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HIGHLIGHTS

- The possibility to use an ethanol-based cross-linker as an additional primer seems to be a promising option in terms of bond strength
- N,N'-dicyclohexylcarbodiimide (DCC) pre-treatment efficiently preserves bond strength over time.
- Matrix-metalloproteinases activity seems to be influenced by the application mode of a universal adhesive

Title Page (with Author Details)

Effect of an ethanol cross-linker on universal adhesive

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RUNNING TITLE: Effect of DCC on dentin bonding over time.

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ABSTRACT

Objectives. To evaluate the effects of N,N'-dicyclohexylcarbodiimide (DCC), an ethanolbased dentin cross-linker, on the immediate and long-term microtensile bond strength (μTBS) and nanoleakage expression of a universal adhesive employed in self-etch mode (SE) or etchand-rinse mode (ER). The effect of DCC on the dentinal MMP activity was also investigated by means of *in-situ* zymography.

Methods: Eighty freshly extracted human molars were sectioned to expose mid-coronal dentin surfaces. The teeth were assigned to one of the following groups, according to the dentin surface priming/adhesive approach : (G1): DCC pre-treatment and Scotchbond Universal (SBU) in ER mode; (G2): SBU in ER mode; (G3): DCC pretreatment and SBU in SE mode; (G4): SBU in SE mode. μTBS test was performed immediately (T_0) or after 1-year aging (T_{12}) in artificial saliva. Ten additional teeth per group were prepared for nanoleakage evaluation (N = 5) and for *in-situ* zymography (N = 5) .

Results. Three-factor analysis of variance revealed significant difference for the variables DCC pretreatment, application mode and aging ($p < 0.05$) for both microtensile bond strength testing and *in-situ* zymography. Nanoleakage analysis revealed reduced marginal infiltration of DCC experimental groups both at T_0 and T_{12}.

Significance. The use of an ethanol-based primer containing DCC appears to be promising in preserving the stability of the adhesive interface of a universal adhesive, especially in the SE mode.

KEY WORDS: cross-linkers; dentin bonding systems; matrix metalloproteinases; N,N'-dicyclohexylcarbodiimide; universal adhesives

1. INTRODUCTION

Adhesion between resin composite restorations and dental substrate is achieved through the infiltration of resin monomers into the demineralized dentin collagen matrix after partial dissolution of the mineral inorganic phase [1]. The need for a retentive cavity became less critical after the advent of adhesive dentistry, with dentin bonding systems provide reasonable immediate bond strength to the dental substrate [2, 3]. Despite nearly six decades of refinement of adhesive materials and protocols, the interdiffusion area between dentin and the adhesive resin, known as the hybrid layer, still remains the weakest region of adhesivelybased restorations [4, 5-8]

The potential reasons behind the degradation of resin-dentin bond over time had been examined by *in vitro* and *in vivo* studies. Hydrolytic or enzymatic breakdown of the polymerized resin compounds and endogenous protease-initiated degradation of the demineralized dentin collagen matrix have emerged as the most likely contributors of interfacial degradation [9-12].

The dentin substrate contains collagen fibrils with bound non-collagenous proteins such as growth factors and endogenous proteases such as matrix-metalloproteinases (MMPs) and cysteine-cathepsin [13-16]. Endogenous proteases play an important role during dentin maturation and become trapped and inactivated after mineralization of the collagen matrix [17]. These enzymes are exposed and reactivated during demineralization of the mineralized dentin, progressively degrading the collagen fibrils that are not protected by adhesive resin within the hybrid layer, eventually resulting in the loss of retention of the adhesive restorations [4, 18-20]. For this reason, much efforts have been devoted to increasing the resistance of the resin-sparse, water-rich collagen fibrils within the hybrid layer against MMPs, with the intention of increasing the longevity of adhesive restorations [21].

Matrix metalloproteinases, particularly MMP-2 and MMP-9, are mostly responsible for the degradation of the dentinal organic matrix [22]. For this reason, much efforts have been devoted to increasing the resistance of the resin-sparse, water-rich collagen fibrils within the hybrid layer against MMPs, with the intension of increasing the longevity of adhesive restorations [4, 11, 12, 23-27].

Different MMP inhibitors such as quaternary ammonium methacrylates [17, 28], and benzalkonium chloride [12] have been used experimentally to increase the durability of the resin-dentin interface [29]. Collagen cross-linkers have also been used to enhance the mechanical properties of the demineralized collagen network as well as bond durability in coronal [11, 30-33] and radicular dentin [34]. Because cross-linking of the collagen matrix is a naturally-occurring phenomenon in dentin, scientists have resorted to the use of chemical substances with cross-linking properties to render the dentin collagen matrix less susceptible to proteolytic attack [4, 35].

Among the cross-linking reagents, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), has demonstrated promising results in the preservation of the integrity of hybrid layers over time. This feature was attributed to the ability of EDC to cross-link peptides without introducing additional linkage groups [11, 32, 36]. N,N'-dicyclohexylcarbodiimide (DCC) is a crosslinker belonging to the same family as EDC, but with a different solubility behavior. Whereas EDC is soluble in water, DCC is soluble in organic solvents such as ethanol or acetone [37]. It is envisaged that the ethanol-based cross-linker agent may help counteract the deleterious effects of water during dentin bonding and preserve the hybrid layer.

Accordingly, the objective of the present *in vitro* study was to evaluate the ability of an ethanol solution of DCC to improve the bond strength of a universal adhesive employed either in self-etch or etch-and-rinse mode, and to stabilize the adhesive interfaces over time.

The DDC was applied before adhesive procedures to cross-link dentinal collagen. The effect of DCC on dentin MMP activity was further investigated by *in-situ* zymography. The null hypotheses tested were that pre-conditioning of dentin with DCC prior to adhesive application 1) does not benefit the immediate bonding performance of a universal adhesive to dentin, 2) does not prevent interfacial degradation over time, and 3) does not inhibit endogenous dentin MMP activity.

2. MATERIALS AND METHODS

Freshly-extracted sound human third molars (N=120) were obtained from anonymous individuals following their signed consent under the protocol ASL_BO N° 0013852, approved on 02/01/2019 by the Ethics Committee.

2.1 Microtensile bond strength test (μTBS)

Eighty teeth were selected to conduct μTBS testing. The occlusal surface of each tooth was cut transversely to the long axis to expose mid-coronal dentin using a low-speed diamond saw (Micromet, Remet, Bologna, Italy) with copious water cooling. A standardized smear layer was created with 600-grit silicon-carbide paper on each tooth surface. The polished teeth were randomly assigned to one of the following groups according to the dentin surface treatment and adhesive approach performed (N = 20; Table 1):

Group 1 (G1): DCC pre-treatment and Scotchbond Universal adhesive (SBU; 3M ESPE, St. Paul, MN, USA used in the etch-and-rinse mode (ER). The dentin surface was etched with 32% H_3PO_4 (Scotchbond Universal Etchant, 3M ESPE) for 15 s, pre-treated with an ethanol solution of 0.5 M DCC for 1 min, air-dried and bonded with SBU according to the manufacturer's instructions (Table 1).

Group 2 (G2): SBU in ER mode. No DCC was applied on dentin; SBU application was the same as G1.

Group 3 (G3): DCC pre-treatment and SBU in self-etch mode (SE). The dentin surface was pre-treated as in G1; instead of etching with 32% H_3PO_4, SBU was applied directly to the smear layer-covered dentin and agitated for 20 s. The adhesive was air-dried. Without lightcuring, DCC was applied for 1 min. The second layer of universal adhesive was subsequently applied, air-dried and light-cured (Table 1).

Group 4 (G4): SBU in SE mode. No DCC was applied on dentin. SBU application was the same as G3.

Each bonded specimen was light-cured for 20 s using a light-emitting diode curing light (Demi^{TM} Plus, Kerr Corp., Brea, CA, USA) after solvent evaporation. Four 1-mm thick layers of a micro-hybrid resin composite (Filtek Z250; 3M ESPE) were incrementally placed over the bonded dentin and individually polymerized for 20 s each to obtain a 4-mm thick composite build up for μTBS testing. Each specimen was serially-sectioned to obtain approximately 1-mm thick sticks, each containing resin composite and dentin and with the adhesive interface in between, in accordance with the non-trimming technique of the μTBS test. The dimension of each stick (0.9 mm × 0.9 mm ± 0.01 mm) was recorded using a pair of digital calipers. The bonded area was calculated for subsequent conversion of microtensile strength values into units of stress (MPa). Sticks from each tooth were randomly assigned to two storage groups: 24 h (T_0) or 1 year (T_{12}) of storage in artificial saliva at 37°C. The artificial saliva consisted of CaCl2 (0.7 mmoles/L), MgCl2 6H2O (0.2 mmoles/L), KH2PO4 (4.0 mmoles/L), KCl (30 mmoles/L), NaN3(0.3 mmoles/L) in HEPES buffer [38].

Each stick was stressed to failure under tension 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 group was recorded, but those null values were not included in the statistical analysis. This is because all premature failures occurred during the cutting procedure and those failures 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 GmbH, Jena, Germany) at 30x magnification. Failure modes were classifieds adhesive failure (A), cohesive failure in dentin (CD), cohesive failure in composite (CC) or mixed failure (M).

Statistical analysis was performed using the tooth as the statistical unit. Bond strength data from each tooth were averaged to obtain the mean bond strength for that tooth. The acquired data were evaluated for compliance with the normality assumption using ShapiroWilk test, and the homoscedasticity assumption using the modified Levene test prior to the use of parametric analytical methods. A three-way analysis of variance (ANOVA) was performed to identify the effects of three variables, DCC pre-treatment (with/without), adhesive application mode (ER/SE) and aging (T_0/T_{12}) and their interactions on bond strength. Post-hoc comparisons were conducted using Tukey test. Additionally, one-way ANOVA was conducted to evaluate differences within each variable. For all tests, statistical significance was pre-set at $\alpha = 0.05$. Statistical analyses were performed using Stata 12.0 software for Mac (StataCorp, College Station, TX, USA).

2.2 Nanoleakage expression

Twenty teeth (N = 5) were used for examination of nanoleakage within the resin-dentin interface. Mid-coronal dentin were bonded in the same manner described for μTBS testing. Each specimen was cut vertically into 1-mm-thick slabs to expose the resin-dentin interface. After storage in artificial saliva at 37 °C for 24 h (T_0) or 12 months (T_{12}), the specimens were immersed in 50 wt% ammoniacal $AgNO_3$ solution for 24 h in the dark, following the protocol described by Tay *et al*. [39]. The specimens were then thoroughly rinsed in distilled water and immersed in a photo-developing solution for 8 h under a fluorescent light to reduce silver ions into metallic silver grains within voids along the bonded interfaces.

For light microscopy, the specimens were fixed, dehydrated, embedded in epoxy resin

(LR White resin, MilliporeSigma, Burlington, MA, USA), fixed on glass slides using cyanoacrylate glue, flattened on a grinding device (LS2; Remet, Bologna, Italy) under water irrigation and polished with a graded series of silicon carbide abrasive papers of increasing fineness (180-, 600-, 1200-, 2400-, and 4000-grit). The presence of the silver tracer was examined along the bonded interface using light microscopy (E800; Nikon, Tokyo, Japan), at 20x magnification. Interfacial nanoleakage expression was scored by two trained investigators based on the percentage of adhesive surface showing $AgNO_3$ deposition, following the method of Saboia *et al.* [40]. A scale 0-4 was used for evaluation: (0) no nanoleakage; (1) <25% surface with nanoleakage; (2) 25-50% surface with nanoleakage; (3) 50-75% surface with nanoleakage; and (4) >75% surface with nanoleakage. Intra-examiner reliability was evaluated using the Cohen's kappa (κ) statistic. Statistical differences among nanoleakage scores were analysed with the chi-square statistic. Statistical significance was pre-set at $\alpha = 0.05$.

2.3 *In-situ* zymography

Twenty freshly-extracted human third molars (N = 5) were used for *in-situ* zymography. One mm-thick slabs of middle/deep dentin were prepared. Each slab was further divided into four parts to test the 4 control land experimental groups on the same substrate (Figure 1). Silicon carbide paper (600-grit) was used to create a standardized smear layer on each dentin surface. One surface of each quarter of a slab was treated with the adhesive systems as described for μTBS testing.

The procedure was performed using the method previously reported by Mazzoni *et al.* [24, 41]. After aging for the designated period (24 h or 1 year), each bonded slab was glued to a glass slide and polished to produce an approximately 40-μm thick section. To produce the substrate, 1.0 mg/mL of a stock solution containing self-quenched fluorescein-conjugated gelatin (E-12055; Molecular Probes, Eugene, OR, USA) was prepared by adding 1.0 mL deionized water to the vial containing the lyophilized gelatin. The substrate was stored at −20

°C until use. The gelatin stock solution was diluted 10 times with dilution buffer (NaCl 150 mm, $CaCl_2$ 5 mm, Tris-HCl 50 mm, pH 8.0), followed by the addition of an anti-fading agent (Vectashield mounting medium with 4′,6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA, USA). Then, 50 µL of the fluorescent gelatin mixture was placed on top of each polished dentin section and protected with a cover slip. The glass slide assemblies were light-protected and incubated in a humidified chamber at 37 °C for 48 h.

Detection of endogenous gelatinolytic enzyme activity within the hybrid layer was based on hydrolysis of the quenched fluorescein-conjugated gelatin substrate. The process was evaluated by examining the glass slides with a multi-photon confocal laser scanning microscope (LSM 5 Pascal; Carl Zeiss), using an excitation wavelength of 495 nm and an emission wavelength of 515 nm. Samples were imaged using a HCX PL APO 40x/1.25 NA oil immersion objective. Series of x-y-z images (0.145*0.145*1 µm3 voxel size) were collected. Laser power and detector gain were set at the beginning of the experiment and kept the same for all specimens in order to have the possibility to compare different groups. Sixteen to 20 optical sections were acquired for each specimen. The stacked images were analyzed, quantified, and processed with ZEN 2009 software (Carl Zeiss). The fluorescence intensity emitted by the hydrolyzed fluorescein-conjugated gelatin was isolated and quantified using Image J (ImageJ; National Institute of Health, Bethesda, MD, USA). The amount of gelatinolytic activity was expressed as a percentage of the green fluorescence within the hybrid layer.

Negative control sections were similarly incubated, with the exception that 250 mL ethylenediaminetetraacetic acid (EDTA) or 2 mM 1,10-phenanthroline was dissolved in the mixture of quenched fluorescein-conjugated gelatin. The EDTA- and 1,10-phenanthrolinecontaining gelatin were used as negative controls. In addition, standard non-fluorescent gelatin was used as the third negative control.

Because the *in-situ* zymography data complied with normality and homoscedasticity assumptions after non-linear transformation, a three-way ANOVA was used to identify the effects of the three variables, DCC pre-treatment, adhesive application mode and aging, on the density of fluorescence signals. Additional one-way ANOVA was conducted to evaluate differences within each variable. Statistical significance was pre-set at $\alpha = 0.05$.

3. RESULTS

3.1 Microtensile bond strength

Microtensile bonds strengths of the four groups tested at T_0 and T_{12} are summarized in Table 2. Three-factor ANOVA revealed significant difference for the variables DCC pretreatment, adhesive application mode and aging ($p < 0.05$), as well as for the interaction between DCC pre-treatment and adhesive application mode ($p < 0.05$). Post-hoc comparisons showed that the use of a 0.5 M DCC-containing ethanol solution before adhesive application improved bond strength of SBU (G1 and G3) *vs* control groups (G2 and G4) ($p < 0.05$), irrespective of the adhesive application mode and aging. Additionally, SBU generated higher bond strength when employed in the ER mode *vs* the SE mode ($p < 0.05$). Aging significantly reduced μTBS among all the adhesive application mode/dentin pre-treatment combinations, except for G3 ($p < 0.05$). One-way ANOVA indicated that at T_0, DCC pre-treatment significantly improved μTBS in both experimental groups compared to the control groups (G1 46.0±15.3; G2 37.1 ± 12.5; G3 39.4 ± 11.1; G4 26.3 ± 11.4). After aging, DCC pre-treatment showed a preservation of the bond strength when SBU was applied in the SE mode (T_0=39.4± 11.1 and T_{12}=35.3 ± 13.9) thus, shoving a final MPa value after aging comparable to that of the SBU ER groups. For all groups at T_0 or T_{12} the predominant failure modes were the adhesive failure and the mixed failure. At baseline, for all groups, except for G2 the adhesive failure were around 70% of the failure. After aging, the number of mixed failure increased between 40% and 55% in the different groups (Table 3).

3.2 Nanoleakage expression

Descriptive statistics of interfacial leakage scores are represented in Figure 2. Statistically significant differences were found among the four groups in the extent of silver nitrate penetration along the adhesive interfaces ($p < 0.05$). Specimens that were pretreated with DCC-containing solution prior to adhesive application showed lower nanoleakage expression in both the ER and SE modes, compared to control groups, at both T_0 and T_{12} ($p < 0.05$). At baseline SBU ER and SBU SE showed an higher percentage of marginal infiltration compared to the experimental groups. However, SBU SE performed worst than SBU ER. After aging in artificial saliva, DCC pretreated groups shoved a significantly lower marginal leakage than the control groups. In addition, DCC SBU groups showed comparable results among them.

3.3 *In-situ* zymography

Representative micrographic images of the different groups are shown in Figure 3 for time T_0 and Figure 4 for time T_{12}. The percentages of hybrid layers exhibiting hydrolysis of the quenched fluorescein-conjugated gelatin at T_0 and T_{12} are shown in Figure 5. For all specimens, the highest enzymatic activity appeared to be concentrated in the hybrid layer and the dentinal tubules underneath the hybrid layer.

Statistical analysis of the *in-situ* zymography identified significant differences for the variables DCC pre-treatment, adhesive application mode and aging ($p < 0$ .05), and for the interaction between application mode and aging ($p < 0.05$). For the variable "DCC pretreatment", post-hoc comparisons showed that DCC pre-treatment significantly reduced fluorescence at the level of the hybrid layer, compared to non-treated groups ($p < 0.05$), irrespective of the adhesive application mode and the aging period. For the variable "adhesive application mode", ER groups resulted in a significantly higher enzymatic activity compared to the SE groups ($p < 0.05$), irrespective of DCC application and aging period. For the variable

"aging period", especially when associated with ER mode, significant increases in fluorescence at the level of the hybrid layer was identified with the ER mode at both T_0 and T_{12} ($p < 0.05$).

One-way ANOVA calculated across all groups indicated that at T_0, there was reduced fluorescence within the hybrid layers, irrespective of the adhesive application mode, when 0.5M DCC solution was applied prior to adhesive application (G1 and G3). However, the decrease was not statistically significant for the SE mode (Figure 5). At T_{12}, both experimental (G3 and G4) and control groups (G1 and G2) showed comparable endogenous enzymatic activity for the ER group (Figure 5). When SBU was applied in the SE mode, 0.5 M DDC solution significantly reduced the activity within the hybrid layer (Figure 5).

No fluorescence was detected in two negative control groups prepared with non-specific inhibitors (EDTA or 1,10-phenanthroline) or when non-fluorescent gelatin was employed (data not shown).

4. DISCUSSION

The results of the present study showed that bond strength was increased by exposing dentin surfaces to 0.5 M DCC treatment prior to bonding with SBU at T_0 , irrespective of the adhesive application mode. Furthermore, dentin specimens pre-treated with the ethanol-based DCC cross-linker exhibited less interfacial nanoleakage compared to specimens in the control groups. Thus, the first null hypothesis that "pre-conditioning of dentin with DCC prior to adhesive application does not benefit immediate tensile bond strength of a universal adhesive to dentin" has to be rejected. Likewise, the application of DCC before SBU resulted in higher bond strength and lower nanoleakage at the adhesive interface in specimens that has been aged for 12 months. Hence, the second null hypothesis that "pre-conditioning of dentin with DCC prior to adhesive application does not prevent interfacial degradation over time" also has to be rejected.

To our knowledge, this is the first study that utilized ethanolic DCC as dentin collagen cross-linker. This is different from the water-based cross-linkers that had been used in previous studies, such as EDC, grape seed extract, riboflavin, proanthocyanidin and chitosan [11, 42-44]. N,N'-dicyclohexylcarbodiimide is an organic compound whose primary use is to couple amino acids during artificial peptide synthesis. Under standard conditions, it exists in the form of white crystals with a heavy, sweet odor. This cross-linker is commonly used as a condensation reagent in amide synthesis or esterification reactions [44]. Unlike EDC, DCC has the distinct advantage of being insoluble in water but is well soluble in other polar organic solvents such as ethanol and acetone. This enables DCC to be more realistically blended with existing adhesive systems, without the need for rinsing off prior to the application ethanol-based universal adhesive.

Each collagen molecule has a primary amine group (-NH2) at the N-terminus, and a carboxyl group (-COOH) at the C-terminus of the polypeptide chain, both of which are on the surface of the protein structure, making them accessible for the conjugation of proteins [45]. EDC and DCC conjugate carboxylates to primary amines directly and cross-link the neighboring collagen molecules, without becoming part of the final cross-linked target molecules. Hence, they are zero-length cross-linking agents [46]. Similarly to an EDC-based dentin primer, [11, 34], the results of the present study showed that the use of an ethanolbased DCC primer improved both bond strength and resin infiltration as well as preserved bond strength and minimized nanoleakage after one year of aging in artificial saliva.

In previous studies, improvement in the bond durability by EDC in both coronal and radicular dentin was not attributed only to strengthening of the demineralized collagen matrix, but also to inactivation of the catalytic sites of endogenous MMPs present in demineralized dentin. The latter is achieved by modifying the three-dimensional conformation of the MMP enzymes [48-51]. Inactivation of MMPs induced by a collagen cross-linker is a non-specific mechanism involving covalent bonds that are claimed to be very stable over time [16, 52]. For this reason, the effect of

0.5 M DCC on MMP activity was further investigated using *in-situ* zymography. At baseline (T_0), Decrease of endogenous enzymatic activity was identified in the baseline (T_0) specimens after the use of DCC, although the reduction was statistically significant only in the ER group. After aging (T_{12}), only the DCC/SE group showed significant reduction in MMP activity. For this reason, the third null hypothesis that "pre-conditioning of dentin with DCC prior to adhesive application does not inhibit endogenous dentin MMP activity" can only be partially-rejected.

In-situ zymography showed clearly detectable gelatinolytic activity within the hybrid layers of the experimental groups both at T_0 and T_{12}. These activities were significantly lower for the groups bonded in the SE mode, irrespective of the storage time or the use of DCC. The gelatinolytic activity within the hybrid layers were either maintained or increased after 1 year of storage in artificial saliva for the control groups without DCC pre-treatment. The effectiveness of DCC used as a conditioning primer was evident in the DCC/SE group (G3), with significantly lower gelatinolytic activity within the hybrid layers after 1 year storage in artificial saliva. It could be speculated that, the DCC primer applied between two layers of adhesive and not after the etching step, as for the experimental ER group, could lead to a longer period of activity of the molecule. Indeed, DCC could remain bound inside the hybrid layer for a longer period of time and slowly released with ageing, similarly to other experimental molecules blended inside the adhesive [12].

The present study also showed that more endogenous MMPs were activated when the universal adhesive was applied in the ER mode [53]. When a multi-mode adhesive is used in the ER mode, acid-etching with phosphoric acid completely removes the mineral components of the dentin matrix, exposes the collagen fibrils completely with increased activation of the endogenous enzymes. Similarly to a 2-step etch-and-rinse adhesive, the enzymatic activity is high and localized at the bottom of the hybrid layer. This is probably due to the inability of the adhesive resin monomers to completely infiltrate the demineralized dentin matrix [24, 54]. Conversely, when

SBU is used in the SE mode, the hybrid layer is simultaneously demineralized and infiltrated by the acidic adhesive resin monomers. In addition, there is less completely denuded collagen fibrils within the thinner hybrid layers. Because the apatite crystallites are only partially-dissolved, here is presumably less activation of the endogenous MMPs [55]. It has been reported that more MMPs are activated when the pH of the dentin matrix is lower [56], as the drop in pH value turns on the cysteine switch mechanism and causes the MMP pro-forms to be activated into fully-functional MMPs [57].

Because dentin MMP activity could be reduced by the application of DCC, the results of the present study suggest that the ethanolic collagen cross-linker stabilizes the adhesive interface by strengthening of the collagen and inactivating endogenous dentin proteases [4]. Severe, albeit non-fatal limitation of the present study is that no experiment was performed to confirm that there was increase in cross-linking of the collagen matrix after pre-treatment with DCC. This should be performed in future studies to identify the type and quantity of cross-links involved and to determine if the cross-linking produced by the use of DCC is reversible or irreversible. It would also be interesting to compare the degree of cross-linking produced by the water-based EDC versus the ethanol-based DCC.

Another important aspect related to the use of DCC is that ethanol is used as the solvent instead of water. The use of an ethanol-based cross-linking agent may result in better infiltration of the collagen matrix by adhesive resin monomers, compared with the use of a water-based cross-lining agent [58]. After acid-etching, the dentin collagen fibrils are in a wet environment saturated with residual water. To substitute water with water-insoluble adhesive resins, a primer agent based on a volatile solvent such as ethanol or acetone is required. Formation of an ideal hybrid layer in dentin requires substitution of all unbound water with resin monomers. However, complete removal of all residual water cannot be achieved due to the presence of bound water that intrinsically wet the collagen fibrils. This process, however, may be improved

by first replacing the unbound water with a non-water-containing polar solvent which is capable of solubilizing the adhesive resin monomers [59]. Previous studies on the "ethanol-wet bonding" concept demonstrated that better resin infiltration occurred with a higher content of ethanol in the adhesive primer [60]. When dentin is fully saturated with ethanol, ethanol-soluble hydrophobic resin monomers may be introduced into a demineralized collagen matrix [61, 62]. Infiltration of hydrophobic monomers decreases water sorption/solubility and resin plasticization, but also, it has been suggested that the elimination of residual water could reduce or eliminate enzyme-catalyzed hydrolytic collagen degradation [63]. The ethanol in the DCC primer used in the present research probably contributes to further dissolution of the adhesive resin monomers, reducing the viscosity and the wettability of the mixture, and facilitating its diffusion into the dentin. In addition, a possible inhibitory effect of ethanol on MMPs activity could not be excluded, although the application of the ethanol-based DCC primer was not performed following the traditional "ethanol-wet" protocol that implies increasing concentrations of ethanol (50%, 70%, 80%, 95% and 3 100% ethanol applications for 30 s each) applied on dentinal substrate.

In light of the favorable results obtained with DCC pre-treatment, it is envisaged that DCC may be incorporated into ethanol-based adhesive systems to produce self-priming adhesives that cross-links the collagen matrix as the adhesive infiltrates the completely- or partially-demineralized dentin. This removes an additional step and further simplifies the bonding procedures. However, further studies on different adhesive systems and protocols are required to clarify the role of DCC on preserving the adhesive bond over time and to validate the possibility to incorporate DCC into current adhesive systems without compromising their adhesive properties.

5. CONCLUSIONS

Within the limitations of the present study, it may be concluded that the use of the ethanolic DCC collagen cross-linker improves bond strength and reduces nanoleakage when a universal adhesive is applied on coronal dentin in the etch-and-rinse mode or self-etch mode. The use of DCC pre-treatment prior to the application of a universal adhesive does not adversely affect baseline MMP activity. When the adhesive is employed in the self-etch mode, the use of 0.5 M DCC further helps in preventing the degradation of dentin organic matrix by inhibiting endogenous dentin matrix metalloproteinases.

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Table 1: Adhesive system, composite composition and application mode

Material	**Composition**	**ER mode**	**DCC + ER mode**	**SE mode**	**DCC + SE mode**
Scotchbond Universal (SBU; 3M ESPE)	1. Etchant: 32% phosphoric acid, water, synthetic amorphous silica, polyethylene glycol, aluminum oxide (Scotchbond Universal Etchant) 2. Adhesive: methacryloyloxydecyl dihydrogen phosphate (MDP) phosphate monomer, dimethacrylate resins, 2hydroxyethyl methacrylate (HEMA), methacrylatemodified polyalkenoic acid copolymer, filler, ethanol, water, initiators, and silane	1. Apply etchant for 15 s 2. Rinse for 10 s 3. Air dry 5 s 4. Apply the adhesive to the entire preparation with a microbrush and rub it in for 20 s 5. Direct a gentle stream of air over the liquid for 5 s until it no longer moves and the solvent is evaporated completely 6. Repeat steps 4 and 5 7. Light-cure for 20 s	1. Apply 0.5 M DCC ethanolbased primer and brush it for 1 min 2. Direct a gentle stream of air over the liquid for 5 s 3. Apply adhesive as for the ER mode	1. Apply adhesive to the entire preparation with a microbrush and rub it in for 20 s 2. Direct a gentle stream of air over the liquid for 5 s until it no longer moves and the solvent is evaporated completely 3. Repeat steps 2 and 3 4. Light-cure for 20 s	1. Apply first coat of adhesive 2.Apply 0.5M DCC ethanolbased primer and brush it for 1 min 3. Direct a gentle stream of air over the liquid for 5 s 3. Apply adhesive as for the SE mode
Filtek Z250 (3M ESPE)	Triethyleneglycol dimetacrylate (TEGDMA) < 1–5%; Bisphenol-A-glycidylmethacrylate (Bis-GMA) < 1–5%; Bisphenol-A polyethylenglycol dietherdimethacrylate (Bis-EMA) 5–10%; Urethane dimethacrylate (UDMA) 5–10% Fillers: Zirconia/silica; 60 vol% inorganic fillers (particle size 0.01 to 3.5 μm)				

Table 2: Results of μTBS test at T_0 and T_{12}. SBU (ER): Scotchbond Universal used in the etchand-rinse mode; SBU (SE): Scotchbond Universal used in the self-etch mode. G1: 0.5 M DCC SBU (ER); G2: SBU (ER), control; G3: 0.5 M DCC + SBU (SE); G4: SBU (SE), control. T_0: Data obtained after 24 h of storage at 37 °C. T_{12}: Data obtained after 1 year of aging in artificial saliva at 37 °C.

Application mode	**SBU (ER)[1]**		**SBU (SE)[†]**	
Pre-treatment	G1	G2	G3	G4
T_0	$46.0 \pm 15.3^{A,a}$	$37.1 \pm 12.5^{A,b}$	$39.4 \pm 11.1^{A,a,b}$	$26.,3 \pm 11.4^{A,c}$
T_{12}	$33.5^{B} \pm 13.9^{B,a}$	$31.0 \pm 11.0^{B,a}$	$35.3 \pm 13.9^{A,a}$	$13.4 \pm 9.1^{B,b}$

[1] Values are means ± standard deviations (in MPa).

Different superscript upper-case letters indicate differences ($p < 0.05$) within the columns. Different superscript lower case letters indicate differences ($p < 0.05$) within the rows.

Table 3: Percentages of failures mode among the different groups.

Application mode	**SBU (ER)**		**SBU (SE)**	
Pre-treatment	G1:	G2:	G3:	G4:
T_0	68% A 32% M	35% A 8% CD 57% M	81% A 1% CD 18% M	74% A 36% M
T_{12}	45% A 55% M	58% A 42% M	53% A 47% M	60% A 40% M

Figure legends

Figure 1. Schematic of tooth preparation for *in-situ* zymography. A dentin disk (1-mm thick) was divided into four quadrants, enabling bonding procedures of the four control and experimental groups to be performed on the same dentin substrate.

Figure 2. Distribution of interfacial nanoleakage (in %) in the resin-dentin interfaces created with the Scotchbond Universal (SBU) adhesive in the etch-and-rinse mode (ER) or the self-etch mode, with or without DCC pre-treatment of dentin. Testing was performed after 24 hours (T_0) or after one year of aging in artificial saliva (T_{12}).

Figure 3: Representative examples of *in-situ* zymography of the resin-dentin interfaces at $T_{0.}$ Dentin treated with SBU adhesive in the SE mode (a,b); SBU (SE) + DCC 0.5 M (c, d); SBU in the ER mode (e, f); SBU (ER) + DCC 0.5 M (g, h). D: dentin; HL: hybrid Layer; R: resin composite.

Figure 4: Representative examples of *in-situ* zymography of the resin-dentin interfaces at T_{12} (aging for 12 months in artificial saliva). Dentin treated with SBU adhesive in the SE mode (a,b); SBU (SE) + DCC 0.5 M (c, d); SBU in the ER mode (e, f); SBU (ER) + DCC 0.5 M (g, h). D: dentin; HL: hybrid Layer; R: resin composite.

Figure 5. Gelatinolytic activity, expressed as the intensity of green fluorescence (pixels/μm2) within the hybrid layers (HL) created with SBU in ER mode or SE mode for the experimental (DCC pre-treatment) and control (no DCC pre-treatment) groups at T0 and T12. Values are means and standard deviations. For comparison of the factor "adhesive application mode", columns labelled with the same upper case letters (T0) or lower case letters (T12) are not significantly different ($p > 0.05$). For comparison of the factor "DCC pre-treatment", columns labelled with the same numerals are not significantly different ($p > 0.05$).

Figure 1

Click here to access/download;Figure;Figure 1.tif

Figure 2

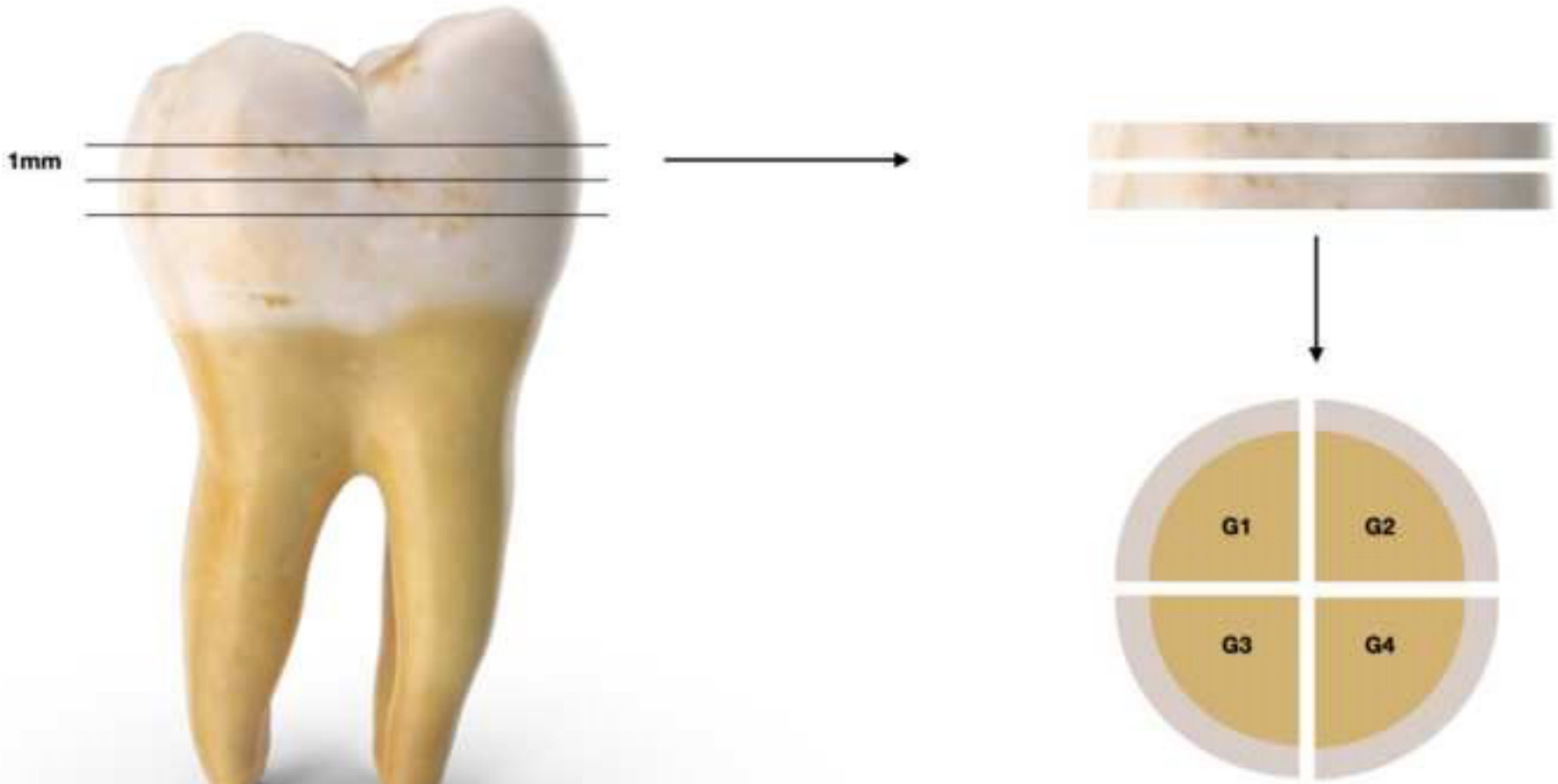

Figure 3

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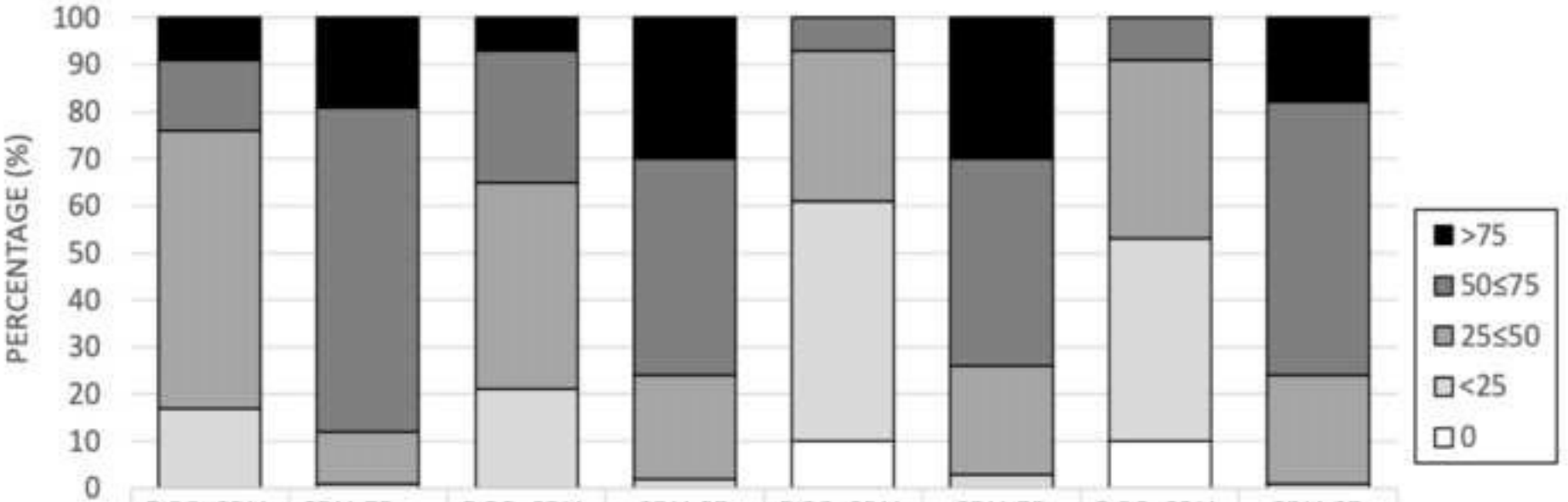

	DCC+SBU ER T0	SBU ER T0	DCC+SBU SE T0	SBU SE T0	DCC+SBU ER T12	SBU ER T12	DCC+SBU SE T12	SBU SE T12
>75	9	19	7	30	0	30	1	18
50≤75	15	69	28	46	7	44	9	58
25≤50	59	11	44	22	32	23	38	23
<25	17	1	21	2	51	3	43	1
0	0	0	0	0	10	0	10	0

Figure 4

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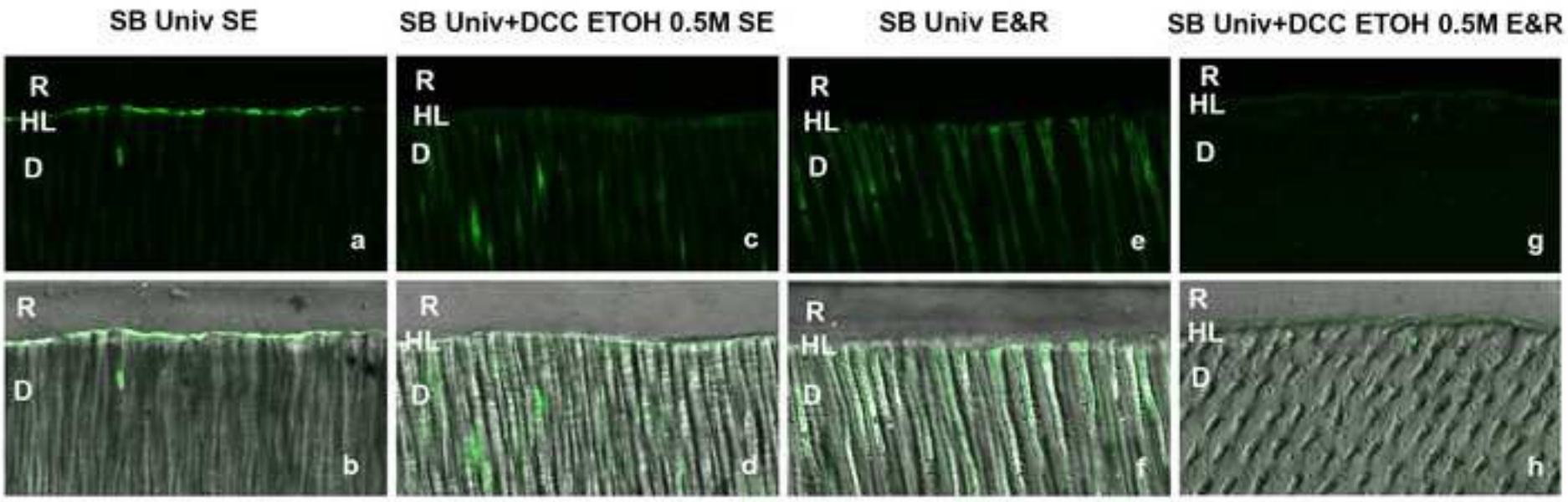

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Figure 5

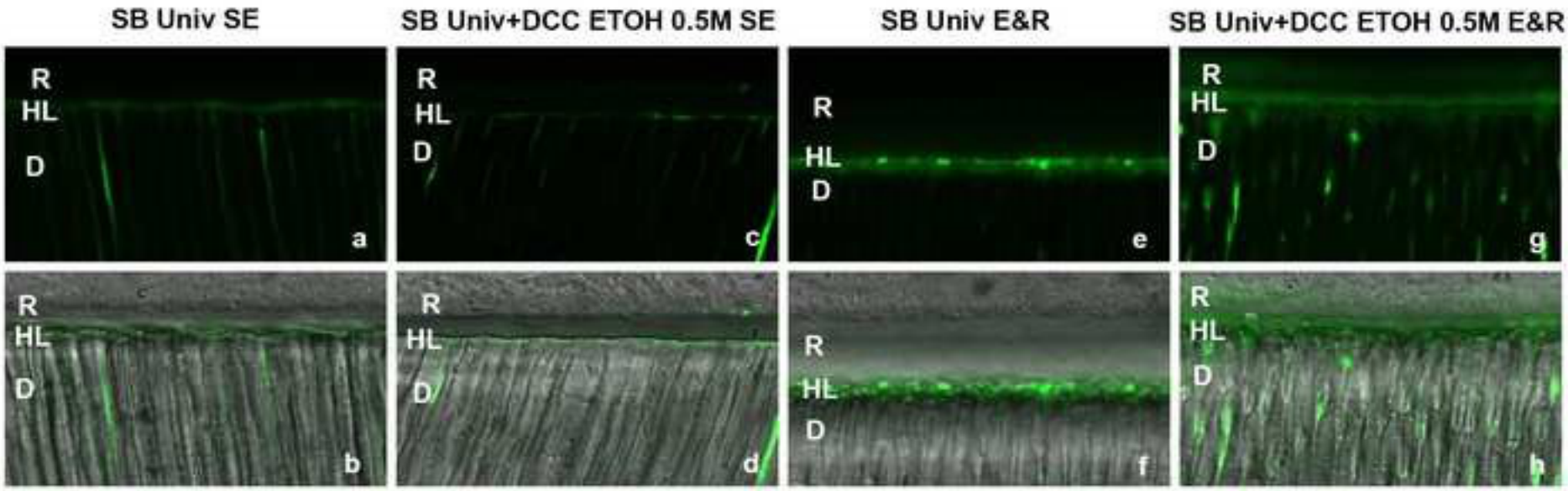

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Figure 6

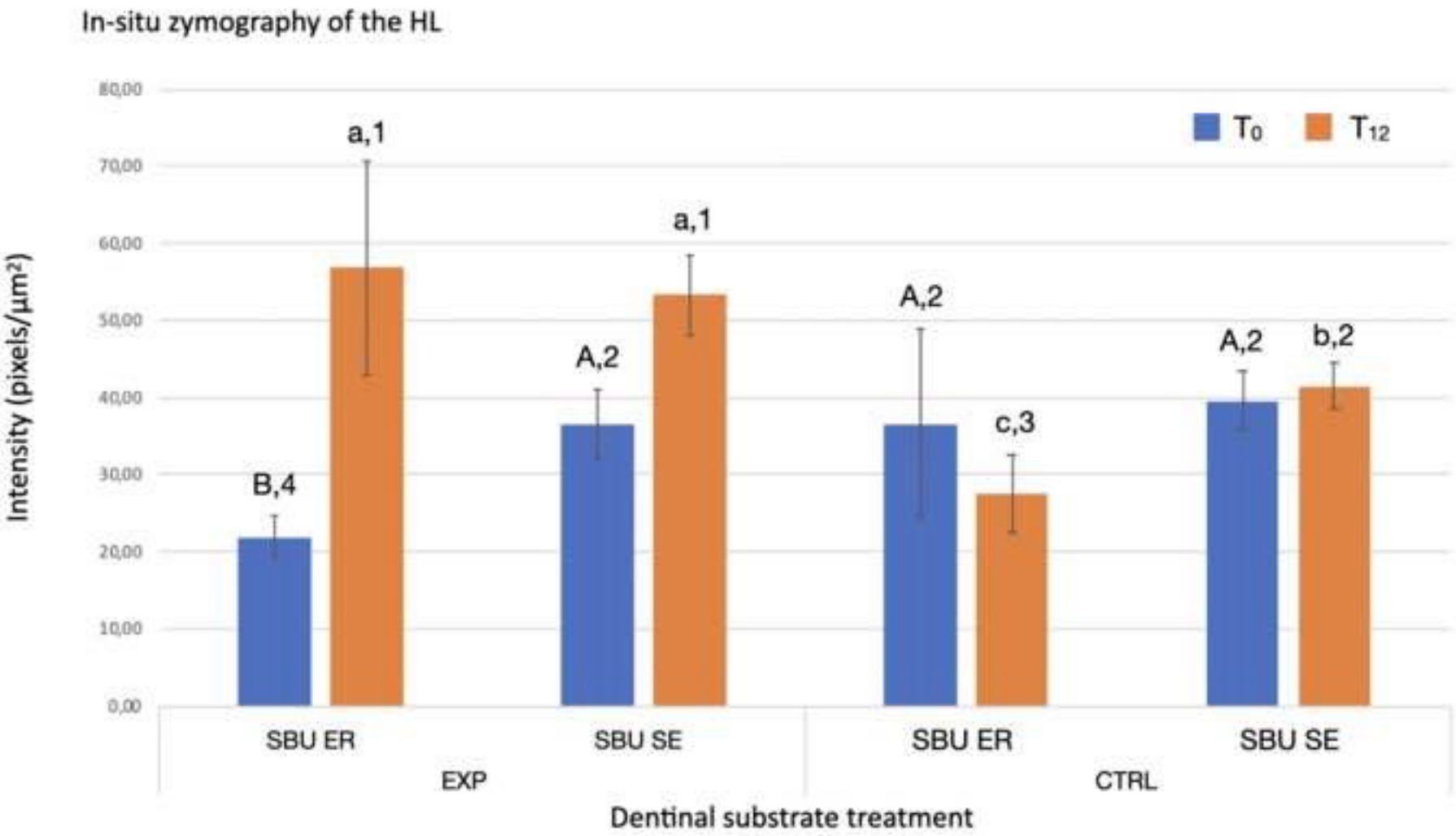
In-situ zymography of the HL
Intensity (pixels/µm²)
80,00
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
T0
T12
a,1
a,1
A,2
A,2
A,2
b,2
B,4
c,3
SBU ER
SBU SE
SBU ER
SBU SE
EXP
CTRL
Dentinal substrate treatment

Table 1

Table 2


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