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Use of crosslinkers to inactivate dentin MMPs

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ABSTRACT

Objectives. This study evaluated the endogenous matrix metalloproteinase (MMP) activity of demineralized dentin matrix following 1 or 5 min pretreatment by various collagen crosslinkers. Generic MMP activity assay, total protein analysis, *in situ* zymography, gelatin zymography and multiplex bead technology were used to evaluate matrix-bound MMP activity.

Methods. Six different crosslinkers; glutaraldehyde, riboflavin/UVA, riboflavin-5-monophosphate/UVA, sumac berry extract, grape seed extract, and curcumin were used. Demineralized dentin beams were pretreated with respective crosslinkers for 1 or 5 min. Demineralized dentin beams with no crosslinker pretreatment served as control. The reduction in the total activity of dentin matrices were measured using generic MMP activity assay. Dentin slabs were used for *in situ* zymography and evaluated by using hydrolysis of self-quenched fluorescein-conjugated gelatin under confocal microscopy. Dentin beam extracts were used for total protein assay and multiplex analysis and powder extracts were used for gelatin zymography.

Results. MMP activity in crosslinker pretreated samples decreased significantly between 21% and 70%, whereas untreated control samples' activity increased up to 84%. Zymograms confirmed a decrease in the gelatinolytic activity and in the amount of extractable total protein content. Multiplex analysis of extracts of crosslinker-treated dentin showed a reduction in the MMP-8, MMP-2 and MMP-9 release.

Significance. The result of this work suggests that the effect of the crosslinkers is source-dependent. The use of crosslinkers for as little as 1 min on demineralized dentin can inactivate the endogenous protease activity of dentin matrices.

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1. Introduction

Enzymatic degradation of resin-infiltrated dentin matrices by endogenous proteases results in the loss of stability of resin–dentin bonds created by contemporary adhesives [1]. Matrix metalloproteinases (MMPs), a family of zinc- and calcium-dependent endogenous proteases, are responsible for the turnover of collagen-based tissues [2]. Although MMPs are inactive in mineralized dentin, the application of etching acids in etch-and-rinse or acidic monomers in self-etch adhesives can uncover and activate these proteases [3–6] resulting in progressive loss of collagen from the hybrid layers over time.

To maintain the durability of bonded restorations, use of synthetic MMP inhibitors has been demonstrated to be effective. Chlorhexidine (CHX), an inhibitor of MMP-2, -9 and -8 [7] has been shown to stabilize the resin–dentin interface [8,9]. However, recent studies have demonstrated that CHX is only electrostatically bound to dentin [10], and the inhibitory effect of CHX on dentin MMPs may be lost in 1.5–2 years [11].

Biomodification of dentin matrices using collagen crosslinkers such as glutaraldehyde or proanthocyanidin improves the mechanical properties of dentin matrix by enhancing intra- and intermolecular crosslinks of collagen [12,13] and inactivates matrix-bound MMPs [14,15]. However, a wide range of crosslinkers are available, and their specific anti-MMP effects are still not clear. In our previous publication [16] we evaluated the effects of pretreatment of completely demineralized dentin matrix by synthetic or natural crosslinking agents on the loss of dry mass and the liberation of ICTP and CTX telopeptide fragments, as indirect measures of matrix degradation over 3, 7 and 14 days of incubation at 37 °C. In the current study we used the same model and same crosslinking agents to pretreat demineralized dentin for 1 or 5 min. The outcomes that were measured were total MMP activity, total extractable protein, gelatin

zymographic activity of dentin extracts and the change in the extractability of MMP-2, -8 and -9 after pretreatment. The tested null hypotheses were that 1 or 5 min application of collagen crosslinkers does not reduce dentin protease activity, that the collagen crosslinkers do not change the release of proteins from dentin matrix, and that crosslinkers do not inhibit dentin matrix MMPs equally.

2. Materials and methods

The source of synthetic and natural collagen crosslinkers and the concentrations used in this study are presented in Table 1. Reagents were purchased from Sigma Chemical (St. Louis, MO, USA) unless otherwise specified. Extracted non-carious human third molars were obtained with informed consent from 18 to 21-year-old patients under a protocol approved by the Ethical Committee of Oulu University, Finland. The teeth were stored at 4 °C in 0.9% NaCl containing 0.02% NaN₃ to prevent microbial growth, and were used within 1 month after extraction.

2.1. Generic MMP assay

A generic colorimetric MMP assay (Sensolyte Generic MMP assay, Anaspec, San Jose, CA, USA) was used to determine if crosslinker could inhibit the total matrix-bound endogenous MMPs in dentin. The enamel and superficial dentin of human molars were removed with a low-speed saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) under water cooling. Dentin specimen with dimensions 0.4 mm × 4 mm × 4 mm was sectioned from the mid-coronal dentin (one square per tooth). Dentin squares were completely demineralized in 10% H₃PO₄ for 24 h at 4 °C and rinsed in distilled water at 4 °C for 1 h. After demineralization, the intrinsic total MMP activity of each specimen was determined by incubating them in 200 µL of chromogenic

Table 1 – Synthetic and natural crosslinking reagent and their composition, solvent, chemical content and manufacturers are shown with their concentrations used in this study.

Group	Composition	Solvent	Description	Manufacturer	Lot No
GA1 GA5	1% Glutaraldehyde (v/v) 5% Glutaraldehyde (v/v)	Distilled water	Protein crosslinker OHC(CH ₂) ₃ CHO	Merck, Finland	S5334503-928
GS1 GS5	1% Grape seed extract (w/v) 5% Grape seed extract (w/v)	Hot water	Vitis vinifera, Natural proanthocyanidin source	Mega-natural gold grape seed extract CA, USA	13682503-01
R1 R5	0.1% (–) Riboflavin (w/v) 0.5% (–) Riboflavin (w/v) with UVA light 365 nm, 7 mW/cm ²	Distilled water	Enzyme cofactor C ₁₇ H ₂₀ N ₄ O ₆	Sigma–Aldrich, Finland	OSOM1704Y
S	10% Sumac (w/v)	Hot water	Rhus coriaria, Natural proanthocyanidin source	Collected natural seed	
RP1	0.1% Riboflavin-5-phosphate (w/v) with UVA light 370 nm, 3 mW/cm ²	Distilled water	C ₁₇ H ₂₀ N ₄ P	Sigma–Aldrich, Finland	24887210
CR20 CR200	20 µM Curcumin (w/v) 200 µM Curcumin (w/v)	0.2% Ethanol in distilled water	(1E,6E)-1,7-Bis(4-hydroxy-3- methoxyphenyl)-1,6- heptadiene-3,5-dione	LKT LAB	458-37-7

thiopeptide substrate and assay buffer, in a 96-well plate for 60 min at 25 °C. After 60 min, the specimens were removed from the wells, and baseline MMP activity of each specimen was measured at 412 nm (Synergy HT; BioTek Instruments, Winooski, VT, USA). After baseline measurements, the specimens were rinsed free of MMP assay substrate and distributed to different groups in such a way that the mean baseline activity of each group was similar ($n=5/\text{group}$). The specimens were then dipped in 300 μL of water (for controls) or respective crosslinkers for 1 or 5 min, rinsed, blot-dried and incubated in fresh chromogenic substrate and assay buffer in the 96-well plate for 60 min at 25 °C. The groups were: Controls (CM) pretreated with distilled water; GA1, treated with 1% glutaraldehyde; GA5, treated with 5% glutaraldehyde; GS1, treated with 1% grape seed extract; GS5, treated with 5% grape seed extract; R1, treated with 0.1% riboflavin activated by Ultraviolet-A light (UVA; Philips, Hamburg, Germany; $\lambda = 370 \text{ nm}$ at $3 \text{ mW}/\text{cm}^2$); R5, treated with 0.5% riboflavin activated by UVA; CR20, treated with 20 μM curcumin; CR200, treated with 200 μM curcumin; S, treated with 10% sumac berry extract; RP1, treated with 0.1% riboflavin-5-phosphate activated by UVA (Table 1). After 60 min of incubation in the buffer, the specimens were removed from the incubation buffer and the MMP activity was reassessed as described above. The total MMP activity was expressed as a percentage of the untreated baseline level of each sample to determine the relative inhibition or activation.

The data from all groups were compared for normality (Kolmogorov–Smirnov test) and equality of variance (modified Levine test). When these statistical assumptions were valid, the differences in the percentage inhibition of MMPs in different groups were analyzed first using two-way ANOVA and Tukey's tests at $\alpha=0.05$ (IBM SPSS v.21, NY, USA). Since the interactions between the groups were significant, one-way ANOVA and Tukey's tests was used to evaluate the differences within each time point.

2.2. Zymographic analysis

One hundred extracted human third molars were ground free of enamel and pulpal soft tissue and roots were removed. Coronal dentin powder was prepared by pulverizing liquid nitrogen-frozen dentin crowns with a steel mortar/pestle (Reimiller, Reggio Emilia, Italy). Dentin powder was demineralized by stirring the powder with 10 wt% H_3PO_4 for 10 min at 4 °C, which was then neutralized with 70 μL 4 N NaOH and centrifuged. Mineralized dentin powder was used as a control.

Analysis of gelatinolytic activity in the dentin powder was performed using gelatin zymography in accordance with Mazzoni et al. [3]. Dentin powder (100 mg in each group) was divided into 12 groups that were treated with 100 μL crosslinker solutions for 1 or 5 min described in Table 1, and with the generic MMP assay above. In addition, mineralized dentin powder (MIN) was used as a negative control. After pretreatment, samples were rinsed twice with 200 μL distilled water and centrifuged to remove the supernatant. Samples were re-suspended in 1.8 mL extraction buffer (50 mM Tris–HCl, pH 6, containing 5 mM CaCl_2 , 100 mM NaCl, 0.1% Triton X-100, 0.1% non-ionic detergent P-40, 0.1 mM ZnCl_2 , 0.02% NaN_3) for 24 h at 4 °C under constant stirring. Each specimen

were then sonicated for 20 min and centrifuged for 20 min at 4 °C (12,000 rpm). Aliquots of samples including protein extracts were concentrated by means of a centrifugal concentrator device (10,000-kDa cut-off, Vivaspin Sartorius Stedim Biotech, Goettingen, Germany) for 30 min at 20 °C (10,000 rpm) until the volume was reduced to a volume of 100 μL . The Bradford assay was performed to determine total protein concentrations of dentin extracts. Sixty microgram of dentin protein aliquots were diluted in Laemmli sample buffer at a 4:1 ratio and subjected to electrophoresis under non-reducing conditions in 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel containing 1 mg/mL gelatin which had been fluorescently labeled with MDPF by the method of O'Grady et al. [17]. A SDS-PAGE standard, mixture of ten recombinant proteins, (Dual Color Standards, Bio-Rad), was used as molecular weight markers. After electrophoresis, the gels were washed for 30 min twice in 2.5% Triton X-100 with agitation, and incubated in activation solution (50 mmol/L Tris–HCl, 5 mM CaCl_2 , 1 mM ZnCl_2 , 150 mM NaCl, pH 7.4) for 48 h at 37 °C. To evaluate gelatinolytic activity, gels were monitored with long-wavelength UV light (Gel Doc XR System, Bio-Rad,) to reveal nonfluorescent bands cleared of gelatin by enzymes.

2.3. In situ zymography

Fourteen freshly extracted intact human third molars were used. The enamel and superficial dentin of each tooth were removed with use of a low-speed saw under water cooling. Dentin disks 0.5 mm thick were sectioned from the mid-coronal dentin. Each dentin disk was glued to a microscope slide with cyanoacrylate glue. The disk surfaces were ground with 600-grit wet silicon-carbide paper, and dentin surface was etched for 10 s with 10% H_3PO_4 and rinsed for 20 s with distilled water. Each etched dentin disk was treated with 50 μL of the relevant crosslinker solutions (Table 1) for 1 or 5 min. Mineralized and demineralized dentin disks without crosslinker pretreatment were used as controls. After crosslinking pretreatment, the dentin surface was blot-dried with absorbent paper. A self-quenched fluorescein-conjugated gelatin was used as the MMP substrate (E-12055, Molecular Probes, Eugene, OR, USA). The substrate was prepared from a 1 mL stock solution of DQ-gelatin (DQ-gelatin, E12055; Molecular Probes, Eugene, OR, USA). An anti-fading agent (Mounting Medium with Dapi H-1200, Vectashield, Vector Laboratories LTD, Cambridgeshire, UK) and fluorescein-conjugated gelatin were diluted 1:1:8 in the dilution buffer (NaCl 150 mM, CaCl_2 5 mM, Tris–HCl 50 mM, pH 8.0). Fifty microliter of the fluorescent-gelatin solution was spread on top of each sample and covered with a coverslip. Samples were incubated in dark in a 100% relative humidity chamber at 37 °C. To observe hydrolysis of quenched fluorescein-conjugated gelatin substrate that resulted from gelatinolytic activity, each sample was examined under a multi-photon confocal microscope, 488 and 530 nm with excitation and emission wavelengths, respectively (Zeiss, LSM 780, Carl Zeiss, Oberkochen, Germany). Optical sections of 85 μm thickness were acquired at different focal planes, and the stacked images were analyzed, quantified, and processed with ZEN 2010 software (Carl Zeiss, Jena, Germany). The images were obtained at 0 and 48 h after incubation.

2.4. Measurement of total extractable total protein

The measurement of total extractable protein in the dentin matrix extracts was performed using phosphoric acid demineralized dentin beams as described above. After pretreatment with crosslinkers, demineralized dentin beams were rinsed with distilled water and incubated in 0.5 mL extraction buffer (50 mM Tris–HCl, pH 6, containing 5 mM CaCl_2 , 100 mM NaCl, 0.1% Triton X-100, 0.1% non-ionic detergent P-40, 0.1 mM ZnCl_2 , 0.02% NaN_3) for 72 h at 4 °C under constant stirring. The demineralized beams dipped in water for 1 or 5 min served as control. Total protein concentrations of the extracts were determined by using Bradford method. The measurements were carried out according to the manufactures instructions (Bio-Rad, Hercules, CA, USA). A 1–50 $\mu\text{g/mL}$ series of bovine serum albumin (BSA) standards (50 μL), and 50 μL aliquots of supernatant from each beam for each time-point were analyzed. After 5 min of incubation, the absorbance was measured using a spectrophotometer (Shimadzu Scientific Instrument Inc., Columbia, MD, USA) at 595 nm wavelength. A linear standard curve was prepared by plotting the average reading for each BSA standard vs. its concentration in $\mu\text{g/mL}$. The standard curve was used to determine the protein concentration of each sample. Data were analyzed by using two-way ANOVA and Tukey's post-hoc test ($p < 0.05$).

2.5. Multiplex bead-based immunoassay

Dentin specimens with dimensions 4 mm $\times$ 6 mm $\times$ 1 mm were sectioned from the mid-coronal dentin (5 beams/group). The specimens were completely demineralized in 10% phosphoric acid as described above and transferred to individually labelled containers and placed in a vacuum desiccator containing dry silica beads for 72 h. The initial dry mass was measured with an analytical balance (XP6 Microbalance, Mettler Toledo, Hightstown, NJ, USA). After the dry mass measurement, the specimens were rehydrated for 1 h in distilled water and dipped in the crosslinker solutions (Table 1) for 1 or 5 min. After crosslinker treatment, the beams were washed three times with 200 μL of distilled water, and placed in corresponding polypropylene tubes with 0.5 mL extraction buffer (50 mM Tris–HCl, pH 6, containing 5 mM CaCl_2 , 100 mM NaCl, 0.1% Triton X-100, 0.1% non-ionic detergent P-40, 0.1 mM ZnCl_2 , 0.02% NaN_3) for 72 h at 4 °C under constant stirring. Demineralized dentin with no crosslinker treatment was used as a control.

The extractable dentinal MMPs in each group were measured using the Fluorescent Microsphere Immunoassay method using human MMP-MAP multiplex kit (R&D Systems, Inc., Minneapolis, MN, USA). The extract supernatants from each beam and reagents were pipetted into wells in duplicate, following the manufacturer's instructions, and incubated for 2 h at room temperature (RT). After washing away any unbound substances, antibodies to MMP-2, -8, and -9 were added to each well and incubated for 1 h at 25 °C. Following a wash to remove any unbound biotinylated antibody, streptavidin–phycoerythrin conjugate (streptavidin–PE) was added to each well and incubated for 0.5 h at RT. After the final wash to remove unbound streptavidin–PE, the microparticles

were resuspended in buffer and measured using a multiplex analyzer (Bio-Plex™ 200 Systems, Bio-Rad, Austin, TX, USA) and associated software. The instrument calculated the mean and the median fluorescence intensity from 100 microspheres of each set. Standard curves were fitted (5 parameters fitting program) and concentrations calculated by Milliplex Analyst software (Millipore Co., Billerica, MA, USA). Data were analyzed by using ANOVA and Tukey's post-hoc test ($p < 0.05$).

3. Results

3.1. Inhibition of MMP activity

The results of the generic total-MMP activity screening assay are shown in Fig. 1. The control group showed an $84.1 \pm 15.8\%$ increase in total MMP activity compared to the demineralized baseline level. All crosslinker pretreatment groups showed significant reductions in the total MMP activity compared to control ($p < 0.05$). After 1 min of pretreatment, the highest inhibition was 64% compared to the baseline activity observed at 5% grape seed group, which corresponds to 177% reduction compared to the activity level of control group. The highest inhibition after 5 min of crosslinking was observed at 1% grape seed group with 69% inhibition, corresponding to a 181% inhibition compared to control. The lowest inhibition was obtained using 1% glutaraldehyde at GA1 and 0.1% riboflavin after 1 min crosslinking, showing 21% and 23% inhibition compared to baseline level, and 78% and 121% respectively compared to control activity level.

3.2. Effect of crosslinkers on extractable total protein

The effect of crosslinkers on the total extractable proteins is shown in Fig. 2. After 72 h of extraction, the non-crosslinked control samples released 40 μg protein/mg dentin dry weight, whereas crosslinker-treated demineralized dentin groups showed extractable protein levels of 10–35 μg protein/mg dry dentin or 20–80% compared to controls. After 1 min of crosslinking, the extractable protein in GA5, GS1, R1, R5, CR20 and S groups were significantly lower compared to control ($p < 0.05$). After 5 min pretreatment, all crosslinker groups showed significantly lower extractable protein compared to control ($p < 0.05$). However, no significant differences were found among different crosslinkers ($p > 0.05$).

3.3. Zymographic analysis

Zymograms of gelatinolytic activities after 1 and 5 min crosslinker treatments are shown in Fig. 3A and B, respectively. Demineralized dentin (DEM) exhibited MMP-2 pro- and active forms (72 and 66 kDa, respectively), and pro- and active form MMP-9 (95–86 kDa) (Fig. 3), whereas mineralized dentin powder showed lower enzymatic activity (MIN, Fig. 3). Gelatinolytic activity was observed as faint or disappeared of MMP-2 active-forms (66 kDa), and fainter bands of MMP-9 active-forms (86 kDa) in crosslinker groups compared with controls. Reduced MMP-9 activity was obvious following both 1 and 5 min crosslinker treatment, especially in GS1, GS5, R1, R5, RP1 and S compared to control.

Fig. 1 – The bar chart shows % of total MMP activity in the control at the positive side, and % inhibition of the dentin MMPs after collagen crosslinker pretreatment or no-treatment control. The activity is expressed as the % change compared to baseline level. (Abbreviations correspond to; glutaraldehyde (GA), grape-seed extract (GS), sumac (S), curcumin (CR), riboflavin/UV (R), riboflavin-5-phosphate/UV (RP1) and untreated control (CM). Groups labeled with different letters are significantly different ($p < 0.05$). The 1 min crosslinker pretreatment groups with the same lower case letter were not statistically different ($p > 0.05$). The 5 min crosslinker pretreatment groups with the same upper case letter are not statistically different ($p > 0.05$). For each crosslinker material or concentration, as well as the control preincubation period, columns connected by a solid black bar were not statistically different from one another ($p > 0.05$).

3.4. In situ zymography

To evaluate whether the gelatinase enzymes remained bound to the dentin matrix, in situ zymography was performed. As can be seen in Fig. 4, there is a clear difference in gelatinolytic

activity between crosslinker treated and non-treated demineralized dentin. In mineralized dentin (MIN), weak activity was observed both initially and after incubation (Fig. 4A and B). Non-crosslinked demineralized dentin matrix (DEM) had the highest enzyme activity indicated by the highest intensity of

Fig. 2 – Extractable protein from various crosslinker treated demineralized dentin beams (72 h of extraction). The group incubated in extraction media (CM) with no crosslinker pretreatment served as the control ($n = 5$). Abbreviations correspond to; glutaraldehyde (GA), grape-seed extract (GS), sumac (S), curcumin (CR), riboflavin/UV (R), riboflavin-5-phosphate/UV (RP1) and untreated control (CM). Groups identified by different letters are significantly different ($p < 0.05$). One minute pretreatment groups with the same lower case letter were not statistically significant ($p > 0.05$). Five minute pretreatment groups with the same upper case letter were not statistically significant ($p > 0.05$). For each crosslinker material or concentration, as well as the control preincubation period, columns connected by a solid black bar were not statistically significant from one another ($P > 0.05$).

Fig. 3 – Zymographic analysis of dentin powder treated with or without crosslinkers for 1 min (A) or 5 min (B). MMP-2 pro- and active-form (72- and 66-kDa respectively) and MMP-9 (92–86-kDa) are labeled with arrows. Lane 1: Molecular masses, expressed in kDa, are reported in the standard lane (STD). Lane 2: MIN, mineralized dentin powder showing the presence of MMP-2 pro- and active-form (72- and 66-kDa, respectively) MMP-9 (95 kDa) in which faint activity was recorded. Lane 3: DEM, demineralized dentin with 10% phosphoric acid (control 2) showed intense activity related to the MMP-2 proform and MMP-9. Lanes 4 and 5: Demineralized dentin powder after treatment with 1% and 5% glutaraldehyde (GA) respectively, reduced MMP-2 pro- and active-form and of MMP-9 compared with the demineralized dentin. Lanes 6 and 7: Treatment of demineralized dentin powder with 1% and 5% grape seed extract (GS) respectively, had a decrease in the activity of MMP-2 and MMP-9 compared with the demineralized dentin. Lanes 8 and 9: Treatment of demineralized dentin powder with 0.1% and 0.5% riboflavin (R), respectively, almost totally inhibited both MMP-2 and -9 activities. Lanes 10 and 11: Treatment of demineralized dentin powder with 20 and 200 μ M curcumin (CR), respectively, caused partial inhibition of the 72-kDa MMP-2 proform and complete inactivation of the 86-kDa MMP-9, in addition a fainter molecular band of approximately 40 kDa was detected. Lane 12: Treatment of demineralized dentin powder with 10% sumac (S), similarly, decreased both active and pro- form of MMP-2 and MMP-9, in addition a fainter molecular band of approximately 40 kDa was detected. Lane 13: Treatment of demineralized dentin powder with 0.1% riboflavin-5-phosphate (RP1) resulted in the partial inactivation of the 66-kDa MMP-2 active and pro- form and inhibition of the 86-kDa MMP-9. As similar to CR (Lanes 10 and 11), a fainter molecular band of approximately 40 kDa was detected.

green fluorescence (Fig. 4B, DEM). Similar to the gelatin zymography results, less gelatinolytic activity was detected for the crosslinked experimental groups (Fig. 4B). Gelatinolytic activity was mainly observed within dentin tubules and slightly on exposed collagen surfaces. The gelatinolytic activity was stronger in dentin tubules before crosslinker treatment than after crosslinker treatment.

3.5. Quantification of MMP-2, -8 and -9 using multiplex bead-based immunoassay

The amount of extractable MMP-2, -8 and -9 in controls vs. crosslinked specimens are presented in Fig. 5. The control specimens showed 25-fold higher levels of MMP-2 (Fig. 5A) compared to MMP-8 (Fig. 5B). After 1 min of crosslinking, all

groups showed a significant decrease in MMP-2 extractability compared to control ($p < 0.05$), which decreased further with 5 min crosslinking.

The amount of MMP-8 extracted from control sample was only 4% as much as MMP-2 and decreased slightly after 1 min of crosslinking, but the reduction was not significant ($p > 0.05$) except for GA1, GS5, R5 and CR200 groups, which showed a significant reduction after 1 min crosslinking ($p < 0.05$). After 5 min of crosslinking, all crosslinked groups showed between 40% and 60% reductions in MMP-8 extractability which was significantly different compared to control group ($p < 0.05$).

MMP-9 extractability from all groups was significantly lower compared to MMP-2 levels. The control groups released 17 pg/mg dentin, and after 1 min crosslinking, all crosslinker groups showed significantly lower quantities ($p < 0.05$).

Fig. 4 – (A) In situ zymography of group with crosslinker following pretreatment (0 h) and (B) 48 h after incubation. Green fluorescence shows gelatinolytic activity localized in dentin surface. Demineralized dentin treated with various crosslinkers and mineralized dentin (negative control) were incubated with quenched fluorescein-labeled gelatin for 48 h at 37 °C; MIN, mineralized (control1) showed less gelatinolytic activity compared with the demineralized dentin; DEM, demineralized (control2) was observed as higher fluorescence density after incubation; GA1, demineralized treated with 1% glutaraldehyde; GA2, demineralized treated with 5% glutaraldehyde; GS1, demineralized treated with 1% grape seed extract; GS2, demineralized treated with 5% grape seed extract; R1, demineralized treated with 0.1% riboflavin; R2, demineralized treated with 0.5% riboflavin; CR1, demineralized treated with 20 μ M curcumin; CR2, demineralized treated with 200 μ M curcumin; S, demineralized treated with (w/v) 20% sumac extract; RP1, demineralized treated with 0.1% riboflavin-5-phosphate; RP1, demineralized treated with 0.5% riboflavin-5-phosphate. All treated groups showed a decrease of gelatinolytic activity after incubation. (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.)

4. Discussion

Collagen crosslinking agents have been reported to increase the resistance of dentin collagen matrix to degradation [14,18,19]. To date, glutaraldehyde is the most commonly used synthetic cross-linker, while proanthocyanidin is frequently reported in dental research as a natural cross-linker [19–22]. In the present study, we compared the effect of various plant-derived natural as well as synthetic cross-linkers on the MMP activity of collagen matrix.

Detection of MMPs activity was accomplished through the use of gelatin zymography, in situ zymography and enzyme activity assays. Additionally, multiplex bead-based assay was used to quantify the degradative enzymes by simultaneously evaluating MMP-8, MMP-2 and MMP-9 levels as collagenolytic, gelatinolytic and telopeptidase MMPs detected in dentin [9].

Notably, all investigated crosslinkers showed different potentials of reducing dentin protease activity, which were

significantly lower compared to no-treatment controls. These results require the rejection of the first null hypothesis.

The highest statistically significant inhibition of total MMP activity was observed using grape seed extract for 1 or 5 min, followed by sumac (S) and by 0.1% riboflavin 5'-phosphate (RP1), and 0.5% riboflavin after 5 min of crosslinking.

Sumac; *Rhus coriaria*, which grows wild in the region from the Mediterranean to the Middle East, is widely used as a medicinal herb in those regions, particularly for wound healing [23]. Previous studies have demonstrated strong antioxidant activity of sumac extracts [24,25] and suggest that extracts are a rich source of hydrolyzable tannins, with gallic acid being the major polyphenol [25]. The tannins are well recognized for their affinity to proteins, as well as the metal ions. Their multiple phenolic hydroxyl groups can form stable complexes with many metal ions [26].

In this study, the total protein concentrations of extracts of control and crosslinked specimens were measured to evaluate the relative efficacy of these agents in crosslinking proteins in general. The results show a decrease of 25–50% in the amount

Fig. 5 – Extractable MMP-2 (A), MMP-8 (B) and MMP-9 (C) amount (pg/mg dry dentin) after 1 or 5 min of crosslinking.

of extractable protein after 1 min of crosslinking, and an almost 70–80% decrease after 5 min crosslinking. The higher protein concentration of extracts of non-crosslinked controls is interpreted as the accumulation of solubilized collagen and released non-collagen products due to MMP activity. It is likely that pretreatment of insoluble dentin matrix by cross-linking agents would produce considerable additional intermolecular crosslinking between adjacent collagen molecules, decreasing the size exclusion cut-off of the collagen. The recent work by Toroian et al. [27] and Takahashi et al. [28] on the size-exclusion characteristics of type I collagen, show that small molecules (up to 6×10^3 Da) can diffuse in and out of collagen fibrils, but that molecules over 48×10^3 Da are excluded from entering or leaving insoluble type I collagen. We speculate that cross-linking demineralized dentin matrix lowers the permeability of soluble molecules, trapping them within the collagen fibrils.

Additionally, the results of multiplex bead analysis confirmed a significant decrease in extractable MMP-2 and MMP-9, even after as short as 1 min of crosslinking. After 5 min of

crosslinking MMP-2, MMP-8 and MMP-9 were significantly less extractable compared to untreated controls. This confirms the speculation that reduced protein extraction in crosslinked groups is correlated with crosslinking non-collagen proteins and collagen matrix, as well as crosslinking of proteases which caused their inactivation.

The binding of polyphenols to collagen and proteases is thought to be mediated by hydrogen bonding between phenolic hydroxyl residues of polyphenol and free amino groups ($-NH_2$) of collagen, or by forming ionic bonds between collagen side chains [29–31]. Proline residues are geometrically preferred polyphenol binding sites, and their interaction consists of a face-to-face stacking of aromatic polyol groups onto proline residues. MMP-9 has several proline dimers, while MMP-2 has only one [32]. However, Streck et al. [33] reported that both enzymes were inhibited to a similar extent by polyphenol-rich extracts. They explained the inactivation mechanism of gelatinases by as being dependent on the proline content, as well as on the amino acid sequences of these enzymes [33].

In the present study, UVA-irradiated 0.1% or 0.5% riboflavin inactivated the total MMPs 46% and 52%, respectively, within 5 min of crosslinking. When 0.1% riboflavin 5'-phosphate was used for crosslinking, it inactivated total matrix bound MMPs 55%, whereas the MMP activity increased 84% in the untreated control. Others have reported that the photo-induced application of 0.1% riboflavin to demineralized dentin increased the bond strength of dentin by stabilizing collagen, and decreased the collagen degradation after bacterial collagenase challenge [34,35]. Riboflavin is a photosensitizer that stimulates the formation of reactive oxygen species when irradiated by UVA light. The release of reactive oxygen species can induce the formation of covalent cross-links through oxidation by UVA [36]. Cova et al. [34] indicated that the degradation of dentin collagen can be reduced by application of UVA-activated riboflavin, and MMPs activity can be inactivated by through direct cross-linking of MMPs or by strengthening the collagen fibrils through cross-linking.

Gelatin zymography has been used for identification of the presence and activity of gelatinases MMP-2 and -9 (gelatinase A and B), are also known as 72 kDa gelatinase and 92 kDa gelatinase, respectively. Both gelatinases have three repeat fibronectin domains which are responsible for the ability of MMP-2 and MMP-9 to bind to gelatin, collagen I and IV, and laminin [37]. In present study, gelatinolytic activities were detected by gelatin zymography. Mazzoni et al. [3] was first to report the presence of both endogenous MMP-2 and -9 are present in latent forms (as pro-enzymes) in mature sound teeth and the presence of several forms of both proforms (92 kDa), and activated MMP-9 (86 kDa) in dentin. Previously, gelatin zymography was used demonstrate MMP-2 and -9 activities [38]. In the current study, proteases were observed in 66 and 86 kDa bands representing active MMP-2 and -9, and the 72 and 92 kDa bands for proMMP-2 and -9, respectively. Following demineralization, the use of crosslinkers as a pretreatment agent decreased the activity of these gelatinases as shown in the fainter 66 and 86 kDa bands.

Most MMPs are secreted as pro-enzymes (zymogens). Once these are activated by proteolytic cleavage, they are converted to their smaller active enzymes [39]. In gelatin zymography, both active and pro-MMPs gelatinolytic activity can be revealed because their molecular structures are distorted by the polyacrylamide gel although quantitation of MMP activity is difficult. *In situ* zymography is another effective method to explore the localization of the gelatin-degrading enzymes in the demineralized dentin matrix, and their relative activities before and after crosslinking [40]. This technique is also used for identification of particular enzymatic activity in physiological and/or pathological conditions. In this study, *in situ* zymography was performed on mineralized and demineralized dentin to screen the effect of crosslinkers compared to untreated controls. Activated MMP -2 and -9 in dentin have the ability to digest gelatin in untreated controls, whereas all crosslinker treated groups were inhibited to varying degrees by crosslinkers, with 5 min exposures inhibiting better than 1 min exposures. In contrast, the enzymatic activity for non-treated controls increased during incubation. The findings of the study confirm that the positive effect of short pretreatment with collagen crosslinkers on inactivation of gelatinases (especially for MMP-2 and -9) in demineralized dentin. Interestingly,

MMP-8 was much more difficult for crosslinkers to inactivate (Fig. 5B). This result requires rejection of the third test null hypothesis. Because MMP-2 and -9 contain triple fibronectin type-II like domains, unlike MMP-8 that is devoid of these domains, it is tempting to suggest that those triple domains may make MMP-2 and -9 more susceptible to crosslinking than MMP-8.

The results of this study indicate that 1 min pretreatments of acid-etched dentin by many different crosslinking agents can inactivate MMP-2, -8 and -9 in a clinically relevant time. Further research is required to determine if such inactivation is permanent over time.

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