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Effect of carbodiimide (EDC) on the bond stability of etch-and-rinse adhesive systems

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Abstract
Objective

Recent studies supported the use of protein cross-linking agents during bonding procedures to inactivate endogenous dentin proteases, preventing dentin collagen degradation thus improving bond durability. The aim of this study was to evaluate the effect of a 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)-containing conditioner on the stability of the adhesive interface created by two etch-and-rinse adhesives.
Methods

Human dentin was etched with 35% phosphoric acid, treated with 0.3 M EDC-containing conditioner followed by a three-step or a two-step etch-and-rinse adhesive. Adhesives were applied to control specimens without EDC pre-treatment. Specimens were subjected to microtensile bond strength test and pulled to failure after 24 h or 1 year of storage and interfacial nanoleakage expression was evaluated and quantified by light microscopy. Additionally, to investigate endogenous dentin matrix metalloproteinase activity a zymographic assay was performed on protein extracts obtained from phosphoric-acid-etched dentin powder with or without EDC treatment.
Results

The use of the EDC-containing conditioner did not affect immediate bond strength to dentin but contributed to preserve the bond strength after 1 year ($p < 0.05$) for both tested adhesives. No difference was found in the interfacial nanoleakage expression that increased after aging irrespective from the treatment. EDC pre-treatment inhibited dentin endogenous MMPs as assayed with the zymography.
Significance

In conclusion, the results of the study provide proof that EDC can produce long-term inactivation of MMPs in acid-etched dentin matrices contributing to bond strength preservation over time. Future studies are needed to support the use of EDC in vivo.
Keywords

Dentin bonding systems; EDC; Protein cross-linkers; Enzymatic degradation; MMPs.

1. Introduction

Consistent evidence supports the hypothesis that exposure and activation of dentin endogenous proteases occurs during dentin bonding procedures. The resultant collagenolytic/gelatynolitic activity is thought to be responsible for the in vitro and in vivo manifestation of thinning and disappearance of collagen fibrils from poorly infiltrated aged hybrid layers [1] and [2]. The application of resin monomer constituents of adhesive blends of either etch-and-rinse or self-etch adhesives have been recently shown to activate these endogenous proteases [3], [4], [5] and [6].

Among these enzymes, matrix-metalloproteinases (MMPs) and cathepsins have been shown to be present in dentin [7], [8], [9] and [10], being responsible for the slow hydrolysis of the collagen

fibrils in hybrid layers that anchor resin composites to the underlying mineralized dentin [11]. This hydrolysis causes a loss of bond strength, allowing gaps to open up between resin composites and tooth structure [1], [2] and [12].

Recently, several efforts have been made to inactivate these proteases during dentin bonding procedures with the attempt to create more durable resin-dentin bonds. The identification of these enzymes as well as the understanding of their functions has prompted innovative approaches to retain the hybrid layer integrity and increase the longevity of dentin bonding over time [1], [2] and [12]. The proposed approaches to prolong the durability of resin-dentin bonds include the use of synthetic MMP inhibitors [13], [14], [15], [16] and [17], quaternary ammonium methacrylates or benzalkonium chloride [18] and [19], as well as other approaches that act indirectly by chemical chelation of calcium ion, collagen cross-linking, ethanol wet bonding, or remineralization to protect the hybrid layer from enzymatic degradation [11], [20] and [21].

Among these strategies, the use of collagen cross-linking agents seems to be very promising [21], [22], [23] and [24]. The potential of cross-linkers is related to the possibility to improve the mechanical strength of the collagen network, improve the resistance to enzymatic degradation, and to inactivate exposed MMPs bound to matrix collagen. Together this leads to a stable dentin matrix network which ultimately determines the formation of a stable hybrid layer [11]. Recently, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) has been proposed to effectively improve the durability of resin-dentin bonds by increasing the mechanical properties of the collagen matrix [21], although the 1–4 h time required for the EDC application step makes it clinically unacceptable. To overcome this problem, a previous in vitro study evaluated the feasibility of reducing the EDC application time up to 1 min, revealing the effectiveness of the cross-linker agent to inactivate soluble rhMMP-9 and matrix-bound dentin proteinases [25]. However, additional data are required to validate the use of EDC (for a clinical reliable time) on demineralized dentin to stabilize the bond inhibiting endogenous MMPs.

Thus, the aim of this study was to evaluate the ability of an EDC-containing conditioner to cross-link dentin collagen (within 1 min of contact time) in order to improve immediate bond strength and stabilize the adhesive interface over time. Since the activity of dentinal MMPs has been implicated in the degradation of resin-dentin bonds, the effect of EDC on the activity of dentin MMPs was also be investigated. The null hypotheses tested were that EDC applied for 1 min to acid-etched dentin before bonding (1) does not affect immediate bond strength and interfacial nanoleakage expression, (2) does not prevent adhesive interface degradation over time, and (3) does not inhibit endogenous dentin MMPs activity.

2. Materials and methods

Reagents were purchased from Sigma Chemical (St. Louis, MO, USA) unless otherwise specified.

2.1. Microtensile bond strength test (μTBS)

Forty freshly extracted, non-carious, human third molars were used. Teeth were collected after obtaining patients' informed consent for research purposes under a protocol approved by the institutional review board of the Georgia Regents University (USA).

Tooth crowns were flattened using a low-speed diamond saw (Micromet, Remet, Bologna, Italy) under water irrigation and a standardized smear layer was created with 600-grit silicon-carbide (SiC) paper. Dentin surfaces were etched for 15 s with 35% phosphoric-acid gel (3 M ESPE, St. Paul, MN, USA) and rinsed with water.

Specimens were then randomly assigned to the following treatments.

Group 1 (G1): acid-etched dentin was pretreated with 0.3 M EDC water-solution for 1 min, air-dried, primed and bonded with Optibond FL (OFL; Kerr, Orange, CA, USA) following the manufacturer's instructions.

Group 2 (G2): OFL was applied on etched dentin without EDC pre-treatment in accordance with manufacturer's instructions.

Group 3 (G3): acid-etched dentin was pretreated with 0.3 M EDC as described for G1 then bonded with Scotchbond 1XT (SB1XT 3 M ESPE).

Group 4 (G4): SB1XT was applied to acid-etched without EDC pre-treatment.

Each bonded specimen was light-cured for 20 s using a quartz-halogen light unit (Curing Light 2500; 3 M ESPE). The irradiance level of the light was monitored periodically with a radiometer (3 M ESPE) to ensure that it remained ≥600 mW/cm2. Four 1-mm-thick layers of microhybrid resin composite (Filtek Z250; 3 M 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 × 0.9 mm × 6 mm) was recorded using a digital caliper (±0.01 mm) and the bonded area was calculated for subsequent conversion of microtensile strength values into units of stress (MPa). Beams were stressed to failure after 24 h (T0) or 1 year (T12) of storage in artificial buffer at 37 °C, prepared in accordance with the protocol of Pashley et al. [26], 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 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.

Because a Kolmogorov–Smirnov test determined that values were normally distributed, data were analyzed using Two-Way (variables: dentin bonding system and storage time) analysis of variance (ANOVA) and post hoc Tukey test. p values of 0.05 were considered to indicate statistical significance.

2.2. Interfacial nanoleakage evaluation

Sixteen additional teeth (N = 4/group) were processed for interfacial nanoleakage evaluation. Middle/deep dentin was selected, acid-etched and bonded for one of the adhesives with or without the EDC-containing conditioner as previously described. A 1-mm-thick flowable composite (Filtek Flow; 3 M ESPE) was applied on the bonded disks and light-cured. Composite-dentin specimens were cut vertically into 1-mm-thick slabs to expose the bonded surfaces and stored for 24 h (T0) or 1 year (T12) in artificial buffer at 37 °C. Specimens were covered with nail varnish, leaving 1 mm exposed at the bonded interface, and processed for interfacial nanoleakage evaluation. Bonded interfaces were immersed in 50 wt% ammoniacal AgNO3 solution in darkness for 24 h according to the protocol described by Tay et al. [27]. After immersion in the tracer solution, specimens were rinsed in distilled water and immersed in photo-developing solution for 8 h under a fluorescent light to reduce silver ions into metallic silver grain within voids along the bonded interfaces. Nanoleakage analysis was performed under light microscopy (LM - Nikon E 800; Tokyo, Japan)

and the degree of interfacial nanoleakage was scored on a scale of 0–4 by two observers as described by Saboia et al. [28]. Interfacial nanoleakage was scored based on the percentage of the adhesive surface showing silver nitrate deposition: 0, no nanoleakage; 1, <25% nanoleakage; 2, 25 to ≤50% nanoleakage; 3, 50 to ≤75% nanoleakage; and 4, >75% nanoleakage.

Statistical differences among nanoleakage group scores (i.e. percentage of specimens falling within each score category) were analyzed using the $\chi 2$ test. All statistical testing was performed at a pre-set alpha of 0.05. Inter-observer agreement was measured using Cohen's kappa test.

2.3. Zymographic analysis

Zymographic analysis was performed in accordance with Mazzoni et al. [6]. In brief, mineralized dentin powder was obtained from eight human third molars by freezing the dentin in liquid nitrogen and triturating it using Retsch miller (Model MM400, Retsch GmbH, Haan, Germany). Aliquots of mineralized dentin powder were treated as follows: G1 – left mineralized (control); G2 – demineralized with 10 wt% phosphoric acid for 10 min to simulate the first step of the etch-and-rinse approach; G3 – demineralized as for G2 and treated with EDC 0.3 M for 30 min.

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% nonionic detergent P-40, 0.1 mM $ZnCl_2$, 0.02% NaN_3) for 24 h at 4 °C, intermittently sonicated for 10 min (ca. ≈30 pulses), centrifuged for 20 min at 4 °C (20,800 G), then the supernatant was removed and re-centrifuged. The protein content was further concentrated using Vivaspin centrifugal concentrator (10,000 kDa cut off; Vivaspin Sartorius Stedim Biotech, Goettingen, Germany) for 30 min at 4 °C (15,000 G for 3 times). Total protein concentration in the dentin extracts was determined by the Bradford assay. Dentin proteins aliquots (60 μg) were diluted in Laemmli sample buffer in a 4:1 ratio and electrophorezed under non-reducing conditions in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 1 mg/mL fluorescein-labeled gelatin. Prestained low-range molecular weight SDS-PAGE standards (Bio-Rad, Hercules, CA) were used as molecular-weight markers. After electrophoresis, the gels were washed for 1 h in 2% Triton X-100 and the gels were incubated in zymography activation buffer (50 mmol/L Tris–HCl, 5 mmol/L $CaCl_2$, pH 7.4) for 48 h. Proteolytic activity was evaluated and registered under long-wave UV light scanner (ChemiDoc Universal Hood, Bio-Rad). Gelatinase activities in the samples were analyzed in duplicate by gelatin zymography.

3. Results

3.1. Microtensile bond strength

Means and standard deviations of microtensile bond strength (in MPa) at T0 and T12 are reported in Table 1. The use of 0.3 M EDC-containing conditioner before adhesives application did not affect the immediate bond strength of either tested adhesives (G1: 44.5 ± 9.8 MPa; G2: 43.3 ± 9.4 MPa; G3: 43.3 ± 9.4 MPa; G4: 40.5 ± 10.3 MPa; $p > 0.05$).

After 1 year of storage, the EDC cross-linked specimens showed no significant loss of bond strength for OFL (G1: 41.2 ± 10.1 MPa) compared to T0 ($p > 0.05$), while the bond strength of control specimens (G2: 33.1 ± 7.9 MPa) was significantly reduced (Table 1; $p < 0.05$). For SB1XT, the EDC pre-treatment resulted in a significant reduction of bond strength after 1 year (G3: 32.5 ± 9.6 MPa) compared to T0 ($p < 0.05$), although EDC-treated specimens performed significantly better when compared to the untreated control specimens (G4: 24.8 ± 8.8 MPa; $p < 0.05$), thus, contributing in the preservation of the HL integrity after aging (Table 1; $p < 0.05$).

3.2. Interfacial nanoleakage expression

The extent of silver nitrate depositions along the bonded interfaces is shown in Table 2.

No difference was found in the interfacial nanoleakage expression between the EDC-treated specimens and controls or between the adhesives. In vitro aging for 1 year in artificial saliva caused an increase in interfacial nanoleakage expression compared to T0 ($p < 0.05$) for all tested groups. Representative images of interfacial nanoleakage expression at T0 and T12 of storage for OFL and SB1XT are shown in Fig. 1 and Fig. 2, respectively.

3.3. Zymographic analysis

Results of zymographic analysis are shown in Fig. 3. Proteins extracted from mineralized and demineralized dentin powder showed the presence of MMP-2 pro- and active-forms (72- and 66-kDa, respectively) and pro-MMP-9 (95 kDa) and an additional band of approximately 50 kDa (Fig. 3, Lanes 1 and 2); after demineralization an increase in the expression of MMP-9 and of the additional band at 50 kDa was observed (Fig. 3, Lane 2) The incubation of demineralized dentin with 0.3 M EDC resulted in complete inhibition of dentin gelatinases (Fig. 3, Lane 3).

Control zymograms incubated with 5 mM EDTA or 2 mM 1,10-phenanthroline showed no enzymatic activity (data not shown).

4. Discussion

The results of this study showed that EDC pre-treatment did not affect immediate bond strength ($p > 0.05$) and that dentin pretreatment resulted in bond strength preservation after 1 year of storage in artificial saliva for both a three-step (OFL) and a two-step (SB1XT) etch-and-rinse adhesives ($p < 0.05$). No differences were found in the nanoleakage expression between EDC-treated specimens and controls that increased after aging irrespective of EDC treatment. Furthermore, complete inhibition of the gelatinolytic activity after 0.3 M EDC pre-treatment of acid-etched dentin was assayed using zymography. Accordingly, these results support the partial rejection of the tested null hypotheses.

Beside the gradual improvements in marketed dentin adhesive systems, the poor stability of the HL is still a matter of concern and the degradation of the collagen matrix at the resin/dentin interface related to the endogenous proteases activity [6] is one of the key factors negatively affecting its longevity [1], [2], [11] and [12]. To stabilize the adhesive interface over time and find a permanent solution to the presence and activity of these endogenous proteases in the dentin matrix, the option to cross-link the activated matrix-bound MMPs inactivating their catalytic side [12] seems to be very promising [21], [25] and [29].

Glutaraldehyde and grape-seed extracts [23] were originally employed as cross-linkers, although the application time required to be effective was not clinically acceptable (from 10 min up to hours), beside the fact that glutaraldehyde is very toxic. To overcome this problem, application of 0.1% riboflavin to acid-etched dentin followed by UVA light for 2 min prior to resin-bonding was tested with the result of inhibiting dentin MMPs and improving the hybrid layer durability [29]. Others showed that 0.1 or 1% riboflavin applied to demineralized dentin in hour-glass shaped specimens treated with UVA or a dental blue light doubled their ultimate tensile strength and reduced the amount of collagen-degradation after bacterial collagenase challenge [30]. Similarly EDC, a cross-linking agent with very low cytotoxicity has been investigated showing potential capabilities to increase the mechanical properties of the dentin matrix [21] and when applied for 1 min on acid-etched dentin it revealed complete inhibition of the endogenous protease activity [25].

Based on these preliminary results, in the present study we tested the use of a 0.3 M EDC-containing conditioner as additional pre-treatment on acid-etched dentin prior to bonding with a three-step (OFL) or a two-step (SB1XT) etch-and-rinse adhesive, with the attempt to reinforce and

strengthen the collagen network within 1 min application. The results of the microtensile study revealed that EDC pre-treatment improves the durability and structural integrity of the resin/dentin interfaces when either etch-and-rinse adhesives (OFL and SB1XT) were applied, although in terms of percentage of bond reduction the three-step system (OFL) seems to be less affected by storage than the two-step adhesive (SB1XT). These data confirm previous in vivo and in vitro findings [30] and [31] supporting the improved stability of the three-step systems vs two-step etch-and-rinse systems due to their increased hydrophobicity [32] and curing ability [33] and [34]. The observed decline in the bond strength can be related to the loss of integrity of resinous components within hybrid layers due to polymer swelling and resin leaching that occurs 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) [2], [35], [36] and [37]. Additionally, the different extend of bond preservation of EDC-treated specimens might also be related to different interaction of the tested bonding agents with the dentin collagen matrix after cross-linking and their susceptibility to endogenous MMPs, that could result in different mode of MMPs activation [3], [4], [6] and [26].

The dentin collagen reinforcement and strengthening through EDC cross-linking might be of importance to improve the bond strength and structural integrity of the resin/dentin interface over time against the enzymatic and/or hydrolytic degradation, through the formation of inter- and intra-molecular crosslinks [22]. EDC contains a functional group with the formula RNdouble bond; length as m-dashCdouble bond; length as m-dashNR. The carbodiimide reacts with ionized carboxyl groups in proteins to form an O-acylisourea intermediate that can react with a nonproteinated amino group and an adjacent protein chain to form a stable covalent amide bond between the two proteins, with the only by-product being urea. This category of crosslinker may inactivate the active sites of dentin proteases by reducing the molecular mobility of the active site or by changing negatively charged ionized carboxyl groups into positively charged amides. Additionally, EDC can cross-link both helical and especially telopeptide domains in collagen and may also prevent telopeptidase activity that would normally remove bulky telopeptides from the specific peptide bond of collagenases [25]. Increases in collagen stiffness may prevent MMPs from “unwinding” collagen peptides [38]. Since this “unwinding” is necessary to allow MMPs catalytic site to cut the peptide [1], [2], [11] and [12], it would also effectively inhibit MMPs functional activity.

Using zymographic analysis in the present study we also tested the effect of EDC on dentinal MMPs activity, demonstrating its efficacy to completely inactivate dentinal gelatinases (Fig. 3). We may speculate that the gelatinases were not extractable due to the EDC cross-linking with dentin collagen, or cross-linking rendered MMPs inactive through allosteric silencing [12]. In fact, although the effects of cross-linking agents on stabilizing dentin matrix degradation have been attributed to their capacity to increase the stiffness of dentin collagen, the results of the present study further support the hypotheses that EDC may inactivate MMPs activity through direct cross-linking of MMPs.

In conclusion, in the present study 0.3 M EDC pre-treatment was found to be effective as an additional conditioner of etch-and-rinse adhesives to improve the bond stability after 1 year, although the extend of improvement was adhesive-dependent. In addition 0.3 M completely inhibit dentinal gelatinases activity as revealed by zymography. Future studies are needed to further assay the efficacy of EDC pre-treatment and promote its potential application in adhesive dentistry as additional conditioner for etch-and-rinse and/or self-etch adhesives.

Conflict of interest

The authors report no conflict of interest and wish to thank Mr. Aurelio Valmori for photographical assistance.

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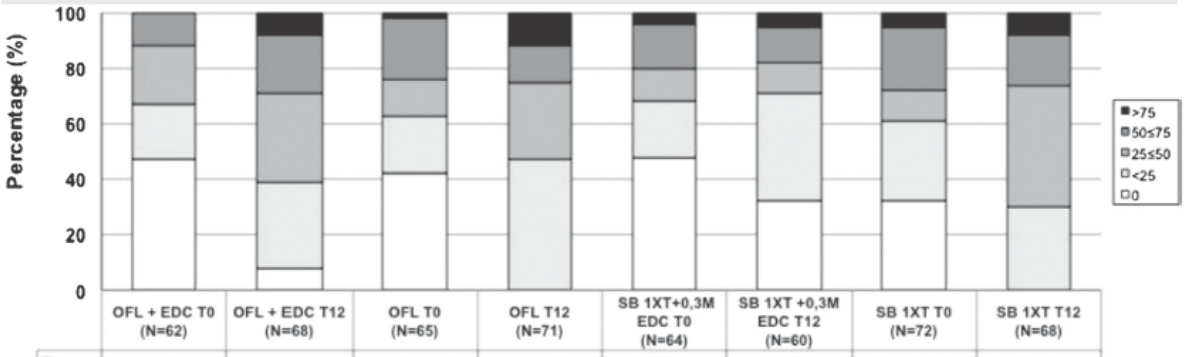
Table 1 – Means and standard deviations [number of premature failed sticks/number of intact sticks tested] of microtensile bond strength (expressed as MPa) obtained by applying OptiBond FL and Adper Scotchbond 1XT with or without 0.3 M EDC as additional therapeutic conditioner for 1 min on the etched 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 (in %) 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.

Treatment group	Storage time		Bond reduction after storage
	T_0	T_{12}	
Group 1 EDC + Optibond FL	44.5 ± 9.8^{a} [5/105] (35A/0CC/10CD/55M)	41.2 ± 10.1^{a} [6/109] (47A/3CC/5CD/45M)	−7.4%
Group 2 Optibond FL	43.3 ± 9.4^{a} [6/108] (32A/8CC/7CD/53M)	$33.1 \pm 7.9^{b,c}$ [4/107] (35A/5CC/10CD/50M)	−23.6%
Group 3 EDC + Adper Scotchbond 1XT	$38.8 \pm 9.8^{a,b}$ [4/102] (30A/5CC/10CD/55M)	32.5 ± 9.6^{c} [5/99] (22A/10CC/19CD/49M)	−16.2%
Group 4 Adper Scotchbond 1XT	40.5 ± 10.3^{a} [6/111] (20A/8CC/25CD/47M)	24.8 ± 8.8^{d} [4/106] (25A/20CC/8CD/57M)	−38.8%

†Premature failures due to preparation procedures were not included in the statistical analysis. Groups with the same superscripts are not statistically different ($p > 0.05$).

Table 2 – Distribution of nanoleakage within the different treatment groups. The numbers indicate the percentage of specimens with respective nanoleakage values observed (from 0 to >75% of the adhesive joint). N = number of sections analyzed. Different letters over the column indicate statistical differences between the groups ($p < 0.05$).



	OFL + EDC T0 (N=62)	OFL + EDC T12 (N=68)	OFL T0 (N=65)	OFL T12 (N=71)	SB 1XT+0,3M EDC T0 (N=64)	SB 1XT +0,3M EDC T12 (N=60)	SB 1XT T0 (N=72)	SB 1XT T12 (N=68)
>75	0	8	2	12	4	5	5	8
50≤75	12	21	22	13	16	13	23	18
25≤50	21	32	13	28	12	11	11	44
<25	20	31	21	47	20	39	29	30
0	47	8	42	0	48	32	32	0

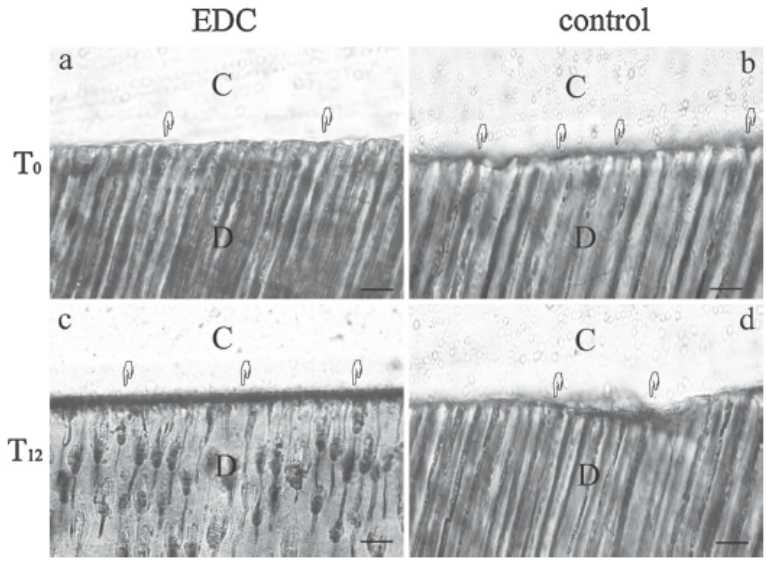


Fig. 1 – Light microscopy images of bonded interfaces created with Optibond FL. The use of EDC as conditioner did not affect nanoleakage expression at T_0 compared to un-treated control specimens (a and b, respectively; pointers). After 1 year of storage in artificial saliva at 37 °C the amount of silver nitrate depositions was significantly increased compared to T_0 specimens (c and d, respectively; pointers). C: composite; D: dentin; Bar = 10 μm.

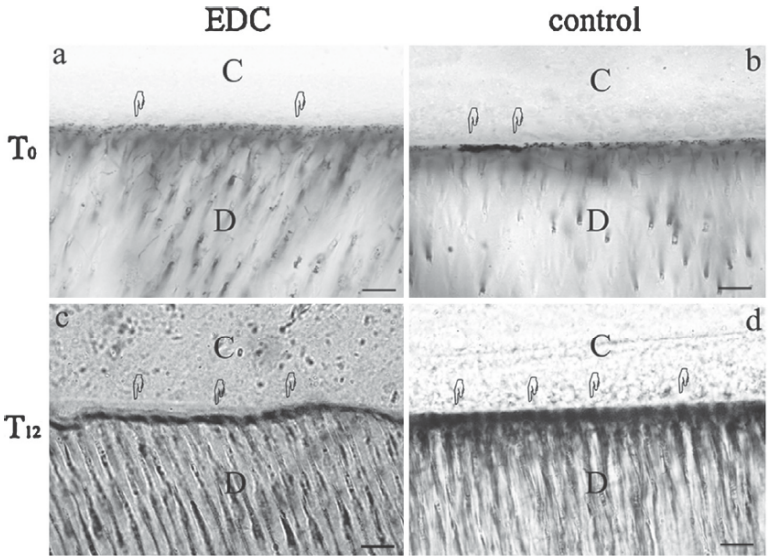


Fig. 2 – Representative light microscopy images of adhesive interfaces created with Adper Scotchbond 1XT. The use of EDC for 1 min did not affect the amount of silver nitrate detected at the adhesive interface either at time 0 (a and b, respectively; pointers), while after 1 year of storage, interfacial nanoleakage expression increased compared to T_0 (c and d, respectively; pointers). C: composite; D: dentin; Bar = 10 μm.

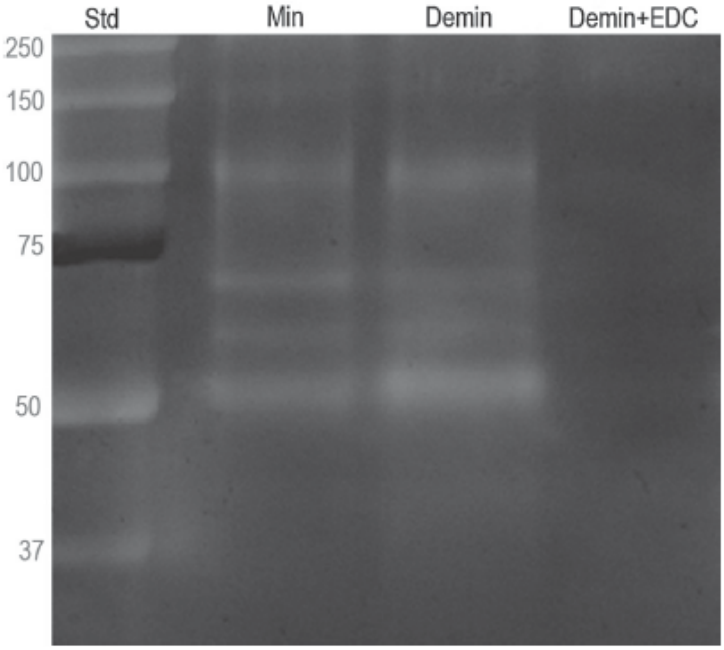


Fig. 3 – Zymographic analysis of proteins extracted from dentin powder. Lane 1: mineralized dentin showing the presence of MMP-2 pro- and active-form (72- and 66-kDa, respectively) and pro-MMP-9 (95 kDa) and an additional band around 50 kDa. Lane 2: proteins extracted from dentin powder demineralized with 10% phosphoric acid, showing similar presence of MMP-2 pro- and active-form and an increase in the expression of MMP-9 and of the additional band at 50 kDa. Lane 3: Demineralized dentin powder after incubation with 0.3 M EDC showing complete inhibition of dentinal MMPs.