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Stabilization of dentin matrix after cross-linking treatments, in vitro

Débora L.S. Scheffel[a], **Josimeri Hebling**[a], **Régis H. Scheffel**[b], **Kelli A. Agee**[b], **Milena Cadenaro**[c], **Gianluca Turco**[c], **Lorenzo Breschi**[c], **Annalisa Mazzoni**[c], **Carlos A. de Souza Costa**[d], and **David H. Pashley**[b,*]

David H. Pashley: dpashley@georgiahealth.edu

[a]Department of Orthodontics and Pediatric Dentistry, Araraquara School of Dentistry, UNESP – Univ Estadual Paulista, Araraquara, São Paulo, Brazil

[b]Department of Oral Biology, College of Dental Medicine, Georgia Regents University, Augusta, GA, USA

[c]Department of Biomedicine, Unit of Dental Sciences and Biomaterials, University of Trieste, Trieste, Friuli Venezia Giulia, Italy

[d]Department of Physiology and Pathology, Araraquara School of Dentistry, UNESP – Univ Estadual Paulista, Araraquara, São Paulo, Brazil

Abstract

Objectives—To evaluate the effect of EDC on elastic modulus (E), MMPs activity, hydroxyproline (HYP) release and thermal denaturation temperature of demineralized dentin collagen.

Methods—Dentin beams were obtained from human molars and completely demineralized in 10 wt% H_3PO_4 for 18 h. The initial E and MMP activity were determined with three-point bending and microcolorimetric assay, respectively. Extra demineralized beams were dehydrated and the initial dry mass (DM) was determined. All the beams were distributed into groups (n = 10) and treated for 30 s or 60 s with: water, 0.5 M, 1 M or 2 M EDC or 10% glutaraldehyde (GA). After treatment, the new E and MMP activity were redetermined. The beams submitted to DM measurements were storage for 1 week in artificial saliva, after that the mass loss and HYP release were evaluated. The collagen thermal denaturation temperature (TDT) was determined by DSC analysis. Data for E, MMP activity and HYP release were submitted to Wilcoxon and Kruskal–Wallis or Mann–Whitney tests. Mass loss and TDT data were submitted to ANOVA and Tukey tests at the 5% of significance.

Results—EDC was able to significantly increase collagen stiffness in 60 s. 10% GA groups obtained the highest E values after both 30 and 60 s. All cross-linking agents decreased MMP activity and HYP release and increased TDT temperature. Significant differences were identified

*Corresponding author at: Department of Oral Biology, College of Dental Medicine, Georgia Health Sciences University, Augusta, GA 30912-1129, USA. Tel.: +1 706 721 2031; fax: +1 706 721 6252.

among EDC groups after 30 or 60 s of cross-linking, 1 M or 2 M EDC showed the lowest MMP activity.

Significance—Cross-linking agents are capable of preventing dentin collagen degradation. EDC treatment may be clinically useful to increase resin-dentin stability.

Keywords

MMPs; Collagen; Dentin; Cross-linkers; Glutaraldehyde; EDC

1. Introduction

Contrary to stable resin-enamel bonds, effective, long-lasting dentin bonds remains a challenge to clinicians [1]. The hybrid layer is considered the main structure responsible for micromechanical retention of resin restorations and also responsible for sealing the dentin [2]. However, this important structure is the most vulnerable area of the adhesive interface [3–5].

Bond degradation has been attributed to hydrolytic breakdown of resin adhesive or dentin collagen, or both. TEM examination of the hybrid layers shows replacement of collagen fibrils by water [6]. This degradation is thought to be due to endogenous MMPs and cathepsins in acid etched dentin. Demineralized dentin contains matrix-bound metalloproteinases-2, -3, -8, -9 and -20 (MMPs) and cathepsins [7,8] in their active forms. These enzymes are exposed and activated by acid-etching and can slowly degrade collagen fibrils [9–12] within the hybrid layer, resulting in a significant loss of bond strength of 36–70% within 12–14 months [13,14].

Cross-linking agents are capable of non-specifically cross-linking protein such as collagen and dentin proteases [15,16]. 1-Ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) is a stable isomer of carbodiimide, capable of cross-linking proteins by activating the carboxyl group of glutamic and aspartic acids and then reacting with ε-amino groups present in protein molecules, resulting in the creation of covalent cross-links [17,18]. Cross-linking increases the mechanical properties of dentin collagen and makes the fibrils more resistant to degradation [15]. Furthermore, EDC has no transdentinal cytotoxic effect (Scheffel et al., unpublished data). However, previous reports of EDC treatment of dentin used 10 min to 4 yrs that are not clinically relevant [15].

Matrix-bound dentin proteases contain numerous residues of glutamic and aspartic acids in their structures. Thus, if EDC reacts with demineralized dentin collagen [15], it should also react with any proteases bound to collagen. Such protease cross-linking should reduce the molecular mobility of dentin MMPs and cathepsins, thereby inactivating them [19]. Additionally, besides bonding between collagen polypeptide chains, it is plausible that EDC could cross-link the catalytic sites of dentin proteases, thereby blocking resin-dentin bond degradation. The purpose of this study was to evaluate the effect of 30 or 60 s EDC application on elastic modulus, total matrix-bound MMPs activity, hydroxyproline release and thermal denaturation temperature of completely demineralized dentin. The null hypotheses were that cross-linker-treated and untreated dentin do not differ regarding elastic

modulus, total MMP activity, amount of hydroxyproline release and thermal denaturation temperature, and the time of application does not influence these properties.

2. Materials and methods

Fifty extracted human third molars were obtained from 18 to 21 year-old patients with informed consent under a protocol approved by the Georgia Regents University. The teeth were stored frozen until required. After thawing, the enamel and superficial dentin were removed using an Isomet saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) under water cooling. One 1 mm-thick dentin disk was produced from the mid-coronal dentin of each tooth.

2.1. Elastic modulus

One-hundred dentin beams (1mm × 1mm × 3 mm) were sectioned from the dentin disks. The beams were completely demineralized in 10 wt% H_3PO_4 (pH 1) for 18 h at 25 °C and rinsed with deionized water for 2 h at 3–4 °C. The initial elastic modulus of each demineralized beam was determined by three-point flexure [20]. An aluminum testing jig with a 2.5 mm span between supports was fixed to the bottom of a glass Petri dish. Specimens were tested under compression, while immersed in distilled water, by means of a testing machine (Vitrodyne V1000, Liveco Inc., Burlington, VT, USA), with a 100 g load cell, at a crosshead speed of 1 mm/min. After maximum displacement, it was returned immediately to 0% stress to prevent creep of the demineralized collagen. Load–displacement curves were converted to stress–strain curves, and the apparent modulus of elasticity was calculated at 15% strain. Then the beams were randomly divided into 10 groups (n = 10), so that the mean initial elastic modulus of each group was statistically similar. To calculate elastic modulus of each specimen, the steepest slope of the linear portion of the stress–strain curve was placed in the following formula:

$$E=\frac{mL^3}{4bd^3}$$

m = slope (N/mm); L = support span (mm); d = thickness of beam (mm); b = width of beam (mm). Because specimen displacement was estimate from cross-head displacement, and the specimens thickness was not one-sixteenth of the length [20] the calculated elastic moduli are approximate. Although both the two supports and the third mid-beam compressive member may have slightly deformed the surface of the specimens, that deformation was the same before and after treatment. We were more interested in changes in modulus of elasticity, rather than their absolute values.

2.2. Pre-treatment MMP activity of dentin

To determine the initial total MMP activity, each beam was placed into 200 μl of a generic MMP substrate (Sensolyte Generic MMP colorimetric assay kit – catalog No. 72095, AnaSpec Inc. Fremont, CA, USA) for 60 min at 25 °C in a 96-well plate. At the end of 60 min, the total MMP activity was determined by measuring the absorbance of the wells at

412 nm in a plate reader (Synergy HT microplate reader, BioTek Instruments, Winooski, VT, USA) against blanks. The substrate is cleaved by MMPs 2, 8 and 9 in dentin and releases a sulfhydryl group that reacts with Ellman's Reagent (5,5′-dithiobis(2-nitrobenzoic acid). The final product of this reaction, 2-nitro-5-thiobenzoic acid (TNB), turns the medium yellow and can be read by a plate reader. All chemicals were purchased from Sigma/Aldrich Chemical Co and used as received.

2.3. Post-treatment MMP activity of dentin

Each completely demineralized dentin beam was dipped for 30 or 60 s into 300 μl of the following solutions: water (positive control), 0.5 M, 1 M or 2 M EDC (EDC-HCl, ProteoChem, Denver, CO, USA) (pH 6.0) or freshly diluted 10 vol% (1 M) glutaraldehyde (GA) (negative control) made from 50 wt% Sigma-Aldrich (St. Louis, MO, USA), followed by abundant rinsing with deionized water for 30 s to dilute the cross-linking agents to near zero and to stop the cross-linking reaction. Immediately after treatment the new elastic modulus and residual total MMP activity were redetermined.

2.4. Hydroxyproline (HYP) assay and dry mass loss

To analyze hydroxyproline release and dry mass loss one-hundred extra beams were prepared and demineralized as described above. The beams were placed in sealed containers of anhydrous calcium sulfate (Drierite, W.A. Hammond Drierite Company, Ltd., Xenia, OH, USA) overnight and the initial dry mass of each beam was measured using a microanalytical balance to the nearest 0.01 mg [21]. The beams were rehydrated in deionized water for 1 h before being treated according to the groups previously described to elastic modulus and MMP activity assay. Immediately after the treatments the beams were incubated for 1 week in 1 ml of artificial saliva.

After 1-week incubation, the dry mass was re-measured for each beam and 200 μl of the 1 ml incubation medium was removed to perform the HYP assay. An equal volume of concentrated 12 N HCl (200 μl) was mixed to the artificial saliva to yield a final acid concentration of 6 N HCl in glass ampoules (Wheaton, Millville, NJ, USA). The vials were automatically sealed using an Ampulmatic Ampule Sealer (Bioscience, Inc., Allentown, PA, USA) and placed in an oil bath at 118 °C for 18 h to hydrolyze the solubilized collagen to amino acids. After cooling, the glass vials were opened and placed in a glass dessicators with NaOH pellets to trap HCl vapor and anhydrous calcium sulfate to trap water vapor. After 3 days in the dry contents of the vials were analyzed for HYP using a colorimetric assay (modified from Jamall et al. [22]). The absorbance of all specimens and standards was measured at 558 nm in a 96-well plate reader.

2.5. Differential scanning calorimetry (DSC) analysis

Dentin slabs (1 mm thick and 6 mm of diameter) were completely demineralized in 10% phosphoric acid aqueous solution for at least 18 h and then rinsed in Milli-Q water under agitation for 24 h. A FTIR-ATR (Fourier transform infra-red attenuated total reflectance, Nicolet 6700, Thermo scientific, Milan, Italy) was used to acquire the spectrum of each slab before and after the demineralization process to ensure that all mineral content was removed, verified by the disappearance of the PO_4^{3-} peak at 1004 cm^{-1}. Each demineralized

slab was sectioned into cubic specimens (1 mm × 1 mm × 1 mm) using a sterile surgical blade. The dimensions of each specimen were individually measured with a digital caliper to the nearest 0.01 mm. The dentin specimens were treated with the treatment solutions as described for the previous tests. Specimens immersed in the GA and EDC solutions were rinsed with Milli-Q water for at least 30 min. The thermal denaturation temperature (TDT) for each specimen was determined using a differential scanning calorimeter (DSC, Q10 TA Instruments, New Castle, DE, USA). All specimens were lightly blot dried and sealed in DSC aluminum pans. Specimens were heated from 30 °C to 250 °C at 10 °C/min in a nitrogen atmosphere. Both the onset and the maximum signal of thermal degradation temperature were measured.

3. Statistical analysis

Data sets for elastic modulus (E), MMP activity, mass loss, HYP release and thermal denaturation of demineralized dentin were evaluated regarding the normality of their distributions. Since the distributions of E, MMP activity and HYP release data sets were not normally distributed, nonparametric tests were selected to analyze these variables. All data sets were submitted to Wilcoxon non-parametric and Kruskal–Wallis tests (for tests involving more than two comparisons) or the Mann–Whitney test for comparing two groups. The percentage of total MMP activity inhibition was calculated based on the untreated water control group MMP activity. Data from % of mass loss and collagen thermal denaturation (°C) were submitted to ANOVA complemented by Tukey tests. All the statistical tests were performed at the 5% level of significance.

4. Results

Completely demineralized dentin beams dipped in water for 30 s or 60 s were used as controls for statistical comparisons (Tables 1 and 2) and were considered as 100% to calculate percent changes in elastic modulus (Table 4) or percent decreases in MMP activity (Table 3) and dry mass in the results. Treatment of demineralized dentin with EDC, in concentrations between 0.5 M and 2 M, did not increase dentin elastic modulus after 30 s treatment. However, demineralized dentin beams treated with 1 M and 2 M for 60 s significantly ($p < 0.05$) increased their moduli of elasticity by 41.2% and 34.2%, respectively. When 10% GA was applied to demineralized dentin beams, the treatment significantly ($p < 0.05$) increased their elastic moduli at both time periods (123.5% for 30 s and 265.8% for 60 s) (Table 4). All cross-linking agents were capable of reducing the total MMP activity of demineralized dentin after 30 and 60 s of topical treatment (Table 3). After 30 s of EDC treatment, the total MMP inactivation among EDC groups was only 64.4%, 48.9% or 64.4% for the 0.5, 1 or 2 M groups and 65.3% in the 10% GA group. None of these values were significantly different from each other. After 60 s treatment of dentin with 1 M or 2 M EDC, the total MMP activity of dentin fell 77.6% and 81.9%, respectively (Table 3). No significant differences were observed between 0.5 M EDC (58.6%) and 10% GA (55.4%) (Table 3). GA was more effective than EDC at increasing elastic moduli in 30 s or 60 s. That is, after 30 s of treatment, the modulus of elasticity increased 123.5%, while after 60 s of treatment, the modulus of elasticity increased 265.8% (Table 4). However, GA

was no more effective at inactivating the total MMP activity of demineralized dentin than was EDC.

Dry mass loss data showed that the treatment of dentin beams with EDC or GA was capable to significantly reduce mass loss (Table 5). After 1-week incubation the control group lost 6.39 ± 0.67% (30 s) and 7.75 ± 1.05% (60 s) of mass, while the beams treated with EDC lost between 2.77 ± 1.25% (2 M EDC for 30 s) and 1.89 ± 0.89% (0.5 M EDC for 60 s). No statistical difference was observed among EDC groups. The beams treated with 10% GA for 30 s and 60 s showed a loss of dry mass of 0.28 ± 1.03% and 1.88 ± 1.38%, respectively.

Hydroxyproline assay detected HYP release in all groups (Table 6). The highest HYP amount was observed to the uncrossed-linked control groups 2571.33 (30 s) and 3271.29 (60 s) ng HYP/mg dentin (90.8% reduction in HYP release in specimens treated with 10% GA for 60 s). EDC and GA treatments reduced HYP release to values between 445.49 (0.5 M EDC for 30 s or 60 s) and 299.75 (10% GA for 60 s) ng HYP/mg dentin. No difference was observed among the groups treated with EDC and GA and the HYP content decrease was not dependent on cross-linker application time (Table 6).

The DSC analysis showed that at the same concentration the treatment time did not influence the collagen thermal denaturation temperature. The control group (water) exhibited a control onset denaturation temperature of 61.43 ± 2.39 °C and a registered significant lower onset (61.43 ± 2.39 °C) maximum thermal denaturation temperature of 67.05 ± 3.87 °C (Table 7). The onset of thermal denaturation of dentin treated with 10% GAfor60 s was 75.61 ± 1.54 °C. However no difference was observed among this group and the specimens treated with 10% GA for 30 s (73.64 ± 3.97 °C), 2 M EDC for 30 s (73.55 ± 1.07 °C) and 60 s (75.22 ± 1.85 °C) and 1 M EDC for 60 s (73.18 ± 1.38 °C). Similar relation was observed to maximum denaturate temperature, with the highest values observed in the 10% GA group (60 s) (78.91 ± 2.28 °C) which was not different from the groups treated with 2 M EDC (30 s and 60 s) or 1 M EDC (60 s).

5. Discussion

The degradation of resin-dentin bonds has been the subject of several recent studies. Host-derived proteases such as metalloproteinases (MMPs) and cysteine cathepsins plays an important role in collagen fibrils degradation via peptide hydrolysis [23]. MMPs binding sites are located in a narrow cleft in collagen approximately 0.5 nm wide [24]. Hence to degrade collagen, MMPs must bind to collagen and unwind collagen molecules in such a way that the enzyme's active site can react and attack the specific glycine-isoleucine peptide bond in peptide chains [25–27] culminating in cleavage of collagen peptides.

Carbodiimide has been investigated as a cross-linking agent that is capable of increasing the stiffness of dentin collagen fibrils, making them more difficult to unwind. Bedran-Russo et al. [15] demonstrated that the treatment of dentin collagen with EDC for periods of time between 10 min to 4 h, increases its mechanical properties. However such reaction times are not clinically applicable. The present study tested much shorter clinically relevant time of EDC application on completely demineralized dentin, and its effects on elastic modulus,

total MMP activity, release of hydroproline peptide fragments and thermal denaturation temperature.

Our results showed that in EDC concentrations between 0.5 and 2 M, applied for only 30 s significantly increase collagen stiffness. However, when the reaction time was increased to 60 s of treatment, EDC significantly ($p < 0.05$) increased the modulus of elasticity of dentin when used at 1 M or 2 M concentrations. On the other hand, all these treatment times and concentrations were sufficient to significantly ($p < 0.05$) reduce matrix-bound MMP activity. Thus, the results require rejection of the tested null hypotheses that cross-linker-treated and untreated dentin do not differ regarding elastic modulus and MMP activity, and that the time of application does not influence these properties.

GA was able to rapidly increase collagen stiffness (123.5–265.8%) more efficiently than EDC (34.2–41.2%). GA, being a dialdehyde, seems to react with amino acids faster than EDC. This may be because the mechanism of the GA reaction with proteins is direct and does not involve the production of an intermediate product as is seen in the EDC reactions [17]. However, GA is considered to be potentially cytotoxic. EDC did not present transdentinal cytotoxic effect on odontoblast-like cells (Scheffel et al., unpublished data). The fast reaction of GA was also observed in its ability to inactivate MMPs. GA was unable to inhibit more MMPs in 60 s than it was in 30 s, suggesting that it completely reacts with MMPs in the first seconds of application. However, the fact that it only inhibited the total MMP activity of dentin 65.3% (Table 3) indicates that cross-linkers may require longer diffusion times to diffuse to the center of 1.0 mm thick specimens.

EDC was a more effective MMPs inactivator, than it was as collagen cross-linker at both periods of time (i.e. 30 s and 60 s). EDC cross-links peptide chains in MMPs, thereby inactivating the enzyme by lowering the molecular mobility of the catalytic sites in these enzymes [19]. Moreover, MMPs-2, -8, -9 and -20 contain glutamic acid in their active sites allowing EDC to activate those free carboxyl groups not involved in peptide bonds. Despite the fact that cross-linking can be done rapidly in MMPs, it seems to occur more slowly in collagen. This suggests that carboxyl and amino groups in collagen may not be as accessible as those groups are in MMPs [28,29], allowing more rapid cross-linking in MMPs than collagen [23].

EDC requires a longer time to increase the elastic modulus of collagen. This study used completely demineralized 1.0 mm thick dentin beams. Such thick zones of demineralization are much deeper than those observed clinically in acid etched bonded teeth (8–10 μm). One mm thick completely demineralized beams must be infiltrated with reagents 500 μm from all sides of the beam to reach the center. We speculate that 10 μm-deep zones of demineralized dentin would be easily saturated by test solutions within seconds. We suggest that EDC could be used clinically to inactivate most of matrix-bound proteases and satisfactorily increase the stiffness of dentin collagen.

The dry mass and the hydroxyproline assay are indirect measures of dentin collagen degradation. Loss of dry mass over time indicates solubilization of collagen matrix by activated endogenous MMPs. The mass loss in the control group was much higher than in

the EDC pre-treated groups, while GA was able to completely stop mass loss after 1-week incubation. Likewise, all cross-linking solutions were able to reduce HYP release for both periods of treatment. These results agree with MMP activity assay that showed that EDC and GA inhibit MMPs, reducing cleavage and solubilization of collagen peptides.

DSC was used to investigate the thermal properties of dentin collagen. All the cross-linking solutions applied for 30 s or 60 s increased the thermal stability of demineralized collagen. Our results agree with those of Safandowska and Pietrucha [30], who treated collagen from fish skin with EDC to be used in medical applications. They observed that EDC was effective to cross-link fish collagen and increase the temperature required to thermal denaturation. In the present study the highest temperatures were observed to the groups treated with 10% GA (30 s and 60 s), 2 M EDC (30 s and 60 s) and 1 M EDC (60 s) similar to that showed by three point-bending test, especially after 60 s of treatment. Future studies will evaluate the effects of shorter application times of cross-linking agents over time in vitro and in vivo, in thinner more clinically relevant specimens.

6. Conclusion

The application of cross-linking agents to demineralized dentin was capable of inactivating matrix-bound MMPs, reducing dry mass loss, decreasing HYP release and in increasing collagen E-modulus and denaturation temperature. The use of EDC in clinically applicable periods of time may prevent resin-dentin degradation. GA showed the highest values of elastic modulus and was more effective to prevent mass loss, although EDC was a better MMP inactivator in high concentrations.

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Table 1

Elastic modulus (MPa) of completely demineralized dentin specimens before and after the application of different cross-linking solutions for 30 s or 60 s.

Dentin treatment	30 s		60 s	
	Initial E-modulus	Final E-modulus	Initial E-modulus	Final E-modulus
Water	1.80 (1.69–2.09)*[a]	1.80 (1.51–2.17)[a]	1.61 (1.37–2.32)[a]	1.62 (1.40–2.40)[a]
0.5 M EDC	1.60 (1.52–2.36)[a]	1.51 (1.20–1.81)[a]	1.94 (1.87–2.02)[a]	1.80 (1.57–1.97)[a]
1 M EDC	1.71 (1.38–2.41)[a]	1.62 (1.23–2.20)[a]	1.62 (1.33–1.85)[a]	2.29 (2.15–2.47)[b]
2 M EDC	1.79 (1.50–2.13)[a]	1.82 (1.39–2.18)[a]	1.74 (1.63–2.03)[a]	2.17 (1.87–2.66)[b]
10% GA	1.94 (1.87–2.17)[a]	4.02 (3.50–5.41)[b]	1.94 (1.54–2.12)[a]	5.93 (4.56–7.25)[b]

*Values are expressed as MPa and represent median (25 percentile–75 percentile), $n = 10$.

Within each dentin treatment solution and application time (initial vs after-treatment E-modulus), medians identified by the same letter are not statistically different (Wilcoxon, $p > 0.05$).

Table 2

Total MMP activity (absorbance) detected in completely demineralized dentin specimens before and after the application of different cross-linking solutions for 30 s or 60 s.

Dentin treatment	Application time			
	30 s		60 s	
	Initial MMP activity	Post-treatment MMP activity	Initial MMP activity	Post-treatment MMP activity
Water	0.227 (0.146–0.279)*[a]	0.265 (0.235–0.334)[a]	0.255 (0.225–0.302)[a]	0.232 (0.176–0.239)[a]
0.5 M EDC	0.183 (0.157–0.229)[a]	0.095 (0.072–0.106)[b]	0.378 (0.332–0.426)[a]	0.100 (0.070–0.160)[b]
1 M EDC	0.255 (0.240–0.319)[a]	0.136 (0.085–0.173)[b]	0.266 (0.236–0.311)[a]	0.052 (0.045–0.079)[b]
2 M EDC	0.252 (0.236–0.279)[a]	0.095 (0.077–0.102)[b]	0.241 (0.194–0.260)[a]	0.042 (0.034–0.061)[b]
10% GA	0.183 (0.141–0.209)[a]	0.092 (0.078–0.108)[b]	0.384 (0.318–0.492)[a]	0.105 (0.100–0.120)[b]

*Values are expressed as absorbance at 412 nm after 60 min of incubation in SensoLyte substrate and represent median values (25 percentile–75 percentile), $n = 10$.

Within each dentin treatment and application time (initial vs after-treatment MMP activity), medians identified by the same letter are not statistically different (Wilcoxon, $p > 0.05$).

Table 3

Percentage of total MMP inhibition in completely demineralized dentin specimens after the application of different concentrations of EDC and 10% glutaraldehyde (GA) for 30 s or 60 s.

Dentin Treatment	Application time		
	30 s	Statistical comparison	60 s
Water	0 (−26.0–11.3)*[b]	ns	−0.7 (–13.8–24.1)[c]
0.5 M EDC	64.4 (60.0–72.8)[a]	ns	58.6 (32.3–71.6)[ab]
1 M EDC	48.9 (34.7–67.9)[a]	**	77.6 (65.9–80.6)[a]
2 M EDC	64.4 (61.5–70.9)[a]	**	81.9 (73.3–85.3)[a]
10% GA	65.3 (59.2–70.6)[a]	ns	55.4 (49.1–59.1)[b]

* Values are expressed as percentage absorbance related to the control and represent median (25 percentile–75 percentile), $n = 10$.

** Indicates statistically significant difference between application times.

Within each application time (columns), groups identified by the same letter are not statistically different (Mann–Whitney, $p > 0.05$).

Table 4

Change in elastic modulus (E) of completely demineralized dentin specimens after the application of different concentrations of EDC and 10% glutaraldehyde (GA) for 30 s or 60 s.

Dentin treatment	Application time		
	30 s	Statistical comparison	60 s
Water	0 (−16.4–20.7)*[b]	ns	0 (−13.6–48.1)[b]
0.5 M EDC	−16.4 (−33.1–0.7)[b]	ns	−11.1 (−3.2–21.5)[b]
1 M EDC	−10.3 (−31.4–22.1)[b]	**	41.2 (−32.7–52.5)[b]
2 M EDC	1.0 (−23–22.1)[b]	**	34.2 (15.7–64.4)[b]
10% GA	123.5 (94–200.6)[a]	**	265.8 (181.3–347.5)[a]

* Values are expressed as percentage change related to the control and represent median (25 percentile–75 percentile), $n = 10$.

** Indicates statistically significant difference between application times.

Within each application time (columns), groups identified by the same letter are not statistically different (Mann–Whitney, $p > 0.05$).

Table 5

Mass loss (%) of total demineralized dentin specimens after treatment with cross-linkers for 30 or 60 s and incubation for one week.

Application time	Dentin treatment				
	Water	**0.5 M EDC**	**1 M EDC**	**2 M EDC**	**10% GA**
30 s	−6.39 ± 0.67[d]	−2.48 ± 0.65[c]	−2.24 ± 0.57[c]	−2.77 ± 1.25[c]	0.28 ± 1.03[b]
60 s	−7.75 ± 1.05[d]	−1.89 ± 0.89[c]	−2.35 ± 0.88[c]	−2.17 ± 0.70[c]	1.88 ± 1.38[a]

Abbreviations = EDC: carbodiimida; GA: glutaraldehyde.

Numbers are mean ± standard-deviation, $n = 10$. Means represented by the same letter do not differ statistically (Tukey, $p > 0.05$).

Table 6

Hydroxyproline (ng HYP/mg dentin) detected after 7-day artificial saliva storage of total demineralized dentin specimens treated with cross-linkers for 30 or 60 s.

Application time	Dentin treatment				
	Water	**0.5 M EDC**	**1 M EDC**	**2 M EDC**	**10% GA**
30 s	2571.33 (2073.11–3053.54)^{a,A}	445.49 (292.71–479.19)^{b,A}	437.47 (343.62–479.19)^{b,A}	386.43 (347.19–450.56)^{b,A}	371.21 (248.98–461.69)^{b,A}
60 s	3271.29 (2390.33–3693.05)^{a,B}	405.41 (351.11–442.39)^{b,A}	383.65 (296.40–457.50)^{b,A}	388.79 (244.09–462.87)^{b,A}	299.75 (280.01–386.13)^{b,A}

Abbreviations = EDC: carbodiimida; GA: glutaraldehyde.

Numbers are median (percentile 25–percentile 75), $n = 10$. Lowercase letters allow comparisons within application time (rows) while uppercase letters allow comparison within treatment (columns).

Medians indicated by the same letter do not differ statistically (Mann–Whitney, $p > 0.05$).

Table 7

Elevations in thermal denaturation temperature of completely demineralized dentin after treatment with increasing concentrations of EDC or glutaraldehyde.

Dentin reatment	Onset temperature		Signal max temperature	
	30 s	60 s	30 s	60 s
Water	61.43 ± 2.39 °C^{e}		67.05 ± 3.87 °C^{e}	
0.5 M EDC	67.49 ± 1.66 °C^{d}	69.79 ± 1.73 °C^{cd}	73.57 ± 1.08 °C^{d}	75.30 ± 0.70 °C^{bcd}
1 M EDC	71.13 ± 1.56 °C^{bc}	73.18 ± 1.38 °C^{ab}	74.69 ± 1.77 °C^{cd}	77.23 ± 1.40 °C^{abc}
2 M EDC	73.55 ± 1.07 °C^{ab}	75.22 ± 1.85 °C^{a}	76.60 ± 0.30 °C^{abcd}	78.48 ± 1.06 °C^{ab}
10% GA	73.64 ± 3.97 °C^{ab}	75.61 ± 1.54 °C^{a}	76.20 ± 3.85 °C^{abcd}	78.91 ± 2.28 °C^{a}

Numbers are mean ± standard-deviation, $n = 10$. Means represented by the same letter do not differ statistically (Tukey, $p > 0.05$). EDC = carbodiimida; GA = glutaraldehyde.