Effect of Metalloproteinase Inhibitors on Bond Strength of a Self-etching Adhesive on Erosively Demineralized Dentin

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Purpose: To analyze the influence of epigallocatechin-3-gallate (EGCG) and chlorhexidine (CHX) on adhesive-dentin bond strength of a self-etch adhesive to sound dentin (SD) and eroded dentin (ED).

Materials and Methods: Thirty-six middle-dentin samples were assigned to six groups (n = 6) according to pretreatment (DW: distilled water, control; 0.1% EGCG; or 2% CHX) and erosive challenge (presence or absence). Specimens were subjected to 2-h acquired pellicle formation, then half of them were exposed to 1% citric acid three times a day for five days. SD and ED were treated with the tested solutions for 60 s, and then Clearfil SE Bond was applied before resin composite buildup. Bonded teeth were longitudinally sectioned into sticks and half were immediately tested, while the remaining specimens were tested after six months. The mode of fracture was examined and the microtensile bond strength (μTBS) measured. Statistical analysis was performed with ANOVA and Bonferroni tests.

Results: At both time periods, regardless of the dentin substrate, EGCG groups did not show bond strengths that were significantly different from those obtained with DW (p > 0.05), while CHX generated lower values than did DW (p < 0.05). On SD, there was a bond strength reduction only in the CHX groups after six months. However, for ED, the bond strength significantly decreased in all groups.

Conclusion: CHX negatively affected both dentin substrates, while the pretreatment with EGCG did not affect μTBS over time on SD. μTBS may be influenced by the substrate over time and EGCG can be used as an alternative to CHX to maintain the bond strength of self-etching adhesives.

Keywords: catechin, chlorhexidine, dentin, matrix metalloproteinase, erosion, adhesive.

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Dental erosion is characterized by chemical dissolution and loss of enamel or dentin by an acidic challenge not caused by bacteria. It is currently being increasingly identified in dental practice and has become an oral health problem. Acids of intrinsic and/or extrinsic origins are associated with this irreversible loss of dental structure, and patients’ biological and behavioral factors can influence dental wear. Thus, the etiology of erosion is multifactorial. Lesions can be localized, generalized, or asymmetric, depending on the etiology. The loss of natural surface morphology and contour are typical signs of erosive tooth wear.

Preventive strategies are extremely important to avoid dental biocorrosion by exogenous and endogenous acids and by biochemical proteolytic enzymes. Dietary counsel-
ing, fluoride treatments, anti-acid products, stimulation of salivary flow rate, or biomimetic products are possible measures to reduce the effects of biocorrosion. However, a restorative treatment may be necessary if the structural integrity of the tooth is threatened.29

Mechanical barriers provided by restorative management can be used to decrease or halt damage progression, reduce symptoms of pain and hypersensitivity, and also restore esthetics and function.7 The main restorative treatment for erosive lesions is the direct adhesive restoration, which provides greater conservation of damaged dental tissues.34 However, adhesion to eroded dentin seems to be compromised compared to the adhesion to sound dentin.14,15,33,43 On sound dentin, despite improvement in adhesive strategies, the efficacy and longevity of dentin adhesion is still a challenge, due to characteristics such as moisture and the chemical and morphological heterogeneity inherent to dental tissue.32 Moreover, degradation of the adhesive-dentin interface over time is one of the main factors involved in the lack of longevity of adhesive bonding.28 Hydrolytic degradation of resin components and the organic matrix of the hybrid layer occurs due to the adhesives’ susceptibility to sorption of water and oral fluids, as well as to the presence of exposed collagen fibrils not covered by the resin material.39 In addition, the intrinsic degradation (proteolysis) of exposed dentin collagen fibrils by host enzymes (matrix metalloproteinases [MMPs]) and cysteine cathepsins are involved in the loss of adhesive bond strength over time.24,26,36 These enzymes also seem to play an important role in the progression of dentin biocorrosion,2 degenerating the organic demineralized dentin layer. This demineralized organic layer, resulting from acid attacks, prevents the diffusion of new acids into dentin. Therefore, MMP action contributes to the progression of erosion, exposing the sound underlying tooth structure to new erosive challenges.21 Hence, the use of MMP inhibitors such as chlorhexidine18,36 and epigallocatechin-3-gallate11 has been accepted as an effective strategy for reducing dentin mineral loss during erosive challenges,23 thus improving the longevity of adhesive restorations.4,5,13,26,35

However, to the best of the current authors’ knowledge, data related to adhesion on eroded dentin1 and its possible improvement using MMP inhibitors are lacking. Only a few studies examined the use of chlorhexidine to improve the adhesion to eroded dentin,16,17 but no study has examined the use of epigallocatechin-3-gallate on eroded dentin. Hence, the purpose of this study was to evaluate the influence of pretreatment with epigallocatechin-3-gallate or chlorhexidine on sound and eroded dentin. The null hypotheses tested were that (a) dentin pretreatment with epigallocatechin-3-gallate or chlorhexidine using a two-step self-etching adhesive does not impair the immediate bond strength to sound and eroded dentin,14,15,33,43 and (b) dentin pretreatment with epigallocatechin-3-gallate or chlorhexidine using a two-step self-etching adhesive does not impair the bond strength to sound and eroded dentin after 6-month water storage.

MATERIALS AND METHODS

Experimental Design

The factors under investigation were: 1. pretreatment on three levels: distilled water, 2% chlorhexidine digluconate, and 0.1% epigallocatechin-3-gallate; 2. erosive challenge on two levels: presence or absence; and 3. aging on two levels: immediate and after six months of water storage. The de-

![Diagram illustrating the erosive cycle.](image-url)
The dependent variable was the bond strength, quantitatively and qualitatively evaluated by microtensile bond strength testing and SEM analyses, respectively. Six specimens were prepared for each pretreatment.

**Tooth Preparation and Sample Selection**

Eighty-six caries-free human third molars were collected after obtaining the patients’ informed consent under a protocol approved by the local research ethics committee (No. 369.345/2013). The specimens were stored in a 0.01% thymol solution for no longer than a month.

For the experiment, a flat coronal dentin surface was exposed by removing the occlusal enamel with a cut perpendicular to the tooth’s long axis in a metallographic cutter (Isomet Buehler; Lake Bluff, IL, USA) at a speed of 300 rpm using a water-cooled diamond saw (Extec, L-12205; Enfield, CT, USA).

In order to standardize the dentin substrate, teeth were selected according to surface microhardness prior to randomization. For this, a second cut parallel to the tooth’s long axis was performed to obtain a coronal fragment of dentin, which was embedded in a circular mold with acrylic resin (Pre 30 Embutidora MI, Arotec; São Paulo, SP, Brazil), ground, and polished (Ecomet/250 Grinder-Polisher, Buehler). The surface microhardness was determined by making five indentations using a Knoop diamond at 25 g for 5 s in the dentin surface (FM100, Future Tech; Tokyo, Japan). Thirty-six samples with Knoop hardness numbers (KHN) ranging from 53.33 to 67.62 were selected and randomly assigned according to a computer generated randomization list into six groups (n = 6). Afterwards, the selected dentin surfaces were further polished on wet #600-grit SiC paper for 60 s to standardize the smear layer (Electric rotary polisher Aropol 2V, Arotec).

**Erosion Cycling Model**

On each day of the experiment, the specimens were subjected to acquired-pellicle formation. Fresh saliva samples were collected from 5 groups of 4 (N = 20) volunteers without active carious lesions, erosions, or salivary dysfunctions. The subjects did not eat or smoke during the 8-h period before sampling. Saliva was stimulated by chewing a piece of paraffin plastic film for 5 min (Parafilm M Laboratory Film, Pechiney Plastic-Packaging; Chicago, IL, USA). Saliva from the first minute of chewing was swallowed, and the remaining (250 ml per day) was collected and deposited into centrifuge tubes. The saliva samples were centrifuged for 10 min at 2000 rpm in a 4°C pre-cooled centrifuge (Model Z 36 HK, Hermle; Wehingen, Germany). The supernatant above the precipitate was pooled and used for pellicle formation. All specimens were immersed in clarified saliva for 2 h under agitation at 100 rpm and a simulated oral cavity temperature of 37°C (Table Oscillating Tecnal TE 143 and bacteriological incubator, Tecnal; Piracicaba, SP, Brazil). After acquired pellicle formation, the specimens were subjected to the experimental cycles. In the erosion cycle, the specimens were immersed in a solution of 1% citric acid (pH 3.75) for 30 s, rinsed with distilled water, and then immersed in artificial saliva (1.5 mM Ca, 0.9 mM PO₄, 150 mM KCl, 0.1M Tris buffer, pH 7) for 2 h. This procedure was repeated three times each day for five consecutive days. All procedures were performed at 37°C under agitation. At the end of each day, the specimens were stored in artificial saliva until the next day (Fig 1). To standardize the procedures, in the non-demineralized groups, the citric acid was replaced by distilled water.

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**Table 1** List of materials used

<table>
<thead>
<tr>
<th>Material</th>
<th>Manufacturer</th>
<th>Composition</th>
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<tbody>
<tr>
<td>Chlorhexidine digluconate</td>
<td>Dentscare, FGM; Joinville, Santa Catarina, Brazil</td>
<td>2% chlorhexidine digluconate, deionized water, volatile surfactant</td>
</tr>
<tr>
<td>Epigallocatechin-3-gallate</td>
<td>EGCG (Sigma Aldrich; St Louis, MO USA)</td>
<td>0.1% aqueous solution</td>
</tr>
<tr>
<td>Clearfil SE Bond</td>
<td>Kuraray Noritake; Tokyo, Japan</td>
<td>Primer: 10-MDP 2-HEMA, dicamphorquinone, N,N-diethanol-p-toluidine, hydrophobic dimethacrylate, water</td>
</tr>
<tr>
<td></td>
<td>Batch number: 062154</td>
<td>Adhesive: 10-MDP bis-GMA, 2-HEMA, dicamphorquinone, N,N-diethanol-p-toluidine, hydrophobic dimethacrylate, colloidal silica</td>
</tr>
<tr>
<td>Composite resin Filtek Z250</td>
<td>3M Oral Care; St Paul, MN, USA</td>
<td>Bis-GMA, TEG-DMA, UDMA, bis-EMA, silica</td>
</tr>
<tr>
<td></td>
<td>Batch number: 1117600319</td>
<td></td>
</tr>
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</table>
Dentin Pretreatment and Adhesive Restorative Procedures

Dentin surfaces were pretreated with 15 μl of each solution (distilled water as the control, 2% chlorhexidine digluco- nate, and 0.1% aqueous solution of epigallocatechin-3-gallate) and scrubbed for 60 s. The excess was removed with absorbent paper (Melitta; Avaré, SP, Brazil). The two-step self-etching adhesive (Clearfil SE Bond, Kuraray Noritake; Tokyo, Japan) was then applied according to the manufacturer’s specifications. The composite Z250 Filtex (3M Oral Care; St Paul, MN, USA) was built up in four 1-mm-thick increments and individually photoactivated for 20 s (Poly Wireless, Kavo; São Paulo, SP, Brazil). The specimens were stored in distilled water at 37°C for 24 h. The materials used are given in Table 1.

Microtensile Bond Strength Test

After 24 h, the specimens were sectioned into sticks with a cross-sectional area about 1 mm². Half of the sticks of each tooth were randomly selected and immediately tested. The remaining sticks were stored in distilled water at 37°C for six months of aging. For testing, the sticks were individually attached to a device with an accelerator (Canada/Zip Kicker, Pacer Technology; Rancho Cucamonga, CA, USA) using cyanoacrylate glue (Superglue Gel, px #45440, Permatex; Hartford, CT, USA) and subjected to microtensile testing (load cell of 50 kgf, DL 2000 N, EMIC; São José dos Pinhais, PR, Brazil) at a speed of 0.5 mm/min until fracture. Bond strength data were calculated in MPa and the mean value of sticks from the same tooth was used as the statistical unit for analysis.

Evaluation of Failure Modes

The fractured surfaces were examined with a stereoscopic microscope (Leica S8AP0, Model MEB 115; Singapore) at 80X magnification. Failure was categorized as cohesive when the fracture occurred exclusively within the dentin (CD) or resin composite (CR), adhesive (AD) when it was at the composite-dentin interface, or mixed (M) when two modes of failure (adhesive and cohesive) occurred simultaneously. Representative fractured specimens of each group were dried at room temperature for 24 h in desiccators, and then sputter-coated with gold (Q150R Rotary-Pumped Sputter Coater/Carbon Coater, Quorum Technologies; Lewes, UK). The surfaces of each fracture site were observed using SEM, and scanning electron micrographs were taken (Inspect 50, FEI; Amsterdam, the Netherlands).

Statistical Analysis

The bond strength means and standard deviations (SD) for each group were tested for normal distribution of errors using the Shapiro-Wilk test. Because the values were normally distributed across the groups, three-way ANOVA (dentin condition, pretreatment, and storage duration) and Bonferroni tests were used for post-hoc comparisons between the groups at a significance level of 5%.

RESULTS

The dentin surfaces after erosion (Fig 2) showed a characteristic dentin demineralized pattern with open dentinal tubules, removed dentin plugs, and some organic portion of intertubular dentin.22

The means and standard deviations of the adhesive-dentin bond strength are shown in Table 2. The results were affected by dentin substrate x aging (p = 0.007; F = 11.7706). The other interactions were not statistically significant (p > 0.05).

At both time periods, regardless of the dentin substrate, the use of epigallocatechin-3-gallate showed no significant difference from the control group (p > 0.05), while the use of chlorhexidine generated lower bond strengths than those of the control group (p < 0.05). On sound dentin after six months, there was no reduction of the bond strength in the control and epigallocatechin-3-gallate groups (p > 0.05). However, on eroded dentin, the bond strength significantly decreased in all groups (p < 0.05).
The fracture pattern distribution (%) is shown in Table 3. For all the tested groups at both times, mixed failure was the most prevalent fracture pattern observed.

A low percentage of adhesive failures was observed after testing immediately (with a somewhat higher percentage in the chlorhexidine groups), but was not observed after 6 months of aging. Water storage affected the bonding interface of the eroded specimens, reducing the proportion of cohesive failures and increasing mixed failures. Representative scanning electron micrographs of the eroded dentin specimens are shown in Figs 2 and 3.

**DISCUSSION**

Data on adhesion to eroded dentin are still scarce, and as are data on the possible effect of MMP inhibitors on the bond strength to this particular substrate. Hence, this study evaluated the effect of pretreatment with epigallocatechin-3-gallate and chlorhexidine digluconate on sound and erosively demineralized dentin with Clearfil SE Bond (CSEB). The present results demonstrated that epigallocatechin-3-gallate did not affect the microtensile bond strength to either substrate at either period of evaluation, while chlorhexidine negatively influenced the bond strength to both SD and ED at both periods. Thus, the null hypotheses were rejected.

To simulate clinical conditions, an in vitro multiple-exposure acid model was used. Before this cycling regime, an acquired pellicle of human saliva was formed and the specimens were maintained in artificial saliva during the intervals. Considering that tooth exposure to intrinsic or extrinsic acids in vivo is generally recurrent but no longer than a few minutes, the current study submitted the specimens to an acid solution for 30 s, three times per day for five days. Although there is no consensus in the literature about the appropriate protocol to be followed, this cyclic model seems to better reflect the effect of acid attacks in the oral cavity due to the presence of saliva remineralization.

Concerning the adhesion on different substrates, this study showed significantly higher bond strengths for specimens subjected to erosive cycling with citric acid in comparison to the non-eroded groups. These results differ from those in the literature, since significantly lower values have been found for the immediate adhesion to in vitro eroded, demineralized dentin. This outcome should probably be attributed to the differences in the erosive cycling protocols used. Studies have shown lower values of microtensile bond strength using protocols with a higher number of cycles (six times per day), longer time (5-10 min), and longer duration (5-10 days), which may have resulted in a thicker zone of demineralized organic matrix. The greater depth of the demineralized dentin layer probably jeopardized the infiltration and photocuring of the adhesive, impairing adhesion. However, the results of de Melo et al. were similar to those of the current study regarding higher bond strength for erosively demineralized groups, and speculated that the demineralization achieved with the erosive cycle protocol possibly promoted further demineralization and increased dentin bond strength due to the weak acidity of the adhesive. Also, another possible explanation of the present outcome is that citric acid (pH = 3.75) produced a less aggressive dentin demineralization pattern, and the thinner organic matrix did not jeopardize the adhesion procedures.

A number of factors make it difficult to establish a clinical protocol for eroded dentin, eg, a lack of studies analyzing morphological characteristics of the adhesive interface on eroded demineralized dentin, the behavior of the adhesive materials on this substrate, and different in vitro erosive protocols. Moda et al. analyzed the micromechanical interaction of the adhesive interface on eroded and sound dentin using confocal microscopy, evaluating the micropermeability and the sealing ability of the resin tags at the adhesive interface. The eroded dentin specimens were submitted to an in vitro demineralization protocol (immersed in a demineralizing solution for 2 min per cycle and remineralizing solution for 10 min per cycle for 9 days). The eroded dentin presented a considerable increase in the density and depth of resin tags in relation to sound dentin.

Despite the high immediate bond strengths, after six months, the bond strength significantly decreased in all eroded groups, regardless of the pretreatment. The present results demonstrated that epigallocatechin-3-gallate and chlorhexidine digluconate positively affected the bond strength to eroded dentin, while the bond strength to sound dentin was not significantly influenced by the pretreatments. These results suggest that these compounds may be considered as possible adhesives for eroded dentin, warranting further investigation.

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**Table 2** Mean (± SD) microtensile bond strengths in MPa (n = 6)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sound dentin</th>
<th>Eroded dentin</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>6 months</td>
</tr>
<tr>
<td>Control</td>
<td>52.44 ± 8.47&lt;sup&gt;A&lt;/sup&gt; ab</td>
<td>47.64 ± 11.67&lt;sup&gt;A&lt;/sup&gt; b</td>
</tr>
<tr>
<td>CHX</td>
<td>40.87 ± 10.23&lt;sup&gt;B&lt;/sup&gt; b</td>
<td>32.77 ± 10.67&lt;sup&gt;B&lt;/sup&gt; c</td>
</tr>
<tr>
<td>EGCG</td>
<td>53.67 ± 6.10&lt;sup&gt;A&lt;/sup&gt; ab</td>
<td>50.02 ± 13.42&lt;sup&gt;A&lt;/sup&gt; abc</td>
</tr>
</tbody>
</table>

Different superscript capital letters in the same column represent statistically significant differences between baseline and 6 months (p < 0.05). Different superscript lowercase letters in the same row represent statistically significant differences in each dentin substrate (p < 0.05).
outcome is in accordance with previous studies that showed more adverse effects for the long-term bond efficacy to eroded dentin. It can be speculated that the pH changes caused by the erosive cycle plus the acidic monomers from the adhesive increased the activation and expression of MMPs and cysteine cathepsins, resulting in an increased digestion of collagen within the hybrid layer. Metalloproteinase inhibitors, such as chlorhexidine and epigallocatechin-3-gallate, have shown promising results on sound dentin, but only a few studies evaluated the

<table>
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<th>Group</th>
<th>Clearfil SE Bond</th>
<th>Baseline</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adhesive Mixed</td>
<td>Cohesive in composite</td>
<td>Cohesive in dentin</td>
</tr>
<tr>
<td>SD+DW</td>
<td>0% 59% 8% 33%</td>
<td>0% 66,67% 17% 17%</td>
<td></td>
</tr>
<tr>
<td>SD+CHX</td>
<td>15% 63% 15% 6%</td>
<td>0% 81% 8% 11%</td>
<td></td>
</tr>
<tr>
<td>SD+EGCG</td>
<td>3% 65% 14% 19%</td>
<td>0% 57% 18% 25%</td>
<td></td>
</tr>
<tr>
<td>ED+DW</td>
<td>0% 69% 19% 12%</td>
<td>0% 91% 5% 5%</td>
<td></td>
</tr>
<tr>
<td>ED+CHX</td>
<td>5% 70% 16% 9%</td>
<td>0% 87% 10% 3%</td>
<td></td>
</tr>
<tr>
<td>ED+EGCG</td>
<td>0% 54% 15% 30%</td>
<td>0% 61% 26% 13%</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: DW: distilled water; CHX: chlorhexidine; EGCG: epigallocatechin-3-gallate; SD: sound dentin; ED: eroded dentin.

Fig 3 Representative scanning electron micrographs of the dentin side of fractured control group specimens (a and d), chlorhexidine group (b and e), and epigallocatechin-3-gallate group (c and f), after erosive challenge and aging for 6 months. White arrow indicates preserved dentin collagen and asterisk indicates the top of the hybrid layer.
the speculation that, as opposed to chlorhexidine, epigallocatechin-3-gallate on bond strength to eroded dentin had not been previously reported until now.

Epigallocatechin-3-gallate is the major polyphenol present in green tea (Camellia sinensis), with properties such as antimicrobial activity, MMP inhibition and dentin cross-linking ability that can potentially be applicable in restorative procedures. The use of 0.1% EGCG as a dentin pretreatment associated with an etch-and-rinse adhesive was first described by Santiago et al., who found no impairment of the immediate bond strength. Regarding self-etching adhesives, the current results for sound dentin corroborate those of a previous study that showed no difference in the immediate bond strength with an epigallocatechin-3-gallate dentin pretreatment.

On sound dentin, bond strength was maintained in both control and ECGG groups after 6 months. Perhaps this result is related to the adhesive chosen for this research. Clearfil SE bond is a two-step self-etch adhesive that contains 10-methacryloxydecyl dihydrogen phosphate (10-MDP), an acidic phosphate monomer capable of bonding to hydroxyapatite, producing adhesive interfaces with different chemical and morphological characteristics. This involves micromechanical and chemical bonding to the tooth surface, which may have a direct impact on its bonding efficacy. Hence, this may explain the higher microtensile bond strengths for immediate testing and the low rate of purely adhesive failures after six months’ aging in the current study. Furthermore, in terms of the 6-month results on sound dentin, epigallocatechin-3-gallate probably showed greater affinity for dentin collagen due to its hydroxyl groups. This makes it a more polar molecule than chlorhexidine, potentially resulting in a more stable bond with dentin over time.

On the other hand, the results with CHX in this study suggest that an unfavorable interaction exists between chlorhexidine and self-etching adhesives, regardless of the type of dentin. This was corroborated by other researchers who showed a negative influence using chlorhexidine as dentin pretreatment. The presence of chlorine ions and crystal-shaped precipitates was previously reported when dentin was pretreated with 2% chlorhexidine and analyzed using energy-dispersive x-ray spectroscopy. It could be hypothesized that chlorhexidine reacts with dentin, and the presence of precipitates possibly reduces the depth of dentin etching, suggesting a chemical and physical interference. Also, a previous study reported that chlorhexidine has higher substantivity on partially or completely demineralized dentin. This possible interference was reflected in more adhesive failures in the immediate groups with chlorhexidine (Table 3). After 6 months, chlorhexidine pretreatment was not able to maintain the bond strength to dentin bond, independent of the type of dentin substrate. The scanning electron micrographs showed the absence of a preserved hybrid layer (Figs 3b and 3e). It is probable that the initial adverse interaction is still reflected in the results after aging. The SEM images also support the speculation that, as opposed to chlorhexidine, epigallocatechin-3-gallate is a nonpolar molecule that does not react with dentin, and does not impair the bonding ability of the adhesive. Figures 3c and 3f show completely closed dentinal tubules and a hybrid layer preserved by epigallocatechin-3-gallate.

CONCLUSION

This study suggests that the comparison of diverse erosive models simulating different causative factors as well as different levels of erosion severity should be the subject of further research. It was concluded that chlorhexidine negatively affected the bond strength, while pretreatment with epigallocatechin-3-gallate did not affect the bond strength to either type of dentin over time using Clearfil SE Bond adhesive.

ACKNOWLEDGMENTS

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REFERENCES


31. Costa et al.

Clinical relevance: Epigallocatechin-3-gallate did not affect the bond strength, while chlorhexidine negatively influenced the bond strength of a self-etching adhesive on erosively demineralized dentin.