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Mechanical Stability and Proteolytic Activity of Resin–dentin Bonds Using the Cross-linked Dry Bonding Technique

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Clinical Relevance

Dentin collagen cross-linking inhibits proteolytic activity, increases the stability of hybrid layers, and improves collagen resistance to air drying, thereby, eliminating the influence of dentin wetness status on dentin bonding, even if a water-free adhesive is used.

SUMMARY

Objective: To evaluate the mechanical stability and the proteolytic activity of bonds created by a two-step, etch-and-rinse adhesive applied to cross-linked and air-dried etched dentin.

Methods: Flat dentin surfaces were produced in 64 extracted sound human molars. The dentin was etched with 35% phosphoric acid for 15 seconds, and then the teeth were divided into groups according to the cross-linking solution applied on the etched dentin. Group 1: 5% grape seed extract (GSE), Group 2: 5% glutaraldehyde, Group 3: Gluma Desensitizer, or Group 4: deionized water

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(control). Solutions were applied for 60 seconds, followed by rinse and blot drying. Then, the teeth were separated into two subgroups where the etched dentin was kept moist or air-dried. The adhesive was applied followed by a composite resin buildup. After 24 hours, the teeth were cut into beams (0.81 mm²) that were tested for microtensile strength immediately or after 12 months of aging in a 37°C saliva-like buffer. Additional teeth (n=32) were bonded as described and cut into 0.5-mm-thick slabs. The slabs were prepared for nanoleakage (scanning electron microscopy) and *in situ* zymography (EnzChek Protease Assay Kit). Bond strength data were submitted to ANOVA and Tukey tests (α=0.05).

Results: Significant reduction in immediate bond strength (*ca* 65%) and increase in proteolytic activity was seen when the etched dentin was air dried without previous cross-linking biomodification. Conversely, bond strengths did not differ from those produced on wet dentin when collagen was cross-linked before air drying, irrespective of the solution applied. For both moist and air-dried etched dentin, collagen cross-linking resulted in mechanically stable bonds and reduced proteolytic activity after 12 months of storage.

Conclusion: Bonds produced by the application of a two-step, etch-and-rinse adhesive to cross-linked, air-dried, etched dentin were mechanically stable and revealed reduced proteolytic activity after 1 year of aging.

INTRODUCTION

The hybrid layer is a microscopic structure that serves two fundamental roles in dentin adhesion—retention of the restorative material and sealing of the intrinsically wet collagen of the dentin matrix. The ideal hybrid layer should contain an impermeable three-dimensional structure composed of the mixture of polymer and collagen, capable of guaranteeing a continuous and stable bond between the dentin and the adhesive material.[1] However, this biocompound contains imperfections created during its formation, such as areas with poor monomer infiltration, hydrophilic and hydrophobic moieties,[2] retention of water and solvent, and the presence of water-filled denuded collagen fibrils.[3] As a result, despite its utmost importance, the hybrid layer has been considered the weak link in resin–dentin bonds.[1] Complete infiltration of the acid-etched dentin by adhesive monomers[3-8] is hindered by residual water between the collagen molecules.[9] Two main factors limit monomer infiltration into these nanospaces: the presence of free water molecules and water molecules bound to demineralized collagen,[10] and the dense packing of the collagen molecules.[9,11-12]

Deterioration of hybrid layers occurs irrespective of the adhesive system used; that is, it occurs in etch-and-rinse and self-etching adhesives,[12-15] and involves hydrolysis of the resin polymer and the breakdown of the exposed collagen—the two main components of this biocompound.[11,12,15] The degradation of the exposed collagen within the hybrid layer by intrinsic proteolytic enzymes bound to collagen is claimed to be the cause of the failure of interfaces produced on dentin.[16,17]

After acid etching dentin, water is important for maintaining the interfibrillar spaces that serve as a pathway for infiltration of the monomers of the adhesive.[18] At the same time, water limits the infiltration of hydrophobic monomers, compromises monomeric conversion,[19,20] and allows hydrolysis of the collagen mediated by proteolytic enzymes such as the matrix metalloproteinases (MMPs) and cysteine cathepsins.[11,12] Nevertheless, attempts to remove the residual water content of etched dentin simply by air evaporation causes collapse of the collagen fibrils and, consequently, loss of the interfibrillar spaces needed for the infiltration of the adhesive monomers.[18,21-23] The stiffness of mineralized dentin is about 20 MPa, which makes it almost incompressible, whereas the stiffness of demineralized dentin is about 0.1 MPa,[24] which is similar to that of cooked spaghetti.

Biomodification of demineralized dentin with cross-linkers has been used to increase the mechanical properties of demineralized dentin collagen to make it more resistant to proteolytic degradation.[25-28] Commonly used cross-linkers are carbodiimide [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)], glutaraldehyde, and polyphenols such as proanthocyanidins (PAs). These agents increase the strength and stiffness of both collagen molecules and collagen fibrils.[24,26,29,30] Also, these agents are powerful inhibitors of matrix metalloproteinases (MMPs) and cysteine cathepsins (CATs), even when applied on the demineralized dentin for only 60 seconds.[31-33] The basic mechanism of cross-linkers is to increase the number of cross-links at the intramolecular, intermolecular (or intrafibrillar), and interfibrillar levels.[34,35]

It has been reported that once biomodified by a proanthocyanidin-rich extract [grape seed extract (GSE)], the reinforced collagen can resist air drying without shrinking and with no detrimental consequences to the immediate bond strength.[23] Conversely, without prior cross-linking of collagen, the immediate bond

strength was significantly reduced after prolonged air-blast drying of the dentin. This new adhesive approach was called cross-linked dry bonding.[23] However, the resistance of cross-linked collagen against air drying was evaluated only 24 hours after bond formation. Its long-term validation is still necessary, since the main challenge of bonding to dentin is the creation of hybrid layers resistant to the mechanisms of hydrolytic and enzymatic degradation over time.

Therefore, the aim of the study was to evaluate the mechanical stability and the proteolytic activity of resin–dentin bonds created by a water-free, two-step, etch-and-rinse adhesive applied to cross-linked, air-dried etched dentin, in comparison to bonds produced using the conventional wet-bonding technique. The null hypotheses tested were that the interfaces produced on biomodified and dry dentin (cross-linked dry bonding) do not differ from interfaces produced using the wet-bonding technique relative to mechanical stability, nanoleakage, and proteolytic activity.

METHODS AND MATERIALS

Specimen Preparation

Sixty-four extracted human third molars were used to evaluate the mechanical stability of interfaces produced on biomodified and dry dentin, using as experimental protocol approved by the Ethics Committee on Research with Human Beings. With a diamond-coated disc coupled to a metallographic cutter (ISOMET 1000, Buehler, Lake Bluff, IL, USA), the enamel and occlusal-third of the tooth crown was removed, exposing a flat dentin surface. The dentin surfaces were manually abraded with 320-grit abrasive paper for 30 seconds, to create a standardized smear layer comparable with those clinically produced with carbide burs.[36,37] The teeth were then randomly distributed into four experimental treatment groups (n=16): 5% GSE, 5% glutaraldehyde (GD) in water, Gluma Desensitizer, and water (control).

Dentin Treatment and Bonding Procedures

The dentin surfaces were etched with 35% phosphoric acid (Ultradent Products INC, South Jordan, Utah, USA) for 15 seconds, followed by water rinsing for 10 seconds and drying with absorbent paper. Then, the dentin was treated with one of the cross-linkers (Table 1) or with deionized water (control group). Twenty microliters (20 μL) of 5 wt% GSE, 5% GD, or deionized water (control) were applied on the dentin surface for 60 seconds and then rinsed off with 5 mL of deionized water. Gluma Desensitizer (Heraeus Kulzer GmbH, Hanau, Germany) was applied following the manufacturer's recommendations. Thus, 20 μL of the product was applied on the etched wet dentin and kept in place for 60 seconds. After this, a jet of air was applied for 5 seconds at a distance of 10 cm from the surface to blow the excess off, and then the surface was washed with 5 mL of deionized water. Then, the teeth of each group were subdivided according to the condition of dentin wetness. In half of the teeth (n=8), the dentin was kept moist by a wet piece of absorbent paper. In the other half (n=8), the dentin surface was dried by a constant jet of air for 60 seconds, with the syringe positioned at a distance of 10 cm from the surface.[23]

The adhesive system Optibond S (Kerr Corporation; Orange, CA, USA) was applied on the entire dentin surface and scrubbed for 15 seconds. Subsequently, a jet of air was applied for 3 seconds at a distance of 10 cm, and the adhesive was light activated for 20 seconds (*ca* 1000 mW/cm^2; LED light Radii Plus, SDI Limited, Bayswater, Victoria, Australia). A block of resin composite (Filtek Z350, 3M Oral Care, St. Paul, MN, USA) was built up in three increments of 1 mm each, which were individually light activated for 20 seconds at a radiant emittance of 1000 mW/cm^2. The teeth were then stored immersed in deionized water at 37°C for 24 hours.

Microtensile Bond Strength

After 24 hours, the restored teeth were sectioned with a metallurgical saw (ISOMET 1000, Buehler Ltd, Lake

Table 1: *Information About the Composition of Agents Used to Biomodify the Dentin Collagen*

Agent	Main Components	Manufacturer	Mixture/pH[a]
5% GD	Glutaraldehyde	Sigma-Aldrich Corp, St Louis, MO, USA	0.5 mL of 50% GD; 4.5 mL of deionized water; pH 6.4
5% GSE	Proanthocyanidin-rich extract from the seed of *Vitis vinifera*	Shaanxi Sinuoti Biotech Co Ltd (Shaanxi, China)	0.25 g of GSE; 5 mL of deionized water; pH = 5.5
Gluma Desensitizer	HEMA (25%-50%) Glutaraldehyde (5%-10%)	Heraeus Kulzer GmbH (Hanau, Germany)	Ready to use; pH = 4.0

Abbreviations: GD, glutaraldehyde; GSE, grape seed extract; HEMA, hydroxyethyl methacrylate.
[a]*Deionized water pH = 6.2.*

Bluff, IL, USA) containing a 0.3-mm thick diamond-coated disc (Diamond Wafering Blade, Buehler Ltd, Lake Bluff, IL, USA), under constant irrigation. A series of 0.9-mm-thick sections were made in both x and y directions to produce rectangular beam-shaped specimens, with a cross-sectional area of approximately 0.81 mm^2. The adhesive areas of these specimens were individually measured with a digital caliper (Mod 500-144b, Mitutoyo Sul Americana Ltda., SP, Brazil) with a resolution of 0.01 mm. The specimens obtained from each tooth were randomly divided into two groups, according to the period of aging. Thus, one group was mechanically tested immediately, and the other group was tested after 12 months of storage at 37°C in a saliva-like buffer solution (12.9 mM KCl, 1.9 mM KSCN, 2.4 mM Na_2SO_4, 3.3 mM NH_4Cl, 1.5 mM $CaCl_2$, 7.5 mM $NaHCO_3$, 0.02 mM $ZnCl_2$, and 5 mM HEPES buffer, pH 7.4).[38]

The microtensile bond test was performed in a mechanical test machine (DL-Digital Line, EMIC, Paraná, Brazil) equipped with a 100 N load cell, operating at a speed of 1.0 mm/min. The specimens were individually fixed to a metal device with cyanoacrylate adhesive (Super Bond Gel and Activator 7456, Henkel Loctite Ltda, São Paulo, Brazil), and the device was adapted to the test machine. The tensile movements were initiated by means of a specific computerized program (Tesc-Test Script, EMIC Equipamentos de Ensaio Ltda, São José dos Pinhais, Paraná, Brazil) until the specimen ruptured. The maximum load values recorded (N) were divided by the area of each specimen (mm^2) to obtain the tensile bond strength values in megapascals (MPa). Immediately after the test, the halves obtained for each specimen were analyzed under a stereomicroscope (Mod SZX7, Olympus, São Paulo, Brazil) at magnifications ranging from 10× to 30×. Failure modes were classified as adhesive, mixed, cohesive in resin, or cohesive in dentin.[37]

Nanoleakage and *in Situ* Zymography

Thirty-two teeth were used for silver nanoleakage and for *in situ* zymography, which were divided into the same groups and treated the same way as was previously described. Using the metallurgical saw, cuts were made perpendicular to the adhesive bond to obtain 0.5-mm-thick slices. Slices cut from the same tooth were equally distributed for nanoleakage and *in situ* zymography studies, and aging periods (24 hours and 12 months). Therefore, the slice (specimen) was the experimental unit, and the number of repetitions considering the interaction cross-linker versus dentin wetness versus period of aging was n=4 for each protocol.

Nanoleakage and Scanning Electron Microscopy (SEM)—

The 0.5-mm-thick slices were submitted to the nanoleakage protocol, as previously published.[39] To rehydrate the specimens, they were kept in distilled water for 20 minutes and afterward immersed in a 50 wt% ammoniacal silver nitrate tracer solution (pH 9.5) in the absence of light for 24 hours. After this, they were washed with deionized water for 5 minutes and immersed in a developing solution (Revelador Kodak, São José dos Campos, São Paulo, Brazil) under a fluorescent light for 8 hours. The specimens were washed in water for 2 minutes and polished with a sequence of silicon carbide abrasive papers of decreasing roughness (400, 600, 1200, 2000, and 4000 grit) moistened with deionized water. Afterward, the slices were rinsed in running water for 3 minutes, dried with absorbent paper, mounted on metal stubs, and stored in a desiccator containing dry calcium sulfate powder for 24 hours. They were imaged by backscattered scanning electron microscopy (SEM) (Inspect S50; FEI Company, Hillsboro, Oregon, USA), and images of the interface were obtained at 1500× magnification.

In Situ *Zymography—*

For *in situ* zymography of the intrinsic gelatinase activity of bonded dentin, the specimen slices were manually abraded with abrasive papers, 1200 and 4000 grit until a thickness of 0.3 mm was attained. Each slice was then glued to an acrylic slide using SuperBonder (Henkel Ltda, São Paulo – SP) and stored in deionized water at 4°C for 24 hours. The *in situ* zymography was performed using the EnzCheck gelatinase/collagenase Assay Kit (Molecular Probes, Eugene, OR, USA). The fluorescein-labeled DQ gelatin was diluted in buffer 1:8 (NaCl 150 mM, $CaCl_2$ 5 mM, *Tris*-HCl 50 mM, pH 8). The gelatin solution was prepared mixing 10 µL of the gelatin stock solution, 80 µL of dilution buffer, and 10 µL of antifade solution. Afterward, 30 µL of the gelatinous solution containing fluorescein were applied on the specimens, which were covered with a glass plate and stored in an oven at 37°C, in 100% humidity, and in the absence of light. After 24 hours of incubation, the samples were evaluated under a fluorescence microscope DM 5500 (ex: 498 nm and em: 530 nm) (Leica Microsystems, Wetzlar, Germany), and photographic images of the interface were obtained at 10× magnification.

Statistical Analysis

For the bond strength data, specimens of the same tooth within each period of aging were averaged using the tooth as the experimental unit. Sample size was

calculated using the software after obtaining the data and considering the bond strength means and standard deviations of the control group for both independent variables (dentin wetness and aging period). Fixed parameters were alpha 0.05, power 0.8, two-tailed test, and N2/N1=1. The bond strength data were initially submitted to a three-way analysis of variance (ANOVA), considering the factors (1) etched dentin treatment, (2) dentin wetness, and (3) period of aging. Then, significant interactions were analyzed by two-way ANOVA complemented by Tukey tests. The level of significance established was 5%. Nanoleakage and *in situ* zymography results were qualitatively analyzed.

RESULTS

Bond Strength

The bond strength values of the various groups are presented in Table 2. The three-way ANOVA revealed no effect of the interaction of the three factors (p=0.170) on bond strength. Conversely, significant effects were shown for etched dentin treatment*dentin wetness (p<0.001), etched dentin treatment*aging period (p<0.001), and dentin wetness*aging period (p<0.001). Therefore, two-way ANOVA was applied to the interactions, and significant differences between groups detected by the Tukey test are given in Table 2.

Without prior cross-linking of the collagen, the immediate bond strength was significantly reduced by 61.7% when the adhesive system was applied on dentin dried with air in comparison with wet dentin. Cross-linking of the etched dentin with the GSE, GD, or Gluma Desensitizer did not interfere in the immediate bond strength values when the dentin was maintained wet before application of the adhesive. However, increased bond strength was observed when the adhesive was applied on dentin dried with air, which had previously been biomodified by GSE, GD, or Gluma Desensitizer, in comparison with dry dentin that was not biomodified. These values became statistically similar to the values obtained for the control group, that is, dentin that was not biomodified but kept wet before application of the adhesive. Thus, cross-linked dentin could be dried without negative influence on the immediate bond strength.

After 12 months of aging in saliva-like buffer, a significant reduction in the bond strength was observed for both non-cross-linked control groups. This reduction was 37.1% when the dentin was kept wet, and 37.3% when the dentin was dried with air before application of the adhesive. Differently, the bond strength values of the groups treated with GSE, GD, and Gluma Desensitizer remained stable, that is, comparable with the respective groups at the 24-hour aging period. Irrespective of the aging period and dentin wetness, there was no difference in performance found among GSE, GD, and Gluma Desensitizer.

Failure Mode

The two halves of the specimens subjected to microtensile bond testing were examined under stereomicroscopy. The distribution of the fracture types is shown in

Table 2: Bond Strength (MPa) to Wet or Air-dried Etched Dentin with No Biomodification (Water-treated Dentin) or with Previously Biomodified with 5% Grape Seed Extract (GSE), 5% Glutaraldehyde (GD), or Gluma Desensitizer[a]

Etched Dentin Treatment	Dentin Wetness	Aging Period[b]	
		24 Hours	12 Months
Water (control)	Wet	43.4 ± 6.4 a	27.3 ± 2.5 Ba*
	Dry	16.6 ± 3.9 b	6.2 ± 1.1 Ca*
5% GSE	Wet	35.5 ± 8.9 a	34.7 ± 5.2 A
	Dry	31.9 ± 3.8 a	30.9 ± 2.5 A
Gluma Desensitizer	Wet	38.5 ± 5.0 a	33.3 ± 7.2 A
	Dry	34.1 ± 5.5 a	33.8 ± 2.6 A
5% GD	Wet	39.7 ± 11.1 a	32.8 ± 5.3 A
	Dry	34.4 ± 10.8 a	37.0 ± 8.5 A

Abbreviations: GD, glutaraldehyde; GSE, grape seed extract.

[a]*Bond strength was evaluated 24 hours and 12 months after storage of bonded specimens in saliva-like buffer.*

[b]*Numbers are mean ± standard deviation, n = 8. Within each aging period, means followed by different letters are statistically different (Tukey, p<0.05). Means followed by an asterisk (*) in the 12-month aging period column indicate statistically significant difference compared to the 24-hour period of aging.*

Figure 1. Adhesive fractures were predominant in all groups, regardless of the treatment of the etched dentin, dentin wetness, or period of aging. In the water-treated etched dentin (Figure 2a), there was an increase in the adhesive-type failure in interfaces produced on dry dentin (90.6%) compared to the dentin (73.3%) at 24 hours. After 12 months of aging, almost all the interfaces failed adhesively (94% for wet dentin and 97% for dry dentin) (Figure 2a). This pattern was not repeated in the groups in which the etched dentin was cross-linked with 5% GSE (Figure 2b), 5% GD (Figure 2c), or Gluma Desensitizer (Figure 2d). When the interfaces of biomodified wet or dry dentin were examined, there was little influence on the distribution of fractures.

Nanoleakage

Silver uptake was observed in all the bonded adhesive interfaces, even after only 24 hours of storage. In that period of aging (Figure 2), drying the etched dentin with air before applying the adhesive resulted in a considerable increase in silver uptake, in comparison with the wet control (Figure 2). In addition, cross-linking of the etched dentin had no influence on the nanoleakage at the interfaces produced on wet dentin. Bonding adhesives to dry dentin (Figure 2) reduced silver nanoleakage of bonded interfaces (Figure 2). In these interfaces, the infiltration of silver ions was similar to that observed at the interfaces of the wet dentin specimens. An increase in the accumulation of silver was observed after 12 months of aging of the interfaces without biomodification of the etched dentin, especially at the interfaces produced on the dry dentin (Figure 3).

In Situ Zymography

The presence of interfacial fluorescence indicative of gelatinolytic activity was identified along the entire length of the interface of the nonbiomodified wet and dry dentin groups, both at the 24-hour (Figure 4) and 12-month (Figure 5) aging periods. The intensity and the linear area of fluorescence did not increase over

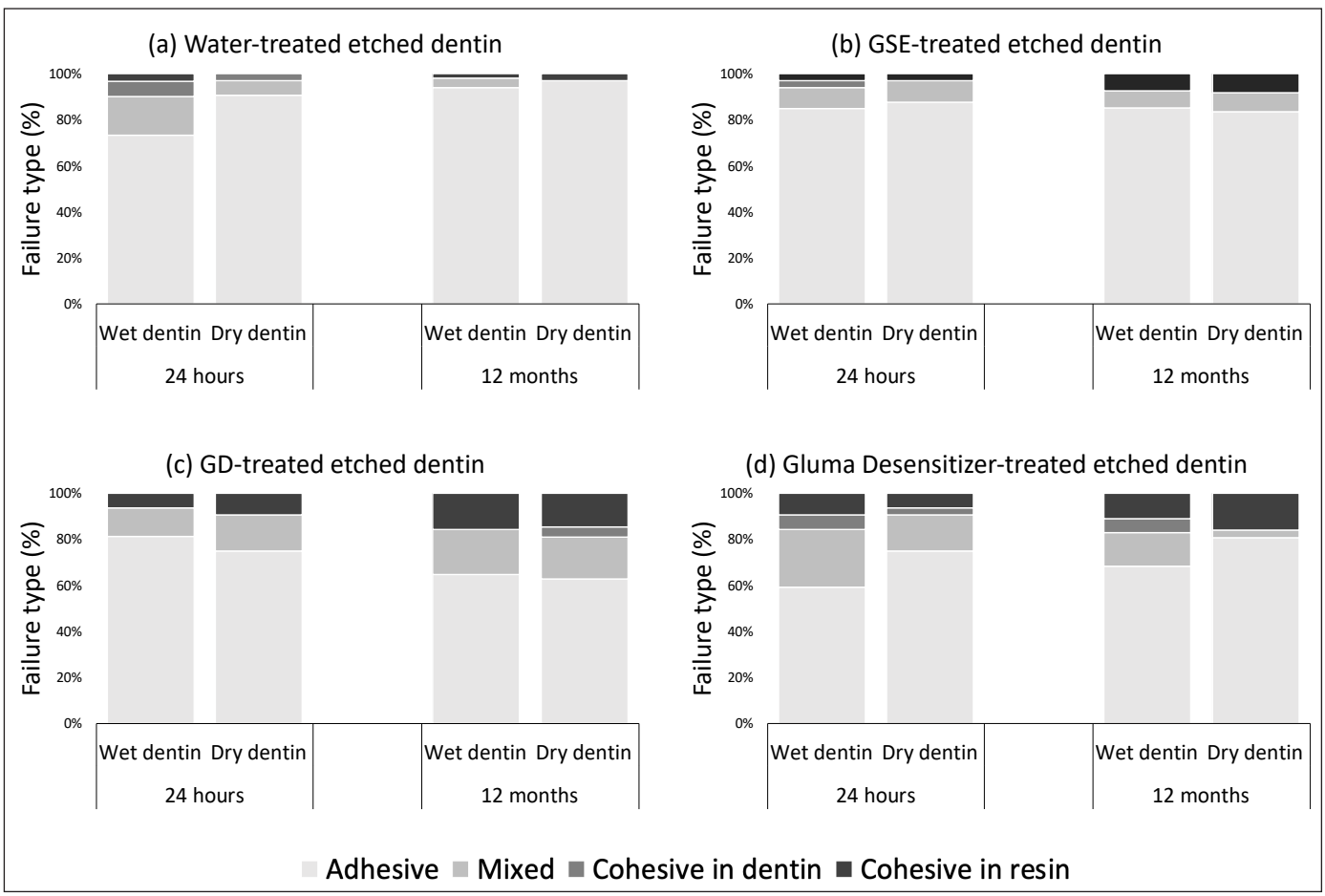

Figure 1. *Distribution of types of failures of mechanically stressed bonded specimens stored in saliva-like buffer for 24 hours or 12 months. The adhesive system was applied to wet or air-dried etched dentin (a) with no biomodification (water-treated dentin) or previously biomodified with (b) 5% grape seed extract (GSE), (c) 5% glutaraldehyde (GD), or (d) Gluma Desensitizer.*

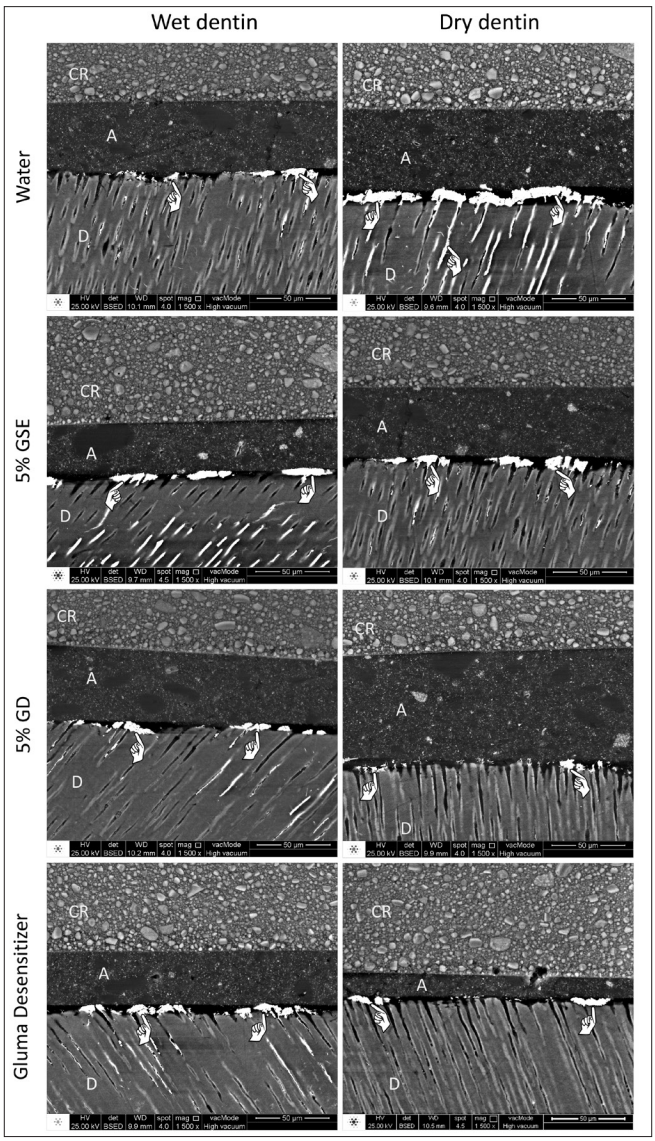

Figure 2. *SEM (scanning electron microscopy) images of silver nanoleakage of resin–dentin bonds stored for 24 hours in saliva-like buffer. The two-step, etch-and-rinse adhesive was applied to wet or air-dried etched dentin previously biomodified with 5% grape seed extract (GSE), Gluma Desensitizer, or 5% glutaraldehyde (GD). A, adhesive layer; CR, composite resin; D, dentin; Pointers: silver nitrate accumulation areas. 500x.*

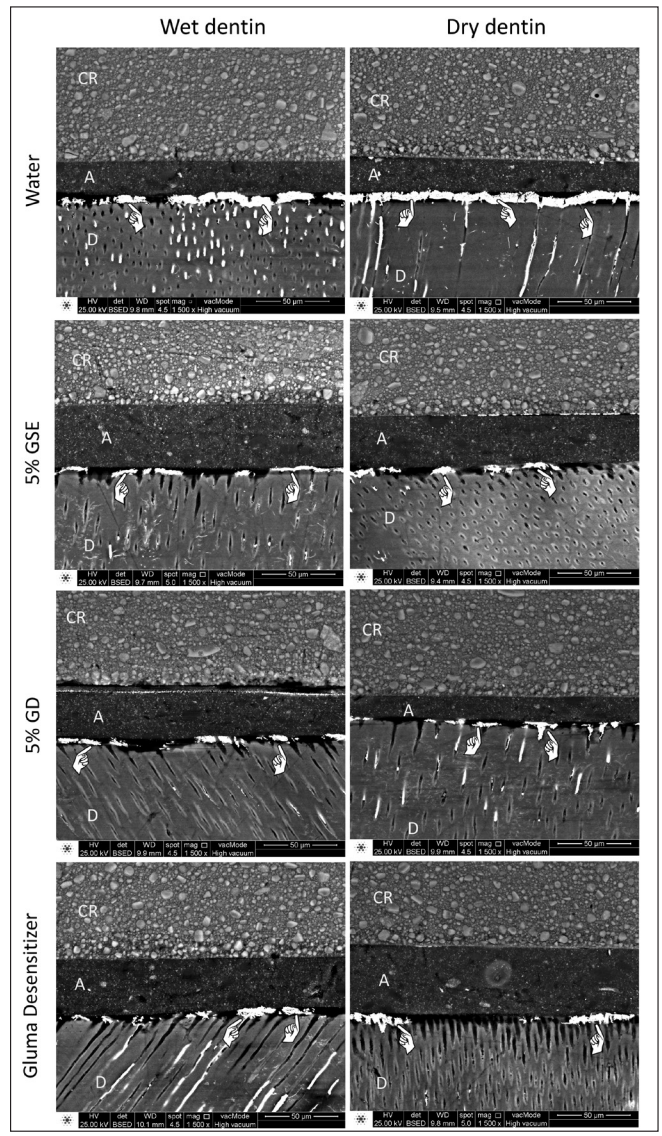

Figure 3. *SEM (scanning electron microscopy) images of silver nanoleakage of resin–dentin bonds stored for 12 months in saliva-like buffer. The two-step etch-and-rinse adhesive was applied to wet or air-dried etched dentin previously biomodified with 5% grape seed extract (GSE), Gluma Desensitizer, or 5% glutaraldehyde (GD). A, adhesive layer; CR, composite resin; D, dentin; Pointers: silver nitrate accumulation areas. 500x.*

time. Conversely, no or little fluorescence signal was detected in isolated areas of the interfaces produced on cross-linked dentin. Interfaces produced after the application of Gluma Desensitizer showed more fluorescence in both aging periods than interfaces produced after the use of GSE and GD.

DISCUSSION

The water-wet bonding technique[40] is still recommended when two- or three-step etch-and-rinse adhesive systems are used. Despite the negative impact of the presence of residual water on nanoleakage[41] and polymerization of adhesive monomers,[19,20] water is important for the maintenance of interfibrillar collagen peptide spaces produced by the removal of hydroxylapatite (HA) nanocrystals by etching with phosphoric acid.[18] The attempt to remove water simply by evaporation with air blasts resulted in a significant reduction of bond strength.[21-23] The same negative interference of drying etched dentin on the immediate bond strength was

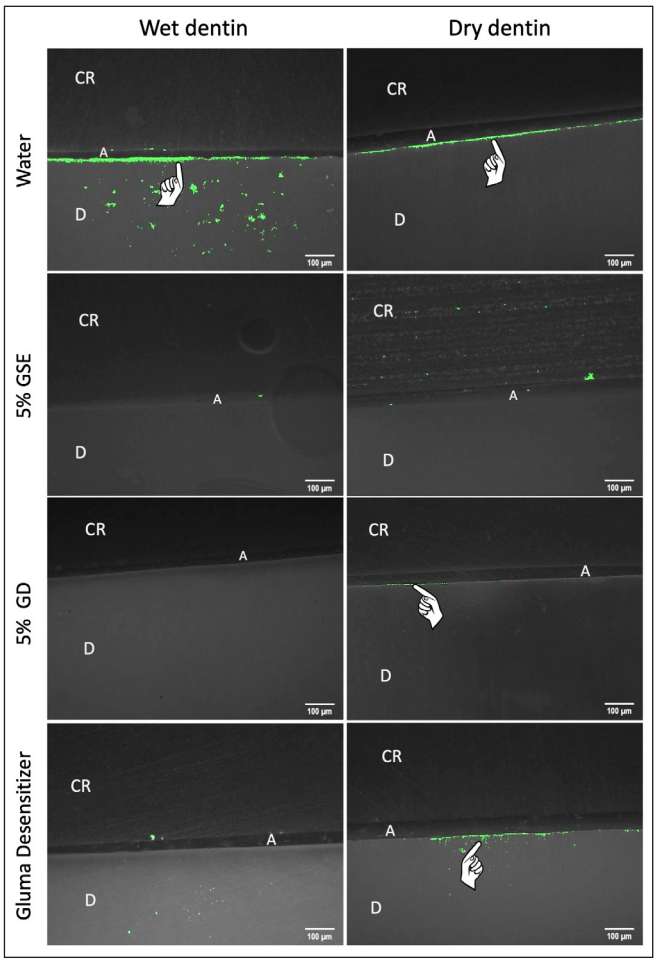

Figure 4. *Fluorescence microscopy images of interfacial gelatinase activity of 24 hour-aged resin–dentin bonds. The two-step etch-and-rinse adhesive was applied to wet or air-dried etched dentin previously biomodified with 5% grape seed extract (GSE), Gluma Desensitizer or 5% glutaraldehyde (GD). Bright green fluorescence indicates collagenolytic activity. All images were captured using the same intensity, 10x. A, adhesive layer; CR, composite resin; D, dentin.*

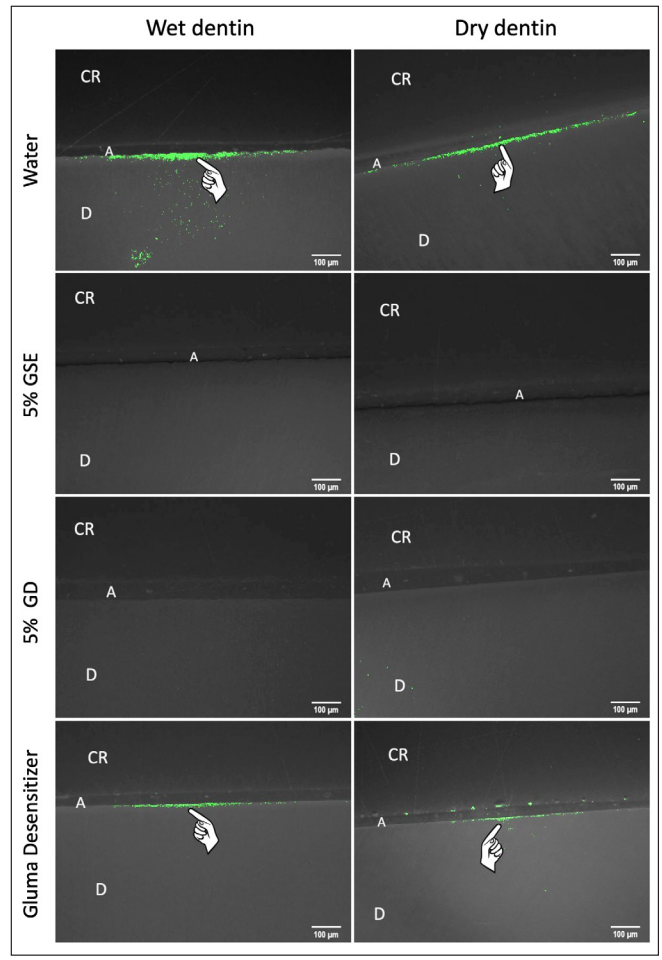

Figure 5. *Fluorescence microscopy images of interfacial gelatinase activity of 12 month-aged resin–dentin bonds. The two-step etch-and-rinse adhesive was applied to wet or air-dried etched dentin previously biomodified with 5% grape seed extract (GSE), Gluma Desensitizer, or 5% glutaraldehyde (GD). Bright green fluorescence indicates collagenolytic activity. All images were captured using the same intensity. 10x, A, adhesive layer; CR, composite resin; D, dentin.*

observed in the present study, with a reduction of 62% in the mean value in comparison to wet dentin. Additionally, interfaces produced on dry dentin showed higher nanoleakage uptake, demonstrating that low bond strength occurred when bonding resin was unable to envelop interfacial collagen.

After washing off the phosphoric acid used for demineralizing the dentin surface, the collagen fibrils depleted of their mineral reinforcement are maintained separated by the presence of water. In this state, the composition of dentin consists of approximately 30% collagen and 70% water, and the elastic modulus of the etched dentin matrix is only 3-5 MPa.[18,26] Simply drying dentin surfaces with air results in collapse of the fibrils,[23] which obliterates the interfibrillar spaces. The close spatial approximation of the collagen fibrils allows the spontaneous formation of hydrogen bonds between the peptides of neighboring fibrils[42] resulting in the shrinkage of the dentin matrix.[18] In addition to obliterating the interfibrillar spaces necessary for the infiltration of resin monomers, the stiffness of the dentinal matrix (Young's modulus) increases with drying (20-30 fold)[43] and may reach 170 MPa.[44-46] Hydrogen bonds between dry collagen fibrils have a cohesive energy of 14.8 $(J/cm^3)^{1/2}$. To rupture such bonds, solvents must have a higher solubility parameter for hydrogen bonds (d_h). Water, for example, has a d_h=40.4 (Hoy's solubility parameter); this fact explains why water is capable of re-expanding dehydrated collagen matrices by 100%.[18] Other solvents present in

the formulations of adhesive systems, such as acetone [11$(J/cm^3)^{1/2}$] and ethanol [20 $(J/cm^3)^{1/2}$], have d_h that are lower or very close to 14.8[18,47]—the strength of hydrogen bonds between adjacent collapsed collagen peptides in air-dried etched dentin.

The adhesive Optibond S used in this study contains ethanol as its solvent. A water-free adhesive was selected to prevent this solvent from interfering with the wetness of the dried dentin. Bond strengths of One-Step (Bisco Inc, Schaumburg, IL, USA), which is an acetone-based adhesive, were negatively affected when applied on air-dried, etched dentin, while a tertiary, butanol-based organic solvent adhesive XP Bond (Dentsply Sirona, Milford, DE, USA) performed equally well in both the wet- and dry-bonding techniques.[48] However, even the application of an adhesive containing ethanol and water as solvents was not capable of countering the negative effects of drying the etched dentin with air.[23] Ethanol has a solubility parameter d_h [20 $(J/cm^3)^{1/2}$] close to the value necessary for rupturing the hydrogen bonds established between dry collagen fibrils [14.8 $(J/cm^3)^{1/2}$].[18,47] Therefore, the capacity of ethanol to re-expand collapsed dentin matrices is limited. The dehydrated acid-etched matrix and its subsequent saturation with ethanol has a thickness of only 30% of that observed for the matrix maintained saturated with water.[47] Other components of the adhesive such as bisphenol A-glycidyl methacrylate (Bis-GMA) (d_h=5.8) and hydroxyethyl methacrylate (HEMA) (d_h=15.2) are incapable of re-expanding the collapsed matrix.[18] In addition to reducing the immediate bond strength value by 62% in comparison with the bond to wet dentin, interfaces produced by the application of adhesive on dentin dried with air were unstable in the long term. After 12 months of aging, the mean bond strength of these interfaces was 37% lower than the immediate bond strength value. The intense uptake of silver observed at these interfaces indicated solubilization of collagen peptides in the hybrid layer in this period and their replacement by water.

The increase in the number of intramolecular, intermolecular, and interfibrillar cross-links promoted by the application of cross-linkers improved the mechanical properties of collagen and, consequently, of the tissues with a high content of this protein.[25,26,28,29] The negative effects promoted by excessive drying of the etched dentin were prevented by the cross-linking of collagen with an extract rich in proanthocyanidins (GSE) prior to the application of the adhesive system.[23] In the same way, all the cross-linkers investigated in the present study were capable of making the collagen more resistant to the point where drying of the biomodified matrix did not have any negative effects on the immediate bond strength. Also, the amount of silver uptake detected became similar to those at the interfaces established on wet dentin, as seen in the SEM images. The bond strength values to dry dentin, when dentin was previously biomodified with GSE, Gluma Desensitizer, or GD, were comparable with the values obtained for dentin that was kept wet. For this condition of hydration, none of the cross-linkers affected the immediate bond strength; that is, the values of all the groups were comparable with those of the wet control without biomodification and similar among them.

After 12 months of aging in a saliva-like buffer, a significant drop in bond strength was seen for interfaces produced on nonbiomodified etched dentin for both wet- and dry-bonding techniques. The overall reduction in bond strength was 37%. One would expect a higher reduction in bond strength for interfaces created on dry dentin, with no previous biomodification of the collagen. Since a similar reduction was seen, it could be assumed that the etched dentin had a lower water content that favored the infiltration of more hydrophobic monomers into the interfibrillar spaces. This may have minimized phase separations[18] and improved monomeric conversion, making the resinous content of the hybrid layer more resistant to degradation. Conversely, bonds produced on wet and dry biomodified dentin remained mechanically stable after 12 months of aging.

Hybrid layers can deteriorate when the resin component is solubilized by esterases,[49] and collagen is solubilized by endogenous MMPs and cysteine cathepsins.[50] These enzymes are hydrolases that use water to break chemical bonds.[16,51,52] Thus, air drying the etched dentin could impair the activity of such enzymes by limiting their access to water, thereby reducing the hydrolytic activity within the hybrid layer. However, the intense collagenolytic activity detected in the interfaces created on dry-etched dentin with no previous biomodification refutes that hypothesis. Most water molecules in the acid-etched dentin are found in a free form (75%-79%), while 21%-25% is bound to collagen and unlikely to be removed.[10] Therefore, even after 60 seconds of air drying, the residual water in the etched dentin was enough to enable the activity of the dentin proteases.

The biomodification of dentin collagen by the use of cross-linkers reduces its enzymatic breakdown. MMPs are unable to cleave the tightly wound triple helix of collagen, because the opening of their catalytic site is smaller (~0.5 nm) than the diameter of the collagen molecule (~1.5 nm).[16,53] To degrade the collagen molecule, MMPs need to unwind or separate each collagen polypeptide chain and cleave each peptide individually. The increased number of secondary

cross-links produced by cross-linkers makes it difficult to separate the chains. Additionally, despite increasing collagen stiffness, cross-linkers also increase the number of secondary cross-links within MMPs and cysteine cathepsins, impairing the exposure and docking of the catalytic site to the substrate receptor.[16] The new cross-links seem to be stable longitudinally, even when the hybrid layer is exposed to exogenous proteases from *Clostridium histolyticum*.[30] Proanthocyanidins are more efficient in inhibiting matrix metalloproteinases (MMP-2, MMP-8, and MMP-9) and cysteine cathepsins (CAT B and CAT K) than is 2% chlorhexidine.[31] The same superiority was seen for 5% glutaraldehyde against MMPs.[32]

Gluma Desensitizer (Kulzer GmbH, Hanau, Germany) is a dentin desensitizer that has been on the market for over 20 years. It is an aqueous solution of 5 wt% glutaraldehyde and 35% HEMA.[54] Its mechanism to treat dentin hypersensitivity is the coagulation of soluble plasma proteins in exposed dentinal tubules. The coagulated proteins in dentinal tubules reduce hydrodynamic fluid movement.[55] The MMPs' inhibitory capacity of Gluma Desensitizer is equivalent to that of 5% glutaraldehyde in water, showing that the presence of HEMA does not affect the antienzymatic effect of glutaraldehyde.[32,54] The use of a 5% glutaraldehyde solution has been shown previously to be effective in producing mechanically stable hybrid layers.[28] In the present study, the application of Gluma Desensitizer on etched dentin allowed air drying with no detrimental effects on the immediate bond strength. Additionally, bonds produced on Gluma Desensitizer-modified dentin were mechanically stable after 12 months of aging, and showed minimal nanoleakage and proteolytic activity.

In situ zymography using fluorescence or *laser* scanning *confocal* microscopy is a well-known technique used to identify gelatinolytic activity in biological tissues. This technique has been used to demonstrate the proteolytic activity in both mineralized and demineralized dentin,[56] and in resin–dentin interfaces.[57-59] Following the demineralization of dentin, intense proteolytic activity was detected in the hybrid layer produced on the nonbiomodified dentin. It has been shown that after the intense initial activity of dentin proteases, there is a progressive reduction in the release of ICTP (carboxyterminal telopeptide of type I collagen) and CTX (C-terminal telopeptides of type I collagen)—biomarkers of type I collagen degradation by MMPs and cysteine cathepsins, respectively,[60] suggesting that these proteases are able to degrade only the substrate within the reach of their molecular mobility. The detection of fluorescence within the hybrid layers after 12 months of aging seems to challenge that statement. Conversely, no or minimal proteolytic activity was seen in aged interfaces produced after the biomodification of the dentin collagen, especially with GSE and GD. The long-term effect of dentin collagen biomodification by proanthocyanidins was also demonstrated by the sustained increased mechanical stability and resistance to degradation by endogenous proteases and bacterial collagenase after 18 months.[30]

CONCLUSIONS

Bonded resin–dentin interfaces produced using the cross-linked, dry bonding technique were as mechanically stable as the interfaces produced using cross-linkers with the current recommended water-wet bonding technique. Nanoleakage was also reduced in biomodified dentin bonds for up to 1 year of aging. As proteolytic activity in specimens treated with cross-linkers in both adhesive techniques was also similar, therefore, all the null hypotheses were accepted. The cross-linking of the etched dentin not only improved the stability of hybrid layers against degradation but also eliminated the sensitivity of the adhesion techniques as to the degree of dentin wetness, even if a water-free adhesive system is used. However, the number of steps and the time necessary for the cross-linked dry bonding technique is much greater, especially if compared with the application of a universal adhesive, even if performing the selective acid etching of enamel with phosphoric acid. The effectiveness of the cross-linked dry bonding technique still needs to be investigated in well-conducted randomized clinical trials. The use of such a multi-step technique should be considered only if the clinical performance over time is proven to be superior to the conventional wet-bonding approach when using etch-and-rinse adhesive systems.

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Regulatory Statement

The author represents that the study was performed in compliance with author's institution's appropriate policies related to the use of animal and/or human subjects and human-derived material.

Conflict of Interest

The authors of the present study certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in the present article.

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