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ABSTRACT

The achievement of a strong and stable bond between composite resin and dentin remains a challenge in restorative dentistry. Over the past two decades, dental materials have been substantially improved, with better handling and bonding characteristics. However, little attention has been paid to the contribution of collagen structure/stability to bond strength. We hypothesized that the induction of cross-linking in dentin collagen improves dentin collagen stability and bond strength. This study investigated the effects of glutaraldehyde- and grape seed extract-induced cross-linking on the dentin bond strengths of sound and caries-affected dentin, and on the stability of dentin collagen. Our results demonstrated that the application of chemical cross-linking agents to etched dentin prior to bonding procedures significantly enhanced the dentin bond strengths of caries-affected and sound dentin. Glutaraldehyde and grape seed extract significantly increased dentin collagen stability in sound and caries-affected dentin, likely *via* distinct mechanisms.

KEY WORDS: dentin, bonding, collagen, cross-linkers, grape seed extract, resin composite, bond strength.

Effects of Chemical Cross-linkers on Caries-affected Dentin Bonding

INTRODUCTION

The achievement of an efficient and stable bond between composite 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 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). While most of the developments in adhesive dentistry have focused on the improvement of bonding agents and technique, limited investigation has explored the contributions of collagen structure/stability to bond strength.

Type-I collagen is present in tissues as fibrils that are stabilized by lysyl-oxidase-mediated covalent intermolecular cross-linking (Yamauchi and Shiiba, 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.*, 2003; Bedran-Russo *et al.*, 2007). Glutaraldehyde, a widely used synthetic cross-linker, can enhance collagen stability; however, it presents some drawbacks, such as toxicity (Cheung *et al.*, 1990; Southern *et al.*, 2000). Grape seed extract (primarily composed of proanthocyanidins) has been reported to be a low-toxicity compound that can induce exogenous cross-links (Han *et al.*, 2003). Moreover, in dentistry, it has been recently reported that glutaraldehyde and grape seed extract-induced cross-linking improved the mechanical properties of dentin (Bedran-Russo *et al.*, 2008).

Studies have used sound dentin as the bonding substrate (Armstrong *et al.*, 2003; De Munck *et al.*, 2003); however, in clinical settings, the substrate being bonded often involves caries-affected dentin, in which the bond strength is lower than that of sound dentin (Nakajima *et al.*, 1995; Yoshiyama *et al.*, 2002; Ceballos *et al.*, 2003). Therefore, potential improvement in the quality of the tooth-restoration complex should consider the effectiveness of different dentin treatments on sound and caries-affected dentin. The objective of this study was to investigate the effects of grape seed extract- and glutaraldehyde-induced cross-linking on the bond strength and stability of caries-affected and sound dentin.

MATERIALS & METHODS

Specimen Preparation for Microtensile Testing

Forty-eight freshly extracted human molars with non-cavitated occlusal caries were used, to standardize the type of lesion. Teeth previously extracted with informed donor consent were used in this study with approval of the Institutional Review Board of the University of North Carolina (#07-0788). The teeth were cleaned, and the occlusal surfaces were ground flat under running water to remove enamel and expose mid-thickness dentin. The protocol

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described previously (Nakajima *et al.*, 1995) was used to obtain caries-affected dentin according to the combined criteria of visual examination, surface hardness, and staining by a caries detector solution (Kuraray Co., Okayama, Japan). All soft, stainable, carious dentin was removed, exposing a relatively hard, caries-affected non-staining dentin. One operator prepared all the specimens, to diminish variability in this somewhat subjective analysis. Dentin surfaces were then ground with 600-grit abrasive paper (Ecomet, Buehler Ltd., Lake Bluff, IL, USA). The flat surfaces were acid-etched for 15 sec with 37% phosphoric acid (3M/ESPE, St. Paul, MN, USA), and teeth were randomly divided according to the treatment and bonding system used, as described by the following.

Control

Bonding protocol followed the manufacturers' instructions for either the Adper Single Bond Plus (3M/ESPE, batch number 70-2010-3672-3) or the One Step Plus adhesive system (Bisco, Schaumburg, IL, USA; batch number 0600006589) (Appendix Table). Specimens were light-cured for 20 sec. After completion of the bonding procedure, a composite build-up (Clearfil APX; Kuraray Co.) was made with three 2-mm increments, each light-cured for 40 sec.

Glutaraldehyde Treatment

The etched dentin surface was treated with 5% v/v glutaraldehyde (Fisher Chemical, Pittsburgh, PA, USA/pH = 7.4) (Bedran-Russo *et al.*, 2007) for 1 hr. After the treatment, the surface was washed with distilled water, followed by the bonding/build-up protocol as described above.

Grape Seed Extract Treatment

The etched dentin surface was treated with 6.5% w/v grape seed extract (MegaNatural; Polyphenolics, Madera, CA, USA/pH = 7.4) (Bedran-Russo *et al.*, 2007) for 1 hr. After the treatment, the surface was washed with distilled water, followed by the bonding/build-up protocol as described above.

Microtensile Bond Strength Test (μ TBS)

After a storage period of 24 hrs in distilled water at 37°C, the restorations were sectioned (Isomet 1000, Buehler Ltd.) perpendicular to the bonded interface to produce beams with a cross-sectional area of approximately 0.64 mm². Beams from each group were mapped, visually examined, and divided into two groups: bonded to either sound or caries-affected dentin. Specimens were fixed to a Ciucchi jig with cyanoacrylate adhesive and tested in tension at a crosshead speed of 1 mm/min until failure (EZ-test, Shimadzu, Kyoto, Japan). Data were analyzed by two- and one-way ANOVA for each adhesive and by Fisher's PLSD test ($\alpha = 0.05$).

Fracture Mode Analysis

After the tensile testing procedure, we fixed the debonded specimens in 10% neutral buffered formalin to examine the morphology of the fractured beams. Selective specimens were gold-sputter-coated (Polaron Equipment, Watford, England), and examined by scanning electron microscopy (S3000 N, Hitachi Ltd., Saitama, Japan).

Knoop Microhardness

To confirm the presence of caries-affected or sound dentin, we embedded debonded specimens in epoxy resin and polished them with abrasive paper (# 600, 800, 1200; Buehler Ltd.) and diamond suspensions (9 μ m, 6 μ m, 3 μ m, 1 μ m; Buehler Ltd.). Specimens were tested for Knoop microhardness (KHN) by means of a microhardness tester (LECO Series 200, St. Joseph, MI, USA), loaded } to 25 g for 15 sec. Measurements were taken 50 μ m below the adhesive/dentin interface (Ceballos *et al.*, 2003). Statistical analysis was performed by one-way ANOVA and Fisher's PLSD test ($\alpha = 0.05$).

Specimen Preparation for Biochemical Analysis

Dentin discs (3 mm thick) were prepared from caries-free human coronal dentin (upper third) with the use of a diamond saw under water cooling (Isomet 1000, Buehler Ltd.). Surrounding enamel was removed, and dentin discs were pulverized and demineralized with 10% phosphoric acid for 5 hrs at 4°C. The insoluble residue was washed with cold distilled water by repeated centrifugation (x 4000 g for 45 min) and lyophilized. This fraction was composed mainly of dentin collagen (> 90%) (Ritter *et al.*, 2001).

Non-cavitated carious molar crowns had their occlusal surfaces ground flat under running water. Thin mesial-distal slices (0.1 mm thick) of dentin were obtained (Isomet 1000, Buehler Ltd.), and a caries detector solution (Kuraray) was used to identify caries-affected dentin (Nakajima *et al.*, 1995). A sharp blade was used to separate caries-affected dentin from the thin slices. The pooled samples were obtained from 15 teeth divided into 3 groups of 5 teeth each to obtain enough dentin powder for analysis. Pooled samples were pulverized, demineralized, and lyophilized following the same protocol as described for sound dentin.

Cross-linker Treatment of Dentin

Demineralized dentin powder from both caries-affected and sound dentin was divided into 3 groups: control group [de-ionized distilled water (DDW)]; glutaraldehyde-treated group (5% v/v, pH = 7.4); and grape seed extract-treated group (6.5% w/v, pH = 7.4). A 1-mg quantity of demineralized dentin powder was immersed in 1 mL of the respective treatment solutions for 1 hr at 37°C. After treatment, the samples were extensively washed with cold de-ionized distilled water (DDW) by repeated centrifugation (5 times), lyophilized, and subjected to the biochemical analysis.

Digestibility with Collagenase

Approximately 1 mg of the dried specimen from each sample (N = 8 for sound/N = 3 for caries-affected pooled samples) was suspended in 1 mL of 0.05 M NH₄HCO₃ (pH = 7.5) and treated with 1% w/w bacterial collagenase (CLSPA; Worthington Biochemical Co., Lakewood, NJ, USA) for 24 hrs at room temperature, and the supernatants and residues were separated by centrifugation, lyophilized, and weighed. The amount of digested matrix was calculated as a proportion of the residues (undigested) to the supernatants (digested) on a dry weight basis (Walter *et al.*, 2008). Statistical analysis was performed by two-way ANOVA and Fisher's PLSD test ($\alpha = 0.05$).

Table 1. Means and Standard Deviations of Microtensile Bond Strength Measurements for Adper Single Bond and One Step Plus

		Control	GA	GSE
Adper Single Bond Plus	Sound	59.62 ($\pm$ 20.0) ^B	71.89 ($\pm$ 25.2) ^A	68.34 ($\pm$ 23.8) ^A
	Affected	36.75 ($\pm$ 8.5) ^C	55.55 ($\pm$ 15.2) ^B	55.9 ($\pm$ 14.0) ^B
One Step Plus	Sound	65.22 ($\pm$ 20.4) ^B	74.30 ($\pm$ 21.9) ^A	73.14 ($\pm$ 17.0) ^A
	Affected	37.38 ($\pm$ 14.8) ^C	57.18 ($\pm$ 16.7) ^B	52.85 ($\pm$ 18.2) ^B

* Different superscript letters equal statistically significant differences between groups ($p < 0.05$). In total, 48 beams were tested *per* group (8 beams *per* tooth, 6 teeth *per* group). GA, glutaraldehyde; GSE, grape seed extract.

Amino Acid Analysis

A total of 1 mg of dried demineralized insoluble dentin matrix was hydrolyzed with 0.3 mL of 6 N HCl and subjected to amino acid analysis as previously described (Yamauchi and Shiiba, 2008), and the composition was calculated as residues/1000. In total, 5 analyses were done.

RESULTS

Two-way ANOVA revealed no interaction between factors (substrate $\times$ treatment) for both adhesive systems (Single Bond Plus, $p = 0.6018$, and One Step Plus, $p = 0.5398$). Both adhesive systems responded positively to the cross-linking treatment, resulting in a significant increase in dentin bond strengths (One Step Plus, $p = 0.0124$, and Single Bond Plus, $p = 0.0023$) (Table 1). Glutaraldehyde and grape seed extract increased the μ TBS of both dentin substrates. All sound dentin groups showed significantly

Table 2. Proportions of Collagen Degradation in Residues and Supernatants after Collagenase Digestion (N = 8)

	DW		GA		GSE	
	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 statistically significant differences among groups. RES, residue (undigested); SUP, supernatant (digested collagen); DW, distilled water; GA, glutaraldehyde; GSE, grape seed extract.

higher dentin bond strengths when compared with their respective caries-affected groups for both adhesive systems ($p < 0.05$) (Table 1). Regardless of the dentin substrate, no statistically significant differences were observed between glutaraldehyde and grape seed extract treatment (Adper Single Bond, $p = 0.3431$; and One Step Plus, $p = 0.4993$).

Photomicrographs (Figs. A, B) of the fracture patterns of the control groups showed mostly cohesive failures at the bottom of the hybrid layer. Glutaraldehyde- and grape seed extract-treated groups showed a similar trend of fracture pattern with mixed failures, mainly cohesive in the resin and on top of the hybrid layer (Figs. C, D). Interestingly, the caries-affected dentin groups performed similarly to sound dentin in terms of mode of failure. The mean hardness (KHN) values found for sound dentin (99.9) were significantly higher ($p < 0.0001$) than those of caries-affected dentin (51.9).

Grape seed extract-treated samples showed significantly lower ($p < 0.001$) collagen digestibility when compared with glutaraldehyde-treated and control samples for both sound dentin and caries-affected dentin (Table 2). The results of amino acid analysis (Table 3) revealed that the contents of Lys (lysine) and Hyl (hydroxylysine) residues were markedly diminished in the glutaraldehyde-treated groups, while they were unchanged in the grape seed extract-treated groups. The mean results of the amino acid analyses comparing all other amino acids did not show any significant differences among their content (data not shown).

DISCUSSION

The present study demonstrated that increased bond strength can be obtained by the use of biochemical cross-linkers in both caries-affected and sound dentin. The increase in bond strength may be attributed to improved dentin collagen stability, due to the higher number of collagen cross-links (Han *et al.*, 2003; Bedran-Russo *et al.*, 2008). Glutaraldehyde has been associated with a decrease in the rate of collagen degradation (Cheung *et al.*, 1990; Han *et al.*, 2003) and improved dentin collagen properties (Bedran-Russo *et al.*, 2008). Glutaraldehyde reacts primarily with the ϵ -amino groups of peptidyl Lys and Hyl residues of collagen fibrils. The amino acid (AA) analysis revealed that the contents of Lys and Hyl residues were significantly diminished in glutaraldehyde-treated groups for sound and caries-affected dentin. The decrease in free (non-cross-linked) Lys and Hyl indicates that glutaraldehyde effectively cross-linked dentin collagen.

Figure. Representative SEM photomicrographs of the debonded surface. **(A)** Dentin side of a fractured specimen in the control sound group (Adper Single Bond) that failed at the bottom of the hybrid layer (black arrow). **(B)** Dentin side of a fractured specimen in the control caries-affected group (Adper Single Bond) that failed at the bottom of the hybrid layer. **(C)** Fracture pattern most commonly found in treated groups: mixed, top of the hybrid layer (black arrow); and cohesive, in adhesive (GA-treated sound dentin—One Step Plus). **(D)** Dentin side in the GSE-treated sound dentin group (One Step Plus), depicting failure at the top of the hybrid layer/adhesive layer. Magnification $\times 2500$; scale bar = 20 μ m.

Table 3. Lysine (Lys) and Hydroxylysine (Hyl) Content (res/1000 total residues) after Cross-linker Treatment

N = 3	Sound Dentin			N = 2	Caries-affected Dentin		
	DDW	GA	GSE		DDW	GA	GSE
Lys	21.6	4.1	15.9	Lys	20.7	5.8	17.4
Hyl	9.6	3.2	9.8	Hyl	9.9	2.7	8.9
Totals	1000.0	1000.0	1000.0	Totals	1000.0	1000.0	1000.0

Hyl, hydroxylysine; Lys, lysine; DDW, distilled and de-ionized water; GA, glutaraldehyde; GSE, grape seed extract.

Grape seed extract treatment also resulted in increased bond strength. Grape seed extract is composed mainly of proanthocyanidin, a naturally occurring cross-linking agent. The use of a proanthocyanidin-based cross-linking agent on demineralized dentin has been associated with a significant improvement in the mechanical and physical properties of dentin (Bedran-Russo *et al.*, 2007, 2008). The 4 proposed mechanisms for interaction between proanthocyanidin and proteins include covalent (Pierpoint, 1969), ionic (Loomis, 1974), hydrogen bonding (Ku *et al.*, 2007), and hydrophobic interactions (Han *et al.*, 2003). Based on amino acid analysis, it is likely that grape seed extract induces cross-links *via* a mechanism different from that of glutaraldehyde, in which Lys and Hyl are involved (Ritter *et al.*, 2001). Amino acid analysis indicated that the cross-links induced by grape seed extract are not stable in acid hydrolysis. Thus, for identification of the grape seed extract-induced cross-links, other approaches should be pursued.

The digestibility tests performed revealed that dentin treated with grape seed extract was significantly less susceptible to collagenase digestion than were the other groups (glutaraldehyde and control). The increased resistance to degradation may be attributed to masking of the recognition site by the cross-links, or to the retention of the cleaved peptide fragments by the newly formed cross-links, or to other, unknown, mechanisms (Charulatha and Rajaram, 2003).

Similar to previously published findings (Nakajima *et al.*, 1995; Yoshiyama *et al.*, 2002; Ceballos *et al.*, 2003), the present study found that untreated caries-affected dentin exhibited lower bond strengths when compared with sound dentin. The results may be due to the altered or partially denatured 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 caries-affected dentin was treated with grape seed extract or glutaraldehyde, the bond strength reached values comparable with those of sound dentin, indicative of increased collagen stability.

The increase in mixed modes of failure at the adhesive/resin interface and at the top of the hybrid layer shown by the bond strength data may indicate that the hybrid layer was strengthened by cross-linkers. Hence, failure at the adhesive layer/top of the hybrid layer in the treated groups may indicate that the cross-linked dentin matrix mechanically strengthened the bottom of the hybrid layer in which demineralized dentin was inadequately infiltrated by resin.

The results of the KHN tests confirmed the presence of caries-affected dentin. Sound dentin was harder than caries-affected dentin, which was consistent with previously reported data (Ceballos *et al.*, 2003; Zheng *et al.*, 2005). Although the tubules were 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 caries-affected compared with sound dentin.

Within the limitations of this *in vitro* study, it was concluded that the application of grape seed extract and glutaraldehyde to dentin significantly improved the microtensile bond strengths to caries-affected and sound dentin. The stabilization of dentin collagen by glutaraldehyde- and grape seed extract-induced cross-links significantly increased resistance to enzymatic degradation in sound as well as caries-affected dentin; likely *via* distinct mechanisms. Future experiments using different concentrations will assess favorable improvements in bond strength using more clinically relevant times. The results of this study may provide insights into the development of novel strategies for efficient and stable dentin bonding with the use of collagen cross-linkers.

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