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Nanomechanical properties of biochemically modified dentin bonded interfaces

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Summary

The effect of biomodification of dentin matrices using collagen cross-linkers, glutaraldehyde (GD) and grape seed extract (GSE), on the reduced modulus of elasticity (Er) and nanohardness (H) of the hybrid layer and underlying dentin was investigated at the dentin-resin bonded interface. The coronal dentin of nine molars were exposed and divided into groups: 5% GD, 6.5% GSE and control. Control samples were etched, bonded with Adper Single Bond Plus and Premise composite. GD and GSE were applied for 1 hour prior to bonding procedures. After 24 hours, samples were sectioned, and resin-dentin beams were either kept in distilled water or exposed to collagenase treatment for 24 hours. Nano-indentations were performed at the hybrid layer and underlying dentin. GD and GSE treatment increased the Er and H of resin-dentin interface structures when compared to the control group ($p < 0.05$), particularly the hybrid layer, and may be a promising novel approach to strengthen the dentin-resin bonded interface structures when using these adhesive system and resin-based composite.

Keywords

collagen; adhesion; mechanical properties; interfaces; dental materials

Introduction

The adhesion of resin based materials to dentin relies on the impregnation of resin into a superficially decalcified dentin zone of exposed collagen fibrils (1). When collagen is not fully enveloped by adhesive or porosity are present in the hybrid layer, micro- and nano-pathways can expedite the process of interface degradation (2). Degradation of resin/dentin interfaces contribute to the reduction in the bond strengths produced by dentin adhesives over time (3), as well as increased leakage at the interface (4). It is believed that the interface

degradation process could be caused by hydrolytic degradation of demineralized collagen matrix and elution of unpolymerizable monomers (3–4).

The degradation of collagen matrices can occur by host-derived matrix metalloproteinases (MMPs) presented in the saliva and released from the dentin matrix (5) or collagenolytic activity from bacteria such as *Streptococcus Mutans* (6). According to Chaussain-Miller et al. host-derived MMPs could be activated by acidic pH and once activated, they are able to digest demineralized dentin matrix even after pH neutralization by salivary buffers (7). In order to stabilize collagen fibrils in biological tissues, some natural or synthetic cross-linkers agents have been studied to maintain, restore and improve dentin function (8). Cross-linkers such as proanthocyanidin based agents and glutaraldehyde have the ability to enhance the strength of dentin and dentin-resin interfaces (8–10) as well as positively affect the remineralization process in artificial root caries (11). However, the direct effect of the agents in the adhesive interface properties is not well established in the literature.

The aim of the present study was to evaluate the effect of two cross-linking agents (glutaraldehyde and grape seed extract, consisting mainly of proanthocyanidin) on the nano-mechanical properties of adhesive interfaces components using a nanoindentation approach. The null hypothesis tested was that collagen cross-linking agents will not affect the reduced modulus of elasticity and hardness of two major components of the adhesive interface (hybrid layer and underlying dentin).

Materials and methods

Nine extracted sound human third molars were used following the Institute Review Board Committee from the University of Illinois at Chicago (protocol # 2006-0229). The teeth were cleaned and kept frozen (–20 °C) for 2–3 weeks. The occlusal surface was ground flat with #180, 320 and 600 grit silicon carbide paper (Buehler, Lake Bluff, IL, USA) under running water to remove enamel and expose dentin surface. The teeth were divided into three groups according to the adhesive procedure:

Control group (C): Surface was etched with 35% phosphoric acid etchant gel (3M ESPE, St. Paul, MN, USA) for 15 sec, rinsed for 30 sec, and excess water was removed with absorbent paper. Two layers of Adper Single Bond Plus (3M ESPE) were applied on the surface, air-dried to allow for solvent evaporation and light-cured for 20 sec with Optilux 501 (Kerr Demetron, Danbury, CT, USA). Premise nanofilled resin composite (Kerr Corporation) was applied and cured in 2.0 mm increments to form 6 mm-high build-ups on the bonded dentin surfaces. Each layer was light-cured for 40 sec.

Glutaraldehyde (GD): Same procedures were carried out as described for C except that after etching and rinsing, the teeth were immersed in 5% GD solution (Fisher Scientific, pH 7.2) for one hour. (9)

Grape Seed Extract (GSE): Same procedures were carried out as described for C, except that after etching and rinsing, the teeth were immersed in 6.5% grape seed extract (Mega-Natural, Polyphenolics, pH 7.2) for one hour (9). The main component of grape seed extract is proanthocyanidins (97.8%).

After 24 hours immersion in distilled water (37°C) the teeth were sectioned from the center of each specimen into 4 beams (approximately 1.5×1.5 mm thick) with a low speed diamond blade (Isomet 1000, Buehler). The beams were embedded in epoxy resin and allowed to cure for 8 hours. The specimens were ground with #180, 320, 600, 800 and 1200-grit silicon carbide paper (Buehler) and polished with 9, 6, 3, 1 and $0.5\mu\text{m}$ polycrystalline diamond suspension (Buehler).

Collagenase challenge

Half of the samples (n=6) were subjected to enzymatic challenge using bacterial collagenase (from *clostridium histolyticum* – Sigma Aldrich) in 0.2M ammonium bicarbonate buffer for 24 hours. The other half of the samples were stored in HBSS (Hank's buffered salt solution) for 24 hours (n=6). A schematic diagram of the experimental treatments is represented on Figure 1.

Nano-mechanical properties

The hardness (H) and reduced modulus (E_r) of dentin and hybrid layer were measured using a Hysitron Custom Triboindenter (Hysitron Inc, Minneapolis, MN). A fluid cell Berkovich tip was used at force $1000\mu\text{N}$ and a standard trapezoidal load function of 5-2-5 seconds. The measurements were performed under hydrated conditions where samples were kept immersed in Hank's solution. Five indentations were done for dentin and hybrid layer in all of the samples. The data of reduced modulus (E_r) and hardness (H) were calculated based on the load-displacement curves according to the following relationships (12):

$$E_r = S \cdot \pi / 2 \cdot A$$

Where S is the initial unloading stiffness and A is the projected contact area between the indenter tip and the sample at maximum load.

$$H = P_{\text{max}} / A$$

Where P_{max} is the maximum load and A is the same projected contact area as described for the calculus of reduced modulus. The percentages of the unloading curve were 20% lower limit and 95% upper limit. Statistical analysis was performed using two-way ANOVA and Fisher's PLSD test ($p < 0.05$).

Results

The indentation image on the dentin and the hybrid layer is illustrated in Figure 2. In the image, we can observe two indentations in underlying dentin and hybrid layer. Because of the differences in the modulus of elasticity of these structures (Tables 1), the indentations in the hybrid layer are bigger than in the underlying dentin, evidencing the distinct mechanical properties in those regions.

Table 1 shows that GD and GSE treatments increased the reduced modulus of elasticity (E_r) values of the hybrid layer when compared to control ($p = 0.001$). GD treatment also resulted in increased E_r of the underlying dentin after enzymatic degradation, followed by GSE and control group ($p < 0.001$) (Table 1). The hardness of dentin was not altered by cross-linkers application (GD or GSE) when compared with control group ($p = 0.738$). However, both GD

and GSE cross-linkers agents increased the hybrid layer hardness when compared to a control group ($p < 0.001$, no digestion and $p = 0.003$, after enzymatic degradation). These values are depicted in Table 2.

Discussion

Both collagen cross-linkers increased the nanomechanical properties of the hybrid layer when compared to a control group. In addition, glutaraldehyde and grape seed extract also increased the reduced modulus of dentin for the samples exposed to collagenase degradation. Therefore the null hypothesis of the present study was rejected. The concentration of GD (5%) and GSE (6.5%) were based on a previous study that report an increase in the stiffness of demineralized dentin compared with other cross-linkers concentrations (10). Samples were tested in Hank's solution to better reflect the hydrated condition on the tooth and oral environment. Hank's balanced salt solution is able to maintain the mechanical properties of dentin, while storage in deionized water or CaCl_2 solution can demineralized the surface, decreasing the modulus and hardness of these structures (13).

The values of reduced modulus and hardness were higher for underlying dentin when compared to the hybrid layer. The mechanical property of dentin was based on nanoindentations made on intertubular underlying dentin, since this structure dictates the elastic behavior of dentin (14). The relative high variation in the data could be explained by the substantial variation in modulus of intertubular dentin which is related to the distribution of apatite in the collagen matrix (15). In the hybrid layer, the collagen and partially demineralized apatite should be filled by adhesive resins (16). The hybrid layer consists of a complex multi-layer structure with morphologies and properties that are highly sensitive to both the demineralization procedure and specific characteristics of the bonding system (17). The properties of hybrid layer are dependent of the strength and relative volumes of both collagen mesh and adhesive resin (18). In this manner, we could expect a hybrid layer with lower values of mechanical properties compared with underlying dentin and also with a high variation in the results as showed in Tables 1 and 2.

Naturally occurring cross-linkers such as proanthocyanidins have been shown to improve the mechanical properties of demineralized dentin, and is believed to function as a dentin matrix stabilizer (8). In the present study, the application of grape seed extract (GSE) increased the mechanical properties of the hybrid layer when compared to a control group, which supports that biomodification of demineralized dentin resulted in stronger hybrid layer. The main component of grape seed extract is proanthocyanidins (97.8%) as provided by the manufacturer. Proanthocyanidin is a mixture of monomers, oligomers, and polymers known as catechins widely used as natural antioxidants and free-radical scavengers proven to be safe in various clinical applications and dietary supplements (19–20). Other natural product as elm cortex, which main active component is proanthocyanidin also showed inhibitory effects against metalloproteinases and proteases in periodontal diseases (21). The primary mechanism of collagen stabilization with proanthocyanidin is the formation of hydrogen bonding between the protein amide carbonyl and the phenolic hydroxyl (22–23). This hydrogen bonding is responsible for the increase in the denaturation temperature of

fixed tissue (22). The proline-rich polypeptides, like collagen, have an extremely high affinity for proanthocyanidin and are precipitated by them most efficiently at pH values near their isoelectric points (24). The application of grape seed extract also increased the bond strength of adhesive system to dentin (9) and enhanced the strength of dentin (8–10).

Glutaraldehyde (GD) treatment not only increased the Er of the hybrid layer but also the underlying dentin properties when compared to a control group (Tables 1 and 2). GD has been widely used as a fixative of biological tissues, and to decrease the rates of tissue degradation (25–26). Furthermore, it can inhibit root caries (23) and prevent dentin demineralization (27–28). Other studies also showed the efficiency of glutaraldehyde to improve the mechanical properties of demineralized dentin (10,29) and ability to increase the dentin-resin bond strengths (9,30). The GD reaction with amino groups of collagen is well documented (31); therefore increased nanomechanical properties observed in the present study are probably due to the GD interactions with dentin collagen. We hypothesize that changes to the underlying dentin are most likely due to ability of GD to infiltrate mineralized tissue as opposed to GSE.

Collagen cross-linkers also have been shown to have a protective mechanism against enzymatic degradation (22,26), therefore collagenase treatment was used to possibly simulate protein degradation at the interface. In this study, the collagenase digestion did not affect the hardness or reduced modulus of dentin and hybrid layer. The bacterial collagenase isolated from *Clostridium histolyticum* belongs to M-31 metalloproteinases family, which is able to hydrolyze triple helical regions of collagen under physiological conditions (32). This is a relatively large protein (ca. 116kDa) and it may not permeate into resin-dentin interface unless there are porosities due to decalcification of non-resin infiltrated dentin layer or resin dissolution over time (33). The short term analysis performed in this study could explain the lack of statistical differences between collagenase digestion and no digestions groups, although collagenase numerically affected the hardness values of GD-treated HL. Hence, the penetration of exogenous collagenase would not be necessary to degrade the resin-dentin interface if endogenous metalloproteinases response could trigger organic matrix destruction (7,34).

The present study demonstrates that collagen cross-linker agents such as grape seed extract and glutaraldehyde improve the mechanical properties of resin-dentin bonded interface components, mainly the hybrid layer. Further studies should assess the long-term behavior on the biomechanically modified interfaces as well as the effects of collagen cross-linkers agents with other adhesive systems and resin-based composites with different compositions. Biomodification of dentin matrix may be a novel approach to improve the properties of main components of the resin-dentin bonds.

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Figure 1.

A schematic diagram of the experimental treatments.

Figure 2.

Representative topography image of the indentations performed at the hybrid layer and underlying dentin components of the interface. (C: resin composite, A: adhesive layer, HL: hybrid layer, D: underlying dentin).

Table 1

Reduced modulus of elasticity (GPa) values (means and standard deviations) of the hybrid layer and underlying dentin following surface treatment.

		Control	GD	GSE
Hybrid layer	No digestion	9.41 ± 3.33 b	12.36 ± 3.56 a	11.68 ± 3.23 a
	Enzymatic digestion	8.81 ± 2.76 b	12.37 ± 3.52 a	11.78 ± 2.98 a
Underlying dentin	No digestion	22.76 ± 4.36 A	23.07 ± 2.75 A	21.27 ± 4.14 B
	Enzymatic digestion	21.34 ± 2.48 C	24.27 ± 3.08 A	22.85 ± 3.02 B

Lower and upper cases indicate statistically significant differences ($p < 0.05$) on each row for hybrid layer and underlying dentin, respectively. GD: Glutaraldehyde; GSE: grape seed extract.

Table 2

Hardness (GPa) values (means and standard deviations) of hybrid layer and underlying dentin following surface treatment.

		Control	GD	GSE
Hybrid layer	No digestion	0.48 ± 0.23 b	0.76 ± 0.25 a	0.63 ± 0.22 a
	Enzymatic digestion	0.47 ± 0.18 b	0.61 ± 0.19 a	0.63 ± 0.25 a
Underlying dentin	No digestion	1.16 ± 0.32 A	1.09 ± 0.24 A	1.10 ± 0.20 A
	Enzymatic digestion	1.10 ± 0.19 A	1.15 ± 0.23 A	1.11 ± 0.20 A

Lower and upper cases indicate statistically significant differences ($p < 0.05$) on each row for hybrid layer and underlying dentin respectively. Glutaraldehyde; GSE: grape seed extract.