Influence of Metalloproteinases on Dentin Hybridization of One-bottle or Self-etch Dental Bonding Systems

1Sérgio Luiz Pinheiro, 2Diana Roberta Pereira, 3Flávia De Milito, 4Karina Teixeira Villalpando

ABSTRACT

Aim: To assess the influence of dentin substrate and chlorhexidine on the marginal seal of composite resin restorations.

Materials and methods: The sample comprised 20 third molars. Four cavities were drilled in the dentin surface, followed by sealing and restoration of sound dentin (n = 10) or carious dentin (n = 10). In the control group, cavities were immediately restored as follows: G1: one-bottle bonding agent (OB) + composite resin (CR); G2: chlorhexidine 2.0% (CLX) + OB + CR; G3: self-etch bonding agent (SE) + CR; G4: CLX + SE + CR. In the experimental group (carious dentin), carious lesions were induced with S. mutans and cavities were restored as in the control group. Five specimens from each group (sound and carious) were stored in brain–heart infusion (BHI) medium for 6 months. All specimens were submerged in methylene blue 0.5% to test for microleakage. The Kruskal-Wallis and Student-Newman-Keuls tests were used to assess results.

Results: On immediate assessment, there were no significant between-group differences in the sound dentin group, whereas in carious dentin, there was less leakage when OB and CLX were used. At 6 months, there was less leakage in the sound dentin group when OB and CLX were used; there were no between-group differences in carious dentin. Comparison of immediate and 6-month assessments showed a significant increase in leakage at 6 months when sound dentin was treated with SE and CLX and when carious dentin was treated with OB and CLX.

Conclusion: To ensure better dentin hybridization and preservation of the organic constituents of the dentin matrix, the properties of chlorhexidine digluconate and the components of the resin matrix must be taken into account.

Clinical significance: Metalloproteinases influence degradation of the hybrid layer in composite resin restorations, regardless of whether the bonding system used is one-bottle or self-etching.

Keywords: Dentin, Matrix metalloproteinases, Hybrid layer, Chlorhexidine, Adhesives, Laboratory research.


Source of support: FAPESP (2010/10275-9) and PUC-Campinas.

Conflict of interest: None

INTRODUCTION

Sound and carious dentin are present in the majority of cavity preparations, but a stable resin-dentin bond is more difficult to achieve in carious tissue. This is attributable to chemical and physiological processes that take place during the progression of the caries process. Proteolytic enzymes known as matrix metalloproteinases (MMPs) are present in greater concentrations in caries-affected substrates. These enzymes are classified as zinc- and calcium-dependent endopeptidases, and catalyze the degradation of components of the extracellular matrix. Five major groups of MMPs have been reported: collagenases, gelatinases, stromelysins, matrilysins and other MMPs. Matrix metalloproteinases are present both in dentin and in saliva, and play an important role in the progression of dentin erosion, which initially consists of mineral dissolution until the organic matrix is exposed. The main characteristics of matrix metalloproteinases include degradation of type I/II/III collagen and of the extracellular matrix. In dental caries, MMPs-2, 8, 9 and 20 are active. Variants in the gene that encodes MMP-20 may increase susceptibility to dental caries by altering enamel development.

During the progression of dentin caries, degradation of the inorganic matrix is a necessary condition for activation of MMPs, which can contribute to the first changes in the noncollagenous organic portion. Moderate acids can activate MMPs, and both one-bottle and self-etch dental bonding systems contain such acids and can release them during the bonding procedure. The durability of the hybrid layer may be negatively affected by MMP-mediated degradation of collagen fibers.

Low-level endogenous collagen degradation activity can be inhibited with protease inhibitors to help preserve...
the hybrid layer. Chlorhexidine has an inhibiting effect on MMPs at low concentrations, and can be used after acid conditioning of dentin. Application of chlorhexidine after acid etching increases the durability of the hybrid layer, regardless of whether the substrate is sound or carious. Chlorhexidine pretreatment does not interfere with the bond strength of the bonding system. The use of chlorhexidine 2% as a protease inhibitor led to increased immediate bond strength with some composite cements.

This study sought to assess the influence of metalloproteinases on dentin hybridization with one-bottle or self-etch dental bonding systems.

MATERIALS AND METHODS

This study was approved by the Pontifícia Universidade Católica de Campinas Research Ethics Committee with protocol no. 784/09.

Sample Selection

Twenty permanent third molars donated by patients of the PUC-Campinas Dental Clinic were selected. All donors had signed a Tooth Donation Form.

Inclusion Criteria

- Permanent third molars
- Absence of cracks, fractures, caries, or hypoplasia.

All teeth were examined under a stereo microscope (Stemi DV4, Carl Zeiss, São Paulo, Brazil) at 10× magnification.

The selected teeth were stored in normal saline (sodium chloride 0.9%) solution containing sodium azide 0.02% (LabCenter, São Paulo, Brazil) at 4°C for no more than 1 month. At the start of the experimental period, teeth were rinsed with normal saline solution containing sucrose 10% (LabCenter, São Paulo, Brazil). The occlusal third was removed with a low-speed, double-sided diamond cutting disk (KG Sorensen, São Paulo, Brazil), under constant cooling, to expose the dentin. The dentin surfaces were wet-polished with 600-grit silicon carbide paper (T223 advance, Norton, São Paulo, Brazil). Four cavities (2 mm wide, 2 mm long, 1 mm deep) were drilled into the dentin of the occlusal surface of each tooth using #1090 round diamond burs (KG Sorensen, São Paulo, Brazil). Burs were replaced after every 10 cavity preparations. Cavities were measured with a periodontal probe (Trinity Periodontia, Jaraguá, SP, Brazil). Teeth were then autoclaved (Sercon, São Paulo, Brazil) for 15 minutes at 121°C and 1 atm.

Under a laminar flow hood (Veco, Campinas, SP, Brazil), specimens were sealed with epoxy resin (Araldite, São Paulo, Brazil) and nail lacquer (Colorama, São Paulo, Brazil), apart from 1 mm around the cavity margins. The specimens were then divided into groups according to the restorations made on sound (n = 5) and carious (n = 5) dentin. In the control group (sound dentin), cavities were restored immediately as follows:

G1: Adper™ Single Bond 2 dental bonding system (3M do Brasil, Campinas, São Paulo, Brazil) + Filtek™ Z350 composite resin (3M do Brasil, Campinas, São Paulo, Brazil).


G3: active application of 10 µl chlorhexidine 2.0% (Fórmula and Ação, São Paulo, Brazil) for 60 seconds after acid etching + removal of excess solution with sterile cotton points + Adper™ Single Bond 2 dental bonding system (3M do Brasil, Campinas, São Paulo, Brazil) + Filtek™ Z350 composite resin (3M do Brasil, Campinas, São Paulo, Brazil).

G4: active application of 10 µl chlorhexidine 2.0% (Fórmula and Ação, São Paulo, Brazil) for 60 seconds + removal of excess solution with sterile cotton points + Adper™ SE Plus self-etch dental bonding system (3M do Brasil, Campinas, São Paulo, Brazil) + Filtek™ Z350 composite resin (3M do Brasil, Campinas, São Paulo, Brazil).

All materials cited above were used in accordance with manufacturer instructions (Table 1).

Preparation of BHI Medium

Briefly, 37 gm of powdered brain-heart infusion (BHI) medium (LabCenter, São Paulo, Brazil) was dissolved in 1 l of distilled water and stirred vigorously for 1 minute until complete homogenization. The resulting broth was autoclaved at 121°C for 15 minutes.

Microbiological Processing

To simulate dentin caries, teeth in the experimental group (carious dentin) were placed in sterile tubes containing prepared BHI medium (LabCenter, São Paulo, Brazil) supplemented with 0.5% yeast extract, 0.5% dextrose (LabCenter, São Paulo, Brazil), and 1% sucrose (LabCenter, São Paulo, Brazil). A reference strain of *Streptococcus mutans* ATCC 25175 (Fundação André Tosello, Campinas, SP, Brazil), adjusted to 0.5 McFarland standard, was added to the BHI medium. Samples were incubated at 37°C for 14 days, in an atmosphere containing 85% nitrogen (N₂), 10% carbon dioxide (CO₂), and 5% hydrogen (H₂), in a laboratory incubator (Fanem Ltda, São Paulo, SP, Brazil). During the 14-day incubation period, BHI medium was renewed every 48 hours. Cavities were restored as in the control group.
Five specimens from each group (carious and sound) were stored in BHI in an anaerobic environment at 37°C. The medium was renewed every 48 hours for 6 months. Every time the medium was replaced, pH was measured as well.

### Assessment of Microleakage

All specimens were submerged in methylene blue 0.5% (LabCenter, São Paulo, Brazil) for 4 hours at 37°C, rinsed in running water to remove excess dye, and bisected along the mesiodistal axis, precisely at the center of the restoration, using a double-sided diamond cutting disc (KG Sorensen, São Paulo, Brazil), for assessment of microleakage.

Specimens were photographed under a stereo microscope (Stemi DV4, Carl Zeiss, São Paulo, Brazil) at 32× magnification. The images thus obtained were evaluated by two blinded and previously calibrated examiners. Microleakage was scored on a scale of 0 to 3 as follows: 0. no dye penetration 1. Dye penetration up to half of the mesial or distal wall of the cavity preparation 2. Dye penetration beyond half of the mesial or distal wall of the cavity preparation 3. Dye reaching the pulp wall.

The kappa coefficient was used to assess interexaminer agreement (Table 2). In case of disagreement between the examiners, the score corresponding to the greatest leakage was taken into account for analysis. The Kruskal-Wallis test, followed by the Student-Newman-Keuls procedure, was applied to the scores.

### RESULTS

#### Immediate Assessment

In sound dentin, there were no significant differences between bonding systems or between chlorhexidine use vs no chlorhexidine use (p > 0.05). In carious dentin, there was less microleakage with the one-bottle bonding system and chlorhexidine vs one-bottle bonding system and no chlorhexidine, self-etch bonding system with chlorhexidine, or self-etch bonding system and no chlorhexidine (p < 0.05). Comparison between sound
was treated with the one-bottle bonding system and chlorhexidine (p < 0.05) (Table 3).

**DISCUSSION**

One of the dental bonding systems used in the present study was the Adper™ Single Bond 2 one-bottle system. On immediate assessment, this system was associated with less microleakage in sound dentin and carious dentin, whether with or without chlorhexidine pretreatment. This is consistent with previous studies\(^{21-23}\) that reported greater collagen exposure in the hybrid layer formed in carious dentin. This may be explained by the greater number of imperfections and greater exposure of collagen fibers at the bond interface, facilitating hydrolytic and enzymatic degradation of the dentin matrix due to the presence of intense proteolytic activity mediated by metalloproteinases that are released during development of the carious lesion.\(^{24}\)

Use of the one-bottle Adper™ Single Bond 2 bonding system with a protease inhibitor, whether on sound or carious dentin, did not yield significant differences on immediate assessment. This is consistent with previous studies\(^{11,13}\) reporting that the hybrid layer of chlorhexidine-pretreated teeth displays structural integrity of collagen fibers, and that specimens stored in artificial saliva containing inhibitors of proteolytic enzymes had preserved collagen fiber diameter.\(^{25}\) In the present study, chlorhexidine 2% was applied for 60 seconds as a protease inhibitor. The results obtained on immediate assessment of the chlorhexidine pretreatment groups were consistent with less infiltration at the bond interface as compared with the untreated groups. This agrees with the literature,\(^{10,25,26}\) in which chlorhexidine has been reported to inhibit MMP-2 and MMP-9 even at low concentrations, preserving the integrity of collagen in the presence of these proteolytic enzymes and slowing degradation of the hybrid layer.

Another bonding system used in this study was the self-etching Adper SE Plus system. When applied to sound or carious dentin after chlorhexidine pretreatment, on immediate assessment, this system was associated with significantly greater leakage in carious substrate. This is consistent with studies\(^{27-31}\) reporting that tubules in caries-affected dentin bear acid-resistant mineral deposits that act as a barrier to infiltration by resin

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**Table 2: Interexaminer agreement (kappa coefficient)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pretreatment</th>
<th>IM</th>
<th>6 months</th>
<th>IM</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>OB/SD</td>
<td>C</td>
<td>1.14 ± 1.35(^a)</td>
<td>1.16 ± 1.29(^A)</td>
<td>1.0</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>CHX</td>
<td>0.57 ± 0.93(^a)</td>
<td>0.71 ± 1.06(^a)</td>
<td>1.0</td>
<td>0.83</td>
</tr>
<tr>
<td>OB/CAD</td>
<td>C</td>
<td>2.15 ± 1.04(^b),(^2)</td>
<td>2.12 ± 1.11(^B)</td>
<td>1.0</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>CHX</td>
<td>1.00 ± 1.23(^a)</td>
<td>1.92 ± 1.18(^2)</td>
<td>1.0</td>
<td>0.83</td>
</tr>
<tr>
<td>SE/SD</td>
<td>C</td>
<td>1.35 ± 0.92(^a)</td>
<td>1.55 ± 0.99(^t)</td>
<td>1.0</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>CHX</td>
<td>0.58 ± 0.68(^a),(^t)</td>
<td>1.94 ± 0.87(^b)</td>
<td>1.0</td>
<td>0.83</td>
</tr>
<tr>
<td>SE/CAD</td>
<td>C</td>
<td>2.33 ± 1.15(^b)</td>
<td>2.04 ± 1.25(^t)</td>
<td>1.0</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>CHX</td>
<td>1.65 ± 0.98(^b)</td>
<td>2.25 ± 1.11(^b)</td>
<td>1.0</td>
<td>0.83</td>
</tr>
</tbody>
</table>

**Table 3: Distribution of marginal leakage across groups**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pretreatment</th>
<th>IM</th>
<th>6 months</th>
<th>IM</th>
<th>6 months</th>
</tr>
</thead>
</table>
| OB: Adper™ Single Bond 2; SE: Adper™ SE Plus; SD: sound dentin; CAD: caries-affect dentin; C: control dentin pretreatment; CHX: chlorhexidine pretreatment; IM: values obtained at the immediate testing period; 6 months: values obtained after 6 months of aging. The same superscript lowercase letters or symbols in the same column denote absence of statistically significant differences (p > 0.05). Different superscript lowercase letters, uppercase letters, numbers and symbols in the same column denote statistically significant differences (p < 0.05). Horizontal arrows denote statistically significant differences (Kruskal-Wallis and Student-Newman-Keuls tests)

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**Assessment after 6 Months**

The least leakage occurred when sound dentin was treated with a one-bottle bonding system and chlorhexidine vs self-etch bonding system and chlorhexidine (p < 0.05). In carious dentin, there were no significant differences between treatment with either bonding system, with or without chlorhexidine (p > 0.05). Comparison between sound and carious dentin revealed a significant difference when the one-bottle bonding system was used on sound dentin, with or without chlorhexidine (p < 0.05) (Table 3).

**Immediate Assessment vs Assessment after 6 Months**

Comparison between the same sample groups revealed a significant increase in leakage after 6 months of aging when sound dentin was treated with a self-etch bonding system and chlorhexidine and when carious dentin was treated with the one-bottle bonding system and chlorhexidine (p < 0.05) (Table 3).
monomers, acids, bacteria and their products, hindering adhesion of the self-etching system. MMPs are present in greater concentrations in carious substrate and are responsible for degradation of the organic matrix, significantly increasing the demineralization process. The degree of demineralization significantly affects the bond strength of the adhesive. Another point that should be discussed regarding the use of these bonding systems is that polymerization of a mixture of monomers, acid, HEMA, and water does not produce a uniform resin layer, but rather results in incompletely polymerized areas. These areas enable water flow within the hybrid layer and, consequently, increase absorption at the bond interface. The presence of water leads to premature loss of the mechanical properties of the polymer, which, over time, leads to hydrolysis of its molecules.

Comparison of the self-etch and one-bottle bonding systems in carious dentin revealed less leakage with use of the one-bottle system at immediate assessment. This finding is consistent with the literature, which showed that phosphoric acid 32 to 37% solubilizes intratubular minerals in caries-affected dentin, contributing to the formation of resin tags and better retention. Prolonging the acid etching time to 45 seconds before application of Single Bond adhesive significantly increases the bond strength in carious substrate, but not in sound dentin. On the other hand, the rationale for the stability provided by one-bottle bonding systems in the dentin substrate is associated with the ethanol/water content of the Single Bond 2 adhesive, which is approximately 30%, promoting greater stability of the bonding agent solution and making it less technique-sensitive. The low-molecular-weight components and hydrophilic monomers present in Single Bond 2 are able to infiltrate demineralized dentin and promote good interlocking with collagen fibers. Analysis of the results obtained after 6 months showed less microleakage in sound dentin as compared with carious dentin when a one-bottle bonding system was used, whether with or without chlorhexidine pretreatment. This is consistent with the literature, which has reported an increase in collagenolytic activity in carious dentin as compared with sound dentin, leading to degradation of collagen fibers by metalloproteinases. Immunohistochemical evidence suggests that type I collagen and proteoglycan antigens are present in caries-affected dentin, preventing remineralization of the demineralized collagen matrix in this substrate. Histologically, there is strong expression of MMP-2 in carious dentin, particularly in areas of denatured collagen. When MMP-2 is activated, it degrades components of the basement membrane, type IV collagen, laminin and proteoglycans. The more intense expression of metalloproteinases in carious dentin and the activation of other proteases, such as cathepsins, is associated with degradation of collagen fibers in the hybrid layer. Compared with that of a healthy substrate, the intertubular dentin of a caries-affected substrate has a lower hardness because it is partially demineralized and more porous, leading to a deeper demineralization and limiting infiltration by resin monomer.

At 6 months, there was less microleakage when sound dentin was treated with the Adper Single Bond 2 system after chlorhexidine pretreatment than with use of a self-etch bonding system and chlorhexidine. This is consistent with the literature, which has reported greater interaction between chlorhexidine and teeth etched with phosphoric acid, due to binding to phosphate groups. Positively charged chlorhexidine molecules interact with peptide groups in demineralized collagen, preserving the structural integrity of collagen fibers and bond strength.

Comparison of the groups assessed immediately and at 6 months showed significant differences when the self-etch bonding system and chlorhexidine were used in sound dentin, with greater leakage after 6 months. In the present study, chlorhexidine was applied as pretreatment before application of the self-etch bonding agent. According to the literature, when chlorhexidine is not removed from the cavity preparation prior to rinsing, despite its antibacterial properties, it has a negative effect on the stability of the hybrid layer. Chlorhexidine has amphipathic properties that can interfere with infiltration of resin-based adhesives, preventing a favorable interaction of chlorhexidine with the dental structure and the self-etch bonding system employed. After 6 months, there was more leakage than at immediate assessment when a one-bottle bonding system and chlorhexidine were used in carious dentin. Greater leakage at the bond interface may be attributable to incomplete filling of some areas by the bonding agent and to the presence of residual solvent. Over time, changes occur in the conformation of adhesive polymers, contributing to an increase in the number of voids within the polymer network. The increase in leakage observed at 6-month assessment in carious dentin treated with a one-bottle bonding system and chlorhexidine in the present study may be related to degradation of the adhesive polymer; this contributes to exposure of collagen fibers, which are then degraded by metalloproteinases, particularly in carious dentin, where MMP activity is more intense.

CONCLUSION
To ensure better dentin hybridization and preservation of the organic constituents of the dentin matrix, the prope-
rties of chlorhexidine digluconate and the components of the resin matrix must be taken into account. Further research is required to devise protocols for application of chlorhexidine as a metalloproteinase inhibitor prior to restoration with self-etch and one-bottle dental bonding systems so as to optimize the potential of this inhibitor and, consequently, increase the longevity of the hybrid layer.

CLINICAL SIGNIFICANCE

Metalloproteinases influence degradation of the hybrid layer in composite resin restorations, regardless of whether the bonding system used is one-bottle or self-etching.

ACKNOWLEDGMENTS

FAPESP (2010/10275-9) for the scientific initiation scholarship.

REFERENCES

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