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Inactivation of Matrix-bound Matrix Metalloproteinases by Cross-linking Agents in Acid-etched Dentin

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Clinical Relevance

Cross-linking agents used in clinically applicable periods of time are capable of inactivating matrix-bound matrix metalloproteinases (MMP) in demineralized dentin. Such treatment may render the hybrid layer less prone to degradation over time and produce long-lasting resin-dentin bonds.

SUMMARY

Objectives: Published transmission electron microscopy analysis of *in vitro* resin-dentin bonds shows that, after 44 months, almost 70% of collagen fibrils from the hybrid layer disappear. Matrix metalloproteinases (MMPs) play an important role in that process and are thought to be the main factor responsible for the solubilization of dentin collagen. Therefore, this study aimed to evaluate the inactivation of matrix-bound MMPs by two different cross-linking agents, carbodiimide (EDC) or proanthocyanidin (PA), or the MMP-inhibitor, chlorhexidine (CHX), on acid-etched dentin using a simplified MMP assay method.

Materials and Methods: Dentin beams (2×1×6 mm) were obtained from mid-coronal dentin of sound third molars and randomly divided into six groups (G) according to the dentin treatment: G1: Deionized water (control); G2: 0.1 M EDC; G3: 0.5 M EDC; G4: 0.5 M EDC + 35% hydroxyethyl methacrylate (HEMA); G5: 5% PA; and G6: 2% CHX. The beams were etched for 15 seconds with 37% phosphoric acid, rinsed, and then immersed for 60 seconds in one of the treatment solutions. The data were expressed both in absorbance values at 412 nm and in MMP-9 activity equivalents. The total MMP activity of dentin was analyzed for one

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hour by colorimetric assay (Sensolyte). Data were submitted to Wilcoxon nonparametric test and Mann-Whitney tests ($p>0.05$).

Results: All experimental cross-linking solutions significantly reduced MMP activity from 79.8% to 95.2% when compared to the control group. No difference was observed among 0.1 M EDC (84.8%), 5% PA (87.6%), and 2% CHX (79.8%). Addition of 35% HEMA to 0.5 M EDC produced inactivation (95.2%) that was similar to that of 0.5 M EDC alone (92.7%).

Conclusion: Dentin treatment with cross-linking agents is effective to significantly reduce MMP activity. Mixing 0.5 M EDC and 35% HEMA did not influence EDC inhibitor potential.

INTRODUCTION

Since the introduction of the total-etching concept by Fusayama[1] in 1980, the effects of acid-etching of dentin have been subject to many studies. Etching dentin with 32-37% phosphoric acid removes the mineral content of the top 10 μm of dentin and exposes the collagen fibrils of the matrix, thereby creating space for monomer infiltration to achieve micromechanical retention of adhesive resins.[2] Although acid-etching of dentin provides satisfactory initial bond strength, those bond strengths fall over time, raising concerns about the long-term stability of adhesive-resin restorations.[3]

Resin/dentin bond degradation is a complex process that is not completely understood, involving the hydrolysis of both the resin and the collagen component of the hybrid layers. Acid-etched dentin contains bound matrix metalloproteinases (MMPs)-2, -3, -8, -9, and -20 and cathepsins[4,5] in their active forms. These enzymes are exposed and activated by acid-etching and can slowly degrade collagen fibrils[6-9] within the hybrid layer, resulting in a significant bond strength loss of 36-70% between 12 and 14 months.[10,11]

In order to reduce the activity of these proteases and preserve the long-term integrity of adhesive interfaces, chlorhexidine (CHX) has been used as a nonspecific inhibitor of MMPs.[6-9,11-13] CHX is also an effective inhibitor of cysteine cathepsins.[14] However, this substance is water-soluble and may undergo leaching from the hybrid layer, which impairs its long-term anti-MMP effectiveness.[13]

A new alternative to the inhibition of proteases by inhibitors is the treatment of demineralized dentin with cross-linking agents that can inactivate the catalytic site of these enzymes.[15] The cross-linker 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) is capable of forming covalent peptide bonds between proteins by activating the free carboxyl groups of glutamic and aspartic acids present in protein molecules.[16,17] This results in the formation of a o-acylisourea intermediate that reacts with the epsilon amino group of lysine or hydroxylysine in an adjacent polypeptide chain to form a stable, covalent amide bond. The only by-product of the reaction is urea,[18,19] which is water-soluble and easily removed from dentin by water rinsing. Furthermore, 0.5 M EDC shows no transdentinal cytotoxicity on odontoblast-like cells (Scheffel and others, unpublished data) and is able to increase the mechanical properties of the collagen matrix.[20]

Other cross-linking agents, such as the proanthocyanidins (PAs), are polyphenolic natural products composed of flavan-3-ol subunits linked mainly through C4-C8 (or -C6) bonds.[21] This substance is widely present in fruits, vegetables, nuts, seeds, flowers, and barks and shows numerous biological activities, such as antioxidant capacity,[22] antimicrobial effects,[23] anti-inflammatory properties,[24] positive effects on cardiovascular diseases,[25] and antiallergic activity.[26]

Thus, the purpose of this study was to evaluate the inactivation of matrix-bound MMPs by topical application of cross-linking agents on acid-etched dentin. The null hypothesis was that cross-linker–treated and –untreated dentin do not differ regarding MMP activity.

MATERIALS AND METHODS

Thirty extracted human third molars were obtained from 18-21-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 (Buehler Ltd, Lake Bluff, IL, USA) under water cooling. One 1-mm-thick dentin disk was produced from the midcoronal dentin of each tooth. Then 60 dentin beams (2×1×6 mm) were sectioned from the dentin disks. One such beam was placed in each well. This represents 40 mm^2 of dentin, which is equivalent to a Class I cavity prepared in a mandibular first molar 2 mm into the dentin and 3×4 mm in dimension. The beams were etched by dipping them into 37% phosphoric acid (pH, −0.5) for 15 seconds and then copiously rinsing with deionized water for 15 seconds. The beams were randomly divided into six groups (n=10) according to the dentin treatment, as

follows: G1: Deionized water (positive control) (pH 6.73); G2: 0.1 M EDC (pH 6.07); G3: 0.5 M EDC (pH 6.24); G4: 35 vol% hydroxyethyl methacrylate (HEMA) in water + 0.5 M EDC (pH 6.34); G5: 5% PA (Polyphenolics Inc, Madera, CA, USA) (pH 5.2) in phosphate-buffered saline (pH 6.0); and G6: 2 vol% CHX digluconate (negative control) in water (pH 6.43). All beams were dipped in the treatment solutions for 60 seconds and rinsed with distilled water for 10 seconds, except for 2% CHX, in which case the beams were only blot dried. After the treatment, each beam was placed in a 200 μL/well containing generic MMP substrate (Sensolyte Generic MMP colorimetric assay kit; catalog No. 72095, AnaSpec Inc, Fremont, CA, USA) for 60 minutes at 25°C in a 96-well plate. At the end of 60 minutes, 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 appropriate blanks. All chemicals were purchased from Sigma/Aldrich Chemical Co. The generic MMP assay uses a proprietary thiopeptide to assay MMP-1, -2, -3, -7, -8, -9, -12, -13, and -14. Thus, the kit measured the total endogenous MMP activity of dentin, with the exception of MMP-20 (enamelysin). A standard curve of absorbance of the substrate vs rh MMP-9 activity (ng) was constructed to permit expression of total MMP activity in MMP-9 equivalents. The rh MMP-9 was activated using trypsin at a final concentration of 10 μg/mL, pH 7.4, at 37°C for two hours. Then the trypsin was inactivated by addition of trypsin inhibitor at a final concentration of 100 μg/mL. Human recombinant MMP-9 was purchased from Calbiochem (catalog No. PF038; Billerica, MA, USA). Its specific activity was 1300 pmoles/mg.

Statistical Analysis

For determination of MMP activity, the absorbance data set was submitted to Wilcoxon nonparametric test and Mann-Whitney test at the 5% level of significance. The percentage of MMP activity inhibition was calculated based on the water control group MMP activity and MMP-9 equivalents (ng/well) based on the rh MMP-9 curve.

RESULTS

When mineralized dentin beams were dipped in 37 wt% phosphoric acid for 15 seconds and then rinsed with water, the top 8-10 μm of the beams were completely demineralized (Figure 1). When etched dentin beams were dipped in water (control) and then dropped into the generic MMP substrate, the absorbance at 412 nm gradually increased to 0.51 (±0.138) over 60 minutes. That value was considered to represent 100% of the total MMP activity in the etched dentin, and it was used to calculate the

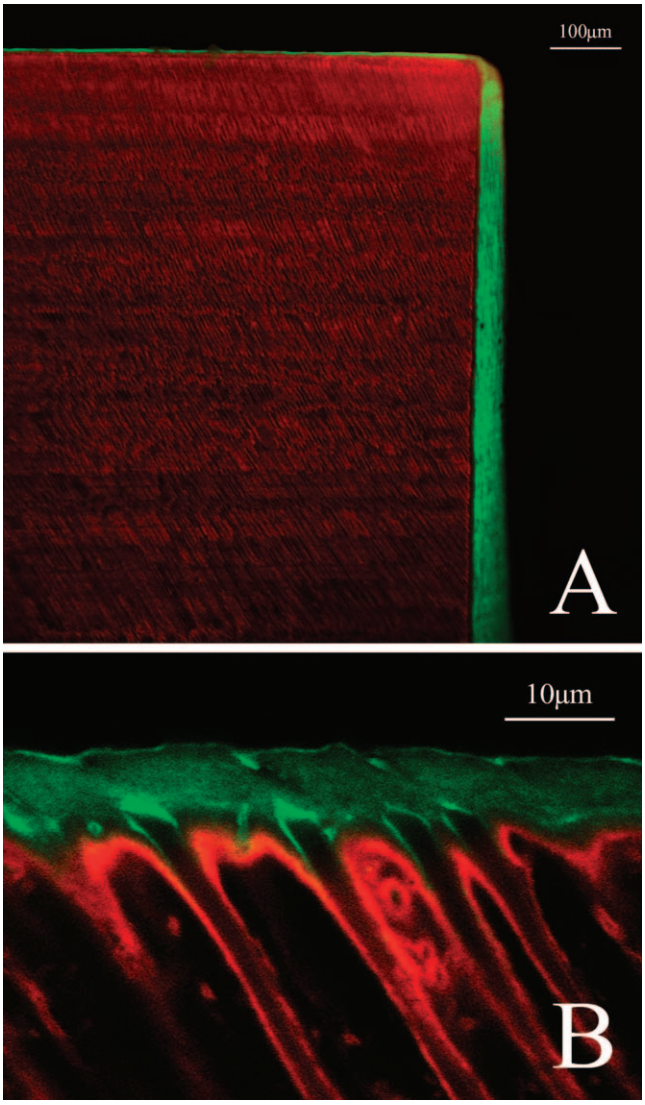

Figure 1. *Confocal laser scanning microscope (CLSM) images of the etched layer shown dyed green on the surface of a dentin beam. Dentin beams were etched in 37% phosphoric acid for 15 seconds and then rinsed with deionized water for 60 seconds and labeled for five hours, respectively, with 1% w/v fluorescein isothiocyanate (FITC) in anhydrous dimethyl sulfoxide (DMSO) and 1% w/v xylenol orange (XO) in water. The two fluorochromes selectively label collagen (FITC) and the mineralized matrix (XO), respectively. Prior to CLSM observation, the slabs were rapidly blotted with absorbent paper to remove the excess of fluorochrome, mounted on glass slides, and promptly examined. Samples were scanned in two-channel fluorescence mode with both 488 nm excitation–525 nm emission (green channel) and 546 nm excitation–580 nm emission (red channel), respectively, for FITC and XO labeling. (A) 10× projection of 53 images (final Z-stack thickness: 346 μm); the sample was intentionally tilted to highlight the peripheral distribution of demineralized collagen. (B) 100× image of the border between demineralized surface collagen fibrils (etched layer) and underlying mineralized dentin matrix.*

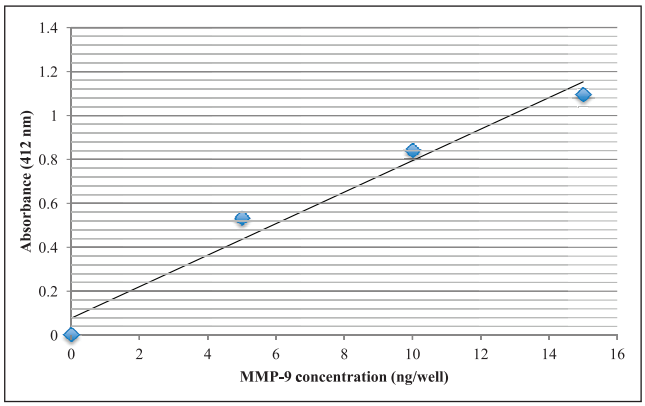

Figure 2. *Standard curve of rh matrix metalloproteinase (MMP)-9 activity (ng/well) vs absorbance at 412 nm after 60 minutes.*

percentage of MMP activity inhibition of the investigated cross-linking agents and CHX. A standard curve of substrate absorbance at 60 minutes vs ng of rh MMP-9 is shown in Figure 2. All cross-linking agents significantly reduced MMP activity in acid-etched dentin after 60 seconds of topical treatment (Table 1). The percentage of MMP inhibition for the EDC solutions, PA, and CHX ranged from 79.8% for 2 wt% CHX to 95.2% for 0.5 M EDC + 35% HEMA (Table 1). There was no statistical difference in MMP activity when 0.1 M EDC, 5% PA, and 2% CHX were compared (Table 1). When 0.5 M EDC was mixed with 35% HEMA to simulate the composition of an adhesive primer, the HEMA did not interfere with that cross-linker in inactivating the total MMP activity of acid-etched dentin. When the absorbances of the MMP activity of acid-etched dentin were expressed in MMP-9 activity equivalents, the total MMP activity of acid-etched dentin was equivalent to 6.10 (±1.93) ng of MMP-9 per 2 × 1 × 6 mm of acid-etched dentin.

DISCUSSION

The conventional method for analyzing the total bound MMP activity using Sensolyte Generic MMP colorimetric assay kit includes the complete demineralization of dentin beams for 18 hours with 10% phosphoric acid.[27] The current study used a simplified MMP assay method in which the dentin was acid-etched for 15 seconds with 37% phosphoric acid. That avoids the complete dentin demineralization and reproduces more closely the surface demineralization of dentin that is completed during etch-and-rinse bonding procedures. The complete demineralization of the dentin beam creates a much deeper collagen area (2×1×6 mm) to be infiltrated by the cross-linking solutions and adhesive resins. Clinically, acid-etching of dentin by 37 wt% phosphoric acid for 15 seconds only demineralizes dentin to a depth of 8-10 μm (Figure 1). Such relatively thin zones of demineralized dentin are easily saturated by test solutions within seconds. Nevertheless, this technique does not reproduce all *in vivo* conditions, such as the presence of pulpal pressure and the outflow of dentinal fluid.

The hybrid layer is composed of 30 vol% collagen[28] (primarily type I), while the other 70% corresponds to resin and residual solvent.[2] The collagen fibril network acts as an anchorage to resin, enabling the retention of adhesive restorations. However, transmission electron microscopy analyses revealed that almost 70% of collagen from the adhesive interface disappears after 44 months of water storage.[29] Proteases such as metalloproteinases (MMPs) and cysteine cathepsins are thought to be responsible for collagen fibril enzymatic degradation via hydrolysis.[30]

Exogenous MMP inhibitors have been tested in order to reduce protease activity and prolong the durability of resin-dentin bonds. CHX was the first

Table 1: *Absorbance, Percent Inactivation/Inhibition of Total Matrix-bound Matrix Metalloproteinase (MMP) Activity in Dentin, and MMP-9 equivalent (ng/dentin beam)*[1]

Demineralized Dentin Treatment	Absorbance (412 nm)	MMP Inhibition, %	MMP-9 Equivalent, ng
Water (control)	0.515 (±0.138) A	0 D	6.10 (±1.93) A
0.5 M EDC	0.038 (±0.014) CD	92.7 (±2.6) AB	0 (±0.19) CD
0.5 M EDC + 35% HEMA	0.025 (±0.016) D	95.2 (±3.0) A	0 (±0.22) D
0.1 M EDC	0.078 (±0.042) BC	84.8 (±8.2) BC	0 (±0.59) BC
5% PA	0.064 (±0.035) BC	87.6 (±6.7) BC	0 (±0.49) BC
2% CHX	0.104 (±0.031) B	79.8 (±6.0) C	0.37 (±0.43) B

Abbreviations: CHX, 2 wt% chlorhexidine digluconate; EDC, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride; HEMA, 2-hydroxyethyl methacrylate; PA, grape seed extract containing proanthocyanidins.

[1] Values are mean (±standard deviation) absorbance, % inhibition of total MMP activity of dentin measured by SensoLyte substrate (AnaSpec Inc, Fremont, CA, USA), and MMP-9-equivalent (ng of MMP-9/dentin beam). Within each column, groups identified by different letters are significantly different (Mann-Whitney, $p<0.05$).

MMP inhibitor proposed for such a purpose during bonding to dentin.[31] It has been largely studied as a nonspecific MMP[12] and cathepsin inhibitor.[14] CHX adsorbs on dentin and decreases hybrid layer degradation *in vitro*[7,32-34] and *in vivo*.[6,8,11,13] However, this inhibitor is soluble in water and can slowly leach from the adhesive interface over time,[13] since no chemical bond is established between the CHX molecule and the collagen fibril.

One of the mechanisms proposed to explain how MMPs degrade collagen is that these proteases unwind collagen molecules when they bind to them. By doing that, the endogenous protease's active site is allowed sufficient space to attack the specific glycine-isoleucine peptide bond in peptide chains.[35-37] Cross-linking agents stiffen collagen polypeptides so that they cannot unwind, and they can also inactivate the catalytic site of proteases[38] by creating a new peptide bond across adjacent peptides. Hence, it is reasonable to expect that MMP inactivation by cross-linking agents should last much longer than the inhibition of proteases by matrix-bound CHX. EDC and PA were first used to increase the modulus of elasticity of collagen and make it more difficult for MMPs to unwind the collagen triple-helix structure. However, EDC and PA are still not capable of increasing the stiffness of collagen in clinically relevant periods of times, such as 30 seconds and 60 seconds (Scheffel and others, unpublished data).

Despite the long application times that cross-linking agents require to increase collagen stiffness,[20] they are effective against MMPs in 60 seconds. The results of this study require rejection of the tested null hypothesis. All investigated solutions significantly decreased MMP activity in acid-etched dentin within 60 seconds. Five percent PA, 0.1 M EDC, and 0.5 M EDC were all able to inactivate more than 84% of the total active MMPs. EDC activates the free carboxylic acid groups of glutamic and aspartic acids without introducing additional methylene groups. MMPs-2 (EC 3.4.24.24), -8 (EC 3.4.24.34), -9 (EC 3.4.24.35), and -20 (EC 3.4.24), the MMPs reported to be in dentin matrix, have glutamic acid in their active sites in positions 404, 218, 402, and 227, respectively, allowing EDC to react to those sites. Additionally, the concentrations of EDC tested in this study did not produce any evidence of transdentinal cytotoxic effect on odontoblast-like cells in separate experiments (Scheffel and others, unpublished data), where they were also used at 0.1 M and 0.5 M, making EDC safe for *in vivo* application. The use of 0.5 M EDC was designed to accelerate its rate of diffusion into demineralized dentin. That EDC concentration is far in excess of the amount of protein in demineralized dentin. It is likely that only 1-2% of the EDC could react with proteins in 60 seconds. This would only generate 0.005-0.01 M of urea, which is not sufficient to denature any proteins. Denaturing concentrations of urea require 2-8 M.[39,40]

PA is a natural plant cross-linking agent. The mechanism of cross-linking is not completely understood. There are four different theories to explain how PA interacts with proteins. They include covalent,[41] ionic,[42] hydrogen bonding,[43] and hydrophobic interactions.[44] This substance has been reported to increase the stiffness of demineralized dentin[45] and to inhibit the progression of artificial root caries.[46,47] Additionally, scanning electron microscopy of demineralized dentin collagen treated with 15% PA for periods shorter than 120 seconds showed a homogeneous and regular collagen fibril arrangement, regardless of the surface moisture condition.[38] That result indicates that, in addition to acting as MMP inhibitor cross-linking agents, it can stiffen demineralized dentin sufficiently to minimize the risk of collagen network collapse resulting from air-drying. However, the PA solution has a dark color, which stains the dentin despite water rinsing. That could be a drawback for the clinical use of this cross-linker. Its rapid, complete inactivation of matrix-bound MMPs in dentin indicates that more research should be done to try to isolate an uncolored fraction of the PA.

When 0.5 M EDC was solubilized in 35 vol% HEMA, there was no reduction in its ability to inactivate all of the MMPs in dentin. That is, it was as effective as 0.5 M EDC alone. Since HEMA is an important component of adhesives, it may be possible to mix EDC with HEMA and other primer components in etch-and-rinse adhesive systems to inactivate MMPs during bonding. However, it is not known whether EDC influences adhesive polymerization. Further studies are still needed to demonstrate the effects of short-time application of cross-linking agents over time *in vitro* and *in vivo*.

CONCLUSIONS

Dentin treatment with cross-linking agents is effective in significantly reducing MMP activity; 0.5 M EDC showed the best results. Mixing 0.5 M EDC and 35% HEMA did not influence EDC cross-linking of MMPs, indicating that EDC could be added to primers in adhesive systems.

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Conflict of Interest

The authors of this manuscript certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

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