Effect of proanthocyanidin incorporation into dental adhesive resin on resin–dentine bond strength

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ABSTRACT

Objectives: This study evaluated the effect of proanthocyanidin (PA) incorporation into experimental dental adhesives on resin–dentine bond strength.

Methods: Four experimental hydrophilic adhesives containing different PA concentrations were prepared by combining 50 wt% resin comonomer mixtures with 50 wt% ethanol. Proanthocyanidin was added to the ethanol-solvated resin to yield three adhesives with PA concentrations of 1.0, 2.0 and 3.0 wt%, respectively. A PA-free adhesive served as the control. Flat dentine surfaces from 40 extracted third molars were etched with 32% phosphoric acid. The specimens were randomly assigned to one of the four adhesive groups. Two layers of one of the four experimental adhesives were applied to the etched dentine and light-cured for 20 s. Composite build-ups were performed using Filtek Z250 (3M ESPE). After storage in distilled water at 37 °C for 24 h, twenty-four bonded teeth were sectioned into 0.9 mm × 0.9 mm beams and stressed to failure under tension for bond strength testing. Bond strength data were evaluated by one-way ANOVA and Tukey’s test (α = 0.05). Interfacial nanoleakage was examined in the remaining teeth using a field-emission scanning electron microscope and analysed using the Chi-square test (α = 0.05).

Results: No significant difference in bond strength was found amongst PA-free, 1% and 2% PA adhesives. However, incorporation of 3% PA into the adhesive significantly lowered bond strength as demonstrated by a greater number of adhesive failures and more extensive nanoleakage along the bonded interface.

Conclusion: Incorporation of 2% proanthocyanidin into dental adhesives has no adverse effect on dentine bond strength.

Clinical significance: The addition of proanthocyanidin to an experimental adhesive has no adverse effect on the immediate resin–dentine bond strength when the concentration of proanthocyanidin in the adhesive is less than or equal to 2%.

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1. Introduction

Despite improved understanding of the adhesion science involved in dentine bonding over the past two decades, the average replacement time of a tooth-coloured resin restoration is only 5.7 years. Biodegradation of the bonded interface is still the major factor responsible for replacement of most aesthetic bonded restorations. The durability of resin–dentine bonds requires stable collagen fibrils within the hybrid layer. However, application of simplified etch-and-rinse adhesives to demineralised dentine activates endogenous collagen-bound and unbound matrix metalloproteinases (MMPs) and results in progressive loss of unprotected collagen fibrils from the hybrid layer. In contrast, the involvement of endogenous MMPs in the bond degradation process is minimal for mild self-etch adhesives.

Biomodification of demineralised collagen matrices with exogenous cross-linking agents plays an important role in improving the biomechanical properties of dentine. Strengthening of collagen fibrils by cross-linking agents decreases enzymatic degradation, which is critical for increasing the stability of the hybrid layer and the durability of bonded restorations. Examples of commonly used cross-linkers in dentistry are glutaraldehyde, genipin, carbodimide and proanthocyanidin (PA).

Proanthocyanidin, a plant flavonoid, is prevalent in pine bark, elm tree and grape seeds. It is also commonly available in vegetables and fruits, but in lower concentrations. Proanthocyanidin is a potent antioxidant and cross-linking agent with low toxicity. Several studies have shown that the use of grape seed extracts mainly composed of PA, improved the ultimate tensile strength and stiffness of dentine collagen and long-term stability of dentine collagen matrices. In addition to its cross-linking effect, PA from elm tree and cranberry extracts has also been shown to inhibit production of MMPs 1, 3, 7, 8, 9, and 13 from macrophages and catalytic activity of MMPs 1 and 9.

Previous studies have shown that pre-treatment of demineralised dentine with PA for 1 h prior to adhesive application significantly improved the nano-mechanical properties (modulus of elasticity and nano-hardness) of the resin–dentine interfaces as well as resin–dentine bond strength. Similarly, application of PA to etched dentine prior to bonding procedures was also found to improve bonding to caries-affected dentine. However, such an application protocol is time-consuming and clinically unrealistic. To simplify the use of PA in clinical situations, PA has recently been incorporated directly into dental adhesives. This allows PA to remain in the hybrid layer for an extended period of time, thereby enhancing the degree of collagen cross-linking and biodegradation resistance. However, no information exists regarding the effect of PA concentrations on resin–dentine bond strength. Hence, the objective of this study was to evaluate the effect of incorporating different concentrations of PA into experimental dentin adhesive systems on the rate of collagen cross-linking and biodegradation resistance. The null hypothesis tested was that PA incorporation has no effect on the immediate bond strength of experimental dental adhesives to dentine.

2. Materials and methods

2.1. Tooth preparation

Forty caries-free human third molars that had been stored in a 0.5% chloramine T solution at 4 °C were used within one month after extraction. The teeth were collected after the patients’ informed consent had been obtained under a protocol reviewed and approved by the Institutional Review Board, the University of Hong Kong [UW 11-242]. The occlusal enamel was removed using a water-cooled slow-speed diamond impregnated disk (Isomet, Buehler Ltd., Lake Bluff, IL, USA). The exposed dentine was polished wet with 180-grit silicon carbide paper for 15 s to create a standardized smear layer on the mid-coronal dentine.

2.2. Preparation of experimental adhesives

A blend of methacrylate resin comonomers consisting of 40 wt% bisphenol A diglycidyl ether dimethacrylate (Bis-GMA), 30 wt% bis(2-(methacryloyloxy)ethyl) phosphate (Bis-PM), 28.80 wt% 2-hydroxyethyl methacrylate (HEMA), 0.26 wt% camphorquinone and 1 wt% ethyl N,N-dimethyl-4-aminobenzoate was used to formulate four hydrophilic experimental adhesives. The experimental adhesives were prepared by combining 50 wt% resin comonomer mixtures and 50 wt% ethanol with different PA concentrations. The PA (Grape Seed Extracts, International Laboratory of USA, SAN BRUNO, CA 94066, USA) was added directly to the ethanol-solvated experimental adhesive to yield four groups of adhesives with PA concentrations of 0, 1, 2 and 3 wt% respectively.

2.3. Bonding procedures

The teeth were randomly assigned to one of the following four adhesive groups (n = 6).

- Group 1: PA-free adhesive (control);
- Group 2: 1% PA-containing adhesive;
- Group 3: 2% PA-containing adhesive;
- Group 4: 3% PA-containing adhesive;

Each dentine surface was acid-etched with 32% phosphoric acid gel (Uni-Etch, Bisco Inc., Schaumburg, IL, USA) for 15 s and rinsed with water for 15 s before bonding. The etched dentine surfaces were blot-dried according to the wet bonding technique. Each adhesive was applied to the dentine surface for 15 s using a microbrush passively. This was followed by a second application of fresh adhesive, giving a total application time of 30 s. Excess solvent was removed with a gentle stream of compressed air for 10 s, followed by light curing of the adhesive for 40 s using a quartz-halogen light-curing unit (Optilux, Demtron-Kerr, Orange, CA, USA) with an output at 600 mW/cm². Resin composite build-ups were performed with a light-cured resin composite (Filtek Z250, 3M ESPE, St. Paul, MN, USA) in three 1 mm increments. Each increment was individually light-cured for 40 s with the same light intensity. The teeth were stored in distilled water at 37 °C for 24 h.
2.4. Microtensile bond strength testing

The bonded teeth were sectioned occluso-gingivally into 0.9 mm × 0.9 mm composite-dentine beams in accordance with the non-trimming technique of the microtensile bond test.21 Five beams were retrieved from the two widest slabs of each tooth for microtensile bond strength testing. The mean microtensile bond strength of the beams originating from each tooth was used for statistical analysis. The cross-sectional area of each specimen was measured with a pair of digital callipers (Model CD-685, Mitutoyo, Tokyo, Japan). Each bonded beam was attached to a Bencor Multi-T device (Danville Engineering, San Ramon, CA, USA) with cyanoacrylate adhesive (Zapit, Dental Ventures of North America, Corona, CA, USA) and stressed to failure under tension with a universal testing machine (Model 4440, Instron, Inc., Canton, MA, USA) at a crosshead speed of 1 mm/min.

2.5. Analysis of failure modes

After bond strength testing, the fractured dentine surfaces were mounted, air-dried, sputter-coated with gold/palladium and examined using a field-emission scanning electron microscope (FE-SEM; Hitachi S-3400N, Hitachi High Technologies America, Inc., Schaumburg, IL, USA). The failure modes were classified as (a) adhesive failure, (b) mixed failure, if the fracture site extended from the adhesive into either the resin composite or dentine; and (c) cohesive failure, if the fracture occurred exclusively within the resin composite or dentine.

2.6. Nanoleakage evaluation

The remaining 16 teeth (n = 4) were prepared and bonded as previously described for nanoleakage evaluation. The bonded specimens were sectioned occluso-gingivally into 1 mm thick slabs after storage in distilled water for 24 h. Two slabs from each tooth were selected. Each slab was blot-dried and painted with two layers of nail varnish, leaving only a 1 mm window at the bonded interfaces. The slabs were immersed immediately in a 50 wt% ammoniacal AgNO₃ solution (pH = 9.5) that was prepared according to the method described by Tay et al.22 After immersing in the tracer solution for 24 h, the specimens were rinsed with deionised water for 5 min and placed in a photodeveloping solution for 8 h under fluorescent light to reduce the diamine silver ions into metallic silver grains. The silver-stained bonded specimens were then polished with 6 μm, 3 μm and 1 μm diamond pastes (Buehler Ltd., Lake Bluff, IL, USA) using a polishing cloth. The slabs were cleaned ultrasonically, air-dried thoroughly and mounted on aluminium stubs. After placing the specimens in a desiccator for 24 h, they were carbon-coated and examined using FE-SEM in the back-scattered electron mode at 15 kV.

Forty-five images of the bonded interfaces were obtained per group at the same magnification. The amount of silver deposition along the bonded interface, which represents the degree of interfacial nanoleakage, was scored on a scale of 0–4 by two examiners using the classification of Saboia et al.23 Interfacial nanoleakage was scored based on the percentage of the bonded interface showing silver deposition: 0, no nanoleakage; 1, <25% nanoleakage; 2, 25 ≤50% nanoleakage; 3, 50 ≤75% nanoleakage; 4, >75% nanoleakage.

2.7. Statistical analysis

The bond strength data were analysed using a statistical package (SigmaStat Version 16, SPSS, Chicago, IL, USA). As the normality (checked by Kolmogorov–Smirnov test) and the equal variance assumptions (checked by modified Levene test) of the data appeared to be valid, one-way ANOVA was used to examine the effect of PA concentrations on microtensile bond strength. Post hoc multiple comparisons were performed using the Tukey–Kramer method at α = 0.05. For nanoleakage evaluation, inter-examiner reliability was assessed using weighted coefficient Kappa. The number of images assigned to the same score was statistically evaluated and differences between the groups were analysed using the chi-square test (p < 0.05).

3. Results

3.1. Microtensile bond strength

The means and standard deviations of microtensile bond strength obtained for the different experimental adhesives are summarized in Table 1. None of the beams debonded during the preparation procedure or bond strength testing. One-way ANOVA showed that the incorporation of PA significantly affected the microtensile bond strength of the experimental adhesives (p < 0.05). No significant difference was observed amongst the bond strengths of PA-free experimental adhesive (36.4 ± 2.7 MPa) and those incorporated with 1% PA (37.0 ± 3.6 MPa) and 2% PA (39.7 ± 1.1 MPa) (p > 0.05). However, the experimental adhesive with 3% PA had a significantly lower bond strength (22.2 ± 4.1 MPa), compared with the other experimental adhesives (p < 0.05).

3.2. Examination of the fractured interfaces

Failure modes of the debonded dentine surfaces were expressed as percentages of the total number of specimens tested in each group and are summarized in Table 2. The experimental adhesive containing 3% PA showed failures predominantly through the adhesive layer; whereas PA-free...
and the experimental adhesives containing 1% and 2% PA showed mainly mixed and cohesive failures.

Fractographic analyses of the debonded dentine surfaces from 2% PA- and 3% PA-containing adhesives are shown in Figs. 1 and 2 respectively. A representative mixed failure from the 2% PA experimental adhesive group is shown in Fig. 1A. The failure mode was classified as mixed failure, because it involved the hybrid layer, adhesive layer and composite. High magnification of the fractured dentine surface revealed failure at the top of the hybrid layer, which was densely infiltrated by adhesive resin (Fig. 1B). A representative adhesive failure from the 3% PA experimental adhesive group is shown in Fig. 2A. The intertubular dentine and dentinal tubules were poorly infiltrated by the adhesive resin (Fig. 2B).

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### Table 2 – Percentage distribution of specimens according to failure mode amongst test groups.

<table>
<thead>
<tr>
<th>Experimental adhesives</th>
<th>PA concentration</th>
<th>Failure mode (%)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>I</td>
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<td>17</td>
</tr>
<tr>
<td>II</td>
<td>1%</td>
<td>25</td>
</tr>
<tr>
<td>III</td>
<td>2%</td>
<td>21</td>
</tr>
<tr>
<td>IV</td>
<td>3%</td>
<td>67</td>
</tr>
</tbody>
</table>

A – cohesive failure in adhesive; M – mixed failure; CD – cohesive failure in dentine; CC – cohesive failure in composite.

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**Fig. 1** – Representative SEM micrographs of the dentine side of fractured specimens from 2% PA containing adhesives. (A) Low magnification view showing mixed failure, involving hybrid layer, adhesive layer and composite. (B) High magnification view of the area indicated by the white rectangle showed that failure occurred through the top of the hybrid layer.

**Fig. 2** – Representative SEM micrographs of the dentine side of fractured specimens from 3% PA. (A) Low magnification view showing the presence of polishing streaks that are indicative of adhesive failure of the resin-dentine interface. (B) High magnification view of the area indicated by the white rectangle showed that fracture occurred through the hybrid layer.

**Fig. 3** – Percentage distribution of nanoleakage amongst the four experimental adhesive groups.
3.3. Nanoleakage evaluation

The inter-examiner reliability was assessed by weighted Kappa test was 0.807. The percentage distribution of nanoleakage in the different experimental adhesive groups is shown in Fig. 3. Representative SEM micrographs from the four adhesive groups are shown in Fig. 4. The 3% PA-containing adhesive showed significantly greater nanoleakage than all the other groups ($p < 0.05$), whilst the 2% PA-containing adhesive showed the least nanoleakage ($p < 0.05$). Silver uptake in the 2% PA-containing adhesive group occurred mainly at the base of the hybrid layer (Fig. 4C). The 3% PA-containing adhesive group showed the most severe interfacial nanoleakage with silver uptake occurring in the hybrid layer, a few of the dentinal tubules and in the adhesive layer (Fig. 4D).

4. Discussion

Type I fibrillar collagen accounts for 90% of the dentine organic matrix, the remaining 10% consists of non-collagenous proteins such as phosphoproteins and proteoglycans.\(^{24}\) Biomodification of the dentine collagen matrix using natural cross-linkers is a novel approach to maintain collagen stability. In comparison with the common synthetic cross-linkers, such as glutaraldehyde and carbodimide, PA from grape seed extract has been shown to be an effective non-toxic cross-linking agent in vitro and in vivo.\(^{25}\)

Proanthocyanidin or condensed tannins is composed of condensed flavon-3-ol subunits, catechin, epicatechin and epicatechin-3-O-gallate and linked mainly through C4–C8.\(^{26}\) These components are responsible for their various biological properties such as free-radical scavenging and antioxidant activities.\(^{27}\) Proanthocyanidin consists of highly-hydroxylated structures that are capable of forming insoluble complexes with carbohydrates and proteins.\(^{28,29}\) Proanthocyanidin has been shown to interact with proline-rich proteins like collagen by covalent linkages,\(^{30,31}\) ionic interaction,\(^{32}\) hydrogen\(^{33}\) and hydrophobic bonding interactions.\(^{34}\) Formation of hydrogen bonds between the phenolic hydroxyl and protein amide carbonyl groups are the major force for stabilizing PA-treated collagen and increasing its mechanical properties.\(^{35}\) Furthermore, the triple helical structure of collagen also facilitates the accessibility of the carbonyl oxygen of the peptide backbone for hydrogen bonding.\(^{35}\) The use of ethanol as the adhesive solvent also promotes PA and collagen interactions by decreasing the dielectric constant of the adhesive and enhancing the stability of hydrogen bonds.\(^{25}\)

By using Fourier Transform Infrared Spectroscopy, PA was found to preserve the triple helical structure of collagen and induce aggregation of collagen microfibrils by displacing water and creating new hydrogen bond interactions with collagen.\(^{36}\) Contact angle measurements have demonstrated that the hydrophobicity of PA-modified collagen films was improved as a result of cross-linking between PA and collagen. The water vapour permeability of collagen/PA films decreased with
increasing PA content due to the formation of a denser structure, which in turn, prevented moisture permeation.\textsuperscript{36}

Proanthocyanidin has recently been used in dentistry as a natural collagen cross-linking agent. Several studies have shown that PA functions as a dentine collagen matrix stabilizer; thereby improving its mechanical properties,\textsuperscript{8,10,13} and increased its resistance to biodegradation.\textsuperscript{12,16} Pretreatment of demineralised dentine with PA increased the bond strength of simplified etch-and-rinse adhesives to both sound\textsuperscript{11,18} and caries-affected\textsuperscript{19} dentine. Furthermore, the nanomechanical properties of bonded interfaces were also improved following the use of PA on etched dentine.\textsuperscript{16,17}

However, the application time of PA in these studies varied from 10 min to 1 h, which is not clinically practical. Recently, PA has been incorporated directly into dental adhesives to increase the substantivity of PA in the hybrid layer for enhancing its collagen cross-linking effect.\textsuperscript{20} The results from that study showed that the presence of PA in an adhesive inhibited the biodegradation of unprotected collagen fibrils in the hybrid layer. The increased resistance of the collagen to degradation by collagenase has been attributed to masking of the cleavage site of collagen by PA released from the experimental adhesive, or decrease in the collagenase activity.\textsuperscript{27} It is speculated that PA hydrogen bonds to multiple sites on the collagen molecules and reduces possible cleavage sites. Furthermore, induction of an interfibrillar cross-link in the dentine collagen matrix also resulted in a decrease in its swelling ratio. This, in turn, could have resulted in decreasing collagenase-sorption and reducing enzymatic degradation of the PA-treated dentine matrix.\textsuperscript{12,13}

The results of the present study showed that the incorporation of 3\% PA into experimental dental adhesive adversely affected resin–dentine bond strength; hence, the null hypothesis which stated that PA incorporation has no effect on the immediate bond strength of experimental dental adhesives to dentine has to be rejected. The addition of 3\% PA reduced the polymerization efficiency of resins, which resulted in lower bond strength and adhesive failures. During the polymerization of resin, linear polymer chains are formed. With the incorporation of 1–2\% PA into adhesive resin, PA can be trapped within the linear chains after curing.\textsuperscript{30} However, with higher concentrations of PA, the formation of linear polymer chains can be disturbed due to the higher density of the PA molecules. This results in inadequate polymerization of the resin and formation of microvoids, thus creating weaker resin–dentine interface and higher frequency of water channels.\textsuperscript{18} Furthermore PA has a free radical scavenging effect, which can disturb the free radical polymerization of the resin. PA amounts in the resin could remove free radicals and inhibit resin polymerization.

For resin–dentine interfaces bonded with etch-and-rinse adhesives, nanoleakage occurs as a result of a permeability problem arising from phase separation of hydrophobic and hydrophilic resin components and the differential diffusion gradient of the two resin components along the depth of demineralised dentine.\textsuperscript{40} It is represented by discontinuous islands of silver deposits located at the base of the hybrid layer. The locations where the silver is deposited represent porous regions within the bonded interface, which provide channels for water sorption and leaching of uncured water-soluble monomers that can initiate hydrolytic degradation of resin–dentine bond.

Significantly less nanoleakage was observed in the hybrid layer of the 2\% PA-containing adhesive group. The greater reduction of nanoleakage observed in this group could be attributed to the reduced hydrophilicity and better resin infiltration of the demineralised dentine. It has been shown that the dense collagen network formed by PA is less susceptible to water sorption.\textsuperscript{36} The swelling ratio of demineralised dentine was lower in samples treated with PA when compared to control groups treated with distilled water and acetone.\textsuperscript{13} Apart from water, the interfibrillar spaces in acid-etched dentine also contain highly hydrated negatively charged proteoglycans that form a hydrogel within that space. These hydrogels may be responsible for “molecular sieving” by restricting hydrophobic resin monomers with high molecular weights to completely diffuse into the interfibrillar spaces that contain residual water. Recently, it has been shown that the glycosaminoglycans (GAGs) content was significantly reduced in PA-treated dentine specimens.\textsuperscript{25} It is speculated that the decrease in GAGs may improve the mechanical properties of the hybrid layer by changing the hydration rates and molecule diffusivity through dentine.\textsuperscript{8}

Increased nanoleakage in the form of large clusters of silver was observed in the hybrid layer formed by the 3\% PA-containing adhesive. Furthermore, the silver deposits were found to extend into the adhesive layer. The incorporation of 3\% PA into the adhesive may have altered the degree of conversion of the polymerized adhesive resin, thereby affecting its mechanical properties. The lower degree of conversion of resin may have allowed some unreacted monomers to leach out of the adhesive and hybrid layers, thereby increasing silver uptake by the porous bonded interface. This also explains why the number of adhesive failures increased when the percentage of PA incorporation in the dental adhesive increased from 2\% to 3\%. Similarly, Green et al.\textsuperscript{20} also reported that a less than suboptimal dentine hybrid layer was formed in an experimental adhesive containing 5\% PA.

At the resin–dentine interface, incomplete resin monomer infiltration of demineralised dentine will result in exposed collagen fibrils within the hybrid layer that are prone to attack by matrix metalloproteinases (MMPs).\textsuperscript{41} These endogenous MMPs are mostly bound to collagen.\textsuperscript{12,43} The application of simplified etch-and-rinse adhesives results in activation of these enzymes.\textsuperscript{4} In addition to its cross-linking effect, cranberry PA has recently been shown to inhibit MMPs production by macrophages.\textsuperscript{14} Clearly, further studies are warranted to investigate the inhibitory effect of PA from grape seed extract on dentine collagen-bound MMPs and the effect of PA incorporation in adhesives on its mechanical properties of resin and durability of resin–dentine bond.

5. Conclusion

Within the limits of this study, it may be concluded that:

1. Incorporation of 3\% proanthocyanidin into an experimental dental adhesive adversely affected dentine bond strength;
2. No significant difference in bond strength was found amongst the proanthocyanidin free, 1% and 2% proanthocyanidin-containing adhesives; and
3. Incorporation of 2% proanthocyanidin into an experimental dental adhesive provided the greatest reduction in nanoleakage at the bonded interface without compromising the 24-h resin–dentine bond strength.

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REFERENCES

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