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Dentin bond optimization using the dimethyl sulfoxide-wet bonding strategy: A 2-year in vitro study

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ABSTRACT

Objective. This study evaluated a new approach, named dimethyl sulfoxide (DMSO)-wet bonding, to produce more desirable long-term prospects for the ultrafine interactions between synthetic polymeric biomaterials and the inherently hydrated dentin substrate.

Methods. Sound third molars were randomly restored with/without DMSO pretreatment using a total-etch (Scocthbond Multipurpose: SBMP) and a self-etch (Clearfil SE Bond: CF) adhesive systems. Restored teeth (n = 10)/group were sectioned into sticks and submitted to different analyses: micro-Raman determined the degree of conversion inside the hybrid layer (DC); resin–dentin microtensile bond strength and fracture pattern analysis at 24 h, 1 year and 2 years of aging; and nanoleakage evaluation at 24 h and 2 years.

Results. DMSO-wet bonding produced significantly higher 24 h bond strengths for SBMP that were sustained over the two-year period, with significantly less adhesive failures. Similarly, DMSO-treated CF samples presented significantly higher bond strength than untreated samples at two years. Both adhesives had significant less adhesive failures at 2 years with DMSO. DMSO had no effect on DC of SBMP, but significantly increased the DC of CF. DMSO-treated SBMP samples presented reduced silver uptake compared to untreated samples after aging.

Significance. Biomodification of the dentin substrate by the proposed strategy using DMSO is a suitable approach to produce more durable hybrid layers with superior ability to

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withstand hydrolytic degradation over time. Although the active role of DMSO on dentin bond improvement may vary according to monomer composition, its use seems to be effective on both self-etch and etch-and-rinse bonding mechanisms.

1. Introduction

Adhesion of resin materials to tooth structure has been a challenge in the history of adhesive dentistry. Currently, the issue of bond durability has attracted significant attention regarding resin–dentin bonding [1–3]. Despite improvements in dental adhesive technology and advances in bonding knowledge, resin–dentin bonding still shows limited durability for both etch-and-rinse and self-etch adhesive systems [1,4–6].

Resin–dentin bonding is a unique form of tissue engineering in which an ultrafine biopolymer, known as hybrid layer, links composites to the underlying mineralized dentin by two substantially different bonding mechanisms produced by the adhesive system used: *etch-and-rinse* or *self-etch* [7,8]. Nevertheless, resin–dentin bonds created by infiltration of hydrophilic resin monomers into demineralized [9,10] and mineralized dentin [10,11] are imperfect and unstable [6,12]. Inadequate polymerization reduces the quality of the hybrid layer [6] leading to lower dentin bond strengths and increased nanoleakage [13]. Moreover, high permeability of the bonded interface and phase separation during adhesive application contribute to hydrolytic degradation of the adhesive resin [6,12]. Insufficient resin impregnation of dentin [9] is associated with the collagenolysis of unprotected collagen fibrils by endogenous matrix metalloproteinases (MMPs) and cysteine cathepsins [3,14]. Irrespective of adhesive type, hydrolytic degradation of the adhesive resin and collagen matrix degradation occur concurrently, for resin elution from hydrolytically unstable polymeric hydrogels within the hybrid layers increases the exposure of unprotected collagen matrix over time.

Several adjunctive procedures have been suggested to prevent biodegradation of hybrid layers over time [3]. Although encouraging results have been produced, the current available techniques do not effectively address both hydrolytic degradation of the adhesive resin and collagen degradation concurrently. The possible exception is the ethanol-wet bonding aiming to remove water from the exposed dentin collagen and to replace it with more hydrophobic resin components [2,8]. Excluding water with high ethanol concentrations would reduce/eliminate the hydrolytic degradation of both the collagen and resin components of the hybrid layer [2,8,15]. Unfortunately, ethanol-wet bonding is clinically unfeasible due to technique sensitivity and increase in application steps and treatment time [3]. Thus, current strategies are at least partially limited in their true potential to optimize the durability of resin–dentin bonding.

Dimethyl sulfoxide (DMSO; $(CH_3)_2SO$) is a polar aprotic solvent with a highly polar S=O group and two hydrophobic CH_3 groups. Its ability to penetrate biological surfaces and tissues makes it the best penetration enhancer for medical purposes [16]. Recent studies have indicated that DMSO may improve the penetration of adhesive into the exposed collagen matrix [10], and improve both immediate [10,17] and long-term [17] dentin bond strength. However, the long-term efficacy has only been demonstrated with a two-step etch-and-rinse adhesive [17]. Therefore, this in vitro study evaluated the effect of DMSO-wet bonding on dentin bond durability, monomer conversion inside the hybrid layer and the quality of aged bonded interfaces of two-step self-etch and three-step etch-and-rinse adhesives after 1 and 2 year storage. The null hypotheses to be tested were that irrespective of adhesive type, the application of 50 vol% DMSO in water on dentin: (i) would not influence monomer conversion at the hybrid layer; (ii) would not affect immediate or long-term dentin bond strength and; (iii) would not improve the adhesive interface quality regarding the formation of nanoleakage channels.

2. Materials and methods

2.1. Teeth selection and preparation

Forty intact human third molars with complete root formation were extracted for surgical reasons with patients' (age 18–25 years) informed consent and approval by the local Ethical Committee under protocol number 110/2014. Teeth were cleaned, disinfected for one week in 0.5% chloramine-T solution at 4 °C, and stored in distilled water at 4 °C for up to one month before use. A flat coronal dentin surface was obtained by sectioning off the occlusal one-third of the crown (Isomet 1000 Precision Saw, Buehler, Lake Bluff, IL, USA). The surface roughness was standardized with 600-grit silicon carbide paper (CarbiMet, Buehler Ltd., Lake Bluff, IL, USA) for 60 s under water cooling and the specimens were randomly assigned to four groups (n = 10) according to the bonding protocols.

2.2. Dentin bonding protocol

Two commercially available unaltered adhesive systems were used (Table 1): a three-step etch-and-rinse adhesive system (Adper Scotchbond Multi-Purpose, 3M ESPE, St. Paul, MN, USA) (SBMP) and a two-step self-etch adhesive (Clearfil SE Bond, Kuraray, Osaka, Japan) (CF). Table 1 lists the mode of application, components and manufacturers of the adhesive systems.

Dentin bonding in control groups was performed according to Table 1. In experimental groups, the DMSO-wet bonding technique was employed, which consisted of light-pressure circular scrubbing movements of a 50 μL of water-based 50% (v/v) DMSO (dimethyl sulfoxide, Sigma–Aldrich, St. Louis, MO, USA) (pH 8.2) for 60 s, using a disposable cavity brush. In SBMP groups, DMSO was applied after dentin etching and water rinsing. In CF groups, DMSO was applied onto smear layer-covered

Table 1 – Adhesive systems, their main components and application modes.

Adhesive system	Components	Application mode (control/DMSO wet-bonding)
Adper Scotchbond Multi-Purpose 3M/ESPE		(1) H_3PO_4 conditioning for 15 s; (2) rinse with water 30 s; (3) blot drying leaving dentin slight moist; (4) active application of 50% DMSO for 60 s (DMSO wet-bonding), or no dentin treatment (control); (5) blot drying; (6) active *Primer* application with a fully saturated brush tip 10 s; (7) gently blow dry 5 s; (8) active *Adhesive* application 10s; and (9) Light cure for 10 s.
Etchant	35% phosphoric acid, fumed silica (pH 0.6)	
Primer	HEMA, polyalkenoic acid methacrylate copolymer, water	
Adhesive	Bis-GMA, HEMA, dimethacrylates, photoinitiators	
Clearfil SE bond Kuraray		(1) Blot drying until no sign of excess visible moisture was observed; (2) active application of 50% DMSO for 60 s (DMSO wet-bonding), or no dentin treatment (control); (3) blot drying until no visible moisture was observed; (4) active *Primer* application with a fully saturated brush tip for 20 s; (5) mild air stream for 5 s; (6) active *Adhesive* application; (7) gentle air stream 5 s; and (8) light cure for 10 s.
Primer	10-MDP; HEMA; CQ; hydrophilic dimethacrylate; water (pH 2.0)	
Adhesive	10-MDP; N,N-diethanol-p-toludine; HEMA; Bis-GMA; silanated colloidal silica; hydrophobic dimethacrylate; CQ	

Abbreviations: HEMA = 2-hydroxyethyl methacrylate; bis-GMA = bis-phenol A diglycidylmethacrylate; 10-MDP = 10-methacryloxydecyl dihydrogen phosphate; CQ = camphoroquinone.

dentin before the primer application. In all groups, SBMP *primer* was applied onto partially wet dentin, while CF *primer* was applied on DMSO wetted dentin. Both adhesive systems were applied actively. Adhesive procedures were carried out in a controlled environment with a temperature of 24 °C and a relative humidity of 55–60%. Resin composite build-ups (Z250, shade A2, 3M ESPE) were built on top of the bonded dentin surfaces in four 1-mm increments that were individually light-cured for 20 s. Light curing of all resin materials was performed using a LED device (Bluephase 20i, Ivoclare Vivadent, Schaan, Liechtenstein) in high power mode (1200 mW/cm^2). All bonding procedures were carried out by a single operator.

2.3. *Specimen preparation*

The restored crown segments were stored in distilled water at 37 °C for 24 h, to allow water sorption and postoperative polymerization of the adhesive and resin composite to take place, and sectioned (Isomet 1000 Precision Saw, Buehler) occluso-gingivally across the bonded interface into slabs measuring approximately 0.8 mm. The slabs were further sectioned into composite-dentin sticks, pursuing a final cross sectional area of approximately 0.7 mm^2 in accordance with the "non-trimming" technique [18] for bond strength testing. A minimum of 24 sticks were obtained from each tooth.

2.4. *Specimen aging*

Sticks were stored for up to two years at 37 °C in artificial solution (pH 7.1) containing (mmol/L): $CaCl_2$ (0.7), $MgCl_2{\cdot}6H_2O$ (0.2), KH_2PO_4 (4.0), KCl (30), NaN_3 (0.3), and HEPES buffer (20) [14]. The storage solution was prepared and changed weekly in accordance with a protocol previously described by Pashley et al. [14].

2.5. *Degree of conversion (DC) inside the hybrid layer measurements*

Two sticks from each tooth (n = 10) were randomly evaluated at 24 h. Sticks were wet-polished with 600; 1000 and 2000-grit SiC paper (Buehler Ltd., Lake Bluff, IL, USA), ultrasonically cleaned for 2 min between polishing steps and 20 min after the last step. Raman spectra were collected using a micro-Raman spectrometer (Senterra, BrukerOptik GmbH, Ettlingen, Baden Württemberg, Germany) to investigate the DC inside the hybrid layer of the adhesive interfaces. The micro-Raman spectrometer was first calibrated for zero and then for the coefficient values using a silicon sample. Samples were analyzed using the following micro-Raman parameters: 20 mW Neon laser with 532 nm wavelength, spatial resolution of approximately 3 μm, spectral resolution approximately 5 cm^{-1}, accumulation time of 30 s with 6 co-additions, and 100× magnification (Olympus UK, London, UK) to a ≈1 μm beam diameter. The spectra were taken in the middle of the hybrid layer, in an arbitrary area of the intertubular dentin. Care was taken to select an area between two dentin tubules. One site was examined in each stick. Spectra of uncured adhesives were taken as reference. Post-processing of spectra was performed using the dedicated Opus Spectroscopy Software version 6.5 (BrukerOptik GmbH, Ettlingen, Baden-Württemberg, Germany). The ratio of double-bond content of monomer to polymer in the hybrid layer was calculated according to the following formula:

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

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

2.6. *Resin–dentin microtensile testing (μTBS)*

Microtensile test was performed at three periods: 24 h, 1 year and 2 years. For each period, six resin–dentin sticks from each restored tooth (n = 10) were randomly chosen and individually attached to a microtensile fixture (OD03d, ODEME Biotechnology, Luzerna, SC, Brazil) using cyanoacrylate adhesive (Super Bonder, Loctite, SP, Brazil). Sticks were tested in tensile forces in a universal testing machine (DL2000, EMIC, São José dos Pinhais, SC, Brazil) at a crosshead speed of

0.5 mm/min until failure. The number of premature failures was recorded and considered as 0 MPa for the statistical analyses. The cross-sectional area of each stick was measured with a digital caliper (Absolute Digimatic, Mitutoyo, Tokyo, Japan) to the nearest 0.01 mm in order to calculate the actual μTBS. Both surfaces of fractured sticks were observed under a stereomicroscope (Olympus 220670; Olympus, Tokyo, Japan) with 40× magnification for fracture pattern classification. The fracture modes were classified as follows: 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). For the failure modes that could not be accurately established under the stereomicroscope, the surfaces were examined with a scanning electron microscope (LEO 435 VP; LEO Electron Microscopy Ltd., Cambridge, UK).

2.7. Nanoleakage evaluation

Four sticks, randomly selected from each tooth (n = 10), were used to evaluate nanoleakage by silver nitrate uptake at the bonded interface at 24 h and two years, two sticks per tooth at each period. These bonded sticks 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. Sticks were rehydrated in distilled water for 10 min prior to immersion in the tracer solution. Ammoniacal silver nitrate (pH 9.5) was prepared according to the protocol previously described [19]. The bonded sticks were immersed in the ammoniacal silver nitrate in darkness for 24 h, rinsed thoroughly in distilled water, and immersed in photo-developing solution for 8 h under a fluorescent light to reduce silver ions into metallic silver grains within voids along the bonded interface. Sticks were wet-polished with 600-, 1000-, and 2000-grit SiC paper and 6, 3, 1, 0.25 and 0.05 μm diamond pastes (Buehler Ltd., Lake Bluff, IL, USA) using a polishing cloth. Specimen were ultrasonically cleaned in distilled water after each polishing step, air dried, mounted on stubs, dried in silica overnight, and carbon-sputtered under high-vacuum ambient (MED 010, Balzers Union, Balzers, Liechtenstein). The samples were analyzed by SEM operating on backscattering mode at 15 kV (LEO 435 VP, LEO Electron Microscopy, Cambridge, UK). A series of sequential micrographs (1000× magnification) were obtained from each stick to include the entire length of the adhesive interface. Silver nitrate uptake was measured using an open-source image software (ImageJ, National Institute of Health, Bethesda, MD, USA) by a single-blinded examiner and the overall extension of silver uptake (μm) was converted into percentage values.

2.8. Statistical analysis

After confirming the normality of the data distribution, Kolmogorov–Smirnov test, and the equality of variances by the Barlett test, μTBS (MPa) and SNU (%) data were subjected to Repeated Measures ANOVA test. DC (%) data was analyzed by one-way ANOVA. Post hoc multiple comparisons were performed with Tukey Test ($\alpha = 0.05$). Tooth was considered the statistical unit. The differences of fracture modes between the groups within each time point was tested with Chi-Square test, and between the different time points within each group with McNemar test ($\alpha = 0.05$).

3. Results

3.1. Degree of conversion (DC) calculation

Raman images based on $R_{1639/1609}$ were generated to show the distribution of unconverted C=C bond content in the hybrid layer. Means and standard deviations of DC (%) obtained from Raman spectra and statistical differences for all groups are reported in Table 2. One-way ANOVA detected significant differences among groups ($p = 0.0008$). DMSO treatment had no influence on DC of SBMP at 24 h ($p = 0.0658$). CF specimens treated with DMSO presented higher DC values than untreated CF specimens ($p = 0.0307$).

3.2. Microtensile bond strength evaluation

The mean cross-sectional area of tested resin–dentin sticks ranged from 0.74 to 0.83 mm^2 and no statistical difference among groups was detected ($p = 0.43$). Microtensile overall means and the respective standard deviations are reported in Fig. 1. Repeated measures ANOVA detected that "adhesive type" ($p = 0.0038$), "dentin treatment" ($p < 0.0001$), and "aging" ($p < 0.0001$) significantly affected dentin bond strength. The interaction between "dentin treatment" and "adhesive type" significantly affected microtensile values irrespective of "aging" ($p = 0.0003$): bond strength of DMSO-treated SBMP specimens were significantly higher (37.4%) than untreated specimens at 24 h ($p < 0.0001$). However, no differences were observed for CF between DMSO treated and untreated specimens at 24 h ($p = 0.6817$). The interaction between "aging" and "adhesive type" was not statistically significant ($p = 0.1196$), showing that aging affected bond strength irrespective of

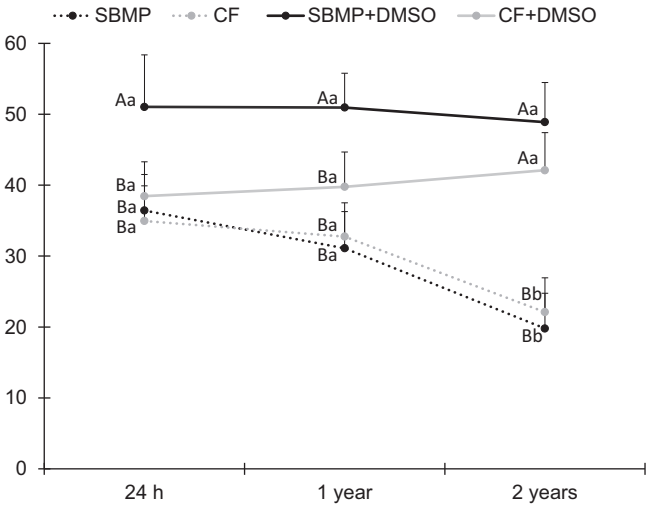

Fig. 1 – Microtensile dentin bond strength values for all groups (n = 10). Different capital letters indicate significant difference according to Tukey's test ($p < 0.05$) for each aging period; different lowercase letters indicate significant difference according to Tukey's test ($p < 0.05$) comparing different aging periods.

Table 2 – Degree of conversion (n = 10) in the hybrid layer and standard deviations for all groups.

Adhesive system	Dentin treatment	Degree of conversion (%)
Adper Scotchbond Multi-Purpose	No treatment	95.19 (2.79)[A]
	DMSO	95.52 (1.91)[A]
Clearfil SE bond	No treatment	86.15 (4.56)[C]
	DMSO	90.72 (4.27)[B]

Different capital letters indicate significant difference between groups according to Tukey studentized range (HSD) test ($p < 0.05$).

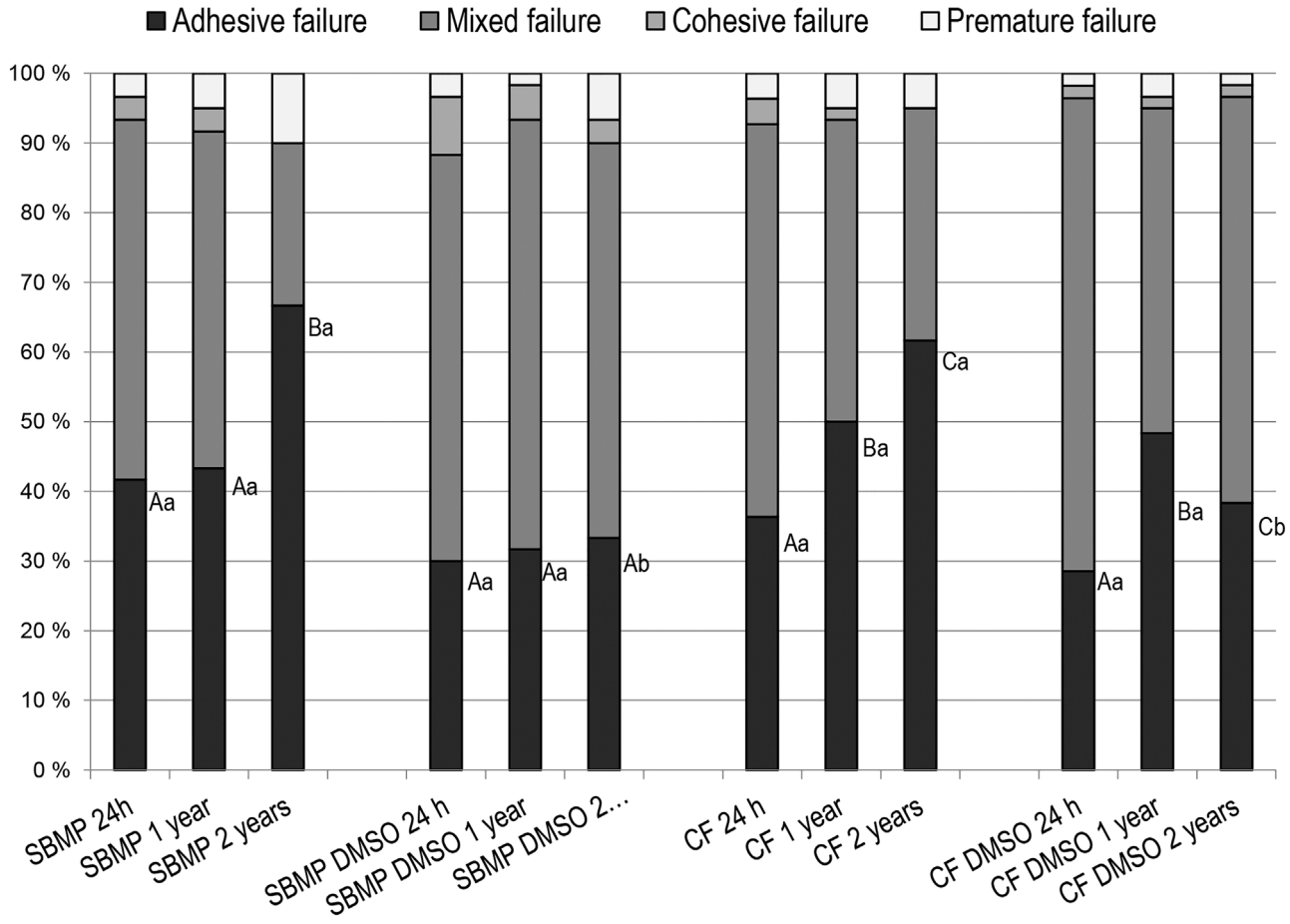

Fig. 2 – Percentage of specimens (%) according to fracture pattern classification and premature failures from each experimental condition (n = 60). The adhesive failures were statistically analyzed: groups with different capital letters indicate statistically significant differences between the different testing periods within the group (McNemar test, $p < 0.05$). The groups with the different lowercase letters indicate statistically significant difference between the groups within each testing period (Chi-Square test, $p < 0.05$).

adhesive type. Moreover, the interaction of "aging" and "dentin treatment" significantly affected dentin bond strength ($p < 0.0001$), showing a different effect between DMSO treatment and no-treatment on bond strength over time. Bond strengths of untreated specimens at one year were not statistically different compared to specimens tested at 24 h, but were significantly lower at two years compared to 24 h and one year values: reduction of 45.7% for SBMP and 36.8% for CF specimens ($p < 0.0001$). No statistical differences were observed for DMSO-treated specimens for both SBMP and CF at the three testing periods.

3.3. *Fracture mode evaluation*

The percentage of specimens with premature failures and the frequency of each fracture pattern mode are shown in Fig. 2. While the adhesive failures increased significantly from 24 h to two years in all groups, the increase was much smaller in DMSO-treated groups. Significantly fewer adhesive fractures were observed in both DMSO-treated groups than in the controls after 2 years ($p < 0.05$), while neither the DMSO-treated nor the control groups showed differences between the adhesives. In other time points, no statistically significant differences were observed (Fig. 2).

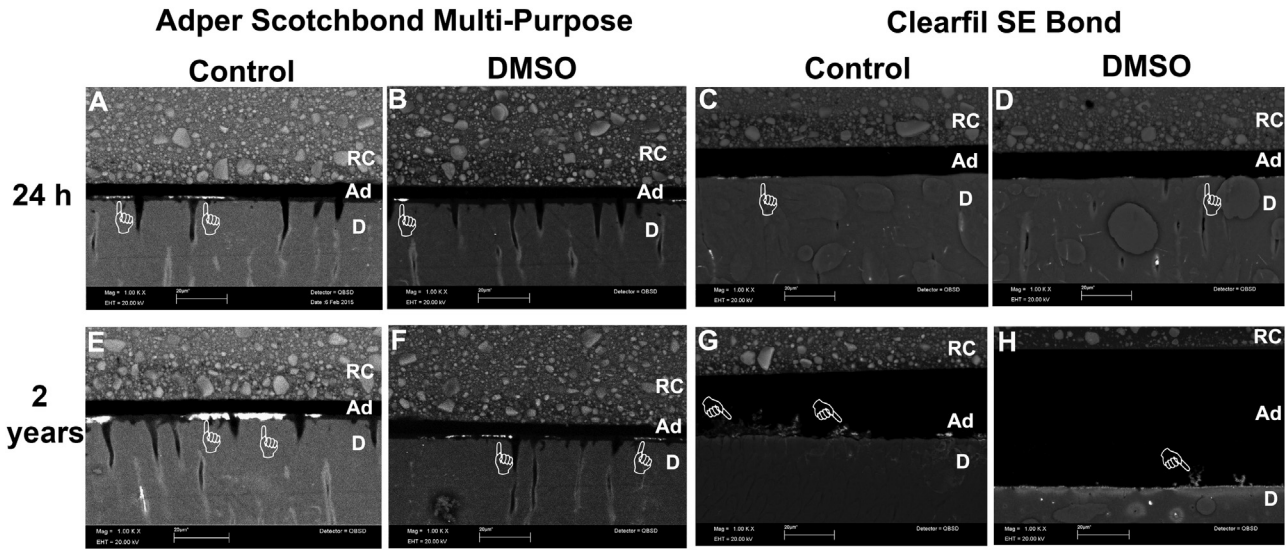

Fig. 3 – Representative SEM micrographs of resin–dentin interfaces in 2 year aged composite-dentin beams bonded with SBMP and CF using the DMSO wet-bonding technique. RC: resin composite; Ad: adhesive layer; D: dentin. Nanoleakage was observed as silver-impregnated areas within the adhesive layer (pointer). (A) SBMP at 24 h showing a dispersed reticular pattern of silver deposits (pointer) at the base of the hybrid layer. (B) DMSO-treated SBMP at 24 h presented a similar reticular nanoleakage pattern (pointer) observed in SBMP control. (C) CF at 24 h showing fine spotted silver deposits predominantly located within the hybrid layer (pointer). (D) DMSO-treated CF specimen at 24 h showing a similar spotted nanoleakage pattern to non-treated CF. (E) Aged SBMP showing increased silver uptake: dense reticular silver deposits (pointer) with increased vertical and horizontal extension within the hybrid layer. (F) Aged DMSO treated SBMP presented a reticular nanoleakage pattern mostly deposited horizontally within the hybrid layer (pointer), but not vertically. (G) Aged CF specimen presenting extensive dendritic water-tree silver deposits (pointer) towards the bulk of the adhesive layer. (H) Aged DMSO treated CF sample showing reduced water tree formation at the bonded interface.

3.4. *Nanoleakage evaluation*

All tested specimens presented traces of silver impregnation in the hybrid layer: representative images of nanoleakage and percentages of silver nitrate uptake along the bonded interface shown in Figs. 3 and 4, respectively. Repeated measures ANOVA revealed that "adhesive system" ($p < 0.0001$), "dentin treatment" ($p < 0.0001$), "time" ($p < 0.00001$) and their interaction ($p = 0.0459$) had significant effects on nanoleakage expression. Overall, SBMP presented higher levels of silver impregnation than CF. DMSO wet-bonding had no influence on immediate silver uptake for both the etch-and-rinse and self-etch adhesives. Irrespective of adhesive system or dentin treatment, storage for two years significantly increased nanoleakage expression. Nevertheless, DMSO wet-bonding significantly reduced silver deposition at the hybrid layer for SBMP after aging but not for CF. It is noteworthy that aside from the amount of silver deposition within the hybrid layer, differences in nanoleakage pattern were clearly observed after aging. SBMP presented a dispersed reticular pattern of discontinuous islands of silver deposits at the base of the hybrid layer (Fig. 3A), which increased both in height (vertically) and length (horizontally) exhibiting a substantial increase in size and density after aging (Fig. 3E). SBMP specimens treated with DMSO presented a similar reticular pattern to non-treated specimens (Fig. 3B), which increased in length but not in height remaining mostly constant after aging, with the exception that a larger extension of the hybrid layer was infiltrated (Fig. 3F).

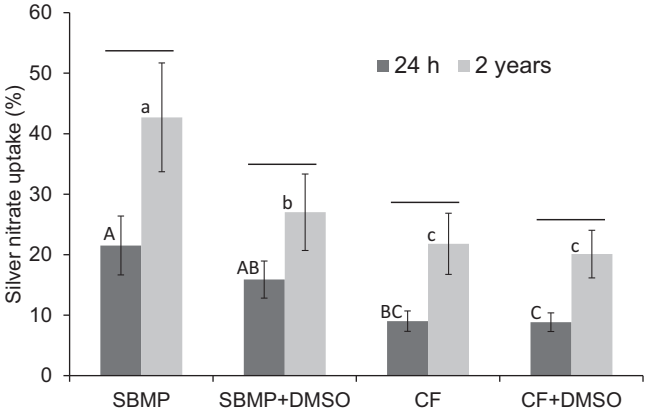

Fig. 4 – Nanoleakage extension (%) along the hybrid layer for all groups after long-term aging. Capital letters indicate significant difference according to Tukey's test ($p < 0.05$) at 24 h. Lowercase letters indicate significant difference according to Tukey's test ($p < 0.05$) at 2 years. Horizontal lines connecting 2-year and 24 h groups indicate significant difference according to Tukey's test ($p < 0.05$), respectively.

DMSO treated and non-treated CF specimens presented similar patterns of fine spotted silver deposits at the base of the hybrid layer at 24 h (Fig. 3C). After aging, dendritic water-tree silver deposits starting at the hybrid layer became more

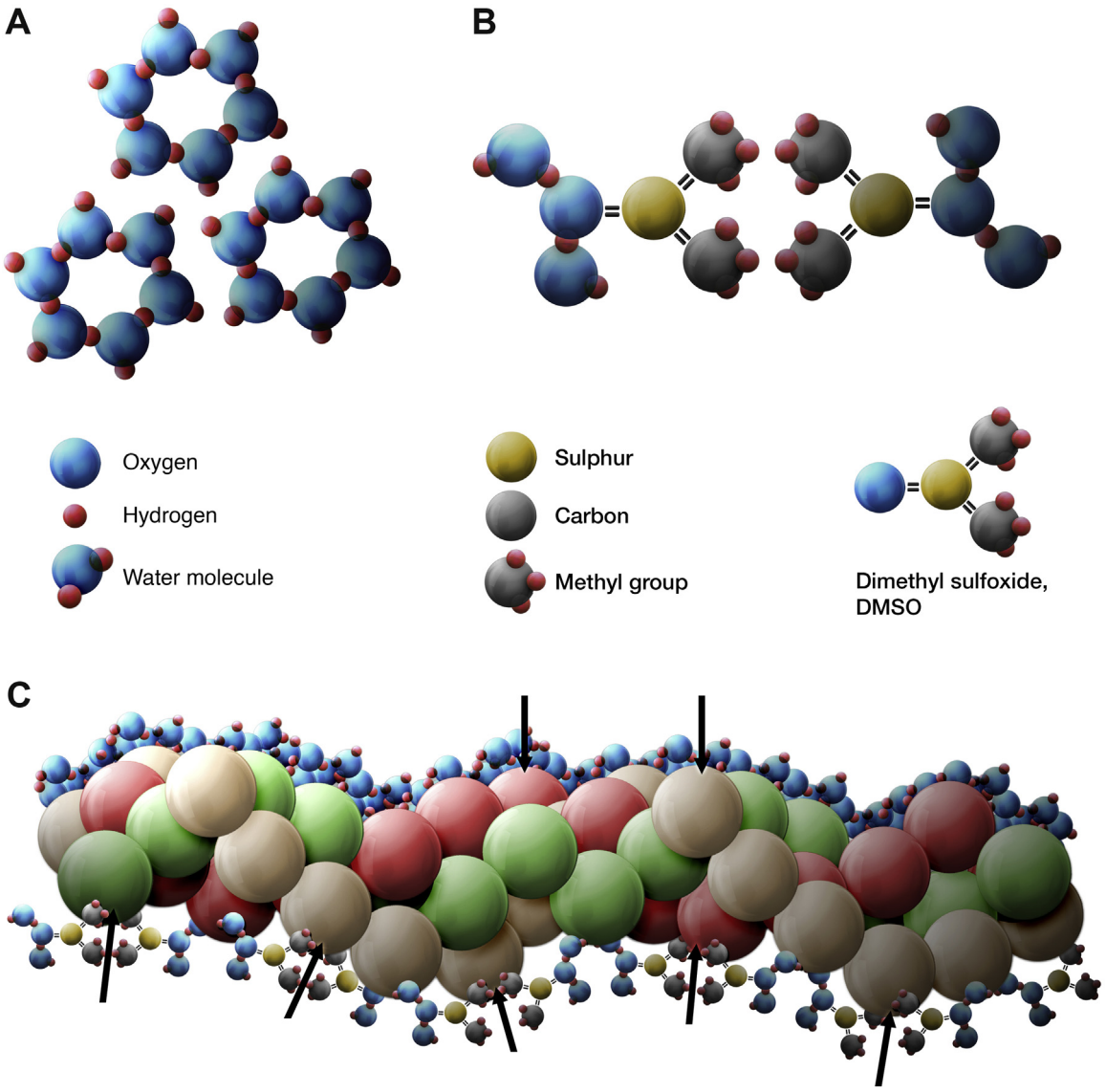

Fig. 5 – Proposed effect of DMSO in dentin matrix. (A) In biological structures, water molecules form hydrogen bonds and behave in a structured form, favoring the cyclic pentamer which has the lowest dipole moment (approximately one half of an individual water molecule [25]). (B) DMSO oxygen forms a hydrogen bond with two water molecules, while the methyl groups form a hydrophobic end, breaking water self-association. (C) In dentin, triple-helical collagen molecules are covered with bound water [40], limiting the chemical bond of functional monomers such as MDP with collagen (upper surface of the molecule). DMSO may disrupt this water layer, exposing more binding sites and allowing more MDP molecules to bond with collagen molecule (lower surface), as it has been shown to happen with MDP-DMSO and MDP-DMSO-HEMA mixtures, but to a lesser extend with MDP-HEMA [22]. Black arrows indicate the exposed binding sites for the functional monomers such as MDP.

evident with larger extensions in non-treated CF specimens (Fig. 3G) compared to DMSO treated specimens (Fig. 3H).

4. Discussion

DMSO pretreatment significantly increased the degree of conversion of CF, but not of SBMP. Therefore, the first hypothesis was partially rejected. While other studies employing micro-Raman to assess the conversion rate in the hybrid layer have also reported high overall DC values [20–22], loss of unpolymerized HEMA over the first 24 h of water storage might have contributed for such high values [20,21]. Nevertheless, assessment of DC at the hybrid layer provided valuable information to begin understanding the interaction between different monomers and DMSO-saturated dentin. The reason for the selective increase in DC may be related to the different composition of the adhesives. Without DMSO, CF DC was similar to that found in a previous study when measured from the hybrid layer [22]. The increase of CF DC with DMSO may relate to the behavior of 10-MDP in DMSO-impregnated collagen.

10-MDP has been indicated to have a hydrophobic interaction with collagen molecule [23,24]. It has been speculated that the monomer hydrophobic force is sufficient to disrupt the hydration surrounding collagen molecule (bound water) to allow the collagen MDP aggregation [23]. It is interesting to note that in those experiments, MDP had been dissolved in DMSO, with final concentration of 20% [23,24]. The authors explained that the increased interaction of MDP-DMSO compared with MDP-HEMA is due to the aggregation of MDP and HEMA, where the MDP molecules are surrounded by HEMA molecules with the hydrophobic regions in the center. Thus, the hydrophobic interaction with collagen molecule is reduced [24]. However, MDP-collagen interaction with MDP-DMSO and MDP-DMSO-HEMA blend is similar [24], which may indicate that the reason for the increased interaction is the presence of DMSO, not the absence of HEMA. In other words, DMSO, via its effects on water behavior, may make hydrated collagen more accessible to hydrophobic monomers such as MDP, and reduce the water-related hindrance of hydrophobic monomer polymerization. The proposed effect of DMSO on the hydrated dentin matrix collagen is illustrated in Fig. 5. Alternatively, or in addition, it may be due to the increased chemical interactions between CF monomers and hydroxyapatite. In mineralized tissues, hydroxyapatite crystal has a hydration layer water adsorbed onto the crystal particle surface [25,26]. If DMSO affects the behavior of this water layer, it might allow more interaction between hydroxyapatite and functional monomers, such as 10-MDP. The increase in DC with Clearfil SE Bond in DMSO-primed hybrid layer may thus relate to the increased 10-MDP-bonding and higher polymerization rate, both due to the effects of DMSO on water self-association [27].

Both tested adhesive systems demonstrated gradual and continuous loss of dentin bond strength very similar to what has been observed in numerous previous studies with all tested systems [3,12]. Since the application of DMSO produced significantly higher immediate bond strength with SBMP and also significantly higher bond strengths with both tested adhesive after two years, the second hypothesis was partially rejected. The findings support and widen recent studies indicating that DMSO pretreatment may indeed have a positive effect on dentin bonding with different bonding systems [10,17].

Improvement in dentin bond strength may be attributed to several factors related to the biomodification of the bonding substrate brought about by DMSO, which most likely enhanced the interaction between adhesive monomers and dentin. DMSO is a polar aprotic solvent that dissolves both polar and non-polar compounds. It is a polyfunctional molecule with a highly polar S=O group and two hydrophobic CH_3 groups, and has the ability to dissociate the highly cross-linked collagen into a sparser network of apparent fibrils [28] also in dentin matrix [17], most likely by the suppression of interpeptide hydrogen bonding [29]. This allows DMSO to efficiently penetrate into biological surfaces [16] and it is widely used as a carrier of medications into tissues. DMSO reduces the exposed collagen at the bottom of the hybrid layer by 86% and 68% with SBMP and Clearfil SE Bond, respectively, and eliminates time-related nanoleakage with Scotchbond 1XT, a two-step etch-and-rinse adhesive [17]. Together with these previous findings, the enhanced penetration of adhesive monomers into the exposed collagen matrix may be the most plausible explanation for the increase and preservation of bond strength observed in this study.

Since the interaction of adhesive monomers and solvents play an important role in proper resin–dentin bonding, the assessment of hybrid layer quality/morphology was performed by nanoleakage evaluation [19] aiming to detected nanometer sized water-filled defects along the bonded interfaces. Residual solvents in the hybrid layer may lead to poor bond strengths [30], so removal of excess solvents prior to polymerization by evaporation of adhesive solvents is usually recommended. However, extensive evaporation is insufficient to completely remove even high vapor pressure solvents [31]. Monomer dilution by residual solvents influences the DC and the rate of conversion of adhesive systems [32]. Excess solvent may dilute monomer concentration and separate growing polymer chains [33,34] leading to the formation of porous permeable zones. DMSO has a low vapor pressure, approximately 25% of pure water in 50% concentration at 25 °C [35]. Considering the proposed DMSO-wet bonding protocol, demineralized dentin remains saturated by DMSO during and after adhesive application. However, the effect of remaining solvents on polymerization kinetics of dental adhesives is concentration dependent [32] and DMSO had not been previously assessed in this manner. In the present study, the DMSO wet-bonding technique had no influence on immediate nanoleakage for both tested adhesives, showing that 50% DMSO does not increase the formation of permeable zones at the hybrid layer for either CF or SBMP at 24 h. Our initial concern regarded the fact that since DMSO was used in a high concentration and it does not evaporate easily from the dentin substrate, remaining DMSO would undermine the adhesive interface on the long-term due to its hydrophilic behavior. Overall, two-year specimens presented higher levels of nanoleakage when compared to 24 h specimens, irrespective of adhesive type and adhesive technique. However, untreated SBMP samples present significantly higher levels of silver uptake than DMSO-treated samples. Therefore, the third null hypothesis that DMSO wet-bonding would not improve the adhesive interface quality regarding the formation of nanoleakage paths was rejected. The overall patterns of nanoleakage also changed after aging for untreated CF and SBMP aged samples, showing the formation of wider permeable regions across the hybrid layer and adhesive layer. In this context, DMSO-wet bonding was able to reduce the formation of water paths within the hybrid layer, producing bonded interfaces with increased hydrolytic stability.

We speculate that enhanced collagen matrix infiltration by hydrophilic monomers in SBMP most likely occurred [36] due to suppression of interpeptide hydrogen bonds within demineralized collagen fibrils produced by DMSO [28,29]. By breaking water's self-associative tendency [37], high DMSO concentrations dissociate collagen fibrils into a sparser network by reversibly destabilizing collagen structure [28] including dentin [17]. Therefore, collagen biomodification associated to DMSO ability to be fully miscible in water and to dissolve most currently known adhesive hydrophilic/hydrophobic monomers, including HEMA and BisGMA [38], may reduce the phase separation dur-

ing dentin hybridization, improving resin monomer diffusion within demineralized dentin. In addition, bond strengths vary directly with the width of interfibrillar spaces within the hybrid layer [36,39]. Since DMSO remains within the collagen matrix during dentin hybridization, most likely maintaining the interfibrillar spaces but without hampering monomer conversion, higher immediate bond strengths can be produced.

Although high bond strengths are crucial to the longevity of resin restorations, they are only meaningful if they are stable over time. This is the first study to evaluate the long-term effect of aging on bond strength of DMSO-saturated hybrid layers. DMSO-wet bonding retained the bond strength up to two years, while control groups showed a 35–50% reduction compared to their 24 h bond strength values, and 50–60% reduction compared to DMSO-treated specimens. The ability of DMSO to prevent dentin bond degradation might be partly attributed to DMSO capacity to reduce gelatinolytic activity produced by endogenous proteases [17]. This becomes more evident when considering the hybrid layer morphology after two-year aging. Even though aging relatively impacted the hybrid layer integrity of DMSO-treated samples, demonstrated by higher silver uptake after two years, major changes occurred neither on nanoleakage pattern nor on bond strength. DMSO ability to reduce gelatinolytic activity might have protected the exposed collagen at the hybrid layer counteracting to some extent the deleterious effects of increased nanoleakage on bond strength over time.

Apart from DMSO endogenous protease inhibition, improvement within the polymer matrix by high DMSO concentration possibly contributed to the stability of the SBMP bonded interface over time. Direct measurements of the mechanical/physical properties of the hybrid layer were not performed in the present study. However, considering the immediate bond strength increase in DMSO treated specimens for SBMP, we speculate that improvements in hybrid layer quality may have reduced the deleterious effects of aging at the adhesive interface. Analysis of fracture patterns at two years comparing DMSO-treated and untreated specimen showed a significant reduction of adhesive failures in DMSO-treated dentin, which suggests a possible improvement of the bonded interface. BisGMA (molecular weight 512) has a limited ability to diffuse across the hybrid layer [40]. As a result, the bulk of the hybrid layers is infiltrated predominantly by HEMA (molecular weight 130) [40,41] subsequently polymerizing mostly into linear poly (HEMA) chains. Since DMSO dissolves both HEMA and BisGMA [38], BisGMA may diffuse deeper into the hybrid layer, favoring polymer crosslinking. Lower degree of polymer crosslinks at the hybrid layer in untreated specimens could have resulted in a higher degree of water sorption [41]. As endogenous proteases require water to function, this could also have expedited the collagenolytic activities within the hybrid layer of untreated specimens. More studies are required to assess differences in etch-and-rinse dentin hybridization produced by DMSO-wet bonding.

Chemical dehydration of dentin with commonly used solvents, such as acetone or ethanol, is virtually impossible especially in clinically relevant times [42]. An attractive alternative to the removal of water from the hybrid layer is to change water behavior in a manner that would allow better adhesive monomer penetration, better protection of exposed collagen with hydrophobic monomers, and in specific cases also higher rates of polymerization. For this, DMSO seems to be an ideal candidate.

5. Conclusions

This study presents compelling evidence that the proposed DMSO-wet bonding technique improves bonding performance of both self-etch and etch-and-rinse adhesives after long-term aging. The introduced new concept of bonding not only prevented bond strength loss, but also produced significantly higher dentin bond strengths over time. Monomer conversion inside hybrid layer on DMSO-saturated dentin was not hampered by remaining DMSO, but benefitted the tested self-etch adhesive degree of conversion. In addition, hybrid layers with higher hydrolytic stability, presenting reduced levels of nanoleakage after long term aging, were produced when the DMSO-wet bonding was employed.

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