Carbodiimide inactivation of matrix metalloproteinases in radicular dentine

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ABSTRACT

Objectives: The present in vitro study evaluated the effect of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), a cross-linking agent used as an additional therapeutic primer for luting fiber posts to radicular dentine to prevent hybrid layer degradation.

Methods: Root canal treatment was performed on 80 extracted single-rooted human teeth. A 10-mm post space was prepared and pecimens were randomly assigned to four groups (n = 20) according to the bonding system: 1) All Bond 3 (Bisco); 2) All Bond 3 + 0.3M EDC; 3) Prime&Bond XP (Dentsply Sirona); 4) Prime&Bond XP + 0.3M EDC. In groups 2 and 4, EDC was applied on phosphoric acid-etched dentine for 1 min. Fiber posts (RelyX Fiber Post, 3M ESPE) were luted with a dual-cured resin cement (Core-X flow, Dentsply Sirona). Slices were prepared for micro push-out test and interfacial nanoleakage evaluation of the coronal and apical region of the canal space after 24 h and 1 year storage in artificial saliva. In-situ zymography was performed to investigate endogenous matrix metalloproteinase activities within the hybrid layer. Results were statistically analysed with three-way ANOVA test or Chi Square test. Statistical significance was set at α = 0.05.

Results: No significant influence was identified between the two adhesives. The use of EDC significantly improved fiber post bond strength at 1 year but not at 24 h. Application of 0.3 M EDC prior to bonding significantly reduced gelatinolytic activities within the radicular hybrid layers.

Conclusions: Carbodiimide was effective in preserving fibre post bond strength over time, through reducing the activities of intra-radicular endogenous proteases.

Clinical Significance: Inhibition of matrix metalloproteinases using EDC over radicular dentin could play an important role in bond strength preservation. However, the clinical relevance of these findings needs to be proven.

1. Introduction

Debonding of fiber post is the most-reported clinical failure modality in post-retained resin composite restorations [1]. Achieving a stable bond between the fiber post and radicular dentine is essential for the longevity of post-retained adhesive restorations [2]. The 3-step etch-and-rinse and the 2-step self-etch bonding approaches well perform in fiber post cementation to radicular dentine [2,3]. In the etch-and-rinse bonding technique, resin monomers have to infiltrate the completely demineralised collagen matrix created by strong acidic etching agents [4]. Previous reports indicate that radicular dentine bond strength values are lower than similar bonds created in coronal

dentine [5]. Additional challenges are encountered when bonding is performed in the root canal space. These challenges include the presence of a secondary smear layer [6], difficulty in reaching the most apical region of the post-space with the curing light [7,8], high polymerisation contraction stresses [9], difficulty in accessing the intraradicular substrate [10] and the sclerotic nature of the root dentine substrate [11].

The susceptibility of resin-dentine bonds to degradation is well-documented in coronal dentine [12]. Acid etching of dentine exposes and activates endogenous matrix metalloproteinases (MMPs) that are embedded within the mineralised dentine during the final stage of dentineogenesis [13]. Proteinases such as MMP-2, MMP-8 and MMP-9

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[14,15] are present in both coronal and radicular dentine [16] and are responsible for the degradation of exposed collagen at the resin-dentine interface [2,17–20].

Several experimental inhibitors have been tested on coronal dentine to reduce enzyme-mediated degradation and improve the stability of the hybrid layer over time. Chlorhexidine is one of the most tested MMP inhibitors [21] for coronal [22] and radicular dentine [23–25]. The biguanide effectively inhibits MMP-2, MMP-8 and MMP-9 [26] as well as cysteine cathepsins [27], and is easy to be applied clinically. However, the long-term efficacy of chlorhexidine in arresting hybrid layer degradation is limited [28]; the solubility of chlorhexidine in water and its inability to co-polymerise with methacrylate resin monomers result in the elution of the MMP inhibitor from hybrid layers over time [12].

A more pragmatic approach in the application of MMP inhibitors to increase the longevity of resin-dentine bonds involves the use of 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC). This zero-length crosslinking agent directly conjugates carboxylates to primary amines without becoming part of the final crosslink (amide bond) between target molecules. Carbodiimide inactivates matrix-bound dentine proteinases in demineralised dentine matrices by altering the three-dimensional conformation of MMP molecules to inactivate their catalytic sites. Several studies of EDC on coronal dentine achieved a durable bonding between adhesive and dentine using one minute application of EDC to acid-etched dentine [16,29]. Furthermore, a recent study clarified the inhibiting effect of EDC on dentine collagenolytic activity within the hybrid layer created on mid coronal dentine treated with etch-and-rinse or self-etch protocols [30]. Previous studies investigated the efficacy of EDC application to radicular dentine when fiber posts were used for post-endodontic restorations [31,32]. However, no data is currently available on the stability of the EDC-crosslinked dentine over time and the efficacy of EDC in inhibiting endogenous enzymes in radicular dentine. Accordingly, the objectives of the present *in vitro* study were to evaluate the effect of the application of EDC during post cementation and to evaluate the stability of the EDC-treated hybrid layers over time. The null hypotheses tested were: (1) EDC has no effect on improving post push-out strength in resin-bonded radicular dentine, (2) EDC has no effect on improving the nanoleakage expression in resin-dentine interfaces created by the two etch-and-rinse adhesives, and (3) EDC has no effect on reducing endogenous enzymatic activities within the hybrid layer created in intraradicular dentine.

2. Materials and methods

2.1. Specimen preparation

Extracted, caries-free, human single-rooted teeth, with similar radicular length and diameter were used for the present study (mostly upper central incisors, upper lateral incisors and upper single-rooted premolars). The specimens were stored in 0.5% chloramine at 4 °C and used within one month after harvesting. After debriding the root surface, each tooth was sectioned at the cementoenamel junction, perpendicular to the longitudinal axis of the tooth, to visualise canal morphology. Among all the collected teeth, sixty-four specimens were selected, each with a circular-shaped canal and at least 12 mm of root length. Root canal treatment was performed using Pathfiles (1-2-3) and ProTaper (S1-S2-F1-F2-F3) (Dentsply Sirona, York, PA, USA) to the working length. During instrumentation, the canals were irrigated with 10 mL of 5% sodium hypochlorite (Niclor 5; Ogna, Muggiò, Italy), alternated with 2 mL of 10% ethylenediamine tetra-acetic acid (Tubuliclean; Ogna). The clean and shaped root canals were rechecked under 20X magnification using an optimal microscope (Zeiss, Germany) to confirm the shape of the coronal part of the canal after instrumentation and to exclude the presence of visible cracks (irregular, oval and cracked canals were discarded). Canals that satisfied those criteria were obturated with gutta-percha in combination with a root canal sealer (AH-Plus; Dentsply Sirona) using warm vertical compaction.

2.2. Post-luting

Each root-treated tooth was placed in a 100% relative humidity chamber for 24 h to facilitate setting of the sealer. A 10 mm post-space was subsequently created in each root-filled canal using fiber post drills (Rely-X Fiber Post, 3 M ESPE, St. Paul, MN, USA). The post space was etched for 15 s with 36% phosphoric acid (Conditioner36, Dentsply Sirona), rinsed for 60 s with air-water spray and dried with paper points. Prior to luting, the correct length of each fiber post (RelyX Fiber Post, Size 2) was verified. The specimens were randomly divided into four groups according to the adhesive protocol employed:

Group 1: All-Bond 3 (AB3, Bisco Inc., Schaumburg, IL, USA), a three-step etch-and-rinse adhesive, was applied on the acid-etched post space following the manufacturer's instructions;

Group 2: The acid-etched dentine was pre-treated with an aqueous solution of 0.3 M EDC for one minute, air-dried, and treated with AB3;

Group 3: Prime&Bond XP (XPB, Dentsply Sirona), a two-step etch-and-rinse adhesive, was mixed with the Self-Cure Activator and applied on the acid-etched post space following the manufacturer's instructions;

Group 4: The acid-etched dentine was pre-treated with 0.3 M EDC as described in Group 2, and then treated with Prime&Bond XP.

Each fiber post was cleaned in ethanol for 30 s prior to the application of a silane coupling agent (Ceramic Primer; 3 M ESPE). The primer-coupled fiber post was air-dried for 5 s. A dual-cure resin luting cement (Core-X Flow, Denstply Sirona) was used according to the manufacturer's instructions and inserted into the post-space with a suitable-sized mixing tip. After the post was inserted into the canal space for one minute, the luting cement was light-cured with a light-emitted diode curing light (Translux Power Blue, Heraeus Kulzer, Hanau, Germany). Light-curing was performed for 40 s each from the cervical surface of the root in the direction of the longitudinal axis, and then obliquely from the buccal and palatal surfaces (total 120 s). After polymerisation, the post-luted specimens were stored in distilled water at 37 °C for 24 h.

Six 1-mm thick slices were prepared from each specimen using a low-speed diamond saw (Micromet, Remet, Bologna, Italy) using water cooling. A mark was placed on the coronal side of each section with an indelible marker. The marked specimens were stored in artificial saliva at 37 °C [33].

2.3. Resistance of fiber post to dislodgement

A micro push-out test (N = 8 specimens/group) was used to evaluate the ability of the fiber posts to resist dislodgement from the bonded canal walls. Testing was conducted after 24 h and after one year of storage in artificial saliva. Push-out was performed by applying an axial load to the post at a crosshead speed of 0.5 mm/min, using an Instron Machine I model 10/D (Sintech, MTS, USA). The apical surface was placed facing the punch tip, ensuring that loading forces were introduced from an apical to coronal direction. Bond failure was manifested by the dislodgment of the fiber post from the root section. Push-out strength data were converted to MegaPascals (MPa) by dividing the load in Newtons by the bonded surface area (SL) in mm^2, and SL was calculated as the lateral surface area of a truncated cone using the formula: SL = $(\pi(R + r))*((h^2 + (R\text{-}r)^2)^{0.5}$ where R is the coronal radius of the canal with the post, r the apical radius and h the thickness of the slice. The wider and the narrowest diameters were digitally measured using ImageJ software on a picture of the slice taken on a millimetre paper to set the scale, while the thickness of the slice were individually measured using a pair of digital callipers with 0.01 mm accuracy. A single observer evaluated the debonded specimens using a stereomicroscope at 40× magnification. Failure modes were classified as: adhesive failure between dentine and cement (AD), adhesive failure between the cement and post (AP), cohesive failure within the cement

Table 1
Push–out strength (mean ± standard deviation) obtained from the four experimental groups at 24 h and after 12 month of aging in artificial saliva. Strength reduction after aging was expressed as a percentage of the 24–hour push–out strength. Root location designation: C, coronal region; M, middle region; A, apical region. Different superscript upper case letters indicate differences within the columns, different superscript lower case letters indicate differences within the rows.

Groups	24 hours (MPa)			12 months (MPa)			Bond change after storage
	Coronal	Middle	Apical	Coronal	Middle	apical	
Group 1: AB3	8.0^{A,a} (± 1.6)	5.5^{A,b} (± 2.4)	4.5^{A,c} (± 2.5)	6,1^{A,d} (± 1.6)	4.1^{A,c} (± 1.2)	3.3^{A,e} (± 1.7)	C - 23.9% M - 25% A -25.7%
Group 2: EDC + AB3	8.3^{A,a} (± 2.5)	6.1^{B,b} (± 3.2)	5.0^{B,c} (± 1.7)	8.1^{B,a} (± 1.5)	5.5^{B,b} (± 1.6)	4.5^{B,b} (± 1.8)	C -2.4% M -10.1% A -9.7%
Group 3: XPB	8.0^{A,a} (± 2.1)	5.4^{A,b} (± 1.6)	4.2^{A,c} (± 1.2)	5.8^{A,b} (± 1.8)	3.3^{C,d} (± 1.1)	2.7^{C,e} (± 1.39)	C -27.1% M -38.5% A -36.1%
Group 4: EDC + XPB	8.1^{A,a} (± 1.1)	5.8^{A,b} (± 1.7)	4.5^{A,c} (± 1.8)	7.0^{C,a} (± 1.8)	4.4^{A,c} (± 1.5)	4.0^{B,c} (± 1.5)	C -13.1% M -24.3% A -10.9%

(CC), cohesive failure within the post (CP) and mixed failure (M). The percentage of each type of failure mode within each group was calculated.

2.4. Nanoleakage

For each group, six additional specimens per group were sectioned into 1mm-thick slices. For each group, 3 specimens were analysed after 24 h, the other three after one year of storage. Slices were immersed in 50 wt% ammoniacal silver nitrate solution for 24 h. The silver ion-infiltrated specimens were subsequently immersed in photo-developing solution to reduce the silver ions into metallic silver grains. The specimens were fixed on glass slides, flattened with silicon carbide paper under running water and observed using a light microscope (Nikon E800; Nikon, Tokyo, Japan). Images of the adhesive interfaces were obtained (original magnification: 100X) and the degree of interfacial nanoleakage was scored using a four-point scale by two observers. Scoring was performed using the methodology described by Saboia et al. [34]. Intra-examiner reliability was evaluated using the kappa (κ) test.

2.5. In-situ zymography of the hybrid layer

Six additional non-carious human premolars with two distinct roots, extracted for orthodontic reasons, were used for *in-situ* zymography. Crown removal and root canal treatment were performed in the manner described in previous sections. A standardised post-space was created in all canals and the radicular dentine was etched for 15 s with 36% phosphoric acid, rinsed for 60 s and dried with paper points. The etched canals were treated according to the aforementioned adhesive procedures. The same adhesive (AB3 or XPB) was used for each tooth; one post-space was pre-treated with 0.3 M EDC while the other post-space was bonded without EDC pre-treatment. The Core-X Flow resin cement was used for luting of the fiber posts in the manner previously described. Six 1-mm thick sections were prepared from each tooth. Each section was fixed to a glass slide using glue and polished with 4000-grit silicon carbide papers with water cooling to obtain specimens with a final thickness of ˜ 50 μm.

In-situ zymography was performed with self-quenched fluorescein-conjugated gelatine as the MMP substrate (E-12055, Molecular Probes, Eugene, OR, USA) [35]. Briefly, the fluorescent gelatine mixture was placed on top of each slab and covered with a glass cover-slip. Each glass slide was light-protected and incubated in a humidified chamber at 37 °C for 24 h. Hydrolysis of quenched fluorescein-conjugated gelatine within the hybrid layer, indicative of endogenous gelatinolytic enzyme activity, was evaluated by examination of the glass slides with a multi-photon confocal laser scanning microscope (TCS SP5-AOBS 5-channel, Leica Microsystems, Buffalo Grove, IL, USA), using an excitation wavelength of 495 nm and an emission wavelength of 515 nm. Images were acquired using a HCX PL APO 40x/1.25 NA oil immersion objective always maintaining the same microscope setting. Optical sections (350 nm thick) were acquired from different focal planes. The stacked images were analysed, quantified, and processed with ImageJ software (National Institute of Health, Bethesda, MD, USA). The fluorescence intensity emitted by the hydrolysed fluorescein-conjugated gelatine was quantified and the amount of gelatinolytic activity assessed through the green signal within the hybrid layer was expressed in arbitrary units.

2.6. Statistical analysis

After ascertaining the normality (Shapiro-Wilk test) and homoscedastic (modified Levene test) assumptions of the data sets, the bond strength data were analysed with three-way analysis of variance to examine the effects of the adhesive system, EDC application and storage time, and the interaction of those three factors on micro push-out bond strength. Post-hoc pairwise comparisons were performed using the Holm-Šidák multiple comparison procedure. Chi-square tests were used to analyse nanoleakage scores and differences in the failure modes. Evaluation of the quantified data obtained from *in-situ* zymography was performed two-way analysis of variance to examine the effect of adhesive and EDC pre-treatment on potential gelatinolytic activities. Pot-hoc comparisons were conducted using the Holm-Šidák statistic. For all tests, statistical significance was pre-set at α = 0.05. All statistical analyses were performed using Stata 12.0 (StataCorp, College Station, Texas, USA).

3. Results

3.1. Push-out strength

Bond strength data were expressed as means and standard deviations and summarised in Table 1.

Results of the three-way ANOVA showed that significant difference was observed for the factors: "EDC treatment" (p = 0.002) and "time" (p = 0.000). The factor "adhesive" had no effect on the push-out bond strength (p > 0.05). The interactions between the factors "EDC treatment" and "adhesive", "adhesive" and "time", "EDC treatment" and "time" were not significant (p > 0.05). Furthermore, also the interaction of the three factors "EDC treatment", "time" and "adhesive" was not significant (p > 0.05).

Table 2

Failure mode of debonded specimens from the four experimental groups at 24 h and after one year of aging in artificial saliva. Data were expressed as percentages of the total number of specimens tested for each group. Failure mode designation; AD, adhesive failure between dentine and cement; AP, adhesive failure between cement and post; CC. cohesive failure within the cement; CP, cohesive failure within the post; M, mixed failure.

Groups	Aging time	AD	AP	CC	CP	M
Group 1:	24 h	75.7	0.7	0	0.2	23.4
AB3	12 months	86.9	0.6	0	0	12.5
Group 2:	24 h	73.5	1.2	0	0	25.3
EDC + AB3	12 months	77.3	1.1	0	0	21.5
Group 3:	24 h	78.6	1.4	0	0	20.0
XPB	12 months	84.1	0.9	0	0	15.0
Group 4:	24 h	77.3	1.3	0	0	11.4
EDC + XPB	12 months	78.9	1.7	0	0	19.4

3.2. Failure mode

Failure modes distribution of the debonded specimens, expressed as percentages of the total number of specimens tested, are summarised in Table 2. More than 95% of the failures in each group were either adhesive failure between dentine and resin cement, or mixed failures. Other failure modes were inconspicuously identified in specimens that were tested after 24 h. After artificial aging for 12 months, Groups 1 (AB3) and 3 (SPB) showed an increased number of adhesive failures between dentine and cement, although the increases were not statistically significant.

3.3. Nanoleakage

Descriptive statistics of interfacial leakage scores are represented in Fig. 1. No statistically significant differences were found among the groups in the extent of silver nitrate penetration (p = 0.052), as shown in Fig. 1.

3.4. In-situ zymography

Gelatinolytic activity, expressed as the percentage of the green fluorescence within the hybrid layer, is represented in Fig. 2.

Results of the two-way ANOVA (Fig.2) showed that significant difference was observed for the factors: "EDC treatment" (p = 0.000) and
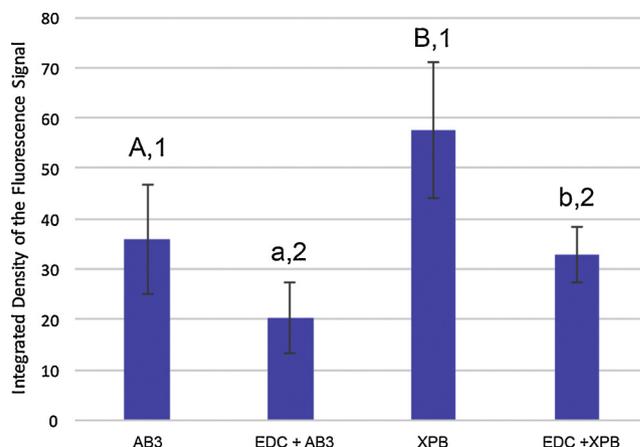

Fig. 2. Gelatinolytic activity, expressed as the percentage of the green fluorescence within hybrid layers created with All-Bond 3 (AB3) or Prime&Bond XP (XPB), with or without carbodiimide (EDC) pre-treatment of the acid-etched dentine. Values are means and standard deviations. For comparison of the factor "adhesive type", columns of labelled with the same upper case letters (24 h) or lower case letters (aging for one-year) are not significantly different (p > 0.05). For comparison of the factor "with or without EDC pre-treatment", columns labelled with the same numerals are not significantly different (p > 0.05) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

"adhesive" (p = 0.000). The interaction between the factors "EDC treatment" and "adhesive" was not statistically significant (p > 0.05).

Application of the two-step etch-and-rinse adhesive XPB to acid-etched dentine resulted in significantly more extensive gelatinolytic activity within the hybrid layer when compared to the application of the three-step etch-and-rinse adhesive AB3 (p < 0.05). For both adhesives, pre-treatment of the acid-etched dentine with 0.3 M EDC prior to adhesive application resulted in significantly reduced gelatinolytic activity within radicular hybrid layer, compared with the corresponding adhesive (p < 0.05). Confocal laser scanning microscopic examination of *in-situ* zymography specimens derived from unaged premolars in the four experimental groups revealed intense green fluorescence within the dentineal tubules of mineralized radicular dentine as well as within the hybrid layer (Fig. 3). Because the original gelatine substrate was heavily quenched with conjugated fluorescein
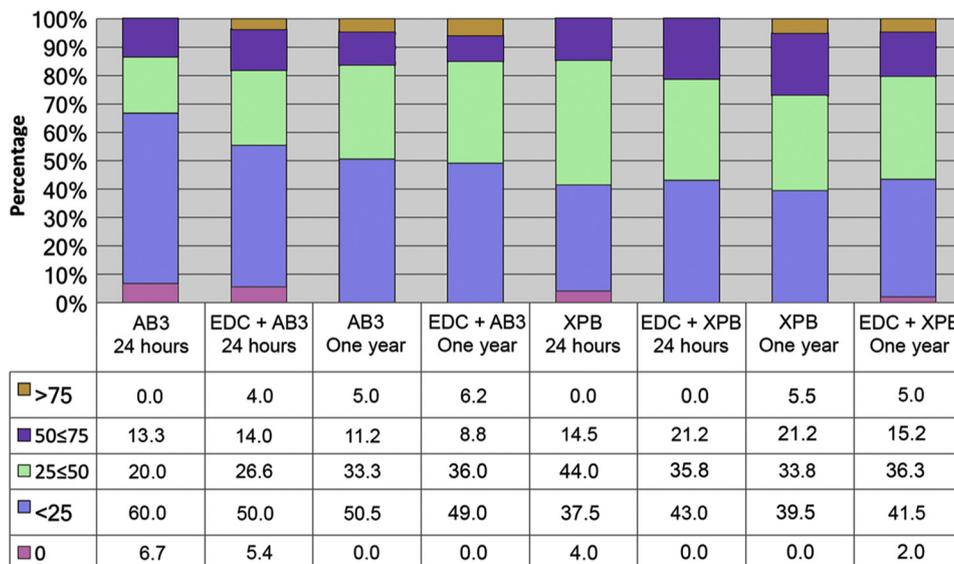

Fig. 1. Percentage of interfacial nanoleakage expression in resin-dentine interfaces created in radicular dentine with All-Bond 3 (AB3) or Prime&Bond XP (XPB), with or without carbodiimide (EDC) pre-treatment of the acid-etched dentine, at 24 h or after one year of aging in artificial saliva.
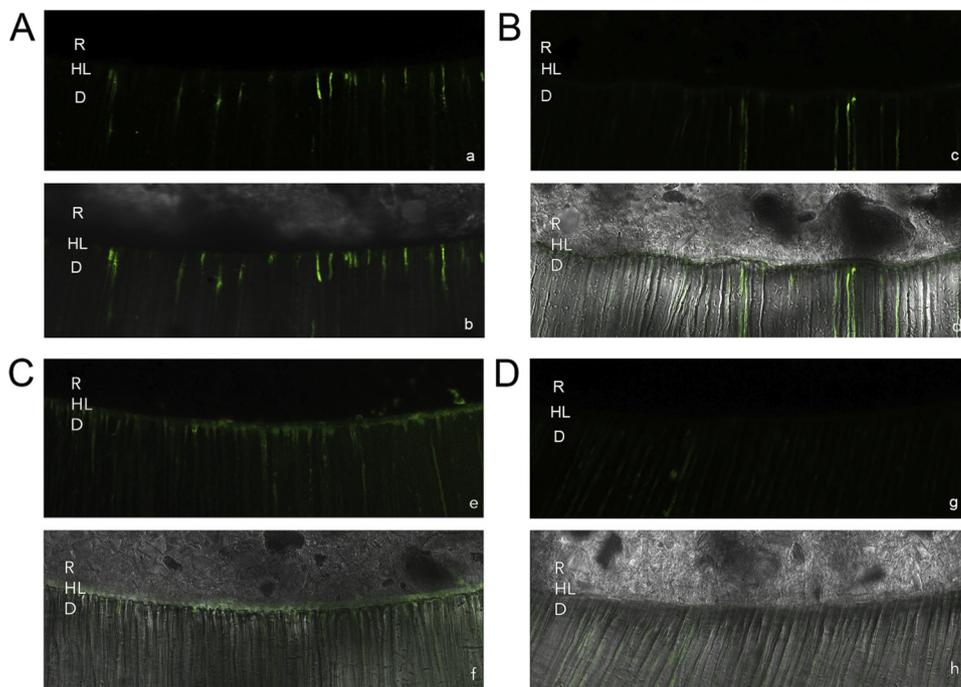
Fig. 3. Representative confocal laser scanning microscopy images of resin-bonded radicular dentine interfaces that were incubated with quenched fluorescein-labelled gelatine. Abbreviations: D, dentine; HL, hybrid layer; R, resin cement. **A.** Dentine bonded with All-Bond 3 without EDC pre-treatment. **B.** Dentine bonded with All-Bond 3 with EDC pre-treatment. **C.** Dentine bonded with Prime&Bond XP without EDC pre-treatment. **D.** Dentine bonded with Prime&Bond XP with EDC pre-treatment. For each set of images, the top image was acquired in the green channel. The green fluorescence represented areas with intense endogenous gelationolytic activity within the dentinal tubules and the hybrid layer. The bottom image was produced by merging the differential interference contrast image (showing the optical density of the resin-dentine interface) and the image acquired in green channel (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

and did not fluoresce, regions in the root slice that exhibit strong fluorescence are indicative of hydrolysis of the extrinsic gelatine substrate, which in turn, released the conjugated fluorescein molecules.

No fluorescence was detected in negative controls prepared with non-specific inhibitors, including: 1) EDTA-treated, 2) specimens incubated with 2 mM 1,10-phenanthroline or 3) with standard non-fluorescent gelatin (data not shown).

4. Discussion

Apart from providing retention to the coronal restoration, bonding of fiber posts to root-treated dentine may be regarded as a coronal seal to prevent reinfection of the canal space [36]. Thus, long-term fiber post adhesion is required to prevent microleakage, improve the prognosis of root canal treatment, and ensure the longevity of fiber-post supported restorations [37]. Results of the present study are supportive of the benefits of pre-treating acid-etched radicular dentine with EDC. Although this procedure takes an additional minute of the clinician's chair time, the use of this extra step is fruitful in preventing deterioration of bonds created in radicular dentine by cross-linking MMPS. Such a process modifies the three-dimensional conformation of the proteolytic enzymes so that their catalytic domains are rendered inactive. Fiber post push-out strength was improved when both adhesives were applied after EDC pre-treatment of the acid-etched dentine. Hence, the first null hypothesis that "EDC has no effect on improving post push-out strength in resin-bonded radicular dentine" has to be rejected. The second null hypothesis that "EDC has no effect on improving the nanoleakage expression in resin-dentine interfaces created by the two etch-and-rinse adhesives" cannot be accepted as no differences were found in silver nitrate penetration in relation to EDC pre-treatment. In-situ zymography clearly showed significant reduction in MMP-induced gelationolytic activity within the hybrid layer after EDC application, irrespective of the type of adhesive. Hence, the third null hypotheses that "EDC has no effect on reducing endogenous enzymatic activities within the hybrid layer created in intraradicular dentine" has to be rejected.

Degradation of exposed collagen by matrix metalloproteinases and cysteine cathepsins at the adhesive interface has been previously reported in root canal dentine [17,19]. Nevertheless, contradictory results

were reported on the preservation of hybrid layers in radicular dentine using chlorhexidine as MMP inhibitor. In some studies, the use of 2% chlorhexidine gel for 5 min on acid-etched radicular dentine preserved the bond strength of the fiber post luted with resin composite to the post space after cyclic loading [38] or after 12 months of water storage [23]. In another study, [39], irrigation with 2% chlorhexidine for one minute did not preserve the bond strength of luted fiber posts after 12 month of aging in artificial saliva, while ethanol-wet bonding was effective in preventing bond degradation. These inconsistencies may be related to different methods of chlorhexidine application or insufficient moisture control in the post space [40].

A recently-reported alternative to the use of chlorhexidine for preventing MMP-induced collagen degradation is EDC. The zero-length crosslinking agent has been shown to increase the mechanical properties of both sound [41] and carious [42] dentine matrix, through the formation of intermolecular and intramolecular crosslinks [43]. This modification purportedly facilitates infiltration of adhesive resin monomers into the demineralised collagen network, thus improving bond strength. However, the results of the present study showed that EDC application did not result in statistically significant difference in immediate bond strength. The reduced quantity of exposed collagen network, due to difficulties in secondary smear layer removal in post spaces [6,44], and the degradation of collagen network in endodontically-treated teeth [33,45] could explain the minimal effect of EDC pre-treatment on immediate fiber post push-out strength from bonded radicular dentine. Indeed, the present findings were in agreement with Mazzoni et al. [46], who stated that EDC pre-treatment has no effect on immediate bond strength to coronal dentine, though reducing the endogenous enzymatic activity [30].

Whereas EDC pre-treatment did not improve immediate bonding performance, the real benefits of EDC pre-treatment can only be realised after aging. After 12 months of storage in artificial saliva, the push-out strength of fiber posts bonded was significantly reduced in the absence of EDC pre-treatment, irrespective of the adhesive system employed. Etching of the intraradicular dentine within the post space exposes the catalytic domains of MMP-2, MMP-8 and MMP-9 bound to the collagen matrix of radicular dentine and activate the pro-forms of these proteolytic enzymes. By altering the configuration of these catalytic domains or allosteric inhibition of other modular domains that co-

participate in collagen degradation [47], EDC preserves fiber post push-out strength over time. These findings were confirmed by in-situ zymography evaluation, which enabled precise localization of MMPs activity within the hybrid layer [35]. Additional evidence of MMP inhibition by EDC is derived from the results of failure mode analysis. Pre-treatment of acid-etched dentine with EDC resulted in almost the same number of adhesive failures before and after 12 months of aging in artificial saliva. This is attributed to the effect of the MMP cross-linker in preserving the integrity of the collagen network. Nevertheless, the effect of EDC on bond strength decreases in the middle and apical portions of the post-space. It is speculated that the effectiveness of EDC is progressively reduced as the amount of collagen is reduced toward the apical portion of the root. Additionally factors such as moisture control, high C-factor inside the canal space [40], limited access and visibility, limited light exposure in the apical portion, as well as differences in the quantity, volume, and orientation of the tubules at different levels of the root canal may also be responsible for this reduction [9,48].

Coronal and radicular dentin differ as to the presence and concentration of MMP-2, -8 and -9 [49]. Because MMPs are also present in radicular dentin, their contribution to the degradation of resin-dentin bonds should be addressed in the development of restorative strategies for the root substrate.

The present study also represents the first attempt in using in-situ zymography to examine the endogenous protease activities of hybrid layer created in radicular dentine with 3-step and 2-step etch-and-rinse adhesives. In-situ zymography [50] was performed on unaged specimens using a much easier to degrade gelatine substrate compared with highly cross-linked dentine collagen [51]. It is a reliable and quantifiable technique for comparing the relative degradation potential of resin-dentine interfaces, without relying on actual degradation of the resin-sparse, water-rich denuded collagen fibrils [35]. Gelatinolytic activities, expressed as percentage area of the hybrid layer fluorescence within the hybrid layer, was significantly reduced when 0.3 M EDC was applied along post-space walls, confirming similar findings obtained on coronal dentine [52]. Intratubular gelatinolytic activities, which were thought to be derived from the proteins that regulate peritubular dentine formation [35], were not taken into account during quantitative evaluation of the gelatinolytic activities of the resin-dentine interfaces [53]. In addition, significantly greater MMP activities were identified in hybrid layers created with the 2-step etch-and-rinse adhesive, being localised at the bottom of the hybrid layer. This phenomenon is probably attributed to the inability of simplified etch-and-rinse adhesives to completely infiltrate the demineralised dentine [54].

5. Conclusions

Within the limits of the present study, EDC was effective in preserving fiber post push-out bond strength overtime. In-situ zymography performed on radicular dentine showed gelatinolytic activity within the hybrid layer, proving to be an effective indicator of sites of potential degradation of incompletely resin-infiltrated collagen. The use of 0.3 M EDC on acid-etched radicular dentine should be taken into consideration as a possible pre-treatment for increasing the durability of fiber post bonded restorations. Additional *in vivo* studies are in order, to clinically validate and promote the use of EDC as additional step during radicular dentine-bonding procedures.

References

[1] K.A. Guldener, C.L. Lanzrein, B.E. Siegrist Guldener, N.P. Lang, C.A. Ramseier, G.E. Salvi, Long-term clinical outcomes of endodontically treated teeth restored with or without Fiber Post-retained single-unit restorations, J. Endod. 43 (2017) 188–193, https://doi.org/10.1016/j.joen.2016.10.008.

[2] A. Mazzoni, G. Marchesi, M. Cadenaro, G. Mazzotti, R. Di Lenarda, M. Ferrari, et al., Push-out stress for fibre posts luted using different adhesive strategies, Eur. J. Oral Sci. 117 (2009) 447–453, https://doi.org/10.1111/j.1600-0722.2009.00656.x.

[3] M. Amaral, M.F. Santini, V. Wandscher, R. Amaral, L.F. Valandro, An in vitro comparison of different cementation strategies on the pull-out strength of a glass fiber post, Oper. Dent. 34 (2009) 443–451, https://doi.org/10.2341/08-113.

[4] D.H. Pashley, F.R. Tay, L. Breschi, L. Tjäderhane, R.M. Carvalho, M. Carrilho, et al., State of the art etch-and-rinse adhesives, Dent. Mater. 27 (2011) 1–16, https://doi.org/10.1016/j.dental.2010.10.016.

[5] I. Radovic, C. Mazzitelli, N. Chieffi, M. Ferrari, Evaluation of the adhesion of fiber posts cemented using different adhesive approaches, Eur. J. Oral Sci. 116 (2008) 557–563, https://doi.org/10.1111/j.1600-0722.2008.00577.x.

[6] C. Serafino, G. Gallina, E. Cumbo, M. Ferrari, Surface debris of canal walls after post space preparation in endodontically treated teeth: a scanning electron microscopic study, Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 97 (2004) 381–387, https://doi.org/10.1016/S1079210403006061.

[7] F. Cerutti, P.A. Acquaviva, M. Gagliani, M. Ferrari, F. Mangani, L.E. Depero, et al., Degree of conversion of dual-cure resins light-cured through glass-fiber posts, Am. J. Dent. 24 (2011) 8–12.

[8] R.H. Halvorson, R.L. Erickson, C.L. Davidson, Energy dependent polymerization of resin-based composite, Dent. Mater. 18 (2002) 463–469.

[9] S. Bouillaguet, S. Troesch, J.C. Wataha, I. Krejci, J.M. Meyer, D.H. Pashley, Microtensile bond strength between adhesive cements and root canal dentin, Dent. Mater. 19 (2003) 199–205.

[10] S. Bouillaguet, B. Bertossa, I. Krejci, J.C. Wataha, F.R. Tay, D.H. Pashley, Alternative adhesive strategies to optimize bonding to radicular dentin, J. Endod. 33 (2007) 1227–1230, https://doi.org/10.1016/j.joen.2007.05.016.

[11] M. Ferrari, A. Vichi, S. Grandini, Efficacy of different adhesive techniques on bonding to root canal walls: an SEM investigation, Dent. Mater. 17 (2001) 422–429.

[12] A. Frassetto, L. Breschi, G. Turco, G. Marchesi, R. Di Lenarda, F.R. Tay, et al., Mechanisms of degradation of the hybrid layer in adhesive dentistry and therapeutic agents to improve bond durability—a literature review, Dent. Mater. 32 (2016) e41–e53.

[13] L. Breschi, P. Martin, A. Mazzoni, F. Nato, M. Carrilho, L. Tjäderhane, et al., Use of a specific MMP-inhibitor (galardin) for preservation of hybrid layer, Dent. Mater. 26 (2010) 571–578, https://doi.org/10.1016/j.dental.2010.02.007.

[14] L.N. Niu, L. Zhang, K. Jiao, F. Li, Y.X. Ding, D.Y. Wang, et al., Localization of MMP-2, MMP-9, TIMP-1, and TIMP-2 in human coronal dentine, J. Dent. 39 (2011) 536–542, https://doi.org/10.1016/j.jdent.2011.05.004.

[15] A. Mazzoni, D.H. Pashley, Y. Nishitani, L. Breschi, F. Mannello, L. Tjäderhane, et al., Reactivation of inactivated endogenous proteolytic activities in phosphoric acid-etched dentine by etch-and-rinse adhesives, Biomaterials 27 (2006) 4470–4476, https://doi.org/10.1016/j.biomaterials.2006.01.040.

[16] A. Tezvergil-Mutluay, M.M. Mutluay, K.A. Agee, R. Seseogullari-Dirihan, T. Hoshika, M. Cadenaro, et al., Carbodiimide cross-linking inactivates soluble and matrix-bound MMPs, in vitro, J. Dent. Res. 91 (2012) 192–196, https://doi.org/10.1177/0022034511427705.

[17] F.R. Tay, D.H. Pashley, R.J. Loushine, R.N. Weller, F. Monticelli, R. Osorio, Self-etching adhesives increase collagenolytic activity in radicular dentin, J. Endod. 32 (2006) 862–868, https://doi.org/10.1016/j.joen.2006.04.005.

[18] I.L. Tersariol, S. Geraldeli, C.L. Minciotti, F.D. Nascimento, V. Pääkkönen, M.T. Martins, et al., Cysteine cathepsins in human dentin-pulp complex, J. Endod. 36 (2010) 475–481, https://doi.org/10.1016/j.joen.2009.12.034.

[19] J. Santos, M. Carrilho, T. Tervahartiala, T. Sorsa, L. Breschi, A. Mazzoni, et al., Determination of matrix metalloproteinases in human radicular dentin, J. Endod. 35 (2009) 686–689, https://doi.org/10.1016/j.joen.2009.02.003.

[20] A. Mazzoni, V. Papa, F. Nato, M. Carrilho, L. Tjäderhane, A. Ruggeri, et al., Immunohistochemical and biochemical assay of MMP-3 in human dentine, J. Dent. 39 (2011) 231–237, https://doi.org/10.1016/j.jdent.2011.01.001.

[21] L. Breschi, Chlorhexidine application to stabilize the adhesive interface: why and how? J. Adhes. Dent. (2013).

[22] L. Breschi, A. Mazzoni, F. Nato, M. Carrilho, E. Visintini, L. Tjäderhane, et al., Chlorhexidine stabilizes the adhesive interface: a 2-year in vitro study, Dent. Mater. 26 (2010) 320–325, https://doi.org/10.1016/j.dental.2009.11.153.

[23] D. Cecchin, J.F.A. de Almeida, B.P.F.A. Gomes, A.A. Zaia, C.C.R. Ferraz, Influence of chlorhexidine and ethanol on the bond strength and durability of the adhesion of the fiber posts to root dentin using a total etching adhesive system, J. Endod. 37 (2011) 1310–1315, https://doi.org/10.1016/j.joen.2011.05.004.

[24] D. Cecchin, A.P. Farina, M. Giacomin, Cde M. Vidal, B. Carlini-Júnior, C.C.R. Ferraz, Influence of chlorhexidine application time on the bond strength between fiber posts and dentin, J. Endod. 40 (2014) 2045–2048, https://doi.org/10.1016/j.joen.2014.08.019.

[25] R.M. Lindblad, L.V.J. Lassila, V. Salo, P.K. Vallittu, L. Tjäderhane, One year effect of chlorhexidine on bonding of fibre-reinforced composite root canal post to dentine, J. Dent. 40 (2012) 718–722, https://doi.org/10.1016/j.jdent.2012.05.002.

[26] R. Gendron, D. Grenier, T. Sorsa, D. Mayrand, Inhibition of the activities of matrix metalloproteinases 2, 8, and 9 by chlorhexidine, Clin. Diagn. Lab. Immunol. 6 (1999) 437–439.

[27] P.M.C. Scaffa, C.M.P. Vidal, N. Barros, T.F. Gesteira, A.K. Carmona, L. Breschi, et al., Chlorhexidine inhibits the activity of dental cysteine cathepsins, J. Dent. Res. 91 (2012) 420–425, https://doi.org/10.1177/0022034511435329.

[28] F.T. Sadek, R.R. Braga, A. Muench, Y. Liu, D.H. Pashley, F.R. Tay, Ethanol wet-bonding challenges current anti-degradation strategy, J. Dent. Res. 89 (2010) 1499–1504, https://doi.org/10.1177/0022034510385240.

[29] A. Mazzoni, P. Scaffa, M. Carrilho, L. Tjaderhane, R. Di Lenarda, A. Polimeni, et al., Effects of etch-and-rinse and self-etch adhesives on dentin MMP-2 and MMP-9, J. Dent. Res. 92 (2013) 82–86, https://doi.org/10.1177/0022034512467034.

[30] A. Mazzoni, V. Angeloni, N. Sartori, S. Duarte, T. Maravic, L. Tjaderhane, et al., Substantivity of carbodiimide inhibition on dentinal enzyme activity over time, J.

Dent. Res. 94 (2017) 22034517708312, , https://doi.org/10.1177/0022034517708312.

[31] D. Cecchin, L.C. Pin, A.P. Farina, M. Souza, Cde M. Vidal, Y.D. Bello, et al., Bond strength between Fiber posts and root dentin treated with natural cross-linkers, J. Endod. 41 (2015) 1667–1671, https://doi.org/10.1016/j.joen.2015.05.022.

[32] F. Shafiei, B. Yousefipour, M. Mohammadi-Bassir, Effect of carbodiimide on bonding durability of adhesive-cemented Fiber posts in root canals, Oper. Dent. 41 (2016) 432–440, https://doi.org/10.2341/15-099-L.

[33] D.H. Pashley, F.R. Tay, C. Yiu, M. Hashimoto, L. Breschi, R.M. Carvalho, et al., Collagen degradation by host-derived enzymes during aging, J. Dent. Res. 83 (2004) 216–221, https://doi.org/10.1177/154405910408300306.

[34] V.P. Saboia, F. Nato, A. Mazzoni, G. Orsini, A. Putignano, M. Giannini, et al., Adhesion of a two-step etch-and-rinse adhesive on collagen-depleted dentin, J. Adhes. Dent. 10 (2008) 419–422 PubMed PMID: 19189671.

[35] A. Mazzoni, F.D. Nascimento, M. Carrilho, I. Tersariol, V. Papa, L. Tjaderhane, et al., MMP activity in the hybrid layer detected with in situ zymography, J. Dent. Res. 91 (2012) 467–472, https://doi.org/10.1177/0022034512439210.

[36] R.S. Schwartz, J.W. Robbins, Post placement and restoration of endodontically treated teeth: a literature review, J. Endod. 30 (2004) 289–301, https://doi.org/10.1097/00004770-200405000-00001.

[37] W.S. Bachicha, P.M. DiFiore, D.A. Miller, E.P. Lautenschlager, D.H. Pashley, Microleakage of endodontically treated teeth restored with posts, J. Endod. 24 (1998) 703–708, https://doi.org/10.1016/S0099-2399(98)80157-X.

[38] D. Cecchin, M. Giacomin, A.P. Farina, C.L. Bhering, M.F. Mesquita, C.C. Ferraz, Effect of chlorhexidine and ethanol on push-out bond strength of fiber posts under cyclic loading, J. Adhes. Dent. 16 (2014) 87–92, https://doi.org/10.3290/j.jad.a30556.

[39] M. Ekambaram, C.K.Y. Yiu, J.P. Matinlinna, J.W.W. Chang, F.R. Tay, N.M. King, Effect of chlorhexidine and ethanol-wet bonding with a hydrophobic adhesive to intraradicular dentine, J. Dent. 42 (2014) 872–882, https://doi.org/10.1016/j.jdent.2014.02.006.

[40] F.R. Tay, R.J. Loushine, P. Lambrechts, R.N. Weller, D.H. Pashley, Geometric factors affecting dentin bonding in root canals: a theoretical modeling approach, J. Endod. 31 (2005) 584–589.

[41] A.K. Bedran Russo, C.M.P. Vidal, P.H. Dos Santos, C.S. Castellan, Long-term effect of carbodiimide on dentin matrix and resin-dentin bonds, J. Biomed. Mater. Res. 94 (2010) 250–255, https://doi.org/10.1002/jbm.b.31649.

[42] A.K. Bedran-Russo, G.F. Pauli, S.-N. Chen, J. McAlpine, C.S. Castellan, R.S. Phansalkar, et al., Dentin biomodification: strategies, renewable resources and clinical applications, Dent. Mater. 30 (2014) 62–76, https://doi.org/10.1016/j.dental.2013.10.012.

[43] A.K. Bedran Russo, P.N.R. Pereira, W.R. Duarte, J.L. Drummond, M. Yamauchi, Application of crosslinkers to dentin collagen enhances the ultimate tensile strength, J. Biomed. Mater. Res. 80 (2007) 268–272, https://doi.org/10.1002/jbm.b.30593.

[44] X.-H. Gu, C.-Y. Mao, C. Liang, H.-M. Wang, M. Kern, Does endodontic post space irrigation affect smear layer removal and bonding effectiveness? Eur. J. Oral Sci. 117 (2009) 597–603, https://doi.org/10.1111/j.1600-0722.2009.00661.x.

[45] M. Ferrari, P.N. Mason, C. Goracci, D.H. Pashley, F.R. Tay, Collagen degradation in endodontically treated teeth after clinical function, J. Dent. Res. 83 (2004) 414–419, https://doi.org/10.1177/154405910408300512.

[46] A. Mazzoni, V. Angeloni, F.M. Apolonio, N. Scotti, L. Tjäderhane, A. Tezvergil-Mutluay, et al., Effect of carbodiimide (EDC) on the bond stability of etch-and-rinse adhesive systems, Dent. Mater. 29 (2013) 1040–1047, https://doi.org/10.1016/j.dental.2013.07.010.

[47] N. Sela-Passwell, G. Rosenblum, T. Shoham, I. Sagi, Structural and functional bases for allosteric control of MMP activities: can it pave the path for selective inhibition? Biochim. Biophys. Acta 1803 (2010) 29–38, https://doi.org/10.1016/j.bbamcr.2009.04.010.

[48] N. Scotti, F.A. Coero Borga, M. Alovisi, E. Bergantin, S. Marchionni, D. Pasqualini, et al., Fiber-post bond strength in canals obturated with a cross-linked gutta-percha core obturator, Eur. J. Oral Sci. 122 (2014) 168–173, https://doi.org/10.1111/eos.12112.

[49] J. Santos, M. Carrilho, T. Tervahartiala, T. Sorsa, L. Breschi, A. Mazzoni, et al., Determination of matrix metalloproteinases in human radicular dentin, J. Endod. 35 (2009) 686–689, https://doi.org/10.1016/j.joen.2009.02.003.

[50] S.J. George, J.L. Johnson, In situ zymography, Matrix Metalloproteinase Protocols, Humana Press, Totowa, NJ, 2010, pp. 271–277, https://doi.org/10.1007/978-1-60327-299-5_17.

[51] Y. Kuboki, G.L. Mechanic, Comparative molecular distribution of cross-link in bone and dentin collagen. Structure-function relationships, Calcif Tissue Int. 34 (1982) 306–308.

[52] A. Mazzoni, F.M. Apolonio, V.P.A. Saboia, S. Santi, V. Angeloni, V. Checchi, et al., Carbodiimide inactivation of MMPs and effect on dentin bonding, J. Dent. Res. 93 (2014) 263–268, https://doi.org/10.1177/0022034513516465.

[53] L. Gu, A. Mazzoni, Y. Gou, C. Pucci, L. Breschi, D.H. Pashley, et al., Zymography of hybrid layers created using extrafibrillar demineralization, J. Dent. Res. 94 (2017) 22034517747264, , https://doi.org/10.1177/0022034517747264.

[54] L. Breschi, A. Mazzoni, A. Ruggeri, M. Cadenaro, R. Di Lenarda, E. De Stefano Dorigo, Dental adhesion review: aging and stability of the bonded interface, Dent. Mater. 24 (2008) 90–101, https://doi.org/10.1016/j.dental.2007.02.009.