Carbodiimide inactivation of matrix metalloproteinases in radicular dentine

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Short Title: EDC inhibits radicular MMPs

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1. Introduction

Debonding of fibre post is the most-reported clinical failure modality in post-retained resin composite restorations (Naumann *et al.* 2008). Achieving a stable bond between the fibre post and radicular dentine is essential for the longevity of post-retained adhesive restorations (Tang *et al.* 2010). The 3-step etch-and-rinse and the 2-step self-etch bonding approaches represent the gold standards in fibre post cementation to radicular dentine. In the etch-and-rinse bonding technique, resin monomers have to infiltrate the completely demineralised collagen matrix created by strong acidic etching agents (Pashley *et al.* 2011). Previous reports indicate that radicular dentine bond strength values are lower than similar bonds created in coronal dentine (Radovic *et al.* 2008). Additional challenges are encountered when bonding is performed in the root canal space. These challenges include the presence of a secondary smear layer (Serafino *et al.* 2004), difficulty in reaching the most apical region of the post-space with the curing light (Cerutti *et al.* 2011, Halvorson *et al.* 2002), high polymerisation contraction stresses (Bouillaguet *et al.* 2003a), difficulty in accessing the intraradicular substrate (Bouillaguet *et al.* 2007) and the sclerotic nature of the root dentine substrate (Ferrari *et al.* 2001).

The susceptibility of resin-dentine bonds to degradation is well-documented in coronal dentine (Frassetto *et al.* 2016). Acid etching of dentine exposes and activates endogenous matrix metalloproteinases (MMPs) that are embedded within the mineralised dentine during the final stage of dentinogenesis (Breschi, Martin, *et al.* 2010). Proteinases such as MMP-2, MMP-8 and MMP-9 (Mazzoni *et al.* 2006, Niu *et al.* 2011) are present in both coronal and radicular dentine (Tezvergil-Mutluay *et al.* 2012) and are responsible for the degradation of exposed collagen at the resin-dentine interface (Tay *et al.* 2006, Mazzoni *et al.* 2009, Tersariol *et al.* 2010, Santos *et al.* 2009, Mazzoni *et al.* 2011).

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 (Breschi 2013) for coronal (Breschi, Mazzoni *et al.* 2010) and radicular dentine (Cecchin *et al.* 2011, Cecchin, Farina *et al.* 2014, Lindblad *et al.* 2012). The biguanide effectively inhibits MMP-2, MMP-8 and MMP-9 (Gendron *et al.* 1999) as well as cysteine cathepsins (Scaffa *et al.* 2012), and is easy to be applied clinically. However, the long-term efficacy of chlorhexidine in arresting hybrid layer degradation is limited (Sadek *et al.* 2010); 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 (Frassetto *et al.* 2016).

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 (Tezvergil-Mutluay *et al.* 2012, Mazzoni, Scaffa *et al.* 2013). Furthermore, a recent study clarified the inhibiting effect of EDC on dentin collagenolytic activity within the hybrid layer created on mid coronal dentin treated with etch-and-rinse or self-etch protocols (Mazzoni *et al.* 2017). Previous studies investigated the efficacy of EDC application to radicular dentine when fibre posts were used for post-endodontic restorations (Cecchin *et al.* 2015, Shafiei *et al.* 2016). 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 hours to facilitate setting of the sealer. A 10 mm post-space was subsequently created in each root-filled canal using fibre post drills (Rely-X Fiber Post, 3M ESPE, St. Paul, MN, USA). The post space was etched for 15 seconds with 36% phosphoric acid (Conditioner36, Dentsply Sirona), rinsed for 60 seconds with air-water spray and dried with paper points. Prior to luting, the correct length of each fibre 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 dentin 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 dentin was pre-treated with 0.3M EDC as described in Group 2, and then treated with Prime&Bond XP.

Each fibre post was cleaned in ethanol for 30 seconds prior to the application of a silane coupling agent (Ceramic Primer; 3M ESPE). The primer-coupled fibre post was air-dried for 5 seconds. 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 seconds 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 seconds). After polymerisation, the post-luted specimens were stored in distilled water at 37 °C for 24 hours.

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 (Pashley *et al.* 2004).

2.3 Resistance of fibre post to dislodgement

A micro push-out test (N=8) was used to evaluate the ability of the fibre posts to resist dislodgement from the bonded canal walls (N = 8). Testing was conducted after 24 hours 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 fibre 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: $SL= (\pi(R+r))*((h^2 + (R-r))^2)^{0.5}$ 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 (CC), cohesive failure within the post (CP) and mixed failure (M). The percentage of each type of failure mode within each group was calculated.

2.3 Nanoleakage

Specimen were sectioned into 1mm-thick slices and immersed in 50 wt% ammoniacal silver nitrate solution for 24 hours. 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.* (2008). Intra-examiner reliability was evaluated using the kappa (κ) test.

2.4 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 seconds with 36% phosphoric acid, rinsed for 60 seconds 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 fibre 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) (Mazzoni *et al.* 2012). 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 hours. 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.

2.5 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 $\alpha = 0.05$. 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 ($p > 0.05$).

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 hours. 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 Figure 1. No statistically significant differences were found among the groups in the extent of silver nitrate penetration ($p = 0.052$), as shown in Figure 1.

3.4 *In-situ zymography*

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

Results of the two-way ANOVA showed that significant difference was observed for the factors: “EDC treatment” ($p = 0.000$) and “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 dentin 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 dentin 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 dentinal tubules of mineralized radicular dentine as well as within the hybrid layer (Figure 3). Because the original gelatine substrate was heavily quenched with conjugated fluorescein 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 nonfluorescent gelatin (data not shown).

4. Discussion

Apart from providing retention to the coronal restoration, bonding of fibre posts to root-treated dentine may be regarded as a coronal seal to prevent reinfection of the canal space (Schwartz & Robbins 2004). Thus, long-term fibre post adhesion is required to prevent microleakage, improve the prognosis of root canal treatment, and ensure the longevity of fibre-post supported restorations (Bachicha *et al.* 1998). Results of the present study are supportive of the benefits of pre-treating acid-etched radicular dentin 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. Fibre 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. Because nanoleakage expression in the experiment groups was independent of EDC pre-treatment, 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 rejected. *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 (Tay *et al.* 2006, Santos *et al.* 2009). Nevertheless, contradictory results were reported on the preservation of hybrid layers in radicular dentin 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 fibre post luted with resin composite to the post space after cyclic loading (Cecchin, Giacomin, *et al.* 2014) or after 12 months of water storage (Cecchin *et al.* 2011). In another study, (Ekambaram *et al.* 2014), irrigation with 2% chlorhexidine for one minute did not preserve the bond strength of luted fibre 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 (Tay et al. 2005).

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 (Bedran Russo *et al.* 2010) and carious (Bedran-Russo *et al.* 2014) dentine matrix, through the formation of intermolecular and intramolecular crosslinks (Bedran Russo *et al.* 2007). 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 deep post spaces (Serafino *et al.* 2004, Gu *et al.* 2009), and the degradation of collagen network in endodontically-treated teeth (Ferrari *et al.* 2004, Pashley *et al.* 2004) could explain the minimal effect of EDC pre-treatment on immediate fibre post push-out strength from bonded radicular dentin. Indeed, the present findings were in agreement with Mazzoni *et al.* (Mazzoni, Angeloni *et al.* 2013), who stated that EDC pre-treatment has no effect on immediate bond strength to coronal dentine, though reducing the endogenous enzymatic activity (Mazzoni *et al.* 2017).

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 fibre 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 (Sela-Passwell *et al.* 2010, Liu *et al.* 2011), EDC preserves fibre 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 (Mazzoni *et al.* 2012). Additional evidence 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 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 (Tay *et al.* 2005), 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 (Bouillaguet *et al.* 2003, Farina *et al.* 2011, Ferrari *et al* 2000, Scotti *et al.* 2014).

The present study 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 (George & Johnson 2010) was performed on unaged specimens using a much easier to degrade gelatine substrate compared with highly cross-linked dentine collagen (Kuboki & Mechanic 1982). It is a reliable and quantifiable technique for comparing the relative degradation potential of resin-dentin interfaces, without relying on actual degradation of the resin-sparse, water-rich denuded collagen fibrils (Mazzoni *et al.* 2012). 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 dentin (Mazzoni *et al.* 2014). Intratubular gelatinolytic activities, which were thought to be derived from the proteins that regulate peritubular dentin formation (Mazzoni *et al.* 2012), were not taken into account during quantitative evaluation of the gelatinolytic activities of the resin-dentin interfaces (Gu *et al.* 2017). 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 (Breschi *et al.* 2008).

5. Conclusions

Within the limits of the present study, EDC was effective in preserving fibre post push-out bond strength overtime. *In-situ* zymography performed on radicular dentine showed gelatinolytic activity within the hybrid layer, an 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 fibre post bonded restorations. Additional *in vivo* studies are in order, to clinically validate and promote the use of EDC as additional step during radicular dentin-bonding procedures.

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