

# Effect of Bioactive Primers on Bacterial-Induced Secondary Caries at the Tooth-Resin Interface

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## Clinical Relevance

Secondary caries is the primary reason for the replacement of resin composite restorations. Using an artificially induced bacterial carious model, this study found that a bioactive primer containing plant-derived proanthocyanidins inhibited secondary caries formation at the dentin-resin interface.

## SUMMARY

**Secondary caries at the tooth-resin interface is the primary reason for replacement of resin composite restorations. The tooth-resin interface is formed by the interlocking of resin**

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material with hydroxyapatite crystals in enamel and collagen mesh structure in dentin. Efforts to strengthen the tooth-resin interface have identified chemical agents with dentin collagen cross-linking potential and antimicrobial activities. The purpose of the present study was to assess protective effects of bioactive primer against secondary caries development around enamel and dentin margins of class V restorations, using an in vitro bacterial caries model. Class V composite restorations were prepared on 60 bovine teeth (n=15) with pretreatment of the cavity walls with control buffer solution, an enriched fraction of grape seed extract (e-GSE), 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide/*N*-hydroxysuccinimide, or chlorhexidine digluconate. After incubating specimens in a bacterial model with *Streptococcus mutans* for four days, dentin and enamel were assessed by fluorescence microscopy. Results revealed that only the naturally occurring product, e-GSE, significantly inhibited the development of secondary caries immediately adjacent to the dentin-resin interface, as indicated by the caries inhibition zone. No inhibitory effects were

**observed in enamel margins. The results suggest that the incorporation of e-GSE into components of the adhesive system may inhibit secondary caries and potentially contribute to the protection of highly vulnerable dentin-resin margins.**

## INTRODUCTION

Resin composite is commonly used for the direct restoration of missing tooth structures. In 2006, the number of resin composite restorations placed in the United States was 121 million, compared with 52.2 million for its alternative, amalgam.<sup>1</sup> In addition to esthetics, resin composites can adhere to tooth structure<sup>2</sup> and allow for a conservative tooth preparation.<sup>3</sup> However, the service life of resin composites is consistently shorter than amalgam.<sup>4,5</sup> A 1%-3% annual failure rate has been reported for resin composite restorations with 50%-75% of failures resulting from secondary caries, followed by postoperative sensitivity and restoration fracture.<sup>2,6-8</sup>

As secondary caries develops at the margins between the restorative material and the tooth substrates, methods to improve the properties of the adhesive interface have been investigated. Resin polymerization reactions are associated with a volumetric shrinkage that induces internal contraction stresses at the interface. The degree of polymerization and shrinkage was positively correlated with interfacial gap size as examined by microtomography.<sup>9</sup> This may lead to marginal breakdown, marginal staining, and possible sites for the development of secondary caries.<sup>10</sup> Furthermore, water absorption into porosities and degradation of resin by esterase activity in saliva contribute to the breakdown of margins over time.<sup>11</sup>

Approaches to strengthen the anchoring dentin matrix have gained increased interest. Specifically, the biomodification of dentin matrix by bioactive agents mediating exogenous collagen cross-linking.<sup>12</sup> Carbodiimide, a synthetic chemical agent, was previously shown to reinforce dentin matrix and stabilize the dentin-resin bond over time by inducing zero-length cross-links.<sup>13</sup> Proanthocyanidins (PACs) are plant-derived polyphenolic compounds derived from catechins and form a structurally complex class of oligomers and polymers. Previous studies have shown that certain PACs can reinforce dentin selectively, improve the dentin-resin bond strength, and are suitable agents for primary caries prevention.<sup>12,14-17</sup>

With the median life span of resin composite restorations being eight years in adults,<sup>18</sup> multiple

replacements are likely in the lifetime of a patient. Every time a replacement is made, more tooth structure is lost, and as a result, repeated failure and replacement of restorations can lead to premature tooth loss. The purpose of the present study was to assess the protective effects of bioactive agents against secondary caries development around enamel and dentin margins of class V resin composite restorations, using an *in vitro* bacterial caries model. The null hypothesis was that bioactive primers do not affect secondary caries development around enamel and dentin margins compared with control groups.

## METHODS AND MATERIALS

### Materials

The chemical agents used in the study were as follows: 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid powder (HEPES, Sigma-Aldrich, St. Louis, MO, USA); 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC/NHS; Thermo Scientific Pierce, Rockford, IL, USA), *N*-hydroxysuccinimide (Thermo Scientific Pierce), and chlorhexidine digluconate (CHX) stock solution (Alfa Aesar, Ward Hill, MA, USA).

Grape seed extract was obtained from Polyphenolics Inc. (Madera, CA, USA) and consisted of PACs with a degree of polymerization (DP) ranging from oligomers (2 to 7) up to polymers (8 to >20). Using a previously published method,<sup>19</sup> the polymeric PACs were selectively depleted from the crude extract to yield an enriched oligomeric mixture (e-GSE). The refined e-GSE material was composed of phenolic acids and phenolic monomers (PACs) that are commonly known as catechins and the oligomeric PACs. Gravimetrically, approximately 70% of the e-GSE fraction consisted of flavan-3-ol monomers; the remaining 30% was oligomeric PACs (OPACs). Among the OPACs in e-GSE, approximately 50% are dimers, and the other half are mid- to high-order OPACs. Both classes of compounds are jointly referred to as PACs in the following.

### Restorative Procedures and Specimen Preparation

Bovine incisors were placed in 0.1% thymol solution for four weeks. Teeth were cleaned to remove debris, periodontal ligament, and cementum of the root surfaces, and then they were visually inspected and excluded if enamel defects and white spots were detected with a magnifying dental loupe. Teeth were sectioned 4 mm above and 4 mm below the cemento-

enamel junction (CEJ) at the mid-mesial and mid-distal surfaces using a diamond wafering blade (Buehler- Series 15LC Diamond, Buehler, Lake Bluff, IL, USA). Teeth were further sectioned into two halves to obtain mesial and distal sections to a final rectangular dimension of 8 mm width  $\times$  8 mm length  $\times$  1.5-2 mm thickness. Class V preparations of 3 mm width  $\times$  3 mm length  $\times$  1 mm depth were cut at the CEJ using a flat-end carbide bur (#558, Brasseler USA Dental, Savannah, GA, USA) in a high-speed handpiece with air/water coolant. Enamel and root dentin margins were prepared at 90° to the tooth surface, and burs were changed every five preparations.

Cavity preparations were randomly assigned to four groups (n=15). For the control group (HEPES primer), cavity walls were etched with 32% phosphoric acid (Scotchbond, 3M ESPE, St Paul, MN, USA) for 15 seconds and rinsed with distilled water for 30 seconds. Preparations were blotted dry with an absorbent tissue (KimWipe, Kimberly-Clark Corporation, Irving, TX, USA) and primed with 20 mM HEPES buffer for one minute, rinsed for 30 seconds, and blotted dry with an absorbent tissue, and a drop of Adper Single Bond Plus (3M ESPE) was actively applied on the preparation surfaces. The adhesive layer was air dried to remove excess solvent and light cured for 20 seconds (Optilux 501 light unit at 830 mW/cm<sup>2</sup>, Kerr, Orange, CA, USA). Preparations were filled with Filtek Supreme Plus Universal composite material (3M ESPE) in two vertical increments and light cured for 40 seconds each. Immediately after the final curing, restorations were polished with coarse-, medium-, and fine-grit aluminum-oxide abrasive discs (Sof-Lex, 3M/ESPE) in a slow speed handpiece.

The experimental groups followed the same restorative sequence, except for the following protocols for each primer: e-GSE primer, priming solution containing 15 w/v% e-GSE was applied for one minute and rinsed with distilled water for 30 seconds, modified from Castellan and others<sup>20</sup>; EDC/NHS primer, priming solution containing 0.3 M EDC/0.12 M NHS was applied for one minute<sup>21</sup> and rinsed for one minute; and CHX primer, priming solution containing 2% chlorhexidine was applied for 30 seconds and blotted dry.<sup>22</sup>

### Artificially Induced Secondary Caries

Cosmetic nail varnish was applied 1 mm away from the margins of the restorations and air dried for 40 minutes. Specimens were disinfected in 70% ethanol for 20 minutes,<sup>23</sup> rinsed with sterile phosphate

buffered saline (PBS) twice, and stored in sterile PBS at 4°C overnight. *Streptococcus mutans* UA159 was aerobically cultured on Brain Heart Infusion (BHI, Difco Laboratories, Detroit, MI, USA) agar, and a colony was inoculated into BHI broth and incubated for 18-20 hours at 37°C. Then, cells were washed twice with PBS and suspended in fresh medium supplemented with 1% sucrose (BHIS) and standardized to  $1 \times 10^8$  cells/mL spectrophotometrically (absorbance of 0.20 at 550 nm; Spectronic 601, Milton Roy, Ivyland, PA, USA). Specimens were inoculated with *S. mutans* suspension in BHIS for 4 hours at 37°C, after which the media were replaced with BHI without sucrose for the next 20 hours (modified protocol from Fontana and others).<sup>24</sup> Wells were gently rinsed with PBS buffer twice following each media change. At the end of a four-day challenge, specimens were removed from the wells and rinsed in running water thoroughly. Specimens were sectioned along the axis of the tooth, through the restorations. Sections were embedded in epoxy resin overnight and polished with #320, #400, #600, #800, and #1200 grit silicon carbide abrasive papers (Buehler) under running water.

### Fluorescence Microscopy Analysis

Specimens were hydrated with distilled water for one hour and stained overnight with 0.1 mM rhodamine B solution (pH 7.2), following the protocol described by Fontana and others.<sup>25</sup> After elapsed time, specimens were rinsed in running water for one minute and blotted dry with absorbent paper. Specimens were examined under a fluorescence microscope (DMI 6000 B, Leica, Buffalo Grove, IL, USA) with a connected digital camera (Hamamatsu, Skokie, IL, USA) and LAS AF software (Leica). Images of light differential interference contrast (DIC) microscopy, as well as red fluorescence at 529 nm, were captured. The same microscope settings were used for all images. Images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Positions of restoration margins were identified in DIC images and transferred to fluorescence images. Lesion depth (LD) was measured 125  $\mu$ m away from the restoration margin as the depth of rhodamine stained from the surface. Secondary caries was measured as total fluorescence (TF; Figure 1),<sup>25</sup> where a fluorescent area was marked and TF was measured as area multiplied by mean fluorescence. For dentin, TF was measured within 250, 100, 50, or 25  $\mu$ m from the restoration. For enamel, TF was measured within 250 or 25  $\mu$ m from the restoration.

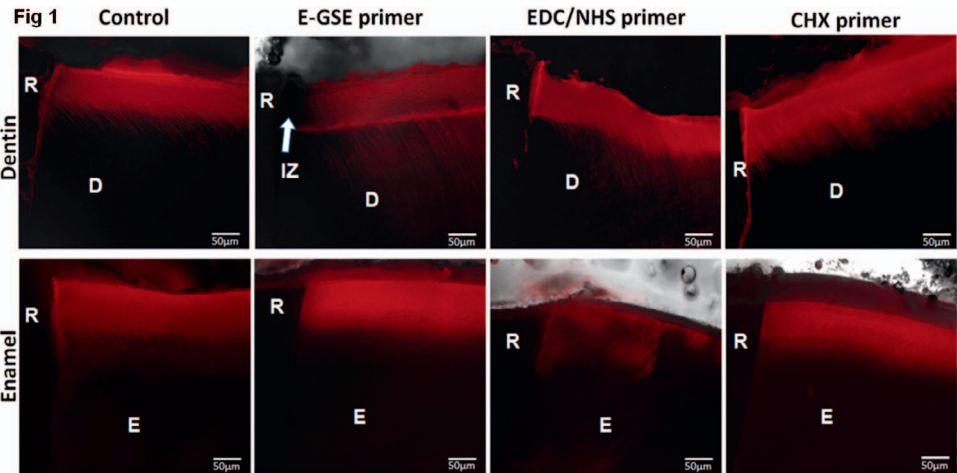


Figure 1. Representative images of rhodamine-B-infiltrated dentin and enamel depicting demineralization around class V restorations restored with bioactive primers. R, resin; D, dentin; E, enamel; IZ, inhibition zone.

Data were analyzed for the effect of treatment on LD and TF by one-way analysis of variance and Bonferroni post hoc test at  $\alpha=0.05$ .

RESULTS

Margins in Dentin

LDs were similar across the treatment groups (Figure 1); there were no statistically significant mean differences in LDs (Table 1;  $p>0.05$ ). Most interestingly, an inhibition zone (IZ) was noted in the e-GSE group, where rhodamine staining was scarce next to the tooth-resin interface (Figure 1). Such IZ was not observed in the control or any other treatment group. When examined up to 250  $\mu\text{m}$  adjacent to the restoration, there was no statistically significant difference in total fluorescence among all treatment groups ( $p>0.05$ ). When the area was limited to 100 or 50  $\mu\text{m}$  adjacent to the restoration, a statistically significant difference was observed between the e-GSE and CHX groups ( $p<0.05$ ). When the area was limited to 25  $\mu\text{m}$  adjacent to the restoration, total fluorescence for the e-GSE group was significantly lower than all the other groups (Table 1). The e-GSE group had the least amount of

demineralization, especially near the restoration margin, which was consistent with the presence of an inhibition zone (Figure 1). The e-GSE primer showed a protective effect immediately adjacent to the dentin-resin interface.

Margins in Enamel

LDs in enamel were similar across the experimental groups as shown in Table 1 ( $p>0.05$ ) and Figure 1. Regardless of the areas examined, 250 or 25  $\mu\text{m}$  adjacent to the restoration, there were no statistically significant mean differences in TF among all groups (Table 1). Enamel demineralization did not differ across the treatment groups ( $p>0.05$ ).

DISCUSSION

An *S. mutans*-induced caries model was used for artificial caries development, which was clinically more relevant than a pH-cycling model. Carious lesions in dentin were more aggressive closer to the restoration (distance 100 vs 300  $\mu\text{m}$ ). The current findings confirm that dentin-resin interface seems to be more susceptible to caries progression around the restoration margin than the enamel-resin interface,

Table 1: Secondary Caries Lesions Depth and Total Fluorescence Calculated by Area and Distances From the Dentin and Enamel Margins <sup>a</sup>								
Cavity primers	Lesion depth (µm)	Margins in dentin				Margins in enamel		
		Total fluorescence (×10 <sup>5</sup> ), mean (SD)				Lesion depth (µm)	Total fluorescence (×10 <sup>5</sup> ), mean (SD)	
		250 µm	100 µm	50 µm	25 µm		250 µm	25 µm
Control	72.5 (9.8)	8.06 a (5.51)	3.37 ab (1.98)	1.82 ab (1.04)	1.04 b (0.57)	80.5 (29.7)	6.20 (6.93)	4.16 (3.71)
e-GSE	74.8 (15.0)	8.23 a (5.57)	2.29 a (1.63)	0.79 a (0.70)	0.27 a (0.30)	67.8 (18.7)	4.06 (3.50)	2.20 (1.37)
EDC/NHS	71.2 (16.4)	10.75 a (6.78)	4.45 ab (3.14)	2.29 ab (2.05)	1.24 b (1.14)	71.7 (22.6)	2.42 (3.90)	2.40 (2.79)
CHX	74.4 (13.1)	12.29 a (6.27)	4.86 b (2.85)	2.69 b (1.81)	1.37 b (0.83)	83.4 (49.3)	5.55 (6.43)	3.21 (3.12)

<sup>a</sup> Different letters indicate statistically significant differences in each column ( $p<0.05$ ).

which may be associated to less effective bonding to dentin compared with enamel. Resin composite's surface roughness and hydrophobicity<sup>26,27</sup> may have favored adhesion of oral streptococci and contributed to more demineralization closer to the dentin margins. The integrity of the tooth-resin interface influences the progression of caries around the restoration margin, with microleakage providing an additional portal for bacterial attack.<sup>28,29</sup>

One of the most interesting results of this study was that the e-GSE primer inhibited secondary caries development immediately adjacent to the dentin-resin interface. The e-GSE protective effect against secondary caries development was clearly represented by the presence of an inhibition zone, as well as the lowest total fluorescence relative to all other groups, measured within 25  $\mu\text{m}$  of the restoration (Table 1). Three possible mechanisms of actions can be considered for e-GSE to inhibit secondary caries in dentin: (1) tissue stabilization, (2) a tighter interfacial seal, and (3) antimicrobial activity. As dentin is relatively porous, the e-GSE PACs were able to diffuse further than a few micrometers of the hybrid layer and protect dentin beyond the interface. Collagen cross-linking has been suggested to stabilize dentin by providing a scaffold for mineralization and a barrier for acid diffusion and mineral loss.<sup>14,16</sup> The observed fluorescence patterns suggest that surface caries lesions progressed to the peripheries until limited by PAC-treated dentin near the interface.

A tighter resin seal is achievable at the dentin-resin interface when the collagen mesh structure is intact and capable of forming a hybrid layer.<sup>30</sup> The e-GSE primer showed these properties, inducing collagen cross-linking and maintaining the collagen mesh structure before application of the bonding agent. A tighter interfacial seal is considered to be more resistant to bacterial leakage and acid diffusion; previous studies reported a positive correlation between the interface gap size and secondary caries.<sup>31-33</sup> Studies have suggested that 250- to 400- $\mu\text{m}$  gaps contribute to the development of secondary caries and do not consider an open margin as an indication for replacement of a restoration.<sup>4</sup> However, gaps of 50  $\mu\text{m}$  and less were previously shown to be colonized by *S. mutans* biofilm and subsequently caries being formed specifically at the interface.<sup>28,29</sup> An additional study will be required to assess the effect of e-GSE on marginal integrity without a caries challenge.

PACs are known for general antimicrobial properties. Specific to cariogenic bacteria, PACs inhibit

surface-adsorbed glucosyltransferases and acid production by *S. mutans*,<sup>34</sup> as well as decrease the growth of *S. mutans* and biofilm formation.<sup>35</sup> Catechins such as epigallocatechin gallate suppress *gtf* genes associated with *S. mutans* biofilm formation.<sup>36</sup> The antimicrobial activity of PACs against cariogenic bacteria likely contributes to the inhibition of dentin demineralization by e-GSE, as the dentin tissue might function as a reservoir for PACs bound to the collagen backbone.

All other agents evaluated in this study had no significant effect on inhibiting secondary caries formation around resin composite restorations. Although EDC has collagen cross-linking activity,<sup>37</sup> it did not have the same effect as e-GSE. EDC's cross-linking ability is known to be less potent than other chemical agents,<sup>13,38</sup> and no effect was observed on interfacial nanoleakage compared with a control.<sup>38</sup> Although EDC was shown to inhibit bacterial membrane ATPases<sup>39</sup> and sugar uptake in oral streptococcal bacteria,<sup>40</sup> EDC does not take part in newly induced cross-linkage and is quickly hydrolyzed in solution, thus limiting a significant antimicrobial protection at the adhesive interface.

CHX was not found to inhibit secondary caries in any of the outcomes. A possible explanation is that CHX does not exhibit a permanent binding mechanism to the dentin structure and that the residual amount of CHX at the interface was too low for exerting bactericidal activity as it is only bacteriostatic at low concentrations.<sup>41</sup> Furthermore, a few studies have suggested weakening of the tooth-resin bond by chlorhexidine.<sup>42,43</sup>

As with any *in vitro* study, cautions remain when extrapolating results to the actual oral environment. The bacterial caries model in this study involved only a single species of cariogenic bacteria (*S. mutans*). However, the present design provides significant and promising findings. Inevitably, the hybrid layer is subjected to degradation and fatigue over time.<sup>30,44</sup> Aging of specimens was not simulated in the present work, but will be pursued in future studies.

## CONCLUSIONS

Bacterial-induced secondary caries develops in enamel and dentin regardless of the composition of the primer solution. Lesions were more aggressive closer to the dentin-resin adhesive margins. An enriched fraction of grape seed extract, e-GSE, significantly inhibited secondary caries development within 25  $\mu\text{m}$  of the restoration margin in dentin.

None of the other treatments inhibited secondary caries development around resin composite restorations. The results suggest that incorporation of e-GSE in the restorative procedure of resin composites may reduce secondary caries development immediately around highly susceptible root dentin margins.

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### Regulatory Statement

This study was conducted in accordance with all the provisions of the local human subjects oversight committee guidelines and policies of the University of Illinois at Chicago.

### Conflict of Interest

The authors of this manuscript certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

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