35.0 mm photomicrographs were taken and carious lesion depth was analyzed using specific software (Image Pro-Plus®) [14]. Digital images were taken, and the lesion depths were measured in micrometers at three sites by Image-Pro Plus software (Version 4.1 for Windows® Media Cybernetics). The depth of caries lesion was measured at 5, 10 and 25 μm from the adhesive interface by the software linear measuring tool (Fig. 2). The resin surface and the adhesive interface were considered as reference points for these measurements.

Statistical analysis was performed using Two-way ANOVA with split-plot at a significance level of 5%. The means and standard deviations of TBS are summarized in Table 1. Two-way ANOVA revealed that there were significant differences for the factor pH Cycling (p = 0.00004). However, no differences were detected for the factor adhesive system (p = 0.94416) nor for the interaction between the two factors (p = 0.39320). Tukey’s test showed significant differences between TBS obtained from control and treated groups (p < 0.05).

Statistical analysis revealed no difference among the adhesive systems in both control and treated groups (Table 1). However, a significant decrease in bond strength values after

2.5. Fluoride release

In order to evaluate fluoride release, standard solutions were prepared from sodium fluoride solution with concentrations of 0.03, 0.06, 0.12, 0.25, 0.50 and 1.00 ppm F\textsuperscript{−} to which was added TISAB III (Total Ionic Strength Adjustment Buffer; Thermo Orion, Beverly, MA, USA) in order to obtain a constant background ionic strength.

Standard solutions were used to plot the calibration graph. Fluoride release was detected using a fluoride-specific electrode connected to a microprocessor ion analyzer (ORION EA-940, Orion Research Inc., Boston, MA 02129). The limit of F\textsuperscript{−} ion measurement for this ion-specific electrode is 0.03 ppm.

Fluoride concentration in De/Re solutions was obtained by collecting 1ml of these solutions and 1ml of TISAB III. Sample readings were captured in millivolts (mV) and transformed in μg F\textsuperscript{−}/ml (ppm F\textsuperscript{−}) by linear regression of the calibration curve.

Fluoride measurements of the De-Re solution were obtained prior to the pH cycling. The initial fluoride concentration was subtracted from the measurements acquired after pH cycling.

3. Results

3.1. Microtensile bond strength

The means and standard deviations of μTBS are summarized in Table 1. Two-way ANOVA revealed that there were significant differences for the factor pH Cycling (p = 0.00004). However, no differences were detected for the factor adhesive system (p = 0.94416) nor for the interaction between the two factors (p = 0.39320). Tukey’s test showed significant differences between μTBS obtained from control and treated groups (p < 0.05).

Statistical analysis revealed no difference among the adhesive systems in both control and treated groups (Table 1). However, a significant decrease in bond strength values after
pH cycling, regardless of the adhesive system tested, was observed. Table 2 shows the distribution of fracture mode pattern for the groups evaluated. A prevalence of mixed fracture was observed for all groups, regardless of treatment (control and treated). However, for SE fluoride-free adhesive system the control group presented fracture patterns divided among interface, mixed and cohesive in dentin. After the pH cycling, all groups depicted cohesive fracture in dentin on the edges (Fig. 3). This could be possibly due to dentinal demineralization by the pH cycling, which may have reduced dentin strength at this site.

3.2. Caries lesions depth analysis

Two-way ANOVA revealed statistically significant differences for the factor adhesive systems ($p = 0.03548$), distance ($p = 0.00001$) and the interaction between both factors ($p = 0.00429$). None of the adhesive systems presented difference in caries depth at 25, 10 and 5 $\mu$m distances, except for OS, which presented lower values of depth of caries at 5 $\mu$m (31.64c), a greater value at 25 $\mu$m (53.91a) and an intermediate value at 10 $\mu$m (45.74b) (Table 3). No evidence of demineralization was found in the specimens from the control groups (Fig. 4A), regardless of the adhesive system.

The microscopy images from the treated samples showed an inhibition zone, also called “acid-resistant” zone, next to the adhesive layer in all adhesive systems (Fig. 4). 3.3. Fluoride release

The ion $F^-$ was detected in low concentrations in the De/Re solutions, below the minimal concentration of the calibration curve ($<0.03$ ppm $F^-$). The adhesives systems that release fluoride, OS and PB, presented a mean concentration of 0.002 ppm $F^-$ (De + Re solutions) and the fluoride-free systems, SB and SE, did not present $F^-$ ions in the solutions.

4. Discussion

Many studies have shown that fluoride has a significant effect in the inhibition of caries [5,7,8,10,17,18]. Based on these findings, restorative materials and adhesive systems that release fluoride have been developed with the purpose of aiding in the prevention of secondary caries development.

In the present study, bovine teeth were used instead of human teeth. According to previous studies [19,20] bovine...
teeth are reliable substitutes of human teeth when used for bond strength (shear and microtensile tests) and microleakage assays. Also, several studies have used bovine dentin substrates in vitro caries research [9,17,21–24]. The major advantage of using bovine teeth is the possibility of controlling the age (average age of 2 years), sclerosis and the amount of wear of the substrate.

In the present study it was observed that the μTBS of the restorations prepared using either fluoride or fluoride-free adhesive systems did not differ statistically when subjected to secondary caries induction (Table 1). However, when comparing treated versus control (Table 1) it was observed that the pH cycling treatment significantly reduced the μTBS of all adhesive systems. A possible explanation for this fact could be that the resulting demineralization around the restorations (Fig. 4) was capable of reducing dentin strength and thus, probably, the adhesive interface, weakening the bond between dentin and restoration, regardless of the presence of fluoride in the adhesive systems. It has been suggested that the fluoride in adhesive systems prevents dentin degradation, which results in stability of the adhesive interface [16]. This fluoride could reduce the solubility of calcium phosphate present in the hybrid layer, which stabilizes dentin bond strength [16]. The mineralized dentin matrix presents many enzymes (alkaline phosphatases, metaloproteases, including collagenase) which can be released and activated during acid conditioning or during tooth storage in water [16]. These enzymes are believed to degrade resin ester bonds [25], collagen [26] or both. However, fluoride can inhibit the activity of some enzymes [27]. According to Nakajima et al. (2003) [16] fluoride released within the hybrid layer might inhibit these enzymes from attacking the components of the hybrid layer. Also, the fluoride could prevent the enzymes from being released from the mineralized matrix due to its remineralization action [16].

Even though it is believed that fluoride-containing adhesive can stabilize the hybrid layer; under secondary caries simulation, it does not seem to influence μTBS, as observed in the present study. The evaluation of the fracture pattern in SEM showed a predominance of mixed fractures, regardless of the adhesive system tested. However, the chemical induction of caries increased the percentage of this pattern. This suggests that alterations in dentin or within the hybrid layer/adhesive layer could have occurred. As shown on Fig. 3, cohesive fracture on dentin at the edges was a common finding in the samples that were submitted to caries induction. This could probably have occurred due to weakening of the dentin caused by demineralization during the DeRe cycling.

No significant differences in the caries depth evaluation, by polarized light microscopy, were observed among the adhesive systems at the distances 10 and 25 μm from the interface (Table 2). Tota et al. (2002) [10] also evaluated by microradiographs the depth of secondary caries formation on dentin restored with fluoride and fluoride-free adhesive systems. The authors did not report any differences amongst the adhesive systems evaluated, which is in agreement with the present study. At the distance of 5 μm from the adhesive interfaces, the OS fluoride-containing adhesive system presented the lowest value of caries penetration when compared to the other systems. Thus, among the fluoride-containing adhesives, OS and PB, only the former one presented reduced values of caries penetration at 5 μm. Total-etching and self-etching adhesives form hybrid layers with different thicknesses: 0.5–1.5 μm for self-etching systems [28] and 4–5 μm for total-etch systems [29]. The hybrid layer is formed by the adhesive infiltration in demineralized dentin and thus, it represents an essential structure for adhesion [1]. It is also considered to be an acid-resistant layer [30]. Montes et al. (2001) [29] showed that the hybrid layer thickness of total-etch adhesive ranges from 6 to 8 μm. These higher values could be a result of different application methods. The manufacturer of OS recommends active application, which facilitates adhesive penetration in the demineralized dentin. Therefore, it can be speculated that the lower values of caries depth formation at 5 μm could be related to the thicker hybrid layer formed by OS, which resisted the acidic conditions of the demineralization solution.

Some studies show that fluoride-containing adhesives are able to form an inhibition zone adjacent to the bonded interface, which is known as “acid-resistant” zone, since it can resist the acid attack during secondary caries formation [10,11,24,31]. This zone is also present in adhesive systems that do not contain fluoride, although it is not as thick as the fluoride containing adhesives [10,18]. This inhibition zone was observed adjacent to the adhesive interface (Fig. 4) by the polarized light microscopy in all adhesive systems. However, it was thicker for the fluoride-containing total-etch adhesive OS. This acid-resistant zone could have also contributed to the lower caries depth found for OS system at 5 μm. It is important to note that regardless of the lower caries depth observed for the OS at 5 μm, the bond strength values of the treated group decreased significantly as compared to its control group.

### Table 3 – Mean caries lesion depth (μm) and standard deviations (S.D.) for the four adhesive systems and different distances evaluated

<table>
<thead>
<tr>
<th>Adhesive systems</th>
<th>5 μm</th>
<th>S.D.</th>
<th>10 μm</th>
<th>S.D.</th>
<th>25 μm</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clearfil Protect Bond</td>
<td>49.75Aa</td>
<td>8.47</td>
<td>55.74Ab</td>
<td>6.94</td>
<td>58.94Aa</td>
<td>7.79</td>
</tr>
<tr>
<td>Clearfil SE Bond</td>
<td>51.64Ab</td>
<td>12.48</td>
<td>45.74Ab</td>
<td>14.08</td>
<td>53.91Aa</td>
<td>9.20</td>
</tr>
<tr>
<td>Optibond Solo Plus</td>
<td>49.58Ab</td>
<td>13.10</td>
<td>53.56Aab</td>
<td>12.78</td>
<td>58.74Aa</td>
<td>11.59</td>
</tr>
<tr>
<td>Single Bond</td>
<td>49.54Ae</td>
<td>8.49</td>
<td>45.74Ab</td>
<td>8.52</td>
<td>58.74Ae</td>
<td>9.95</td>
</tr>
</tbody>
</table>

Means designated by different letters (capital letters in columns and lower case letters in rows) are significantly different according to Tukey’s test (p<0.05).
Itota et al., in 2002 [10], observed that only a fluoride-containing adhesive associated with a restorative material that also contains fluoride is able to reduce artificial secondary caries depth due to the high concentration of fluoride present in the restorative material. These authors also reported that the same adhesive system used with a restorative material without fluoride was not effective against caries reduction. Specific electrode was not able to detect fluoride ions since the De/Re solutions presented very low concentrations of fluoride ions released by the adhesive system. This technique presents some limitations because the electrode is not able to precisely measure low fluoride concentrations. Studies [10,32,33] proposed the use of adhesive system discs attempting to overcome this technique limitation, however,
this methodology does not ideally mimic the condition in which the restorative material is present in the restoration. The low fluoride concentration released from adhesive systems may be justified by the fact that the discharge is restricted to a small portion of the restoration exposed to the solutions, i.e. adhesive layer only. In addition, the fluoride ions would probably be confined in the adhesive polymerized resin matrix and/or at the hybrid layer and therefore would no be released to the environment. Toba et al. [2000] [24] suggested that since fluoride in adhesive systems is surrounded by resin matrix, its contact with water would be restricted since its movement might be limited by the matrix. However, further investigation is required in order to understand fluoride release mechanisms from dentin adhesive materials. Studies have shown that fluoride-containing restorative materials help prevent secondary caries [10,17,18]. Nevertheless the role of fluoride in adhesive systems remains unclear. The present study shows that fluoride-containing adhesive systems had a minimal effect upon the chemically induced secondary caries process and on the bond strength to dentin. Further investigation is required to evaluate the effect of fluoride within the bonding system upon cariogenic plaque microorganisms, as well as in vivo or in situ studies to evaluate its behavior in intra-oral conditions.

5. Conclusions

Within the limitations imposed on this in vitro study, the following conclusions may be drawn: (1) secondary caries significantly reduces μTBS of adhesive restorations; (2) adhesive systems with and without fluoride presented similar significantly reduces secondary caries process and on the bond strength to dentin. Further investigation is required to evaluate the effect of fluoride within the bonding system upon cariogenic plaque microorganisms, as well as in vivo or in situ studies to evaluate its behavior in intra-oral conditions.

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