EFFECT OF CHEMICAL CROSS-LINKING AGENTS ON DENTIN BONDING

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ABSTRACT

Georgia Macedo, DDS: Effect of Chemical Cross-linking Agents on Dentin Bonding.
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This study investigated the effects of Glutaraldehyde (GA) and Grape Seed Extract (GSE)-induced cross-linking on: 1. the stability of dentin collagen, and 2. dentin bond strengths of sound and caries-affected dentin. Our results demonstrated that the treatments of dentin collagen with GA and GSE significantly increase dentin collagen stability (resistance to collagenase degradation) in sound and caries affected dentin, likely via distinct mechanisms. In addition, the application of chemical cross-linking agents (GA and GSE) to etched dentin prior to bonding procedures significantly enhances dentin bond strengths for sound and caries-affected dentin.

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DEDICATION

To my parents, Dr. Silvio Macedo Jr. and Mrs. Sandra Macedo for their guidance, support, extraordinary courage and unconditional love. Thank you, always.

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CHAPTER ONE

INTRODUCTION

Stable and reliable bonding of composite resin to dentin remains a challenge in restorative dentistry (Dos Santos *et al.*, 2005; Breschi *et al*., 2008). The bonding is apparently most effective when the hybrid layer formed between resin monomers and collagen fibrils is structurally stable (Nakabayashi *et al.*, 1991). Over the last two decades, dental materials have been substantially improved attaining a better handling and bonding. However, little attention has been paid on the contribution of collagen structure/stability to the bond strength. Studies have shown that over time collagen in the hybrid layer is affected by enzymatic degradation; leading to bond failure (Okuda *et al.*, 2002; Armstrong *et al*., 2004; Koshiro *et al.*, 2005). This indicates that the improvement of dental materials alone is not sufficient to achieve a successful long-term bonding. Obviously, the stability of collagen fibrils should be maintained for a long period of time. In this study, we propose the hypothesis that induction of cross-linking in dentin collagen improves dentin collagen stability and dentin bond strength. It is well known that the biomechanical properties of type-I collagen can be improved by treatment of specific chemical agents likely due to the increase in the number of cross-links within the fibrils (Rao *et al*., 1983; Cheung *et al*., 1990; Sung *et al*., 1999; Ritter *et al*., 2001; Han *et al*., 2002; Bedran-Russo *et al*., 2007a). Thus, in this study, we investigated: 1. the effects of glutaraldehyde (GA) and grape seed extract (GSE) on dentin collagen stability, and 2. the effects of GA- and GSE-cross-linked dentin collagen on bond strength.

In order to achieve a better understanding of the present project, the study was divided in 2 articles. Each article (chapter 2 and 3) presents its own introduction, materials and methods, results and discussion sections.

CHAPTER II

STUDY ONE

Effect of chemical cross-linkers on stability of sound and caries-affected dentin collagen

INTRODUCTION

Dentin, the most abundant mineralized tissue in the human tooth, is basically composed of two phases: an inorganic phase of carbonate-rich hydroxyapatite crystals and an organic phase that is primarily composed of type-I collagen representing 90% of the organic matrix (Marshall *et al.*, 1997). It has been recently proposed that the degradation of dentin collagen contributes to the weakened bond strength between adhesive-dentin (Hashimoto *et al.*, 2003; Pashley *et al.*, 2004; Carrilho *et al*., 2007). Type-I collagen is present in tissues as fibrils that are stabilized by lysyl oxidase-mediated covalent intermolecular cross-linking (Yamauchi *et al*., 2002). The biomechanical properties of type-I collagen, however, can be improved by introducing more cross-links within and/or between the fibrils with the treatment of specific chemical agents. Glutaraldehyde (GA) is the most extensively used reagent for cross-linking collagen (Charulatha *et al*., 2003). GA, however, is known to elicit toxicity, thus, limiting its use to clinical settings. Therefore finding a collagen cross-linking reagent that exhibits low cytotoxicity and can form biocompatible cross-linked products is desirable (Han *et al*., 2003).

Grape Seed Extract which is a natural occurring cross-linker mainly composed of proanthocyanidins (PA), could be a good candidate to fulfill such role (Sung *et al*., 1999; Han *et al*., 2003). PAs are commonly used as dietary supplements (Facino *et al*., 1999). They are natural plant metabolites and shown to possess low toxicity and the ability to induce exogenous cross-links (Han *et al*., 2003). In addition, it has been recently reported that PA-based compounds improve dentin collagen physical properties (Bedran-Russo *et al*., 2007a,b).

In dentistry, most studies have evaluated sound dentin (Armstrong *et al*., 2003; De Munck *et al*., 2003). However in the clinical settings, after caries excavation, the majority of the prepared surface is composed of caries-affected dentin (CA dentin). CA dentin has been shown to have lower mechanical properties when compared to sound dentin (Nakajima *et al*., 1995; Yoshiyama *et al*., 2002; Ceballos e*t al.* 2003). The mechanisms involved in the premature failure of the adhesive bond to these clinically relevant dentin substrates remain unclear. It has been suggested that structural changes in collagens fibrils may cause incomplete infiltration of resin leading to inferior long-term durability of the dentin bond. For example, upon exposure, the structurally altered collagen may be more susceptible to further collagen disorganization or denaturation in the clinical environment (Wang *et al*., 2007). Therefore studies attempting to evaluate the quality of dentin should also assess altered forms of dentin, such as CA dentin.

The use of chemical cross-linking agents such as GSE and GA to increase the stability of dentin collagen fibrils could improve the stability of collagen organic matrix, thus, less susceptible to enzymatic degradation. The objective of this study was to evaluate the effects of GSE and GA treatment on demineralized human dentin collagen

obtained from sound and CA dentin at biochemical levels (by means of enzymatic digestion and amino acid analysis).

MATERIALS AND METHODS

Specimen preparation for sound dentin

Freshly extracted molars were used with approval of the Institutional Review Board of the University of North Carolina (#07-0788) Dentin discs (N=8) of 3mm thickness were prepared from human coronal dentin (upper third) by means of a diamond saw under water cooling (Isomet 1000, Buehler Ltd, Lake Bluff, IL, USA). Surrounding enamel was removed by wet-grinding the peripheries of discs with a polishing machine and 600-grit SiC paper discs (Buehler Isomet, Buehler Ltd, Lake Bluff, IL, USA).

Dentin discs were pulverized using a Spex-Freezer Mill in liquid nitrogen (Metuchen, NJ, USA). Subsequently the samples were lyophilized and later demineralized with 10% phosphoric acid for 5 hours at 4^{o}C. The insoluble residue was washed extensively with cold distilled water by repeated centrifugation (x4,000g for 45min), and lyophilized. This fraction is mainly composed of dentin collagen (>90%)(Ritter *et al.*, 2001).

Specimen preparation for caries-affected dentin

Freshly extracted human third molars presenting non-cavitated occlusal caries were used in order to standardize the type of lesion. The teeth were cleaned of debris, roots were cut, their occlusal surfaces were ground flat under running water, enamel was removed and the middle dentin was exposed. For this group, caries detector solution

(Kuraray Dental Co., Okayama, Japan) was applied to the surface in order to distinguish sound (no staining) from CA dentin (slightly stained pink) (Nakajima et al., 1995) in which 600 grit abrasive paper (Buehler Isomet, Buehler Ltd, Lake Bluff, IL, USA) was used until caries-affected dentin was exposed. Thin slices of caries-affected dentin were made by means of a sharp blade. Due to the limited amounts of CA dentin per tooth, CA dentin from five teeth was pooled and analyzed for each treatment group.

Pooled samples were pulverized, demineralized and lyophilized following the same protocol described for sound dentin.

Cross-linker treatment of dentin

Demineralized dentin powder from both sound and CA dentin groups was divided into 3 groups each according to the treatment received:

1. Control Group: 1mg of demineralized dentin powder was immersed in 1mL of deionized distilled water (DDW) for 1 hour at 37^{o}C;

2. GA-treated Group: 1mg of demineralized dentin powder was treated with 1mL of 5% glutaraldehyde (v/v%)(Fisher Chemical, Pittsburgh, PA / pH=7.4) (Bedran-Russo *et al*., 2007a) for 1 hour at 37^{o}C;

3. GSE-treated Group: 1mg of demineralized dentin powder was treated with 1mL of 6.5% grape seed extract (w/v%)(Mega Natural Polyphenolics, Madera, CA/ pH=7.4) (Bedran-Russo *et al*., 2007a) for 1 hour at 37^{o}C.

After treatment, the samples were extensively washed with cold deionized distilled water (DDW) by repeated centrifugation (5 times), lyophilized and subjected to the biochemical analysis.

Enzymatic digestion

The stability of demineralized dentin collagen was analyzed based on the digestibility of collagen with bacterial collagenase. Approximately 1 mg of the dried materials from each sample was suspended in 1ml of 0.05M NH_4HCO_3 (pH=7.5), and treated with 1% w/w bacterial collagenase (Worthington Biochemical Co., Lakewood, NJ) for 24h at room temperature. The supernatants and residues were separated by centrifugation (x14,000g for 45min), lyophilized and weighed. The amount of digested collagen was estimated as a proportion of the residues (undigested) to the supernatants (digested).

Amino acid analysis

A total of 1 mg of dried demineralized insoluble dentin matrix from each group was hydrolyzed with 0.3 ml of 6N HCl, after flushing with N_2, for 22h at 105ºC. The hydrolysates were dried by a speed vacuum concentrator (Savant Instruments Inc., NY, USA). The dried hydrolysates were reconstituted with 300 μL of DDW and filtered by a 0.22 mm cellulose acetate filter membrane (Millipore, Bedford, MA, USA). An aliquot of each hydrolysate was subjected to amino acid analysis. The amino acids were separated by a strong cation exchange column (AA911; Transgenomic, San Jose, CA, USA) on a high-performance liquid chromatography (HPLC) system (Varian 9050 and 9012; Varian, Walnut Creek, CA, USA). The quantification for each amino acid was done by ninhydrin color development at 135ºC. The amino acid composition of the dentin matrix was expressed as residues per 1000 total amino acids.

Statistical Analysis

Data were subjected to 2- and 1-way ANOVA to compare mean amino acid contents and collagen digestibility for the different substrate and dentin treatments.

RESULTS

Data analysis from enzymatic degradation showed that almost all collagen was recovered in the supernatant in the control groups demonstrating that the untreated dentin collagen was almost completely digested by bacterial collagenase; however in the GA- and GSE-treated groups the amount of digested collagen was markedly lower for both sound and caries-affected dentin than those of the untreated groups.

GSE-treated groups presented significantly lower ($p<0.001$) collagen degradation rates when compared to GA-treated and control groups for both types of dentin (sound and CA). Table 1 summarizes the collagen degradation amounts.

After amino acid analysis of each sample, it was observed that the contents of lysine (Lys) and hydroxylysine (Hyl) residues were significantly diminished in the GA-treated groups while they were unchanged in the GSE-treated groups in both sound and caries-affected dentin. The mean results of the amino acid analyses comparing all other amino acids did not show any significant differences among their contents independently of the different treatments (Table 2).

DISCUSSION

The susceptibility of untreated (control) and cross-linked dentin collagen to bacterial collagenase digestion was assessed. The rationale of this study was to stabilize the demineralized dentin collagen by means of application of cross-linkers (GSE and GA) to sound and CA dentin.

The data obtained from this *in vitro* bacterial collagenase experiment demonstrated an increased resistance of GA- and GSE-cross-linked samples to enzymatic degradation. It has been reported that increased number of collagen cross-links contribute to improvement of physical properties of dentin (Bedran-Russo *et al*., 2007a,b). Studies have shown lower bond strengths to CA dentin when compared to sound dentin (Nakajima *et al*., 1995; Yoshiyama *et al*., 2002; Ceballos e*t al.* 2003). However the factors and mechanisms involved in the premature failure of the adhesive bond to this substrate (CA dentin) remains unclear (Wang *et* al., 2007). It has been suggested that this may be due to the weakness of the CA dentin matrix (predominantly type-I collagen) (Yoshiyama *et al*., 2002). Carrilho *et al*. (2007) recently reported a significant decrease in resin-dentin bond strength after 14 months *in vivo* as a result of collagen degradation in the hybrid layer by collagenolytic enzymes. From a clinical perspective, it would be advantageous to be able to prevent the degradation of collagen by collagenolytic enzymes such as matrix metalloproteinase-2 (MMP-2) (Boushell *et al*., 2008) present in dentin hybrid layers. In this study, bacterial collagenase was used to evaluate the stability of collagen because of the specificity and speed of the digestion. The results clearly demonstrated that the treatment of collagen with GA and GSE significantly increased the resistance against this enzymatic digestion. Therefore the

potential increase in the stability of dentin collagen (in sound and especially in CA dentin), may conceivably enhance their resistance to enzymatic degradation and may consequently improve the longevity of the tooth-restoration complex.

It has been reported that stability of type-I collagen can be improved by the application of specific cross-linking agents such as GA (Charulatha *et al*., 2003, Han *et al*., 2002), due to an increase in the number of cross-links within and between the collagen fibrils involving the ε-amino groups of peptidyl Lys and Hyl residues (Ritter *et al*., 2001). By an *In vitro* bacterial degradation assay often used to examine the degree of collagen cross-linking (Han *et* al., 2003) showed that the GA-treated dentin collagen was more resistant to digestion by bacterial collagenase than the untreated collagen groups. This data confirmed the efficacy of GA-induced cross-linking in collagen previously reported (Cheung *et al*., 1990; Han *et al*., 2002). Although GA application can lead to considerable improvements in collagen matrix properties, its potential cytotoxicity remains a concern. Thus in this study, as an alternative material, the effect of GSE on collagen stabilization was evaluated and compared to GA.

Grape Seed Extract compounds (primarily composed of Proanthocyanidins) have been reported to be a low toxic reagent (120 times less toxic than GA) that can induce exogenous cross-links (Han *et al*., 2002). Proanthocyanidins (PA) are natural products with polyphenolic structures that have the potential to give rise to stable hydrogen bonded structures and generate non-biodegradable collagen matrices (Facino *et al*., 1999). The four proposed mechanisms for interaction between PA and proteins include covalent interactions (Pierpoint *et al.*, 1969), ionic interactions (Loomis et al., 1974), hydrogen bonding interactions (Ku *et al*., 2007), or hydrophobic interactions (Han *et al*.,

2002). In dentistry, it has been recently reported that GSE-induced cross-linking improves physical properties of dentin (Bedran-Russo *et al*., 2007a,b). Our data showed that GSE-treated groups were significant less susceptible to collagenolytic activity than the GA-treated groups in both sound and CA dentin samples. This increased resistance to degradation may be attributed to masking of the recognition site by the cross-links, or to the retention of the cleaved peptides fragments by the newly formed cross-links, or to other unknown mechanisms (Charulatha *et al*., 2003).

The results of the amino acid analysis revealed that the contents of lysine (Lys) and hydroxylysine (Hyl) residues were significantly diminished in GA groups, suggesting the formation of new cross-links involving those amino acids. Amino acid analysis also indicated that the mode of cross-linking by GSE is distinct from GA-induced cross-linking in which Lys and Hyl are involved. Amino acid analysis indicated that the cross-links induced by GSE are not stable in acid hydrolysis, thus, to identify the GSE-induced cross-links other approaches should be pursued. From our results of amino acid analysis, we were unable to detect any differences in Lys and Hyl levels in GSE-treated groups compared to the untreated groups (control).

CONCLUSION

Within the limitations of this *in* vitro study, it was concluded that the stabilization of dentin collagen by GA- and GSE-induced cross-links significantly increased resistance to enzymatic degradation in sound as well as CA dentin; likely via distinct mechanisms.

The use of chemical cross-linking agents such as GSE and GA could potentially improve the long-term stability of the dentin-resin interface, thereby enhancing the

longevity of the tooth-restoration complex. Long-term studies and clinical research in this area still necessary.

CHAPTER III

STUDY TWO

Effect of Chemical Cross-linking Agents on Caries-affected Dentin Bonding

INTRODUCTION

Achieving a strong and stable bond between composite resin and dentin remains a challenge in restorative dentistry (Dos Santos *et al.*, 2005; Breschi *et al*., 2008). Dentin, the most abundant mineralized tissue in the human tooth, is basically composed of two phases: an inorganic phase of carbonate-rich hydroxyapatite crystals, and an organic phase of predominantly type-I collagen (Marshall *et al.*, 1997). Dentin bonding is apparently most effective when the hybrid layer formed between resin monomers and collagen fibrils is structurally stable (Nakabayashi *et al.*, 1991).

Studies have shown that over time collagen in the bonded interface is affected by the endogenous matrix metalloproteinases such as MMP-2, thereby leading to bond failure (Okuda *et al.*, 2002; Armstrong *et al*., 2004; Koshiro *et al.*, 2005). While most development and understanding of dentin bonding relies on the improvement of bonding agents and technique, little attention has been paid to the contribution of collagen structure/stability on the dentin bond strengths.

Type-I collagen is present in tissues as fibrils that are stabilized by lysil oxidase-mediated covalent intermolecular cross-linking (Yamauchi *et al*., 2002). Chemical cross-linkers have been reported to further stabilize collagen fibrils in several connective

tissues (Rao *et al*., 1983; Cheung *et al*., 1990; Sung *et al*., 1999; Ritter *et al*., 2001; Han *et al*., 2002; Bedran-Russo *et al*., 2007a). Glutaraldehyde (GA), a synthetic cross-linker, can enhance collagen stability, however they present drawbacks such as toxicity (Cheung *et al*., 1990; Ritter *et al*., 2001, Southern *et al*., 2001). Grape Seed Extract (primarily composed of Proanthocyanidin) compounds have been reported to be a low toxicity natural compound that can induce exogenous cross-linkings (Han *et al*., 2002). Grape Seed Extract (GSE) is widely used in the medical field as natural antioxidants, free-radical scavengers (Facino *et* al., 1999) and has proven to be safe as dietary supplements (Skovgaard *et* a*l*., 2006). Moreover, in dentistry, it has been recently reported that GA and GSE-induced cross-linking improved the mechanical properties of dentin (Bedran-Russo *et al*., 2007a,b).

Most studies have used sound dentin as the bonding substrate (Armstrong *et al*., 2003; De Munck *et al*., 2003) however in the clinical settings, the substrate being bonded usually involves altered forms of dentin such as caries-affected dentin, in which the bond strength is lower than that of sound dentin (Nakajima *et al*., 1995; Yoshiyama *et al*., 2002; Ceballos e*t al.* 2003). Therefore potential improvement in the quality of the tooth-restoration complex should consider the effectiveness of different dentin treatments to both sound and caries-affected dentin.

Increased stability of the collagen fibrils could conceivably enhance mechanical properties of dentin (Bedran-Russo *et al*., 2007a,b). Moreover enhanced collagen matrix properties may contribute to improved dentin bond strength. We tested the hypothesis that the use of collagen cross-linkers will increase the dentin bond strength of caries-affected dentin, regardless of cross-linker and adhesive system investigated.

MATERIALS AND METHODS

Specimen preparation

Freshly extracted human molars presenting non-cavitated occlusal caries (n=8 teeth for each group) were used in order to standardize the type of lesion. Teeth were used with approval of the Institutional Review Board of the University of North Carolina (#07-0788). The teeth were cleaned of debris, and the occlusal surfaces were ground flat under running water to remove enamel and to expose middle dentin. Caries detector solution (Kuraray Dental Co., Okayama, Japan) was applied to the surface in order to distinguish sound (no staining) from caries-affected dentin (slightly stained pink)(Nakajima *et al*., 1995), in which 600 grit abrasive paper (Buehler Isomet, Buehler Ltd, Lake Bluff, IL, USA) was used until caries-affected dentin was exposed. Then, the flat dentin surfaces were acid etched for 15 seconds using 37% phosphoric acid (3M/ESPE) and teeth were randomly divided into six groups according to the treatment and bonding system used as described below:

1. **Control SB:** Bonding protocol followed the manufacturers' instructions for Adper Single Bond Plus adhesive system – 3M/ESPE, St. Paul, MN, (Table 1) and light-cured for 20 seconds. After completion of the bonding procedure, a composite build-up (Clearfil APX – Kuraray Co.. Okayama, Japan) was made with three 2-mm increments, each light-cured for 40 seconds;
2. **Control OS:** Bonding protocol followed the manufacturers' instructions for One Step Plus adhesive system – Bisco, Schaumburg, IL, (Table 1) and light-cured for 20 seconds. Build-up protocol was done as described for Control SB group;

3. **GA-treated SB:** The etched dentin surface was treated with 5% glutaraldehyde (v/v%)(Fisher Chemical, Pittsburgh, PA / pH=7.4) (Bedran-Russo *et al*., 2007a) for 1 hour. After the treatment, the surface was washed with distilled water, followed by the bonding/build-up protocol as described for Control SB group;
4. **GA-treated OS:** The etched dentin surface was treated with 5% GD (v/v%) for 1 hour. After the treatment, the surface was washed with distilled water, followed by the bonding/build-up protocol as described for Control OS group;
5. **GSE-treated SB:** The etched dentin surface was treated with 6.5% grape seed extract (w/v%)(Natural Polyphenolics, Madera, CA/ pH=7.4) (Bedran-Russo *et al*., 2007a) for 1 hour. After the treatment, the surface was washed with distilled water, followed by the bonding/build-up protocol as described for Control SB group;
6. **GSE-treated OS:** The etched dentin surface was treated with 6.5% GSE (w/v%) for 1 hour. After the treatment, the surface was washed with distilled water, followed by the bonding/build-up protocol as described for Control OS group.

Microtensile Bond Strength Test (μTBS)

After a storage period of 24h in distilled water at 37^{o}C, the restorations were sectioned (Isomet 1000, Buehler Ltd, Lake Bluff, IL, USA) perpendicular to the bonded interface to produce beams with a cross-sectional area of approximately 0.64mm^2. Beams from each group were visually examination and divided into two groups: bonded to sound or caries-affected dentin. Specimens were fixed to a Ciucchi jig with cyanoacrylate adhesive and tested in tensile at a crosshead speed of 1 mm/min until failure (EZ-test,

Shimazu, Kyoto, Japan). Data were analyzed using 2 and 1-way ANOVA for each adhesive and Fisher's PLSD test at α=0.05.

Fracture Mode Analysis

After the tensile testing procedure, the debonded specimens were fixed in 10% neutral buffered formalin to examine the morphology of the fractured beams. The dentin and resin sides of the fractured specimens were trimmed, placed on stubs, followed by desiccation at room temperature, gold sputter-coated in a 5100 sputter-coater (Polaron Equipment, Watford, England), and examined at 2500-5000× in the Scanning electron microscopy (Hitachi).

Knoop microhardness

In order to confirm the presence of CA dentin and sound, debonded specimens were embedded in epoxy resin, ground and polished with a series of 600, 800 and 1200-grit SiC paper discs (Buehler) in a polishing machine. The final polishing was conducted with a series of 6, 3 and 1 microns compound diamond pastes (Buhler) on a felt cloth. Specimens were tested for Knoop microhardness (KHN) using a microhardness tester (Micromet 2100, Buehler Ltd, Lake Bluff, IL, USA) loaded to 25 g with an indentation time of 15 seconds. Measurements were done 50µm below the adhesive/dentine interface (Ceballos *et* al., 2003). Statistical analysis was performed using One-way ANOVA.

RESULTS

The μTBS (MPa ±SD) values for both adhesive systems are summarized in Table 2 and 3. Statistical analysis compared the groups within each adhesive system. Comparisons between adhesives were not performed. Two-way ANOVA revealed no interaction between factors (substrate x treatment) for both adhesive systems (SB- $p = 0.6018$ and OS- $p = 0.5398$).

Both adhesive systems responded positively to the cross-linking treatment resulting in a significantly increase in dentin bond strengths (OS- $p = 0.0124$ and SB- $p = 0.0023$). In addition, GA and GSE increased the μTBS of both dentin substrate sound and caries-affected dentin. All sound dentin groups showed significantly higher dentin bond strengths when compared to their respective caries-affected groups for both adhesive systems ($p < 0.05$).

Regardless of the dentin substrate type (sound and CA), GA- and GSE-treated groups were not statistically different (SB, $p = 0.3431$; and OS, $p = 0.4993$). Caries-affected dentin groups treated with GA or GSE increased dentin bond strengths to values that were statistically similar ($p > 0.05$) to untreated sound dentin (control) group.

Photomicrographs of the fracture patterns of the control groups showed mostly cohesively failures in the bottom of the hybrid layer as shown in Figure 1 and 2. SEM micrographs of the GA- and GSE-treated groups showed a similar trend of fracture pattern that was distinct from the control groups. Mixed failures, mainly at cohesive in resin and on the top of the hybrid layer were observed for both adhesive systems (Figure 3 and 4). Interestingly, the CA dentin groups performed similarly to sound dentin. For the treated groups, mixed failures (cohesive in resin and on the top of the hybrid layer)

occurred, and for the control groups, cohesively failures in the bottom of the hybrid layer were observed.

The mean hardness (KHN) values recorded for sound dentin (99.9) was significant higher ($p<0.0001$) than the mean KHN of caries-affected dentin (51.9).

DISCUSSION

This study investigated effect of two cross-linking agents - glutaraldehyde (GA) and grape seed extract (GSE), on dentin bond strengths of caries-affected dentin. Among all experimental groups, post-etch application of GSE and GA led to significantly higher dentin bond strengths, regardless of the type of adhesive system used. The relative increase in bond strength may be attributable to the improved dentin collagen stability, owing to the higher number of collagen cross-links (Han *et al*., 2002; Bedran-Russo *et al*., 2007b).

GA has been widely used as a fixative of biological tissues, and its use has been associated with a decrease in the rate of collagen degradation (Cheung *et al*. 1990; Han *et al*., 2002) and improved dentin collagen properties (Bedran-Russo *et al*., 2007b). GA reacts primarily with the ε-amino groups of peptidyl lysine and hydroxilysine residues of collagen fibrils. The amino acid (AA) analysis revealed that the contents of lysine and hydroxylysine residues were significantly diminished in GA-treated groups, confirming the formation of such cross-links (Macedo et al, under review). An increase in the stiffness of GA-treated dentin matrix (Bedran-Russo *et al*., 2007b) is also attributed to the increase in the number of cross-links.

GSE treatment also resulted in an increase in the bond strength. GSE is mainly composed of PA, a naturally occurring cross-linking agent The use of a PA-based (grape seed extract) cross-linking agent on demineralized dentin has been associated with a significant improvement in the mechanical and physical properties of dentin, including ultimate tensile strength and modulus of elasticity (Bedran-Russo *et al*., 2007a,b). The four proposed mechanisms for interaction between PA and proteins include covalent interactions (Pierpoint *et al.*, 1969), ionic interactions (Loomis et al., 1974), hydrogen bonding interactions (Ku *et al*., 2007), or hydrophobic interactions (Han *et al*., 2002). Based on our preliminary amino acid analysis, it was concluded GSE apparently induces cross-links via a mechanism different from that of GA (Han *et al,* 2003). We believe that the enhanced cross-links could have positively affected dentin bond strengths and its resistance to enzymatic degradation. The digestibility tests performed in our previous study revealed that GSE-treated groups were significantly less susceptible to collagenase digestion than the other groups (GA and control) (Macedo et al, under review).

The bond strength to caries-affected (CA) dentin was the primary focus of this study, due to the fact that CA dentin is frequently encountered in restorative dentistry, especially along the pulpal floor of cavity preparations (Marshall *et al*., 2001). Similar to the findings of Nakajima *et al*.(1995), Yoshiyama *et al*. (2002) and Ceballos *et al*. (2003), the present study found that untreated caries-affected dentin exhibited lower bond strengths when compared to sound dentin, This may be due to the altered or partially denaturated dentin matrix (predominantly type-I collagen) (Yoshiyama *et al*., 2002) and/or the relative paucity of resin tags in mineral-filled tubules (Marshall *et al*., 2001). However when CA dentin was treated with either GSE or GA, the bond strength was

significantly to the level compared to sound dentin. This indicates that the stability of collagen in CA dentin can be restored by inducing cross-linking by both GA and GSE.

Results of the SEM fracture analysis revealed differences between control and treated samples. The increase in mixed failures at adhesive/resin and top of the hybrid layer may indicate that the hybrid layer was strengthened by the different treatments. The superior integrity of the cross-linked dentin-resin interface could have potentially resulted in a stronger bond for the treated groups, thus explaining the predominantly mixed modes of failure. The usual failures at the bottom of the hybrid layer (rich in collagen) were not often seen in the GSE and GA treated samples. Increases in failures at the adhesive layer and top of the hybrid layer in the treated groups, indicate that cross-linked dentin matrix strengthen the bottom of the hybrid layer where more collagen fibrils are present.

The results of the Knoop Hardness test (KHN) confirmed the presence of CA dentin. Sound dentin was harder than CA dentin which is observation was consistent with previously reported data (Ceballos *et al*., 2003; Zheng *et al*., 2005). Although the tubules are filled with mineral, the relative softness of caries-affected dentin may be attributable to the partial demineralization of the intertubular dentin (Ceballos *et al*., 2003). The blockage of the dentin tubules by mineral deposits might have interfered with the acid etching process and subsequently incomplete resin monomer penetration thus explaining the lower bond strengths of CA when compared to sound dentin.

The present study has demonstrated that increased bond strength can be obtained by the use of biochemical cross-linkers in both caries-affected and sound dentin. It has been recently proposed that the deterioration of dentin collagen fibrils contributes to the mechanism responsible for bond degradation (Hashimoto *et al.*, 2003; Pashley *et al.*,

2004; Carrilho *et al*., 2005). The use of chemical cross-linking agents such as GSE and GA could also potentially improve the long-term stability of the dentin bond, thereby enhancing the longevity of the tooth-restoration complex.

CONCLUSION

Within the limitations of this *in vitro* study, it is concluded that the application of GSE and GA to dentin significantly improved the microtensile bond strengths to sound, and more importantly, to caries-affected dentin. The results of this study may provide insights into developing novel strategies for efficient and stable dentin bonding, with the use of naturally occurring and synthetic cross-linking agents.

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TABLES

Table 1. Proportions of collagen degradation in residues and supernatants after collagenase digestion.

Table 1	DW		GA		GSE	
N=8	**RES**	**SUP**	**RES**	**SUP**	**RES**	**SUP**
SOUND	8% ^{C}	92% ^{a}	68% ^{B}	32% ^{b}	85% ^{A}	15% ^{c}
CARIES-AFFECTED	9% ^{C}	91% ^{a}	71% ^{B}	29% ^{b}	83% ^{A}	17% ^{c}

*Different superscript letters represent statistical significant differences among groups.

Table 2. Amino acid analysis of sound and CA dentin after cross-linker treatment

	Sound dentin (Mean)				Caries-affected dentin (Mean)		
N=3	**DDW**	**GA**	**GSE**	N=2	**DDW**	**GA**	**GSE**
hyp	150.9	129.2	90.1	**hyp**	95.8	83.6	97.5
asp	33.6	40.8	51.1	**asp**	35.2	30.3	38.7
thr	21.6	15.2	18.1	**thr**	20.1	19.2	19.9
ser	41.6	32.2	37.7	**ser**	39.5	39.2	38.4
glu	58.9	66.5	80.0	**glu**	63.0	62.1	73.4
pro	223.6	148.4	112.4	**pro**	109.0	118.4	107.2
gly	247.3	343.5	330.2	**gly**	332.8	352.0	336.1
ala	78.5	106.6	114.1	**ala**	116.6	123.9	117.3
val	15.3	20.0	26.2	**val**	28.2	28.5	27.6
met	3.9	0.9	1.0	**met**	0.0	0.8	8.5
ile	14.3	8.7	10.6	**ile**	11.3	10.3	20.2
leu	19.4	24.1	29.8	**leu**	31.9	36.0	14.5
tyr	8.6	3.6	5.4	**tyr**	5.1	6.8	9.1
phe	9.1	13.1	15.3	**phe**	18.6	20.0	10.5
his	2.2	1.7	4.7	**his**	10.5	6.1	8.8
hyl	9.6	3.2*	9.8	**hyl**	9.9	2.7*	8.9
lys	21.6	4.1*	15.9	**lys**	20.7	5.8*	17.4
arg	39.9	38.2	47.6	**arg**	51.5	54.2	48.8
Totals	1000.0	1000.0	1000.0	**Totals**	1000.0	1000.0	1000.0

Asterisks (*) indicates statistical significant differences in Hyl and Lys contents among groups

Table 3. Materials, Components and Manufacturers

MATERIALS	COMPONENTS	BATCH No.	MANUFACTURER
ONE-STEP PLUS	Primer/Adhesive: Bis-GMA, BPDM, HEMA, CQ, *p*-dimethylaminobenxoic acid (co-initiator), acetone, 8.5% glass fillers.	0600006589	Bisco, Inc, Schaumburg, IL, USA
ADPER SINGLE BOND PLUS	Primer/Adhesive: BisGMA, HEMA, dimethacrylates, ethanol, water, methacrylate functional copolymer of polyacrylic and polyitaconic acids, and photoinitiator	70-2010-3672-3	3M ESPE, St Paul, MN, USA

Table 4. Mean and Standard deviation of microtensile bond strength for Adper Single Bond.

SINGLE BOND PLUS	CONTROL	GA	GSE
SOUND DENTIN	59.62 (± 20.0) ^{B}	71.89 (± 25.2) ^{A}	68.34 (± 23.8) ^{A}
AFFECTED	36.75 (± 8.5) ^{C}	55.55 (± 15.2) ^{B}	55.9 (± 14.0) ^{B}

* Different superscript letters equals statistical significant difference between groups.

Table 5. Mean and Standard deviation of microtensile bond strength for One Step Plus

ONE STEP PLUS	CONTROL	GA	GSE
SOUND DENTIN	65.22 (± 20.4) ^{b}	74.30 (± 21.9) ^{a}	73.14 (± 17.0) ^{a}
AFFECTED	37.38 (± 14.8) ^{c}	57.18 (± 16.7) ^{b}	52.85 (± 18.2) ^{b}

* Different superscript letters equals statistical significant difference between groups.

FIGURES

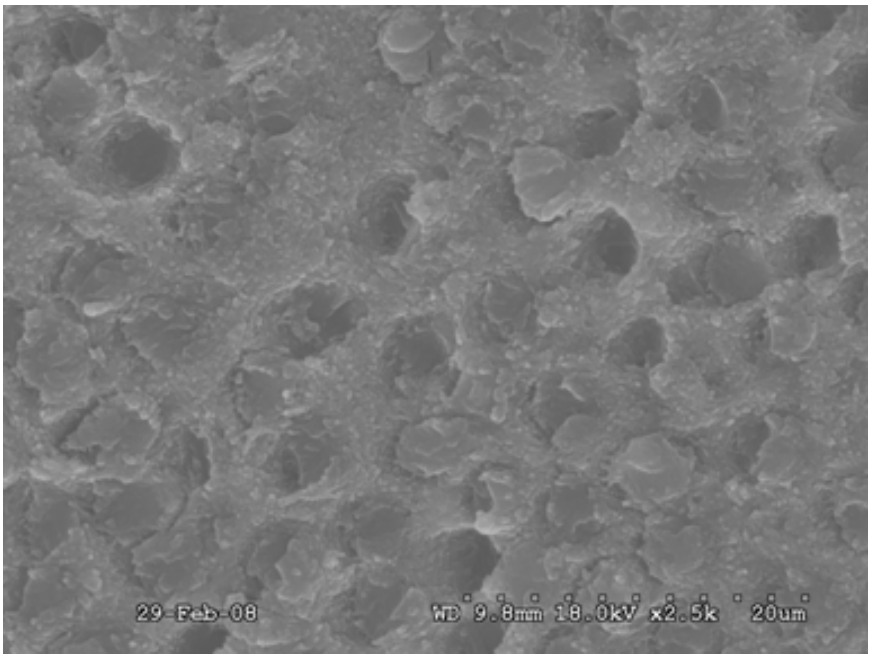

Fig. 1. SEM photomicrograph showing the dentin side of a fractured specimen for Control sound group (Adper Single Bond) that failed at the bottom of the hybrid layer

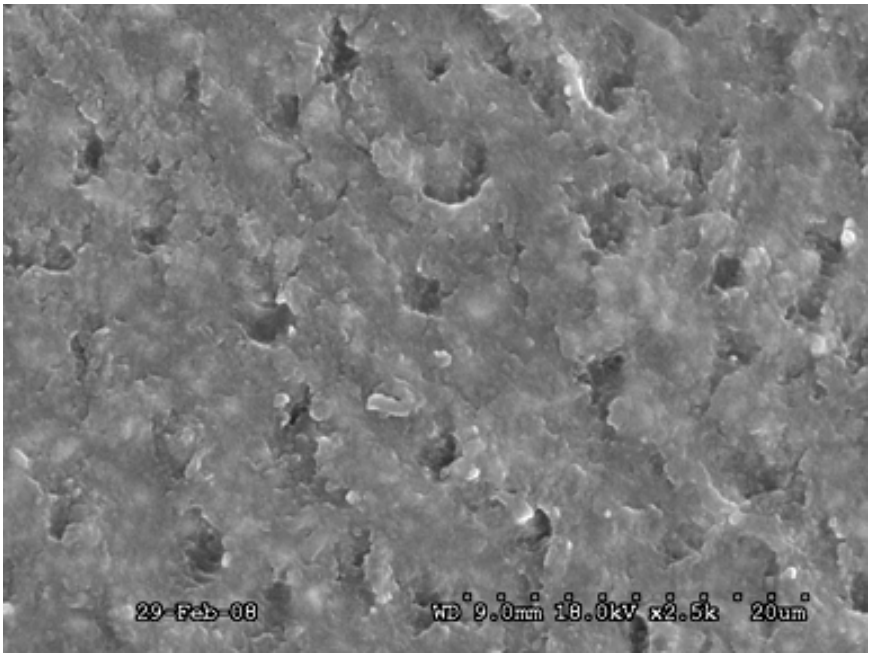

Fig. 2. SEM photomicrograph showing the dentin side of a fractured specimen in group Control caries-affected (One Step Plus) that failed at the bottom of the hybrid layer

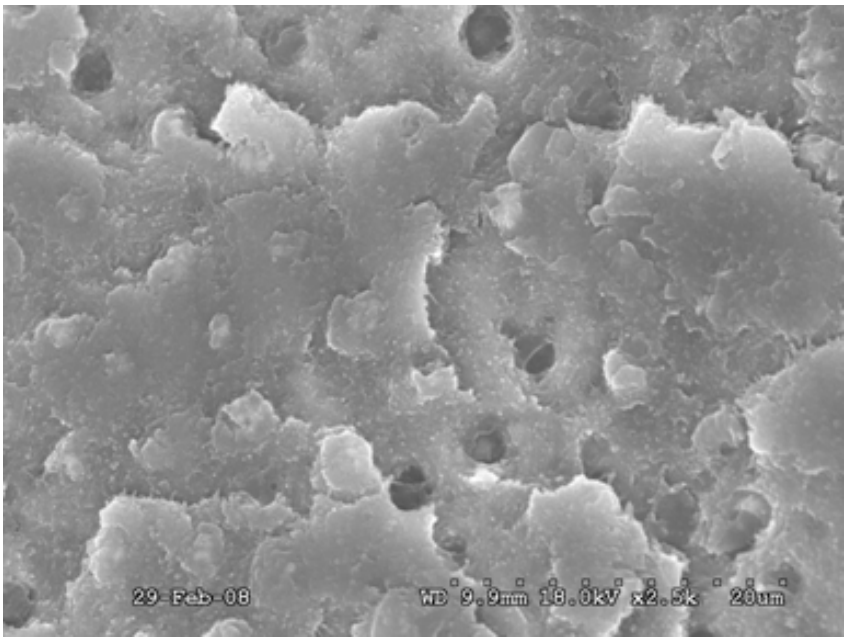

Fig. 3. SEM photomicrograph of fracture pattern mostly commonly found in treated groups - mixed – cohesive in adhesive and top of the hybrid layer (GA-treated sound dentin - One Step Plus)

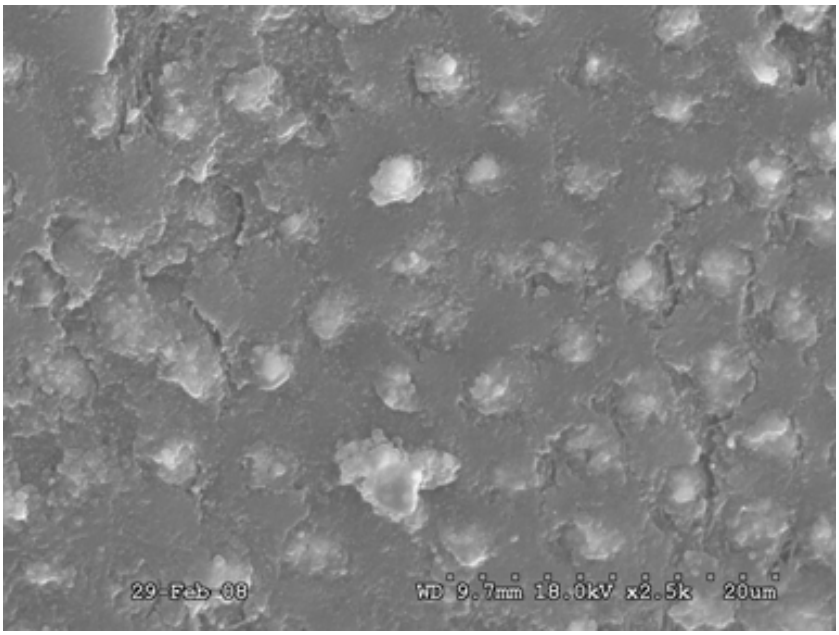

Fig. 4. SEM photomicrograph of a fractured dentin side in group GSE-treated sound dentin (One Step Plus) - top of the hybrid layer/adhesive layer

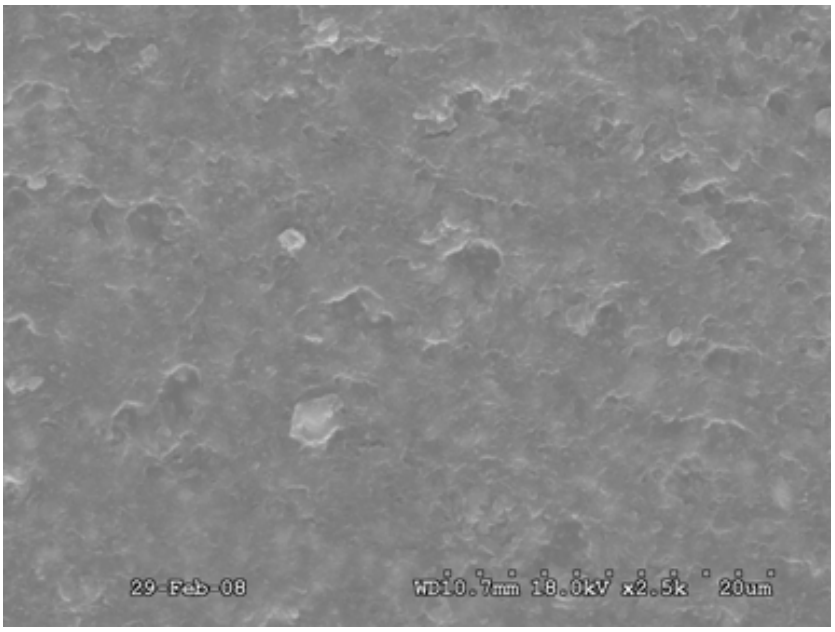

Fig. 5 SEM photomicrograph of a fractured dentin side in GA-treated caries-affected dentin (One Step Plus) - top of the hybrid layer/adhesive layer.

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