Cross-linking effect on dentin bond strength and MMPs activity

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ABSTRACT

Objective. The objectives of the study were to evaluate the ability of a 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide (EDC)-containing primer to improve immediate bond strength of either self-etch or etch-and-rinse adhesive systems and to stabilize the adhesive interfaces over time. A further objective was to investigate the effect of EDC on the dentinal MMPs activity using zymographic analysis.

Methods. Freshly extracted molars (n = 80, 20 for each group) were selected to conduct microtensile bond strength tests. The following groups were tested, immediately or after 1-year aging in artificial saliva: G1: Clearfil SE (CSE) primer applied on unetched dentin, pretreated with 0.3 M EDC water-solution for 1 min and bonded with CSE Bond; G2: as G1 but without EDC pre-treatment; G3: acid-etched (35% phosphoric-acid for 15s) dentin pre-treated with 0.3 M EDC, then bonded with XP Bond (XPB); Group 4 (G4): as G3 without EDC pre-treatment. Further, gelatinase activity in dentin powder treated with CSE and XPB with and without EDC pre-treatment, was analyzed using gelatin zymography.

Results. The use of 0.3 M EDC-containing conditioner did not affect the immediate bond strength of XPB or CSE adhesive systems ($p > 0.05$), while it improved the bond strength after 1 year of aging ($p < 0.05$). Pre-treatment with EDC followed by the application of CSE resulted in an incomplete MMPs inactivation, while EDC pretreatment followed by the application of XPB resulted in an almost complete inactivation of dentinal gelatinases.

Significance. The μTBS and zymography results support the efficacy of EDC over time and reveal that changes within the dentin matrix promoted by EDC are not adhesive-system-dependent.

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1. Introduction

Currently, adhesive dental restorations are an essential part in everyday dental practice [1]. However, despite evolution of adhesive protocols, the hybrid layer (HL) remains the weakest point of resin–composite restorations. The structure of this connecting layer is responsible for the retention of the resin restorations. However, it is also the most vulnerable area of the adhesive-resin bond [2]. Previous *in vitro* and *in vivo* studies revealed that degradation of resin dentin bonds over time is caused by hydrolytic breakdown of the resin or of dentinal collagen fibrils [3,4], identifying the important contribution of host derived proteinases in the deterioration of the hybrid layer over time [5–8]. To date, several matrix metalloproteinases (MMPs) and cysteine cathepsins have been identified in dentin; while their role is still unclear in sound dentin, they could synergistically digest collagen fibrils exposed at the adhesive interface [8].

Collagen fibrils not completely encased by resin polymers during the bonding procedure are highly susceptible to enzymatic hydrolysis over time [9]. Furthermore, polymer degradation leads to the exposure of more collagen. The unprotected collagen fibrils at the base of the hybrid layer are slowly destroyed by proteases that are bound, directly or indirectly to the fibrils, causing the loss of the anchoring function of the HL with the consequent loss of bond strength [10]. A significant fall in bond strength of 36–70% after 1 year of storage has been reported [4,11]. Thus, attempts to increase the resistance of collagen against enzymatic deterioration, and the inactivation of these proteases are fundamental approaches to enhance the quality and the longevity of dental restorations. The inhibition enzymes activity is crucial to prolong the resin–dentin bond strength over time [8,12].

The use of synthetic MMP-inhibitors [13,14], quaternary ammonium methacrylates, benzalconium chloride [15] or other reagents has been proposed to increase the durability of resin dentin bonds. Among these different approaches, the use of cross-linkers has recently attracted the interest of investigators.

Endogenous cross-linkers are naturally present in collagen structure in the form of intra- and inter-molecular covalent or ionic bonds which provide the fibrillar resistance against enzymatic degradation as well as greater tensile properties [16,17].

The biomodification of dentinal collagen has been proposed through the application of exogenous cross-linking solutions prior to the adhesive procedures. Such procedures have shown improvement of the mechanical properties of collagen, thus increasing its resistance to degradation, resulting in superior ultimate tensile strength and in an enhancement of resin–dentin bond durability [7,18].

Among the available cross-linking reagents, 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide (EDC), has shown promising results due to its ability to cross-link peptides without introducing additional linkage groups [19]. Recent *in vitro* studies have demonstrated that the application of EDC to etched dentin surfaces for 60 s inactivates matrix MMPs [20]. However, although EDC have shown promising results at baseline, information on the behavior of EDC and its capability of inactivating MMPs over time are still missing.

Thus, the aim of this study was to evaluate the ability of a EDC-containing primer applied during adhesive procedures to cross-link the dentinal collagen, in order to improve the immediate bond strength of either self-etch or etch-and-rinse adhesive systems, and to stabilize the adhesive interfaces over time. Furthermore, the effect of EDC on the dentinal MMPs activity was investigated by means of zymographic analyses. The null hypotheses tested were that: pre-conditioning of dentin with EDC before adhesive system application (1) does not affect immediate bond strength, (2) does not preserve adhesive interface degradation over time, and (3) does not inhibit endogenous dentin MMPs activity.

2. Materials and methods

2.1. *Microtensile bond strength test (μTBS)*

Freshly extracted sound human third molars were obtained from anonymous individuals following their signed consent under a protocol approved by the University of Trieste (Italy). Eighty tooth crowns (n = 20 for each group) were selected to conduct microtensile bond strength tests, flattened using a low-speed diamond saw (Micromet, Remet, Bologna, Italy) under water cooling, and a standardized smear layer was created with 600-grit silicon-carbide (SiC) paper on each tooth surface.

Specimens were then randomly assigned to four different groups as according to the adhesive procedure performed:

- Group 1 (G1): Clearfil SE primer (Kuraray Dental, Osaka, Japan; abbreviation: CSE) was applied on unetched, smear layer-covered dentin according to the manufacturers' instructions. Then the dentin surface was pretreated with an aqueous solution of 0.3 M EDC for 1 min, air-dried and bonded with Clearfil SE Bond (Kuraray) according to the manufacturer's instructions;
- Group 2 (G2): CSE was applied on unetched dentin without EDC pre-treatment as per manufacturer's instructions;
- Group 3 (G3): dentin was etched for 15 s with 35% phosphoric-acid gel (3 M ESPE, St. Paul, MN, USA) and rinsed with water. The acid-etched dentin was than pretreated with the 0.3 M EDC solution for 1 min, air-dried and then bonded with XP Bond (Dentsply DeTrey GmbH, Konstanz, Deustche; abbreviation:XPB) following the manufacturer's instructions;
- Group 4 (G4): XPB was applied on etched dentin without EDC pre-treatment as per manufacturer's instructions.

Each bonded specimen was then light-cured for 20 s using a LED curing light (Demi Light, Kerr). Four 1-mm-thick layers of microhybrid resin composite (Filtek Z250; 3M ESPE) were placed and polymerized individually for 20 s. Specimens were serially sectioned to obtain approximately 1 mm-thick beams in accordance with the microtensile non-trimming technique. The dimension of each stick (ca. 0.9 mm × 0.9mm × 6 mm) was recorded using a digital caliper (±0.01 mm) and the bonded area was calculated for subsequent conversion of microten-

sile strength values into units of stress (MPa). Beams were stressed to failure after 24 h (T0) or 1 year (T12) of storage in artificial saliva at 37 °C [6] using a simplified universal testing machine (Bisco, Inc., Schaumburg, IL, USA) at a crosshead speed of 1 mm/min. The number of prematurely debonded sticks in each test group was recorded, but these values were not included in the statistical analysis because all premature failures occurred during the cutting procedure and they did not exceed the 3% of the total number of tested specimens and were similarly distributed within the groups. A single observer evaluated the failure modes under a stereomicroscope (Stemi 2000-C; Carl Zeiss Jena GmbH) at magnifications up to 50× and classified them as adhesive, cohesive in dentin, cohesive in composite, or mixed failures.

As values were not normally distributed (Kolmogorov–Smirnov test), the collected data were statistically analyzed with the non-parametric Kruskal–Wallis test followed, when significant, by pair-wise comparisons using the Mann–Whitney *U* test. The Chi-square test was used to analyze differences in the failure modes. For all tests, statistical significance was pre-set at $\alpha = 0.05$. Statistical analysis was performed using SPSS 21.0 software for Mac (SPSS Inc., Chicago, IL, USA).

2.2. *Zymographic analysis*

The zymographic analysis was performed according to the protocol of Mazzoni et al. [21]. In brief, mineralized dentin powder was obtained from additional 16 human third molars. Teeth were ground free of enamel, pulpal soft tissue, and cementum; dentin powder was obtained by freezing the dentin in liquid nitrogen and triturating it by means of a Retsch mill (Reimiller, Reggio Emilia, Italy). The fine mineralized dentin powder was pooled, dried, and kept frozen until use. Aliquots of mineralized dentin powder were divided into 6 groups as follows:

- Group 1 (Lane 1 — L1): dentin powder (DP) left untreated as mineralized control;
- Group 2 (Lane 2 — L2): DP treated with 1 ml of 10%/wt phosphoric acid for 10 min to simulate the etching procedure as the first step of the etch-and-rinse bonding technique and used as demineralized control (DDP);
- Group 3 (Lane 3 — L3): DP treated with 100 μl of 0.3 M EDC for 30 min, then gently dried and treated with CSE primer for 30 min in the dark;
- Group 4 (Lane 4 — L4): DP mixed with 100 μl of CSE primer for 30 min in the dark;
- Group 5 (Lane 5 — L5): DDP treated with 0.3 M EDC as for L4, followed by XPB application for 30 min in the dark;
- Group 6 (Lane 6 — L6): DDP mixed with 100 μl of XPB for 30 min in the dark.

From each group, the adhesive was extracted from the dentin-treated powder with 1 ml of acetone and centrifuged (20,800 × *g* for 20 min), then re-suspended in acetone and re-centrifuged 2 more times for removal of additional unpolymerized comonomers [21]. For protein extraction, dentin powder aliquots were re-suspended in 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 and sonicated every 20 s for 10 min (30 pulses), centrifuged for 20 min at 4 °C (20.800 g), after which the supernatant was removed and re-centrifuged. The protein content was further concentrated in a Vivaspin centrifugal concentrator (10,000 kDa cut-off; Vivaspin Sartorius Stedim Biotech, Goettingen, Germany) for 30 min at 4 °C (15,000 × *g*, 3 times). Total protein concentration of dentin extracts was determined by Bradford assay (Bio-Rad, Hercules, CA, USA). Dentin protein aliquots (60 μg) 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 gel electrophoresis (SDS-PAGE) containing 1 mg/ml fluorescein-labeled gelatin. Pre-stained low-molecular-weight SDS-PAGE standards (Bio-Rad) were used as molecular-weight markers. After electrophoresis, the gels were washed for 1 hr in 2% Triton X-100, and then were incubated in zymography activation buffer (50 mmol/l Tris–HCl, 5 mmol/l CaCl2, pH 7.4) for 48 h. Proteolytic activity was evaluated and registered under long-wave UV light scanner (ChemiDoc Universal Hood, Bio-Rad). Gelatinase activity in the samples was analyzed in duplicate by gelatin zymography.

Zymographic bands were identified and quantified with Bio-Rad Quantity One Software (Bio-Rad).

3. Results

3.1. *Microtensile bond strength test (μTBS)*

Means and standard deviations of microtensile bond strength (in MPa) at times T0 and T12 months are reported in Table 1. The use of the 0.3 M EDC-containing conditioner before adhesive application did not affect the immediate bond strength of either XPB or CSE adhesive systems ($p > 0.05$; Table 1). That is, both adhesives showed comparable bond strength values when employed with or without EDC pretreatment.

However, after incubating the bonded sticks for 12 months, the two control groups (2 and 4) showed significant ($p < 0.05$) reduction in μTBS compared to EDC-treated experimental groups (1 and 3).

Group 2 specimens bonded with Clarefil SE Bond fell 35%, while Group 4 specimens bonded with XP Bond fell 51%.

Specimens pretreated with EDC and bonded with Clarefil SE Bond showed only an 11% decrease in bond strength compared to the 35% reduction seen in Group 2. Similarly, specimens pretreated with EDC before being bonded with XP Bond (Group 3) showed only 21% decrease in μTBS compared to the 51% decrease seen in non EDC-treated specimens (Group 4) (Fig. 1).

A predominance of mixed failures was detected in all groups, except for XPB control that resulted in increased adhesive fracture at the bonded interface either at T0 and T12.

3.2. *Zymographic analysis*

Zymographic analysis and densitometric evaluation of bands, expressed as percentage of increase/decrease of MMPs activ-

Table 1 – Means and standard deviations of microtensile bond strength (expressed as MPa) obtained by applying primer for 1 min on the etched dentin surface. T_0 and T_{12} indicate specimens that were tested after storage for 24 h or 12 months in artificial saliva, respectively. Distribution of failure mode among tested groups is also reported in square rounds and classified as: A: adhesive; CD: cohesive failure in dentin; CC: cohesive failure in resin composite; M: mixed. Bond reduction after storage report the percentage of mean bond reduction after 1 year of storage. Clearfil SE Bond (CSE) and XP Bond (XPB) with or without 0.3 M EDC as additional therapeutic.

Treatment group	Storage time		Bond reduction after 1 year of storage
	T_0	T_{12}	
Group 1	$30.1 \pm 6.3^{a,A}$	$26 \pm 8.0^{a,A}$	−11.45%
0.3 M EDC + Clearfil SE	(35A/11CC/12CD/42 M)	(48A/5CC/7CD/36 M)	
Group 2	$32.8 \pm 4.4^{a,A}$	$21.4 \pm 5.7^{b,B}$	−34.79%
Clearfil SE	(35A/0CC/10CD/55 M)	(42A/8CC/5CD/45 M)	
Group 3	$36.5 \pm 7.1^{b,A}$	$28.6 \pm 6.4^{a,B}$	−21.63%
0.3 M EDC + XP Bond	(32A/8CC/CD12/48 M)	(30A/4CC/7CD/59 M)	
Group 4	$37.6 \pm 5.9^{b,A}$	$18.1 \pm 4.9^{b,B}$	−51.0%
XP Bond	(69A/8 CC/3CD/20 M)	(59A/5CC/0CD/36 M)	

Premature failures due to preparation procedures were not included in the statistical analysis. Groups with the same superscripts are not statistically different ($p > 0.05$). Different superscript lower-case letters (in rows) indicate statistical differences between storage time. Different superscript upper-case letters (in columns) indicate statistical differences between different adhesive protocol. The distribution of failure mode are shown within the parentheses and were classified as A: adhesive; CD: cohesive failure in dentin; CC: cohesive failure in resin composite; M: mixed failure. Reduction in bond strength after storage are reported as percentages of mean bond reduction after 1 year of storage.

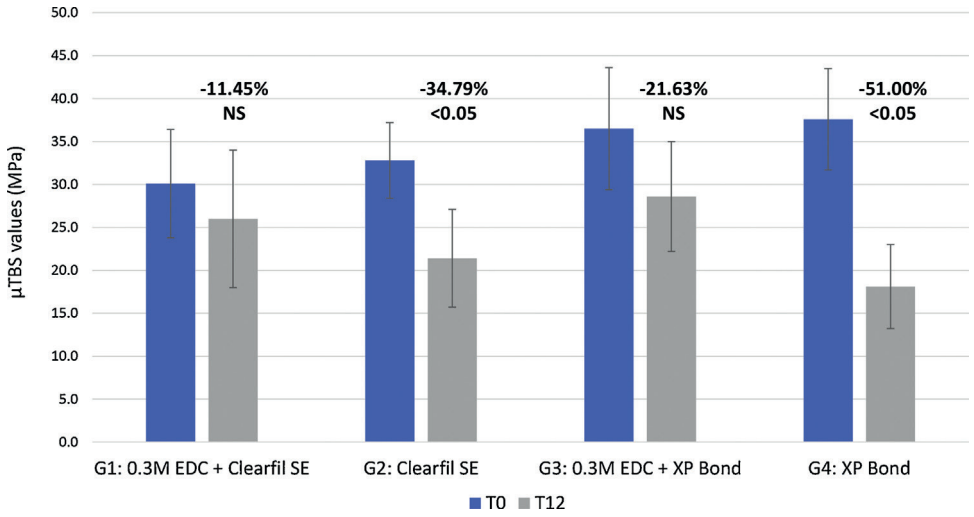

Fig. 1 – Graph summarizing the bond strength values (MPa) reported in Table 1.

ity among the different treatment groups compared with mineralized dentin, considered as baseline, are shown in Figs. 2 and 3. Proteins extracted from mineralized and demineralized dentin powder (Lane 1, Lane 2, Fig. 2) showed the presence of MMP-2 pro- and active-forms (72- and 66-kDa, respectively) and pro-MMP-9 (100 kDa). Mineralized dentin powder treated with CSE and XPB resulted in enzymatic activation (Lane 4, Lane 6 Fig. 2), especially for the XPB where the activity of MMP-2 and -9 are clearly visible, while for CSE MMP-2 activity was almost absent. Pre-treatment with EDC followed by the application of CSE resulted in incomplete inhibition of MMPs, and the presence of a band corresponding to the active MMP-9 was still detectable (Lane 3, Fig. 2). Pre-treatment with EDC followed by the application of XPB resulted in an almost complete inactivation of dentinal gelatinases (Lane 5, Fig. 2).

4. Discussion

The results of the study showed that the application of 0.3 M EDC pretreatment prior to adhesive application did not affect the immediate bond strength for either tested adhesives, requiring acceptance of the first tested null hypothesis. Application of 0.3 M EDC to either adhesives resulted in bond strength preservation after 1 year of storage, in addition to a reduction of MMP-2 and -9 activities, requiring rejection of the second and the third tested null hypotheses.

Over the last few years, the experimental use of collagen cross-linking agents to increase the longevity of resin–dentin bonds has gained increased popularity [22–24].

The use of cross-linkers can be considered as a biological tissue engineering approach, where dentin tissue repair/regeneration is the development of a biomimetic strat-

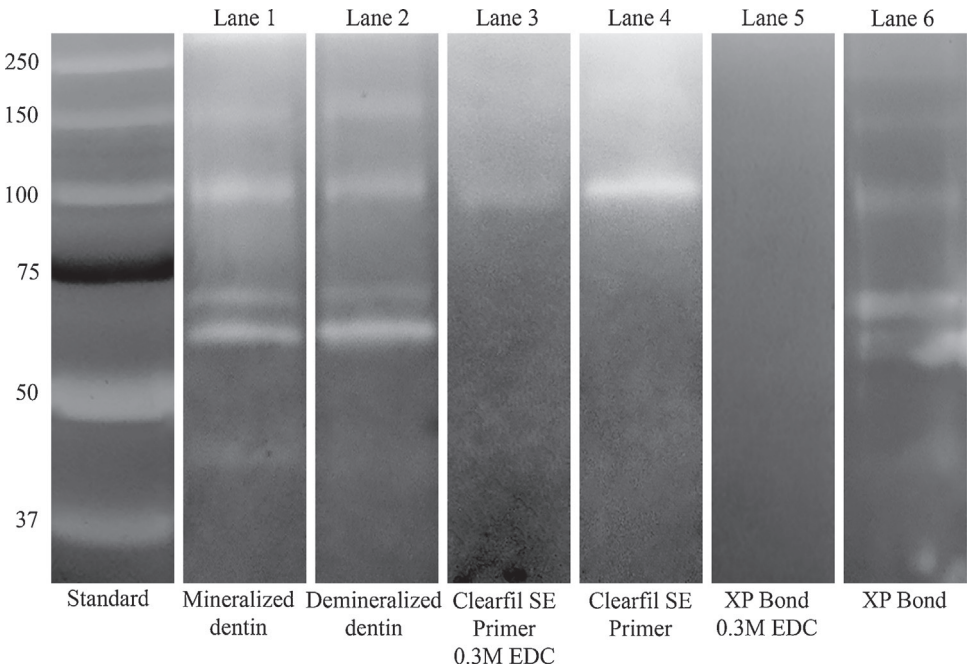

Fig. 2 – Zymographic analysis of proteins extracted from dentin powder. Std: Standards (Std.) are reported in lane Std. Lane 1: mineralized dentin showing the presence of MMP-9 pro-form, MMP-2 pro- and active-form (≈92, 72 and 66 kDa, respectively) and an additional band around 45 kDa. Lane 2: proteins extracted from dentin powder demineralized with 10% phosphoric acid, showing an increase of MMP-2 active-form and a slight decrease in the expression of gelatinases pro-forms, and of the additional band at 45 kDa. Lane 3: demineralized dentin powder after incubation with 0.3 M EDC followed by CSE showing a decrease in the activity of MMP-9 pro-form and complete inactivation of dentinal MMP-2 pro- and active forms. Lane 4: demineralized dentin powder treated with CSE showing a slight decrease in the activity of MMP-9 pro-form and complete inactivation of dentinal MMP-2 pro- and active forms. Lane 5: proteins extracted from demineralized dentin powder pre-treated with 0.3 M EDC followed by XP Bond application showing complete inactivation of dentinal gelatinases. Lane 6: demineralized dentin powder treated with XP Bond showing enzymatic activation of both MMP-2 and −9 and of the additional band at approx. 45 kDa.

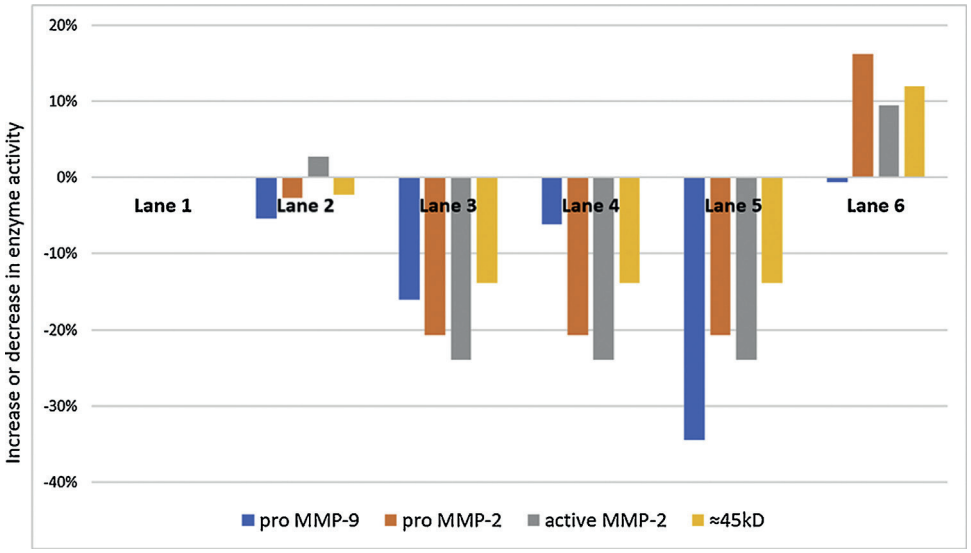

Fig. 3 – Graph illustrating the densitometric evaluation of bands obtained from the zymographic analysis of proteins extracted from dentin powder. The densitometric evaluation of bands is expressed as percentage increase/decrease of MMPs activity among the different treatment groups compared with mineralized dentin (considered as baseline).

egy to enhance the substrate properties by modifying the chemistry of the tissue [16]. Previous studies investigated the use of different cross-linkers, such as glutaraldehyde, genepin, proanthocidin and EDC, as biomodifier agent, although the application time required to be effective (10 min to several hours [25,26]) could not be considered clinically acceptable. For this reason, more recent studies concerning the use of EDC were conducted to evaluate the capabilities to increase the mechanical properties of the etching-dentin matrix within 1 min application time, revealing that this short application time is sufficient to inactivate endogenous protease activity of dentin without significantly stiffening the collagen matrix [6]. These findings were further confirmed by a recent study conducted by Mazzoni et al. [7] demonstrating that 1 min is an adequate timing to positively influence the durability of resin–dentin bond over time. According to these findings, in the present study, EDC was applied for 1 min on the dentin surfaces. The results demonstrated that increased bond strengths could be obtained, compared to controls, with the use of EDC in association with a self-etching *versus* an etch-and-rinse adshesive.

The μTBS results showed that EDC pretreatment can improve the durability and the structural integrity of the resin/dentin interfaces created either with etch-and-rinse (XPB) or self-etch adhesive systems (CSE) (Table 1). The results of the μTBS of the EDC experimental groups showed that bond strength values, even when at baseline were comparable to the control groups, remained stable over time, or at least more stable than the control groups (Table 1). Furthermore, in terms of percentage of bond strength reduction, the self-etch adhesive (CSE) lost less bond strength following EDC pretreatment, compared to etch-and-rinse adhesive system (XPB). These data further confirm previous *in vitro* findings that showed the improved stability of the 2-step self-etch system *versus* the two-step etch-and-rinse system due to the increased hydrophobicity [8] and curing ability [9,10]. The observed decline in bond strengths of the present study can be related to the loss of integrity of resinous components within the hybrid layer due to polymer swelling and resin leaching that occur after water/oral fluid sorption, which is recognized to be more pronounced for simplified (two-step) etch-and-rinse adhesives than unsimplified systems (three-step) [27,28]. The 2-step self-etch adhesive is considered the most durable bond [28]. This can be due to the fact that the self-etch adhesives do not completely expose the dentin collagen matrix [28,29]. Self-etch adhesives maintain more residual hydroxyapatite crystal in their hybrid layers which minimizes activation of dentin MMPs [21,30,31]. Those calcium ions that are released from the matrix during self-etching are thought to form relatively insoluble calcium salts with the functional monomers like 10-MDP (10-methacryloyloxydecyl dihydrogen phosphate) in SE Bond. The slow solubilization of the insoluble salts over 12 months may allow control SE Bond to lose more bond strength than their EDC-pretreated experimentals [21,30,31]. The present μTBS results revealed that bonded dentin interfaces created with EDC pretreatment improves the durability of the resin–dentin bonds. These results are similar to a recently published study in which a 3-step etch-and-rinse adhesive (Optibond FL) and a 2-step self-etch adhesive (CSE) were tested using chlorhexidine as a conditioning primer and MMP inhibitor [21,30,31]. The results of that study showed that the use of chlorhexidine stabilized bond strength values over time for both tested adhesives. Unlike chlorhexidine which only binds to dentin electrostatically [21,30,31], EDC reacts with collagen to forming covalent bonds [21,30,31].

The results of the zymographic analysis performed in the present work are in accordance with previous findings [21,30,31]; thus, the use of the adhesive systems tested resulted in an increase in MMP-2 and -9 activity, but EDC pretreatment resulted in reduction or almost complete inhibition of the gelatinolytic activity as shown in Figs. 2 and 3.

Based on the outcomes of the present project, the effectiveness of EDC in improving both the mechanical properties of collagen over time, and in inhibiting the gelatinolytic activity within the HL has been successfully demonstrated. Previous studies suggested that this may be attributed to silencing mechanism of MMPs and probably other exogenous collagen degradation enzymes *via* conformational changes in the enzyme 3-D structure [32]. The use of cross-linking agents may create multiple cross-links between amino acids within their catalytic sites that irreversibly alter the 3-D conformation or flexibility of the cleft-like catalytic domain and prevent its optimal recognition and complexing with the type I collagen substrate [33]. Although there is no evidence that the catalytic domain of collagenolytic MMPs can be cross-linked to inactivate their functions, it has been hypothesized that the use of cross-linking agents may also contribute to MMPs silencing *via* allosteric control of non-catalytic domains [34]. For example, the catalytic domains in collagenolytic MMPs can cleave non-collagen substrates, but the hemopexin-like domain of these enzymes is crucial to initially unwind and subsequently cleave the three triple-helical fibrillar elements of the collagen molecule in succession [23]. For MMP-2, there are three fibronectin-like repeats that form a domain for binding to collagen or gelatin substrates. This collagen-binding domain binds preferentially to the α1 chain and mediates local unwinding and gross alteration of the triple helix prior to the cleavage of the β2 chain [24]. Regardless of which of the two collagen-binding mechanisms is involved, cross-linking of either the hemopexin-like or fibronectin-like domains may contribute to inactivation of the associated MMPs and reduction in their collagenolytic efficacy. Cross-linking may also affect MMP activities known to be modified by non-collagenous proteins [35]. In dentin, MMP activities and resistance to degradation may be regulated by fetuin-A [36] and the SIBLINGs Bone Sialoprotein (BSP) and Dentin Matrix Protein-1 (DMP-1) [37], all of them being present in dentin. Thus, cross-linking of these non-collagenous proteins may indirectly block MMPs *via* inactivation of the functional domains of these glycoproteins. Since MMPs do not turn over in peripheral dentin, their inactivation by cross-linking agents should last for a long time and may be even more effective than inhibitors such as chlorhexidine [13,31].

In conclusion, the μTBS and zymography results support the efficacy of EDC over time and revealed that changes within the dentin matrix promoted by EDC are not adhesive-system-dependent. Further *in vivo* studies are necessary to clinically validate and promote the use of EDC as additional step during dentin-bonding procedures.

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