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# EFFECT OF NATURALLY DERIVED COLLAGEN CROSS-LINKERS ON DENTIN BONDING AT CLINICALLY RELEVANT EXPOSURE TIMES

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## *ABSTRACT*

**Purpose:** To investigate the effect of treatment the demineralized dentin with two naturally derived collagen cross-linking agents on resin-dentin shear bond strength using three clinically relevant treatment times.

**Materials and methods:** 60 mid-coronal dentin specimens were randomly divided into two divisions (n=30) and either was treated with one of two cross-linking agents: 25% grape seed extract (GSE) and 25% ascorbic acid (ASA). The teeth of each division were further divided into three groups (n=10) according to the time of cross-linking agent application: 30 sec, 60 sec, and 90 sec. Additionally, 10 teeth were used as a control group (no treatment). After cross-linkers treatment and adhesive application, composite (Filtek Z250 XT) cylinders (3 mm diameter 2 × mm length) were built on all dentin surfaces. All specimens were stored in distilled water for 24 hours at 37°C and then subjected to shear stress in a universal testing machine. Failure patterns were observed using a light microscope at 10X. The micromorphology of the fractured surfaces of selected specimens was evaluated using SEM. SBS data (MPa) were statistically analyzed by two-way ANOVA and t-test.

**Results:** Both cross-linking agents resulted in a significant ($P<0.0001$) increase in resin-dentin SBS in comparison to control, regardless of application time. SBS values of both cross-linking agents were significantly increased by increasing their application time from 30 to 90 sec, while they were not affected, between other treatment times. There was no significant ($P = 0.5$) differences in SBS values between the two cross linkers at each corresponding treatment time.

**Conclusions:** Resin-dentin SBS can be improved after treatment of demineralized dentin by GSE, and ASA cross-linking agents at a clinically relevant treatment times.

**KEYWORDS:** Dentin, collagen, cross-linking agents, resin composite, bond strength.

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## INTRODUCTION

In the latter 20 years a remarkable improvement in adhesive systems and aesthetic restorative materials formulations has occurred. Adhesive restorations are regularly used to substitute lost tooth structure. The bonding of the current adhesive systems to dentin based on the formation of a collagen-resin interface layer called hybrid layer.[1] After acid-etching, the three dimensional network of demineralized collagen receive primers and adhesive resin which infiltrate the collagen interfibrillar spaces producing mechanical retention through hybrid layer formation after resin polymerization.[2] In spite of the substantial improvement of the adhesive systems, the hybrid layer remains the weakest area of the bonded interfaces where most failures take place.[3,4] However, it seems actually that adhesive/dentin bonding is highly efficient if the hybrid layer is compact, homogenous and structurally stable.[5,6] Although bonding to enamel has been indicated to be consistent over-time, the achievement of a durable bond to dentin still remains a challenge.[7]

Dentin forms the major part of the tooth structure and a strong and stable bond is necessary for the success of adhesive restorations.[8] Dentin is composed of two phases: an inorganic phase of hydroxyapatite crystals, and an organic matrix phase of type-1 collagen.[2] Type 1-collagen constitute 90% of the dentin organic matrix and present in dentin as fibrils that are stabilized by covalent intra- and inter-molecular cross-links.[9,10] This collagen cross-links is responsible for providing the collagen fibrils with stability, tensile strength, viscoelasticity, and resistance against enzymatic degradation.[11] A durable effective resin-dentin bonding relies mainly on the structural stability and mechanical properties of demineralized collagen fibrils.[12] Improvement in resistance to enzymatic degradation and mechanical properties of collagen fibrils can be achieved by increasing the formation of intra- and inter-molecular collagen cross-links.[13]

Many synthetic; such as glutaraldehyde and carbodiimides; and natural; such as proanthocyanidin, tannic acid, sodium ascorbate and genipine; exogenous collagen cross-linkers have been reported to increase the formation of inter- and intra-molecular collagen cross-links.[11,14-16] Proanthocyanidin (PA), widely present in fruits, vegetables, nuts, seeds, flowers, is a protein anti-oxidant cross-linking agent and it is well documented that grape seed is one of the richest sources of PA.[8,17] Ascorbic acid is a potent naturally occurring antioxidant ant it plays a vital role in collagen synthesis and skin regeneration. It has been proposed that exogenous ascorbic acid application contributes to strengthening the collagen framework by increasing the formation of collagen cross links.[18] Recent investigations have stated that grape seed extract, mainly composed of PA, improved the mechanical properties of demineralized dentin,[13,19] and increased the resin-dentin bond strength after 1 h treatment.[8,11] Other studyhas shown that a significant improvement in bond strength of self-etch adhesive to deep dentin was achieved when the dentin pretreated with 10% sodium ascorbate or 6.5% Proanthocyanidin for 5 and 10 min.[20]

Previous studies have used clinically unpractical treatment times of collagen cross-linkers varying from 10 min to 1 hr.[8,11,13,16,20] In an attempt to decrease the treatment time to reproduce a more clinical relevant condition, this study aimed to evaluate the resin-dentin bond strength after treatment of demineralized dentin (acid-etched layer) with two naturally occurring collagen cross-linking agents (GSE and ASA) using three clinically applicable treatment times (30, 60 and 90 sec). The null hypothesis tested was that the use of cross-linking agents would not affect the resin-dentin bond strength at any of the used treatment times compared to control (non-treated group). Further, increasing treatment time would not affect the bond strength and also no difference in bond strength

would be noticed between the two cross-linkers at each corresponding treatment time.

## MATERIALS AND METHODS

Two naturally derived collagen cross-linking agents (GSE and ASA; 25%) were used in this study as a treatment of demineralized dentin surfaces before bonding a composite restorative material. The materials used in this study, as well as their manufacturers, types, and compositions are listed in table 1.

### Cross-linking agent preparation

25% GSE and 25% ASA cross-linking agent solutions were prepared by dissolving 25 gm powder from each material in 100 cc distilled water. Both solutions had pH adjusted to 7.4 using sodium hydroxide. The concentration of cross-linking agents used were based on a previous study.[11]

### Specimen preparation

Seventy sound extracted human molars were collected, cleaned from debris and soft tissues, and stored in 0.5% chloramine solution at 4 ºC for not more than two months until used in this study. The occlusal third segment of the crown of each tooth was removed using a water-cooled diamond saw (Isomet 1000, Buehler Ltd, Lake Bluff, IL, USA) to expose a flat superfacial coronal dentin. Then, all teeth were mounted vertically in self-cured acrylic resin up to their exposed occlusal dentin surfaces. For the purpose of standardization, teeth were ground using 600-grits carborundum discs with water irrigation on a grinding machine (Ecomet 3, Buehler, Lake Bluff, IL, USA) to expose mid-coronal dentin.

60 of the prepared dentin surfaces were randomly divided into two divisions (n = 30) and either treated with one of the two cross-linking agents. The teeth of each division were further divided into three groups (n=10) according to the cross-linking agent treatment time: 30 sec, 60 sec, and 90 sec. The remaining 10 teeth were used as a control group (non-treated group).

TABLE (1) Specifications of materials used in the study.

| Material | Type* | Manufacturer | Lot* | Composition* |
|---|---|---|---|---|
| Grape seed extract | Collagen cross-linking agent | YunDao Biotech Production, China | | 95% Proanthocyanidin. |
| Ascorbic acid | Collagen cross-linking agent | Medex UK, Pharmaceuticals-chemicals,Naseby, Northants, UK. | 11409075 | 100 % Ascorbic acid. |
| Filtek Z 250 XT | Nano hybrid composite | 3M ESPE, St Paul, MN, USA. | N713125 | Resins: BisGMA, UDMA, BIS-EMA, PEGDMA, TEGDMA. Fillers (81.8% by wt): zirconia/silica (0.1-10 m) and silica particles (20 nm). |
| Adper single bond 2 | Total etch, visible-light activated dental bonding agent | 3M ESPE, St Paul, MN, USA. | N709579 | BisGMA, HEMA, dimethacrylates, ethanol, water, photoinitiator, a methacrylate functional copolymer of polyacrylic and polyitaconic acids, 10 % by wt of 5nm silica filler. |
| Scotchbond universal etchant | Etching gel | 3M ESPE, St Paul, MN, USA. | 591896 | 37% Phosphoric acid, silica thickener. |

**** Information provided by manufacturers.***

The dentin surface of each tooth was etched using 37% phosphoric acid gel for 15 s, thoroughly rinsed with water for 15 s, and gently air dried. The dentin surfaces of each group were then treated with their respective cross-linking solution for the recommended time. Each treated dentin surface was thoroughly rinsed with distilled water for 30 sec and excess water was removed by blotting with a cotton pellet, leaving dentin surface moist. Adper Single Bond 2 adhesive was applied, gently air dried, and photo-polymerized for 10 s using LED curing unit (Mini LED, Satelec, Acteon Group, France). The exposed dentin surfaces of the control group were prepared in the same manner except that no cross-linking agent applied.

Filtek Z250 XT composite (shade A3) build-up (3 mm diameter and 2 mm high) was made over the bonded surfaces by using a teflon mold and light-cured for 20 sec using LED curing unit with 1200 mW/cm$^2$ output power. The output intensity of the curing unit was verified with the built-in radiometer. Each dentin-composite interface was checked with a magnifying lens (Q Optics, Dentaltown L.L.C., Phoenix, USA) at X5 magnification and defective samples were excluded. Then, all specimens were stored in distilled water at 37$^o$C for 24 hours.

**Shear bond strength (SBS) testing**

After storage, the diameter of each composite cylinder in close proximity to the dentine surface was verified with a digital caliper (Mitutoyo digital calipers, Mitutoyo Corp., Kawasaki, Japan) with an accuracy of 0.01 mm before testing. A special design was fabricated to hold the acrylic embedded tooth specimen with its own bonded composite cylinder, which was secured to the lower fixed compartment of a universal testing machine (Instron, System ID No. 3345J8621, USA). An orthodontic wire (0.014 inch diameter) loop was wrapped around the bonded composite cylinder as close as possible to its base and aligned with the loading axis of the upper movable compartment of the testing machine (Fig. 1). A shearing load with tensile mode was applied at a crosshead speed of 1 mm/min until failure occurred. The SBS was calculated in MPa as the load on failure (N) divided by the cross-sectional area (mm$^2$).

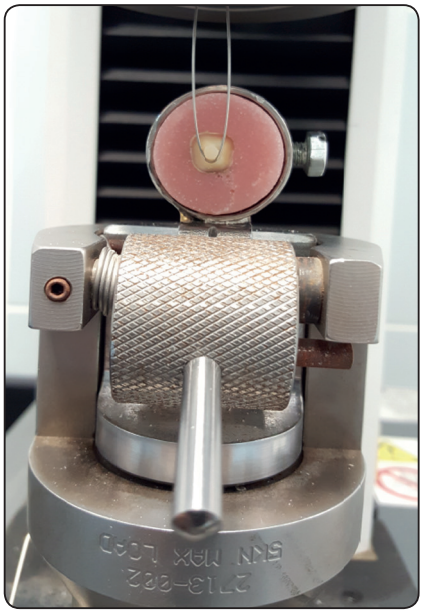

Fig. (1) Specimen in Instron testing machine during shear bond strength testing.

**Failure pattern evaluation**

Failure modes were evaluated under a light stereomicroscope (Olympus SZ 6045 TR Zoom, Olympus Optical Co, Osaka, Japan) at X10 magnification and classified as adhesive (if it occurred at dentin or composite /adhesive interface), cohesive (if it occurred within composite or dentin) or mixed (if adhesive and cohesive failures occurred concurrently).

**Scanning electron microscopy (SEM)**

The fractured dentin side of selected debonded specimens from each group were subjected to micromorphological analysis. Specimens were mounted on aluminum stands, left to dry for 24 hours and gold sputter coated (Fine coat, Ion sputter JFC-1100, JEOL Ltd, Tokyo, Japan). The micromorphology of the fractured surface was examined using a scanning electron microscope (JEOL, JSM-6360LV, JEOL Ltd, Tokyo, Japan).

**Statistical analysis**

T test with Bonferroni adjustment for multiple comparisons was used to compare mean SBS between control and each treated group. Two ways ANOVA was used to assess the effect of treatment time (30, 60 and 90 sec) and agent type (GSE and ASA) and their interaction on shear bond strength. Adjusted means were calculated and Bonferroni correction was used for multiple comparisons. Multinominal regression test was used to investigate the effect of agent and time on failure modes. Statistical significance was defined at P< 0.05. All Statistical analysis were carried out using Statistical Package for the Social Science (SPSS Inc., Chicago, IL) version 16.0 program.

**RESULTS**

The mean values and standard deviations of SBS as a function of cross-linking agents treatments are listed in table 2. The two cross-linking agents used resulted in a significant (P < 0.0001) increase in SBS in comparison to the control group regardless of the application time.

The mean and standard deviation values of SBS comparison as a function of treatment time and agent type are recorded in table 3. Two-way ANOVA indicated that treatment time ($p < 0.05$) but not agent type ($p > 0.05$) significantly affected the SBS. The effect of treatment time of both cross-linking agents on SBS was identical. SBS values of both cross-linking agents were significantly (P = 0.03) increased by increasing their application time from 30 to 90sec, while they were not affected (P < 0.05) if the application time increased from 30 to 60 or from 60 to 90 sec. On the other hand, no statistically significant differences (P = 0.5) in SBS values were observed between the two cross-linking agents at each corresponding application time. Two-way ANOVA also revealed that there were no significant interactions between the factors evaluated (treatment agent vs. application time, p = 0.95).

The frequent distribution (%) of failure modes are presented in table 4. It was shown that the predominant failure pattern of GSE- and ASA-treated groups was the mixed failure, regardless of the treatment time; while the predominant failure pattern for the non-treated group (control) was adhesive failure. There were no statistically significant differences among failure modes of all groups either for the effect of agent type ($X^2$ = 6.07, P = 0.19) or for the effect of treatment time ($X^2$ = 6.22, P = 0.18) as indicated by multinomial regression test.

TABLE (2) Descriptive statistics of SBS values of control and cross-linker treated groups.

| Treatment | Group | Treatment time (s) | Mean (MPa) | Standard deviation | 95% confidence interval for mean | | *P* value |
|---|---|---|---|---|---|---|---|
| | | | | | Lower bound | Upper bound | |
| GSE | 1 | 30 | 33.37* | 4.22 | 31.17 | 35.57 | < 0.0001 |
| | 2 | 60 | 35.31* | 3.53 | 33.11 | 37.51 | < 0.0001 |
| | 3 | 90 | 36.87* | 3.50 | 34.67 | 39.08 | < 0.0001 |
| ASA | 4 | 30 | 32.95* | 4.06 | 30.76 | 35.16 | < 0.0001 |
| | 5 | 60 | 34.85* | 3.25 | 32.65 | 37.05 | < 0.0001 |
| | 6 | 90 | 35.79* | 3.45 | 33.59 | 37.99 | < 0.0001 |
| Control | 7 | | 18.05 | 1.86 | 15.84 | 20.25 | |

***GSE: grape seed extract; ASA: ascorbic acid; s: second*** ****: significantly different from control ($p < 0.0001$).***

TABLE (3) Mean (SD) SBS (MPa) values of treated groups as function of application time and agent type.

| Treatment time (s) | GSE | ASA |
|---|---|---|
| 30 | 33.37 (4.22) [a] | 32.95 (4.06)[a] |
| 60 | 35.31 (3.53) [ab] | 34.85 (3.25)[ab] |
| 90 | 36.87 (3.50) [b] | 35.79 (3.45)[b] |

***Within each column or raw, values with identical letters represent no statistically significant difference ($p > 0.05$).***

***SD: Standard deviation; s: second***

TABLE (4) Frequent distribution (%) of failure modes for all groups.

| Treatment | Group | Treatment time (s) | Failure modes (%) | | |
|---|---|---|---|---|---|
| | | | Adhesive | Cohesive | Mixed |
| GSE | 1 | 30 | 40 | | 60 |
| | 2 | 60 | 30 | 10 | 60 |
| | 3 | 90 | 20 | 10 | 70 |
| ASA | 4 | 30 | 30 | 10 | 60 |
| | 5 | 60 | 20 | 20 | 60 |
| | 6 | 90 | 20 | | 80 |
| Control | 7 | | 70 | 10 | 20 |

***GSE: grape seed extract; ASA: ascorbic acid; s: second***

Representative SEM photomicrographs of deboned dentin surfaces are shown in Fig 2A-2F. The fractured surfaces of the control group showed mostly adhesive failures at the bottom of the hybrid layer (Fig. 2A). However, the fractured surfaces of GSE- and ASA-treated groups showed a similar trend of fracture pattern with mostly mixed failures, mainly interfacial failures at the top of the hybrid layer (Fig. 2B–2E).

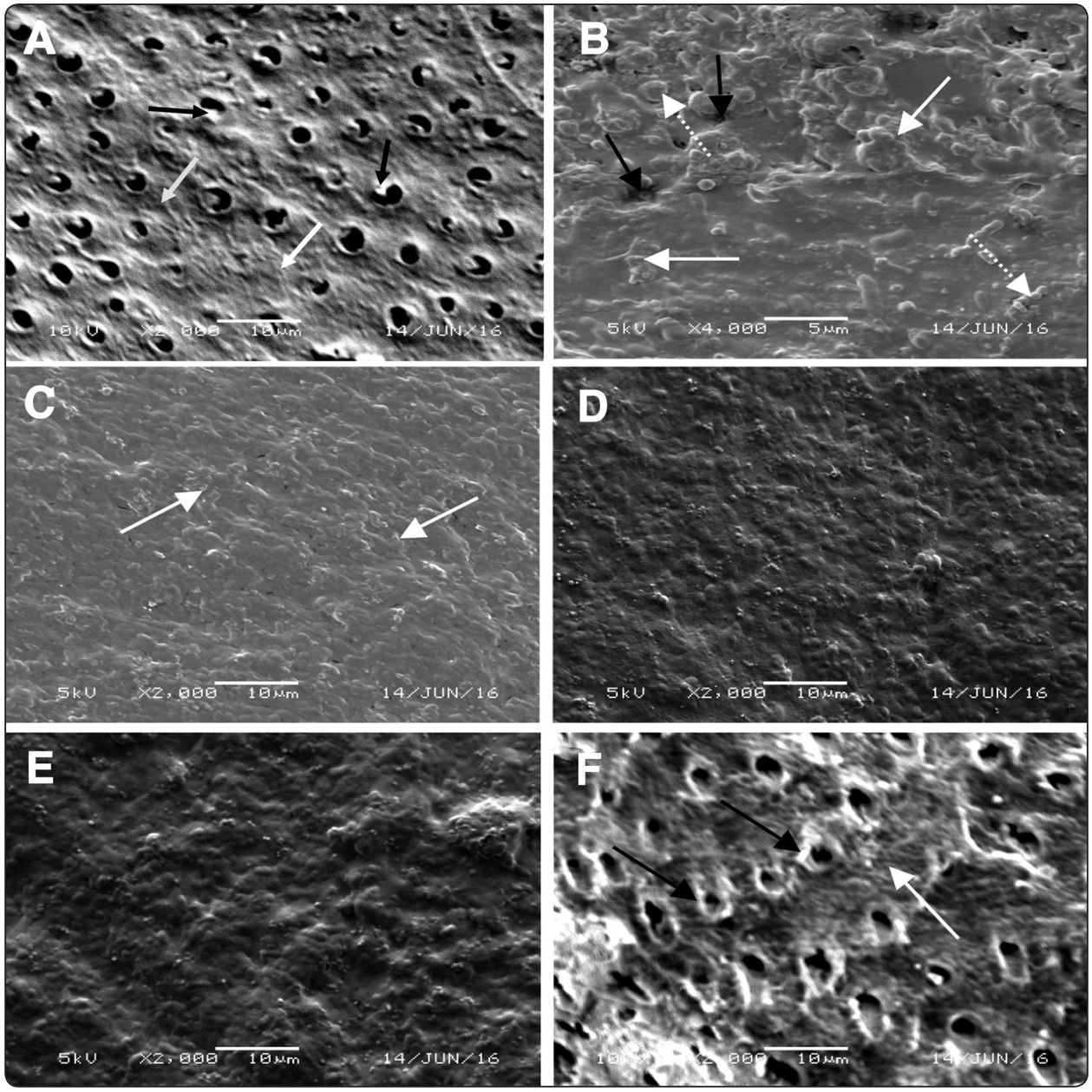


Fig. (2) Representative SEM photomicrographs from debonded dentin surfaces of control and cross-linkers treated specimens. (A) Dentin surface of a fractured specimen in the control group. The most common failure was at the bottom of the hybrid layer with remaining resin tags into dentinal tubules. Black arrows point out resin tags, while white arrows indicate hybrid layer. (B and C) Fractured dentin surfaces of specimens in group 1 (GSE-treated, 30 sec.) and group 4 (ASA-treated, 30 sec.) respectively, depicting failure at the adhesive layer/top hybrid layer. Black arrows indicate dentinal tubules, white arrows indicate adhesive resin, and dash white arrows point to resin tags. (D and E) Debonded dentin sides in group 2 (GSE-treated, 60 sec.) and group 5 (ASA-treated, 60 sec.) respectively, showing tags and adhesive layer covering dentin, which illustrates similar failures at the top of the hybrid layer. (F) Dentin side of a fractured specimen in group 6 (ASA-treated, 90 sec.) representing failure at the adhesive layer/ bottom hybrid layer. Black arrows indicate peritubular dentin and white arrow indicate hybrid layer.

## DISCUSSION

The durability of the adhesive interface depends on a compact and homogenous hybrid layer. Furthermore, hybrid layer is supposed to be necessary as stress-relieving medium under mechanical loading of the restorations.[21] There are lasting shortcomings associated with adhesive systems such as incomplete infiltration of demineralized dentin with resin monomer[22] and loss of unpolymerized resin monomers from interfibrillar spaces,[23] leaving exposed and disorganized collagen fibrils that lead to degradation of the hybrid layer over time.[24] Thus, if strong and more stable collagen layer can be achieved by tissue engineering and/or biomimetic agents, the resultant hybrid layer will be stronger and less liable to degradation.[25] Collagen in biological tissue is strengthened by inter- and intra-molecular cross-links and these links are the basis for the stability, tensile strength, form, cohesiveness, and viscoelasticity of collagen fibrils.[11,26] To promote additional stabilization of collagen fibrils in biological tissue, numerous exogenous cross-linkers have been used to induce extra intra- and inter-molecular cross-links.[8,11,13-16]

The present study was conducted to explore the capability of using natural exogenous cross-linkers to improve resin-dentin bond strength at clinically applicable exposure times. According to the results, the treatment of demineralized dentin surface using GSE or ASA for 30, 60 and 90 sec produced a significant increase in resin-dentin SBS compared to control, regardless of the treatment time. Increasing treatment time has a significant effect on SBS for both cross-linkers. There was no significant difference in SBS values between the two cross-linkers at each corresponding treatment time. Therefore, the null hypothesis was partially rejected.

PA, the main component of the GSE, are a class of bioflavonoids that are naturally occurring plant metabolites and can be obtained in high concentrations from natural sources such as grape seed extract, cocoe beans, pine bark extract, cranberries, lemon tree bark, and hazel nut tree leaves.[8,11,13,15,17] PA has been recognized and used securely as an antioxidant in several clinical therapies and food supplements.[27] PA is a mixture of monomers, oligomers, and polymers used as natural potent antioxidants and free-radical scavengers.[28] OPC (oligomericproanthocyanidin complex) contains multiple electron donor sites (hydroxyl sites) that allow it to bind to unstable molecules called free radicals by donating its hydrogen atoms.[29] PA is known to stabilize and increase the cross-linkage of type-I collagen fibrils.[11,13,15] Though not well defined, the primary mechanism of collagen stabilization/cross-linking with PA is the formation of hydrogen bonding between the protein amide carbonyl and the phenolic hydroxyl.[15]

Ascorbic acid and its sodium salt, sodium ascorbate, are also potent antioxidants and they play a vital role in collagen synthesis and skin regeneration.[18] Ascorbic acid and sodium ascorbate have a principle role in the synthesis of hydroxyproline and hydroxylysin of collagen. Hydroxyproline aid to stabilize the collagen triple helix and hydroxylysin is necessary for the formation of native collagen intermolecular cross-links.[30]

In this study, the use of GSE significantly increased the bond strength at all treatment times compared to control. This is in an agreement with the findings of previous studies done by Al-Ammaret. al.,[8] Bedrran-Russo et. al.,[11,13] and Han et. al.[15] These studies showed that the application of 6.5% GSE for 1 hour to demineralized dentin surface significantly improved the resin-dentin micro-tensile bond strength ($\mu$TBS). Macedo et al.,[16] also indicated that treatment of dentin matrix with 6.5% GSE for 1 hour resulted in an increase in $\mu$TBS of composite to both caries-affected and sound dentin. In another study,[25] the effect of dentin treatment with

GSE for 10 min and 60 min on resin-dentin $\mu$TBS was assessed and it was observed that the $\mu$TBS significantly increased at both treatment times compared to control, while no significant difference was noted between the treatment times. In all these previous studies, it was reported that the treatment of demineralized dentin with Proanthocyanidin-based cross-linking agents produced a significant enhancement in the mechanical properties and structural stability of dentin matrix and hence increased dentin bond strength. Four different mechanisms have been proposed for the interactions between Proanthocyanidin and proteins: covalent interaction,[31] ionic interaction,[32] hydrogen bonding interaction,[33] or hydrophobic interactions.[15] Therefore, GSE has high affinity to interact with collagen fibrils and consequently affect the stiffness of dentin.[19] In addition it has been stated that the stiffness of dentin matrix can be influenced by the concentration and treatment time of GSE.[19] The increase in stiffness of dentin matrix between approximately 9 to 30 times following GSE-treatment for 10 min. and 2 hrs, respectively, indicates an ability of the agent to stiffen the demineralized dentin matrix.[11] It was reported that proline-rich proteins like collagen have a very high ability for interaction with PA based components, forming a Proline-PA complex.[15,34] Therefore, the high SBS of GSE treated dentin surfaces are most likely due to increased formation of collagen cross-links, which caused an improvement in the dentin collagen stability and an increase in its mechanical properties and consequently the hybrid layer.[8]

The results of this study also indicated that treatment of demineralized dentin with ASA result in a significant increase in resin-dentin bond strength in comparison to control, regardless the treatment time. It can be proposed that the increased collagen cross-links induced by exogenous ASA cross-linker were sufficient to improve the structural stability and mechanical properties of dentin matrix, which in turn lead to an increase in the SBS. This is in accordance with outcomes of Srinivasulu et. al.,[20] who showed that the treatment of deep dentin with 10% sodium ascorbate for 5 and 10 min significantly increased the SBS at both times. They also attributed this to the improved collages stability achieved by increasing collage cross-links.

Unlike previous studies[8,11,13,15,16,20,25] where the used treatment times of collagen cross-linkers were too long to be applied clinically, application times of 30, 60, and 90 sec, which are more clinically feasible were chosen in the present study. Statistical analysis showed that the increase in SBS for both cross-linking agents was time dependent. The results indicated that the SBS values of both agents were significantly increased when the treatment time increased from 30 to 90 sec only, however they were not affected from 30 to 60 or from 60 to 90 sec. In spite of the too short treatment times employed in this study in comparison to previous investigations, the resultant SBS values were comparable or even more than those obtained from preceding studies. This is may be attributed to the high concentration (25%) of cross-linking agents used in the present study. It can be noted that the bond strength increased by about 83%, 93%, and 104% after 30, 60, and 90 application times, respectively; for both cross-linkers. At the same time, there were no significant differences in SBS between the two cross-linking agents at each corresponding treatment time. This is might be explained on the bases that both GSE and ASA biochemical cross-linking agents are potent antioxidants[18,28] and could induce comparable collagen cross-links at the same treatment time.

Failure pattern data showed a predominance of mixed failure mode of GSE- and ASA-treated groups at all application times. When the fractured surfaces of these treated groups were examined under SEM, it was noted that the failures were mainly in the adhesive interface and the top of the hybrid layer (Fig. 2B-2F), regardless of application time. However, the failure pattern of the untreated

specimens (control group) were mostly observed at the bottom of the hybrid layer (Fig. 2A). The changes in the failure pattern of GSE- and ASA-treated specimens, as observed in SEM images, in comparison to control could be due to improvement in the mechanical properties of the hybrid layer caused by dentin matrix biomodification with collagen cross-linkers, specifically at the bottom of the hybrid layer in which the acid-etched (demineralized) dentin usually poorly infiltrated by resin adhesive. These observations are in line with previous investigations.[8,16]

The use of cross-linking agent solutions with pH 7.4 followed previous studies[8,11,13,16,19] in which cross-linking solutions had their pH adjusted to a neutral level. Such pH is necessary and desired, since it would have low effect on the dentin matrix and also on the mechanism of cross linking agent-collagen interaction.[33] It has been reported that the interaction of proanthocyanidin with proline-rich proteins like collagen is pH-dependent, in which the greatest interaction was found to be near the neutral pH level.[35] Therefore, a pH 7.4 may be indicated with the use of cross linking agents during bonding procedures.

## CONCLUSIONS

The natural and biocompatible cross-linking agents used in this study seem to be very promising pretreatment agents during adhesive procedures. The use of GES and ASA, as collagen cross-linkers of demineralized dentin, significantly increased the resin-dentin shear bond strength at all clinically relevant treatment times used when compared to the control. SBS values of both cross-linking agents were significantly increased by increasing their application time from 30 to 90 sec, while they were not affected, between other treatment times. No significant difference were found in SBS values between the two cross-linking agents within each corresponding treatment time. Further experiments may be needed to explore the long-term validity of these collagen cross-linkers on bond strength.

## CLINICAL RELEVANCE

The results indicated that the use of GSE and ASA collagen cross-linkers as a treatment of demineralized dentin at more clinically applicable times (tens of seconds) may lead to the development of new methodologies for improving resin-dentin bond strength.

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