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The pursuit of resin-dentin bond durability: Simultaneous enhancement of collagen structure and polymer network formation in hybrid layers

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ABSTRACT

Objective. Imperfect polymer formation as well as collagen's susceptibility to enzymatic degradation increase the vulnerability of hybrid layers over time. This study investigated the effect of new dimethyl sulfoxide (DMSO)-containing pretreatments on long-term bond strength, hybrid layer quality, monomer conversion and collagen structure.

Methods. H_3PO_4-etched mid-coronal dentin surfaces from extracted human molars (n = 8) were randomly treated with aqueous and ethanolic DMSO solutions or following the ethanol-wet bonding technique. Dentin bonding was performed with a three-step etch-and-rinse adhesive. Resin-dentin beams (0.8 mm^2) were stored in artificial saliva at 37 °C for 24 h and 2.5 years, submitted to microtensile bond strength testing at 0.5 mm/min and semi-quantitative SEM nanoleakage analysis (n = 8). Micro-Raman spectroscopy was used to determine the degree of conversion at different depths in the hybrid layer (n = 6). Changes in the apparent modulus of elasticity of demineralized collagen beams measuring 0.5 × 1.7 × 7 mm (n = 10) and loss of dry mass (n = 10) after 30 days were calculated *via* three-point bending and precision weighing, respectively.

Results. DMSO-containing pretreatments produced higher bond strengths, which did not change significantly over time presenting lower incidence of water-filled zones. Higher uniformity in monomer conversion across the hybrid layer occurred for all pretreatments. DMSO-induced collagen stiffening was reversible in water, but with lower peptide solubilization.

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Significance. Improved polymer formation and higher stability of the collagen-structure can be attributed to DMSO's unique ability to simultaneously modify both biological and resin components within the hybrid layer. Pretreatments composed of DMSO/ethanol may be a viable-effective alternative to extend the longevity of resin-dentin bonds.

1. Introduction

Despite all significant advances in dental adhesion during the past decades, resin-dentin bonding remains a challenge [1–3]. Dentin is a biologically active histologic tissue characterized by an intrinsically hydrated and complex structure containing several endogenous proteases. Bonding to this highly mineralized dynamic structure constitutes a unique form of tissue engineering in which demineralized collagen is used as the scaffold for resin infiltration to couple restorative composites to the underlying mineralized dentin. Unfortunately, hybrid layers formed during collagen hybridization with methacrylate resin monomers are often referred to as the weakest link in resin-dentin interfaces [4,5]. Poorly infiltrated demineralized collagen between hybrid layers and mineralized dentin is inherent to the traditional etch-and-rinse mechanism [5,6]. It is present in even less aggressive self-etch dentin bonding approaches, albeit to a lesser extent [6,7]. Irrefutably, the presence of such vulnerable collagen network further challenges dentin-bond durability [2,3]. No resin-dentin bonding protocol created hitherto is able to produce perfectly hybridized dentin interfaces within clinically reasonable time [1,2,8]. Furthermore, activation of endogenous enzymes during bonding contributes to rapid collagen hydrolysis over time [3] damaging the link between resin components and dentin substrate. Since collagen fibrils serve as reinforcement against cyclic crack growth within the resin-dentin interfaces, collagen degradation invariably creates detrimental weak links [4]. In this context, maintenance of collagen integrity over time is crucial to the anchorage and durability on resin-dentin interfaces [1–3,9].

The quality of the polymer-network within the hybrid layer can also be problematic [10,11]. Even though exceedingly high degree of conversion values in the hybrid layer have been commonly reported for different adhesive systems [12], some well above 80% [13,14], the reality may be unfulfilling [11,15]. Herein, preservation of the demineralized collagen network over time protected by a well-formed polymer-network is of utter importance for the durability of hybrid layers [1–3].

Considering such drawbacks, new resin-based adhesives and countless bonding techniques have been proposed to overcome such limitations, but only few protocols have been truly considered for clinical use. The ethanol wet-bonding technique was proposed in 2007 [16] following etch-rinse bonding and it is considered one of the most effective strategies [1,17] to improve hybrid layer formation *in vitro*, especially when combined with hydrophobic-bonding monomers [18–20]. However, it is technique sensitive and clinically impractical due to the time needed for successive ethanol applications for adequate dentin dehydration [21,22] and detrimental effects on composite durability *in vivo* have been reported [23]. Reduction of the water-contamination effect as well as technique simplification could aid the implementation of such bonding approach to truly benefit resin-dentin bonding [1,17,24].

Recently, dimethyl sulfoxide (DMSO) has emerged as a new potential solvent in adhesive dentistry [25] displaying innumerous properties that may benefit resin-dentin bonding [6,25–28]. DMSO [$(CH_3)_2SO$] is a polar aprotic colorless solvent classified as an organosulfur complex, which is capable of dissolving both polar and nonpolar compounds [29]. It is miscible in several organic solvents, in resin monomers commonly used in dentistry [30] and in water [31]. DMSO can: (*i*) hydrogen-bond to proteins; (*ii*) increase collagen fibril interspacing [32]; (*iii*) improve dentin wettability [33]; (*iv*) facilitate monomer penetration within demineralized dentin [6]; (*v*) lower the activity of endogenous hydrolytic enzymes [25,28] and (*vi*) reduce the technique sensitivity in etch-and-rinse dentin bonding, even under drastic air-drying conditions [28]. DMSO also facilitates radical polymerization in solvent-monomer mixtures [34]. However, the mechanisms involving hybrid layer improvement by DMSO are not yet fully understood. For instance, monomer polymerization *in loco* across the hybrid layer and its impact on the mechanical properties of collagen remain unknown. Therefore, the aim of this study was to examine the central hypothesis that both resin and collagen components of the hybrid layer may be simultaneously optimized by binary solutions of DMSO, containing water or ethanol, at different concentrations. The tested null hypotheses were that the etch-and-rinse bonding approach associated to DMSO-pretreatments: (i) would have no effect on the long-term resin-dentin bond strength; (ii) the mechanical properties/collagen integrity and (iii) the polymer network formed within the hybrid layer would not be affected.

2. Materials and methods

Extracted sound human third molars were obtained with informed consent from patients (18–32 years) under a protocol approved by the University of Oulu, Finland (#23-2003) in accordance with local regulations. The primary indications for tooth extractions were not related to the present study. After the extractions, teeth were stored at 4 °C in 0.9% NaCl containing 0.02% NaN_3 to prevent microbial growth and were used within 1 month.

2.1. Experimental design and bonding procedures

Specimen preparation followed the Academy of Dental Materials guidance of *in vitro* testing for non-trimmed microtensile bond strength testing [35]. Teeth were coronally sectioned under water cooling to expose flat mid-coronal dentin surfaces using a slow speed diamond saw (Isomet, Buehler Ltd, Lake Bluff, IL, USA). Absence of remaining enamel on the dentin surfaces was verified with a stereomicroscope (Leica M60, Leica Microsystems, Wetzlar, Germany) at 40× magnifications. Roots were removed 1 mm below the cervical line and discarded. Exposed dentin surfaces were standardized by wet-polishing with 320-grit SiC paper for 60 s. Crown segments (n = 8/group) were randomly allocated into 4 groups following a study design with two factors: (i) "dentin treatment" in four levels consisting of no treatment, ethanol wet-bonding and use of 50 vol% DMSO (Dimethyl Sulfoxide, Sigma-Aldrich, St Louis, MO, USA) dissolved in either water (DMSO/H_2O) or ethanol (Sigma-Aldrich) (DMSO/EtOH); and "storage time" in two levels 24 h and 2.5 years in artificial saliva. Dentin surfaces were initially etched for 15 s with H_3PO_4 32 wt% (Scotchbond Universal Etchant, 3M ESPE, St. Paul, MN, USA) and rinsed for 15 s. The control group was conventionally wet bonded following manufacturer's instructions. After rinsing, dentin surfaces were blot dried with lint-free absorbent paper until water uptake by capillarity was no longer detected. Dentin surfaces remained partially moist but not overwet. In experimental groups, dentin was treated with DMSO-containing solutions or followed the ethanol-wet bonding technique. For the ethanol-wet bonding [17,18], etched dentin surfaces were dehydrated with a series of increasing ethanol concentrations (50%, 70%, 80%, 95% and 3 × 100%, 30 s each; *i.e.* 3 min 30 s in total). During this procedure, dentin surfaces were kept fully immersed in the ethanol solutions to avoid collapse of demineralized collagen. The 50% DMSO (v/v) solutions were prepared by mixing equal volumes of DMSO in distilled water or ethanol. 50 μL of DMSO/H_2O or DMSO/EtOH solutions [6,27,28] were applied on the etched-dentin surfaces, left undisturbed for 60 s and blot dried until paper filters presented no moisture absorption from the bonding surface by capillarity. Dentin surfaces presented no visible superficial moisture before bonding. For dentin hybridization, one gold-standard three-step etch-and-rinse adhesive (Scotchbond Multi-Purpose: SBMP, 3 M ESPE) was used following manufacturer's instructions. SBMP's *Primer* and *Bond* were applied sequentially for 10 s and air dried for 10 s each. Both were actively applied with manual pressure of approximately 4.0 g ± 1.6 [36]. Adhesive procedures were carried out in a controlled environment with a temperature of 24 °C and a relative humidity of 45–55%. Composite blocks were built with a nanofilled composite resin (Filtek Z350 XT, 3M ESPE, Shade A2) in 3 increments of 1.5 mm. Each increment was light-cured for 20 s using a LED light-curing unit (Elipar Deepcure, 3 M ESPE) at 1200 mW/cm^2. All bonding procedures were carried out by a single operator. The restored crown segments were stored in distilled water for 24 h at 37 °C to allow water sorption and postoperative polymerization. Resin–dentin beams were produced with a cross sectional area of approximately 0.9 mm^2 by sectioning the restored crowns longitudinally in mesio-distal and buccal-lingual directions perpendicular to the bonded interface with a slow-speed diamond saw (Isomet, Buehler Ltd).

2.2. Resin-dentin beam storage

Resin-dentin beams were randomly selected for the microtensile test under two conditions: after 24 h of storage in distilled water at 37 °C and long-term aging for 2.5 years in artificial saliva (pH 7.4) at 37 °C containing 5 mM HEPES, 2.5 mM $CaCl_2{\cdot}H_2O$, 0.05 mM $ZnCl_2$, and 0.3 mM NaN_3 [37] and changed biweekly in accordance with protocols previously described. In order to obtain a research design balanced by tooth dependency [35], resin-dentin beams from the same tooth were submitted to both testing periods (*i.e.* 24 h and 2.5 years). The nanoleakage evaluation included beams aged for 2.5 years to focus on the effect of aging on hybrid layer integrity.

2.3. Microtensile bond strength (μTBS)

Resin-dentin bond strength evaluation followed the Academy of Dental Materials guidelines for non-trimmed *μTBS* testing [35]. A minimum of 8 beams per tooth were tested on each storage period. Specimens were tested by a blinded operator. Beams were individually attached to a custom made micro-tensile testing jig using a cyanoacrylate adhesive (Loctite 416, Henkel Corp., Dublin, Ireland) and tested under tensile forces (Bisco, Schaumburg, IL, USA) at a crosshead speed of 0.5 mm/min until failure. Pretesting failures were recorded and considered as 0 MPa for the statistical analyses. The cross-sectional area of each beam was measured with a digital caliper for *μTBS* calculation in MPa. Tooth was considered the statistical unit, the bond strength average of beams tested at each time period representing the *μTBS* for each tooth (n = 8). Both surfaces of fractured resin-dentin beams were analyzed by scanning electron microscopy (SEM) (Phenom ProX, Phenom-World, Eindhoven, Netherlands) to determine fracture patterns. The fracture modes were classified as: cohesive (failure exclusive within dentin or resin composite); adhesive failure (failure at resin/dentin interface); and mixed failure (failure at resin/dentin interface with cohesive failure of the neighboring substrates).

2.4. Nanoleakage evaluation

Three resin-dentin beams per tooth (n = 8) aged for 2.5 years were randomly selected to evaluate the integrity of hybrid layers through the measurement of silver nitrate uptake at the bonded interface. Nanoleakage evaluation was performed according to a protocol previously described by Tay et al., [38]. Briefly, resin-dentin beams were initially wet-polished with 2000-grit SiC paper and coated with two layers of nail varnish applied up to 1 mm of the bonded interfaces. After rehydration in distilled water for 1 h, beams were immersed for 24 h in 50% (w/v) ammoniacal silver nitrate (pH 9.5), and thoroughly rinsed in distilled water for 120 s. Samples were then immersed in photo-developing solution (Kodak Professional D-76 developer, Kodak Rochester, NY) for 8 h under a fluorescent light to reduce silver ions into metallic silver grains within the water-filled voids along the bonded interface. Beams were embedded in epoxy resin, wet-polished

with 600-, 1000-, and 2000-grit SiC paper (Carbimet, Buehler Ltd.,) and 1, 0.25 (MetaDi, Buehler Ltd) and 0.05 μm (MasterPrep, Buehler Ltd) polishing pastes. Resin-dentin beams were ultrasonically cleaned in distilled water after each polishing step, air dried for 2 h, mounted on stubs, dried in silica overnight and carbon-sputtered. Nanoleakage extension was semi-quantified using SEM imaging on backscattering mode at 10 kV (Phenom ProX, Phenom-World). A series of sequential micrographs (2000× magnification) were obtained from each beam to detect silver deposition within the adhesive interface. Total silver nitrate uptake was measured on the 2D images using open-source image software (ImageJ, National Institute of Health, Bethesda, MD, USA) by a single-blinded examiner. The overall extension of silver uptake (μm) along the bonded interface was calculated and converted into percentage values. Silver uptake patterns were evaluated at higher magnifications (4000−10000×).

2.5. Collagen apparent modulus of elasticity (E)

One disc measuring 0.51 (±0.06) mm from the mid-coronal dentin region of each tooth (n = 10/group) were sectioned perpendicularly to the its long axis using a slow speed diamond saw (Isomet, Buehler Ltd,) under water cooling, wet-polished with 600-grit SiC paper to remove superficial imperfections from the low speed diamond blade and to fine adjust their thickness. The discs were then sectioned mesial-distally to produce rectangular dentin beams measuring approximately 0.5 mm thickness × 1.7 mm width × 7 mm length [39,40]. A dimple was made on the corner of each beam on the occlusal surface allowing repeated measures to be performed on the same surface. Beams were then demineralized in 10 wt% phosphoric acid [37,39–41] for 7 h at 24 °C under constant stirring and thoroughly rinsed with distilled water for 10 min. Digital radiography was used to document the absence of residual minerals. Digital images were obtained on a stereo microscope (Leica M60, Leica Microsystems) and thickness and width dimension of the demineralized beams were precisely measured using ImageJ. Beams were placed on a 3-point flexure jig with 5 mm span length between supports fully immersed in distilled water and tested under flexure to 3% strain [39] with a displacement rate of 0.5 mm min^{-1} using a 5 N load cell (SMT1-5N, Interface, Scottsdale, AZ, USA) mounted on a universal testing machine (Autograph AGS-X, Shimadzu, Japan). After maximum displacement, the load was immediately returned to 0% stress without further holding to prevent creep of the demineralized collagen matrix. Specimens were tested by a blinded operator. Load-displacement curves were converted to stress-strain curves and the apparent modulus of elastic (*E*) in MPa was calculated using the following formula: $E = mL^3/4bh^3$ where m is the steepest slope of the linear portion of the load- displacement curve (N/mm), L is the span length (5 mm), b is the width of the test specimen and h is the beam thickness. The 3-point bending test was selected for evaluation of the apparent elastic modulus because it is nondestructive and allows repeated measurements to be performed. After the baseline measurements, dentin beams (n = 10/group) were distributed into 5 balanced groups, so that their mean values were not statistically different. To allow proper solvent diffusion throughout the entire sample extension [39,40], demineralized beams were then immersed for 2 h in the DMSO solutions used for the bonding protocols (*i.e.* 50% DMSO/H_2O and 50% DMSO/EtOH), in EtOH, following the sequential EtOH wet-bonding dehydration protocol used except that the total time in the final EtOH step was 2 h. Undiluted DMSO was also included as a treatment. Beams were retested fully immersed in their respective solutions. After 15 min of rehydration, beams were retested in distilled water. Following this immediate testing in their respective treatment solutions, samples were stored for 7 and 30 days in artificial saliva at 37 °C and retested. Demineralized dentin beams without any treatment served as a negative control group.

2.6. Loss of dry mass (L_{dm})

Fifty demineralized dentin beams were dehydrated in silica under vacuum during 72 h at 24 °C and then desiccated to a constant weight (variations lower than 0.1 mg over 6 h; approximately 48 h in total). The initial dry mass (M1) was followed gravimetrically to the nearest 0.01 mg using an analytical balance (XS 105, Mettler Toledo, Hightstown, NJ, USA). After the initial dry mass measurement, each dried-shrunken dentin beam was hydrated in water for 2 h before being immersed in the treatment solutions for 2 h: EtOH following the ethanol-wet bonding protocol, pure DMSO, DMSO/H_2O and DMSO/EtOH (n = 10). Untreated collagen beams served as control. Subsequently, treated beams were rehydrated in artificial saliva for 2 h and placed in separate polypropylene tubes containing 3 ml of artificial saliva. Samples were placed in a shaking-bath to facilitate artificial saliva diffusion within collagen fibrils. After 30 days of incubation at 37 °C, the beams were rinsed with water for 10 min and sonicated for 5 min in distilled water to remove media salts. Determination of the dry mass was repeated under the same conditions after dehydration in silica (*M2*). Specimens were tested by a blinded operator. Loss of dry mass (L_{dm}) was calculated according to the formula: $Ldm\,(\%) = \frac{M2-M1}{M1}$. Loss of dry mass over time provides an indirect measurement of collagen solubilization by endogenous enzymes.

2.7. Adhesive conversion in the hybrid layer (DC)

Additional resin-dentin beams were created for each group (n = 6) following the previously described bonding protocols with the exception that samples were kept in 100% humidity for 24 h at 37 °C before testing instead of water-immersion. The rationale for this change was to limit HEMA elution from the bonded interface to reduce the discrepancy between "apparent" and "real" adhesive conversion values [11]. In order to more precisely locate the hybrid layer, samples were embedded in epoxy-resin and wet-polished with 600, 1000 and 2000-grit SiC paper (Buehler Ltd., Lake Bluff, IL, USA), ultrasonically cleaned for 2 min after the last step. Since water is a weak Raman scatterer, samples were not dehydrated. Raman spectra of resin-dentin interfaces were collected using Raman microscope (Thermo DXR2xi, Thermo Fisher Scientific, Madison, USA) equipped with a 785 nm laser and 400 lines/mm grating resulting in approximately 5 cm^{-1} spectral resolution and 3300–50 cm^{-1} spectral range. Spectra were obtained with a 100× objective at the top half and bottom half of the

hybrid layer, in arbitrary areas of intertubular dentin. Care was taken to select areas between dentin tubules. Instrumental calibration was performed according to manufacturer's specifications before each experiment. Specimens were tested by a single blinded operator. The reactive peak at 1639 cm^{-1} is attributed to the methacrylate CC= in both HEMA and Bis-GMA monomers [42,43]. Upon polymerization, the area of this peak diminishes as C=C are converted into CC— to form the polymer chain. An unchanging reference peak at 1609 cm^{-1} is correlated with the aromatic C=C [42,43]. The ratio of double-bond content of monomer to polymer in the hybrid layer was calculated according to the following formula:

$$DC\,(\%) = 1 - \frac{R^{(Cured)}}{R^{(Uncured)}} \times 100$$

where "R" is the ratio of aliphatic and aromatic peak intensities at 1639 cm^{-1} and 1609 cm^{-1} in cured and uncured adhesives.

2.8. Statistical analyzes

Data regarding bond strength, nanoleakage extension along the hybrid layers, elastic modulus of collagen and loss of dry mass were analyzed separately. Since microtensile data was normally distributed (Kolmogorov–Smirnov test $p = 0.085$) and homoscedastic (Levene's test $p = 0.15$), bond strength values were analyzed by two-way ANOVA followed by Tukey Test. Nanoleakage data (Kolmogorov–Smirnov test $p = 0.075$ and Levene's test $p = 0.285$) was analyzed by one-way ANOVA and Tukey test. Elastic modulus data concerning the effect of "dentin treatments" and "storage time" (Shapiro–Wilk test $p = 0.0840$ and Brown-Forsythe $p = 0.089$) was analyzed by two-way repeated measures ANOVA and Tukey test. Since data regarding the loss of dry mass and the elastic modulus of collagen violated the normality and homoscedasticity assumptions for parametric analysis, Kruskal–Wallis followed by Dunn-Bonferroni comparison tests were employed. As the normality (Shapiro–Wilk; $p = 0.203$) and homoscedasticity (Brown–Forsythe; $p = 0.9$) assumptions of data were not violated, degree of conversion was evaluated by two-way ANOVA and Tukey test. A significance level (α) of 0.05 was used for all statistical tests. Analyses were carried out using IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, NY, USA) and SigmaPlot for Windows, version 14.0 (Systat Software, San Jose, CA, USA).

3. Results

3.1. Microtensile bond strength test

Two-way ANOVA showed that "dentin treatment" ($p < 0.0001$; = 0.717), "storage time" ($p = 0.039$; = 0.74) and their interaction ($p = 0.002$, = 0.226) significantly affected bond strengths. The mean cross-sectional area of tested resin-dentin beams ($0.86 \pm 0.1\ mm^2$) ranged from 0.79 to 0.92 mm^2 without significant differences between groups regarding specimen size ($p = 0.621$). Resin-dentin bond strength values for all groups and standard deviations are shown in Fig. 1**A**. EtOH wet-bonding had no effect on immediate dentin bonding, while DMSO/H_2O and DMSO/EtOH significantly increased bond strengths in the order of 30% and 40%, respectively. Untreated samples had a significant 34% reduction in bond strength after 2.5 years, while DMSO-treated groups, regardless of dentin treatment, presented no significant reductions after long-term aging. Fracture patterns (Fig. 1**B**) were predominantly mixed for all groups. Untreated samples presented a substantial increase in adhesive failures after long-term aging, while the experimental groups presented only a minor increase.

3.2. Nanoleakage

One-way ANOVA revealed that "dentin treatment" ($p < 0.0001$; = 0.854) had significant effects on nanoleakage extension along the hybrid layer after storage in artificial saliva for 2.5 years. Nanoleakage extension for all groups in % of the total analyzed bonded interface and standard deviations are shown in Fig. 2**A**. The control group (*i.e.* untreated samples) presented the highest levels of silver uptake, roughly a 2-fold increase when compared to treated groups. No significant differences were observed between samples submitted to EtOH or DMSO/EtOH wet-bonding, producing the lowest levels of silver infiltration. DMSO/H_2O wet-bonding produced significantly higher nanoleakage levels than EtOH or DMSO/EtOH wet-bonding; however values were still significantly lower than in the control group.

Dentin treatments also produced different nanoleakage patterns (Fig. 2B). Different patterns of silver uptake within the same group were invariably identified by SEM, albeit predominant patterns were evident according to the dentin treatment. Untreated control samples were characterized by heavy deposits of silver located within the hybrid layers into the overlying adhesive layers. Distinction between the hybrid and adhesive layers was often impossible due to heavy silver deposition. EtOH wet-bonding produced spotted silver deposits sparsely scattered within the hybrid layer, which was also the most predominant pattern in the DMSO/EtOH group. DMSO/H_2O produced light reticular silver deposits characterizing water-trees.

3.3. Collagen apparent elastic modulus (E)

The average elastic modulus of dentin collagen considering all samples at baseline (n = 50) was 6.41 MPa ± 1.45. Variations in elastic modulus as a result of the different dentin treatments followed by rehydration are shown in Fig. 3**A**. Kruskal–Wallis revealed that "dentin treatments" had a significant effect on the elastic modulus of collagen ($p < 0.001$). EtOH produced a significant 13.8-fold increase in elastic modulus followed by a 12.3-fold increase by DMSO/EtOH. DMSO/H_2O and DMSO produced significantly lower 3.8 and 3.1-fold increases, respectively. Rehydration significantly reduced elastic modulus to baseline values, regardless of dentin treatment.

Changes in elastic modulus over time after the 30-day period are shown in Fig. 3**B**. Repeated measures two-way ANOVA revealed that "dentin treatment" ($p = 0.001$; = 0.1563) and "storage time" ($p < 0.001$; = 0.2149) significantly affected the elastic modulus of dentin collagen. Significant interactions between "dentin treatment" and "storage time" were identified ($p < 0.001$; = 0.1635). No significant differences in elastic modulus occurred between baseline and 7 days of

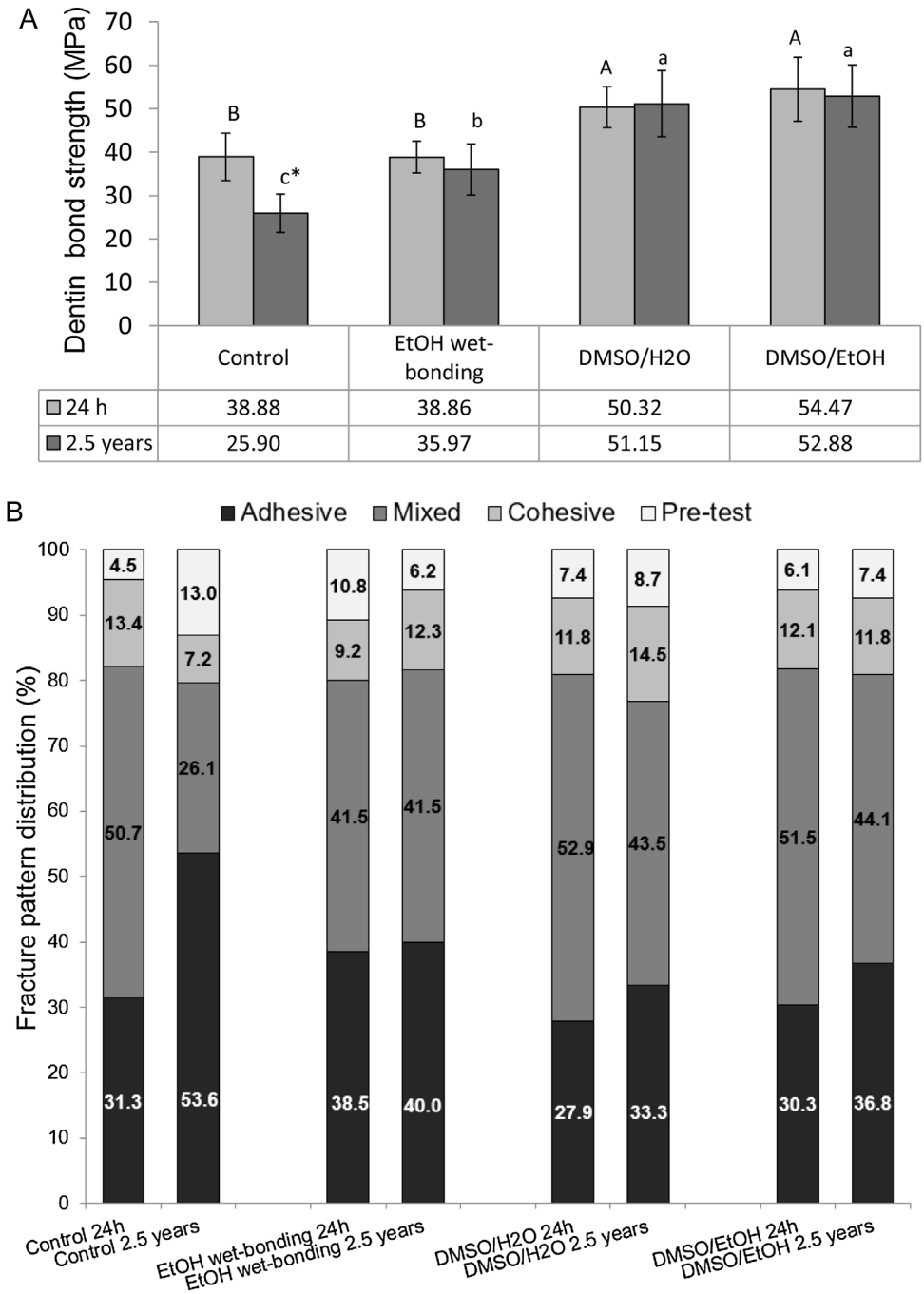


Fig. 1 – (A) Microtensile bond strength (MPa) means and standard deviations of resin-dentin interfaces (n = 8) bonded (Scotchbond Multipurpose, 3M ESPE) with aqueous and ethanolic DMSO-solutions or following ethanol-wet bonding as dentin pretreatments at 24 h or 2.5 years of ageing in artificial saliva at 37 °C. Different upper case letters indicate significant differences between groups at 24 h. Different lower case letters indicate significant differences between groups at 2.5 years. * indicates significant differences between ageing periods within treatments. (B) Fracture patterns (%) for all groups.

storage regardless of dentin treatment. At 30 days, untreated samples and samples treated with EtOH, following the EtOH wet-bonding technique, presented a significant reduction in elastic modulus of roughly −55%. DMSO produced a significant reduction in the order of −74% at 30 days. Differently, collagen samples treated with DMSO/H_2O and DMSO/EtOH presented no significant reductions in elastic modulus at 30 days.

3.4. *Loss of dry mass*

Kruskal–Wallis revealed that "dentin treatments" ($p < 0.0001$) had significant effects on loss of dry mass from demineralized collagen beams after 30 days of storage at 37 °C in artificial saliva. Loss of dry mass reductions in % and standard deviations are shown in Fig. 4. Untreated samples presented large

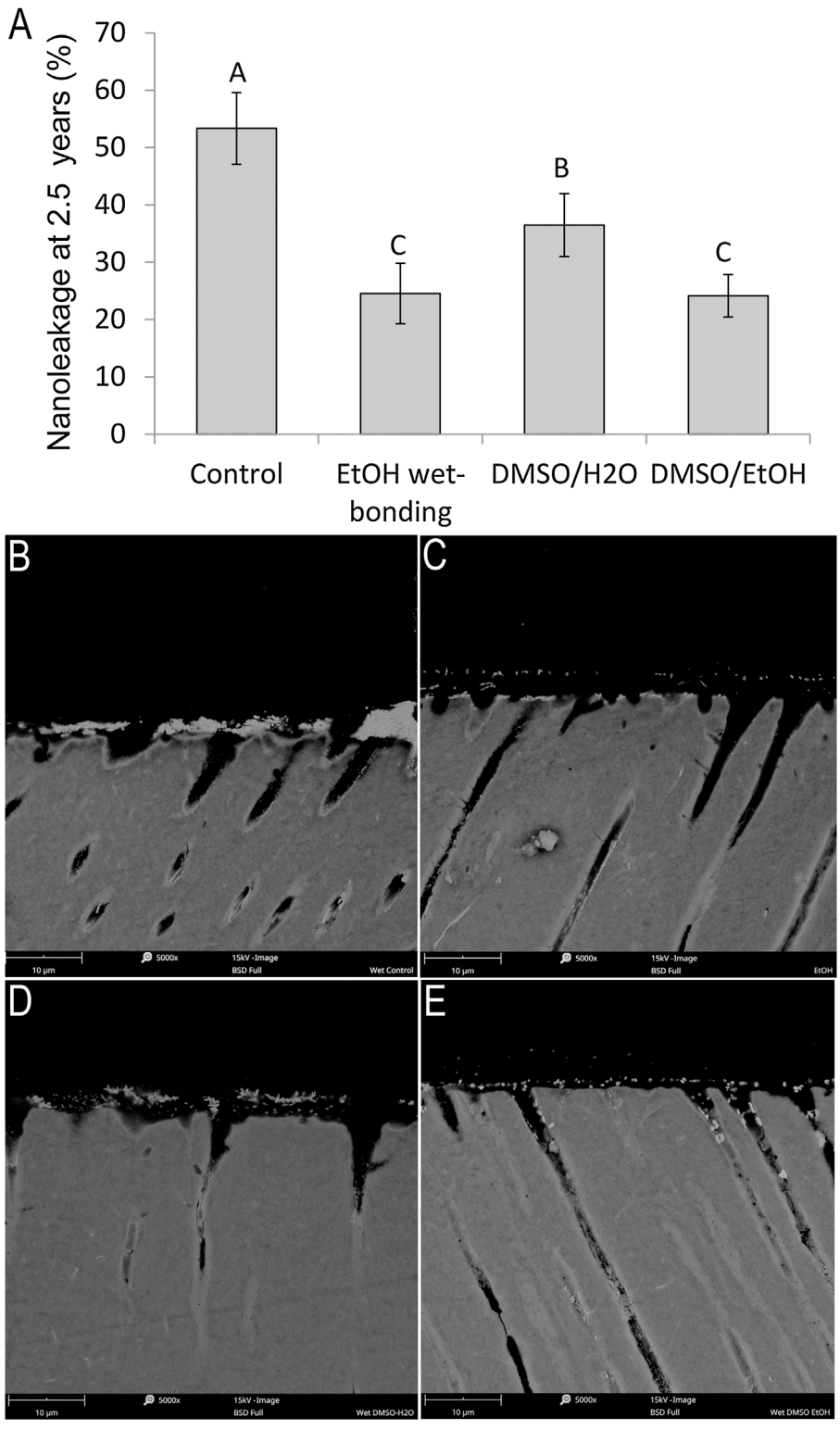
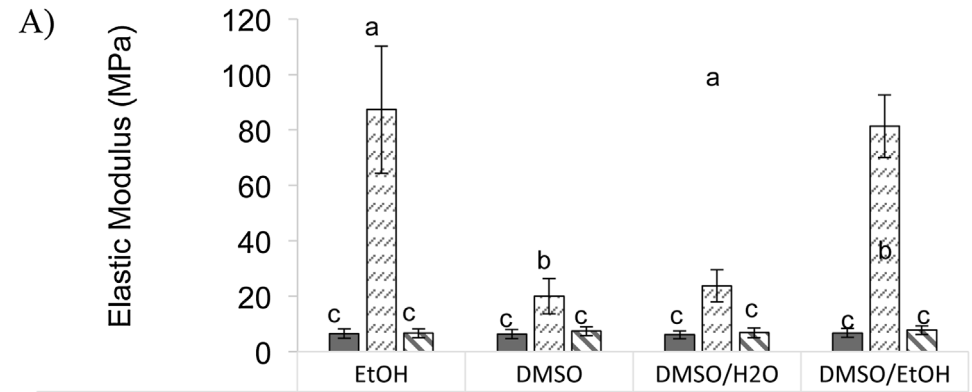
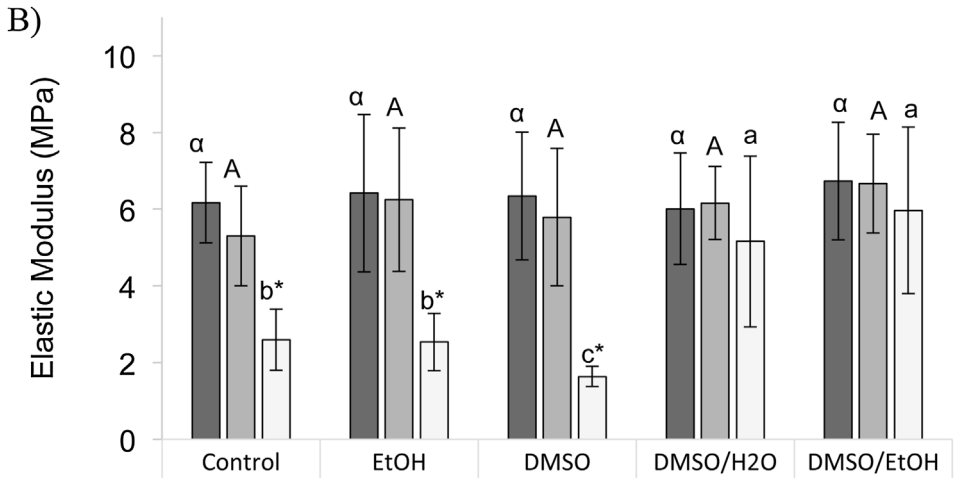


Fig. 2 – (A) Means and standard deviations of the overall nanoleakage extension (%) along resin-dentin interfaces (n = 8) bonded (Scotchbond Multipurpose, 3 M ESPE) with aqueous and ethanolic DMSO-solutions or following ethanol-wet bonding after 2.5 years of storage in artificial saliva at 37 °C. Different letters indicate significant differences between groups. Representative SEM micrographs of nanoleakage patterns for (B) untreated dentin; (C) following the ethanol wet-boding approach; (D) DMSO/H_2O; (E) and DMSO/EtOH.



	EtOH	DMSO	DMSO/H2O	DMSO/EtOH
Baseline	6.42	6.35	6.01	6.73
Treatment (2 h)	87.31	19.98	23.73	81.37
Rehydration (15 min)	6.59	7.33	6.77	7.73



	Control	EtOH	DMSO	DMSO/H2O	DMSO/EtOH
Baseline	6.17	6.42	6.35	6.01	6.73
7 days	5.30	6.25	5.79	6.16	6.67
30 days	2.60	2.54	1.64	5.16	5.97

Fig. 3 – Elastic modulus (MPa) means and standard deviations of demineralized dentin beams (n = 10) submitted to (A) different dentin treatments followed by rehydration for 15 min. Different letters inidicate significant differences between groups. (B) Elastic modulus means and standard deviations of previously treated and rehydrated beams after incubation in a calcium- and zinc-containing ageing medium for 7 and 30 days at 37 °C. Different Greek letters indicate significant differences between groups at baseline. Different upper case letters indicate significant differences between groups at 7 days. Different lower case letters indicate significant differences between groups at 30 days. No significant differences were detected between baseline and 7 days. * indicate significant differences between baseline and 30 days within treatments.

reductions in dry mass (−58.7%), which was not significantly different from EtOH (−51.7%). DMSO-containing treatments produced significantly lower reductions in dry mass when compared untreated and EtOH treated samples. Loss of dry mass of roughly −30% were observed in DMSO/EtOH and DMSO/H_2O; pure DMSO resulted in −37% reduction without significant differences among each other.

3.5. *Adhesive conversion in the hybrid layer (DC)*

Two-way ANOVA revealed that the interaction between "dentin treatments" and "hybrid layer depth" ($p < 0.001$; $\eta_p^2 =$ 0.246) significantly affected the degree of conversion in the hybrid layer. The degree of conversion of Scotchbond Multi-Purpose Bond polymerized on a Mylar strip (71.25% ±4.57) served as a reference value (n = 6). Degree of conversion means and standard deviations for all groups are shown in Fig. 5. No significant differences between EtOH, DMSO/H_2O or DMSO/EtOH were detected on the top of the hybrid layer: conversion values ranged from 33 to 44%. Nonetheless, monomer conversion was not uniform across the uppermost and lowest portions of the hybrid layer: significant reductions, were observed for the control group, roughly -75%, but to lower extensions for DMSO/H_2O, roughly -34%. EtOH, DMSO/H_2O

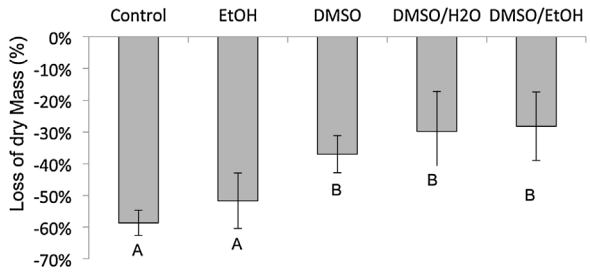


Fig. 4 – Loss of dry mass (%) means and standard deviations of completely demineralized dentin beams submitted to different dentin treatments after incubation in a calcium- and zinc-containing ageing medium for 30 days at 37 °C. The loss of dry mass from each beam (n = 10) was calculated as a percentage of the dry mass of that beam at baseline. Groups with different upper case letters were statistically significant.

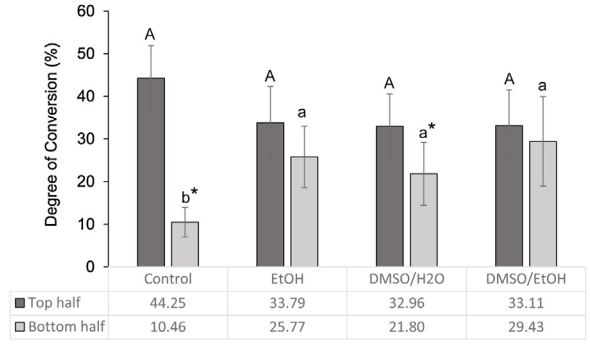


Fig. 5 – Degree of conversion (%) in the top and bottom halves of hybrid layers (n = 6) bonded (Scotchbond Multipurpose, 3 M ESPE) with aqueous and ethanolic DMSO-solutions or following ethanol-wet bonding as dentin pretreatments. Different upper case letters indicate significant differences between groups in the top half of the hybrid layer. Different lower case letters indicate significant differences between groups in bottom half of hybrid layer. * indicates significant differences between the top and bottom halves of hybrid layers within pretreatments.

and DMSO/EtOH produced a 2.5-fold increase in monomer conversion at the bottom of the hybrid layer when compared to the control group. Dentin treatments containing ethanol (EtOH-wet bonding and DMSO/EtOH) produced more uniform conversions with no significant differences in degree of conversion between the top and bottom portion of the hybrid layer.

4. Discussion

DMSO-containing pretreatments produced higher dentin bond strengths without significant changes over time. Hence, the first null hypothesis was rejected. The outstanding ability of DMSO to improve resin-dentin bonding has been shown previously [26–28,44]. Nonetheless, the longer aging period employed in this study suggests that the DMSO's protective effect on hybrid layers created by the etch-and-rinse approach may last longer than previously expected. DMSO is certainly one of the most versatile solvents to be used in adhesive dentistry on account of its multiple positive interactions with the constituents of the resin-dentin bonded interface including water [31], collagen [32,33], methacrylate monomers [29,34,44] and endogenous enzymes [28,45]. Since DMSO produces minor cytotoxic effects on the pulp tissue [46], it is reasonable to assume its safe use for dental applications regarding resin-dentin bonding. Although a few mechanisms have been proposed to explain the superior resin-dentin bonding performance caused by DMSO, such as improved dentin wettability [33], disruption of the water layers surrounding collagen [27], increased collagen interfibrillar spacing [32], higher monomer uptake [6] and lower nanoleakage in the immediate [26,27] and aged samples [25,27], the enhancement of hybrid layers produced by DMSO is not yet fully understood.

Clearly, the presented DMSO-bonding protocols differ conceptually from the classic ethanol wet-bonding. The latter approach relies on chemical dehydration by replacing residual water within the demineralized collagen network with ethanol, while DMSO-bonding additionally focuses on disrupting residual water conglomerates due to the strong DMSO-water interaction [31]. Increase in collagen interfibrillar spacing occurs in both scenarios either by shrinkage of collagen fibrils, as a result of water removal by ethanol [47] or by direct modifications in collagen structure produced by DMSO [32]. Furthermore, both approaches aim to improve dentin wetting [20,33], increase the infiltration of hydrophobic monomers [17,47] and reduce phase separation of hydrophobic-hydrophilic comonomers into the intrinsically wet dentin substrate [48], which play important roles in resin-dentin bonding [17,24]. Naturally, higher resin-dentin bond strengths have been reported as a consequence of ethanol-wet bonding [48,49], albeit increase in bond strengths is material dependent [20]. Unlike the DMSO-containing dentin pretreatments, ethanol-wet bonding was unable to significantly increase the immediate bond strength of the tested three-step etch-and-rinse commercial adhesive. Nonetheless, no significant changes occurred throughout the long-term aging as previously reported [18,24], showing that ethanol wet-bonding can indeed prevent long-term degradation of resin-dentin bonds *in vitro*. Unfortunately, when tested *in vivo*, ethanol-wet bonding is unable to replicate the immediate benefits observed *in vitro* [23]. However, ethanol wet-bonding can be compromised by water contamination: as little as 5% water can reduce bond strengths by 25% [50]. The same effect was not verified for pretreatments containing DMSO, which were not sensitive to water "contamination" under normal wet-bonding conditions. For instance, the ability of DMSO/H_2O to increase immediate bond strengths and maintain it over time was not affected by the water used as a cosolvent (*i.e.* 50% v/v) in the pretreatment solution. Similar results were obtained by the water-free DMSO/EtOH, pretreatment implying that DMSO-bonding protocols are not only less technique sensitive, but also easier to implement in comparison to ethanol-wet bonding due to markedly fewer steps.

Dentin etching with H_3PO_4 causes collagen fibrils to be literally suspended in water [17,24]. Depending on the test method and the strain used, the elastic modulus of dentin collagen can range between 0.2 MPa [51] to 6 MPa [39,40].

The latter agrees with our measurements using the three-point bending method at 3% strain, which resulted in 6.41 MPa ± 1.45. The soft and flexible nature of demineralized collagen makes it highly susceptible to spontaneous hydrogen bonding between adjacent collagen peptides once water is removed [17] or when solvents present in the bonding resin compete with water for interpeptide hydrogen bonding [52]. Dehydration results in stiffening of the demineralized matrix in a collapsed state followed by reduction in the width of interfibrillar spaces, greatly compromising monomer diffusion through such densely packed collagen network [17]. Unlike water, methacrylate monomers do not possess the ability to expand collapsed collagen matrix [17]. Consequently, stabilization of the collagen matrix prior to hybridization is critical to resin-dentin bonding and DMSO probably plays an important role on it.

It is known that saturation of demineralized dentin with polar organic solvents (*i.e.* acetone, ethanol, propanol and methanol) stiffens collagen [53–55]. Our results showed that both neat solvents (EtOH and DMSO) and the diluted versions of DMSO in either water or ethanol significantly increased the elastic modulus of dentin collagen, so the second null hypothesis was rejected. The solvent-induced stiffening effect was produced by dehydration, allowing collagen interpeptides to form weak associations mainly through hydrogen bonding [17,54]. The degree of stiffening caused by solvent dehydration seems to depend on the solubility parameter for hydrogen bonding (δh) of the solvent [54,55]. In general, the higher the δh the lower the degree of stiffening [54,55]. This is due to the fact that solvents with higher δh tend to preferentially interact with collagen, preventing interpeptide hydrogen bonding and thus resulting in lower matrix stiffening [54]. DMSO seems to be an exception to the rule. To the best of our knowledge, the effect of pure DMSO on the mechanical properties of human-dentin collagen has not been previously investigated. Hence, it seemed reasonable to include pure DMSO as a control group for elastic modulus and loss of dry mass analyses, albeit it was not used as a bonding protocol. Pure ethanol (δh 20) produced a 13.6-fold increase in elastic modulus, while pure DMSO (δh 13.11) produced a 3.15-fold increase. Diluted pretreatment solutions also significantly increased the elastic modulus of collagen. DMSO/EtOH (δh 16.56) produced a 12.1-fold increase certainly due to the additional water scattering ability of ethanol allowing interptide hydrogen bonding similarly to pure ethanol. Even though water (δh 40.4) effectively plasticizes collagen by rapidly breaking interptide hydrogen bonding, DMSO/H_2O (δh 26.77) also produced a 3.95-fold increase in the elastic modulus of collagen. This indicates that the presence of water (50% v/v) does not affect collagen stiffening by DMSO. Curiously, the increase in collagen stiffening by aqueous-DMSO solutions was unexpected considering the morphological changes caused by DMSO to type I collagen [32]. DMSO alters the continuous fibrous collagen structures replacing it by a rather discontinuous pattern of larger interfibrillar spacing [32]. The DMSO-induced collagen stiffening implies that collagen dehydration also takes place in DMSO-saturated collagen through increasing interpeptide hydrogen bonding [56]. The extent of stiffening was reduced when compared to higher δh solvents (*i.e.* ethanol), likely due to simultaneous collagen dissociation produced by DMSO, which is not observed with ethanol. It is tempting to speculate that DMSO pretreatments not only improve dentin wetting [33] and monomer infiltration [6], but could also stiffen demineralized collagen conferring higher dimensional stability without significantly compromising interfibrillar spaces. Although the DMSO-induced stiffening effect was reversible by water-immersion, similarly to a variety of organic solvents [17], it is likely that the interfibrillar spaces may be sufficiently maintained for resin infiltration. This could help explain reductions in technique sensitivity of the etch-and-rinse approach by DMSO, even under extensive air-drying prior to hybridization [28].

Beyond just adequate collagen morphology before hybridization, the durability of resin-dentin bonds is strongly affected by the preservation of the collagen structure over time [1–3]. Hydrolysis of exposed demineralized collagen by endogenous proteases in dentin matrices accounts for significant impairment of resin-dentin interfaces [1,3]. Endogenous proteases gradually lower the mechanical properties of collagen as peptides are slowly degraded [57,58]. In the present study, indirect evaluation of the impact of endogenous proteases on collagen structure was performed by the three-point bending to determine mechanical stability and loss of dry mass to assess collagen solubilization over time. Moreover, loss of dry mass can serve as an index of matrix degradation [59]. At 30 days, the control group presented a 50% reduction in elastic modulus, which was not statistically different from the EtOH group. Together with a large increase in dry mass loss, the reduction in elastic modulus indicates that lower mechanical properties of untreated and ethanol-treated collagen are due to modifications in collagen structure related to peptide solubilization. The poor inhibition of matrix metalloproteinases produced by ethanol [59] explains the comparable collagen stiffening to untreated samples. Differently, DMSO solutions (50% v/v) were not only effective to preserve the mechanical properties of collagen, but they also reduced collagen solubilization regardless of the cosolvent used. DMSO can bind to enzyme's hydrophobic moieties leading to protein unfolding and thus denaturation [45]. Reduction in metalloproteinase activity has been previously reported for DMSO [25,28]. Hence, DMSO's ability to inactivate enzymes certainly contributed to improved collagen integrity over time resulting in no significant changes in elastic modulus. Surprisingly, neat DMSO was unable to prevent collagen plasticization, albeit lower levels of collagen solubilization were similarly detected. Therefore, such reductions in elastic modulus were not entirely caused by matrix degradation followed by peptide solubilization. Since DMSO can bind to proteins [45,56], we speculate that collagen treated by neat DMSO may slowly uptake water over time, gradually producing a plasticizing effect on the peptide structure.

Conversion of monomers into polymers is a key factor in successful resin-dentin bonding [12,24]. The third null hypothesis was rejected for DMSO-containing pretreatments significantly affected monomer conversion at the hybrid layer. The overall lower monomer conversion values obtained in this study can be explained by the reduced elution of unpolymerized monomers [11,15], mostly HEMA, from the bonded interfaces before collecting Raman spectra. Since samples were only briefly submerged in water for cleaning, the

majority of unpolymerized monomers were included in the calculations. At the top half of the hybrid layer, the tested etch-and-rinse adhesive produced conversion values of roughly 50%, which is accordance with previous studies (*i.e.* 58%) using a similar methodology [11,15]. Moreover, drastic reductions of roughly −75% in monomer conversion at the lower half of the hybrid layer emphasizes that polymerization of methacrylate monomers in wet environments can be even more challenging than commonly believed. At lower portions of the hybrid layer, the hybridization efficiency accounts for no more than 25% considering BisGMA/HEMA infiltration under wet-bonding conditions [60]. Reduced availability and increased spacing between monomers hamper conversion efficiency further compromising adequate polymer formation. Nonetheless, higher monomer infiltration produced by DMSO [6] most likely facilitated monomer conversion at the lower half of the hybrid layer. The positive correlation between degree of conversion and bond strength indicates that higher conversion tends to produce higher bond strengths [12]. Since a bonded interface is only as strong as its weakest link, the more uniform degree of conversion across the hybrid layer with fewer areas of lower conversion contributed to the improved resin-dentin bonding produced by the DMSO pretreatments. The same effect was not observed for the ethanol-wet bonding approach where only long-term improvements in bond strengths were detected, albeit higher monomer conversion. Higher conversion does not necessarily reflect the overall quality of polymer structures. DMSO lowers the termination rates in poly-methacrylate free radical polymerization [34] producing longer chains. This increases the likelihood of crosslinking between adjacent chains, which additionally reinforces the polymer structure.

5. Conclusions

Saturation of demineralized dentin with polar solvents produce different outcomes on both collagen and hybrid layer formation according to solvent type and concentration. Dentin bonding with three-step etch-and-rinse adhesives may be greatly improved by the use of pretreatment solutions composed of DMSO diluted with either water or ethanol (50% v/v) prior to collagen hybridization. Traditional ethanol-wet bonding preserved the long-term resin-dentin bond integrity. Nevertheless, the use of diluted DMSO additionally improved the mechanical stability and reduced solubilization of demineralized collagen. Ethanol alone was unable to produce such protective effects on collagen. Diluted DMSO solutions were also effective to maintain the higher immediate dentin bond strengths after long-term storage with reduced water-filled zones within the hybrid layer. It could be implied that the higher uniformity in monomer conversion across the hybrid layer contributed to the higher stability of the resin-dentin bonded interface over time. Disruption of collagen structure by undiluted DMSO with gradual water uptake negatively affects the long-term mechanical properties of collagen, contraindicating the use of neat DMSO. Hence, the proposed DMSO-bonding protocols, especially the DMSO-ethanol pretreatments, may constitute viable-effective alternatives to the imminent need of identifying new uncomplicated methods for extending the quality and thus longevity of resin-dentin bonds.

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