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Mechanical characterization of proanthocyanidin-dentin matrix interaction

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Abstract

Objectives—To characterize the properties of dentin matrix treated with two proanthocyanidin rich cross-linking agents and their effect on dentin bonded interfaces.

Methods—Sound human molars were cut into 0.5 mm thick dentin slabs, demineralized and either treated with one of two cross-linking agents (grape seed - GSE and cocoa seed - COE extracts) or left untreated. The modulus of elasticity of demineralized dentin was assessed after 10 or 60 min and the swelling ratio after 60 min treatment. Bacterial collagenase was also used to assess resistance to enzymatic degradation of samples subjected to ultimate tensile strength. The effect of GSE or COE on the resin-dentin bond strength was evaluated after 10 or 60 min of exposure time. Data were statistically analyzed at a 95% confidence interval.

Results—Both cross-linkers increased the elastic modulus of demineralized dentin as exposure time increased. Swelling ratio was lower for treated samples when compared to control groups. No statistically significant changes to the UTS indicate that collagenase had no effect on dentin matrix treated with either GSE or COE. Dentin-resin bonds significantly increased following treatment with GSE regardless of the application time or adhesive system used.

Significance—Increased mechanical properties and stability of dentin matrix can be achieved by the use of PA-rich collagen cross-linkers most likely due to the formation of a PA-collagen complex. The short term dentin-resin bonds can be improved after 10 minutes dentin treatment.

Keywords

dentin; collagen; natural cross-link; bond strength; mechanical properties

Introduction

Adhesive restorations are routinely used to replace carious dental tissue, fractured tooth, and replacement of defective restorations. Despite significant improvement of adhesives systems, the bonded interface formed by a mixture of dentin organic matrix, residual hydroxyapatite

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crystallites, resin monomers and solvents, still remains the weakest area of adhesive restorations [1]. Moreover, failure at the bonded interface may lead to the formation of pathways in which oral fluid, bacterial products and endogenous proteolytic enzymes can degrade the components. Deterioration of the dentin collagen fibrils has been suggested as a possible mechanism responsible for adhesive bonds degradation [2].

Fibrillar type I collagen accounts for 90% of the dentin organic matrix while the remaining 10% consists of non-collagenous proteins such as phosphoproteins and proteoglycans [3]. Lower biodegradation rates and high mechanical properties of collagen are desirable for many *in vivo* applications, such as restorative dentistry procedures. The induction of exogenous collagen cross-links has been proposed as a mechanism to improve the mechanical stability and reduce the biodegradation rates of collagen [4]. Several synthetic (glutaraldehyde, carbodiimides and others) and natural occurring (genipin, proanthocyanidin from grape seed extract and others) agents can induce exogenous collagen cross-links [5,6].

Proanthocyanidin (PA) is a natural collagen cross-linker [7] well known to readily precipitate proline rich proteins (such as collagen) due to hydrogen and covalent bonds [8]. PAs are considered one of the most important classes of secondary metabolites in the plant kingdom available in fruits, vegetables, nut, seeds, flowers, and barks [6]. It is well documented that cocoa and its products, along with grape seed are among the richest sources of PAs [9]. Recent studies have shown that a PA-based cross-linker agent (grape seed extract) increased the mechanical properties of demineralized dentin matrix [7,10] and enhanced the resin-dentin bond strength after 1 hour treatment [11].

In an attempt to reduce treatment time to reproduce a more clinical relevant procedure, this study compared the effect of two different PA-based cross-linker (cocoa and grape seed extracts) on resin-dentin bonded interface using two different treatment times (10 min and 60 min); and characterized the effect of different sources of PA on the mechanical properties and resistance against enzymatic degradation of demineralized dentin matrices. The null hypothesis tested was that the use of PA-based cross-linkers would not affect the dentin bond strength and properties of demineralized dentin when compared to a non-treated group.

Materials and Methods

The use of seventy eight sound molars was approved by the Institutional Review Board Committee from the University of Illinois at Chicago (protocol # 2009–0198). Cocoa seed extract (*Theobroma cacao* – Foratero) was obtained from Barry Callebaut (Lebbeke-Wieze, Belgium). Grape seed extract (*Vitis vinifera*) was obtained from Polyphenolics (Madera, CA, USA). Bacterial collagenase from *Clostridium histolyticum* (type I, ≥ 125 CDU/mg solid) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Modulus of elasticity of demineralized dentin

Seven teeth were sectioned into 0.5 ± 0.1 mm thick slabs ($n = 7$ slabs per tooth) in the mesio-distal direction with a slow speed diamond wafering blade (Buehler-Series 15LC Diamond) under constant water irrigation. The sections were further trimmed to a final rectangular dimension of 0.5 mm thickness $\times$ 1.7 mm width $\times$ 7.0 mm length using a cylindrical high speed diamond bur (#557D, Brasseler, Savannah, GA). A dimple was made at one end of each sample to allow for repeated measurements to be performed on the same surface. Specimens were immersed in 10% phosphoric acid solution (LabChem, Pittsburgh, PA) for a period of 5 hours and thoroughly rinsed with distilled water for 10 min. Demineralized specimens ($n=12$) were treated with one of two different cross-linkers, grape seed extract (GSE) or cocoa seed extract (COE), with the same concentration (6.5%) and dissolved in distilled water and acetone-water (30:70) respectively. All solutions had the pH adjusted to 7.4 using NaOH. Two control groups

(no cross-linking agents) were tested, one in distilled water and other in acetone-water following the same protocol described for the experimental groups. Specimens were immersed in water for baseline measurements and then in their respective solutions for either 10 or 60 min.

An aluminum alloy fixture with 2.5 mm span where specimens were tested in 3-point bending while immersed in liquid using a 1 N load cell mounted on a universal testing machine (EZ Graph, Shimadzu, Kyoto, Japan) at crosshead speed of 0.5 mm/min. Displacement (D) during compression was displayed in millimeters and calculated at a maximum strain of 3%. The modulus of elasticity (E) was obtained as previously described [10] and calculated as follow:

$$E = PL^3 / 4DbT$$

Where P is the maximum load, L is the support span, D is the displacement, b width of the specimen, and T is the thickness of the specimen. The data were collected and statistically analyzed using a General Linear Model SPSS program for ANOVA followed by Post-Hoc Tukey test at a 95% confidence interval.

Swelling ratio

Dentin samples ($0.5 \times 1.7 \times 7.0$, $n=12$) were treated for 1 hour in either cross-linking solution (GSE and COE) or both control groups (distilled water and acetone) as described above. After treatment, samples were swollen in water and equilibrated overnight in PBS (pH 7.4) at room temperature. The specimens were removed quickly blotted with filter paper to remove excess surface water and weighed immediately. Dentin samples were then placed in a large volume of deionized water to remove the buffer salts and air dried to constant weight. The swelling ratio (Q) was calculated as the ratio of the weight of swollen sample to that of dry sample [12]. Data was analyzed using a General Linear Model SPSS program for ANOVA followed by a Pos-Hoc Tukey's test at a 95% confidence interval.

Resistance against enzymatic degradation – Ultimate tensile strength (UTS)

Eleven teeth were sectioned into 0.5 mm thick slabs and trimmed to an hour-glass shaped sample with neck area of $0.5 \pm 0.1 \times 0.5 \pm 0.1$ mm at middle dentin using a cylindrical diamond bur (#557, Brasseler). Samples were fully demineralized using 10% phosphoric acid solution (LabChem, Inc) for 5 hours, thoroughly rinsed and immediately randomly divided into three treatments ($n=12$): control (distilled water), 6.5% GSE in distilled water and 6.5% COE in acetone-water (30:70). Specimens were kept in their respective solutions for 1 hour, thoroughly rinsed and either subjected or not to enzymatic challenge for 24 hours using bacterial collagenase (100 µg/ml) in 0.2M ammonium bicarbonate buffer (pH= 7.5) or 24 hours in buffer only. Enzymatic activity of bacterial collagenase has already been proved to successfully challenge dentin matrix [13]. For UTS evaluation, the specimens were glued with a cyanoacrylate adhesive (Loctite Superbond, Henkel, Avon, OH, USA) to a jig, which was mounted on a microtensile tester machine (Bisco, Schaumburg, IL, USA) and subjected to a tension force at a crosshead speed of 1mm/min. Means and standard deviations were calculated and expressed in MPa. Statistical analysis was performed using a General Linear Model SPSS program for ANOVA two-way (treatment and collagenase challenge) followed by a Post-Hoc Tukey's test at a 95% confidence interval.

Resin-dentin microtensile bond strength

The occlusal surfaces of 60 molars were ground flat using #180, 320 and 600 grit silicon carbide papers (Buehler, Lake Bluff, IL) under running water to expose middle coronal dentin. The

prepared dentin surfaces were divided into six groups (n=20): control distilled water for 10 minutes, control distilled water for 60 min, 6.5% GSE for 10 min, GSE for 60 min and 6.5% COE for 10 min and COE for 60 min. Each group was either restored using Adper Single Bond Plus (SB, 3M ESPE, St Paul, USA) or One Step Plus (OS, Bisco, Schaumburg, USA). Dentin surfaces were etched with the respective system's etchants for 15 seconds, rinsed, treated with cross-linking solutions and then thoroughly rinsed. The bonding procedures were performed following manufacturers' instructions. The control group followed the same protocol but distilled water was used instead of cross-linking solutions. Filtek supreme (3M ESPE) was used to build a 5 mm crown incrementally. Specimens were stored in distilled water at 37° C for 24 h.

Teeth were sectioned into $0.7 \times 0.7 \pm 0.1$ mm resin-dentin beams that had their edges glued with cyanoacrylate to a jig, and were then tested in a microtensile tester machine (Bisco, Schaumburg, IL, USA) at a crosshead speed of 1.0 mm/min. Five beams with at least 2 mm remaining dentin thickness were tested per tooth. Microtensile bond strength (MPa) was determined by dividing the fracture load by the cross-sectional area of the interface. Mean bond strength values for both adhesives systems were analyzed using a General Linear Model SPSS program for two-way ANOVA (treatment and exposure time) followed by a Post-Hoc Tukey's test at a 95% confidence interval.

Results

The demineralized dentin matrix modulus of elasticity data as a function of time and different treatments are depicted on Table 1. A statistically significant interaction was observed between the factors (treatment and time) ($p < 0.001$). Dentin treatment and different exposure times significantly affected the values ($p < 0.001$). Exposure time did not significantly affect the elastic modulus of both control groups ($p = 0.161$ distilled water, $p = 0.861$ acetone), but values significantly increased as exposure time to GSE and COE increased ($p < 0.001$, for both).

Figure 1 presents the results of the swelling ratio. Different treatments significantly affected the swelling ratio ($p < 0.001$). The use of cross-linking agents reduced the swelling ratio when compared with control groups. A comparison of the swelling ratio of all groups reveals that COE causes minimum weight gain when swollen followed by GSE treated samples.

In vitro enzymatic degradation assay revealed that untreated collagen samples exposed to bacterial collagenase was found completely digested after 24 hours (Table 2). UTS values significantly increased following treatment with both cross-linking agents ($p < 0.001$). GSE- and COE- treated samples were highly resistant to digestion with collagenase and the UTS values were not significantly affected by collagenase exposure ($p = 0.198$ /GSE; $p = 0.109$ /COE).

Resin-dentin microtensile bond strength data is depicted on Table 3. Two-way ANOVA revealed no statistically significant interaction among the factors evaluated (surface treatment and exposure, $p = 0.674$ /SB and $p = 0.422$ /OS). Treatment of the surface with GSE resulted in statistically significant increase in the μ TBS values when compared to a control group and COE ($p < 0.001$, SB and OS). There were no statistically significant differences between 10 or 60 min exposure time ($p = 0.168$ /SB; $p = 0.984$ /OS).

Discussion

There are enduring challenges associated with adhesive dentistry such as the poor infiltration of wet dentin with resin monomers that yield a weaker hybrid layer [1]. We hypothesize that if a stronger and more stable collagen layer is chemically engineered, the resultant hybrid layer will be stronger and less prone to degradation. In the present study, PA based cross-linking agents were examined to assess its potential effect as a biomimetic agent to enhance the

mechanical properties of demineralized dentin and bond strength when compared to a non-treated group. According to our results, the null hypothesis was partially rejected.

The time dependant enhancement of the mechanical properties of demineralized dentin treated with PA confirms previous findings [7,10]. Different mechanisms of interactions of PA-based agents and proteins are most probably involved in the remarkable increase in the demineralized dentin stiffness when compared to the control groups (Table 1). Interestingly, the source of PA had a statistically significant effect on the stiffness values. Tannins from grape seed consist of a complex mixture of oligomers and polymers composed of the monomeric flavan-3-ols catechin, epicatechin and their gallates [14]. While cocoa is composed mainly of epicatechin units [15]. This simplicity in procyanidin composition makes it possible to detect the higher oligomers in cocoa by high performance liquid chromatography and mass spectrometry (HPLC/MS) [15,16]. The much more complex composition of the grape seed procyanidins reveals numerous diastereomers and as the molecular weight increases, the number of isomers becomes so large that the separation and detection of individual isomers become challenging by HPLC/MS [15]. The chemical structure of grape seed and cocoa flavan-3-ols dictates their physical properties and reactivity, as well as their interactivity with collagen [17]. Since, different tannins show variations in interaction with a given protein [18] it is important not only to analyze GSE that has already been proved efficient but other PA rich extracts, such as cocoa.

The bond strength of 1 hour GSE-treated dentin restored with 2 commercial adhesives systems (Single Bond and One Step) has already been assessed [11]. 6.5% GSE-treatment produced the highest bond strength values that were statistically higher than glutaraldehyde treated and a control group [11]. The authors suggested that GSE has far more interactive ability with collagen than glutaraldehyde. The present study observed the effect of cross-linking agents after 10 minutes treatment since 1 hour application time is not clinically feasible. No statistically significant difference was observed for both treatment times for both GSE and COE, but only GSE presented higher values of bond strength when compared to an untreated group. COE has been described as a potential collagen cross-linker with increased time exposure [19] and this may be due to the diversity of structural composition, as already described, and proanthocyanidin content of extracts. The lower content of PA (values provided by manufacturers) present in COE (approximately 45%) when compared to GSE (at least 95%) could delay or even decrease the interactive ability with collagen.

The cross-linking mechanism between PA and collagen, though not well defined, may be primary by the formation of hydrogen bonding between the protein amide carbonyl and the phenolic hydroxyl [20]. The relative stability of these cross-links compared with other types of polyphenols suggests that only some PA-molecules, would interact with collagen protein [6]. Therefore, COE not only contains less PA, but could also have less active compounds. The complexity of natural products, their chemical structures and extraction mode resulted on the use of different solvents for each extract. These findings were concluded in a previous study (data not presented).

Both cross-linkers presented high ultimate tensile strengths which were not affected by collagenase digestion. The resistance to enzymatic degradation is a crucial property of a PA treated dentin matrix, since it indicates an increase of stability and possibly a protective mechanism over a long period of time. Tannic acid is also a polyphenol that has been shown to decrease enzymatic rates most likely by masking the cleavage sites or decreasing the enzymatic activity [21]. The induction of exogenous cross-link in dentin matrix also leads to a decrease in the swelling ratio [22,12,23]. The network formed by exogenous cross-links would be dense, and impaired water absorption by the matrix. This phenomenon was observed in this study for both collagen cross-linkers. A statistically significant difference was pointed

between GSE and COE when compared to their respectively controls. The low swelling ratio for the treated groups may indicate that not only masking of the cleavage sites or decrease in the collagenase activity may affect the degradation of the treated dentin matrix. Low swelling ratio may indicate a decrease in the collagenase absorption [22] and therefore assist on the matrix resistance against enzymatic degradation. Lower swelling values for acetone control groups could be explained by the increased ability of the acetone-saturated collagen fibrils to form interpeptide bonds that also stiffens the collagen matrix, by reducing the plasticizing effect of water [24]. Apparently the equilibration overnight in PBS could not eliminate the acetone effect in the dentin matrix. Both assays, swelling ratio and resistance to enzymatic degradation, were only performed after 60 minutes of treatment with the cross-linkers due to quantity of samples and teeth needed.

It is well accepted that bond strength and durability rely on the quality of the hybrid layer rather than on the thickness or morphology of hybrid layer/resin tags [25]. Major concerns have been recently expressed regarding interfacial aging due to degradation of the hybrid layer, water absorption, hydrolysis of the resin and disruption of the collagen network [1]. The demineralized dentin treated with PA-based agents decreased collagenase degradation, increase mechanical properties and reduced water absorption. The dense collagen matrix would be potentially less susceptible to creep rupture [26] or cyclic fatigue rupture [27] after prolonged intraoral function. It appears in our study that there is a notable correlation between the results of stiffness and bond strength. The stiffer the dentin matrix becomes the more suitable it is as a collagen substrate for hybridization.

Conclusions

GSE and COE agents have shown to increase the mechanical properties of dentin matrix, reduce the rate of water absorption and diminish collagen degradation. Changes to the dentin matrix positively affected the μ TBS. The quantity and types of PA may influence its interaction with collagen fibrils and consequently the resin-dentin bond strength.

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Figure 1.

Swelling ratio of demineralized dentin samples following different treatments.

Different letters indicate statistically significant ($p < 0.05$) differences.

DW- distilled water; GSE- grape seed extract; AC- acetone; COE AC- cocoa seed extract dissolved in acetone water.

Table 1

Modulus of elasticity (MPa) means and standard deviations of the demineralized dentin matrix following different treatments and exposure times.

Treatment	Exposure Time		
	Baseline	10 min	60 min
Control DW	6.99 (1.85) ^{A,a}	5.86 (1.47) ^{A,a}	6.02 (1.17) ^{A,a}
Control AC	6.76 (2.05) ^{A,a}	6.85 (2.01) ^{A,a}	7.17 (2.33) ^{A,a}
GSE	6.59 (2.59) ^{A,a}	39.64 (17.95) ^{B,b}	73.66 (36.96) ^{C,b}
COE	6.28 (3.08) ^{A,a}	18.82 (9.06) ^{B,c}	33.50 (17.10) ^{C,c}

Different lower case letters indicate statistically significant ($p < 0.05$) differences within each column.

Different upper case letters indicate statistically significant ($p < 0.005$) differences within each row.

DW- distilled water; GSE- grape seed extract; AC- acetone; COE- cocoa seed extract.

Table 2

In vitro dentin matrix resistance against enzymatic degradation for dentin treatments. Ultimate tensile strength means [MPa] and standard deviations are presented.

Treatment	Enzymatic degradation	
	Buffer	Buffer with collagenase
GSE	19.5 (6.06) ^a	18.17 (4.54) ^a
COE	16.33 (2.60) ^b	17.15 (4.32) ^b
Control	11.53 (5.27) ^{c*}	0.00 ^{c*}

Different letters indicate statistically significant ($p < 0.05$) differences among dentin treatments.

Asterisk (*) indicates statistically significant ($p < 0.05$) difference after collagenase digestion.

GSE- grape seed extract; COE- cocoa seed extract.

Table 3

Microtensile bond strength values (standard deviation) (MPa) for adhesive systems following use of collagen cross-linkers.

Adhesive	Treatment	Exposure Time	
		10 min	60 min
Single Bond	GSE	57.44 (11.43) ^a	61.08 (6.34) ^a
	COE	49.97 (5.8) ^b	51.25 (2.96) ^b
	Control	52.42 (19.55) ^b	57.87 (18.56) ^b
One Step	GSE	63.47 (5.49) ^A	64.12 (2.5) ^A
	COE	54.44 (4.3) ^B	55.59 (9.18) ^B
	Control	50.39 (12.19) ^B	55.44 (5.26) ^B

Different lower and upper case letters indicate statistically significant ($p < 0.05$) differences between groups for SB (single bond) and OS (one step) respectively.

GSE- grape seed extract; COE- cocoa seed extract.