

**Effect of Hard Tissue Modification  
on Bacterial-Induced Secondary Caries  
at the Tooth-Resin Interface**

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THESIS

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This thesis is dedicated to my family and friends for their love and support along the way.

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## LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AR	Adhesive Resin
BHI	Brain Heart Infusion
BHIS	Brain Heart Infusion with Sucrose
CEJ	Cementoenamel junction
CHX	Chlorhexidine digluconate
CLSM	Confocal Laser Scanning Microscopy
D	Dentin
Ds	Distal
DIC	Differential Interference Contrast
E	Enamel
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
GSE	Grape Seed Extract
GSE-UP	An enriched fraction of Grape Seed Extract
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HL	Hybrid Layer
IF	Interface
IZ	Inhibition Zone
LD	Lesion Depth
M	Mesial
NHS	N-hydroxysuccinimide
PAC	Proanthocyanidin



PBS	Phosphate Buffered Saline
R	Restoration
RC	Resin Composite
SD	Standard Deviation
SEM	Scanning Electron Microscopy
T	Resin Tag
TF	Total fluorescence

## 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 interlocking of resin material with hydroxyapatite crystals in enamel and collagen mesh structure in dentin. Efforts have been made to strengthen the tooth-resin interface, and dentin biomodification agents have been previously identified with collagen cross-linking potential and antimicrobial activities. The purpose of the current study was to assess protective effects of dentin biomodification agents against secondary caries development around enamel and dentin margins of a class V restoration, using a bacterial caries model.

Class V composite restorations were made on sixty bovine tooth samples (n=15) with pre-treatment of cavity walls with either control buffer solution, an enriched fraction of grape seed extract (GSE-UP), 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide/N-hydroxysuccinimide (EDC/NHS), or chlorhexidine digluconate (CHX). After incubating specimens in a bacterial model with *Streptococcus mutans* for 4 days, caries lesions were evaluated by microhardness test and confocal laser scanning microscopy with rhodamine B staining. Data was statistically analyzed by three-, two-, or one-way ANOVA, followed by post-hoc Bonferroni tests.

Results of the study revealed that GSE-UP significantly inhibited secondary caries development immediately adjacent to the dentin-resin interface, as indicated by

## **SUMMARY (continued)**

absence of rhodamine B staining around the restoration margin in dentin. Results suggest that incorporation of biomodification agents, specifically GSE-UP, into adhesive systems may inhibit secondary caries and thereby increase the longevity of resin composite restorations.

## I. INTRODUCTION

### A. Background

Secondary caries, caries that forms around an existing restoration, is an ongoing problem in dentistry. Teeth need restorations for reasons of caries, fracture, wear, esthetics, and malocclusion. Commonly used restorative materials are amalgam, resin composite, metal, and ceramic. When these materials are placed, there is a junction, also called a margin, between the restoration and the remaining tooth structure. Because this junction between two different materials is not perfect, there is a risk of gap formation that can increase chances of new caries developing at the margin. Moreover, if a patient needed a restoration for caries, there are dietary, hygiene, and genetic risk factors for secondary caries. Secondary caries is frustrating for both patients and clinicians, as replacement of a restoration is undesirable.

Resin composite is commonly used for direct restoration of missing tooth structure. In 2006, the number of resin composite restorations placed in the U.S. was 121 million, compared to 52.2 million for its alternative, amalgam (American Dental Association, 2007). In addition to its esthetic tooth shade, its main advantage is the ability to adhere to tooth structure (Demarco et al., 2012), providing a clinically acceptable bond strength (Saygili and Mahmali, 2002) and allowing a conservative tooth preparation (Cenci et al., 2005). Yet, its reported longevity is consistently shorter than amalgam: two versus three years in primary teeth (Mjör et al., 2002) and eight versus eleven years in permanent teeth (Mjör et al., 2000). 1-3% of annual failure rate has been reported for resin composite restorations with 50-75% of failures resulting from

secondary caries, followed by post-operative sensitivity and restoration fracture (Lempel et al., 2015; Pallesen et al., 2013; 2014; Kopperud et al., 2012; da Rosa Rodolpho et al., 2006; Demaco et al., 2012).

As secondary caries develops at the interface between the restorative material and the tooth cavity wall, methods to improve the properties of the interface have been investigated. When resin undergoes its polymerization reaction, there is a volumetric shrinkage towards its bulk, creating internal contraction stress at the interface and leading to marginal breakdown with signs and symptoms of post-operative sensitivity, marginal staining and secondary caries (Schneider et al., 2010). Degree of polymerization shrinkage was positively correlated with interfacial gap size as examined by microtomography (Kakaboura et al., 2007). Silorane-based resin composite and ormocers are examples polymer structures with less polymerization shrinkage (Burke et al., 2011; Efes et al., 2006). Furthermore, water absorption into porosities and degradation of resin by esterase activity in saliva, contribute to marginal breakdown over time (Braden, 1977; Kermanshahi et al., 2010). As the filler content provides mechanical strength, different filler loading and sizes have been tested (Da Rosa Rodolpho et al., 2011; Ernst et al. 2006).

While methods to strengthen the resin side of the interface continue, methods to strengthen the tooth side of the interface have gained increased interest. An example of enamel modification is treatment with fluoride, which binds to hydroxyapatite structure and increases its stability and resistance to demineralization (Takagi et al., 2000).

Dentin biomodifications have mainly involved cross-linking and stabilization of collagen network, with the possibility of additional interactions with non-collagenous proteins in dentin (Bedran-Russo et al., 2014). Carbodiimide is a synthetic chemical agent with collagen cross-linking potential, previously shown to strengthen dentin and stabilize the dentin-resin bond over time (Bedran-Russo et al., 2010; 2013). Proanthocyanidins (PACs) are naturally occurring collagen cross-linkers, previously shown to strengthen dentin, improve the dentin-resin bond, and promote remineralization of dentin (Bedran-Russo et al., 2008; Castellan et al., 2013; Al-Amman et al., 2009; Xie et al., 2008).

**B. Statement of the problem**

With the median age of resin composite restorations being 8 years in adults (Mjör et al., 2000), multiple replacements are likely in a 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 loss of teeth. In order to reduce the rate of replacement, its most frequent reason, secondary caries, needs to be addressed and prevented (Burke et al., 2001; Kopperud et al. 2012).

**C. Purpose of the study**

The purpose of this study is to assess protective effects of dentin biomodification agents against secondary caries development around enamel and dentin margins of a class V resin composite restoration, using a bacterial caries model. Dentin biomodification agents to be tested are proanthocyanidins (PACs) and 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide (EDC). The null hypothesis is that these dentin

biomodification agents do not affect secondary caries development around enamel and dentin margins when compared to the control.

**D. Significance of the problem**

Despite technological advancements, dental materials wear and fail over use and misuse. Although restorations are desired to last a long time, failures occur in a harsh oral environment with moisture, bacterial challenge, and occlusal load. For resin composite restorations, failure is most frequent by secondary caries around the restoration margins, and strengthening the tooth-resin interface has been a continuous challenge. Protecting the interface can reduce the failure rate and increase the lifetime of a restoration, ultimately reducing the dental costs for the society and emotional stress of patients.

**E. Significance of the study**

Previous studies have shown benefits of hard tissue biomodification in strengthening the hard tissue (Bedran-Russo et al., 2007; 2008; 2013; Castellan et al., 2010; 2011; Vidal et al., 2014) as well as the dentin-resin bond (dos Santos et al., 2011; Bedran-Russo et al., 2010; Green et al., 2010; Macedo et al., 2009; Al-Ammar et al., 2009; Castellan et al., 2010; 2013; Mazzoni et al., 2013; 2014). Benefits of hard tissue modification in inhibiting primary caries have been investigated (Xie et al., 2008; Pavan et al., 2011; Walter et al., 2008) but not for secondary caries. Current study uses a bacterial model to simulate intraoral conditions and obtain results that are more clinically relevant, unlike many studies that use chemical means to induce caries. If the proposed

treatments show benefits in secondary caries inhibition, their incorporation in clinical procedures may be considered. An improved tooth-resin interface will further assure clinicians' choice for resin composite restorations. Study results may be extended to resin composite cements.



## **II. CONCEPTUAL FRAMEWORK AND RELATED LITERATURE**

### **A. Conceptual Framework**

#### **1. Enamel and dentin**

A tooth can be divided into a crown portion that is above the gingiva and a root portion that is below the gingiva. The crown has an outer layer called enamel. The root has an outer layer called cementum. Underneath the outer layers is dentin, and underneath the dentin is pulp. Enamel and dentin are different in their composition, structure, and properties.

Enamel is composed of 95% hydroxyapatite, 3% water, and 1% non-collagenous proteins, lipids and ions, by weight (LeGeros, 1991). Hydroxyapatite crystals are densely packed in enamel prisms running parallel to each other (Meckel et al., 1965; Kennedy et al., 1951). Dentin is composed of 70% mineral, 20% organic component and 10% fluid by weight. 90% of the organic component is fibrillar type I collagen and 10% is non-collagenous proteins such as phosphoproteins and proteoglycans (Linde, 1985; Birkedal-Hansen et al., 1977).

Mantle dentin is a thin layer of dentin underneath enamel or cementum that is mostly atubular, less mineralized, and 30-60  $\mu\text{m}$  in thickness (Goldberg et al., 1993). Circumpulpal dentin forms the majority of dentin. Predentin is the premature dentin near the pulp. Dentinal tubules are 0.5-1.5  $\mu\text{m}$  in diameter, and they are more densely packed, closer to the pulp (Garberoglio and Brannstrom, 1976). Secondary tubules interconnect primary tubules (Powers and Sakaguchi, 2006), and tubules are filled with

odontoblastic processes called Tomes' fibers (Kennedy et al., 1951; Cate, 1998). Highly mineralized peritubular dentin lines the lumen of the tubules, and intertubular dentin with a high composition of collagen fibrils forms the majority of circumpulpal dentin (Goldberg et al., 1993).

Type I collagen in dentin is fibrillar, strong, elastic and highly organized (Marshall et al., 1997). Collagen molecules are triple helices with repeated trimers of Gly-X-Y, where X is usually proline and Y is usually hydroxyproline. Collagen molecules undergo cleavage and post-translational modifications to form fibrils. Monomers of 300nm arrange in a staggered form with repeated overlaps with neighboring molecules (Gelse et al., 2003).

Scanning electron microscopy (SEM) of dentin shows inorganic and organic components occupying intertubular dentin (Figure 1A). When dentin is demineralized, the delicate mesh-like network of collagen fibrils becomes apparent (Figure 1B). Collagen matrix has an open form when it is moist and a collapsed form when it is dehydrated. An intact collagen matrix can act as a scaffold for tissue remineralization and as a substrate for resin bonding (Bedran-Russo et al., 2014). Covalent cross-links between fibrils are the final post-translational modifications, and they are the basis for stability, tensile strength, viscoelasticity, biodegradability and thermal stability of collagen network (Silver et al., 2001; Knott and Bailey, 1998).

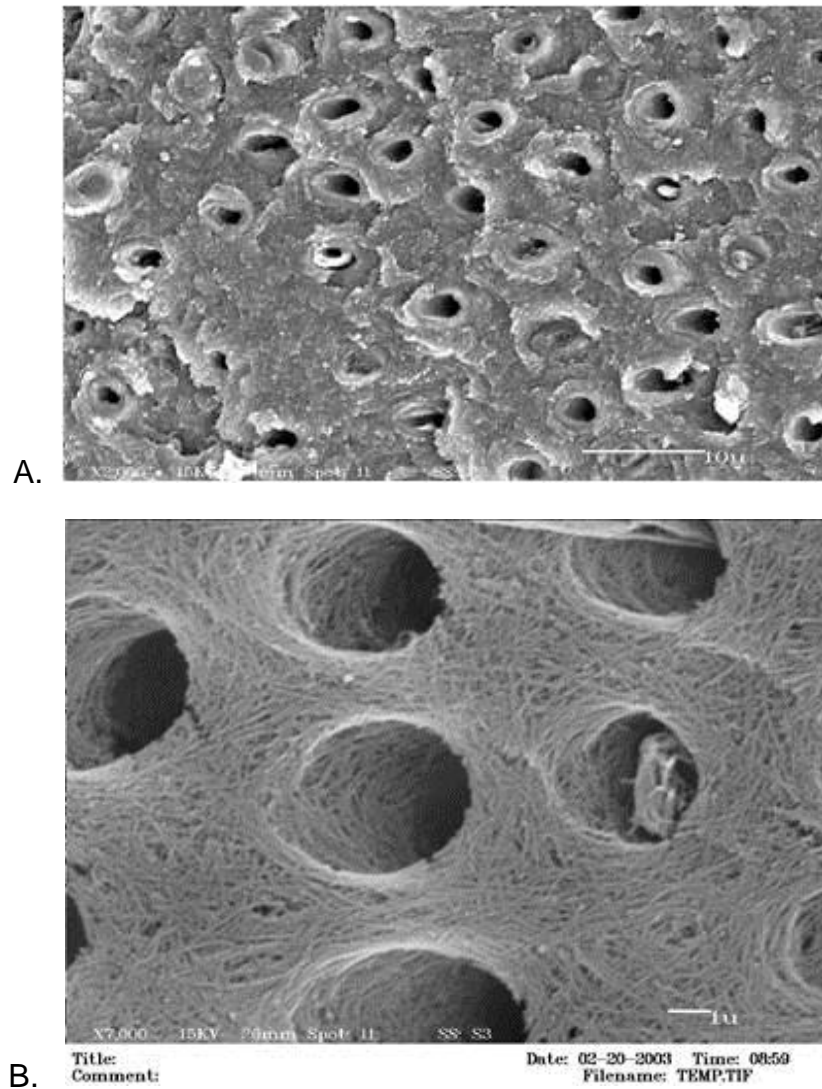


Figure 1. SEM images of dentin.<sup>a</sup>  
1A. Mineralized.  
1B. Demineralized.

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<sup>a</sup> Courtesy of Dr. Ana Bedran-Russo

## **2. Resin composite**

Development of modern resin composites came around 1960s. Resin composites provide an esthetic tooth-colored solution to missing tooth structures and allow more conservative tooth preparation with less dependence on the mechanical retention form (Powers and Sakaguchi, 2006).

A composite material is defined as having two or more materials combined. For direct restorations of teeth, a “particulate-reinforced polymer matrix composite” is used, with aromatic bis-GMA (2,2-bis[4-(2-hydroxy-3-methacryloyloxypropoxy)phenyl] propane) or urethane dimethacrylate frequently as the polymer part and inorganic silica fillers as the particulates. Other essential components include a coupling agent and an initiator-accelerator system. The coupling agent reacts with both the fillers and the polymers, and a light-cure system is generally used. (Powers and Sakaguchi, 2006)

Resin composite materials are categorized by the filler size and volume. In general, the greater the filler size and the volume, the greater the strength. The smaller the filler size, the greater polishability and improved esthetics. Filtek Supreme Plus Universal, the resin composite restorative material used in this study, has nanoclusters developed for both strength and esthetics (3M ESPE, 2005).

## **3. Tooth-resin interface**

Long-term success of resin composites depends on the mechanical properties as well as the quality of the bond between tooth and resin composite (Carrilho et al., 2005).

In enamel, acid-etching eliminates the smear layer and dissolves enamel crystals in prisms, leaving surface irregularities and porosities to which adhesive interlocks. Increased surface area by acid-etching in enamel is stable and makes interlocking efficient.

In dentin, acid-etching similarly dissolves the smear layer and hydroxyapatite crystals in the intertubular and peritubular dentin (van Meerbeek et al., 1992) and exposes collagen fibrils and non-collagenous proteins (Linde, 1989). Adhesive resin material flows in and forms a hybrid layer by interlocking with collagen mesh network (Nakabayashi et al. 1991; Figure 2). Such interlocking provides micromechanical retention, yet dentin bonding is more technique sensitive than enamel bonding because collagen structure is subjected to collapse upon dehydration (Marshall et al., 1997). The presence of collagen network is essential for bond strength (Yamazaki et al., 2008). Resin tags into the tubules were shown to contribute less to bond strength (Gwinnett, 1993).

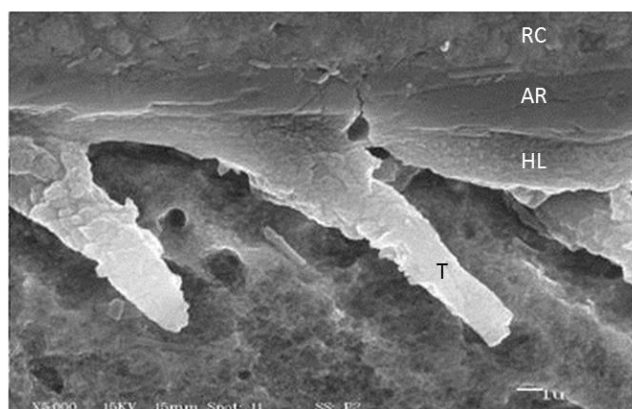


Figure 2. Hybrid layer at the dentin-resin interface.<sup>a,b</sup>

<sup>a</sup> AR: adhesive resin; HL: hybrid layer; D: dentin; T: resin tags.

<sup>b</sup> Courtesy of Dr. Ana Bedran-Russo

History of adhesives goes back to the 1960s and 70s with the first- and second-generation adhesives with no acid-etching. The third generation introduced acid-etching and a primer step, increasing bond strength to 12-15MPa from 2-6MPa. Primers were introduced as hydrophilic monomers that wet the surface, so that hydrophobic oligomers of adhesives could penetrate better. The fourth-generation system of the early 1990s introduced wet bonding and formation of a hybrid layer. Bond strength in low- to mid- 20 MPa was achieved, and the seal was improved. The fifth generation combined the primer and the adhesive in one bottle, while maintaining the bond strength. Self-etching primers were introduced in the sixth generation to reduce post-operative sensitivity. The seventh-generation combines etching, priming and bonding all in one bottle. Although self-etch systems are more convenient to use, they do not provide the same bond strength durability. (Nazarian, 2007)

Adper Single Bond Plus, bonding system used in this study, is a light-cured fifth-generation bonding agent. It requires a total-etching technique with 30-40% of phosphoric acid with a pH of 0.1-0.6, and the primer and the adhesive come in a single bottle. It is composed of BisGMA, HEMA, dimethacrylates, ethanol, water, a photo initiator, methacrylate moisture-resistant copolymer, and 10% by weight spherical silica nano-fillers that stay in suspension rather than sinking to the bottom. The shear bond strength for composite to enamel and dentin is around 30MPa. (Farah and Powers, 2008; 3M ESPE, 2004) Nanofillers have shown to stabilize the hybrid layer to a degree upon dehydration (Inoue et al., 2000).

#### 4. Secondary Caries

Caries is the most common infectious disease in humans (World Health Organization, 2003). It is a multi-factorial problem involving diet, nutrition, microbes, and host responses (Forssten et al., 2010). Process starts with plaque formation on tooth surface that acts as an adhesion site for bacteria (Loesche, 1986). Salivary molecules including glycoproteins, mucins, proline-rich proteins adhere to the tooth surface. Then, bacteria cell-to-surface interactions follow (Davey and O'toole, 2000), with primary colonizers, *Streptococcus sanguinis* and *Actinomyces viscosus* (Lamont et al., 1991). *Streptococcus mutans* is the secondary colonizer that adheres by cell-to-surface and cell-to-cell interactions, consumes sucrose, and produces acid (Davey and O'toole, 2000). When the acid level goes beyond saliva's buffering capacity, demineralization of tooth surface results, while *S. mutans* continues to grow in the presence of acid (Loesche, 1986). *Lactobacillus* is another culprit bacteria producing strong acid (Tanzer et al., 2001).

Sucrose is the only sugar that can be transformed into extracellular polysaccharides (EPS). The EPS structure creates a favorable microenvironment for further growth of bacteria with desirable pH, temperature and nitrogen source (Paes Leme et al., 2006). Sticky glucans also allow *S. mutans* particularly good at adhering to tooth surface (Trahan et al., 1985). Brain-heart infusion media has been shown most effective in the production of EPS. (Forssten et al., 2010)

Secondary caries is defined as a “positively diagnosed carious lesion, which occurs at the margins of an existing restoration” (Federation Dentaire Internationale, 1962). Lesions are thought to have an outer surface portion and an inner wall portion (Hals et al., 1974; 1971). “Secondary caries can occur in two independent locations: at the surface and along the wall (Thomas et al., 2007).” The outer lesion occurs in the same way as primary caries, while the wall lesion occurs along the wall and has been discussed in relation to microleakage. Bacteria and fluids enter the gap at the tooth-resin interface, and caries extends further than the outer lesion. Hydrogen ions diffuse into the gap, or bacteria themselves colonize in the gap, and the gap acts as a reservoir (Diercke et al., 2009).

However, there is controversy over development of a wall lesion, whether it starts at the tooth-resin interface or results as an extension of a nearby surface primary lesion. It is often difficult to study the inner and the outer lesions separately. Some believe that a wall lesion without an outer lesion is infrequent (Hals et al., 1974; Mjör and Toffenetti, 2000), while others argue that a wall lesion can form on its own. Diercke et al. (2009) attempted to isolate the wall lesion from the surface lesion, by applying acid-resistant varnish over the outer tooth surface. They created various sizes of gaps and showed that wall lesions still formed in the absence of surface lesions. Seemann et al. (2005) showed that saliva-contaminated tooth-resin interfaces were associated with greater depths of wall lesions. The type of adhesive material also influenced wall lesion development in gaps (Kuper et al. 2015).



The influence of the interface quality can also be discussed by comparing resin composite and amalgam restorations. The risk of secondary caries was found to be 3.5 times greater for composite restorations than amalgam restorations when followed up to seven years (Bernardo et al., 2007). Amalgam restorations were shown to survive longer than composite restorations (Mjör et al., 2000; Soncini et al., 2007). Although some say there is not enough evidence (Sarrett, 2005), greater incidence of secondary caries around resin composite restorations may partly be explained by the material's polymerization shrinkage and resulting gap formation. Despite controversy, a tight seal at the tooth-resin interface is desirable.

## **B. Review of Related Literature**

### **1. Use of bovine teeth**

Bovine teeth are used instead of human teeth in *in vitro* studies for many reasons. Human teeth are difficult to obtain in a large quantity and good quality (Mellberg, 1992). Unless extracted teeth are for orthodontic or periodontal treatments, they are often decayed. It is also difficult to control the source and age of the samples, and human teeth are small and curved (Zero, 1995).

Among many animals from which teeth can be obtained, bovine teeth are most widely used. They can be collected in a large quantity and good quality. They are more uniform in composition than human teeth. Teeth are also large with flat surfaces. (Yassen et al., 2011)

A review paper by Yassen et al. (2011) searched and included 68 papers and examined bovine teeth's similarity to human teeth in terms of morphology, chemical composition, physical properties, dental caries, dental erosion/abrasion, bonding/adhesive strength, and marginal microleakage. The average diameters of enamel crystallites were larger in bovine teeth (Arends and Jongebloed, 1978). The number of dentinal tubules was the same (Schilke et al., 2000). The calcium/phosphate ratio of the enamel surfaces, fluoride uptake, enamel matrix proteins were the same or similar (Feagin et al., 1969; Gwinnett et al., 1972; Fincham, 1980). No significant differences were found for the ultimate tensile strength, modulus of elasticity, or radiographic density (Schmalz et al., 2001; Tanaka et al., 2008). Bovine teeth of any

age had Knoop hardness for dentin similar to human teeth around 53 kgf/mm<sup>2</sup> (Fonseca et al., 2004). Older bovine teeth had Knoop hardness for enamel similar to human around 250 kgf/mm<sup>2</sup>, while younger bovine teeth had lower values for Knoop hardness (Fonseca et al., 2004). Authors further concluded that results were inconsistent for caries, bonding, and microleakage (Yassen et al., 2011). Extrapolation of results to humans may not be perfect, but bovine teeth are one of the closest alternatives to use before testing on humans.

## **2. In vitro bacterial caries model**

As caries is ultimately demineralization of hard tissue by acid, pH-induced caries models have been popular. However, caries is known as a multifactorial dynamic process, and incubating teeth in a highly acidic solution for an extended period of time is an over-simplification of the process (Holly and Gray, 1968). Studies have shown different results depending on whether a chemical model or a bacterial model was used (De Carvalho et al., 2009). Using bacteria to induce caries is clinically more relevant, and antibacterial chemicals can only be tested in such models.

The concept of an *in vitro* artificial mouth system arose to mimic the *in vivo* oral environment, creating plaque and carious lesions indistinguishable from those of the oral cavity (Tang et al., 2003). Studies have used different media such as saliva substitutes, tryptic soy broth, or brain heart infusion. Caries was successfully simulated in all. Some used automatic systems with continuous flow of media in and out (Kermanshahi et al., 2010). Others used simpler culture systems in cell plates

(Giacaman et al., 2012). Comparison of single- and multi- species models suggested that using either *S. mutans* or *S. sobrinus* was sufficient at inducing *in vitro* carious lesions similar to clinical observations (Steiner-Oliveira et al., 2007).

The tooth-resin interface has been studied using these models. Fontana et al. (1996) provided a protocol for studying secondary caries formation using *S. mutans* and *Lactobacillus casei* in TSB. With confocal laser scanning microscopy, they showed development of incipient surface lesions as well as wall lesions in as few as 7 days. They pulsed sucrose supplementation three times a day for 30 minutes each. For the rest of the time, specimens were in mineral buffer solution. The same group used 4-day exposure for early secondary lesions, applied fluoride varnish, incubated for another 4 days to show remineralization (Fontana et al., 2002). Similar protocols were used to study effects of interface gaps (Totiam et al., 2007) and to compare adhesive systems (Hayati et al., 2011).

### **3. Hard tissue biomodification**

As bonding to resin composite is more problematic in dentin than in enamel, hard tissue modification has focused on strengthening dentin. Knowing that collagen comprises most of the organic composition of dentin and that collagen structure is crucial in the formation of hybrid layer at the dentin-resin interface, biomodification of dentin has focused on stabilizing collagen structure by the use of cross-linking agents (Bedran-Russo et al., 2014). Endogenous cross-linking occurs through enzymatic activation and oxidation and glycation processes in forming Lys-Lys covalent bonds

(Knott and Bailey, 1998). Exogenous means include a physical methods such as UV radiation and chemical methods such as glutaraldehyde, carbodiimide, genipin, proanthocyanidins, or other polyphenols (Bedran-Russo et al., 2014). A synthetic carbodiimide and a proanthocyanidin-rich fraction from grape seed extract were used in this study.

Carbodiimide is a synthetic agent that is less toxic than glutaraldehyde. It is water-soluble and activates carboxylic groups of glutamic and aspartic acid residues in collagen molecules, which in turn react with amine groups of lysine or hydroxylysine residues in covalent bonds within and between fibrils. Addition of N-hydroxysuccinimide (NHS) increases the rate of cross-linking by preventing hydrolysis of activated carboxylic groups (Olde Damink et al. 1996). Proanthocyanidins (PACs) are naturally occurring agents that are condensed tannins, capable of forming hydrogen bonds with amide carbonyl groups in proteins (Hagerman and Klucher, 1986) with structural specificity (Hagerman and Butler, 1981). In addition, hydrophobic interactions decrease dielectric constant and further stabilize hydrogen bonds (Han et al., 2003).

Previous studies have shown that demineralized dentin treated with GSE had an increased ultimate tensile strength (Bedran-Russo et al., 2007) and increased modulus of elasticity (Bedran-Russo et al., 2008; 2013), which were stable over time (Castellan et al., 2011) and against collagenase challenge (Castellan et al., 2010; Vidal et al., 2014). At the dentin-resin interface, tensile bond strength increased and remained stable over time (Marcedo et al., 2009; Al-Amman et al., 2009; Castellan et al., 2010),

with the underlying dentin exhibiting greater elastic modulus and nanohardness (dos Santos et al., 2011). Root caries was also significantly inhibited and remineralized by PACs (Xie et al., 2008; Pavan et al., 2011; Walter et al., 2008). Similarly, EDC/NHS-treated dentin showed increased stiffness and stable ultimate tensile strength against collagenase challenge (Bedran-Russo et al. 2010). The tensile bond strength of the EDC/NHS-treated hybrid layer remained stable after aging in water (Bedran-Russo et al., 2010; Mazzoni et al., 2013).

Collagen cross-linking is the main mechanism of dentin biomodification. Cross-linking improves the stability and strength of collagen fibrils (Knott and Bailey, 1998), enhances mechanical properties (Macedo et al., 2009, Koide et al., 1997), and lowers enzymatic degradation (Chaussain-Miller et al., 2006). Intact collagen structure provides a framework for mineralization (Xie et al., 2008) and acts as a mechanical barrier to acid diffusion and mineral release (Pavan et al., 2011). Additional mechanisms have also been suggested. PACs decreased proteoglycan content in dentin, enhancing diffusivity (Torzilli et al., 1997; Bedran-Russo et al., 2011). Carbodiimides inhibited MMP activity (Mazzoni et al., 2014). Cross-linking agents have shown anti-bacterial activity against various pathogens (Corrales et al., 2009; Mayer et al., 2008). Most interestingly, proanthocyanidins decreased growth of *S. mutans* and inhibit formation of biofilm (Zhao et al., 2014). Carbodiimides are known to inhibit bacterial membrane ATPases (Abrams and Baron, 1970) and sugar uptake in oral streptococcal bacteria (Keevil et al., 1984).

### III. METHODS

#### A. Study design

Samples: Sixty bovine incisors (n=15)

Treatment groups: (1) Negative control buffer solution  
(2) An enriched fraction of grape seed extract with a high concentration of proanthocyanidin (GSE-UP)  
(3) 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide/  
N-hydroxysuccinimide (EDC/NHS)  
(4) Chlorhexidine digluconate (CHX)

Assessments: Microhardness test, and confocal laser scanning microscopy (CLSM) (Figure 3)

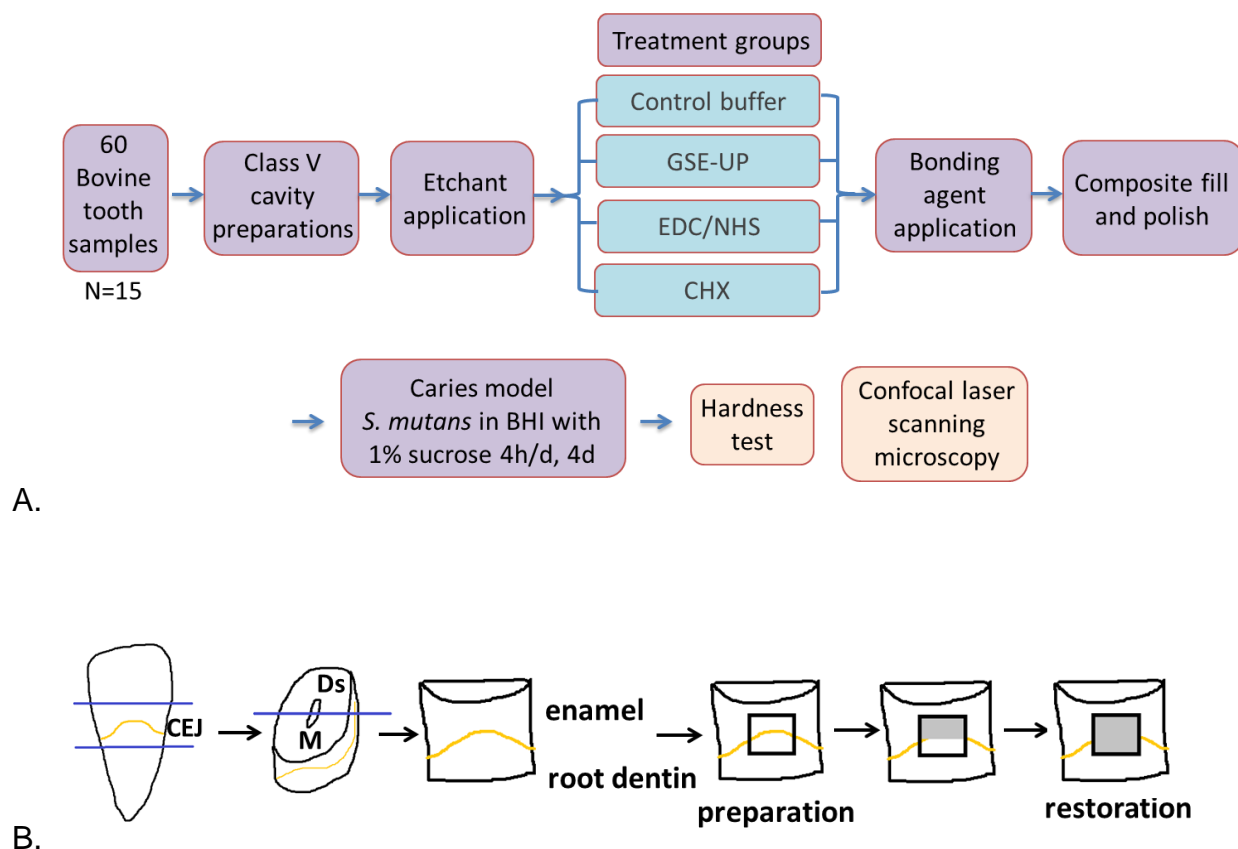


Figure 3. Study design.

3A. Four treatment groups and two outcome measures.

3B. Cutting, cavity preparation and restoration of bovine incisors.<sup>a</sup>

<sup>a</sup> CEJ: cemento-enamel junction; M: mesial; Ds: distal.



**B. Specimen preparation**

Bovine incisors were purchased from a slaughterhouse (Tri-State Beef Co., Cincinnati, OH) and delivered in 0.1% thymol/distilled water solution. Teeth were scaled with a scalpel to remove debris, calculus, extraneous soft tissue as well as cementum on the root surfaces. Teeth were visually inspected, and teeth with caries or white spots were excluded. Teeth were mounted on a base using sticky wax (Kerr, Orange, CA) and cut 4mm above and 4mm below the cemento-enamel junction (CEJ) at the mid-mesial and mid-distal surfaces using a diamond wafering blade in cooling water (Buehler-Series 15LC Diamond, Buehler, Lake Bluff, IL) (Figure 3B). Teeth were cut in half to obtain a mesial section and a distal section and further trimmed using a cylindrical diamond bur (557D, Brasseler, Savannah, GA) to a final rectangular dimension of 8 mm width x 8 mm length x 1.5-2 mm thickness. Any remaining pulp tissue was removed.

**C. Cavity preparation**

In the center of the specimens, class V preparations of 3mm width x 3mm length x 1 mm depth were made using a flat-end carbide bur (558, Brasseler, Savannah, GA) on a high-speed handpiece (KaVo Dental, Charlotte, NC) with air/water coolant (Figure 3B). Preparation walls were made at 90 degrees to the tooth surface, and burs were changed every five preparations. Dimensions were checked with a periodontal probe (Hu-Friedy, Chicago, IL). The top half of the restoration was in enamel and the bottom half of the restoration was in root dentin.

## D. Experimental groups

### D1. Preparation of treatment solutions

Four treatment solutions were prepared as follows:

- Group 1 (Buffer control): 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid powder (HEPES, Sigma-Aldrich, St. Louis, MO) was dissolved in distilled water to obtain a 20mM buffer solution as the negative control.
- Group 2 (GSE-UP): An enriched fraction of grape seed extract with a high concentration of bioactive proanthocyanidins was obtained by two phase solvent system composed of methyl acetate/water as described by Phansalkar, et al. (2014). Powder was measured in a 2mL Eppendorf tube (Celltreat, China) using an analytical balance (XP504, Mettler Toledo, Columbus, OH), dissolved in 20mM HEPES buffer, and adjusted to pH of 7.2-7.4 using a pH-/Cond-/DO-/ISE-Meter (Mettler Toledo, Columbus, OH), for a final concentration of 30% weight/volume.
- Group 3 (EDC/NHS): 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (Thermo Scientific Pierce, RockFord, IL) and N-hydroxysuccinimide (Thermo Scientific Pierce, RockFord, IL) powers were measured in separate 2mL Eppendorf tubes using an analytical balance, dissolved in 20mM HEPES buffer, combined, and adjusted to pH of 7.2-7.4, for final concentrations of 0.3M EDC/0.12M NHS.

- Group 4 (CHX): 20% stock chlorhexidine digluconate solution (Alfa Aesar, Ward Hill, MA) was diluted to 2% using 20mM HEPES buffer.

## **D2. Restorative protocol**

Cavity preparations were randomly assigned to four groups (n=15):

- Group 1 (Buffer control): Cavity walls were etched with 32% phosphoric acid etchant (Scotchbond, 3M ESPE, St Paul, MN) for 15 sec and rinsed with distilled water for 30 seconds. Preparations were blotted dry with an absorbent tissue (KimWipe, Kimberly-Clark Corporation, Irving, TX) and filled with HEPES buffer for 1-minute application. Specimens were rinsed with distilled water for 30 seconds.
- Group 2 (GSE-UP): Cavity walls were etched with 32% phosphoric acid etchant for 15 sec and rinsed with distilled water for 30 seconds. Preparations were blotted dry with an absorbent tissue and filled with 30% GSE-UP for 1-minute application. Specimens were rinsed with distilled water for 30 seconds (modified from Castellan et al., 2010).
- Group 3 (EDC/NHS): Cavity walls were etched with 32% phosphoric acid etchant for 15 sec and rinsed with distilled water for 30 seconds. Preparations were blotted dry with an absorbent tissue and filled with 0.3M EDC/0.12M NHS for 1-minute application (Mazzoni et al., 2013).

- Group 4 (CHX): Cavity walls were etched with 32% phosphoric acid etchant for 15 sec and rinsed with distilled water for 30 seconds. Preparations were blotted dry with an absorbent tissue and filled with 2% CHX for 30-second application (Sartori et al., 2013).

Preparations were blotted dry with an absorbent tissue, and a drop of Adper Single Bond Plus (3M ESPE, St Paul, MN) was applied on preparation surfaces for 20 seconds with a microbrush (Microbrush, Grafton, WI). Excess material was gently dried with an air syringe, and the adhesive was cured for 20 seconds using an Optilux 501 light unit at  $830 \text{ mW/cm}^2$  (Kerr, Orange, CA). Preparations were filled with Filtek Supreme Plus Universal composite material (3M ESPE, St Paul, MN) in two increments and light cured for 40 seconds each. The first increment was for the top half of the cavity on the enamel side and the second increment was for the bottom half of the cavity on the root dentin side (Figure 3b). Excess material was carefully removed before curing. Immediately after final curing, the restorations were polished with coarse-, medium-, and fine-grit aluminum-oxide abrasive discs (Sof-Lex, 3M/ESPE, St Paul, MN) on a slow speed handpiece (KaVo Dental, Charlotte, NC).

#### **E. In vitro secondary caries bacterial model**

Cosmetic nail varnish (Revlon, New York, NY) was applied 1mm away from the restorations and air-dried for 40 minutes. Specimens were disinfected in 70% ethanol (Decon Lab, King of Prussia, PA) and agitated for 20 minutes (Hayati et al., 2011) and

rinsed with sterile phosphate buffered saline (PBS, Chicago, IL) twice before storage in sterile PBS at 4°C overnight.

*Streptococcus mutans* UA159 was aerobically cultured on Brain Heart Infusion (BHI, Difco Laboratories, Detroit, MI) agar, and a colony was inoculated into BHI broth and incubated for 18-20hrs 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 550nm, Spectronic 601, Milton Roy, Ivyland, PA).

Specimens were placed into 6-cell plates (Corning Life Sciences, Tewksbury, MA), with each well containing five specimens of the same treatment group. Specimens were inoculated with *S. mutans* suspension in BHIS for 4 hours at 37°C, and then the media was changed to BHI without sucrose for the next 20 hours. Every time media was changed, wells were gently rinsed with PBS buffer twice. Specimens were subjected to 4 hours in BHIS and 20 hours in BHI for three more days, for a total of four days in the bacterial caries model (modified protocol from Fontana et al., 1996). At the end of four-day challenge, specimens were taken out of the wells and rinsed in running water thoroughly.

A pilot study was performed to optimize the conditions of bacterial challenge. Initially 5 and 7 days were tested with 1% sucrose for 24 hours/day. As the caries

lesions were considered too large for the purpose of the study, the total incubation time was adjusted to 4 days with sucrose supplement for 4 hours/day.

## **F. Secondary caries evaluation**

### **F1. Specimen embedding**

Specimens were sectioned in two halves, along the axis of the tooth, through the restorations. One section was designated for microhardness test and the other for confocal laser scanning microscopy (CLSM). Sections were aligned and embedded in epoxy resin (Buehler, Lake Bluff, IL) overnight. Epoxy blocks were ground flat with no. 320, 400, 600, 800, and 1200 grit silicon carbide abrasive papers (Buehler, Lake Bluff, IL) under running water.

### **F2. Microhardness test**

After hydration with distilled water for 1 hour, specimens were tested for Knoop microhardness using a microhardness tester (LECO Series 700, St. Joseph, MI, USA), loaded to 100 grams for 5 seconds for enamel and 25 grams for 5 seconds for root dentin (modified from Cury et al., 2000; Diamanti et al., 2011; Vale et al., 2011). Twelve indentations were made in enamel and dentin (Figure 4).

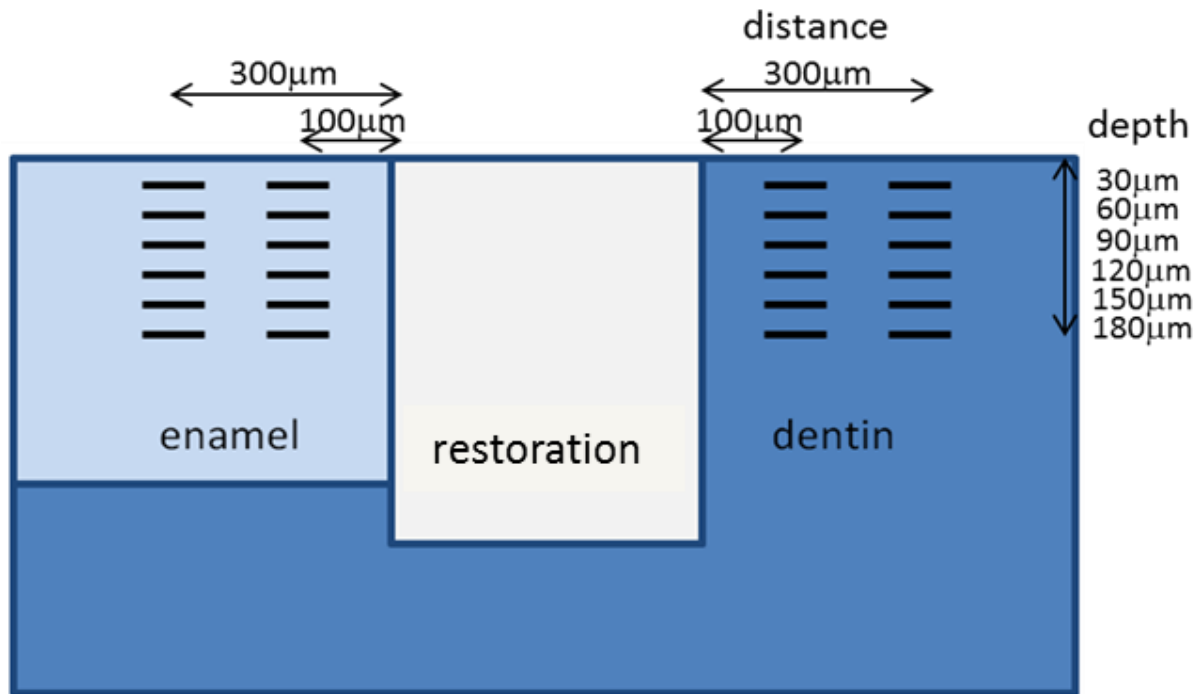


Figure 4. Twelve test positions for microhardness in dentin and enamel.

Two different distances were tested, 100 $\mu$ m and 300 $\mu$ m away from the restoration margin. Six different depths were tested, 30, 60, 90, 120, 150, 180 $\mu$ m from the tooth surface. Surface and indentations were imaged using ConfiDent software (LECO, St. Joseph, MI, USA). Knoop hardness was automatically calculated by the tester as load in kgf divided by impression area mm<sup>2</sup>.

### **F3. Confocal laser scanning microscopy**

Specimens were hydrated with distilled water for one hour and stained overnight with 0.1mM rhodamine B, following the protocol described by Fontana et al. (2002). Solution was prepared by dissolving rhodamine B power (Sigma-Aldrich, St. Louis, MO) in 1x PBS buffer (Thermo Scientific, Rockford, IL) and pH was adjusted to 7.2-7.4. After staining, specimens were rinsed in running water for 1 minute and blotted-dry with absorbent paper. Specimens were examined under a confocal laser scanning microscope (Leica DMI 6000 B, Buffalo grove, IL) with a connected digital camera (Hamamatsu, Skokie, IL) and LAS AF software (Leica, Buffalo grove, IL). Images of light differential interference contrast (DIC) microscopy as well as red fluorescence at 529nm were captured. The same microscope settings were used for all images.

Images were analyzed using ImageJ software (NIH, Bethesda, MD). 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 rhodamine stained from the surface (Figure 5). Secondary caries was also measured as total fluorescence (TF) within a certain distance from the restoration



(Figure 6, Fontana et al., 2002). 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.

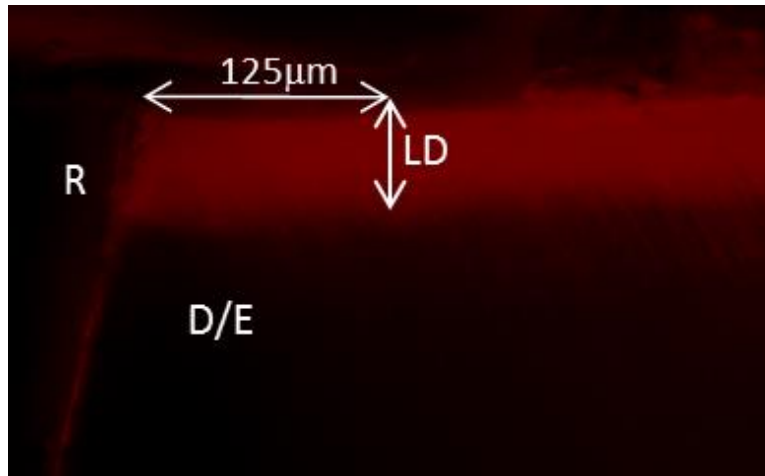


Figure 5. Measurement of lesion depth (LD).<sup>a</sup>

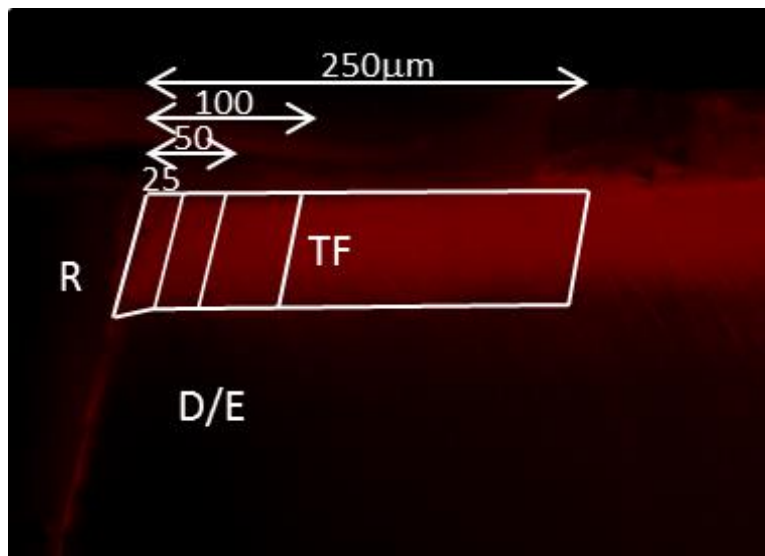


Figure 6. Measurement of total fluorescence (TF) to represent secondary caries in a given area.<sup>b</sup>

<sup>a</sup> R: restoration; D/E: dentin or enamel.

<sup>b</sup> R: restoration; D/E: dentin or enamel; TF: total fluorescence.

**F4. Statistical analysis**

Microhardness data for dentin and enamel were organized by treatment, depth, and distance and analyzed by three-way, two-way and one-way ANOVA for statistical significance at  $p < 0.05$ , followed by post-hoc Bonferroni test, using SPSS Statistics 22 software (Chicago, IL). Microhardness values for different treatment groups were also compared at each of the twelve indentation positions described in Figure 4 separately, by one-way ANOVA and post-hoc Bonferroni test at  $p < 0.05$ .

CLSM data were analyzed for the effect of treatment on lesion depth (LD) and total fluorescence (TF) by one-way ANOVA and post-hoc Bonferroni test at  $p < 0.05$ .

## IV. RESULTS

### A. Microhardness results

#### A1. Microhardness results for dentin

In all treatment groups, demineralization was most severe at the surface as represented by the lowest hardness values at 30 $\mu$ m depth from the surface (Figure 7). Demineralization gradually subsided and plateaued as the depth increased from the surface.

A three-way ANOVA did not show statistically significant interaction among the three factors: treatment, depth and distance ( $p>0.05$ ). A two-way ANOVA showed a statistically significant interaction only between depth and distance ( $p<0.05$ ). Student t-test showed that the mean microhardness at distance 100 $\mu$ m was significantly lower than the mean microhardness at distance 300 $\mu$ m from the restoration ( $p<0.05$ ), indicating that dentin demineralization was more severe closer to the restoration.

For depth, one-way ANOVA and post-hoc Bonferroni showed statistically significant mean differences between all pairs of the levels ( $p<0.05$ ), except between depths 150 $\mu$ m and 180 $\mu$ m ( $p>0.05$ ).

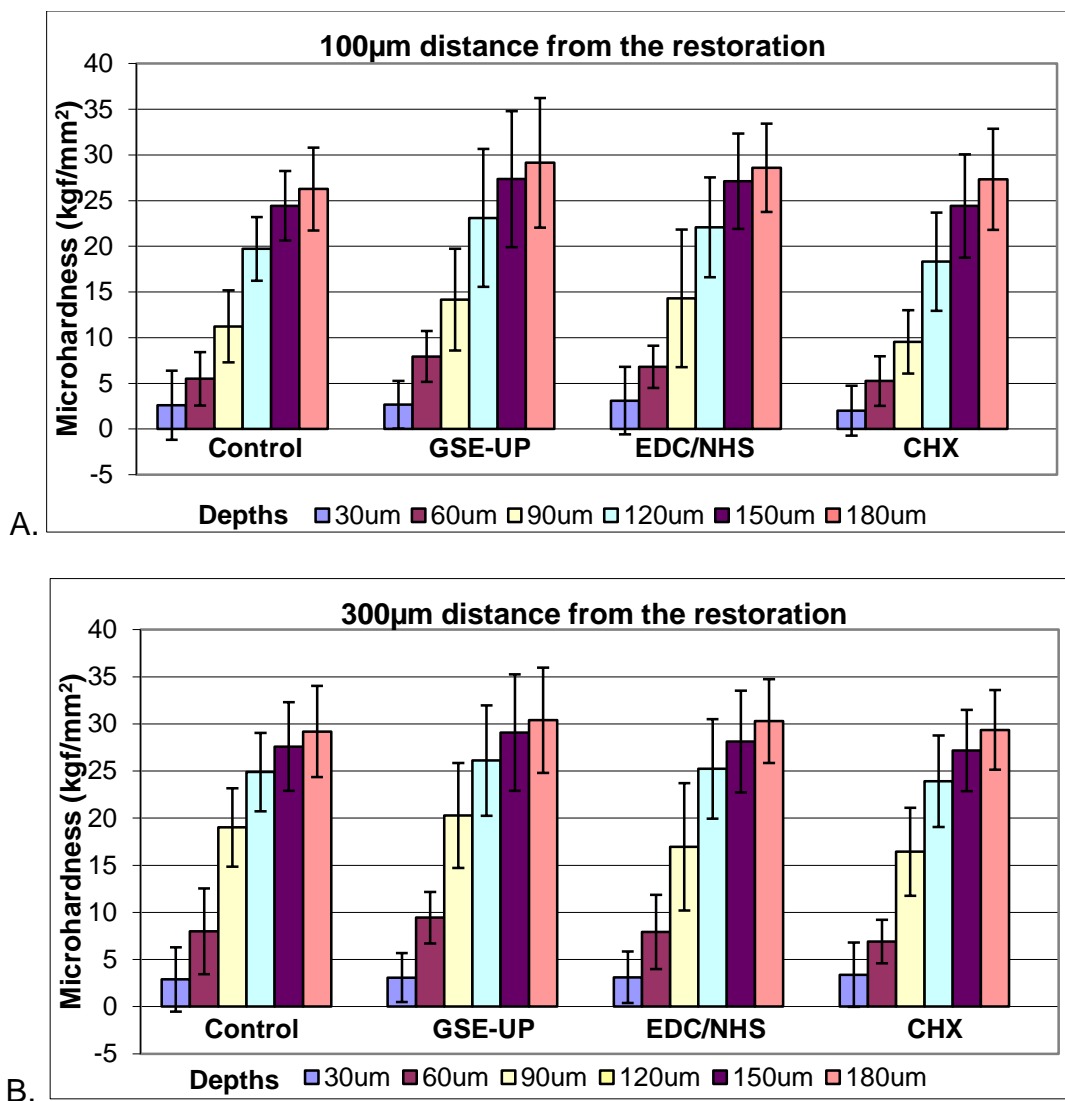


Figure 7. Microhardness of dentin at distance (A) 100 $\mu$ m and (B) 300 $\mu$ m from the restoration according to treatments.<sup>a,b</sup>

<sup>a</sup> Test positions are described in Figure 4.

<sup>b</sup> Error bars indicate SD.

For treatment, one-way ANOVA did not show statistically significant mean differences among the treatment groups ( $p>0.05$ ). Also, when each of the twelve test positions (Figure 4) was considered separately, there were no statistically significant differences among the treatment groups at any of the positions.

## **A2. Microhardness results for enamel**

Demineralization was induced in enamel as well (Figure 8). A three-way ANOVA did not show statistically significant interaction among the three factors: treatment, depth and distance ( $p>0.05$ ). A two-way ANOVA did not show a statistically significant interaction between any pairs of the factors ( $p>0.05$ ). Student t-test, similarly, did not show statistically significant mean difference between distance 100 $\mu$ m and distance 300 $\mu$ m from the restoration ( $p>0.05$ ), indicating that enamel demineralization was not more severe closer to the restoration.

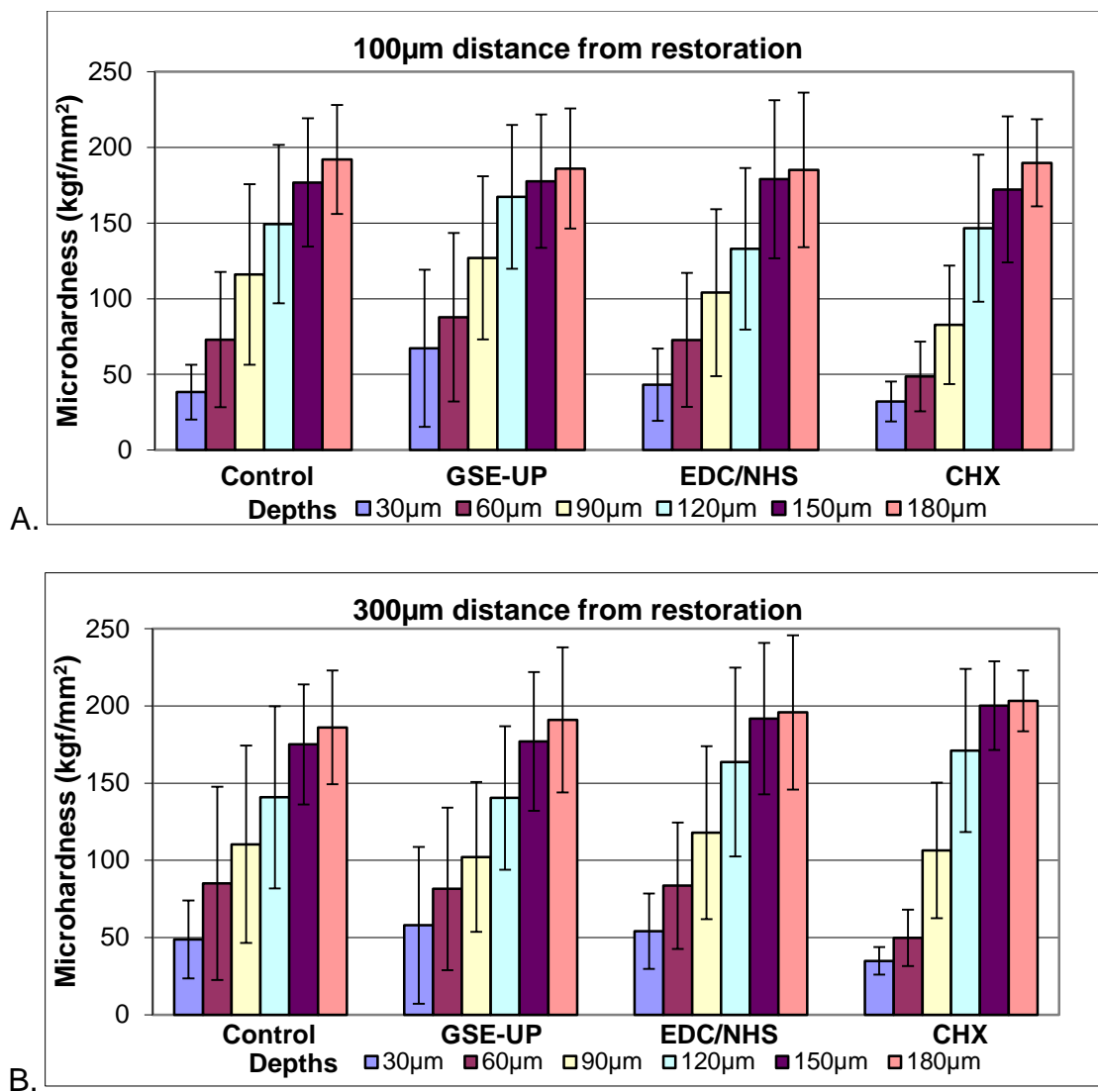


Figure 8. Microhardness of enamel at (A) 100 $\mu$ m and (B) 300 $\mu$ m distance from the restoration according to treatments.<sup>a,b</sup>

<sup>a</sup> Test positions are described in Figure 4.

<sup>b</sup> Error bars indicate SD.

For depth, one-way ANOVA and post-hoc Bonferroni tests showed statistically significant mean differences between all pairs of the depth levels ( $p < 0.05$ ), except for the pair between 150 $\mu\text{m}$  and 180 $\mu\text{m}$  ( $p > 0.05$ ).

For treatment, one-way ANOVA did not show statistically significant mean differences among the treatment groups ( $p > 0.05$ ). Even when each of the twelve test positions (Figure 4) was considered separately, there were no statistically significant differences among the treatment groups at any of the positions.

## **B. Confocal laser scanning microscopy**

### **B1. CLSM results for dentin**

Red fluorescence from rhodamine staining depicted tissue demineralization. Lesion depths (LDs) were similar across the treatment groups as shown in Figure 9, and one-way ANOVA confirmed that there were no statistically significant mean differences in LD (Table 1,  $p > 0.05$ ). Most interestingly, an inhibition zone (IZ) was noted in the GSE-UP-treated group, where rhodamine staining was absent next to the tooth-resin interface (Figure 9). IZ was not observed in the control or any other treatment groups.



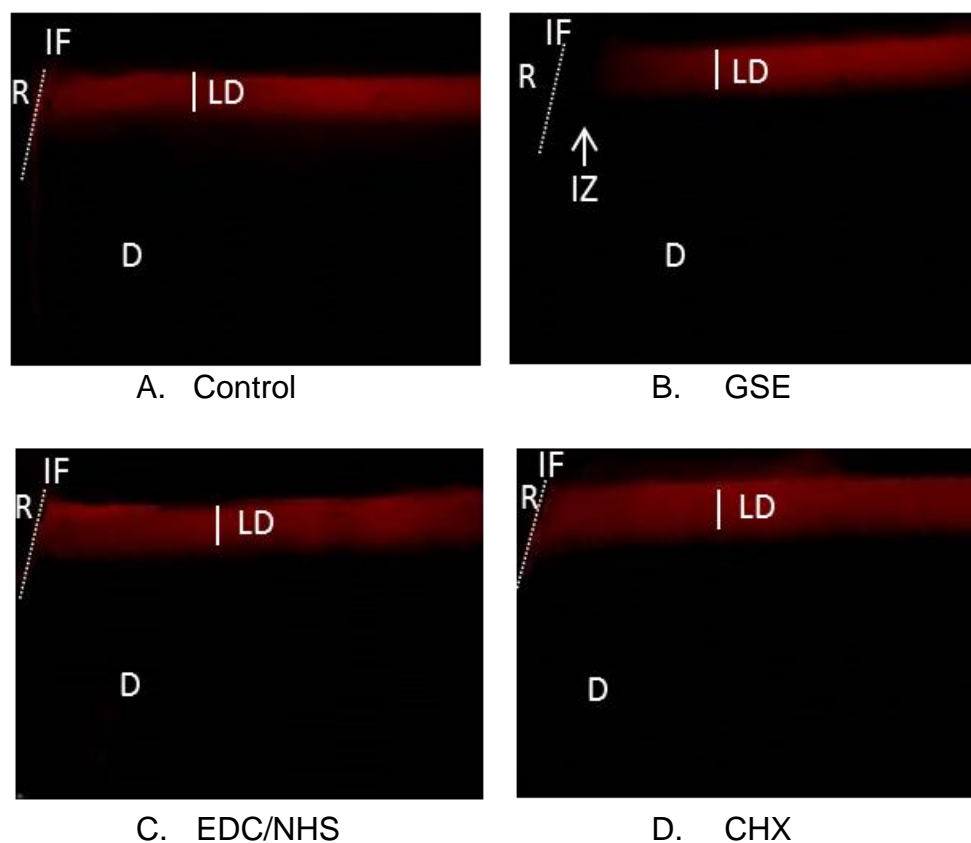


Figure 9. Dentin demineralization represented by red fluorescence in CLSM images.<sup>a</sup>

<sup>a</sup> R: restoration; D: dentin; LD: lesion depth; IF: interface; IZ: inhibition zone.

**TABLE I**  
**LESION DEPTHS IN DENTIN<sup>a</sup>**

<b>Treatment</b>	<b>Lesion depth (μm)</b>	<b>n</b>
<b>Control</b>	72.5 ± 9.8	15
<b>GSE-UP</b>	74.8 ± 15.0	15
<b>EDC/NHS</b>	71.2 ± 16.4	15
<b>CHX</b>	74.4 ± 13.1	15

<sup>a</sup> Mean ± SD; p>0.05.

To quantify demineralization, total fluorescence in a given area was measured as described in Figure 6. When examined up to 250μm adjacent to the restoration, there was no statistically significant difference in total fluorescence among all treatment groups (Table 2, second column). When the area was limited to 100μm or 50μm adjacent to the restoration, the GSE-UP group showed statistically significant difference from the CHX group (Table 2, third and fourth columns). When the area was limited to 25μm adjacent to the restoration, total fluorescence for the GSE-UP group was significantly lower than that of all the other groups, including the control (Table 2, fifth column). The GSE-UP group had the least amount of demineralization, especially near the restoration margin, which was consistent with the presence of an inhibition zone in Figure 9. GSE-UP showed a protective effect immediately adjacent to the dentin-resin interface.

TABLE II

DENTIN DEMINERALIZATION REPRESENTED BY TOTAL FLUORESCENCE<sup>a,b,c</sup>

Treatment	Total fluorescence in area			
	250µm along (x10 <sup>5</sup> )	100µm along (x10 <sup>5</sup> )	50µm along (x10 <sup>5</sup> )	25µm along (x10 <sup>5</sup> )
<b>Control</b>	8.06 ± 5.51 (A)	3.37 ± 1.98 (AB)	1.82 ± 1.04 (AB)	1.04 ± 0.57 (B)
<b>GSE-UP</b>	8.23 ± 5.57 (A)	2.29 ± 1.63 (A)	0.79 ± 0.70 (A)	0.27 ± 0.30 (A)
<b>EDC/NHS</b>	10.75 ± 6.78 (A)	4.45 ± 3.14 (AB)	2.29 ± 2.05 (AB)	1.24 ± 1.14 (B)
<b>CHX</b>	12.29 ± 6.27 (A)	4.86 ± 2.85 (B)	2.69 ± 1.81 (B)	1.37 ± 0.83 (B)

<sup>a</sup> Refer to Figure 6 for measurement method.<sup>b</sup> Different letters indicate statistically significant differences with  $p < 0.05$  in each column.<sup>c</sup> Mean ± SD.**B2. CLSM results for enamel**

Lesion depths (LDs) in enamel were similar across the treatment groups as shown in Figure 10, and one-way ANOVA confirmed that there was no statistically significant mean differences in LD ( $p > 0.05$ , Table 3). Interestingly, an inhibition zone (IZ) was not observed in enamel for any of the treatment groups.

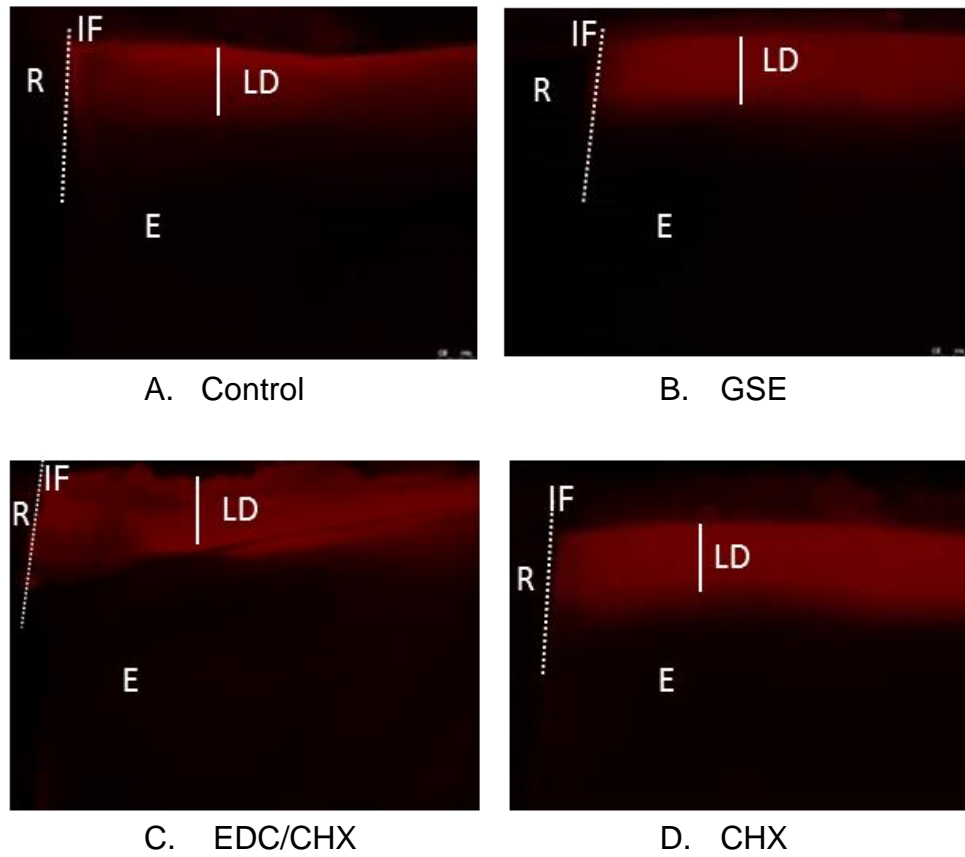


Figure 10. Enamel demineralization represented by red fluorescence in CLSM images.<sup>a</sup>

<sup>a</sup> R: restoration; E: enamel; LD: lesion depth; IF: interface.

**TABLE III**  
**LESION DEPTHS IN ENAMEL<sup>a</sup>**

<b>Treatment</b>	<b>Lesion depth (μm)</b>	<b>n</b>
<b>Control</b>	80.5 ± 29.7	15
<b>GSE-UP</b>	67.8 ± 18.7	15
<b>EDC/NHS</b>	71.7 ± 22.6	15
<b>CHX</b>	83.4 ± 49.3	15

<sup>a</sup> Mean ± SD; p>0.05.

Regardless of the areas examined, 250μm or 25μm adjacent to the restoration, there were no statistically significant mean differences in total fluorescence among all the treatment groups (Table 4). Enamel demineralization did not differ across the treatment groups.

**TABLE IV**  
**ENAMEL DEMINERALIZATION REPRESENTED BY TOTAL FLUORESCENCE<sup>a,b,c</sup>**

<b>Treatment</b>	<b>Total fluorescence in area</b>	
	<b>250μm along (x10<sup>5</sup>)</b>	<b>25μm along (x10<sup>4</sup>)</b>
<b>Control</b>	6.20 ± 6.93	4.16 ± 3.71
<b>GSE-UP</b>	4.06 ± 3.50	2.20 ± 1.37
<b>EDC/NHS</b>	2.42 ± 3.90	2.40 ± 2.79
<b>CHX</b>	5.55 ± 6.43	3.21 ± 3.12

<sup>a</sup> Refer to Figure 6 for measurement method.

<sup>b</sup> In each column, no significant differences were found (p>0.05).

<sup>c</sup> Mean ± SD.

## V. DISCUSSION

### A. Inhibition of secondary caries

Secondary caries developed in all experimental groups, as represented by a decrease in microhardness and an increase in rhodamine staining in dentin and enamel (Figure 7, 8, 9, 10). All groups exhibited similar patterns of demineralization with gradual changes in microhardness (Figures 7, 8), and CLSM images revealed similar lesion depths across the treatment groups ( $p>0.05$ , Tables 1, 3). Caries formation and progression away from the tooth-resin interface were consistent in all groups, both in dentin and enamel (Figure 9, 10).

Cariious lesions in dentin were found more aggressive closer to the restoration (distance 100 $\mu$ m vs. 300 $\mu$ m in Figure 4, Student t-test,  $p<0.05$ ). As oral streptococci are known to favor adhesion to resin composite for its roughness and hydrophobicity (Hahnel et al., 2010; Lee et al., 2011), resin composite restorations may be considered cariogenic. In addition, 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 (Diercke et al., 2009; Seemann et al., 2005). In enamel, microhardness values did not differ at distances 100 $\mu$ m and 300 $\mu$ m from the restoration (Student t-test,  $p>0.05$ ). Bonding to resin is less effective in dentin than in enamel (Powers and Sakaguchi, 2006), and as a result, the dentin-resin interface was more susceptible to caries progression around the restoration margin than the enamel-resin interface.

One of the most interesting results of this study was that proanthocyanidins (PACs)-rich fraction of grape seed extract (GSE-UP) inhibited secondary caries development immediately adjacent to the dentin-resin interface. This finding was best represented by the presence of an inhibition zone in CLSM images (Figure 9B) and the lowest total fluorescence measurement, compared to all other groups, within 25µm of the restoration ( $p < 0.05$ , Table 2). Differences in microhardness data were subtle, as the scale of the indentations (~100µm) was approximately four times larger than the area of interest (~25µm). GSE-UP was consistently on the higher end of the hardness spectrum, but statistically significant differences were not found compared to the control.

Three possible mechanisms of actions were considered for GSE-UP against secondary caries in dentin: tissue stabilization, a tighter interfacial seal, and antimicrobial activity. As dentin is relatively porous, PACs were able to diffuse further than a few microns 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 (Xie et al., 2008; Pavan et al., 2011). Fluorescence patterns in Figure 9B suggest that surface caries lesions progressed to the peripheries until limited by PACs-enhanced dentin near the interface.

A tighter seal is achievable at the dentin-resin interface, when collagen mesh structure is intact for the formation of a hybrid layer (Nakabayashi et al., 1991). GSE-UP induced collagen cross-linking and maintained the collagen mesh structure before application of the bonding agent. A tighter interfacial seal is hypothetically more

resistant to bacterial leakage and acid diffusion. Previous studies have reported a positive correlation between the interface gap size and secondary caries (Totium et al., 2007; Nassar and Gonzalez-Cabezas, 2011; Cenci et al., 2009). Some suggest that only very large gaps of 250-400µm make a difference in the development of secondary caries and do not consider an open margin as an indication for replacement of a restoration (Mjör et al., 2000; Özer, 1997; Kidd et al., 1995). However, gaps of 50µm and less were previously colonized by *S. mutans* biofilm, and caries formed specifically at the interface (Seemann et al., 2005; Diercke et al., 2009). A future study may assess the effect of GSE-UP on marginal microleakage without a caries challenge.

Lastly, PACs are known to be antimicrobial. Previous studies have shown that PACs inhibit surface-adsorbed glucosyltransferases and acid production by *S. mutans* (Duarte et al. 2006) and decrease the growth of *S. mutans* and formation of a biofilm (Zhao et al., 2014). Because of PACs' high affinity for proteins (Han et al., 2003) and dentin's high organic content, in addition to porosity in dentin, PACs molecules were able to diffuse and remain bound to dentin. PACs' antimicrobial activity against cariogenic bacteria contributed to inhibition of dentin demineralization by GSE-UP. In this study, only a single concentration of GSE-UP was used. A future study may assess concentration-dependent effects of GSE-UP on secondary caries.

All other agents had no effect on inhibiting secondary caries formation around resin composite restorations. While EDC had collagen cross-linking activity (Olde Damink et al., 1996), it did not have the same effect as GSE-UP (Figure 9, Table 2).



EDC's cross-linking ability is known to be less potent than PACs, as previous studies reported on an increase in the immediate bond strength by GSE (Castellan et al., 2013) but not by EDC (Mazzoni et al., 2013; Bedran-Russo et al., 2010), and a nanoleakage study using  $\text{AgNO}_3$  solution showed no protective effects by EDC compared to the control (Mazzoni et al., 2013). Cross-links induced by PACs may be more favorable in keeping the collagen mesh structure than EDC. Furthermore, carbodiimides are known to inhibit bacterial membrane ATPases (Abrams and Baron, 1970) and sugar uptake in oral streptococcal bacteria (Keevil et al., 1984), but a recent study reported that EDC inhibited growth of *L. acidophilus* but not *S. mutans* at concentrations up to 2M (Estrêla, 2013). In addition, EDC does not take part in newly induced cross-links and it is subjected to hydrolysis over time. Since EDC may not remain at the interface, its antimicrobial effect may be diminished.

CHX was not found to inhibit secondary caries in any of the outcomes (Figure 7-10, Tables 1-4). A possible explanation is that CHX did not have a mechanism to stay bound to the tooth structure, and the residual amount of CHX at the interface for bactericidal activity was likely low. CHX is only bacteriostatic at low concentrations (Russel, 1986). Furthermore, a number of studies have suggested weakening of the tooth-resin bond by chlorhexidine (Elkassas et al., 2014; Ercan et al., 2009). In future studies, fluoride or glass ionomer may serve as a positive control instead.

In enamel, none of the treatments had significant effects on secondary caries (Figure 10, Table 4). Possible explanations are that because enamel is less porous than

dentin, diffusion of agents was limited, and because enamel is 97% mineral, the protein content was limited for binding of PACs.

The hybrid layer is subjected to degradation and fatigue over time (Hashimoto et al., 2001; Okuda et al., 2002; Yamazaki et al., 2008; van Strijp et al., 2003). Aging of specimens was not simulated in this study but would be relevant in future studies. PACs' inhibitory effects may or may not be stable over time. Greater differences may be found among the treatment groups after aging of specimens. Aging is most commonly performed by thermocycling, cyclic loading, and water storage (de Mattos Pimenta Vidal et al., 2013; Erhardt et al., 2008), while Fontana et al. 2002 skipped etching and bonding steps intentionally. Recently, marginal microleakage was also induced by the use of resin-degrading esterases (Kermanshahi et al., 2010).

## **B. Strengths and Limitations**

This *in vitro* study was designed to test three different agents for their ability to protect hard tissue against secondary caries. GSE-UP and EDC were known collagen cross-linkers with additional antimicrobial activities. CHX was an antibacterial agent without cross-linking potential. A buffer solution was included as a negative control. The sample size and standard deviations were within the range of published studies (Diamanti et al., 2011; Fontana et al., 2002), and the adhesive system and the composite material were current technology. A bacterial model was utilized for artificial caries development, which was clinically more relevant than using a pH-induced model. A moderate size of lesions was achieved in four days. Microhardness test and CLSM

were validated means of studying hard tissue demineralization (Diamanti et al., 2011; Fontana et al., 2002).

There were limitations still. Specimens could not be autoclaved as the tooth-resin interface would be damaged. Ultraviolet or gamma radiations were rejected as they would lead to cross-linking of proteins. Ethylene oxide appeared least disturbing (Itota et al., 2002), but the procedure involved drying of the specimens for multiple hours, damaging the collagen structure. In this study, 70% ethanol was used, as in other studies (Hayati et al., 2011). It is not a sterilizing agent. However, when tested for bacterial contamination by culturing the buffer, no growth was observed.

As with any *in vitro* study, cautions remain when extrapolating results to the actual *in vivo* situation. The *in vitro* study environment can never mimic entirely what is happening in the oral cavity. For example, the bacterial caries model in this study involved only a single species of cariogenic bacteria (*S. mutans*). In addition, saliva was not included in the model. Significant differences found in an *in vitro* study may not translate to the clinical settings. However, it represents a novel approach and the results of the current study are promising.

### **C. Implications for clinical practice**

Oral health plays an important part of the overall health and quality of life (Gift and Redford, 1992). Preventing caries and reducing the need for replacement of restorations are important aspects of maintaining oral health. Results of the current

study suggest that incorporation of bioactive ingredient in the existing adhesive systems may inhibit secondary caries near the tooth-resin interface. Daily home care is still necessary to remove surface biofilm. The brown color of GSE-UP, reflected by higher molecular weight PACs and their oxidation products (Vidal et al., 2014), may cause staining and needs to be managed before its clinical use.

#### **D. Conclusion**

Secondary caries developed in enamel and dentin, regardless of treatment. The lesion depths were the same across the treatment groups in enamel and dentin, as severity changed gradually with respect to depth from the surface. Lesions were more aggressive closer to the dentin-resin interface, but not closer to the enamel-resin interface. Most interestingly, GSE-UP significantly inhibited secondary caries development within 25µm of the restoration margin in dentin. All other treatments had no inhibitory effects on secondary caries development around resin composite restorations. At the enamel-resin interface, none of the treatments had an effect on secondary caries development. Results suggest that incorporation of biomodification agents, specifically GSE-UP, into adhesive systems may increase the longevity of resin composite restorations by reducing secondary caries development around their dentin margins.

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3M ESPE.: Filtek Supreme Plus Universal Restorative—collection of scientific results. St. Paul, 2005.

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