Research Article

Effects of carbodiimide dentin surface treatment on resin-dentin bonding

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ABSTRACT: **Purpose:** To assess the effects of ethanol-based 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) dentin surface treatment on resin-dentin bonding and dentin collagen fibril biodegradation. **Methods:** Acid-etched dentin surfaces were pretreated with different concentrations of ethanol-based EDC solutions (0.01-2M) for 60 seconds, followed by two-step etch-and-rinse dentin adhesive application and resin composite bonding. Dentin surfaces pretreated with either ethanol alone or no pretreatment were used as controls. The specimens were subjected to microtensile bond strength testing after storage in 0.9% NaCl solution at 37°C for either 24 hours or 90 days. Furthermore, demineralized dentin slabs with and without ethanol-based EDC pretreatment were exposed to a collagenase solution for 24 hours, and subsequent hydroxyproline release was measured using ELISA. Data were analyzed with ANOVA and multiple comparison tests at $\alpha = 0.05$. **Results:** The bond strength values were significantly lower for dentin surfaces pretreated with 1 and 2 M ethanol-based EDC than for the control surfaces ($P < 0.05$). The 0.01, 0.1, and 0.3 M ethanol-based EDC pretreated groups obtained significantly higher bond strength values at 90 days compared to controls. Hydroxyproline release measurements revealed that there were significantly lower levels released in the 0.3 and 1 M ethanol-based EDC pretreated specimens than for controls ($P < 0.05$). (*Am J Dent* 2016;29:208-212).

CLINICAL SIGNIFICANCE: Pretreatment of dentin surfaces with ethanol-based EDC solution ≤ 0.3M before resin composite bonding can improve the stability of the resin–dentin bond and prevent dentin collagen fibril biodegradation.

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Introduction

The durability of resin-dentin bonding is directly affected by the continuity of the interface between the resin composite and adjacent tooth structure.[1] Dentin bonding strength mostly relies on the micromechanical retention of bonding resins on etched dentin, and its long-lasting effectiveness remains a challenge to clinicians.[2] Studies[3,4] have consistently shown that endogenous dentin proteases are activated during the acid-etching procedure. Collagen fibrils from poorly infiltrated hybrid layers degrade gradually because of the collagenolytic/gelatinolytic activities of these activated proteases, which results in a significant decrease in the resin-dentin bonding strength. The use of collagen cross-linkers during bonding procedures has gained much attention in recent years.[5,6] Cross-linkers are capable of inducing nonspecific cross-linking among proteins such as collagen and dentin proteases.[7,8] The aforementioned cross-linking reaction not only increases the mechanical properties of dentin collagen fibrils but also prevents collagen fibrils from degradation.[9,10] Glutaraldehyde is a synthetic cross-linker widely used as a fixative.[11] Previous studies have reported that glutaraldehyde can improve dentin collagen properties[12] and increase the resin-dentin bond strength.[13] Unfortunately, glutaraldehyde is a highly cytotoxic agent.[12,13]

1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) has been used as an alternative cross-linking agent because of its relatively low cytotoxicity.[14] EDC can also cross-link peptides without introducing additional linkage groups.[7] Both inter- and intra-molecular cross-linking results lead to reinforcement and strengthening of dentin collagen fibrils, which are important for preserving the resin-dentin bond strength and structural integrity of the hybrid layer over time.[15] Bedran-Russo et al[7] treated demineralized dentin with 0.3 M EDC/0.12 M N-hydroxysuccinimide for different exposure times and reported that the cross-linking activity reached a plateau after 1-hour treatment, with enhanced mechanical properties of the dentin matrix and slowed collagen degradation significantly. Although a 1-hour treatment is clinically impractical, the procedure provides the potential to enhance the bonding interface stability. Furthermore, carbodiimide was recently found to be an effective cross-linker for preservation of the resin-dentin bond strength and inhibition of dentin matrix-bound matrix metalloproteinases (MMPs).[7,16,17] With its unique properties, EDC may enhance cross-linking of collagen fibrils in the dentin matrix, and thereby improve dentin stability.

A clear protocol of dentin surface treatment and guidelines to bond resin composites to dentin surfaces is crucial in order to maximize bond strength and assure the durability of the resin-dentin bond. The purposes of this study were to assess the effects of ethanol-based EDC dentin surface treatment on the resin-dentin bond strength and dentin collagen biodegradation, using different surface treatment protocols using a clinically practical exposure crosslinking time. The hypotheses were that there are no differences in the resin-dentin bond strength values and collagen resistance to collagenase-mediated degradation with regard to different concentrations of ethanol-based EDC dentin surface treatment.

Materials and Methods

Ethanol-based EDC solution preparation - EDC powder (Ekar[a]) was dissolved and diluted with dehydrated ethanol (99.7%, Sinopharm[b]) to prepare five different concentrations (0.01 M, 0.1 M, 0.3 M, 1 M, and 2 M) of ethanol-based EDC solution.

Specimen preparation - Forty caries-free third molars were collected from patients who had provided written informed con-

sent for the use of their teeth in this investigation. The molars were stored at 4°C in 0.9% (w/v) NaCl containing 0.02% sodium azide to prevent bacterial growth for no more than 1 month before use. The selected teeth were first sectioned using a low-speed diamond saw (Isomet 1000[c]) under water irrigation to prepare a flat mid-coronal dentin surface. The prepared surfaces were then polished using wet 600-grit silicon carbide papers to create a smear layer on the occlusal dentin surface. The polished dentin surfaces were etched with 35% phosphoric acid gel (Gluma Etch 35 Gel[d]) for 15 seconds, rinsed thoroughly with deionized water, and kept moist in accordance with the wet-bonding technique.[18] Excess water was removed from the surface using an absorbent mini-sponge. Specimens with acid-etched surfaces were randomly divided into seven groups according to the tested ethanol-based EDC concentrations (0.01 M, 0.1 M, 0.3 M, 1 M, and 2 M) and two control groups (no ethanol-based EDC pretreatment and ethanol alone pretreatment). In the no pretreatment control group, Single Bond 2[e]) adhesive was directly applied according to the manufacturer's instructions to the wet acid-etched dentin surface. In the ethanol alone pretreated control group, dehydrated ethanol (99.7%) was applied to the dentin surface for 60 seconds, which was kept moist before adhesive application. The acid-etched dentin surfaces in the five experimental groups were pretreated with solutions containing different concentrations of ethanol-based EDC for 60 seconds. Excess solution was removed using an absorbent mini-sponge, and Single Bond 2 adhesive was applied, per manufacturer's instructions. Four 1 mm-thick increments of resin composite (Filtek Z250[e]) were built on the treated dentin surfaces incrementally and light-cured individually for 20 seconds using an LED light unit (Elipar FreeLight 2[e]) with an output intensity of 1200 mW/cm^2. The specimens were then stored in 0.9% (w/v) NaCl solution within a 37ºC water bath for 24 hours.

Microtensile bond strength (μTBS) test - The specimen teeth were longitudinally sectioned across the bonded interface using a slow-speed diamond saw (Isomet 1000) to fabricate beam specimens measuring 0.9 mm × 0.9 mm × 8 mm for μTBS testing. The dimension of each beam was measured and recorded using a pair of digital calipers (Absolute Digimatic CD-6CS[f]). The bonded area was calculated for the subsequent conversion of failure load forces (N) into units of μTBS values (MPa). The beams were loaded until failure after either 24-hour or 90-day incubation in 0.9% (w/v) NaCl solution at 37°C. Each beam was individually fixed to a custom-made testing jig with cyanoacrylate glue (Loctite 495[g]) and subjected to tensile load using a universal testing machine (EZ-L[h]) with a crosshead speed of 1 mm/minute. All failure modes were evaluated under a stereomicroscope (CF-2000c[i]) at ×40 magnification and classified as adhesive or cohesive failure in dentin, cohesive failure in composite, or mixed failure.[19]

Data were analyzed using SPSS[j] software (version 13.0). Two-way ANOVA, post-hoc Tukey and Dunnett-t tests were used to compare the effects of the surface treatments and storage periods on μTBS values. Statistical significance was preset at $\alpha = 0.05$ for all tests.

Scanning electron microscopy (SEM) observation - Additional three resin-dentin slabs from each group were prepared as described above for observation of the resin–dentin bonding interface morphology using SEM. The specimens were stored in 0.9% (w/v) NaCl solution for 24 hours, etched with 35% phosphoric acid gel for 15 seconds, rinsed for 15 seconds with distilled water, fully deproteinized using 5.25% sodium hypochlorite solution for 20 minutes, and thoroughly washed with de-ionized water three times for 2 minutes each. Then, the specimens were immersed in de-ionized water and cleaned in an ultrasonic cleaner (KQ218[k]) for 15 minutes; dehydrated in 70%, 80%, 90%, and 100% ethanol solutions; dried in a critical point dryer (EM CPD300[l]); mounted onto aluminum stubs; sputter-coated with platinum-palladium alloy; and examined using a scanning electron microscope (S-4800[m]) with an acceleration voltage of 15 kV. Representative areas of the resin-dentin bonding interface were imaged.

Resistance against enzymatic degradation test - Twenty-eight dentin slabs with dimensions of 4.5 mm × 3.5 mm × 0.5 mm were prepared from the mid-coronal dentin, completely demineralized in 10% (w/w) phosphoric acid (Sigma[n]) for 5 hours at 37°C, and rinsed three times with deionized water for 10 minutes each. Complete demineralization was verified by x-ray. The demineralized specimens were divided into experimental groups corresponding to four ethanol-based EDC concentrations (0.01 M, 0.1 M, 0.3 M, and 1 M) and treated with their respective solutions for 60 seconds. Surfaces not pretreated with ethanol-based EDC solution, (those treated with ethanol alone for 60 seconds, and those treated with 2.5% glutaraldehyde[n] for 60 seconds) were used as controls (n= 4 specimens per group). All specimens were exposed to 100 μg/mL of type I bacterial collagenase (C0130, 125 CDU/mg[n]) in tricine buffer (0.1 M Tris-HCl, 5 mM $CaCl_2$, pH 7.4, Sigma[n]) at 37°C for 24 hours. After centrifuging at 3,000 rpm for 10 minutes (Centrifuge 5417R[o]), a 30-μL aliquot of the supernatant was collected and hydrolyzed in 6 N HCl (Sigma[n]) at 110°C for 24 hours.[20] After collagenase degradation of collagen, the amount of hydroxyproline release was quantified using an ELISA kit according to the manufacturer's instructions. The aliquots were evaporated until dry and dissolved in 30 μL of deionized water. A 10 μL aliquot of the product from the test and control groups was transferred to a 96-well plate, and 40 μL of sample diluents were added to the testing sample wells. A 50 μL aliquot of standard was added to the standard well. Moreover, a 100 μL aliquot of hydroxyproline-conjugate reagent was added to each well. The wells were then covered with a plate sealer and incubated for 60 minutes at 37°C. The wells were subsequently washed five times by filling with wash solution. A 50 μL aliquot of chromogen solution A and a 50 μL aliquot of chromogen solution B (R&D systems[p]) were added to each well, followed by incubation for 15 minutes at 37°C. A 50 μL aliquot of stop solution (R&D systems) was added to each well, and a microplate reader (Enspire[q]) was used to measure the absorbance at a 450 nm wavelength. Standard curves for hydroxyproline release were constructed. The amount of hydroxyproline released from each specimen was averaged from duplicate measurements. The data were analyzed by SPSS software. One-way ANOVA with Bonferroni correction for multiple comparisons was used for between group comparisons.

Table 1. Means and standard deviation values for the microtensile bond strength after 24-hour storage of test and control specimens in 0.9% NaCl solution at 37°C.

Treatment groups	N	μTBS (MPa)*	Failure mode (%)
2 M EDC	16	22.17 ± 13.31[a]	A:81; CC:0; CD:0; M:19
1M EDC	34	45.31 ± 17.80[b]	A:41; CC:9; CD:12; M:38
0.3 M EDC	34	65.68 ± 18.54[c]	A:26;CC:15; CD:18; M:41
0.1 M EDC	26	64.84 ± 19.76[c]	A:27;CC:31; CD:11; M:31
0.01 M EDC	33	57.23 ± 17.44[b,c]	A:27;CC:31; CD:24; M:18
Ethanol	18	52.94 ± 14.86[b,c]	A:28;CC:22; CD:22; M:28
Untreated	23	59.06 ± 17.33[c]	A:26;CC:30; CD:22; M:22

*Microtensile bond strength values are expressed as means ± standard deviations. Groups with the same superscript letters are not significantly different (P> 0.05). The failure modes observed in the microtensile bond strength test and evaluated by stereomicroscopy were classified as follows: A: adhesive, CC: cohesive failure in composite, CD: cohesive failure in dentin, M: mixed failure.

Table 2. Mean and standard deviation values for the microtensile bond strength after 24-hour and 90-day storage of test and control specimens in 0.9% NaCl solution at 37°C.

EDC Treatment groups	Storage time*		% Bond strength decrease after 90-day storage
	24-hour	90 days	
0.3 M	65.68 ± 18.54[Aa]	63.77 ± 12.17[Aa]	-2.91%
0.1 M	64.84 ± 19.76[Aa]	61.80 ± 10.76[ABa]	-4.69%
0.01 M	57.23 ± 17.43[Aa]	56.63 ± 9.76[ABa]	-1.05%
Untreated	59.06 ± 17.33[Aa]	52.98 ± 10.88[Ba]	-10.29%

*Numbers are expressed as means ± standard deviations and percentage bond strength decrease after 90-day incubation. Sample size = 15 specimens/group. The lowercase letters indicate comparison with regard to storage time. The uppercase letters indicate comparison with regard to treatment. Groups with the same superscript letters are not significantly different (P> 0.05).

Results

Microtensile bond strength testing - The mean and standard deviation values for μTBS after 24-hour storage in 0.9% NaCl solution and the percentage of specimens exhibiting each pattern of bond failure are shown in Table 1. The 1 M and 2 M ethanol-based EDC groups obtained significantly lower μTBS values (P< 0.05) with higher rates of adhesive failure than those for the other test groups.

Fifteen beams collected from the 0.3 M, 0.1 M, and 0.01 M ethanol-based EDC groups and control groups were subjected to μTBS tests after 90-day storage in 0.9% NaCl solution (Table 2). Compared with the 24-hour bond strength values, the 90-day values were lower by 1.05%-10.29%. The untreated control group displayed a significant (>10%) decrease in bond strength values (Table 2).

SEM of bonding interfaces - Representative SEM micrographs of the resin-dentin interfaces obtained from the specimens pretreated with different concentrations of ethanol-based EDC before bonding procedure, following 24-hour storage in 0.9% NaCl solution, are shown in Fig. 1. Insufficient infiltration of the adhesive and short, conical resin tags were observed in specimens from the 1 M and 2 M ethanol-based EDC groups (Figs. 1a, b). A uniform hybrid layer with long, well-formed resin tags and lateral branches was observed in the 0.3 M, 0.1 M, and 0.01 M groups (Figs. 1c, d, e). Lateral branches were not found in the ethanol-treated alone and no surface pretreated control groups (Figs. 1f, g).

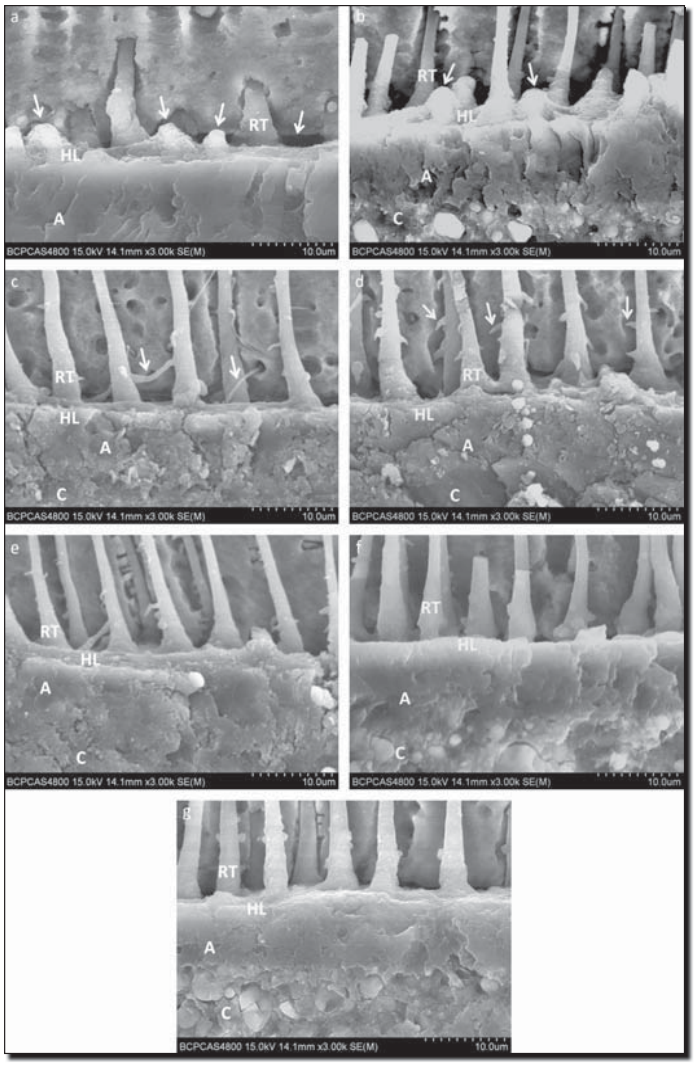

Fig. 1. Representative SEM micrographs of the resin-dentin interface obtained after 24-hour storage of specimens treated with different concentrations of ethanol-based EDC in 0.9% NaCl solution. Orig. mag ×3000. (a) and (b): Insufficient infiltration of the adhesive layer (A) and short, conical (arrows) resin tags (RT) can be observed in the hybrid layer (HL) of specimens treated with 2 M (a) and 1 M (b) ethanol-based EDC solution. (c) to (g): A uniform hybrid layer with long, well-formed resin tags and lateral branches (arrows) as well as a composite resin layer (C) can be observed in the 0.3 M (c), 0.1 M (d), and 0.01 M (e) ethanol-based EDC groups, no lateral branches found in the ethanol-treated (f) and untreated (g) control groups.

Amount of hydroxyproline release - Amount of hydroxyproline release was significantly lower in the 0.3 M and 1 M ethanol-based EDC groups as well as the 2.5% glutaraldehyde alone pretreated group than the untreated and ethanol-treated groups (P< 0.05) (Fig. 2). There were no significant differences among the 0.3 M and 1 M ethanol-based EDC groups as well as the 2.5% glutaraldehyde alone pretreated group (P> 0.05) (Fig. 2) with regard to the amount of hydroxyproline release into medium.

Discussion

The results of this study suggested that the dentin surface pretreated with the 0.3 M ethanol-based EDC solution for 60 seconds not only provides significantly high immediate bond strength but also maintains a relatively high resin-dentin bond strength value after water storage for 90 days. Furthermore, measurement of hydroxyproline release from dentin collagen after exposure to collagenase indicated that dentin surface pre-

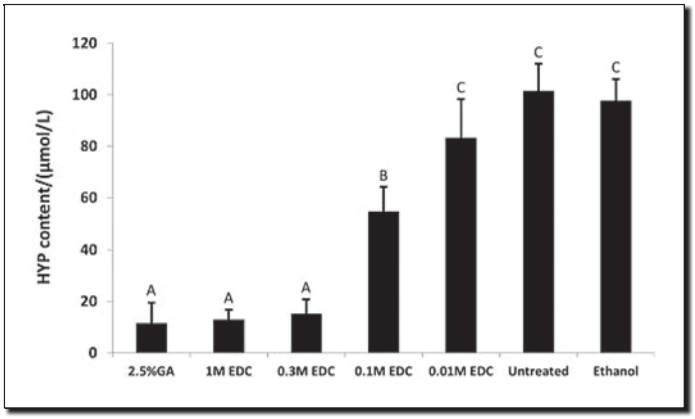

Fig. 2. Hydroxyproline (HYP) content in the supernatant (μmol/L) after 24-hour exposure to collagenase (n = 4 specimens/group). Groups pretreated with 2.5% glutaraldehyde (GA) solution and ethanol for 1 minute and the untreated group served as controls. Groups with the same superscripts are not significantly different (P> 0.05).

treated with the 0.3 M ethanol-based EDC solution improved collagen resistance to collagenase-mediated degradation, which may lead to stabilization of the hybrid layer and improve the resin–dentin bond durability. Therefore, the null hypotheses proposed in this study were all rejected.

Biomodification of the dentin substrate is an important and promising approach for the improvement of biomechanical and biochemical properties of the dentin tissue. Inter- and intramolecular cross-linking induced by cross-linkers was reported to increase the resistance of dentin collagen fibrils to biodegradation.[5] Recently, the use of cross-linking agents during dentin bonding procedures has been found to improve the resin-dentin bond durability.[5,9,21] These procedures can be classified as physical methods (photo-oxidative) or chemical methods. The photo-oxidative methods typically use light exposure, particularly ultraviolet radiation, which requires the presence of singlet oxygen.[22] However, safety issues regarding the use of ultraviolet radiation have arisen especially for dental use.[21]

Carbodiimide was introduced as a chemical treatment and considered one of the least cytotoxic cross-linkers compared with other chemicals such as glutaraldehyde. This chemical forms not only relatively stable cross-linking results,[6] but also provides the potential to enhance the mechanical properties of the dentin matrix.[7] In order to determine the optimal concentration of carbodiimide for dentin surface pretreatment use before bonding procedure, we formulated a series of ethanol-based EDC solutions and applied them onto the acid-etched dentin surfaces for 60 seconds using wet-bonding technique. In general, the presence of moisture left on the etched dentin surface results in plasticization of hydrophilic components and lowers the degree of cross-linking within the bonding interface during bonding procedure. However, ethanol-based EDC solutions were formulated and could be used to identify the nanoscopic phase domains and changes during dentin bonding.[23] Basically, the vapor pressure of ethanol is much higher than that of water, thus allowing better evaporation of residual water droplets within the dentin structure.[24] The replacement of water within the interfibrillar spaces by using ethanol-based chemicals or dentin adhesives is considered to have a positive influence on the resin-dentin bond durability.[25] Accordingly, we used ethanol as the solvent to prepare EDC solutions because the dental adhesive used in the study was also an ethanol-based component. Ethanol pretreated dentin surfaces have been reported to show dentin collagen fibril shrinkage when applied to demineralized dentin surfaces. Furthermore, results of this pretreatment procedure will provide an increase of interfibrillar spaces and hydrophobicity of the dentin matrix.[26] In addition, there will be no phase separation when the resin monomers infiltrate into the dentin matrix which is already saturated with ethanol because the adhesive used for bonding procedure is an ethanol-based material.[27] The results of the μTBS test revealed that the dentin bond strength values of the groups 0.3 M, 0.1 M, and 0.01 M ethanol-based EDC pretreatment preserve similar strengths between 24-hour and 90-day storage. However, in the 1 M and 2 M ethanol-based EDC pretreated dentin surface groups, once the ethanol solvent evaporated from the highly concentrated EDC solute, the EDC deposited in the dentin matrix. We speculate that these deposits physically interfere in the infiltration of the adhesive comonomers in the interfibrillar spaces, as illustrated by SEM images (Figs. 1a,b) showing short resin tags within hybrid layers.

Dentin contains bound MMPs and cathepsins, which have a negative effect on the resin–dentin bond durability.[4] Acid-etching of dentin, followed by the application of an etch-and-rinse adhesive, exposes endogenous MMPs through removal of the mineral component, thereby contributing to an activation process related to the acidity of the etchant and adhesive.[28] The collagenolytic and gelatinolytic activities in dentin are involved in the disruption of collagen fibrils within the hybrid layer, and this plays a key role in aging of the resin-dentin bond with time.[3,28] Previous studies[12,21,29] have shown that the mechanical and biological stability of dentin collagen can be enhanced after treatment with a variety of cross-linkers. Carbodiimide cross-linker penetrated very rapidly in acid-etched dentin, which comprises 30% (v/v) collagen and 70% (v/v) water.[4] We speculate that carbodiimide inhibits MMP activity through direct cross-linking of MMPs and strengthening of the collagen fibrils through cross-linking. Previous studies have indicated that even a short pretreatment time of 60 seconds is sufficient to inactivate endogenous protease activity within an acid-etched dentin matrix.[16,30] In this study, the resin-dentin bond strength was preserved after aging of dentin surfaces pretreated with EDC-ethanol solutions for 60 seconds. MMP cross-linking is more rapid than collagen cross-linking,[16] probably because the carboxyl and amino groups in dentin collagen are not as accessible as those in MMPs.[31] We therefore speculate that MMP inhibition plays an important role in bond strength preservation.

The measurement of hydroxyproline release can be considered an indirect evaluation of the effect of the cross-linker on the resistance of dentin collagen to collagenase-mediated biodegradation. Accordingly, lower hydroxyproline release in the supernatant may indicate higher collagen content and higher resistance to dentin collagen biodegradation. Previously published studies have reported a significant increase in the resistance of dentin collagen cross-linked with riboflavin/UVA[32] or glutaraldehyde to enzymatic degradation.[33] As a dialdehyde, glutaraldehyde reacts with amino acids directly without the formation of an intermediate product, unlike EDC.[7] This suggests that glutaraldehyde reacts faster than EDC. However, the hydroxyproline release measured in the glutaraldehyde-treated group was not significantly different from that measured in the 0.3 M and 1 M ethanol-based EDC groups. In addition, the hydroxyproline release in the supernatant was lower in the 0.3 M

and 1 M ethanol-based EDC groups than in the untreated group, suggesting that using either 0.3 M or 1.0 M ethanol-based EDC solution for dentin surface pretreatment before bonding procedure can significantly increase the resistance of dentin collagen to biodegradation within a clinically relevant time.

Within the limitations of this in vitro study, the results suggest that ethanol-based EDC surface treatment for 60 seconds preserves the resin-dentin bond strength and increases the resistance of dentin collagen to biodegradation. The 0.3 M ethanol-based EDC solution may be an optimal concentration for dentin surface treatment. Further studies are recommended to validate the feasibility of incorporating ethanol-based EDC as a component in etch-and-rinse or self-etch adhesives in order to support its in vivo application.

a. Ekar, Shanghai, China.
b. Sinopharm Chemical Reagent Co., Ltd, Beijing, China.
c. Buehler, Lake Bluff, IL, USA.
d. Heraeus-Kulzer Inc, Hanau, Germany.
e. 3M ESPE, St. Paul, MN, USA.
f. Mitutoyo Co., Ltd, Kanagawa, Japan.
g. Henkel Adhesives, Shantou, China.
h. Shimadzu Co., Ltd, Kyoto, Japan.
i. Tongfang, Shanghai, China.
j. SPSS Inc, Chicago, IL, USA.
k. Shumei, Kunshan, China.
l. Leica Microsystems Inc, Buffalo Grove, IL, USA.
m. Hitachi, Tokyo, Japan.
n. Sigma, St Louis, MO, USA.
o. Eppendorf, Hamburg, Germany.
p. R&D Systems, Minneapolis, MN, USA.
q. PerkinElmer, Seer Green, UK.

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