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Optimization of the etch-and-rinse technique: New perspectives to improve resin–dentin bonding and hybrid layer integrity by reducing residual water using dimethyl sulfoxide pretreatments

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ABSTRACT

Objective. To determine whether bonding effectiveness and hybrid layer integrity on acid-etched dehydrated dentin would be comparable to the conventional wet-bonding technique through new dentin biomodification approaches using dimethyl sulfoxide (DMSO).
Methods. Etched dentin surfaces from extracted sound molars were randomly bonded in wet or dry conditions (30 s air drying) with DMSO/ethanol or DMSO/H_2O as pretreatments using a simplified (Scotchbond Universal Adhesive, 3M ESPE: SU) and a multi-step (Adper Scotchbond Multi-Purpose, 3M ESPE: SBMP) etch-and-rinse adhesives. Untreated dentin surfaces served as control. Bonded teeth (n = 8) were stored in distilled water for 24 h and sectioned into resin–dentin beams (0.8 mm^2) for microtensile bond strength test and quantitative interfacial nanoleakage analysis (n = 8) under SEM. Additional teeth (n = 2) were prepared for micropermeability assessment by CFLSM under simulated pulpar pressure (20 cm H_2O) using 5 mM fluorescein as a tracer. Microtensile data was analyzed by 3-way ANOVA followed by Tukey Test and nanoleakage by Kruskal–Wallis and Dunn-Bonferroni multiple comparison test ($\alpha = 0.05$).

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DMSO
Ethanol
Dentin
Adhesives

Results. While dry-bonding of SBMP produced significantly lower bond strengths than wet-bonding ($p < 0.05$), DMSO/H_2O and DMSO/ethanol produced significantly higher bond strengths for SBMP irrespective of dentin condition ($p < 0.05$). SU presented significantly higher nanoleakage levels ($p < 0.05$) and micropermeability than SBMP. Improvement in hybrid layer integrity occurred for SBMP and SU for both pretreatments, albeit most pronouncedly for DMSO/ethanol regardless of dentin moisture.

Conclusion. DMSO pretreatments may be used as a new suitable strategy to improve bonding of water-based adhesives to demineralized air-dried dentin beyond conventional wet-bonding. Less porous resin–dentin interfaces with higher bond strengths on air-dried etched dentin were achieved; nonetheless, overall efficiency varied according to DMSO's co-solvent and adhesive type.

Clinical significance. DMSO pretreatments permit etched dentin to be air-dried before hybridization facilitating residual water removal and thus improving bonding effectiveness. This challenges the current paradigm of wet-bonding requirement for the etch-and-rinse approach creating new possibilities to enhance the clinical longevity of resin–dentin interfaces.

1. Introduction

Despite the evolution in adhesive dentistry over the past decades [1–3], degradation of tooth-bonded interfaces [2] still contributes to the reduced long-term clinical success of adhesive restorations [4–6]. Currently, resin–dentin bonding relies on effective adhesive penetration into demineralized collagen matrix for proper hybrid layer formation [2,7]. The hybrid layer is characterized by the creation of complex collagen-resin biopolymers aiming to provide a continuous and stable link between the bulk adhesive and dentin substrate [1,2]. In face of the limitations of most current clinically-feasible bonding protocols and inherent drawbacks of the etch-and-rinse approach *per se* [1] an ideal resin-enveloped collagen scaffold is unlikely to be produced in a consistent manner. In dentin hybridization, adhesive infiltration is far from perfect [1,8,9] resulting in poorly formed hybrid layers. Replacement of all 70 vol% residual water in etched-dentin [1] with monomers is hardly achieved. For this reason, the hybrid layer may be considered as the weak link in resin–dentin bonds [10,11]. All in all, improvements in resin–dentin bonding effectiveness using simple time-efficient bonding protocols [9,11,12] aiming to eliminate the presence of residual water during dentin hybridization are still required.

The etch-and-rinse dentin bonding approach still relies on traditional wet-bonding technique to couple relatively hydrophilic adhesives to the hydrated dentin substrate in clinically relevant protocols. A partially wet dentin substrate has been consensually advocated to maintain the demineralized collagen matrix expanded for proper resin infiltration by relatively hydrophilic monomers [13]. Nevertheless, management of adequate moisture is not easily accomplished, and either excess or lack of dentin moisture may compromise resin–dentin bonding [14–16]. Although adequate resin–dentin bonding is usually immediately achieved, reduced bonding efficiency occurs with time [2,4–7]. Such lack of durability may be partly attributed to the involvement of excess residual water with poorly formed hybrid layers [17,18] for water: (i) causes phase separation of adhesive components [19] during hybridization, (ii) accelerates hydrolysis of polymers containing ester linkages [20] on the long run; and (iii) allows endogenous host-derived collagen-hydrolytic enzymes (*i.e.* matrix metalloproteinases and cathepsins) to degrade demineralized collagen. In this sense, creation of less porous hybrid layers with reduced water content and affinity could indeed improve dentin bonding.

The main problem lies on how to remove excess water without compromising resin–dentin interaction. Previous attempts to remove excess residual water from the bonded interface using dry-bonding protocols have generally produced inadequate resin–dentin interfaces [14]. Collapse of air-dried collagen drastically reduces the interfibrillar spaces that serve as diffusion paths for resin infiltration [13] and produces a surface more resistant to wetting [21]. To overcome the drawbacks of excessive air-drying, the ethanol wet-bonding technique was proposed to gradually replace free water from the dentin substrate before resin bonding. Even though encouraging *in vitro* results have been presented [1,13], the technique proved to be clinically unfeasible and highly technique sensitive [1] due to the extra bonding steps and higher likelihood of demineralized collagen matrix collapse after ethanol evaporation. Recently, dimethyl sulfoxide (DMSO) has been introduced as a new potential solvent to be used in adhesive dentistry [8,22]. DMSO [$(CH_3)_2SO$] 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 methyl groups, fully miscible in most solvents and monomers used in adhesive dentistry [23]. DMSO is perhaps the best currently known penetration enhancer for medical purposes [24] with the ability to dissociate the highly crosslinked collagen into a sparser network of apparent fibrils [25] concentration dependently. In addition, dissociation of water self-associative tendency by DMSO [26] improves wettability of demineralized dentin [27,28], monomer diffusion into the collagen matrix [8] and concomitantly re-expands collapsed collagen to a fairly modest level [28].

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The possibility of combining DMSO and ethanol to displace and reduce water from the bonded interface and improve resin–dentin interaction brings up unexplored possibilities regarding resin–dentin bonding to dry dentin. The aim of this study was to examine the central hypothesis that biomodification of the dentin substrate produced by DMSO mixtures would permit adequate resin bonding to dehydrated demineralized dentin. Therefore, the effect of new dentin bonding approaches, consisting of DMSO/H_2O and DMSO/ethanol pretreatments applied onto wet and extensively air-dry dentin, on the bond strength micropermeability and nanoleakage of three-step and two-step *etch-and-rinse* adhesives was investigated. The null hypotheses to be tested were that irrespective of dentin moisture, pretreatments containing DMSO would have no effect on (i) bond strength and (ii) and hybrid layer integrity.

2. Material and methods

2.1. Tooth preparation and bonding procedures

Sound human third molars were extracted for surgical reasons under an informed consent from the patients (age: 18–24 years) reviewed and approved by the Ethics Committee of the University of Oulu, Finland (19/2006). The teeth were cleaned and stored in 0.5% chloramine-T at 4 °C for one week, rinsed and stored in distilled water (pH 7.1) until use for no more than 3 months at 4 °C. Teeth were sectioned 1 mm beneath the cemento-enamel junction using diamond wafering blades in a slow-speed saw (Isomet 1000 Precision Saw, Buehler, Lake Bluff, IL, USA) and occlusal enamel was subsequently removed with a parallel cut to expose flat midcoronal dentin surfaces. Bonding surfaces were standardized using P320 silicon carbide paper (CarbiMet, Buehler) for 60 s under water cooling. Teeth were randomly divided into 12 groups (n = 8) following a study design composed of three studying factors: (i) "adhesive system" in two levels composed by a three-step *etch-and-rinse* (Adper Scotchbond Multi-Purpose: SBMP, 3M ESPE, St Paul, MN, USA) and a universal adhesive in etch-and-rinse mode (Scotchbond Universal Adhesive: SU, 3M ESPE); (ii) "dentin moisture" in two levels using the dry- and wet-bonding approach; and (iii) "dentin pretreatment" in three levels consisted of no treatment following manufacturer instructions and application of two DMSO solutions for 60 s after dentin etching. The 50%(v/v) solutions were prepared immediately before use mixing DMSO (Dimethyl Sulfoxide, Sigma-Aldrich, St Louis, MO, USA) in distilled water or ethanol (Ethanol, Sigma-Aldrich). The rationale for using 50%(v/v) DMSO was based on previous studies that employed the same concentration, albeit only in aqueous solutions, reporting significant improvements in resin–dentin interactions under wet conditions [29–31]. Table 1 displays the mode of application, components and batch number of the adhesives. For the wet-bonding protocols, blot-drying with paper filters was carefully performed leaving the dentin surface visibly moist. Dry-bonding was performed by blot-drying until no signs of visible moisture were detected followed by continuous air blast using a 3-way syringe at a distance of 10 cm for 30 s. Dentin pretreatments consisted of active application of 50 μL DMSO/H_2O or DMSO/ethanol solutions on etched-dentin followed by blot drying until paper filters were no longer able to remove liquids from the bonding surface by capillarity. Adhesive systems were applied with slight rubbing action onto the demineralized dentin surface totaling 20 s for SU and 10 s for each of SBMP *Primer* and *Bond* with manual pressure of approximately 4.0 g ±1.6 [32]. Adhesive procedures were carried out in a controlled environment with a temperature of 24 °C and a relative humidity of 45–55%. Resin composite restorations (Z250, shade A2, 3 M ESPE) were built on top of the bonded dentin surfaces in four 1-mm increments that were individually light-cured for 40 s. Light curing of all resin materials was performed using a LED device (Bluephase 20i, Ivoclare Vivadent, Schaan, Liechtenstein) that delivered 1100 mW/cm^2.

2.2. Microtensile bond strength (μTBS) test

After storage in distilled water at 37 °C for 24 h, the restored crown segments were longitudinally sectioned into 0.9-mm slabs across the adhesive interface with a water-cooled diamond wafering blade. The slabs were further sectioned into resin–dentin beams with cross-sectional area of approximately 0.8 mm^2 in accordance with the "non-trimming" technique [33]. Beams were individually attached to a microtensile fixture (OD03d, ODEME Biotechnology, Luzerna, SC, Brazil) with a cyanoacrylate adhesive (Zapit, Dental Ventures of America, Corona, CA, USA) and submitted to the test (DL2000, EMIC, São José dos Pinhais, PR, Brazil) in tension, at a crosshead speed of 0.5 mm/min until failure. The cross-sectional area of each beams was measured with a digital caliper (Absolute Digimatic, Mitutoyo, Tokyo, Japan) to the nearest 0.01 mm to calculate the actual bond strength (MPa). The failure pattern modes were evaluated at 40× magnification under a stereomicroscope (Leica M60, Leica Microsystems, Heidelberg, Germany) and classified as cohesive (C) when exclusively within dentin or resin composite, adhesive (A) when failure occurred at the dentin/resin interface, or mixed (M) when two modes of failure happened simultaneously.

2.3. Assessment of micropermeability with confocal laser scanning microscopy (CLSM)

Further teeth for each group (n = 2) were prepared for hybrid layer micropermeability analysis under simulated pulpar pressure. The pulpal tissue was carefully removed with tweezers, after sectioning off the roots 1 mm below the cemento-enamel junction. Bonding procedures were performed as previously described except that the adhesives were doped with 0.1 wt% Rhodamine B (Sigma-Aldricht, Louis, MO, USA) [34]. The fluorescent dye used to trace the water-filled spaces along the bonded interface was a solution of Sodium Fluorescein 5 mM (Sodium Fluorescein, Sigma-Aldricht) under simulated pulpar pressure (20 cm H_2O) for 3 h [34,35]. Then, the teeth were rinsed in ultrasonic bath for 60 s, and sectioned into six 0.4 mm mesio-distal slabs using a slow-speed water-cooled diamond wafering blade. Both sides of the resin–dentin slabs were slightly polished using 1200-grit SiC paper for 30 s followed by ultrasonic bath for 60 s. The bonded interfaces of all slabs were completely

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Table 1 – Adhesive systems, main components, bonding protocols and mode of application.

Adhesive system/composition		Bonding protocol	Mode of application[1]
Adper Scotchbond Multi-Purpose (3M/ESPE)		Wet-bonding	a, b, c, e, f, g, f and i
Universal Etchant	Water, 32% phosphoric acid, synthetic amorphous silica, polyethylene glycol, aluminium oxide.	Wet-bonding DMSO/H_2O	a, b, c, d, c, e, f, g, f and i
Batch# 501113			
Primer	HEMA, polyalkenoic acid methacrylate copolymer,	Wet-bonding DMSO/ethanol	a, b, c, d1, c; e, f, g, f and i
Batch#N751217	water	Dry-bonding	a, b, c1, e, f, g, f and i
Bond	BisGMA, HEMA, dimethacrylates	Dry-bonding DMSO/H_2O	a, b; c1,d, c, e, f, g, f and i
Batch#N668837	photoinitiators	Dry-bonding DMSO/ethanol	a, b, c1; d1; c, e, f, g, f and i
Scotchbond Universal Adhesive (3M/ESPE)		Wet-bonding	a, b, c, h, f and i
		Wet-bonding DMSO/H_2O	a, b, c, d, c, h, f and i
Adhesive	MDP phosphate monomer,	Wet-bonding DMSO/ethanol	a, b, c, d1, c, h, f and i
Batch#506848	dimethacrylate resins, HEMA,	Dry-bonding	a, b, c1, h, f and i
	methacrylate-modified	Dry-bonding DMSO/H_2O	a, b, c1, d, c, h, f and i
	polyalkenoic acid copolymer, filler,	Dry-bonding DMSO/ethanol	a, b, c1, d1, c, h, f and i
	ethanol, water, initiators, silane		

Abbreviations: MDP: 10-methacryloyloxydecyl-dihydrogen-phosphate; BisGMA: bisphenyl-glycidyl methacrylate; HEMA: 2-hydroxyethyl methacrylate.

[1] a: etching for 15 s; b: rinse with water for 15 s; c: blot drying; c1: continuous air drying for 30 s; d: active application of 50% DMSO/H2O 60s; d1: active application of 50% DMSO/Ethanol 60s; e: *Primer* application 10 s; f: gentle blow dry 5 s; g: *Bond* application 10 s, h: *Adhesive* application for 20 s; i: light cure for 10 s.

investigated and representative images of the most common patterns of micropermeability were randomly recorded. Two experienced blinded examiners evaluated all slabs. The imaging procedures were performed using a confocal laser scanning microscope (Leica SP5 TCS-CLSM, Leica Microsystems) equipped with a 63 × 1.4 NA oil immersion lens using 488 nm Argon and a 633 nm Helium–Neon ion laser illumination. CLSM fluorescence images were obtained from 20 μm optical sections using a 0.5 μm z-step, starting 1 μm below the surface. The z-axis scans were compiled into a single and topographic projections using Leica SP5 CLSM image-processing software (Leica, Microsystems).

2.4. *Nanoleakage evaluation*

Two resin–dentin beams from each tooth (n = 8 teeth/group) were randomly selected and submitted to nanoleakage evaluation according to a protocol previously described by Tay et al. [36]. Briefly, beams were placed in the ammoniacal silver nitrate in dark for 24 h, rinsed thoroughly in distilled water and immersed in photo-developing solution for 8 h under fluorescent light to reduce silver ions to metallic silver grains within the nanosized water filled voids along the bonded interface. Resin–dentin beams were gently wet-polished using 600, 1000, 1200 and 4000-grit SiC paper followed by 6, 3, 1 and 0.25 μm diamond pastes (Buehler) and ultrasonically cleaned for 5 min between polishing steps. Samples were dried in silica overnight and carbon coated (CA7625 Carbon Accessory, Quorum Technologies Ltd, United Kingdom). The bonded interfaces were analyzed in a scanning electron microscope (Phenom ProX, Phenom-World, Eindhoven, Netherlands) in backscattered electron mode. Magnifications ranging from 2500× to 6000× were used to qualitatively characterize the nanoleakage patterns. Sequential micrographs (2500× magnification) including the entire length of the adhesive interface were obtained from each resin–dentin beam. Silver nitrate uptake was quantitatively assessed using 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) for each group was converted into percentage values.

2.5. *Statistical analysis*

Nanoleakage and μTBS data were evaluated separately. Tooth was considered the statistical unit. For the μTBS data, the average value of a minimum of 8 beams per tooth was used for statistical analysis. The number of beams with premature failures were recorded and included as 0 MPa. Data from the μTBS test were normally distributed (Kolmogorov–Smirnov Test, $p = 0.200$) and homoscedastic (Levene Test, $p = 0.529$). Three-way ANOVA was performed to evaluate the statistical interactions of the independent variables "adhesive system", "dentin moisture" and "dentin pretreatment". Microtensile bond strength (MPa) was considered the dependent variable. Statistical significance level was set in advance at $\alpha = 0.05$. *Post hoc* multiple comparisons were performed with Tukey's studentized range (HSD) test. As the normality assumption of the nanoleakage data was violated, data was analyzed by Kruskal–Wallis followed by Dunn-Bonferroni multiple comparison test. Statistical significance was preset at $\alpha = 0.05$ using SAS statistical software (SAS 9.4 Software, SAS Institute, NC, USA).

3. Results

3.1. *Microtensile bond strength*

Three-way ANOVA showed that the interactions between "adhesive system" * "dentin pretreatment" ($p < 0.0001$) and "adhesive system" * "dentin moisture" ($p = 0.005$) had signif-

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Table 2 – Microtensile bond strength values of wet- and dry-bonding protocols using DMSO/H_2O and DMSO/ethanol solutions as dentin pretreatments.

	Scotchbond Multi-Purpose		Scotchbond Universal	
	Wet-bonding	Dry-bonding	Wet-bonding	Dry-bonding
Control	30.13 ± 4.99^{Ba} [64–25/35/4] (4.5%)	17.53 ± 2.81^{Bb} [58–44/12/2] (20.5%)	27.46 ± 4.01^{Aa} [69–28/39/2] (5.5%)	27.58 ± 4.55^{Aa} [64–22/40/2] (5.9%)
DMSO/H_2O	43.68 ± 7.03^{Aa} [64–21/38/5] (3%)	40.47 ± 4.29^{Aa} [67–24/35/8] (4.3%)	30.31 ± 3.35^{Ab} [64–23/36/5] (7.2%)	31.13 ± 3.66^{Ab} [67–22/42/3] (6.9%)
DMSO/ethanol	42.86 ± 5.87^{Aa} [65–22/41/2] (4.4%)	41.80 ± 4.73^{Aa} [69–22/41/6] (2.8%)	32.91 ± 3.29^{Ab} [66–21/36/9] (4.3%)	31.78 ± 5.61^{Ab} [66–19/43/4] (5.7%)

Dentin bond strength (MPa) means and standard deviation for all groups (n = 8). Similar superscripts capital letters indicate no significant differences within each group (columns) and similar superscript lowercase letters indicate no significant differences between the groups with the same treatment (rows) according to Tukey's studentized range (HSD) test ($p > 0.05$). The total number of tested resin–dentin beams and their failure modes for each group are expressed into brackets as [total number of tested beams—adhesive/mix/cohesive failures]. The percentage of premature failures is indicated in parentheses.

icant effects on dentin bond strength. The triple interaction "dentin pretreatment" * "adhesive system" * "dentin moisture" ($p = 0.1$) was not significant. The mean cross-sectional area of tested resin–dentin beams (0.76 mm^2 ±0.2) ranged from 0.71 to 0.84 mm^2 with no significant differences between the groups ($p = 0.64$). Microtensile means (MPa), standard deviations, pretest failures and fracture pattern distribution for all groups are reported in Table 2. Wet-bonding SBMP with DMSO/H_2O and DMSO/ethanol pretreatments provided significantly higher bond strengths ($p < 0.05$) compared to SBMP wet-bonding control group roughly by 45%. Dry-bonding significantly reduced SBMP bond strength by over 40% ($p < 0.05$). However, when DMSO/H_2O and DMSO/ethanol pretreatments using SBMP were performed on dehydrated dentin, significantly two-fold higher bond strengths were obtained compared to the dry-bonding control group ($p < 0.05$), without significant differences between the DMSO-pretreatments. No significant differences in SBMP dentin bond strengths occurred when DMSO/H_2O or DMSO/ethanol treatments were used irrespective of dentin condition. No significant differences were observed between SBMP and SU when manufacturer's instructions using the wet-bonding technique were followed. Unlike SBMP, dry-bonding had no significant impact on SU bond strength. DMSO/H_2O and DMSO/ethanol pretreatments had no significant influence on SU bond strength irrespective of whether wet or dry-bonding approaches were used.

Mixed failure was the most common fracture pattern. Dry-bonding produced higher percentages of adhesive failures for SBMP compared to wet-bonding over 60%, on top of producing a total of 20% pre-test failures. No substantial changes occurred when both DMSO pretreatments were used with SBMP under dry and wet conditions, producing similar failure modes to SBMP control. Dry-bonding had no impact on SU failure modes. Wet-bonding of SU with DMSO/ethanol produced 20% reduction in adhesive failures compared to control group; dry-bonding with DMSO/H_2O and DMSO/ethanol reduced approximately 20% and 30%, respectively.

3.2. *Hybrid layer micropermeability evaluation using CLSM*

Representative CLSM images of resin–dentin interfaces are shown in Fig. 1. All samples presented some degree of fluorescein penetration (i.e. micropermeability of water from the dentinal tubules) along the hybrid layer and/or around resin tags with differences in the extent of micropermeability levels in accordance with the bonding protocol used. SBMP wet-bonding (Fig. 1A) produced micropermeability sites throughout the entire bottom and lower half of the hybrid layer: fluorescein easily penetrated around resin tags extending throughout the hybrid layer thickness. Porous zones 3–4 μm wide immediately below the hybrid layer were identified. SU (Fig. 1C) produced a similar micropermeability pattern except that heavy fluorescein deposits around resin tags extending towards the hybrid layer were more evident. In general, wet-bonding of SU produced wider fluorescent bands below the hybrid layer depicting higher micropermeability compared to SBMP, irrespective of dentin pretreatment. While DMSO/H_2O had no considerable impact on micropermeability levels of wet-bonded SBMP (Fig. 1E), it reduced the extension of fluorescein deposits for SU in wet-bonding (Fig. 1G). Conversely, DMSO/ethanol markedly reduced fluorescent sites nearby the hybrid layer for wet-bonded SBMP and SU: for SBMP (Fig. 1I) fluorescein uptake was limited to minimal deposits sparsely located mostly around resin tags; while for SU (Fig. 1K), diffused-scattered fluorescent sites were observed.

Dry-bonding of SBMP (Fig. 1B) produced extensive fluorescein deposits throughout most of the hybrid layer and around resin tags forming wide fluorescent bands (roughly 7–10 μm). Differently from SBMP, similar levels of fluorescein uptake in dry-bonded SU (Fig. 1D) and SU control group were evident, but with reduced fluorescein accumulation around dry-bonded resin tags. DMSO/H_2O (Fig. 1F) and DMSO/ethanol (Fig. 1J) pretreatments reduced micropermeability in dry-bonded SBMP samples, with the latter producing only sparse fluorescein deposits mainly around resin tags. For dry-bonded SU interfaces, both DMSO pretreatment solutions generally reduced the extension of fluorescein bands regardless of dentin moisture. Micropermeability levels for dry-bonded SU using DMSO/H_2O (Fig. 1H) were lower than dry-bonded SU (Fig. 1D). DMSO/H_2O produced comparable results in both wet and dry dentin; however, DMSO/ethanol (Fig. 1L) reduced fluorescein deposition within the hybrid layer when SU was applied on dry dentin. Dry-bonding of SU with DMSO/ethanol produced thin fluorescent bands (1–3 μm) several microns

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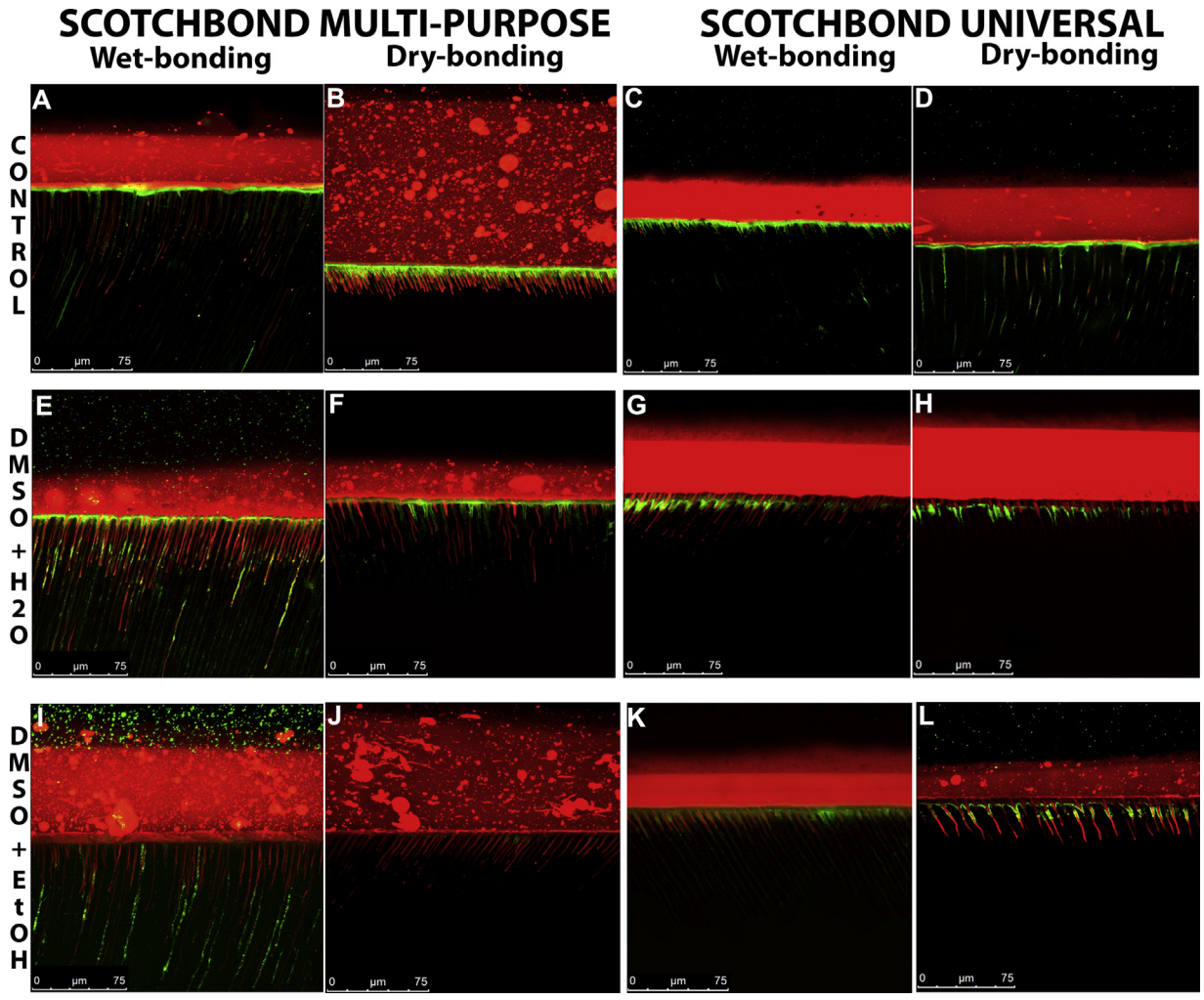

Fig. 1 – Representative micropermeability confocal laser scanning micrographs of DMSO-treated etched-dentin bonded with SBMP and SU following wet- and dry-bonding protocols. Sodium fluorescein under simulated pulpar pressure was used as a tracer solution to evaluate the dentin sealing ability of the proposed bonding protocols.

away from the hybrid layer. Nevertheless, dry-bonding of SU using DMSO/H_2O and DMSO/ethanol generally produced higher micropermeability levels compared to the respective dry-bonded SBMP groups.

3.3. *Nanoleakage evaluation*

Representative backscattered SEM micrographs showing the most common nanoleakage patterns/levels for all groups are presented in Fig. 2. The distributions of nanoleakage percentages along the hybrid layer for all groups are quantitatively shown in Fig. 3. All analyzed beams presented silver deposits within the resin–dentin interfaces. While wet-bonded SBMP presented few discontinuous areas of reticular silver deposits mostly located at the base of the hybrid layer (Fig. 2A), dry bonding produced a significant two-fold higher silver uptake composed mostly of dense reticular deposits along the entire extension of the bulk of hybrid layer (Fig. 2B). Regardless of dentin conditions (i.e. dry or wet) prior to bonding, pretreatment with DMSO/H_2O produced similar patterns nanoleakage levels (Fig. 2E and F) compared to SBMP control group (Fig. 2A) without significant differences in nanoleakage extension. Significantly lower silver uptake occurred when DMSO/ethanol pretreatments were performed on wet (−54%), but especially on dry dentin (−71%). High-magnification views of SBMP DMSO/ethanol specimens revealed the existence of isolated minor silver grains along the deepest part of the hybrid layer for both wet and dry bonding protocols (Fig. 2I and J). In general, SU presented significantly higher silver uptake when bonded to acid-etched dentin compared to SBMP. In SU wet-bonding control group, spotted silver grains could be identified along most of the extension of the hybrid layer with few areas presenting reticular deposits (Fig. 2C). SU wet-bonded to DMSO/H_2O pretreated dentin also presented mostly a spotted pattern with sparse reticular silver deposits occupying most of the bulk of the hybrid layer without significant differences in silver uptake compared to SU wet-bonding control group. Significant lower extents of nanoleakage occurred on dry-bonding with (−22%) and without DMSO/water (−28%) compared to SU wet-bonding control group. Similar spotted patterns (Fig. 2H and D) to the SU wet-bonded group (Fig. 2C) were observed, but with reduced silver grain size and over-

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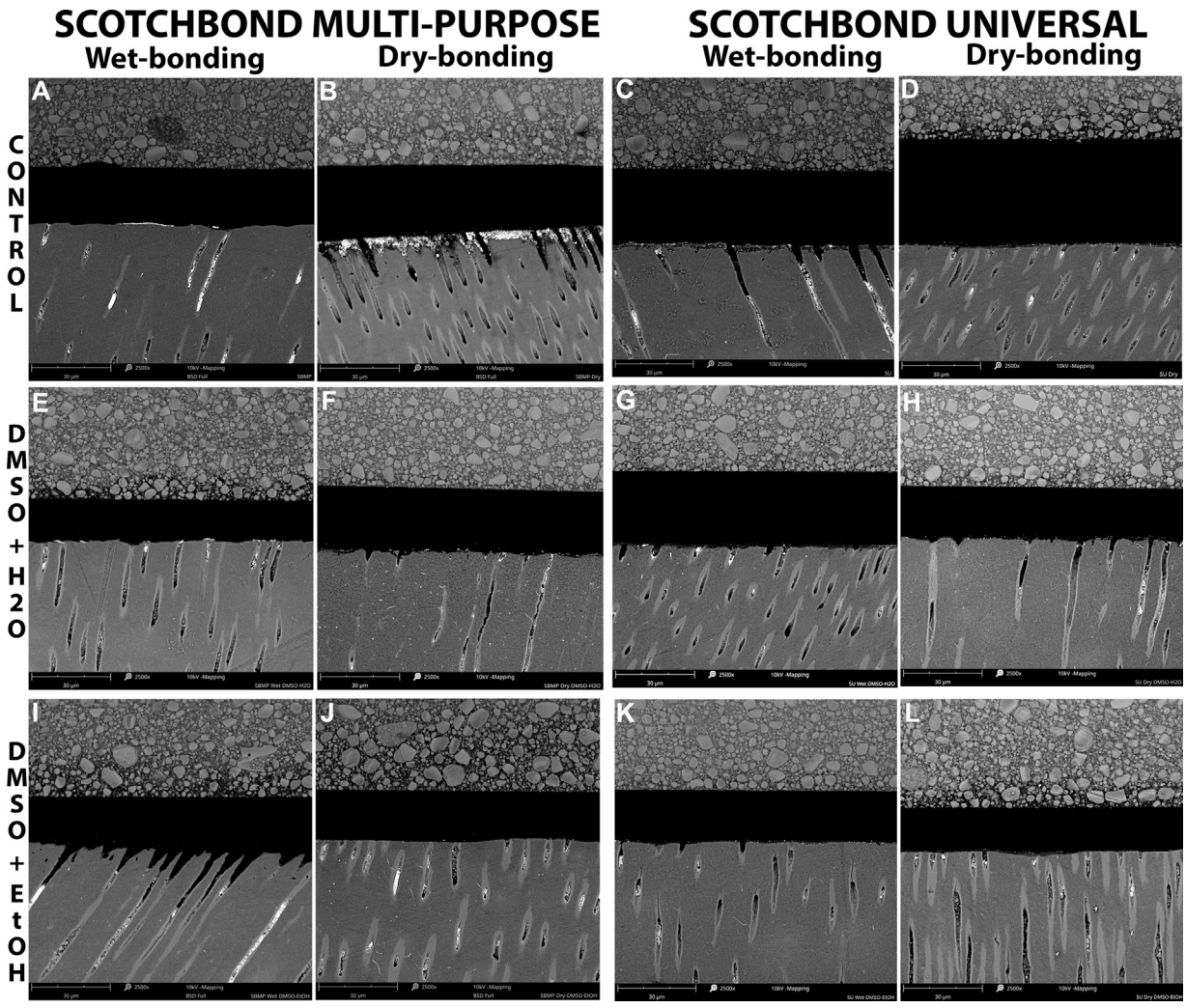

Fig. 2 – Representative nanoleakage backscattered SEM micrographs of DMSO-treated acid-etched dentin with SBMP and SU following wet- and dry-bonding protocols. Silver deposits within the hybrid layer depict the formation of porous water-filled interfaces.

all density. DMSO/ethanol pretreatment also produced sparse spotted silver deposits with significantly 30% lower nanoleakage expression compared to SU wet-bonding control group, irrespective of either wet- (Fig. 2K) or dry-bonding protocol (Fig. 2L).

4. Discussion

Since the interactions between the tested "dentin pretreatments" containing DMSO and "dentin moisture" ($p = 0.005$) had a significant impact on dentin bond strength, the first null hypothesis was rejected. In this context, the effects of "dentin pretreatment" and "dentin moisture" on dentin bond strength were adhesive-dependent. Both DMSO/H_2O and DMSO/ethanol pretreatments produced higher bond strengths irrespective of whether dry- or wet-bonding were performed when the multi-step etch-and-rinse adhesive was used. The mechanism in which DMSO-pretreatments affect dentin bonding is still not yet fully understood. However, DMSO improves adhesive infiltration into demineralized dentin [8] most likely due to biomodification of the collagen matrix as a result of: (i) increased spacing between the collagen microfibrils [22,25]; (ii) improvement in dentin wettability [28]; (iii) reduction in water's self-associative tendency [26]; and (iv) DMSO's ability to act as a penetration enhancer [24]. Therefore, DMSO/H_2O dentin pretreatment improved the monomer-dentin interaction contributing to enhanced bond strengths as previously presented [8].

The ability to bond relatively hydrophobic adhesives to dry demineralized collagen matrix in a clinically relevant time frame is somewhat controversial. According to Raoult's law, removal of excess water by evaporation before it has been mixed with solvated monomers allows rapid residual water removal [37,38]. Hydrogen bonding between water and monomers hinders effective water removal by evaporation [37]. For this reason, the dry-bonding approach used with etch-and-rinse adhesives to eliminate water before hybridization has regained attention as an efficient method to reduce water affinity of resin–dentin bonds [14,39–41]. The main limitation is that chemical or physical dehydration of demineralized

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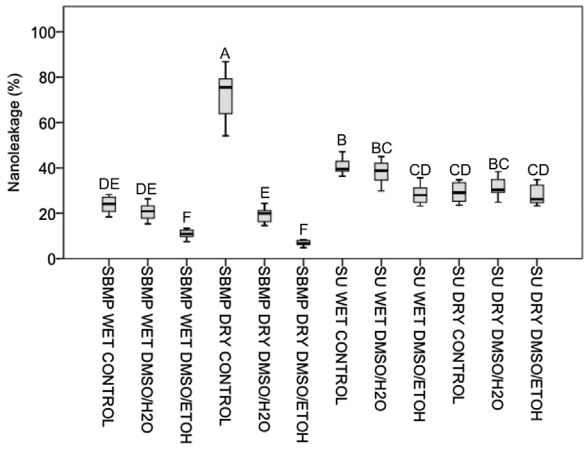

Fig. 3 – Boxplot of nanoleakage extension (%) within the hybrid layer of wet- and dry-dentin samples (n = 8) bonded with SBMP and SU using DMSO solvated in either water (DMSO/H_2O) or ethanol (DMSO/EtOH) as pretreatments. The box contains 50% of the data and the middle line of the box represents the median nanoleakage percentage distribution. The whiskers extend between the minimum and maximum value measured. Different capital letters indicate significant differences in nanoleakage percentages according to Dunn-Bonferroni post-hoc test ($p < 0.05$).

Table 3 – Hoy's solubility parameters of common solvents used in adhesive dentistry including their mixtures for the tested pretreatment solutions (v/v), monomers and collagen in different conditions.

Substance	δ_d	δ_p	δ_h	δ_t
Water	12.2	22.8	40.4	48.0
Ethanol	12.6	11.2	20.0	26.1
DMSO	13.1	16.2	13.1	24.6
50% DMSO/H_2O	12.6	19.4	26.8	35.4
50% DMSO/ethanol	12.8	13.7	16.6	25.0
BisGMA	16.6	13.4	5.8	22.1
HEMA	13.3	12.3	15.2	23.6
Wet collagen	11.8	15.3	22.5	30.1
Dry collagen	11.7	12.1	14.8	22.5

Values are in $(J/cm^3)^{1/2}$. δ_d—Hoy's solubility parameter for dispersive forces; δ_p—Hoy's solubility parameter for polar forces; δ_h—Hoy's solubility parameter for hydrogen bonding forces; δ_t—Hoy's total solubility parameter.

dentin brings collagen fibrils into contact facilitating hydrogen bonding between polypeptide chains. This shrinkage phenomenon reduces collagen interfibrillar spaces that serve as diffusion channels for resin infiltration. If the air-dried shrunk collagen matrix is not re-expanded before adhesive application, bonding to dry dentin can be severely jeopardized [13,14]. This is in agreement with the present results showing that in general dry-bonding of *etch-and-rinse* adhesives produce lower microtensile values compared to conventional wet-bonding. Previous attempts to enable dry-bonding of *etch-and-rinse* adhesives to demineralized dentin (*e.g.* vigorous adhesive application) produced at best comparable bond strengths to the conventional wet-bonding protocol [40,42]. Nevertheless, the collapse of dried collagen matrix caused by air-drying can be reversible [13,40] as previously reported with the use of DMSO-water solutions [28] in a concentration dependent manner. Hoy's solubility parameters of the tested pretreatment solutions (Table 3) and their interaction with wet or dry collagen provide a plausible insight to stablish the ability of treatment solution re-expand collapsed collagen [13]. To re-expand dried collagen allowing proper adhesive penetration, interpeptide hydrogen bonding (hydrogen bonding force (δ_h) 14.8 $(J/cm^3)^{1/2}$) must be broken [13]. 50% DMSO/H_2O (δ_h 26.8) and 50% DMSO/ethanol (δ_h 16.6) dentin pretreatments actively applied for 60 s acted as re-expanding solutions with higher δ_h than air-dried collagen and thus were likely able to break such hydrogen bonds resulting with matrix re-expansion [13]. Even though the maximal expansion of dried dentin organic matrix using 50% DMSO/H_2O is lower than pure water, the overall re-expansion of collapsed collagen is around 70% [28]. In the present study, higher dentin bond strengths were obtained for SBMP when air-dried dentin was pretreated by either one of the DMSO solutions. Biomodification of the collagen matrix [8,22] by DMSO pretreatments containing either water or ethanol improved multi-step etch-and-rinse adhesives' bond effectiveness [8] irrespective of dentin moisture. Increased and faster dentin wettability with a fairly modest re-expansion ratio of collapsed collagen [28] may explain the higher bond strengths. To the best of our knowledge, production of higher immediate bond strengths on completely air-dried dentin compared to the conventional wet-bonding has not been previously presented.

In this new proposed bonding approach, application of DMSO/ethanol pretreatment-solution for 60 s on either wet or dry dentin produced higher bond strengths than the conventional wet-bonding protocol when SBMP was used. When the amphiphilic DMSO/ethanol pretreatment was performed on wet dentin, ethanol dehydrated the collagen matrix to some extent contributing to water removal. However, the presence of remaining DMSO (vapor pressure 0.6 mmHg at 25 °C) most likely changed the residual water behavior due to DMSO's ability to break water self-associative tendency and displace water molecules [26]. In this sense, the mechanism involved in the DMSO/ethanol wet-bonding protocol aggregates water displacement when compared to the original ethanol-wet bonding technique, certainly making water replacement/displacement more effective in a relatively short application period. In addition, DMSO competes with water molecules in collagen interpeptide hydrogen bonding [26] and increases collagen interfibrillar spacing [25]. The triple-helical collagen molecules are covered with bound water [43], which limits the interaction of hydrophobic crosslinking monomers with collagen. The oxygen atom in DMSO hydrogen bonds with two [26] or three [44] water molecules, while the methyl groups form a hydrophobic end displacing water from demineralized collagen matrix [28]. Therefore, DMSO may disrupt this layer of water and improve the interaction between collagen and hydrophobic monomers irrespective of the vehicle used in the pretreatment solutions. Indeed, DMSO improves resin infiltration [8] and bond strength [8,22], as shown also in this study.

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SU is a universal etch-and-rinse adhesive containing ethanol and silane in addition to water having part of the original dimethacrylate monomers substituted by the functional monomer 10-methacryloyloxydecyl dihydrogen phosphate (10-MDP). This invariably reduces the overall availability of hydrophobic dimetacrylates crosslinking monomers. Although adhesives with higher crosslinking rates present improved mechanical properties [45], no significant differences in dentin bond strength were observed between SBMP and SU wet-bonded control groups. This is supported by previous findings [46] suggesting that universal adhesives should not perform differently from previous generations of etch-and-rinse adhesives, at least in immediate conditions. Unlike SBMP, dry-bonding did not reduce SU bond strength which is in accordance with previous studies [47,48]. The longer application time of SU (10 s for SBMP vs. 20 s for SU) apparently produced re-expansion of collagen fibrils by the water present in the adhesive composition producing similar immediate bond strengths to wet-bonded samples. Even though SBMP presented higher bond strengths irrespective of dentin moisture for both pretreatments, DMSO pretreatments had no significant effect on immediate bond strength of SU. Similarly, it has been reported that the immediate bond strength of a simplified etch-and-rinse adhesive was not affected by the same DMSO/H_2O pretreatment [31]. Normally, diffusion of high molecular weight monomers across the extension of demineralized dentin is deficient [49]. For instance, the absolute molar concentration of BisGMA at the middle of the hybrid layer is only about 10% of the expected concentrations in an ideally infiltrated hybrid layer [50]. Since DMSO is an effective penetration enhancer [24], it possibly improved BisGMA diffusion across the hybrid layer contributing to polymer chain crosslinking contributing to higher bond strengths for SBMP. Due to reduced availability of crosslinking dimetacrylates monomers in SU, solely favoring their penetration with DMSO limited the improvement of bond strength to the same extent as in SBMP. Reduction of flexible poly-HEMA gel formation in deeper portions of the hybrid layer [49,51] was most likely not as efficient which might explain similar bond strengths in SU treated and untreated groups. More studies using Raman spectroscopy and nanoindentation should be performed to evaluate the monomer composition and mechanical properties of DMSO-treated hybrid layer.

The presence of excess water during dentin hybridization increases the formation of hydrogels at the HEMA-rich lower half of hybrid layer [49,50] resulting in water permeable resin–dentin interfaces. Silver [36] and fluorescein [34,35] deposition on the bonded interface reflected the presence of such water-rich zones revealing inconsistent resin-infiltration of demineralized collagen in both adhesives. While nanoleakage analyses in SEM permits a precise interpretation with higher resolution images of the silver deposition within the microporosities at the hybrid layer, micropermeability provides an indication of the relative sealing of the bonded interface [52,53]. Moreover, using a water-based fluorescein tracer solution under simulated pulpar pressure and CLSM for micropermeability assessment allows direct fluid movement visualization with minimal specimen preparation reducing possible artifacts [53]. Since the extension of fluorescein and silver deposition along the bonded interface of SBMP and SU samples was affected by dentin moisture and the DMSO solutions used, the second null hypothesis was rejected. While dry-bonding severely compromised the sealing ability of SBMP, no negative effects were observed on SU. However, DMSO pretreatments reduced micropermeability and nanoleakage levels for both adhesives, reducing the extension of the porous water-rich poorly impregnated resin layer immediately below the hybrid layer even when dry-bonding was performed. The vehicle used for DMSO application also had an impact on micropermeability and nanoleakage levels: DMSO/ethanol pretreatment produced the lowest leakage levels irrespective of the adhesive used, especially in dry-bonding. Extensive air-drying followed by application of DMSO/ethanol solution invariably reduced the amount of water present at the hybrid layer and produced a favorable dentin bonding substrate. Therefore, the combination of residual water evaporation by extensive air drying and DMSO/ethanol pretreatment seems to be a highly promising method to reduce microporosities at the hybrid layer irrespective of dentin moisture condition.

5. Conclusion

This study presents compelling evidence that residual water removal from resin–dentin interfaces of simplified and three-step *etch-and-rinse* adhesives may be possible by air drying in a clinically realistic time frame without compromising – bonding effectiveness. Furthermore, DMSO/H_2O and DMSO/ethanol pretreatments on either dry or wet dentin improved resin–dentin interfaces by increasing SBMP bond strength and reducing overall nanoleakage and micropermeability levels of both SBMP and SU bonded interfaces. The proposed DMSO-pretreatments may have potential benefits on the etch-and-rinse bonding mechanism producing improved hybrid layers with reduced defective and vulnerable degradation sites irrespective of whether dry- or wet-bonding techniques are employed.

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New perspective to improve dentin–adhesive interface stability by using dimethyl sulfoxide wet-bonding and epigallocatechin-3-gallate

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ABSTRACT

Objectives. To determine whether dentin–adhesive interface stability would be improved by dimethyl sulfoxide (DMSO) wet-bonding and epigallocatechin-3-gallate (EGCG).

Methods. Etched dentin surfaces from sound third molars were randomly assigned to five groups according to different pretreatments: group 1, water wet-bonding (WWB); group 2, 50% (v/v) DMSO wet-bonding (DWB); groups 3–5, 0.01, 0.1, and 1 wt% EGCG-incorporated 50% (v/v) DMSO wet-bonding (0.01%, 0.1%, and 1%EGCG/DWB). Singlebond universal adhesive was applied to the pretreated dentin surfaces, and composite buildups were constructed. Microtensile bond strength (μTBS) and interfacial nanoleakage were respectively examined after 24 h water storage or 1-month collagenase ageing. In situ zymography and*Streptococcus mutans* (*S. mutans*) biofilm formation were also investigated.

Results. After collagenase ageing, μTBS of groups 4 (0.1%EGCG/DWB) and 5 (1%EGCG/DWB) did not decrease ($p > 0.05$) and was higher than that of the other three groups ($p < 0.05$). Nanoleakage expression of groups 4 and 5 was less than that of the other three groups ($p < 0.05$), regardless of collagenase ageing. Metalloproteinase activities within the hybrid layer in groups 4 and 5 were suppressed. Furthermore, pretreatment with 1%EGCG/DWB (group 5) efficiently inhibited *S. mutans* biofilm formation along the dentin–adhesive interface.

Significance. This study suggested that the synergistic action of DMSO wet-bonding and EGCG can effectively improve dentin–adhesive interface stability. This strategy provides clinicians with promising benefits to achieve desirable dentin bonding performance and to prevent secondary caries, thereby extending the longevity of adhesive restorations.

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1. Introduction

To minimize tooth preparation and acquire satisfactory aesthetic outcome, adhesive technique has become one of the greatest inventions in clinical dentistry in the 20th century. The durability of dentin bonding, however, remains limited despite its immediate efficiency [1]. Insufficient bonding durability may lead to frequent replacement of restorations and extra cost [2]. Therefore, effective measures to improve dentin bonding durability are urgently needed, thus extending the service life of adhesive restorations.

The decline of dentin bond strength is principally caused by the degradation of the hybrid layer at dentin–adhesive bonding interfaces [3]. Evidence has proven that adhesive hydrolysis, enzymolysis from cysteine cathepsin and matrix metalloproteinases (MMPs), inadequate penetration of resin monomers, and secondary caries are potential threatens for hybrid layer degradation [4,5]. Strategies, including application of collagen cross-linkers, MMP inhibitors, ethanol wet-bonding, and biomimetic remineralization, have been designed to preserve the integrity of dentin–adhesive interfaces for reliable bonding stability [6,7].

Wettability plays a vital role in facilitating dentin bonding performance [8]. Water wet-bonding method was once widely used in dentin bonding [9]. However, controlling the humidity is technology-sensitive because excessive wetting or drying may cause a negative effect on bond strength [10]. Ethanol wet-bonding was introduced since it enables ethanol to replace water in dentin matrix and support demineralized collagen fibers, thereby prompting the penetration of hydrophobic adhesive monomers and avoiding collagen collapse to achieve desirable bonding efficiency [11]. Whereas, ethanol has high vapor pressure, the replacement of adequate water is time-consuming, which seems impractical in clinical applications [12].

Dimethyl sulfoxide (DMSO), an aprotic polar solvent with a highly polar S=O group and two hydrophobic CH_3 groups, can dissolve polar and nonpolar compounds [13]. Compared with ethanol, DMSO possesses lower volatility and stronger permeability. These characteristics compensate for the high technique susceptibility of ethanol wet-bonding. DMSO can dissociate the highly cross-linked dentin collagen into a discrete fibril network, thereby improving the wettability of demineralized dentin and the penetration of monomer into etched dentin matrix and re-expanding collapsed collagen to a moderate level [14,15]. Previous studies have proven that the application of a 50% (v/v) DMSO aqueous solution can significantly improve the dentin bonding durability and hybrid layer stability [16,17]. These findings suggest DMSO as an ideal solvent to achieve long-term dentin bond strength.

Considerable attention has been focused on MMP inhibitors in recent decades because of their clinical feasibility and ability to prevent the degradation of collagen fibrils with incomplete resin infiltration [18]. Owing to concerns on drug resistance and potential cytotoxicity of chemical synthetics, such as chlorhexidine [19,20], naturally sourced materials are highly desired and widely investigated. Epigallocatechin-3-gallate (EGCG), a sort of green tea extract, has become popular on account of its anti-inflammatory, antioxidant, antibacterial, and anticancer efficacy [21]. EGCG possesses favorable biocompatibility and suppresses MMP-2 and MMP-9 activities to prevent dentin collagen degradation [22]. The formation of cariogenic bacteria biofilm can be effectively inhibited by EGCG [23,24], which is beneficial to the stability of the dentin–adhesive bonding interfaces. However, the biological functions of EGCG may be limited because of its low solubility in water [25]. Although EGCG is more soluble in ethanol than in water, the technical sensitivity of ethanol wet-bonding and the rapid volatilization of ethanol when exposed to air remain a conundrum. Fortunately, DMSO is of particularly low volatility and EGCG can be well dissolved in DMSO aqueous solution at room temperature.

To maximize the efficacy of DMSO and EGCG in stabilizing the dentin–adhesive bonding interfaces, the application of DMSO wet-bonding combined with EGCG may be a promising strategy for improving dentin bonding durability and preventing secondary caries. To the best of our knowledge, no relevant information is available.

Therefore, the present study aimed to investigate the synergistic effects of DMSO wet-bonding and EGCG on improving the dentin–adhesive interface stability. The null hypotheses were that the adjunctive application of DMSO wet-bonding and EGCG on dentin (i) would not affect the immediate or long-term dentin bonding performance and (ii) would not reduce bacterial biofilm growth along the dentin–adhesive interface.

2. Materials and methods

2.1. Specimen preparation and bonding protocols

Sixty-five sound third molars were obtained under an informed consent from the donors reviewed and approved by the Ethics Committee for Human Studies of the School and Hospital of Stomatology, Wuhan University, China [no. 2011(067)]. These teeth were preserved in 0.5% (w/v) thymol solution at 4 °C before use. A 50% (v/v) DMSO aqueous solution was immediately prepared before use by mixing DMSO (Sigma-Aldrich, St. Louis, MO, USA) in sterilized deionized water. EGCG powder (Sigma-Aldrich, St. Louis, MO, USA) was freeze-dried and dissolved into the 50% (v/v) DMSO aqueous solution to obtain four experimental solutions at concentrations of 0, 0.01, 0.1, and 1 wt%, respectively.

Thirty of the sixty-five teeth were sectioned below the enamel-dentinal junction by using a diamond saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) with low speed to expose flat mid-coronal dentin surfaces under water-cooling. These dentin surfaces were burnished by using a 600-grit silicon carbide paper for 1 min under water irrigation to yield a standardized smear layer. After etched with 35% phosphoric-acid gel (3 M ESPE, St. Paul, MN, USA) for 15 s, each dentin surface was thoroughly sprayed with deionized water and blot-dried. These teeth were randomly assigned to five groups ($n = 6$ teeth each group) based on different treatment protocols:

- Group 1: deionized water (water wet-bonding, WWB group).
- Group 2: 50% (v/v) DMSO aqueous solution (DMSO wet-bonding, DWB group).

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- Group 3: 0.01 wt% EGCG-incorporated 50% (v/v) DMSO aqueous solution (0.01%EGCG/DWB group).
- Group 4: 0.1 wt% EGCG-incorporated 50% (v/v) DMSO aqueous solution (0.1%EGCG/DWB group).
- Group 5: 1 wt% EGCG-incorporated 50% (v/v) DMSO aqueous solution (1%EGCG/DWB group).

The dentin surface of each group was pretreated with the corresponding solution via a microbrush for 60 s, respectively, and then blot-dried with filter papers to generate a visible moist surface which liquids were no longer able to remove. The specimens were bonded with a Singlebond Universal (3 M ESPE, St. Paul, MN, USA) adhesive in accordance with the manufacturer's instructions by the same proficient dentist. After 15 s polymerization by a Bluephase Style light-curing unit (Ivoclar-Vivadent, Amherst, NY, USA), build-ups of resin composite (Charisma, Haraeus Kulzer, Hanau, Germany) were constructed on top of the bonded surfaces in four 1-mm increments, and each increment was light-cured for 20 s.

2.2. Microtensile bond strength (μTBS) test

After 24 h storage in deionized water at 37 °C, the bonded teeth were sectioned perpendicular to the bonding interfaces into slabs of 0.9 mm thickness. Six middle slabs randomly selected from each group were stored for interfacial nanoleakage assessment (n = 4 slabs each group) and in situ zymography of the hybrid layer (n = 2 slabs each group). The remaining slabs were further sliced into beams of 0.9 mm × 0.9 mm. Unqualified beams either situated peripherally or accompanied enamel residual were excluded, and ten qualified beams were selected from each tooth. Five of them (n = 30 each group) were immediately tested for μTBS, and the other five (n = 30 each group) were examined after 1-month collagenase ageing. The ageing solution of collagenase was obtained by dissolving collagenase of *Clostridium histolyticum* (Sigma-Aldrich, St. Louis, MO, USA) into artificial saliva (20 mM HEPES buffer, 30 mM KCl, 4.0 mM KH_2PO_4, 0.2 mM $MgCl_2{\cdot}6H_2O$, 0.7 mM $CaCl_2$, 0.3 mM NaN_3, pH 7.4 [26]) to achieve a concentration at 0.1 mg/mL. The ageing specimens were stored in this collagenase solution at 37 °C, protected from light.

Beams used for μTBS were individually fixed to a machine for microtensile testing (Bisco Inc., Schaumburg, IL, USA) via a Zapit adhesive (Dental Ventures of America, Corona, CA, USA), and were loaded in tension at a cross-head speed of 1 mm per min until fracture. After recording the maximum fracture load, a digital caliper was used to measure the cross-sectional area of each beam and the actual bond strength values were calculated in Megapascal (MPa).

2.3. Fracture pattern analysis

After μTBS test, the fractured surface of each beam was collected, desiccated, sputter-coated with Au-Pd alloy (JFC-1600, JEOL, Tokyo, Japan), and then examined by a field-emission scanning electron microscopy (FESEM, Carl Zeiss Sigma, Jena, Germany). The fracture patterns were distributed into four groups: (*A*) adhesive failure; (*CC*) cohesive failure in composite; (*CD*) cohesive failure in dentin; and (*M*) mixed failure [27].

2.4. Interfacial nanoleakage evaluation

Four of the stored middle slabs selected from each group were randomly classified to be treated immediately or after 1-month collagenase ageing (n = 2 slabs each subgroup). After coated with two layers of nail varnish which left 1 mm away from the bonded interface, the slabs were soaked in a 50% (w/v) ammoniacal silver nitrate solution for 24 h in darkness, followed by thoroughly rinsing in deionized water. The slabs were further dipped in a photo-developing solution for another 8 h exposure to fluorescent light, then wet-ground using 600, 800, 1200, and 2000-grit silicon carbide papers and 0.25 μm diamond paste, ultrasonically cleaned, desiccated, and sputter-coated with carbon (JFC-1600, JEOL, Tokyo, Japan).

The interfacial nanoleakage was evaluated with FESEM under a back-scattered electron mode. Ten fields-of-view along the bonding interface of each slab were randomly captured (20 images for each subgroup). NIH Image J software (Bethesda, MD, USA) was used to compute the nanoleakage percentage of silver nitrate deposition within the dentin–adhesive layer. The percentage was scored by two examiners on a range of 0–4 according to a protocol previously described as below [28]: 0, no nanoleakage; 1, <25% nanoleakage; 2, 25%≤50% nanoleakage; 3, 50%≤75% nanoleakage; and 4, >75% nanoleakage. Consistency between the results of observers was determined by Kappa test (K = 0.86).

2.5. In situ zymography of the hybrid layer

Two of the stored middle slabs randomly selected from each group were utilized for in situ zymography test. A mixture of fluorescein-conjugated gelatin (E-12055, Molecular Probes, Eugene, OR, USA) was prepared immediately before use according to the manufacturer's protocols. The slabs were wet-ground to approximately 50 μm thickness and placed on a microscope slide. The gelatin mixture was then dropped individually on each slab, followed by covering a coverslip. All the slabs were incubated in a humidified chamber protected from light at 37 °C for 24 h. Each slab was visualized with a confocal laser scanning microscope (CLSM) (Fluoview FV1200, Olympus, Tokyo, Japan) in fluorescence mode by using 40 × objective lens of 0.95 NA under the excitation/emission wavelengths of 488/530 nm. Three images obtained from the same z layer were randomly captured for each slab. All images (n = 6 images each group) were analyzed and quantified using a NIH Image J 1.8.0 software (Bethesda, MD, USA) to inspect the hydrolysis of the fluorescein-conjugated gelatin substrates; this process can be suggestive of the activity of the endogenous gelatinolytic enzyme based on the value of relative green fluorescence [29].

2.6. Contact angle measurement

Twenty of the sixty-five third molars were sectioned below the enamel-dentinal junction by using the diamond saw with low speed to produce dentin disks (0.5 mm thickness). All the disks were wet-ground with 600, 800, 1200, and 2000-grit silicon carbide papers and 0.25 μm diamond paste, ultrasonically cleaned, and etched with 35% phosphoric-acid gel for 15 s. After rinsed with deionized water and blot-dried, these disks were randomly assigned to five groups (n = 8 disks each

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group) in a same procedure as descried in Section 2.1.: group 1 (WWB); group 2 (DWB); group 3 (0.01%EGCG/DWB); group 4 (0.1%EGCG/DWB); group 5 (1%EGCG/DWB). Contact angle measurement was conducted by using a Contact Angle System OCA (Dataphysics Instruments; Filderstadt, Germany). A droplet of 5 μL of Singlebond universal adhesive was deposited on each dentin disk and the optical data of the droplet was captured and measured with a digital-imaging camera. All parameters were kept constant, especially the distance between the dentin surface and the tip.

2.7. Antibacterial activity

2.7.1. Specimens preparation and bacterial culture

Streptococcus mutans (*S. mutans*, ATCC 25175), provided by the School of Stomatology, Wuhan University, was cultivated in a Brain Heart Infusion (Becton-Dickinson & Co., Sparks, MD, USA) broth at 37 °C for 24 h in an anaerobic environment. The concentration of the bacterial suspension was confirmed at 10^8 colony forming units (CFU)/mL before use.

According to the same procedure as described in Section 2.1., the remaining fifteen of the sixty-five third molars were sectioned, wet-ground, etched, rinsed, blot-dried, and randomly distributed into five groups (n = 3 teeth each group) : group 1 (WWB); group 2 (DWB); group 3 (0.01%EGCG/DWB); group 4 (0.1%EGCG/DWB); group 5 (1%EGCG/DWB). The dentin surface of each tooth was then bonded with Singlebond Universal adhesive, light-cured, and constructed with 4 mm resin composite build-ups. After 24 h storage in deionized water at 37 °C, the bonded teeth were longitudinally sectioned into a series of slabs of 0.9 mm-thick across the adhesive interface. Eighteen qualified middle slabs were collected from each group, among which, nine of them randomly selected from each group were immediately tested for the antibacterial activity, and the rest nine slabs were tested after 1-month ageing in sterile deionized water at 37 °C.

The inoculation medium of *S. mutans* was obtained by diluting previously prepared medium with BHI broth supplementing 1% (w/v) sucrose. Each slab was positioned in one well of a 24-well plate and injected with inoculation medium of 1 mL. After 24 h anaerobic incubation at 37 °C for biofilm growth, the biofilm-coated slabs were gently rinsed thrice using sterile phosphate buffer solution (PBS) to wash away non-adherent bacteria.

2.7.2. Live/dead staining of biofilms

Six biofilm-coated slabs (three for immediate test, three for ageing test) randomly selected from each group were stained with a live/dead bacterial viability kit (Molecular Probes, Invitrogen, Eugene, OR, USA). This kit includes two dyes which are SYTO-9 and propidium iodide (PI), enabling live and dead bacteria to be stained to emit green and red fluorescence, respectively [30]. CLSM was used to examine live and dead *S. mutans* biofilm along the dentin–adhesive interface in fluorescence mode by using 40 × objective lens of 0.95 NA under the excitation/emission wavelengths of 488/500 nm for SYTO-9 and 594/635 nm for PI. Three representative stacks (Z-stack) of each biofilm image were captured at a Z-step of 2 μm, starting from the bottom (contacting with the treated surface) to the top of the biofilm. A Bitplane Imaris 7.4.2 software (Zurich, Switzerland) was employed to analyze confocal images obtained from the first 10 layers of each Z-stack and to investigate the inhibitory efficacy on biofilm formation for each group.

2.7.3. FESEM observation

Six biofilm-coated slabs (three for immediate test, three for ageing test) randomly selected from each group were utilized to survey *S. mutans* adhesion and biofilm formation along the dentin–adhesive interface. Each slab was fixed with 2.5% glutaraldehyde for 4 h then gradient dehydrated by ethanol (30%, 50%, 70%, 80%, and 90% for 20 min, respectively, and 100% for 20 min twice). After desiccated and sputter-coated with gold (JFC-1600, JEOL, Tokyo, Japan), all slabs were observed by FESEM under a secondary electron (SE2) mode at 5 kV in a high vacuum mode. Three fields-of-view were randomly captured for each slab.

2.7.4. MTT assay

The remaining six biofilm-coated slabs (three for immediate test, three for ageing test) obtained from each group were used to determine the cellular viability of *S. mutans* biofilm. Each slab was transferred to one well of a new 24-well plate containing 1 mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) solution (0.5 mg/mL), and anaerobically incubated at 37 °C for 4 h. After that, the MTT solution of each well was taken out and replaced by 1 mL of DMSO to dissolve the blue/purple formazan, followed by gently shaking for 20 min. The supernatant of each well was detected at 570 nm via a PowerWave XS2 spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). Four readings of each slab were recorded for each group (n = 12). MTT assay was performed in triplicate.

2.8. Statistical analysis

An IBM SPSS Statistics 20.0 software (Armonk, NY, USA) was applied for statistical analysis. For μTBS test, statistical analysis was performed using tooth as the statistical unit; the mean μTBS obtained from the 5 beams of per tooth was used to represent the bond strength of the specific tooth. After the normal distribution of μTBS, gelatinolytic activity, contact angle, and MTT data was confirmed, a two-factor (variables: pretreatment method and collagenase ageing) analysis of variance (ANOVA) with *post-hoc* Tukey's test was used to analyze the values of μTBS test. A Kruskal-Wallis test with Dunnett's *post-hoc* test was utilized to analyze the statistical differences among the interfacial nanoleakage groups in their scores, while inter-examiner reliability was determined by Cohen's kappa test. A one-factor ANOVA with *post-hoc* Tukey's test was conducted to analyze the data of gelatinolytic activity, contact angles and MTT assay. The significance level for all tests was defined at 0.05.

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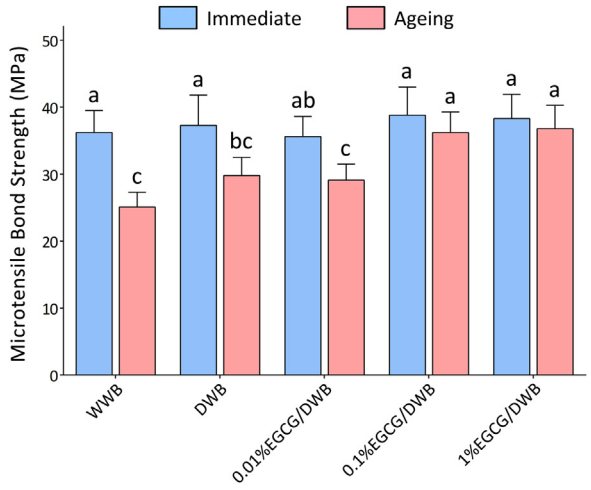

Fig. 1 – Means and standard deviations of microtensile bond strength (μTBS) from each group (n = 6 teeth). Columns identified with different letters are significantly different ($p < 0.05$).

3. Results

3.1. Microtensile bond strength

The mean μTBS values for all groups are represented in Fig. 1. Two-factor ANOVA indicated that the variables of pretreatment method (F = 10.607, p = 0.000) and collagenase ageing (F = 46.425, p = 0.000) significantly influenced the bond strength. The interaction of pretreatment method × collagenase ageing was significant (F = 4.160, p = 0.006), suggesting that the variation of μTBS values among the five groups were dependent on the aforementioned two factors.

For immediate bond strength, there was no significant difference among the five groups ($p > 0.05$). For ageing bond strength, the values of groups 4 (0.1%EGCG/DWB) and 5 (1%EGCG/DWB) were significantly higher than those of the other three groups ($p < 0.05$). Collagenase ageing did not significantly decrease the values in groups 4 and 5 ($p > 0.05$).

3.2. Fracture pattern analysis

The frequency distribution chart of fracture patterns is plotted in Fig. 2. Adhesive failure was the foremost fracture pattern in the immediate groups. After collagenase ageing was implemented, the frequency of adhesive failure reduced in all the five groups, while the incidence of mixed failure increased. Representative images of fracture patterns captured by FESEM are shown in Fig. 3.

3.3. Interfacial nanoleakage evaluation

Table 1 summarizes statistical data of the interfacial nanoleakage. The result of the Kruskal–Wallis test revealed that, irrespective of collagenase ageing, interfacial nanoleakage in groups 4 (0.1%EGCG/DWB) and 5 (1%EGCG/DWB) was significantly less than that in the other three groups ($p < 0.05$).

Fig. 4 exhibits the typical FESEM pictures of nanoleakage from different groups. For immediate evaluation, extensive silver deposits were observed within the hybrid layer in group 1 (WWB, Fig. 4a). By contrast, the silver deposits decreased to some extent in groups 2 (DWB, Fig. 4b) and 3 (0.01%EGCG/DWB, Fig. 4c). Notably, groups 4 (0.1%EGCG/DWB, Fig. 4d) and 5 (1%EGCG/DWB, Fig. 4e) showed apparent fewer silver deposits than the other three groups. After collagenase ageing, the silver uptake in each group did not change substantially (Figs. 4A–4E).

3.4. In situ zymography of hybrid layer

Fig. 5a summarizes the relative percentages of gelatinolytic activity of hybrid layers that exhibited green fluorescence after being labeled with fluorescein-conjugated gelatin mixture. Fig. 5A–E refer to the representative CLSM images of in situ zymography from different groups. Green fluorescence had extensively emerged within the hybrid layer in group 1 (WWB, Fig. 5A) which was considered as having the highest fluorescence intensity of gelatinolytic activity among the five groups ($p < 0.05$). Groups 2 (DWB, Fig. 5B) and 3 (0.01%EGCG/DWB, Fig. 5C) manifested relatively decreased green fluorescence compared to group 1. Scarcely any green fluorescence had emerged within the hybrid layer in groups 4 (0.1%EGCG/DWB, Fig. 5D) and 5 (1%EGCG/DWB, Fig. 5E), and their fluorescence values were significantly lower than those of the other three groups ($p < 0.05$).

3.5. Contact angle measurement

The contact angle value of each group and the corresponding anterior view were plotted in Fig. 6. Group 1 (WWB application) exhibited the highest contact angle among the five tested groups ($p < 0.05$). The low contact angles were observed in DMSO-containing groups (2–5), indicating enhanced permeability of the adhesive into the etched and pretreated dentin.

3.6. Antibacterial activity

Fig. 7 reflects representative images of live/dead *S. mutans* biofilm developed on the surfaces of the dentin–adhesive slabs from different groups. For immediate evaluation, the total amount of bacteria grown along the dentin–adhesive interface in groups 4 (0.1%EGCG/DWB, Fig. 7d) and 5 (1%EGCG/DWB, Fig. 7e) was less than that in the other three groups (Figs. 7a–7c). After 1-month ageing in water, the total amount of bacteria in each group was much more than that in the immediate groups. Furthermore, the lowest number of bacteria among the five ageing groups was observed in group 5 (1%EGCG/DWB, Fig. 7E). A certain degree of red fluorescence could be identified in the dentin–adhesive interface above, which may be induced by the blending of the air-inhibition layer of the cured adhesive with the resin composite during composite polymerization [31].

The representative FESEM images of biofilm formation on the surfaces of the dentin–adhesive slabs from different groups are shown in Fig. 8. In general, the tendency of biofilm formation is in accordance with the CLSM results. Regardless of ageing, group 5 (1%EGCG/DWB, Figs. 7e and 7E) presented

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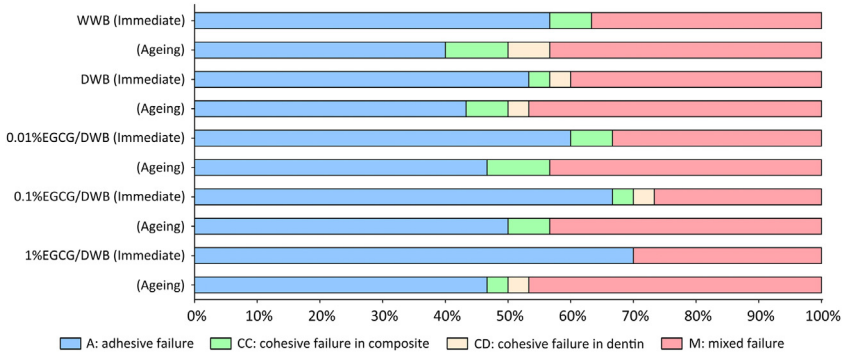

Fig. 2 – Frequency distribution of fracture patterns for each group following microtensile bond strength test.

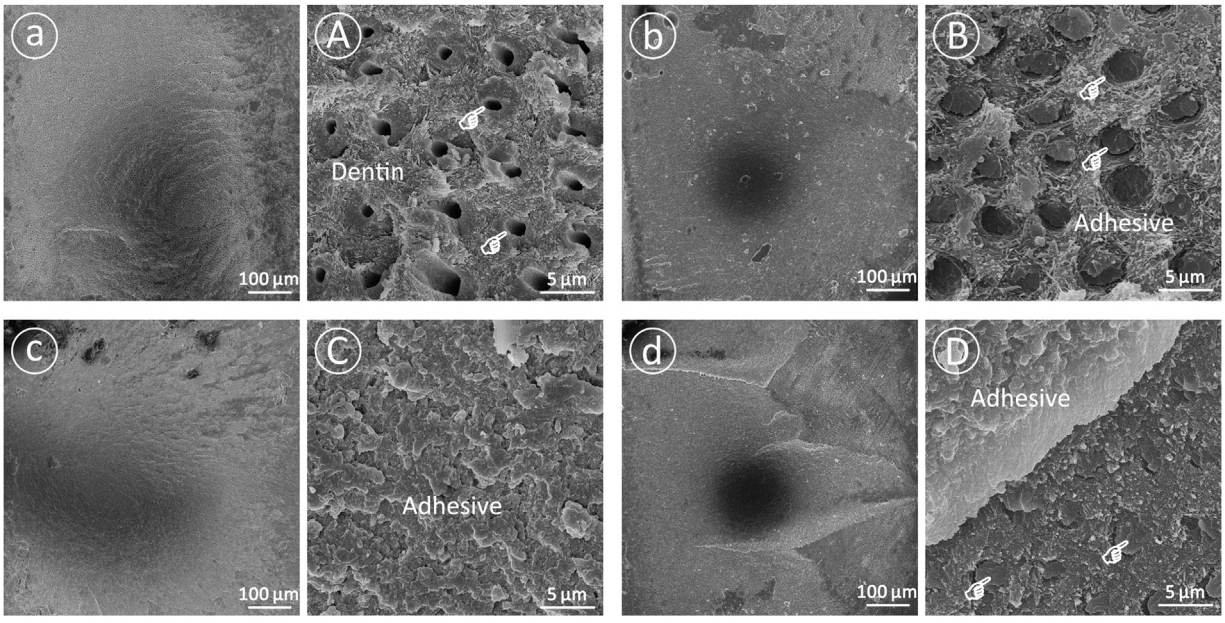

Fig. 3 – Representative FESEM images of fractured surfaces following microtensile bond strength test (a–d, low-magnification of general morphology of fractured surfaces at 100×; A–D—, high-magnification of a–d at 3000×). (A) cohesive failure in dentin, pointers indicating open dentinal tubules; (B) cohesive failure in composite, pointers indicating obstructed tubules; (C) adhesive failure; (D) mixed failure, pointers indicating obstructed tubules.

Table 1 – Percentage distribution of nanoleakage scores from each group for immediate and after 1-month collagenase ageing.

Groups	Time	Score percentage (%)					Statistical difference
		0	1	2	3	4	
WWB	Immediate	0	5	20	40	35	ab
	Ageing	0	0	10	40	50	a
DWB	Immediate	0	15	45	30	10	bc
	Ageing	0	5	30	45	20	abc
0.01%EGCG/DWB	Immediate	0	20	45	25	10	c
	Ageing	0	10	40	35	15	bc
0.1%EGCG/DWB	Immediate	10	70	15	5	0	d
	Ageing	0	65	30	5	0	d
1%EGCG/DWB	Immediate	15	80	5	0	0	d
	Ageing	5	70	20	5	0	d

The Kruskal–Wallis test with Dunnett's *post-hoc* test. Groups identified with different letters are significantly different ($p < 0.05$).

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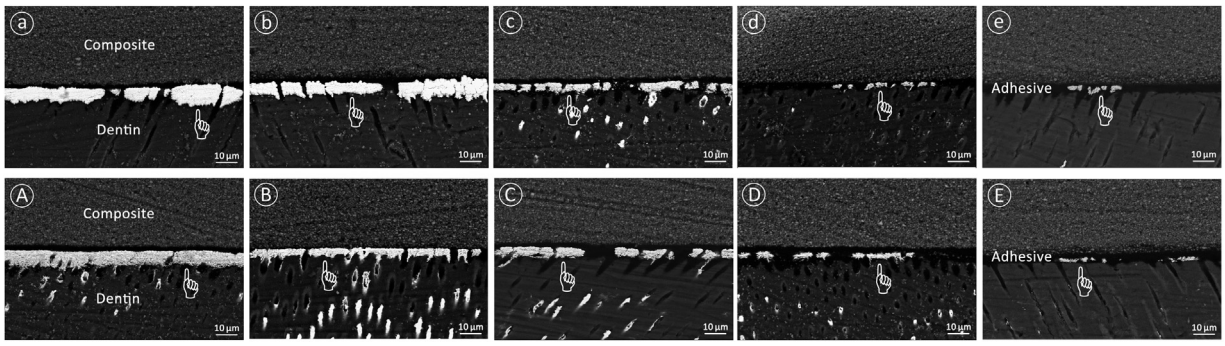

Fig. 4 – Representative FESEM images of interfacial nanoleakage for each group (a–e, immediate; A–E—, collagenase ageing): Group 1 (WWB, a and A); Group 2 (DWB, b and B); Group 3 (0.01%EGCG/DWB, c and C); Group 4 (0.1%EGCG/DWB, d and D); Group 5 (1%EGCG/DWB, e and E). Pointers indicating silver deposits.

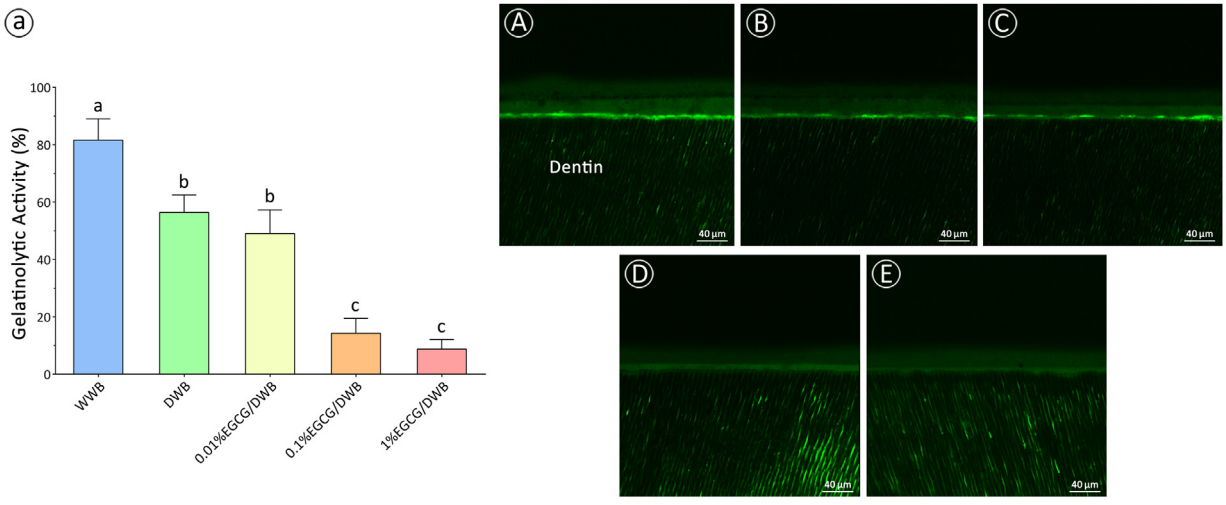

Fig. 5 – (a) Means and standard deviations of the relative percentages of gelatinolytic activity within the dentin–adhesive interface from in situ zymography for each group ($n = 6$). Columns identified with different letters are significantly different ($p < 0.05$). (A–E) Representative CLSM images of in situ zymography labeled with fluorescein-conjugated gelatin mixture for each group: Group 1 (WWB, A); Group 2 (DWB, B); Group 3 (0.01%EGCG/DWB, C); Group 4 (0.1%EGCG/DWB, D); Group 5 (1%EGCG/DWB, E).

obviously less bacterial growth than the other four groups. The high-magnification image of group 5 in Fig. 7E+ further revealed that scarcely any *S. mutans* adhered and aggregated along the dentin–adhesive interface.

The result of MTT assay in Fig. 9 shows that bacteria derived from group 5 (1%EGCG/DWB) presented a statistically lower cellular viability than those of the other four groups ($p < 0.05$), irrespective of 1-month ageing.

4. Discussion

In the present study, the synergistic effect of DMSO wet-bonding and EGCG on improving dentin–adhesive interface stability was investigated. Pretreatment with 0.1%EGCG/DWB and 1%EGCG/DWB preserved dentin bond strength, reduced interfacial nanoleakage after 1-month collagenase ageing, and suppressed MMP activity within the hybrid layer. A desirable dentin bonding performance was achieved. Thus, the first null hypothesis was rejected.

Poor bond durability is primarily caused by hybrid layer degradation at the dentin–adhesive interfaces [32]. The exposed resin matrix is vulnerable to hydrolysis, while collagen is degraded by enzymes [33]. Collagenase derived from *Clostridium histolyticum* can damage collagen chains and prompt collagen fibrils degradation [34]. In vitro ageing test was conducted in the bacterial collagenase solution storage to challenge dentin–adhesive bonds. Although immediate μTBS among the five tested groups showed no statistical difference ($p > 0.05$), the values in groups 4 (0.1%EGCG/DWB) and 5 (1%EGCG/DWB) after collagenase ageing were significantly higher than those in the other three groups ($p < 0.05$). Collagenase ageing did not significantly decrease the values in groups 4 and 5 ($p > 0.05$). This outcome can be attributed to several factors. First, excessive moisture in water wet-bonding (group 1) may compromise the polymerization of adhesive resin monomers, and the limited miscibility of resin monomers in

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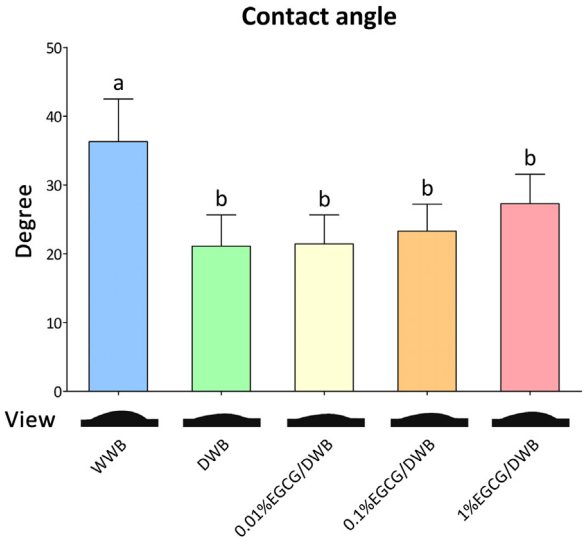

Fig. 6 – Means and standard deviations of contact angle for each group (n = 8). Columns identified with different letters are significantly different ($p < 0.05$). Corresponding anterior view of each group was shown below.

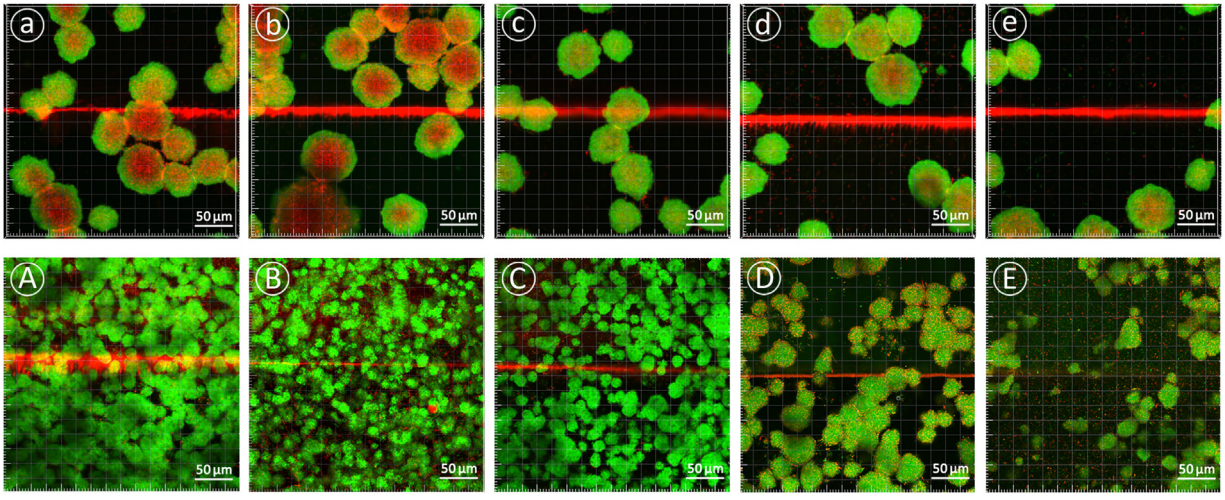

Fig. 7 – CLSM images (2D overlay projections) of representative live/dead stained *S. mutans* biofilm (live bacteria, green; dead bacteria, red) for each group (a–e, immediate; A–E—, 1-month ageing in deionized water): Group 1 (WWB, a and A); Group 2 (DWB, b and B); Group 3 (0.01%EGCG/DWB, c and C); Group 4 (0.1%EGCG/DWB, d and D); Group 5 (1%EGCG/DWB, e and E) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

water can cause phase separation of hydrophobic resins [35]. What's more, although the moisture of dentin surface is modified and the penetration of adhesive monomers is enhanced, DMSO wet-bonding alone may still not be strong enough to protect the integrity of dentin–adhesive interface for long term in the face of bacterial collagenase, endogenous MMPs, and various cariogenic bacteria grown in artificial saliva. Besides, EGCG can stabilize collagen depending on hydrogen bond and hydrophobic interactions with collagenases for improving dentin bonding strength [36]. Compared with ethanol, DMSO can enhance the dissolution of EGCG to achieve a higher incorporated concentration. For these reasons, the adjunctive use of DMSO and EGCG (especially 0.1% and 1%) can develop a synergistic positive effect not only on immediate bond strength but also for long-term bonding stability.

The expression of nanoleakage along the bonding interface is regarded as a crucial criterion to evaluate bonding durability and stability because water infiltration or bacteria invasion in these gaps generally leads to bonding failure [37]. Kruskal–Wallis test (Table 1) indicated that pretreatments with 0.1%EGCG/DWB and 1%EGCG/DWB (groups 4 and 5) significantly decreased interfacial nanoleakage compared with the other three groups ($p < 0.05$), irrespective of collagenase ageing. The details in Fig. 4 further demonstrate fewer silver deposits in these two groups. Probable reasons for this outcome are as follows. DMSO has the ability to break down the self-associative tendency of water, which then releases water molecules trapped between the polymeric chains, thus increasing the conversion degree of resin monomers. The relative decrease in free water and increase in post-curing bond

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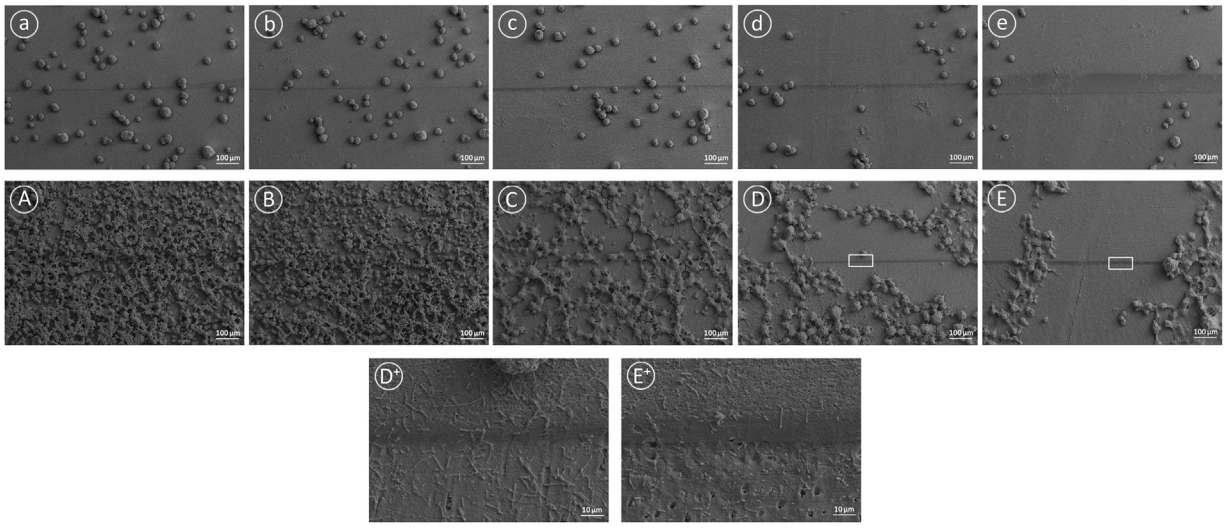

Fig. 8 – Representative FESEM images of *S. mutans* biofilm formation on the surfaces of the dentin–adhesive slabs for each group (a–e, immediate; A–E, 1-month ageing in deionized water): Group 1 (WWB, a and A); Group 2 (DWB, b and B); Group 3 (0.01%EGCG/DWB, c and C); Group 4 (0.1%EGCG/DWB, d and D); Group 5 (1%EGCG/DWB, e and E). Panels D+ and E+ are high-magnification images of panels D and E.

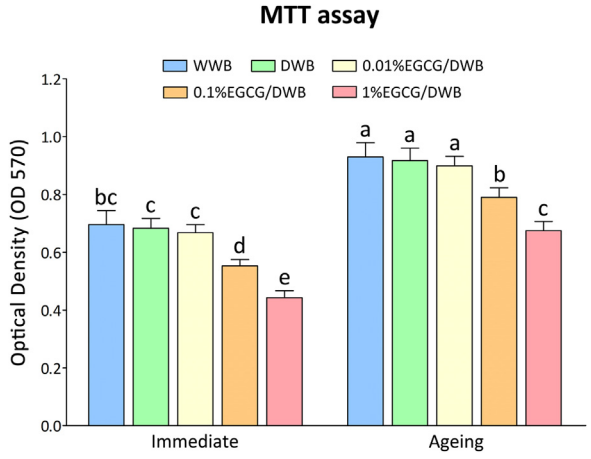

Fig. 9 – MTT assay of *S. mutans* biofilm after 24 h or 1-month ageing in deionized water of incubation on the surfaces of the dentin–adhesive slabs for each group. Data are expressed as mean ± SD (n = 12). Columns identified with different letters are significantly different ($p < 0.05$).

strength suppress the hydrolytic degradation of adhesive in or above the hybrid layer [38]. The mechanism of EGCG to decrease the interfacial nanoleakage may be attributed to the possibility of reduction in dentin permeability, which prevents the penetration of silver nitrate at the bond interface, especially at a relatively high concentration in 50% DMSO aqueous solution [23]. It should be highlighted that both EGCG and DMSO are inhibitors of MMPs, and adjunctive use strengthen both effects.

Several studies confirm that the decrease of μTBS is accompanied by the increase in nanoleakage [39,40]. To the contrary, other studies show that μTBS is not related to nanoleakage expression [41,42]. Over the past decades, various attempts have been made to determine the accurate nanoleakage at dentin–adhesive interface [43–45]. Specimens from different teeth may produce distinct nanoleakage expression due to variation of dentin substrate [46]. As a result, data of nanoleakage among different studies may not be compared fairly. Furthermore, the use of diverse statistical methods and observation approaches, including light microscope, FESEM, transmission electron microscope, and CLSM, may potentially influence the result of nanoleakage even though the specimens are derived from the same tooth [46]. Thus, the relationship between interfacial nanoleakage and dentin bond strength should be explored in the future.

Sufficient infiltration of adhesive resin monomers into the demineralized dentin collagen network is crucial to dentin bonding stability [4]. Considering the amphiphilicity and strong permeability of DMSO, the contact angle was measured in this study. Fig. 6 shows that irrespective of incorporating with EGCG, the DMSO wet-bonding groups (2–5) exhibited lower contact angles than the water wet-bonding group (1). The low contact angles of the universal adhesive on DMSO-pretreated demineralized dentin indicated that the infiltration of hydrophobic adhesive to the demineralized dentin substrate was enhanced. Triple-helical dentin collagen molecules are concealed with bound water, limiting the chemical bond of functional monomers, such as 10-methacryloxydecyl dihydrogen phosphate (MDP) with collagen [47]. The methyl groups in DMSO molecule act as a hydrophobic end, while the oxygen atom forms a hydrogen bond with two water molecules, interfering with water self-association [17]. In this way, DMSO can disrupt the layer of bound water, thereby providing additional binding sites for the MDP molecules to bond with collagen molecules [48]. This result echoes the findings of recent studies, in which the contact angle decreases when 50% DMSO aqueous solution is applied on the etched dentin surface [16].

In addition to exogenous enzymes, such as bacterial collagenase, dentin collagen may be also degraded by endogenous

enzymes, including MMPs. Therefore, the in situ zymography of the hybrid layer was examined. Scarcely any green fluorescence was observed in groups 4 (0.1%EGCG/DWB) and 5 (1%EGCG/DWB) within the hybrid layer (Fig. 5), and their fluorescence values were significantly lower than those of the other three groups ($p < 0.05$), implying that MMP activity was significantly inhibited. The outcome can principally be attributed to the effects of DMSO and EGCG that effectively inhibited endogenous MMP-2 and MMP-9. According to Tjaderhane et al., 5% or higher DMSO solution concentration apparently inhibit human gelatinases MMP-2 and MMP-9 [38]. The possible mechanism of EGCG on inactivating MMP activity is the high affinity of EGCG to metal ions, especially its chelation with zinc, which is associated with MMP recognition and activation [49]. Once MMP recognition is blocked, collagen degradation is likely to be prevented. Taken together with the aforementioned results, the synergistic action of DMSO wet-bonding and EGCG (particularly at 0.1% and 1%) may provide desirable dentin–adhesive interface stability by preserving dentin bond strength, reducing interfacial nanoleakage, and inhibiting MMP activity. The methods adopted in this study to verify the bonding stability of the dentin–adhesive interface were consistent with those of previous studies [50,51].

The integrity of the dentin–adhesive bonding interface is generally challenged by a complex intra-oral environment, where cariogenic bacteria accumulate along the resin-based restorations, thereby causing secondary caries [52]. The collagenase excreted by bacteria could degrade collagen and accelerate ageing [28]. Moreover, cariogenic bacteria (especially *S. mutans*) can metabolize carbohydrates to generate acid and adhere to tooth surface to form biofilm, which initiates caries development [53]. Therefore, a material with anti-biofilm ability is essential to dentin bonding. Given that bonding stability is always desired, the retained anti-biofilm capability of the material on the hybrid layer is also needed. In this study, irrespective of 1-month ageing, pretreatment with 1%EGCG/DWB (group 5) effectively inhibited *S. mutans* growth and biofilm formation along the dentin–adhesive interface, as shown by the CLSM and FESEM images (Figs. 7 and 8). The lowest cellular metabolic activity among the five groups was observed in group 5 as revealed by the MTT assay results. Thus, the second hypothesis was rejected.

The remarkable anti-biofilm ability is mainly attributed to the function of EGCG. The possible mechanisms are as follows: (i) EGCG suppresses *S. mutans* growth and its adhesion to the dentin surface, preventing the formation of biofilm cultures and (ii) EGCG inhibits relevant cariogenic virulence factors to reduce acidogenicity and compromise acid tolerance [54]. In the present study, the high concentration of 1% EGCG was obtained by applying 50% DMSO aqueous solvent and preferable antibacterial activity at the resin–dentin bonding interface was achieved when EGCG concentration was increased. DMSO served as an excellent solvent and assisted EGCG in maintaining favorable stability. The synergistic effects of EGCG and DWB may exert long-term anti-biofilm capability owing to the cross-linking effect of EGCG on dentin collagen and the remarkable solubility and permeability of DMSO. Thus, the leaching rate of EGCG into saliva is maintained on the hybrid layer to function.

The versatile functions of EGCG and DWB show satisfactory potential in the application of universal adhesive. However, the pretreatment step did not simplify the dentin bonding procedure. The combined effects of EGCG and DMSO on dentin collagen fibrils should be further studied to verify their cross-linking effect or ability to remove residual free water. Further studies are also needed to explore the mechanisms of EGCG and DMSO function at dentin–adhesive interfaces.

5. Conclusions

This study suggested that the synergistic action of DMSO wet-bonding and EGCG can effectively improve dentin–adhesive interface stability. Pretreatment with EGCG/DWB preserved dentin bond strength, reduced interfacial nanoleakage, suppressed MMP activity, and inhibited *S. mutans* growth and biofilm formation along the dentin–adhesive interface. Therefore, the proposed strategy provides a promising approach to achieve desirable dentin bonding performance and prevent secondary caries, thereby extending the longevity of adhesive restorations.

Acknowledgments

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A novel dry-bonding approach to reduce collagen degradation and optimize resin-dentin interfaces

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In dentistry, the wet-bonding approach relies on water to maintain demineralized collagen expanded for proper resin infiltration; nevertheless, hydrolytic instability of the resin-dentin interface is inevitable with current bonding techniques. Considering dimethyl sulfoxide's (DMSO) ability to "biomodify" collagen and precipitate enzymes, the aim was to test whether the use of DMSO would permit adequate resin bonding to H_3PO_4-etched dehydrated dentin and assess its impact on collagen degradation by host-derived enzymes. Etched dentin surfaces from extracted sound human molars were randomly bonded in wet or dry conditions using aqueous or ethanolic DMSO solutions as pretreatments and bonding resins with or without DMSO. Bonded teeth were sectioned into resin-dentin slabs for confocal *in situ* zymography and beams for microtensile bond strength test. Demineralized powdered dentin was incubated in the tested DMSO -media and a hydroxyproline assay evaluated dissolution of collagen peptides. Zymography was performed on protein extracts obtained from dry and wet H_3PO_4-ecthed dentin powder treated with the DMSO- media. The correlative biochemical analysis demonstrated that reduction of water content during dentin hybridization by the innovative dry-bonding approaches with DMSO is effective to inactivate host-derived MMP-2 and MMP-9 and thus reduce collagen degradation while simultaneously optimizing resin-dentin bonding.

Resin-dentin bonding is a revolutionary form of *in situ* tissue engineering in which intrinsically hydrated demineralized collagen[1] serves as a scaffold for resin infiltration to couple dental adhesives to the underlying mineralized dentin[2,3]. Nonetheless, limitations in this complex bonding process contribute to formation of imperfect hybrid layers invariably fated to failure after prolonged function[2–4]. Incomplete[5] and suboptimal[6] resin infiltration, the inability of current bonding resins to completely replace free and loosely bound water within collagen matrix[7], hydrolytic instability of hydrophilic methacrylate monomers[8] and collagen degradation[2,9] remain as major drawbacks to the longevity of resin-dentin bonds[4]. Curiously, such processes can be considered as highly correlative since their resultant degradative effects occur exclusively in the presence of water[2].

Water actively participates in resin-dentin bond degradation[10] serving as a functional medium for the hydrolysis of resin matrices by esterases[11] and collagen by both endogenous and exogenous collagenolytic and gelatinolytic enzymes (*i.e.* matrix metalloproteinases and cysteine cathepsins)[2,12]. Notably, especially the etch-and-rinse approach requires a partially wet substrate to maintain adequate interfibrillar spaces within demineralized collagen for proper resin infiltration[13]. There is a general consensus that the presence of unprotected water-filled fibrils[14] creates a weak link highly prone to hydrolytic degradation[2–4,12]. Hence, the main questions reside on how to eliminate free and loosely bound water[1] from demineralized collagen in clinically feasible time frames without jeopardizing resin-dentin bonding and whether the reduction of such water-filled zones would have an impact on collagen degradation.

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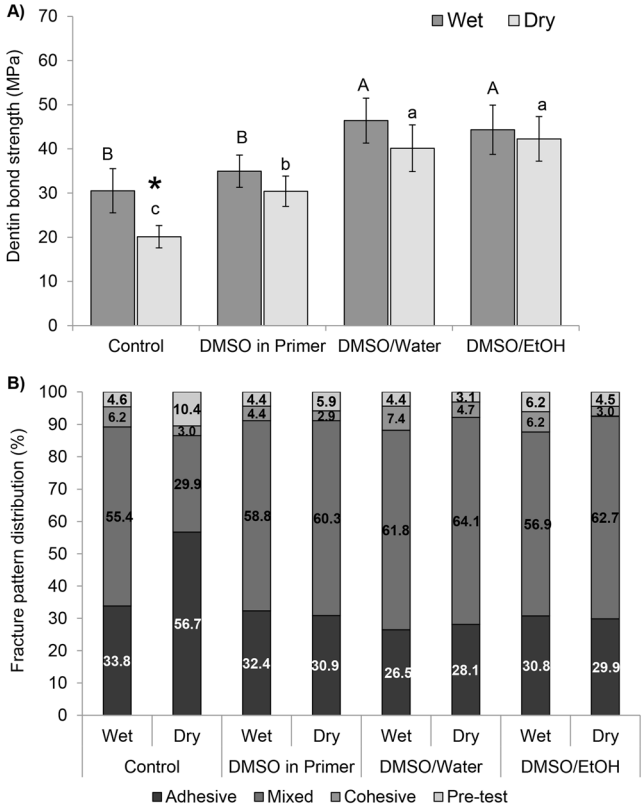

Figure 1. (**A**) Microtensile bond strength of wet- and extensively air-dried demineralized dentin bonded with SBMP using DMSO solutions as dentin pre-treatments or incorporated in the bonding agent (DMSO/SBMP). Pretreatment solutions consisted of DMSO dissolved in either water (DMSO/H2O) or ethanol (DMSO/EtOH). Heights of bars indicate the mean values (MPa) of 8 teeth per group (n = 8) and standard deviations. Columns identified by different capital letters represent significant differences according to Tukey Test ($p < 0.05$) for wet-dentin groups. Columns identified by different lowercase letters represent significant differences for dry-dentin groups. * represent significant differences between wet- and dry-dentin for each pre-treatment. (**B**) Graphical presentation of proportional prevalence of fracture modes for all experimental groups.

Dimethyl sulfoxide (DMSO) is one of the most versatile solvents in biological sciences with a unique ability to "biomodify" demineralized collagen[15,16], thus favoring resin-dentin bonding[17]. DMSO's interaction with water[18] and its capacity to bind, precipitate and unfold hydrophobic proteins[19] may synergically contribute for this matter. The aim of this study was to examine the central hypothesis that binary solutions of DMSO, dissolved in either ethanol or water, and a DMSO-containing *primer* would permit adequate resin bonding to dehydrated demineralized dentin while reducing collagen degradation by host-derived enzymes. The tested null hypotheses were that: (i) dry-bonding using DMSO would have no impact on dentin bond strength; and (ii) the relative proteolytic activity of H_3PO_4 etched-dentin would not be affected by DMSO-pretreatments.

Results

Dentin bond strength. Two-way ANOVA revealed that "dentin condition" ($p = 0.00004$; $\eta^2_p = 0.318$) and the "use of DMSO" ($p < 0.0001$; $\eta^2_p = 0.761$) had significant effects on bond strength regardless their interaction ($p = 0.0786$). The mean cross-sectional area of tested resin-dentin beams (0.81 $mm^2 \pm 0.2$) ranged from 0.77 to 0.88 mm^2 without significant statistical different regarding specimen size ($p = 0.793$). The bond strengths are reported in Fig. 1A. Dry-bonding caused a significant 33% reduction of bond strength ($p < 0.05$), while incorporation of DMSO into SBMP *primer* produced bond strength values similar to wet-bonding. Pretreatments with DMSO solvated in either water or ethanol significantly increased immediate bond strengths ($p < 0.05$) and were not statistically different from each other irrespective of dentin condition ($p < 0.05$). Fracture patterns (Fig. 1B) were mostly mixed for all groups with the exception of the control dry-bonded control group which presented a substantial 60% increase in adhesive failures compared to the conventional wet-bonding protocol.

***In situ* zymography.** Representative CLSM images of the tested resin–dentin interfaces exhibiting hydrolysis of the FITC-conjugated collagen by the endogenous enzymatic activity are shown in Fig. 2. Signs of collagenolytic activity were detected in all samples at the hybrid layer, underlying intertubular dentin and inside dentinal tubules. Untreated groups presented substantial collagen breakdown in both wet and dry dentin (Fig. 2A2,E2). DMSO/H_2O (Fig. 2C2,G2) and DMSO/EtOH (Fig. 2D2,H2) produced fewer areas with collagenolytic signals

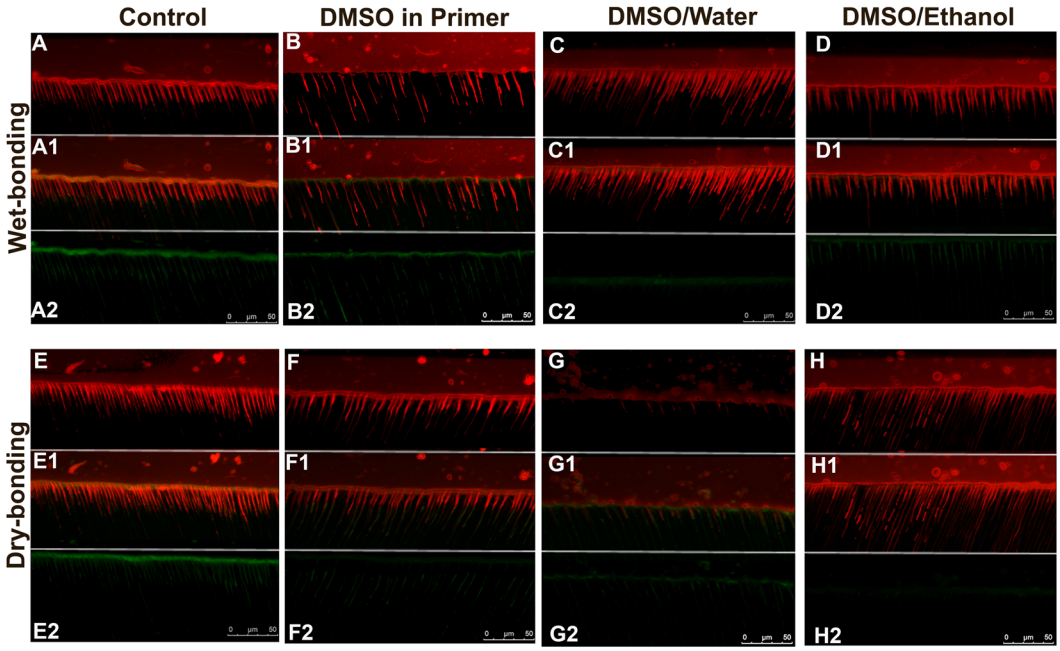

Figure 2. Representative CLSM scans (63x/1.4NA oil immersion objectives) for in situ zymography of wet- and extensively air-dried demineralized dentin bonded with SBMP. DMSO was solvated in water (DMSO/Water) or in ethanol (DMSO/Ethanol) and used as dentin pre-treatments or incorporated in the bonding agent (DMSO in Primer). Isolated red fluorescence signals, originated from Rhodamine B in adhesive, delineate the morphology of adhesive interface (**A**–**H**). Green fluorescence signals designate collagenolytic activity originated from quenched FITC-conjugated collagen breakdown by endogenous enzymes. (A1-H1) depict the localization of collagenolytic activity on the hybrid layer and surrounding areas. (A2-H2) show isolated FITC fluorescence revealing different levels of collagenolytic activity according to the different pretreatments and dentin conditions. While untreated control groups (A2 and E2) exhibited higher FTIC fluorescence signals, pretreatments with DMSO/H2O (C2 and G2) and DMSO/EtOH (D2 and H2) indicate reduced endogenous enzymatic activity especially on dry dentin (F2 and G2). Incorporation of DMSO in the bonding resin produced similar FITC fluorescence signals (D2) to control group in wet condition; however, a slight reduction of enzymatic activity in the hybrid layer was observed for the dry-bonding protocol (H2).

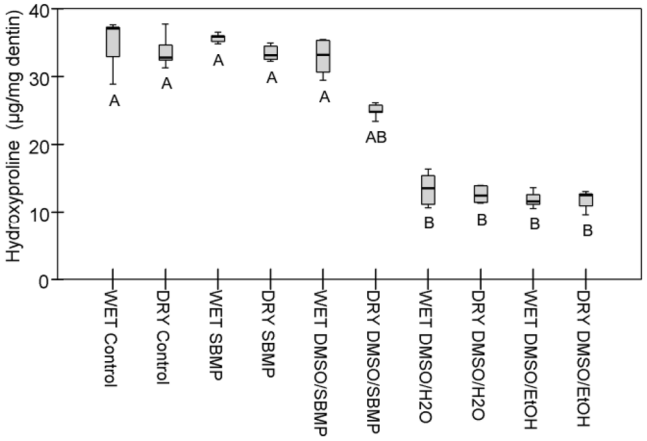

Figure 3. Hydroxyproline content derived from wet- and dry- demineralized dentin powder (n = 5) treated with DMSO after incubation for 7 days at 37 °C. Control groups were incubated in distilled water (WET Control and DRY Control). SBMP also served as a control incubation solution (Wet SBMP and Dry SBMP). Experimental treatments consisted of DMSO solvated in water (DMSO/H2O), ethanol (DMSO/EtOH) or incorporated in the SBMP primer (DMSO/SBMP). Dissolved collagen from the demineralized dentin was expressed as µg hydroxyproline per mg dry mass of the baseline demineralized dentin powder. Groups with different upper ase letters were significantly different ($p < 0.05$) according to Dunn's multiple comparison test.

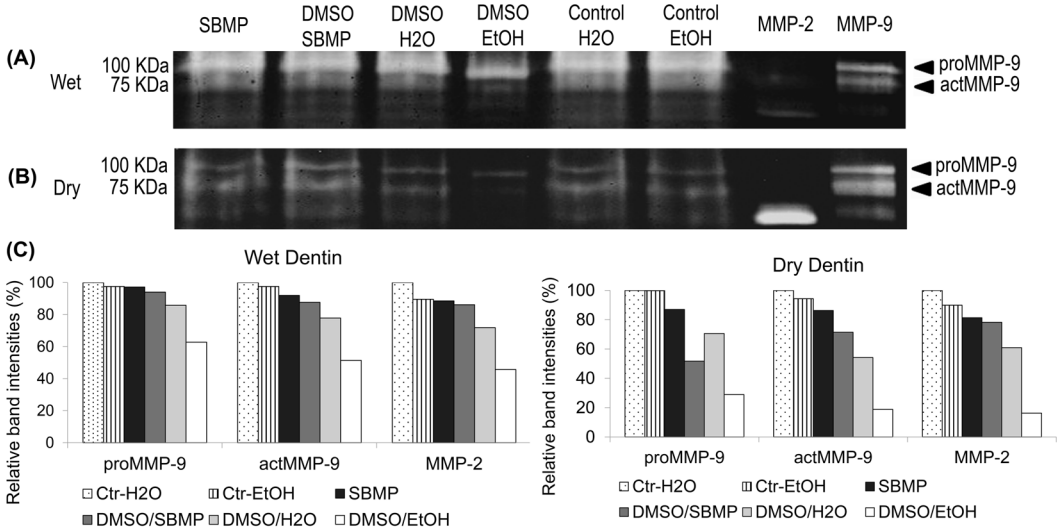

Figure 4. Gelatin zymograms of wet (**A**) and dry (**B**) demineralized dentin powder treated with DMSO solvated in water (DMSO/H2O), ethanol (DMSO/EtOH) or incorporated in SBMP. Control groups consisted of untreated dentin powder (Control H2O), SBMP and ethanol. Pure MMP-2 and MMP-9 extracts from odontoblasts were used as specific enzyme molecular mass standards. Molecular masses, expressed in kDa, are reported in the standard lane (Std). The graph (**C**) shows band intensities for proMMP-9, actMMP-9 and actMMP-2 calculated according to the peak area method. Complete inhibition of actMMP-2 and MMP-9 activity was not observed for neither of the DMSO treatments. Nevertheless, fainted bands indicate partial inactivation of MMP-2 and -9.

compared to wet and dry control groups, respectively. DMSO/EtOH on dry dentin presented the lowest levels of enzymatic activity of all groups (Fig. 2H2). Dentin condition influenced the collagenolytic activity of DMSO incorporated in the bonding resin and DMSO/EtOH producing slightly better inactivation levels in dry conditions than in wet groups, respectively. For wet dentin, incorporation of DMSO in the bonding resin (Fig. 2B2) produced fluorescence levels almost similar to control groups; however, a clear reduction was observed on dry dentin (Fig. 2F2).

Collagen dissolution. The amount of hydroxyproline (μg/mg dry dentin) released from the demineralized dentin matrix is shown in Fig. 3. "Incubation solutions" had a significant effect on hydroxyproline release ($p < 0.0001$; Kruskal-Wallis). Hydroxyproline release from wet and dry demineralized dentin powder incubated in dH_2O and SBMP *primer* were not statistically different ($p < 0.05$), showing that SBMP *per se* had no impact on collagen dissolution, irrespective of dentin condition. In contrast, both DMSO solutions significantly reduced collagen breakdown over 66% when compared to the controls ($p < 0.05$). No significant differences between the DMSO solutions were observed irrespective of dentin condition ($p > 0.05$). The DMSO-containing *primer* had a moderate reducing effect on hydroxyproline release without statistically significant differences to any other group ($p > 0.05$).

Gel Zymography. Zymograms of wet and dry demineralized dentin treatments are shown in Fig. 4A,B, respectively. Demineralized dentin exhibited pro- (92 kDa) and active (86 kDa) forms of MMP-9, MMP-2 in active form (66 kDa) and other minor bands with lower molecular weights (not shown). Analysis of band intensities using the peak area method (Fig. 2C) revealed a consistent partial inactivation of MMP-2 and −9 regarding the tested dentin conditions and treatments. Intensities of pro- and active MMP-9 and MMP-2 bands were similar for the wet dentin-treatments apart from DMSO/EtOH, which exhibited fainter active MMP-9 and MMP-2 bands compared to untreated demineralized dentin. A similar trend occurred for DMSO-treatments performed on dry-dentin, with the exception that DMSO/H_2O presented fainter active MMP-9 and MMP-2 bands compared to control.

Discussion

While the conventionally accepted wet-bonding approach requires residual water to maintain demineralized collagen expanded for resin infiltration, the proposed DMSO-protocols produced improved resin bonding to dry dentin. In fact, the DMSO-containing hydrophilic *primer* (*i.e.* 10 wt%) produced comparable bond strengths to wet-controls and dentin pretreatments with higher DMSO concentration (*i.e.* 50 vol%) improved immediate dentin bonding of dry-dentin beyond conventional wet-bonding. Therefore, the first null hypothesis was rejected. The rationale for testing a bonding resin with relatively low DMSO-content is that incorporation of high DMSO concentrations may hamper the mechanical properties of dimethacrylate bonding polymers[20], which in turn could compromise the bonded interface. According to the hydrogen bonding force (δh: $(J/cm^3)^{½}$) of Hoy's solubility parameters, 50% DMSO/H_2O (δh 26.8) and 50% DMSO/EtOH (δh 16.6) acted as collagen re-expanding solutions due to their higher δh than air-dried collagen (δh 14.8). DMSO's ability to "biomodify" collagen structure,

increasing spaces between collagen microfibrils[16] and improving dentin wettability[15] support the improved bonding effectiveness even under dry-conditions. Since DMSO is able to break water self-associative tendency[21], 10% DMSO incorporation into the water-containing SBMP *primer* certainly increased dried-collagen re-expansion rate producing comparable bond strengths to conventional wet-bonding. It is evident that this simplified use of DMSO or, to a better extent, its use as a dentin pretreatment reduced technique sensitivity of the etch-and-rinse approach[13] concomitantly allowing water removal from the bonded interface by the proposed dry-bonding technique. Optimized bonding efficiency combined with reduced water-content during dentin hybridization could greatly contribute to clinical long-term durability; however, further studies are necessary to test such hypothesis.

This work brings up compelling evidence that DMSO may not only enhance resin-dentin interaction as previously reported[15,22], but it may also incorporate a new protective factor to the resin-dentin bonded interface: DMSO-pretreatments considerably reduced dentin endogenous collagenolytic activity. *In situ* zymography showed no effect of dry-bonding on collagen breakdown indicating that removal of residual water by air-drying is insufficient to interfere on endogenous proteinase activity within bonded interfaces. However, DMSO-pretreatments performed on both wet and dry dentin produced lower levels of collagen degradation at the hybrid layer, even after incubation in artificial saliva. A similar trend was observed in the activity levels of MMP-2 and MMP-9 and overall collagen solubilization indicating that the presence of DMSO partially inactivated endogenous enzymatic activity. This led to rejection of the second null hypothesis for the relative proteolytic activity of etched-dentin was reduced by DMSO. Since the effect of DMSO on the collagenolitic activity within the hybrid layer was affected by dentin hydration levels especially for DMSO/EtOH and DMSO in the *primer*, DMSO's strong interaction with remaining water likely played an important role on DMSO-enzyme interaction. Determining the exact mechanism in which DMSO reduced collagen breakdown is beyond the scope of this study. However, it is important to note that the markedly low solubility of proteins in polar solvents[23] does not apply to DMSO, which promptly dissolves common proteins[24]. Therefore, we speculate that DMSO's ability to solubilize and interact with proteins concentration-dependently[19] supports the possibility of enzymatic debinding from the collagen matrix by the DMSO solutions[16], which would explain the reduction of collagen breakdown. In fact, in high DMSO-water concentrations (*i.e.* 40–70%; depending on the protein structure and hydrophobicity), enzymes may have a stronger affinity to DMSO than pure water producing what is known as preferential DMSO interaction[19].

This condition in which DMSO binds to protein hydrophobic moieties leading to protein unfolding and thus denaturation[19] could explain the enzymatic inactivation findings on DMSO-treated dentin. Preferential binding of DMSO is affected by the bulk concentration of DMSO, substrate hydration and protein polarity[19]. Less polar proteins tend to bind more DMSO as the solvent concentration increases and as the substrate hydration diminishes[19]. Therefore, reduced water availability in dry-dentin pretreated with DMSO/EtOH most likely maximized DMSO binding to endogenous enzymes explaining the highest enzymatic inactivation levels for the *in situ* and gel zymography. Nonetheless, the specific interactions between DMSO and endogenous dentin enzymes must be further evaluated.

The *in situ* zymography results are in accordance with the hydroxyproline quantification where dentin pretreatments with 50 vl% DMSO reduced collagen solubilization more efficiently than the 10 wt% DMSO-containing primer. The exception was that dentin condition (*i.e.* wet vs. dry) had no impact on collagen solubilization after DMSO-treatments. Unlike the DMSO application time of one minute for the *in situ* zymography analyses, for hydroxyproline quantification demineralized dentin powder was incubated in the different DMSO-containing media for 7 days at 37 °C to allow collagen breakdown by the endogenous enzymes. The longer incubation period and larger volume of incubation medium kept under constant shaking certainly potentialized DMSO ability to debind endogenous proteases from the demineralized collagen, irrespective of water-content within collagen. From a clinical stand point, longer DMSO application times are unfeasible so the amount of residual water should be considered in order to enhance the inactivation of endogenous enzymes by DMSO pretreatments.

This study demonstrates the proof of concept that bonding current relatively hydrophilic resins to extensively air-dried demineralized dentin becomes viable when DMSO is used as a pretreatment or incorporated in the bonding resin. Apart from immediate resin-dentin bonding optimization, DMSO partially inactivates endogenous MMPs at the hybrid layer, thus reducing collagen solubilization of dry dentin. The ability to remove water from bonded interface and simultaneously reduce collagen breakdown in a clinically relevant time frame brings new possibilities to create resin-dentin interfaces with higher longevity.

Methods

Extracted sound human third molars were obtained with informed consent from patients (age 18–21) under a protocol approved by the University of Oulu, Finland (#23-2003). Tooth collections were performed in accordance with relevant guidelines and regulations. Original clinical indications for tooth extractions were not related to the present study. After extraction, 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.

Bonding procedures. Specimen preparation followed the general guidance for testing of dental composite bonding effectiveness[25]. Teeth were coronally sectioned under water cooling to remove occlusal enamel and to expose flat midcoronal dentin surfaces followed by root removal 2 mm below the enamel-dentin level. Exposed midcoronal dentin surfaces were wet-polished with 320-grit SiC paper for 60 s to create standardized smear layers. Crown segments (n = 8/group) were randomly allocated to 8 groups following a study design with two factors: (i) "dentin condition" in two levels composed of dry- and wet-bonding protocols; and (ii) "DMSO treatment" in four levels consisting of no treatment, use of 50 vol% DMSO (Dimethyl Sulfoxide, Sigma-Aldrich, St Louis, MO, USA) dissolved in either water (DMSO/H_2O) or ethanol (DMSO/EtOH), and incorporation of 10 wt% DMSO in the bonding resin (Adper Scotchbond Multi-Purpose: SBMP, 3 M ESPE, St Paul, MN, USA).

In order to produce the DMSO-containing bonding resin, SBMP *primer* was evaporated at room temperature to remove 10 wt% of the original solvent composition and thus avoid changes in the original monomer-solvent ratio. Subsequently, 10 wt% DMSO was added to the evaporated aliquot followed by ultrasonic mixing for 60 s. Dentin surfaces were acid-etched with 32 wt% H_3PO_4 for 15 s (Scotchbond Universal Etchant, 3 M ESPE) and rinsed for 15 s with water. For the wet-bonding protocols, blot-drying with paper tissue was carefully performed leaving the dentin surface slightly moist. Conversely, dry-bonding was performed by continuous air blasts using a 3-way syringe at a distance of 10 cm for 30 s. After dentin etching and humidity control, dentin pretreatments were performed consisting of active application of 50 µL DMSO/H_2O or DMSO/EtOH solutions on etched-dentin followed by blot drying until paper filters no longer absorbed liquids from the bonding surface by capillarity. SBMP, with or without DMSO, was applied totaling 20 s and light cured for 10 s. Composite build ups (Filtek Supreme, 3 M ESPE) were performed in two 2 mm increments and individually light-cured for 40 s. Light curing of all resin materials was performed using a LED device (Bluephase 20i, Ivoclare Vivadent, Schaan, Liechtenstein) delivering 1100 mW/cm^2.

Microtensile bond strength. The restored crown segments (n = 8/group) were stored in distilled water for 24 h to allow water sorption and postoperative polymerization of the adhesive and resin composite to take place. Samples were then longitudinally sectioned into bar-shaped resin-dentin beams with cross-sectional area of approximately 0.8 mm^2 [26]. Each specimen was individually fixed to a testing jig with cyanoacrylate glue and subjected to tensile load at a crosshead speed of 0.5 mm/min until failure (DL2000, EMIC, São José dos Pinhais, PR, Brazil). The force (N) required to fracture the sample and the dimensions of the cross-sectional area (mm^2) were recorded with a digital caliber to the nearest 0.01 mm and the tensile bond strength (MPa) was calculated. The bond strength of a minimum of 8 resin-dentin beams was averaged to represent the bond strength of each tooth. Since the data was normally distributed (Shapiro-Wilk; $p = 0.604$) and homoscedastic (Levene test $p = 0.321$), they were analyzed by two-way ANOVA followed by Tukey test ($\alpha = 0.05$). Failure modes were evaluated at 40× magnification under a stereomicroscope (Leica M60, Leica Microsystems) and classified as cohesive, adhesive, or mixed failures[17].

***In situ* zymography.** *In situ* zymography was used to identify collagenolytic activity within the hybrid layer Two teeth per group were prepared for qualitative analyses of collagenolytic activity at the bonded interface[27]. Freshly reconstituted FITC-conjugated collagen (D-12060, Molecular Probes, Eugene, USA) was actively applied for 60 s on etched-dentin after DMSO-treatments were performed or previously to the application of the DMSO-incorporated SBMP *primer*. Bonding procedures were carried on for all groups as previously described except that SBMP *primer* and *adhesive* were doped with Rhodamine B powder 0.1 wt%. The samples were stored at 37 °C for 7 days in calcium- and zinc-containing artificial saliva (5 mM HEPES, 2.5 mM $CaCl_2$·H2O, 0.05 mM $ZnCl_2$, and 0.3 mM NaN_3, pH 7.4), individually sectioned into a minimum of four 0.6 mm thick slabs per tooth, wet-polished with 600, 1200 and 2000 SiC paper and ultrasonically cleaned for 5 min after the polishing steps. The entire resin-dentin interface was examined using a multiphoton confocal laser microscope (CLSM: Leica SP5, Heidelberg, Germany) equipped with 63×/1.4NA oil immersion lens using a 488 nm argon laser (490–540 nm band pass filter) and a 563 nm laser (580–630 bandpass filter). The z-stack scans (0.5 µm) were compiled into single projections until 20 µm final volume. The emission of FITC signal allowed the identification of areas at the bonded interface presenting collagenolytic activity as a result of breakdown by endogenous enzymes. Sequential images of the bonded interface were recorded and qualitatively analyzed for the intensity and extension of FITC-conjugated collagen hydrolysis.

Enzyme activity assays. Ninety extracted human third molars were ground free of enamel, the roots were sectioned off and pulp soft tissues were removed. Dentin fragments were frozen in liquid-nitrogen for 5 min followed by trituration at 24 Hz for 2 min in a ball-mill (Model MM400, Retsch, Newtown, PA, USA). Dentin powder was then sieved (Advantech Sonic Sifter, Advantech Mfg., New Berlin, MN, USA) to uniform particle size at $<$300 µm.

Collagen solubilization was assessed by hydroxyproline quantification. Five g of dentin powder were demineralized in 10 wt% H_3PO_4 (pH ≈ 0.4) for 10 min, centrifuged at 12000 rpm for 20 min at 4 °C and rinsed twice with 1 ml dH_2O. Demineralized dentin powder was dehydrated in a silica desiccator for 72 h at 4 °C in order to remove loosely-bound water and divided (25 mg/sample) into 12 groups (n = 5). Half of the samples were rehydrated with 5 µL/sample dH_2O. Wet and dry samples were incubated in a shaking bath for 7 days at 37 °C in 1 mL of tested pretreatment solutions (*i.e.* DMSO/H_2O and DMSO/EtOH) and in the DMSO-containing *Primer* (DMSO/SBMP). Dry control samples were incubated in dH_2O or SBMP *Primer*. At the end of the incubation, 25 µL of the media was collected from each vial, freeze-dried for 72 h (Alpha 1–5, Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany) for solvent removal, subsequently re-suspended in 75 µL of dH_2O and transferred to individually labeled ampules. Solubilized collagen peptide fragments were assessed following a previously described hydroxyproline quantification protocol[28]. The specimens were re-suspended with 25 µl water after freeze-drying. Aliquots of standard hydroxyproline (2–20 µg) prepared from stock solutions and test samples containing hydroxyproline under 10 µg/ml were mixed with 25 µl of 4 N sodium hydroxide (2 N final concentration) in a total volume of 50 µl in 2 ml Nalgene O-ring tubes. The samples were hydrolyzed by autoclaving at 120 °C for 20 min. 450 µl of chloramine-T was added to hydrolyzed tubes and mixed gently to allow oxidation for 20 min at room temperature. 500 µl Ehrlich's aldehyde reagent was added to each specimen for chromophore formation by incubating the specimens at 65 °C for 20 min. Absorbance values were obtained in a spectrophotometer (Model UV-A180, Shimadzu, Tokyo, Japan) at 550 nm and plotted against the standard hydroxyproline curves to determine the hydroxyproline release (µg/mg of dry dentin). Data was analyzed by Kruskal–Wallis one-way ANOVA on ranks and Dunn's multiple comparison tests ($\alpha = 0.05$).

A gel zymographic assay evaluated the effect of solvent and adhesive components on gelatinolytic activity of demineralized dentin extracts. Zymography was performed in accordance with Mazzoni *et al.*[29]. Briefly, demineralized dentin powder (200 mg/sample) was divided into 12 groups (n = 4) according to dentin condition (wet vs. dry) and treatment solutions (DMSO/H_2O, DMSO/EtOH, DSMO/SBMP). dH_2O, ethanol and SBMP *Primer* served as controls. For dry groups, demineralized dentin was dehydrated in a desiccator for 72 h. Dentin powder was treated with 400 μL of the DMSO solutions and vortexed for 60 s and centrifuged to remove the supernatant. Samples were re-suspended in 1.8 mL extraction buffer for 24 h at 4 °C under constant stirring, sonicated for 20 min and centrifuged at 12000 rpm for 20 min at 4 °C. Sample aliquots were concentrated using a centrifugal concentrator device (10,000-kDa cut-off, Vivaspin Sartorius Stedim Biotech, Goettingen, Germany) for 30 min at 20 °C (10,000 rpm) until the volume was reduced to 20 μL. The Bradford assay was performed to determine total protein concentrations. One hundred micrograms of protein aliquots were diluted in Laemmli sample buffer and subjected to electrophoresis under non-reducing conditions in 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel containing 1 mg/mL gelatin which had been fluorescently labeled with MDPF. A SDS-PAGE molecular weight standard (Dual Color Standards, Bio-Rad), was used along with purified MMP-2 and MMP-9[16] to allow specific match of corresponding MMP bands. After electrophoresis, the gels were washed for 30 min twice in 2.5% Triton X-100 with agitation, and incubated in activation solution for 48 h at 37 °C. The gels were monitored with UV light (Gel Doc XR System, Bio-Rad) to reveal the gelatinolytic bands in triplicate samples. Band intensities were calculated according to the peak area method with digital image-analysis software (ImageJ, National Institute of Health, Bethesda, MD, USA).

Data Availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

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Author Contributions
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Dry bonding to dentin: Broadening the moisture spectrum and increasing wettability of etch-and-rinse adhesives

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ABSTRACT

Objective. To determine whether the effect of dentin moisture on the etch-and-rinse bonding may be minimized by dry-bonding protocols utilizing aqueous or ethanolic dimethyl sulfoxide (DMSO) pretreatments.

Methods. H_3PO_4-etched mid-coronal dentin surfaces from human molars were randomly blot- or air-dried for 30 s and pretreated with DMSO/H_2O or DMSO/EtOH solutions. Untreated samples served as control. Moisture control was performed by either blot- or air-drying. Samples were bonded with a multistep etch-and-rinse adhesive. Restored crown segments (n = 8/group) were stored in distilled water for 24 h and sectioned for microtensile bond strength testing. Resin-dentin beams (0.8 mm^2) were tested under tension until fracture (0.5 mm/min) after 24 h and two years of storage in artificial saliva at 37 °C.

SEM nanoleakage evaluation was performed on aged samples. Collagen wettability was also measured by sessile drops of the hydrophilic and hydrophobic bonding resins (n = 8/group). Data were examined by factorial ANOVA followed by the Tukey test ($\alpha = 0.05$).

Results. Dry bonding to untreated collagen produced inferior immediate and long-term bond strengths than wet bonding ($p < 0.05$). Regardless of initial hydration and moisture control, DMSO-dry bonding produced initially higher and stable bond strengths after aging ($p < 0.05$). DMSO-pretreated groups presented improved collagen wettability with lower silver uptake ($p < 0.05$).

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Significance. Despite the common belief that etch-and-rinse adhesives must be applied onto moist collagen, DMSO-dry bonding protocols not only improved bonding performance and hybrid layer integrity, but also brought more versatility to collagen hybridization by reducing overdrying-related issues.

1. Introduction

Resin-dentin bonding has greatly evolved in the past decades in search for more durable interfaces. Nevertheless, bonding to the biologically active dynamic dentin substrate still presents several unsolved challenges [1]. Dentin is an intrinsically hydrated mineralized tissue that imposes several obstacles to long-term resin bonding.

The two main factors involved in etch-and-rinse bonding are adequate wetting of the dentin surface by the adhesive components and subsequent micromechanical interlocking of resin monomers to the demineralized collagen fibrils upon curing [1,2]. To do so, maintenance of collagen interfibrillar spaces is critical for proper monomer penetration [1,3–5]. There is a general consensus that by maintaining a state of hydration previously to adhesive application, collagen interfibrillar spaces are preserved and thus improved bonding outcomes are achieved [6–8]. This was referred to as the wet-bonding technique [9] and it has been the standard protocol for etch-and-rinse bonding for the past three decades [1,4,5]. However, control of dentin moisture prior to adhesive application is not a simple procedure [6,10]. Optimal dentin moisture degree varies among different adhesive systems depending on their solvent composition [8,11], making proper clinical use of the wet bonding technique even more challenging. In addition, controlling dentin moisture in a reproducible manner is virtually impossible by current means, which further complicates the proper use of such technique-sensitive wet-bonding approach [1].

The high sensitivity of etch-and-rinse systems to dentin moisture, including both overwet and overdry conditions, strongly affects adhesive performance [12–14]. Detrimental effects of excessive water on the formation of highly cross-linked polymer chains [15,16] within hybrid layers contribute to the unpredictability and complexity of the wet-bonding approach [17]. Water entrapment within the collagen matrix limits the diffusion of cross-linking hydrophobic monomers deeply into hybrid layers [18], while phase separation is also likely to occur [8]. Monomer conversion may also be negatively affected by excessive residual moisture resulting in mechanically weaker polymers [19]. Even with theoretically ideal moisture conditions, wet-bonded interfaces are still prone to degradation over time [6,20]. Furthermore, residual water may also participates in collagen hydrolyses by endogenous enzymes (*i.e.* matrix metalloproteinases and cysteine cathepsins) [21,22] contributing to long-term resin-dentin bond degradation. Reduction or even elimination of such water-content benefits resin-dentin bonding [10,17,23,24], as long as collagen hybridization and polymer formation is not be jeopardized [25,26].

Undoubtedly, a simpler alternative to standardize dentin moisture and potentially reduce the detrimental effects of water entrapment would be the classic dry-bonding approach. The inability of resin-solvent blends to re-expand dried-collapsed collagen limits the dry-bonding approach [3,25]. Several attempts have been proposed to overcome such limitations and reestablish the dry-bonding approach with various degree of success [17,23,27–30]. Recently, dimethyl sulfoxide (DMSO) has emerged in the field of adhesive dentistry as a polar aprotic solvent capable of improving different aspects in resin-dentin bonding. Unlike previously proposed attempts, DMSO may simultaneously act in several fronts to facilitate dry bonding to dentin conventionally etched with H_3PO_4. Higher monomer diffusion [31], better hybrid layer formation [24,31,32] and even lower endogenous collagenolytic activity [23,33] have been attributed to DMSO. DMSO-dry bonding protocols are not only effective to produce higher initial bond strengths [24,34], but they preserve long-term bond strengths producing interfaces with lower levels of residual water [23,24]. Nonetheless, the necessity to re-wet air-dried collagen with DMSO pretreatments prior to hybridization raises concerns about the true ability to bond methacrylate monomers to dry and fully demineralized collagen. The combination of both collagen re-expansion by water-based DMSO pretreatments [35] and their ability to subsequently stiffen collagen [34] may confer dimensional stability to demineralized collagen fibrils. This could allow collagen air-drying at different bonding stages before adhesive application. The possibility to air-dry fully demineralized collagen before or after DMSO pretreatments would constitute a pivotal modification, characterizing a dry-bonding technique more realistic, reliable and reproducible. Moreover, the proposed disruption of residual-water layers surrounding collagen fibrils by DMSO [32,36] could further facilitate the infiltration of hydrophobic monomers in such dry state to strengthen hybrid layers.

Therefore, the primary aim of this study was to investigate the possibility of air-drying etched dentin as the sole form of moisture control in attempt to reduce moisture-related issues of resin-dentin bonding. This would discard the current necessity of maintaining etched dentin moist to preserve demineralized collagen interfibrillar spaces prior to hybridization. The objectives were to determine the effect of DMSO pretreatments and the degree of collagen moisture, prior and after pretreatments, on the long-term bond strength, hybrid layer quality and collagen wettability of a water-based three-step etch-and-rinse adhesive system. The tested null hypotheses were that conventional dry bonding and the pro-

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posed variations in DMSO-dry bonding approach would not affect: (i) resin-dentin bonding performance, (ii) hybrid layer integrity and (iii) collagen wettability of a water-based etch-and-rinse adhesive system.

2. Materials and methods

Extracted sound human third molars were obtained with informed consent from patients (18–26 years) under a protocol approved by the University of Oulu, Finland (#23-2003) in accordance with local regulations. Indications for tooth extractions were not related to the present study. After 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

The experimental design was composed of four study factors defined as: (i) initial collagen hydration condition at two levels (wet or dry bonding); (ii) dentin pretreatments at three levels (no pretreatment, DMSO/H_2O and DMSO/EtOH); (iii) collagen moisture condition prior to hybridization at two levels (blot- or air-drying) and (iv) storage time at two levels (24 h and 2 years). Control groups received no DMSO pretreatments following the conventional wet- or dry-bonding approaches tested at both storage times. Teeth were coronally sectioned under water-cooling to expose flat midcoronal 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× magnification. Roots were removed 1 mm below the cervical line and discarded. Exposed dentin surfaces were wet polished with 320-grit SiC paper for 60 s for smear layer standardization. Crown segments (n = 8/group) were randomly allocated to 10 groups according to dentin condition prior to hybridization, dentin pretreatments and moisture control after pretreatments. Dentin surfaces were etched for 15 s with 32% phosphoric acid (Scotchbond Universal Etchant, 3 M ESPE, St. Paul, MN, USA), rinsed for 30 s and either blot-dried, leaving the surface partially wet (moist dentin), or air-dried for 30 s (dry dentin). The 50% DMSO (v/v) solutions were prepared by mixing equal volumes of DMSO (Dimethyl Sulfoxide, Sigma-Aldrich, St. Louis, MO, USA) in distilled water or ethanol (Ethanol 99.8%, Sigma-Aldrich). 50 μL of DMSO/H_2O or DMSO/EtOH solutions [23,31,32] were actively applied on the etched-dentin surfaces for 60 s. Moisture control was performed by either blot-drying, until paper filters presented no visible moisture or by air-drying for 30 s [23,31,32]. One water-based three-step etch-and-rinse adhesive (Scotchbond Multi-Purpose: SBMP, 3 M ESPE) was used following manufacturer's instructions. SBMP's *Primer* was applied for 10 s and gently evaporated for 10 s with air-streams. The *Bond* was subsequently applied and gently air-blown for 5 s to produce a more uniform adhesive layer. Both were actively applied with manual light pressure of approximately 4 g, equivalent to a slight rubbing pressure [10,37]. Adhesive procedures were carried out in a controlled environment with a temperature of 24 °C and a relative humidity of 45–55%. Adhesives were light cured for 10 s using a LED light-curing unit (Elipar Deepcure, 3 M ESPE) at 1200 mW/cm^2. Composite blocks were built with a nanofilled composite resin (Filtek Z350, 3M ESPE) in two increments of 2 mm. Each increment was light-cured for 20 s. 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 0.8 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). A minimum of 18 resin-dentin beams were produced per tooth.

2.2. Resin-dentin beam storage

Resin-dentin beams were randomly selected for the microtensile test under two conditions: immediate testing after 24 h of storage in distilled water at 37 °C and long-term aging after two-years in artificial saliva (pH 7.4) at 37°C composed by 5 mM HEPES, 2.5 mM $CaCl_2$·H2O, 0.05 mM $ZnCl_2$, and 0.3 mM NaN_3 [38]. The storage media was changed biweekly to prevent possible pH changes. In order to obtain a research design balanced by tooth dependency [39], resin-dentin beams from the same tooth were submitted to both testing periods (*i.e.* 24 h and 2 years). For the nanoleakage evaluation, resin-dentin beams aged for 2 years were selected to focus on the effect of aging on hybrid layer integrity.

2.3. Microtensile bond strength (μTBS)

Microtensile bond strength evaluation followed the Academy of Dental Materials guidelines for non-trimmed μTBS testing [39]. A minimum of 7 beams per tooth (n = 8 teeth/group) were tested on each storage period. Beams were individually attached to a custom-made testing jig using a cyanoacrylate adhesive (Loctite 416, Henkel Corp., Dublin, Ireland) and tested under tension on a mechanical testing machine (Bisco, Schaumburg, IL, USA) at a crosshead speed of 0.5 mm/min until failure to obtain the maximum load (*P*) in N. The cross-sectional area (*CA*) in mm^2 of each beam was measured with a digital caliper to nearest 0.01 mm. The formula $\mu TBS = P/CA$ was used to calculate μTBS values in MPa. Pre-test failures were considered as 0 MPa for the statistical analyses. Since tooth was considered as the statistical unit, bond strengths of resin-dentin beams from each tooth, tested at each period, were averaged to represent the bond strength of each tooth [39]. Both surfaces of fractured resin-dentin beams were analyzed with a stereomicroscope (Leica MD60, Leica Microsystems) at 40× magnification to determine fracture patterns. Unidentifiable samples were examined by scanning electron microscopy (SEM) (Phenom ProX, Phenom-World, Eindhoven, Netherlands). 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).

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2.4. Nanoleakage evaluation

Three resin-dentin beams per tooth (n = 8/group) stored for 2 years in artificial saliva were randomly selected to measure silver nitrate uptake at the bonded interface after long-term storage. Nanoleakage evaluation was performed according to a protocol previously described by Tay et al. [40]. 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 in 50% (w/v) ammoniacal silver nitrate (pH 9.5) for 24 h and thoroughly rinsed in distilled water for 120 s. Subsequently, samples were immersed in photo-developing solution (Kodak Professional D-76 developer, Kodak Rochester, NY, USA) for 8 h under a fluorescent light to reduce silver ions into metallic silver grains. 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. Embedded samples were ultrasonically cleaned in distilled water after each polishing step for 5 min, air-dried for 2 h, mounted on aluminum stubs, dried in silica overnight and carbon sputtered. Nanoleakage extension was qualitatively analyzed using SEM imaging on backscattering mode at 10 kV (Phenom ProX, Phenom-World). Silver uptake patterns and extensions were blindly evaluated by an experienced operator at magnifications ranging from 1000−10000×.

2.5. Contact angle measurements

Dentin discs measuring approximately 2.5 mm in thickness (n = 8/group) from the midcoronal section of sound third molars were transversally sectioned under water cooling (Isomet, Buehler Ltd). Occlusal surfaces were inspected for the absence of remaining enamel with a stereomicroscope (Leica M60, Leica Microsystems) at 40× magnification and polished with 600-grit SiC paper for 60 s. H_3PO_4-etching (Scotchbond Universal Etchant, 3 M ESPE) was performed for 15 s and rinsed for 30 s. Moisture control and DMSO pretreatments were performed as previously described for the bond strength measurements. In order to investigate the wettability properties, contact angle measurements were performed using the sessile drop method. Dentin discs were placed on top of a water droplet and a goniometer (Attension Thetha Lite 101, Biolin Scientific, Espoo, Finland) was used to measure contact angles of the hydrophilic (Primer, Scotchbond Multi-Purpose: SBMP, 3 M ESPE) and hydrophobic (Bond, Scotchbond Multi-Purpose: SBMP, 3 M ESPE) bonding resins. Droplets (approximately 3 μL) were deposited on the etched-dentin surfaces with a micropipette after the respective dentin pretreatments and drying conditions. Contact angles θ were measured up to 240 s after the drop. Images were captured at 0.1 s intervals during the initial 20 s, 0.5 s during the subsequent 20 s and after 5 s intervals for the remaining 200 s to evaluate spreading times. Left and right contact angles were automatically averaged by the goniometer software (OneAttension Version 2.9 (r5612), Biolin Scientific, Finland). A logarithmic fitting model [41] of the contact angles over time was used to calculate the spread rate constant k for the bonding resins according to DMSO pretreatments and moisture conditions.

2.6. Statistical analysis

Bond strength data were normally distributed (Shapiro-Wilk; $p = 0.2$) and homoscedastic (Levene Test; $p = 0.24$). Four-way ANOVA followed by the Tukey test were employed with statistical significance set at $\alpha = 0.05$ using IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, NY, USA.

3. Results

3.1. Microtensile bond strength test

The mean cross-sectional area of the resin-dentin beams (0.81 ± 0.1 mm^2) ranged from 0.72 to 0.89 mm^2 without significant differences between groups regarding specimen size (p = 0.251). Four-way ANOVA showed that "initial collagen hydration" ($p < 0.0001$; $\eta_p^2 = 0.263$), "dentin pretreatments" (p = 0.0001; $\eta_p^2 = 0.871$), "collagen moisture prior to hybridization" ($p = 0.0001$; $\eta_p^2 = 0.045$) "storage time" ($p = 0.0001$; $\eta_p^2 = 0.164$), the interactions between "initial collagen hydration * dentin pretreatments" ($p = 0.0001$; $\eta_p^2 = 0.341$) and "dentin pretreatment * storage" ($p = 0.0001$; $\eta_p^2 = 0.246$) significantly affected bond strengths. Resin-dentin bond strength values are shown in Fig. 1 and Table 1. Dry bonding produced significantly lower immediate bond strength (approximately -48%) compared to the traditional wet bonding approach. Irrespective of initial dentin hydration (wet or dry) or moisture control (blot- or air-drying), DMSO pretreatments produced significantly higher bond strengths (ranging from 30% to 45%) compared to the traditional wet bonding protocol. No significant differences were detected between DMSO pretreated groups. Aging produced significant reductions (approximately −40%) in samples following the traditional wet bonding protocol. Dry bonding presented the lowest bond strengths after aging with a significant reduction of −85%. No significant changes were observed for the DMSO pretreated groups after aging irrespective of initial dentin hydration or moisture control. Fracture pattern distributions for all groups are shown in Fig. 2. At 24 h, the predominant pattern was mixed, except for the dry-bonded samples, which were mostly characterized by adhesive failures. After aging, samples bonded following the wet bonding protocol presented adhesive failures as the predominant pattern. Aged dry-bonded samples presented 71% pretest failures. Aged DMSO pretreated samples did not present substantial changes in fracture patterns compared to samples tested at 24 h.

3.2. Nanoleakage evaluation

Representative SEM micrographs of silver uptake and nanoleakage patterns for all groups after long-term aging are shown in Fig. 3. Nanoleakage was invariably identified at the peritubular region around resin tags in all groups with no marked differences in their extension between DMSO treatments. Different nanolekage patterns between the control and DMSO-pretreated groups were identified within the hybrid layer. Dry-bonded control samples presented the highest levels of silver uptake, with the hybrid layers nearly fully impregnated by heavy silver deposits, depicting extremely

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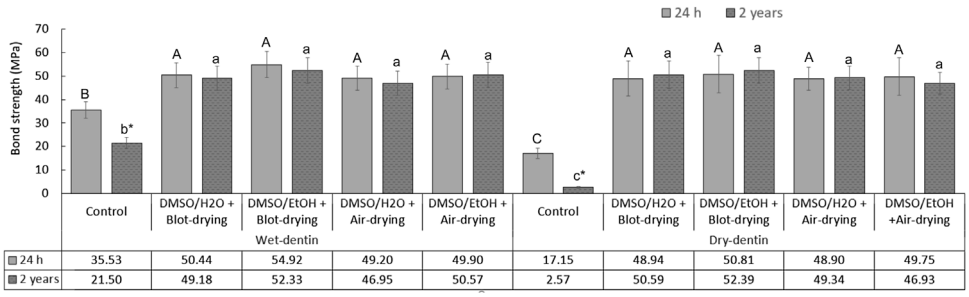

	Wet-dentin					Dry-dentin				
	Control	DMSO/H2O + Blot-drying	DMSO/EtOH + Blot-drying	DMSO/H2O + Air-drying	DMSO/EtOH + Air-drying	Control	DMSO/H2O + Blot-drying	DMSO/EtOH + Blot-drying	DMSO/H2O + Air-drying	DMSO/EtOH +Air-drying
24 h	35.53	50.44	54.92	49.20	49.90	17.15	48.94	50.81	48.90	49.75
2 years	21.50	49.18	52.33	46.95	50.57	2.57	50.59	52.39	49.34	46.93

Fig. 1 – Microtensile bond strength (MPa) means and standard deviations of resin-dentin interfaces bonded to wet or dry dentin using aqueous or ethanolic DMSO solutions as pretreatments. Moisture control after pretreaments was performed by blot- or air-drying. Samples were tested at 24 h or after 2 years of aging in artificail saliva at 37 °C. Tooth was considered the statiscial unit (n = 8/group). Different upper case letters indicate significant differences beween groups within the 24 h testing period. Different lower case letters indicate significant differences beween groups after aging for 2 years. * inidicates significant differences between aging periods within similar treatments. Statistical comparisons were performed by the Tukey test (α = 0.05).

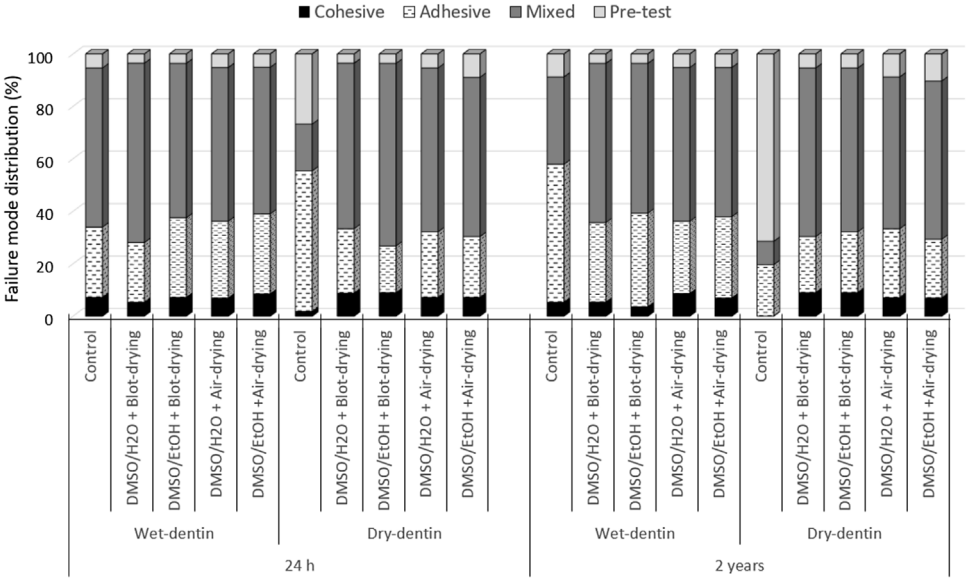

Fig. 2 – Fracture patterns in percentages (%) of tested specimens after the bond strength test at 24 h and 2 years of aging in artificail saliva at 37 °C. Resin-dentin interfaces were created by bonding a multistep etch-and rinse adhsieve (SBMP) to wet or dry dentin, using DMSO pretreatments with different moisture controls (blot- or air-drying). Fracture patterns were classified as: cohesive failure = 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.

porous bonded interfaces. Wet-bonded control samples presented lower extensions of silver deposits compared to dry-bonded samples. Nanoleakage patterns were characterized by reticular silver deposits extending mainly at the bottom of the hybrid layers. Areas of complete silver uptake through the full thickness of the hybrid layer were also observed, but to a much lesser degree than in the dry-bonded samples.

Irrespective of the initial dentin hydration (wet or dry) or moisture control (blot- or air-drying) after DMSO pretreatments, both pretreatments (DMSO/H_2O and DMSO/EtOH) produced clearly lower levels of silver uptake at the bonded interfaces compared to the wet and dry control groups. Nanoleakage extensions were similar in DMSO-pretreated groups, characterized by spotted silver deposits sparsely distributed across the hybrid layers. Areas with complete hybrid layer silver impregnation were hardly identified within DMSO-pretreatment dentin.

3.3. *Contact angle measurements*

A rapid decrease in contact angles for both bonding resins occurred during the initial 5 s, followed by a slower but still considerable decrease rate until 20 s. Contact angles then decreased slowly and reached a nearly constant value at approximately 180 s for the hydrophobic resin and 210 s for the hydrophilic resin. Fig. 4 illustrates the variation in contact angles over time observed for both resins applied on the flat dentin surfaces submitted to the different DMSO treatments with different moisture conditions. Contact angles from each

Table 1 – Microtensile bond strength means (MPa), standard deviations (±SD) and fracture modes.

	Wet dentin				Dry dentin			
	Control	DMSO/H_2O +Blot	DMSO/EtOH +Blot	DMSO/H_2O +Air	DMSO/EtOH +Air	Control	DMSO/H_2O +Blot	DMSO/EtOH +Blot	DMSO/H_2O +Air	DMSO/EtOH +Air
24 h	35.53 ± 3.50 (4/15/34/ 3/56)	50.44 ± 5.35 (3/13/39/2/ 57	54.92 ± 5.49 (4/17/33/2/ 56	49.20 ± 5.16 (5/17/36/2/ 60	49.90 ± 5.29 (6/19/34/2/ 61	17.15 ± 2.21 (1/30/10/15/ 56)	48.94 ± 7.50 (5/14/36/2/ 57	50.81 ± 8.02 (5/10/41/2/ 58	48.90 ± 4.97 (4/14/35/3/ 56	49.75 ± 7.96 (4/13/34/5/ 56
2 years	21.50 ± 2.24 (3/30/19/5/ 57)	49.18 ± 5.17 (3/17/34/2/ 56	52.33 ± 5.41 (2/20/33/3/ 58	46.95 ± 5.16 (5/16/34/3/ 58	50.57 ± 5.32 (4/18/33/3/ 58	2.57 ± 0.34 (0/11/5/40/ 56)	50.59 ± 5.75 (5/12/38/3/ 58	52.39 ± 5.41 (4/13/36/3/ 56	49.34 ± 5.02 (5/13/35/3/ 56	46.93 ± 4.59 (4/13/35/6/ 58

Tooth was considered the statistical unit (n = 8). Numbers in parentheses represent the total number of specimens following the fracture mode classification (1/2/3/4/5): (1) cohesive failure; (2) adhesive failure; (3) mixed failure; (4) pre-test failure and (5) total number of tested specimens.

group followed a logarithmic decay model, allowing the determination of the kinetics parameters listed as the spreading rate constants during the initial 20 s in Table 2. Factorial ANOVA showed that the factors "initial collagen hydration" ($p < 0.0001$; $\eta_p^2 = 0.19$), "dentin pretreatment" ($p < 0.0001$; $\eta_p^2 = 0.601$), "collagen moisture prior to hybridization" ($p < 0.0001$; $\eta_p^2 = 0.152$), "resin" ($p < 0.0001$; $\eta_p^2 = 0.54$), "time" ($p < 0.0001$; $\eta_p^2 = 0.77$) and the interactions "dentin pretreatment * resin * time" ($p < 0.017$; $\eta_p^2 = 0.77$), "dentin pretreatment * collagen moisture prior to hybridization * resin" ($p < 0.002$; $\eta_p^2 = 0.27$) significantly affected the contact angles. For the control groups, the hydrophilic resin produced significantly lower contact angles on wet than on dry dentin at both time periods (0.1 s and 20 s). In contrast, the hydrophobic resin presented no significant differences between wet-untreated or dry-untreated dentin at the same time periods. The hydrophobic resin produced significantly higher contact angles (roughly 90%) than the hydrophilic resin when deposited on untreated dentin at 0.1 s. Similarly at 20 s, hydrophobic resins also produced higher contact angles than the hydrophilic resin (roughly 85% higher) under wet conditions; however, no significant differences between resins occurred on air-dried dentin at 20 s. For the hydrophilic resin, DMSO pretreatments produced significantly lower contact angles than their respective dry-control group on both time periods irrespective of the initial collagen hydration (wet or dry) or moisture control (blot- or air-drying). Such contact angles were not statistically different from their respective wet-control group. Unlike the hydrophilic resin, the hydrophobic resin produced significantly lower values on DMSO-pretreated collagen when compared to their respective dry- and wet-control groups. These significant reductions were in the order of 30–50% at both time periods and occurred regardless of the initial dentin hydration (wet or dry) or moisture control (blot- or air-drying).

4. Discussion

Since dry bonding negatively affected resin-dentin bond strengths and hybrid layer stability while DMSO-dry protocols improved bonding, the first hypothesis was rejected. Bonding a three-step etch-and-rinse adhesive system to air-dried dentin produced inferior outcomes compared to the traditional wet-bonding technique, which is in accordance with previous reports [10,25,28,42]. Our findings reinforce the concept that etch-and-rinse adhesives must be preferably bonded to moist dentin to avoid collagen collapse and thus minimize issues related to matrix shrinking [3]. The main problem with dry bonding resides on collagen collapse, an active and rapid process involving the rapid spontaneous development of hydrogen bonds between adjacent collagen peptides and decreasing interfibrillar spaces [3]. Solvents may fully (*i.e.* water) or partially (*i.e.* ethanol, propanol, and acetone) re-expand collapsed collagen depending on whether their hydrogen bonding solubility parameters (δh) exceed 14.8 $(J/cm^3)^{1/2}$ [3]. Methacrylate-based bonding agents do not always promote adequate re-expansion of dried collagen [3]. As a result, diffusion of methacrylate monomers through such densely-packed collagen meshes is inefficient [3], which greatly compromises dentin bonding [10,28,42]. When com-

Table 2 – Wettability kinetics of hydrophilic (Primer) and hydrophobic (Bond) resins deposited onto DMSO-pretreated dentin with different moisture levels: contact angles at 0 s, 20 s, 240 s, standard deviation and spreading rate constant (*k*) at the initial 20 s.

Resin	Initial dentin condition	Dentin pretreatment	Moisture control (drying)	0.1 s	20 s	*k*
Hydrophilic (*Primer*)	Wet	(control)	Blot	23.2 (3.3) ^{CD}	13.1 (2.1) ^{CD}	1.85
	Dry	(control)	Air	30.5 (3.4) ^{B}	20.9 (3.2) ^{B}	1.71
	Wet	DMSO/H_2O	Blot	16.9 (2.9) ^{D}	9.9 (1.9) ^{D}	2.98
			Air	21.6 (4.9) ^{D}	12.4 (2.7) ^{D}	3.29
		DMSO/EtOH	Blot	16.8 (3) ^{D}	10.6 (4.3) ^{D}	2.23
			Air	18.9 (2.9) ^{D}	12.6 (3.8) ^{D}	2.7
	Dry	DMSO/H_2O	Blot	18.3 (3.9) ^{D}	11.9 (3.6) ^{D}	2.34
			Air	22.1 (3.4) ^{D}	11.8 (3.7) ^{D}	2.93
		DMSO/EtOH	Blot	18.8 (3.7) ^{D}	11.3 (3.1) ^{D}	2.27
			Air	20.8 (3.3) ^{D}	11.2 (2.3) ^{D}	2.21
Hydrophobic (*Bond*)	Wet	(control)	Blot	44.1 (5.3) ^{A}	24.3 (3.6) ^{A}	3.62
	Dry	(control)	Air	45.5 (3.4) ^{A}	22.4 (3.1) ^{AB}	3.98
	Wet	DMSO/H_2O	Blot	28.3 (3.5) ^{BC}	15.5 (3.4) ^{C}	4.99
			Air	30.3 (6.7) ^{B}	16.1 (3.4) ^{C}	4.64
		DMSO/EtOH	Blot	22.4 (3.5) ^{CD}	10.2 (1.6) ^{D}	4.65
			Air	30.7 (3.5) ^{B}	11.1 (3.6) ^{D}	5.22
	Dry	DMSO/H_2O	Blot	28.3 (4.3) ^{BC}	14.7 (3.1) ^{C}	4.93
			Air	30.8 (3) ^{B}	16.4 (1.9) ^{C}	5.7
		DMSO/EtOH	Blot	24.1 (4.1) ^{C}	10.6 (1.8) ^{D}	4.5
			Air	30.3 (5.1) ^{B}	14 (1.5) ^{CD}	4.34

Contact angles with different superscript letters indicate significant difference according to Tukey test ($p < 0.05$) when analyzed per column.

bined with HEMA, the re-expansion potential of solvents tends to drop, except for HEMA-water mixtures [3,43,44]. HEMA-water mixtures, commonly found in commercial bonding resins [45], may re-expand dried collagen up to 92%; however, subsequent solvent evaporation substantially shrinks the matrix again [44]. Water evaporation results in interpeptide hydrogen bonding, which may expel HEMA from within the collagen matrix [46]. Such instability of the collagen matrix prevents optimal resin-dentin bonding. Although HEMA-water mixtures may re-expand dried collagen quite effectively [3,43,44], additional resources, such as vigorous adhesive application, are necessary to produce similar outcomes to wet bonding [10].

The antagonic effects of water on resin-dentin bonding have been well documented [3,5,13]. While the lack of moisture compromises interfibrillar spaces, excess moisture may be detrimental to polymer formation [15]. Etch-and-rinse adhesives present a small window of opportunity regarding optimal surface moisture [6,11]. It is virtually impossible to consistently determine the ideal surface moisture in a clinical situation. Hence, broadening the moisture spectrum of etch-and-rinse adhesive systems to substantially drier levels would not only prevent potential problems associated to overwetting, but also allow a more consistent moisture control. Air-drying is a far simpler and more consistent moisture control approach if compared to blot-drying. The safe use of air-drying could greatly facilitate bonding procedures of etch-and-rinse bonding. Since comparable bond strength results were obtained on wet or dry collagen, regardless of initial dentin hydration or moisture control, it is evident that DMSO pretreatments eliminated the negative short and long-term effects of air-drying on resin-dentin bonding.

The benefits of using DMSO solutions as dentin pretreatments have been previously reported for wet [31,32,47] and air-dried [23,24] collagen. Our previous studies regarding DMSO-dry bonding [23,24,34] also made use of extensive air-drying for resin-dentin bonding to aid in the reduction of water content within the bonded interface. Nonetheless, DMSO pretreatments were blot-dried before adhesive application to avoid possible problems with collagen collapse after the application of DMSO pretreatments. In the present study, prolonged air-drying before or after DMSO pretreatments had no negative effects on bond strengths of etched dentin. DMSO-pretreated collagen may be blot- or extensively air-dried immediately before adhesive application without any negative effect on long-term bond strengths. This brings new possibilities to facilitate DMSO-bonding protocols and to reduce residual water from hybrid layers more efficiently by air-drying DMSO-pretreated collagen. Noteworthy, residual water is more effectively removed from collagen matrixes prior to the addition of bonding resins containing HEMA [48]. Hydrogen bonding between water and methacrylate monomers hinders effective water removal by evaporation [49]. In theory, prolonged air-drying after DMSO-pretreatments could potentialize water removal from hybrid layers. In previous attempts to combine DMSO and dry bonding, the pretreatment solutions were blot dried [23,24] to prevent possible collagen collapse as proposed for the ethanol-wet bonding technique [3]. It is important to note that saturation of demineralized dentin with polar organic solvents tends to stiffen collagen [50–52]. DMSO is a polar aprotic solvent with considerably low vapor pressure [53]. We speculate that DMSO-pretreated collagen may present sufficient increase in stiffness to prevent collagen collapse following prolonged air-drying [34], without any comprise in polymer formation. Unlike ethanol, which readily evaporates from collagen after air-drying, DMSO's low vapor pressure prevents full evaporation. Residual DMSO molecules may contribute to the maintenance of interfib-

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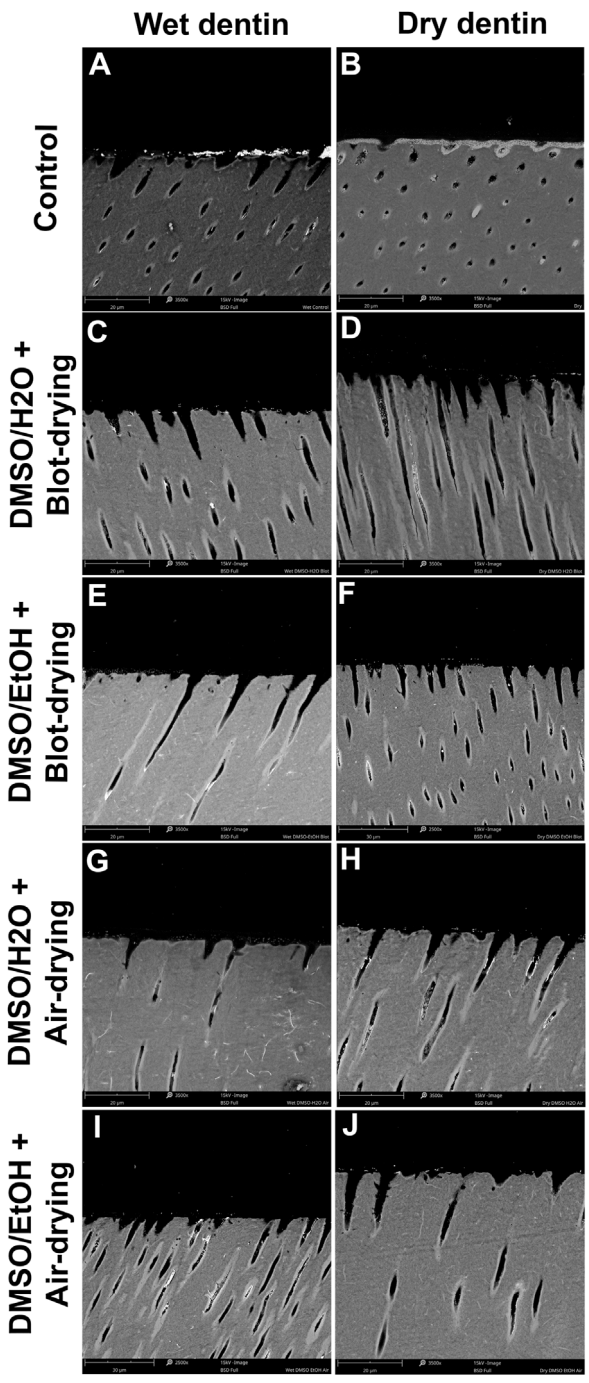

Fig. 3 – Representative SEM nanoleakage micrographs of aged resin-dentin interfaces bonded to wet or dry dentin using aqueous or ethanolic DMSO solutions as pretreatments. Moisture control after the application of pretreaments was performed by blot- or air-drying.

rillar spaces allowing appropriate monomer diffusion even after prolonged air-drying. Reduction of the post-collapsing effect of solvent volatilization could explain the higher bond strengths produced by DMSO pretreatments. Further studies should validate this hypothesis.

The rationale for testing different moisture controls (blot- and air-drying) at different stages (before or after pretreatments) was to determine whether DMSO-dry bonding would be possible and to identify which combinations would produce the most favorable long-term outcomes. Surprisingly, the initially higher bond strengths produced by bonding protocols containing DMSO were not affected by long-term aging irrespective of moisture controls at the different drying stages. Since the hybrid layer integrity was substantially improved in DMSO-pretreated samples after aging and bonded interfaces created on dry-untreated collagen were extremely porous, the second null hypothesis was rejected. DMSO-pretreatments not only improved bond strengths, but also produced hybrid layers with lower porosity after aging regardless of moisture control. This highlights the ability of DMSO-pretreatments to reduce technique sensitivity of resin bonding on etched dentin by broadening the spectrum of moisture towards a drier state. DMSO-water (δh 26.8 $(J/cm^3)^{1/2}$) and DMSO-ethanol (δh 16.6 $(J/cm^3)^{1/2}$) pretreatments present δh values superior to dry collagen (δh 14.8 $(J/cm^3)^{1/2}$). Although the overall expansion of dried collagen varies among solvents [3] and solvent mixtures [35], δh values higher than 14.8 $(J/cm^3)^{1/2}$ indicate the ability of both DMSO/H_2O and DMSO/EtOH to break interpeptide hydrogen bonds and thus re-expand collagen. DMSO/H_2O is clearly more effective in re-expanding dry collagen due to its higher δh. However, the use of water-free DMSO pretreatments further facilitates the overall removal of residual water from bonded interfaces. Furthermore, air-drying DMSO/EtOH from dentin surfaces may increase water removal as ethanol evaporates. Water evaporation is more efficient when performed before adhesive application [49]. Therefore, air-drying may potentialize water removal compared to routinely employed bonding protocols that rely exclusively on adhesive solvents to chase water molecules within demineralized collagen. To the best of our knowledge, this is the first attempt to successfully bond methacrylate monomers to demineralized collagen via ethanol saturation followed by extensive air-drying to eliminate solvents prior to adhesive application.

One of the first requirements for good adhesion is the optimum wettability of the bonding surface, allowing spontaneous spreading of the bonding agent on the surface. An intimate contact between the bonding agent and the surface is thereby of paramount importance to produce reliable bonding. A positive correlation exists between dentin wetting and bond strengths [54]. High wettability relates to intimate resin-dentin contact, which leads to enhanced adhesion. This is strongly dependent on the physicochemical parameters attributed to the bonding agent and the bonding surface such as chemical composition, viscosity, polarity, surface roughness and hydration. Measurement of contact angles of a liquid over a surface is the most common method to investigate wettability [41,54–56]. In this study, 20 s was established as a cutoff point, since most of the reduction in contact angles took place before this time point. In addition, 20 s is well within clinically acceptable time during bonding procedures. The rationale for measuring contact angles of the hydrophilic and hydrophobic resins separately was to determine the specific effects of the DMSO-bonding protocols on their wettability. Naturally, the hydrophobic resin produced higher contact angles than the hydrophilic resin on untreated dentin. Hydrophobic bonding resins contains higher ratios of high molecular weight

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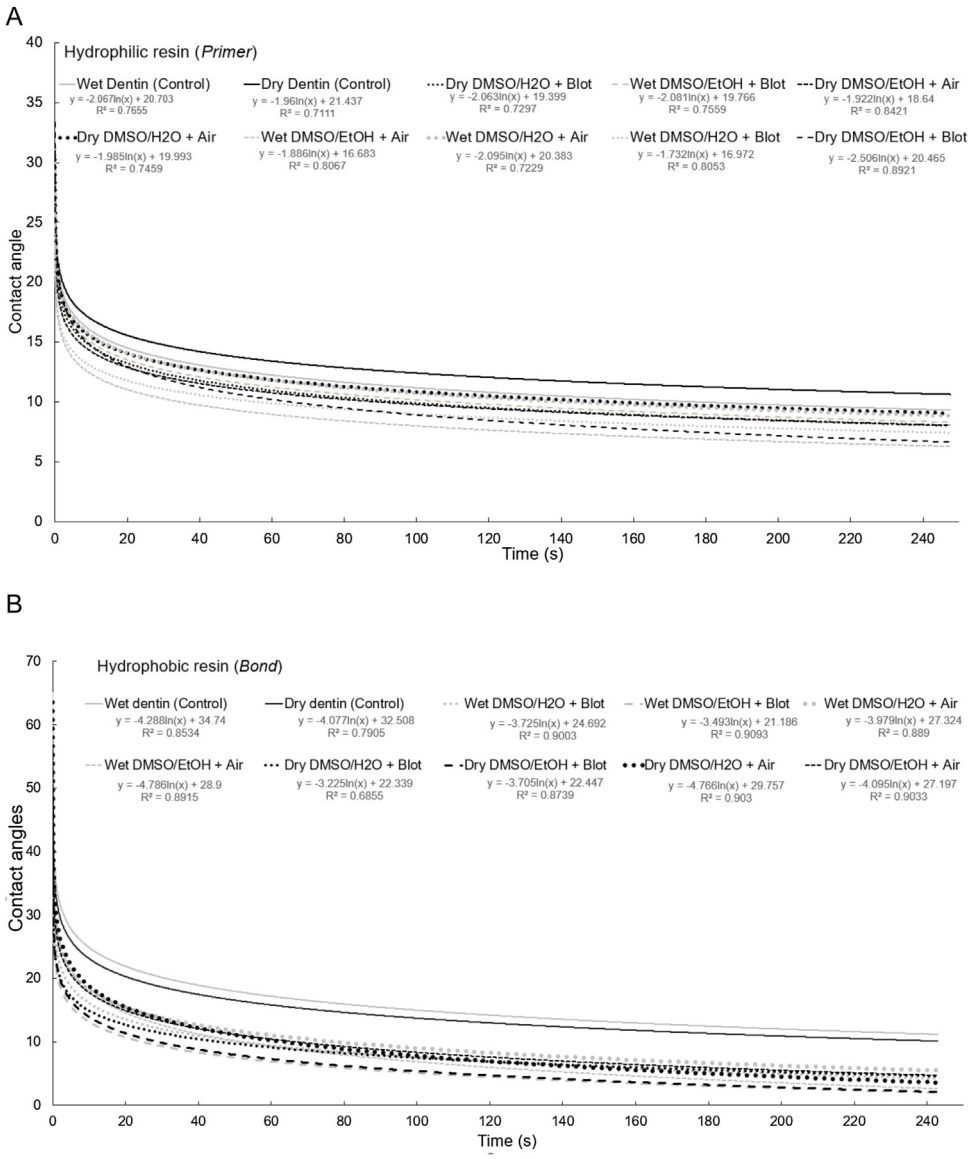

Fig. 4 – Contact angle evolution up to 240 s of hydrophobic (A; Bond) and hydrophilic (B; Primer) resins deposited onto wet or dry H_3PO_4-etched dentin pretreated with aqueous or ethanolic DMSO solutions followed by blot- or air-drying. Trend lines for each group (n = 8 measurements/group) were determined by the logarithmic decay model.

monomers [45]. Such cross-link monomers (*i.e.* bis-GMA) produce strong intermolecular hydrogen-bonding interactions between the hydroxyl groups (OH) and the carbonyl groups (C═O) in distinct bis-GMA monomers [57]. This accounts for the higher viscosity of the commoner, which reduces overall monomer mobility and consequently hinders collagen wetting. Moreover, dentin hydrophobicity increases with air-drying [56]. The notion that air-drying reduces the wettability of demineralized collagen was demonstrated over two decades ago [54–56] and further complicates resin-dentin bonding. Our bond strength data corroborates the latter. The high water content of the hydrophilic resin did not compensate for the lack of collagen moisture. Higher contact angles were observed for the HEMA-based hydrophilic primer on air-dried dentin collagen confirming the worse interaction between them [56].

Clinical adhesive procedures usually fall short of adequate resin spreading times [41,54,56], which compromises optimal resin-dentin wetting. This was corroborated by our findings where hydrophobic and hydrophilic resins achieved near equilibrium contact angles only at 180 and 210 s, respectively. From a clinical perspective, waiting for the bonding resins to completely wet dentin surfaces is unfeasible. The effect of DMSO on the specific spreading behavior of bonding resins onto collagen remained unknown until now. DMSO-pretreatments reversed wettability issues of air-dried collagen, so the third hypothesis was rejected. DMSO has the ability to reduce contact angles between water and dentin collagen [33,35], implying wettability improvements. Altogether, DMSO pretreatments accelerated resin spreading. Spreading rate constants (*k*) were 20–60% higher during the initial 20 s for DMSO-pretreated dentin. Regardless of initial dentin hydra-

tion (wet or dry) or moisture control (blot- or air-drying), DMSO increased wettability of the hydrophilic resin to levels similar to those of wet dentin, eliminating the negative impact of air-drying on collagen wetting. An even more profound effect was observed for the hydrophobic resin, with reductions in contact angles ranging from 30 to 50 % at 20 s. It is important to note that the lowest contact angles at 20 s occurred for ethanol-containing DMSO pretreatments. While DMSO pretreatments had no effect on contact angles of the hydrophilic resin applied on wet dentin, they greatly facilitated the spread of the hydrophobic resin on dry or wet collagen. Replacing water by ethanol, as the cosolvent in the pretreatment solution, had a positive impact in the wettability of the hydrophobic bonding resin. Water is generally poorer as a solvent for methacrylate monomers compared to ethanol, especially considering high molecular weight monomers such as bis-GMA present in hydrophobic formulations. It is tempting to speculate that priming of etched dentin with only DMSO-based cosolvents containing no hydrophilic monomers would be possible. Adhesive spreading indeed occurs simultaneously with monomer diffusion across demineralized collagen, albeit not necessarily at the same relative rate. The diffusion of high molecular weight monomers through dried-collapsed collagen is a demanding process, which may be facilitated by DMSO pretreatments. Nonetheless, more studies are necessary to evaluate both the feasibility and stability of resin-dentin bonded interfaces produced with a priming step free from hydrophilic monomers.

5. Conclusion

The ability to safely air-dry demineralized collagen and yet produce more stable resin-dentin bonding and less porous hybrid layers over time can be considered a major step towards technique-sensitive reduction of etch-and-rinse adhesive systems. DMSO not only improved long-term resin-dentin bonding, but also provided an added versatility to minimize overdrying-related issues in etch-and-rinse bonding. Moreover, the proposed DMSO-ethanol pretreatment followed by the air-drying approach constitutes a feasible alternative to reduce residual water from resin-dentin interfaces by broadening the moisture spectrum of demineralized dentin to drier levels without compromising collagen wettability.

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The influence of dimethyl sulfoxide on resin–dentin bonding: A systematic review

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ABSTRACT

Objective: This article aims to systematically review published literature about the influence of dimethyl sulfoxide (DMSO) on resin–dentin bonding.

Materials and methods: Online databases (Medline (PubMed), Embase, Web of Science, and the Cochrane Library) were searched on this topic. Gray literature was searched using Google Scholar. Published works until December 2020 were collected, and pertinent articles were selected in accordance with the eligibility criteria. Quality assessment was conducted via the modified Consolidated Standards of Reporting Trials (CONSORT) checklist and the modified Cochrane risk of bias tool.

Results: Thirty-eight studies were identified, twelve of which satisfied the eligibility criteria were included in this review. Micro tensile bond strength (MTBS) was set as the main outcome. Secondary outcomes include nano-leakage evaluation, the effect of DMSO on dentin collagen fibrils, and protease inhibition. Several studies proved the promotion of DMSO on long-term dentin bond strength of etch-and-rinse adhesives. However, evidence about its effect on bond strength of self-etch adhesives was insufficient. Different concentrations of DMSO solution also cause diverse impact on dentin bonds.

Conclusion: Based on the included studies, DMSO considerably affects resin–dentin bonds by dissociating demineralized dentin matrix, inhibiting matrix metalloproteinases (MMPs), and enhancing penetration of resin monomers. The influence of DMSO on dentin bonding may offer inspirations for dental clinical practice to optimize resin–dentin bonds.

1. Introduction

Among direct tooth-colored restorative materials, resin composites are the most frequently introduced [1]. In adhesive dentistry, dentin bonding is regarded as a form of tissue engineering, which involves water, adhesive resin composite, and organic collagen fibrils. After etching, demineralized collagen fibrils interact with polymerized adhesive resins by micromechanical force and form a structure of hybrid layer, a bio-composite containing dentin collagen, and polymerized resin adhesive [2]. The integrity of hybrid layer faces challenges in ageing process although it has achieved satisfactory immediate bond strength. Excessive demineralization caused by acid etching [3], insufficient infiltration of adhesive monomers [4], hydrolysis of resin composites [5], and degradation of dentin collagen fibrils [6] are the main factors leading to hybrid layer degradation [7,8].

Various approaches to preserve hybrid layer have been introduced over the past decades, including extrafibrillar demineralization [9], the application of cross-linking agents [10,11], inhibition of the enzymatic activity [12], removal of the residual water [13], and biomimetic remineralization [14]. It has been reported that surface wetting or moisture of dentin may have great impact on its bonding performance [15–17]. Thus, water wet bonding technique was developed to maintain a stiff state of collagen fibril network and expand the microporous structure to promote the penetration of the resin monomer [18]. However, it is technically sensitive to control dentin humidity in clinical practice because excessive wetting or drying may adversely affect the bonding effect [19]. To optimize water wet bonding technique, ethanol was introduced in etch-and-rinse (E&R) adhesive system to expand

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dentin collagen fiber network and improve the bond strength of dentin [20]. In the laboratory, the so-called ethanol wet-bonding technique, in which ethanol is used to replace water, presents satisfactory results when applied in incremental concentrations in hydrophobic adhesives [21]. However, it also brings about problems of premature volatilization during clinical practice when the bottle of adhesive is opened repeatedly, thus compromising bonding effectiveness. In addition, pretreatment with a series of increasing ethanol concentrations is time consuming and impractical for clinical operation [22,23].

Dimethyl sulfoxide (DMSO), a highly dipolar aprotic organic liquid, can dissolve polar and nonpolar compounds. It is generally used in organic synthesis and chemical industry and is well-known as a universal solvent [24]. In the bioengineering and pharmaceutical industry, it has been widely used as a solvent in biological study as well as a vehicle for drug delivery [25]. It has also been discovered as an excellent enhancer of tissue penetration/membrane permeability [26] and interacts actively with collagen fiber [27]. In cellular biology, DMSO works as an efficient cryoprotectant of stem cells; it prevents cellular damage during freeze–thaw [28]. Various primary pharmacological functions of DMSO have been documented in laboratory studies and successively proven in clinical medications, including treatment of dermatological diseases [29], interstitial cystitis [30], increased intracranial pressure [31], and membrane transport purposes [32].

In terms of the chemical and biological activities of DMSO and the nature of hybrid layer, researchers found that DMSO is not only a competitive candidate for the solution of dental adhesives, but also a multifunctional adhesion primer in dentin bonding [33,34]. DMSO has been used as a solvent of several dental monomers and other adhesive components in toxicological testing of adhesive components [35,36]. It is favored for its outstanding performance of dissolving hydrophilic and hydrophobic components [35,37]. DMSO possesses stronger permeability and lower volatility than ethanol. Thus, a concept of DMSO wet bonding was gradually developed in dentin bonding, attracting a number of investigators to evaluate its effectiveness [37–39]. According to previous studies, DMSO could improve dentin surface moisture [38], stabilize collagen fibrils [40], and suppress the activity of MMPs [33], thereby preserving hybrid layer integrity and promoting long-term resin–dentin bonds. However, the application time, methods, and toxicity bring about serious concerns, limiting the utilization of DMSO in adhesive dentistry [26,37,41]. To the best of our knowledge, however, no review of the existing literature has been made to systematically analyze different study results involving DMSO in resin–dentin bonding.

Therefore, this study aims to conduct a systematic review describing the influence of DMSO on resin–dentin bonding, including dental adhesives, dentin collagen fibrils, and endogenous collagenolytic enzymes.

2. Methods

2.1. Search strategies

This systematic review was conducted in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guideline [42], registered in the International Prospective Register of Systematic Reviews (PROSPERO) database (ID number: CRD42021271810). The PICOS question for systematic review were set as follows:

Participants: Human extracted teeth.

Intervention: Application of DMSO on dentin surface before resin bonding.

Comparison: Untreated control or water as a primer on dentin surface before resin bonding.

Outcome: Micro tensile bond strength.

2.2. Study design: in vitro studies

Two independent reviewers (Z. Z. and K. L.) were designated for literature search to identify articles in the databases Medline (PubMed), Embase, Web of Science, and the Cochrane Library until December 2020. Gray literature was searched using Google Scholar. Strategies for literature search involved the following keywords:

1#: “dimethyl sulfoxide” OR “Dimethyl sulphoxide” OR “dimethylsulfoxide” OR “dimethylsulphoxide” OR “DMSO” OR “polar aprotic solvent” OR “dipolar aprotic solvent”
2#: “dentin bonding” OR (“bond*” AND “strength” AND “dentin*“) OR “hybrid layer”
3#: 1# AND 2#

Fig. 1 illustrates the study selection process of this systematic review. The identified articles from the selected databases were checked for duplication. Two independent reviewers (Z. Z. and K. L.) screened the titles and abstracts of the potential eligible articles after removing the duplicates and hand-searched the reference lists of the included articles for additional papers.

2.3. Eligibility criteria

Inclusion criterion: The articles which reported the effect of DMSO application on the bond strength of human dentin to resin composite were included.

Exclusion criterion: Articles were excluded if (1) the study was not about resin–dentin bond strength, (2) the tested dental substrate was not resin–dentin bio-composite, (3) the method to evaluate bond strength was not micro tensile test, (4) no blank control (such as water or saline) was set, and (5) other kind of organic solvent (such as ethanol or acetone) was incorporated with DMSO.

Subsequently, full texts of the remaining articles were retrieved to exclude articles without reporting bond strength values. The following materials and methods were extracted from the included studies: types of adhesive, application strategy of DMSO, and ageing method. The values of the bond strength were set as the main outcome. Secondary outcomes include nanoleakage evaluation, the effect of DMSO on dentin collagen fibrils, and protease inhibition. The same two independent reviewers (Z. Z. and K. L.) performed data extraction. A discussion with a third independent investigator (J. Y.) was conducted to arrive at a consensus if there’s disagreement on the study inclusion or data extraction. Cohen Kappa value was calculated to define the inter-rater agreement in the study selection process.

2.4. Data extraction

The data were extracted with all of the trial documents containing demographic data (year, country), adhesive systems, DMSO application protocol, ageing methods, and outcomes evaluated. The authors of the included studies were contacted by e-mail to retrieve the missing data. If no response was obtained after the first e-mail message sent for two weeks, a second e-mail would be resent for confirmation. The missing information would not be included in this systematic review if the authors did not reply with an answer within one month. The data of MTBS values from selected articles can be viewed in supplementary information (Table S1).

2.5. Quality assessment

Quality assessment of the included studies was performed by the two independent reviewers (Z. Z. and K. L.) via a modified CONSORT checklist of the items for reporting in vitro studies of dental materials (Table 1) [43,44] to evaluate the fulfillment for each of the quality assessment parameters or items considered in the checklist. Risk of bias was assessed using the modified Cochrane risk of bias tool [45,46]. Fig. 2 describes the classification scheme for risk of bias assessment. An article received a zero if all sections were clear; 1, if insufficiently or unclearly

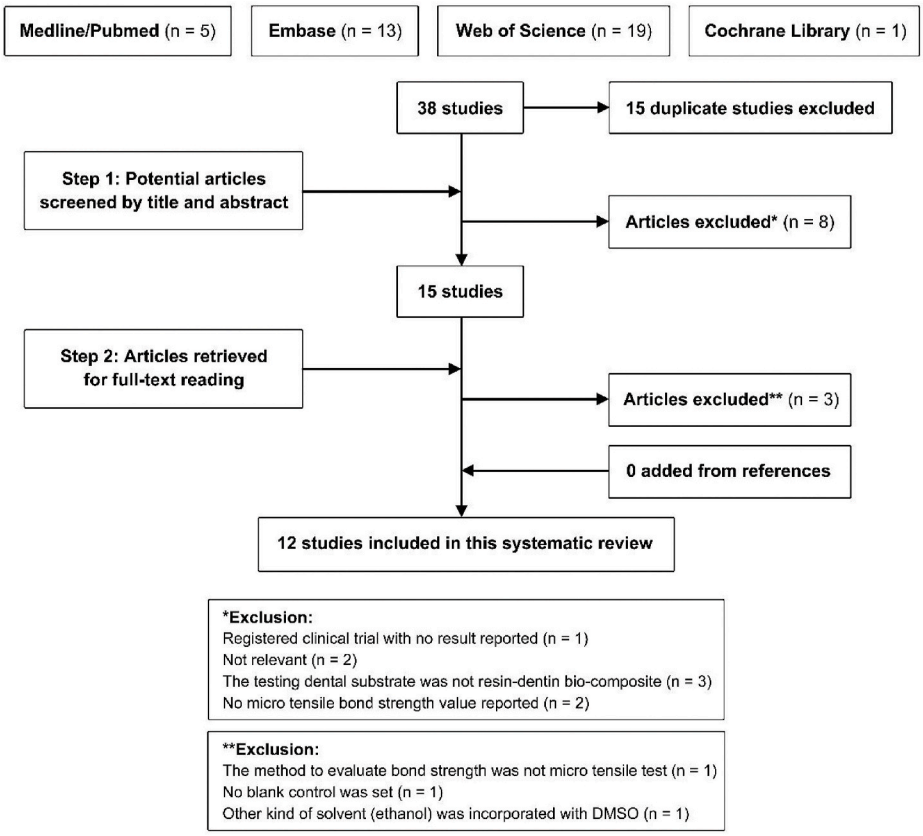

Fig. 1. Flow chart of the study selection process.

defined; and 2, if unrevealed. Articles with scores of 0–3 were graded low risk, 4–7 moderate or unclear risk, and 8–10 high risk of bias.

3. Results

A total of 38 articles were identified from the selected databases, and 15 duplicated articles were removed (Fig. 1). After screening the title and abstract, two articles were excluded because they were unrelated to DMSO in dentin bonding. One article was excluded as a registered clinical trial in Cochrane Library without result reported. Three articles were excluded because the testing dental substrate was not a resin–dentin bio-composite. Two articles were excluded for not reporting bond strength test. Fifteen articles were retrieved for full-text reading. One article was excluded for using shear test to measure bond strength between dentin substrate and resin composite. Two articles were excluded because no blank control was set (n = 1) and ethanol was incorporated in DMSO (n = 1), respectively. Finally, 12 articles were included in this review. The Cohen κ ± standard deviation (SD) range was 0.91 ± 0.03. The primary characteristic of the included studies is exhibited in Table 2.

Micro tensile bond strength (MTBS) was the dominant criterion to assess dentin bonding quality. All studies included in this review used mid-coronal dentin disks from extracted human teeth (third molar or premolar). Four studies [48–50,52] evaluated the influence of naturally derived substances (baicalein, mussel-inspired molecule, epigallocatechin-3-gallate (EGCG), and galardin) on dentin bond strength, in which DMSO served as solvent and better dissolved these organic compounds. In these four studies, groups subjected to DMSO treatment alone were set as control groups (blank control groups were also used).

Eight studies evaluated nanoleakage across the resin–dentin interface and found that the degree of nanoleakage in DMSO-treated groups was lower than or equal to that in the control groups [33,38,39,41,47–50]. Seven studies described the influence of DMSO on dentin collagen fibrils [33,37,38,48–51]. Tjäderhane et al. found that 100% DMSO induced collagen dissociation [33]. Stape et al. and Guo et al. discovered that 50% DMSO decreased collagen exposure at the bottom of the hybrid layer [37,38]. However, Li et al. revealed that 100% DMSO induced collagen dissociation and hampered the formation of the hybrid layer [49]. With regard to endogenous protease inhibition, two studies reported that DMSO inhibited MMPs activity by zymographic analysis [33,51]. Other studies found that DMSO can suppress gelatinase by in situ zymography [38,50,51]. One study revealed that 1% DMSO had no effect on inhibiting gelatinase but can inhibit collagenase [48].

Self-etch (SE) adhesive system was used in three of the included studies [37,39,52]. Two studies showed that no significant increase was found in immediate MTBS value by applying 50% DMSO aqueous solution [37,39]. After storage in artificial solution for two years, DMSO treatment produced significant higher bond strength than the control. One study found that 100% DMSO irrigation had no evident effect on bonding performance after 500 thermal cycles [52].

All included studies adopted E&R adhesives, among which are three-step, two-step, or universal adhesive in E&R mode. For immediate MTBS, five studies [37,39,41,48,51] reported improvement in dentin bond strength by DMSO treatment, and seven studies [33,38,47,49,50,52,53] found no significant increase in dentin bond strength. Nine studies [33,38,39,47–50,52,53] tested bond strength after ageing, and their ageing methods and duration varied from storage in artificial saliva for 3, 6, 12, and 24 months to 500 or 10,000 thermal cycling, and only one study used storage in collagenase containing artificial saliva for one month. Six studies [33,38,39,47,48,53] reported improvement in bonding stability after ageing by DMSO treatment, with the concentration ranging from 0.004% to 50%. Three studies [40,49,52] revealed that the bond strength of DMSO-treated groups decreased to the same degree as of the control groups after the ageing procedure. In these studies, the concentrations of DMSO were 50% [50] and 100% [49,52].

Table 1
Modified CONSORT checklist of items for reporting in vitro studies of dental materials.

Section/Topic	Item No.	Checklist item
Abstract	1	Structured summary of trial design, methods, results, and conclusions
Introduction		
Background and objectives	2	a. Scientific background and explanation of rationale b. Specific objectives and/or hypotheses
Methods		
Intervention	3	The intervention for each group, including how and when it was administered, with sufficient detail to enable replication
Outcomes	4	Completely defined, pre-specified primary and secondary measures of outcome, including how and when they were assessed
Sample size	5	How sample size was determined
Randomization: Sequence generation	6	Method used to generate the random allocation sequence
Allocation concealment mechanism	7	Mechanism used to implement the random allocation sequence (for example, sequentially numbered containers), describing any steps taken to conceal the sequence until intervention was assigned
Implementation	8	Who generated the random allocation sequence, who enrolled teeth, and who assigned teeth to intervention
Blinding	9	If done, who was blinded after assignment to intervention (for example, care providers, those assessing outcomes), and how
Statistical methods	10	Statistical methods used to compare groups for primary and secondary outcomes
Results		
Outcomes and estimation	11	For each primary and secondary outcome, results for each group, and the estimated size of the effect and its precision (for example 95% confidence interval)
Discussion		
Limitations	12	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses
Other information		
Funding	13	Sources of funding and other support (for example suppliers of drugs), role of funders
Protocol	14	Where the full trial protocol can be accessed, if available

The in vitro studies included were assessed using the modified CONSORT checklist [43] (Table 3). All of studies presented a well-organized abstract (item 1) and an introduction, which provided sufficient background information about previous application of DMSO for medical or dental purposes (item 2a), with specific objectives and hypotheses (item 2b). All included studies had explicit description of methodology and variable synthesis that allows for replication (items 3 and 4). However, the calculation of the sample size was missing, and the allocation sequence used was not mentioned (items 5–9). Every study mentioned statistical method for analysis (item 10), except that in one study, statistical methods used to analyze data were not described in detail (the specific type of analysis of variance (ANOVA) test was not mentioned, and two kinds of variance were found) [53]. Only one study reported 95% confidence interval by supplementary in the online version via a link (item 11) [33]. The authors tended to provide a brief report of the critical results and a comparison with correlated findings from other published articles. Only three studies described the possible limitations (item 12) [33,49,50]. Most studies indicated the sources of their funding (item 13), and only four studies failed to provide explicit information about potential conflicts of interest [38,39,47,53]. No study included references to full trial protocols (item 14). Risk of bias assessment is illustrated in Fig. 2. The bias was related to allocation concealment, sample size calculation, blinding, testing methodology, and selective reporting. Quality assessment (Table 4) showed that all the included studies had moderate risk of bias.

4. Discussion

In this systematic review, MTBS was set as the main outcome to investigate the effect of DMSO on resin–dentin bonds. The advantages of the MTBS test are increased adhesive failures and decreased cohesive failures, measurement of high interfacial bond strengths, and calculation of means and variances for a single tooth. Given that the tested surface area is approximately 1 mm^2, it facilitates SEM/TEM examinations of failed bonds [54]. However, the drawback is that the number of flaws becomes critical and would affect load in small bonding areas [55]. To minimize heterogeneity among different studies in this systematic review, we only included researches and statistics involving MTBS and excluded other types of mechanical tests. Since the objective of this study is to review the effect of DMSO on the bond strength between adhesives and dentin, studies which incorporated DMSO and ethanol in dentin pretreatment were excluded because they bring confounding factors.

SE adhesive system was used in three of our included studies [37,39,52]. The mild acidity of SE adhesive limits its potential to demineralize the highly mineralized components of dentin. DMSO would be entrapped in large intratubular spaces if applied prior to priming, offering a tendency to dilute the primer and weaken its already mild demineralization capacity [34]. The insufficient etching results in relatively lower degree of collagen exposure. DMSO may not infiltrate the exposed collagen matrix efficiently since it was applied on the smear layer-covered dentin, and the hydrophilic monomer, 10-methacryloxydecyl dihydrogen phosphate (10-MDP), might dissolve and incorporate the smear layer into a narrow hybrid layer simultaneously [39]. This condition might limit the capacity of DMSO to increase the micromechanical retention of SE systems [51]. However, there is still a lack of studies investigating the influence of DMSO on SE adhesive systems, and more evidence are required. Different from SE adhesives, all included studies adopted E&R adhesives. In E&R adhesive systems, 37% phosphoric acid is sufficient to etch and dissolve mineralized component. Basically, DMSO was applied after acid-etching and caused no interference to demineralization. When the inorganic substrates are removed, DMSO could better interact with protein peptides like collagen fibrils and MMPs. The secondary outcomes included in this review give clues on the mechanism of DMSO's function.

Optimized wetting of the demineralized dentin surface can be achieved with the DMSO pretreatment. Guo et al. reported that contact angles of 50% DMSO-pretreated dentin were significantly smaller than those of the water-wet dentin [38]. A significant decline in water contact angle was observed when 1%–5% DMSO was applied to acid-etched dentin [40], and there was a negative correlation between the DMSO concentrations and surface tension of DMSO/H_2O mixtures. Lowered contact angle is a reflection of higher surface energy and better contact of resin with the dentin surface as well as enhanced penetration into the hybrid layer [33,37]. The DMSO wet bonding treatment manifested significantly reduced width of exposed dentin collagen compared with the water and ethanol wet bonding treatments. This finding denotes that DMSO better promotes resin monomer infiltration into the collagen matrix, thereby leaving less unprotected collagen [37,38]. DMSO pretreatment exhibited much longer resin tags and increased infiltration depth into dentin [38,41]. Increased length of resin tags and decreased width of exposed collagen facilitate the integration between adhesive resins and collagen fibrils, improving the stability of the hybrid layer (**Fig. 3**). A "wet" surface is always difficult to control in clinical practice. Thus, continuous suspicion lies in that wet bonding is highly technically sensitive. Stape et al. applied DMSO on dry dentin surface and discovered that irrespective of wet or dry dentin surface, 50% DMSO significantly raised immediate bond strength [41,51], which is also a reflection of enhanced resistance to moisture change by DMSO. This condition

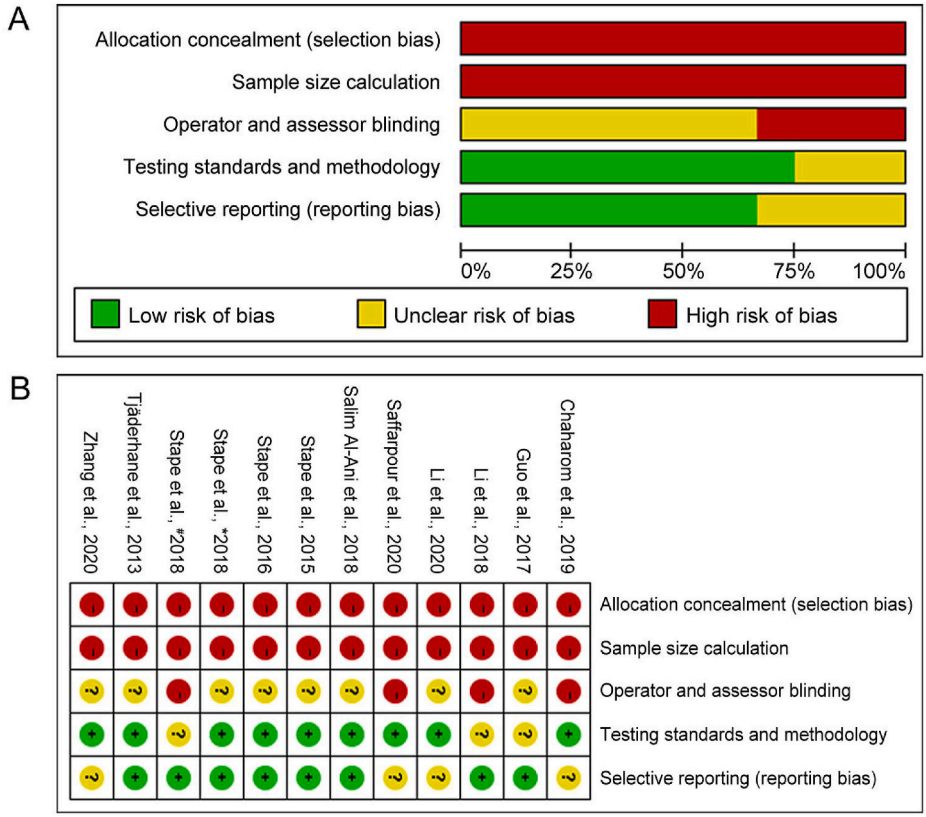

Fig. 2. Risk of bias graph assessment of the included studies. Green circles indicate low risk of bias; yellow circles indicate unclear risk of bias; red circles indicate the high risk of bias. A, Risk of bias for each included study. The studies were assessed for five types of bias; B, The overall summary of bias of the included 12 studies (*corresponds to Ref. [41]; #corresponds to Ref. [51].). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

enables DMSO to be user-friendly because it is adapted to different wetting degrees of demineralized dentin.

The partial negative charge on the oxygen atom favors the formation of hydrogen bonds with water molecules given that DMSO has a highly polar S═O group and two hydrophobic CH_3 groups [56]. Typically, DMSO forms two hydrogen bonds with water molecules that are four times more stable than hydrogen bonds inside water molecules [33]. In this condition, DMSO could break down water's self-associative tendency and result in decrease in surface tension or cohesion in DMSO aqueous solution. A balance between polarity and low-surface tension and a relatively high dielectric constant causes prominent surface wetting, especially for porous anomalous surface such as demineralized dentin. Collagen matrix expansion measurements exhibited an enhanced resistance of collapsed collagen network to dehydration and wetting after incubation in DMSO (with concentration up to 80%) [40]. Impregnation of demineralized dentin disks in pure DMSO for 30 min endowed transparent disks, and further immersion in water for 15 min returned the turbidity of the disks [33]. Thus, the decrease in collagen matrix expansion with the optical clearing effect indicated that DMSO dissociates collagen fibril bundles into a sparser network by reversibly destabilizing the collagen structure.

In biological structures, the triple-helical dentin collagen molecules are concealed by bound water [57] that limits the chemical bond of functional monomers, such as 10-MDP [39]. DMSO may expose more binding sites and allow more MDP molecules bond with collagen molecules by disrupting the water layer bound to collagen molecules [58]. The hydrated collagen might be more accessible to hydrophobic monomers, and the water-related hindrance of hydrophobic monomer polymerization might be reduced by DMSO [39]. The 10-MDP chemical bonding may be.

Enhanced due to the effects of DMSO on resin monomers and collagen [59]. Therefore, DMSO/H_2O priming strengthens the diffusion of functional monomers across the hybrid layer prompting polymer chain crosslinking and monomer–dentin interaction to improve the hybrid layer integrity and enhance the bond strength [37,41]. Notably, the infiltration of adhesive system is partially related to the variation of the hydrophobic/hydrophilic component ratio [37]. The separation of hydrophilic and hydrophobic monomers affects the ability of the adhesive systems on diffusing into the dentin substrate appropriately [60]. Phase separation hampers hydrophobic monomer (such as Bis-GMA) infiltration into sites, where residual water is present throughout the width of the demineralized dentin matrix and inhibits integration of collagen–polymers [61]. As a result, the quality of the hybrid layer is low [62]. Better monomer diffusion and improved collagen encapsulation were achieved by DMSO saturation on demineralized dentin matrix due to the amphiphilic nature of DMSO, thereby enhancing the formation of integrated hybrid layer by reducing phase separation **(Fig. 3)** [60,63].

The nanoleakage expression along the resin–dentin bonded interface is deemed as a significant standard to assess the bonding stability and durability and integration of the hybrid layer [64,65]. Voids left after acid etching was incompletely filled with adhesive resins because of insufficient infiltration of adhesives into etched dentin [66]. These nano-sized gaps lie beneath or within the hybrid layer and allow water infiltration or bacteria invasion, leaving unprotected collagen more susceptible to degradation over time [67,68]. DMSO holds the ability to preserve the stability of the hybrid layer and maintain the integrity of resin–dentin bonds by enhancing adhesive infiltration into etched dentin matrix. In this systematic review, only one study evaluated the nanoleakage of the SE adhesive with or without DMSO treatment and found that there was no significant difference [39]. However, eight included studies reported that the nanoleakage of the DMSO-treated groups which used E&R adhesives was less or no more than those of the control groups, especially when the hybrid layer had undergone ageing procedure [33,38,39,41,47–50]. Decreases in nanoleakages indicate prohibition of water permeability and obstruction of bacteria invasion, thereby guaranteeing long-term stability of resin–dentin bonds.

MMPs are a series of host-derived collagenase that degrades

Table 2
Characteristics of the articles included in this systematic review, along with main and secondary outcomes.

Authors (Year)	Teeth	Adhesives	Protocol	Ageing method	Main outcome	Secondary outcome		
					Bond strength	Nanoleakage	Collagen	Protease inhibition
Tjäderhane et al. (2013)[33]	Third molar	E&R: Scotchbond 1XT	0.004% DMSO as additional primer for 30 s	6/12 months storage in artificial saliva	Immediate: DMSO = Control Aged: DMSO > Control	Immediate: Control = DMSO 6-month: Control > DMSO	100% DMSO induced collagen dissociation	5% DMSO inhibited MMPs activity
Stape et al. (2015)[37]	Third molar	E&R: Scotchbond Multi-Purpose SE: Clearfil SE Bond	50% DMSO pretreatment for 60 s	NA	E&R: DMSO > Control SE: DMSO = Control	NA	50% DMSO decreased the collagen exposure at the bottom of the hybrid layer with both adhesives tested	NA
Stape et al. (2016)[39]	Third molar	E&R: Scotchbond Multipurpose SE: Clearfil SE Bond	50% DMSO pretreatment for 60 s	12/24 months storage in artificial saliva	Immediate: E&R + DMSO > E&R = SE = SE + DMSO 12-month aging: E&R + DMSO > E&R = SE = SE + DMSO 24-month aging: E&R + DMSO = SE + DMSO > E&R = SE	Immediate: E&R and SE: DMSO = Control 24-month: E&R: DMSO < Control SE: DMSO = Control	DMSO may disrupt the water layer which covers the collagen surface, providing more exposed binding sites of the collagen fibril for the functional monomers to attach	NA
Guo et al. (2017)[38]	Third molar	E&R: Adper Single Bond 2	50% DMSO pretreatment for 60 s	10,000 times thermocycling	Immediate: DMSO = Control Aged: DMSO > Control	Immediate: DMSO = Control Aged: DMSO < Control	50% DMSO reduced the amount of collagen exposure at the bottom of the hybrid layer	50% DMSO decreased the collagenolytic activity in the hybrid layer
Stape et al. (2018)[41]	Third molar	E&R: Adper Scotchbond Multi-Purpose & Scotchbond Universal Adhesive	50% DMSO pretreatment for 60 s on wet/dry dentin surface	NA	Multi-Purpose: DMSO > Control (in both wet & dry bonding) Universal: DMSO = Control (in both wet & dry bonding)	Multi-Purpose: DMSO = Control (wet bonding) DMSO < Control (dry bonding) Universal: DMSO = Control (in both wet & dry bonding)	NA	NA
Salim Al-Ani et al. (2018)[47]	Third molar	E&R: Adper Single Bond Plus	0.001, 0.01, 0.1, 1, 5, 10, and 20% DMSO pretreatment for 30 s	6 months storage in artificial saliva	Immediate: DMSO = Control 6-month aging: DMSO (>0.01%) > Control	Immediate: DMSO (5% & 10%) < Control 6-month: DMSO (>0.01%) < Control 5% DMSO showed the lowest value for both time points	NA	NA
Stape et al. (2018)[51]	Third molar	E&R: Adper Scotchbond Multi-Purpose	50% DMSO/H_2O or 10% DMSO/ SBMP primer applied on wet/ dry dentin surface	NA	Wet surface: 50% DMSO/ H_2O > 10% DMSO/ primer = Control Dry surface: 50% DMSO/ H_2O > 10% DMSO/ primer > Control	NA	50% DMSO/H_2O significantly reduced collagen breakdown, while 10% DMSO/ primer had no significant difference	50% DMSO pretreatment inhibited the activity of MMPs in both wet and dry conditions
Li et al. (2018)[48]	Third molar	E&R: Adper Single Bond 2	1% DMSO pretreatment for 120 s	3/6 months storage in artificial saliva	Immediate & aged:	Immediate & Aged:	NA	1% DMSO had no effect on inhibiting gelatinase, but

(*continued on next page*)

Table 2 (*continued*)

Authors (Year)	Teeth	Adhesives	Protocol	Ageing method	Main outcome	Secondary outcome		
					Bond strength	Nanoleakage	Collagen	Protease inhibition
					DMSO > Control	DMSO < Control		could inhibit collagenase
Chaharom et al. (2019)[52]	Third molar	E&R: All-Bond 3 & One-Step Plus SE: Clearfil SE Bond & Clearfil S3 Bond	100% DMSO pretreatment	500 times thermocycling	DMSO = Control of all the tested adhesives	NA	NA	NA
Li et al. (2020)[49]	Third molar	E&R: Adper Single Bond 2	100% DMSO pretreatment for 60 s	10,000 times thermocycling	Immediate & Aged: DMSO = Control	Immediate & Aged: DMSO = Control	100% DMSO induced collagen dissociation and hampered the formation of the hybrid layer	NA
Saffarpour et al. (2020)[53]	Premolar	E&R: Adper Single Bond 2	5% DMSO pretreatment for 60 s	10,000 times thermocycling	Immediate: DMSO = Control Aged: DMSO > Control	NA	NA	NA
Zhang et al. (2020)[50]	Third molar	E&R: Single Bond Universal	50% DMSO pretreatment for 60 s	1-month collagenase aging	Immediate &Aged: DMSO = Control	Immediate & Aged: DMSO = Control	NA	50% DMSO inhibited the activity of the gelatinase

Abbreviations: DMSO, dimethyl sulfoxide; E&R, etch-and-rinse; SE, self-etch; NA, not applicable.

Table 3
Results of the assessment of included studies by the use of the modified CONSORT checklist.

Item	Studies											
	Tjäderhane et al. (2013)[33]	Stape et al. (2015)[37]	Stape et al. (2016)[39]	Guo et al. (2017)[38]	Stape et al. (2018)[41]	Salim Al-Ani et al. (2018)[47]	Stape et al. (2018)[51]	Li et al. (2018)[48]	Chaharom et al. (2019)[52]	Li et al. (2020)[49]	Saffarpour et al. (2020)[53]	Zhang et al. (2020)[50]
1	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
2a	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
2b	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
3	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
4	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
5	No	No	No	No	No	No	No	No	No	No	No	No
6	No	No	No	No	No	No	No	No	No	No	No	No
7	No	No	No	No	No	No	No	No	No	No	No	No
8	No	No	No	No	No	No	No	No	No	No	No	No
9	No	No	No	No	No	No	No	No	No	No	No	No
10	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No[1]	Yes
11	Yes[2]	No	No	No	No	No	No	No	No	No	No	No
12	Yes	No	No	No	No	No	No	No	No	Yes	No	Yes
13	Yes	Yes	Yes	No	No[3]	No[3]	Yes	Yes	Yes	Yes	No	Yes
14	No	No	No	No	No	No	No	No	No	No	No	No

1. The statistical methods used to analyze data was not described in detail.
2. The 95% confidence interval was supplemented in the online version via a link.
3. The information on potential conflicts of interest was insufficient.

Table 4
Quality assessment (adapted and modified from Cochrane risk of bias tool).

Authors (Year)	Allocation concealment	Sample size calculation	Operator and assessor blinding	Testing standards and methodology	Reporting bias	Risk of bias
Tjäderhane et al. (2013)[33]	2	2	1	0	0	Moderate
Stape et al. (2015)[37]	2	2	1	0	0	Moderate
Stape et al. (2016)[39]	2	2	1	0	0	Moderate
Guo et al. (2017)[38]	2	2	1	1	0	Moderate
Stape et al. (2018)[41]	2	2	1	0	0	Moderate
Salim Al-Ani et al. (2018)[47]	2	2	1	0	0	Moderate
Stape et al. (2018)[51]	2	2	2	1	0	Moderate
Li et al. (2018)[48]	2	2	2	1	0	Moderate
Chaharom et al. (2019)[52]	2	2	2	0	1	Moderate
Li et al. (2020)[49]	2	2	1	0	1	Moderate
Saffarpour et al. (2020)[53]	2	2	2	0	1	Moderate
Zhang et al. (2020)[50]	2	2	1	0	1	Moderate

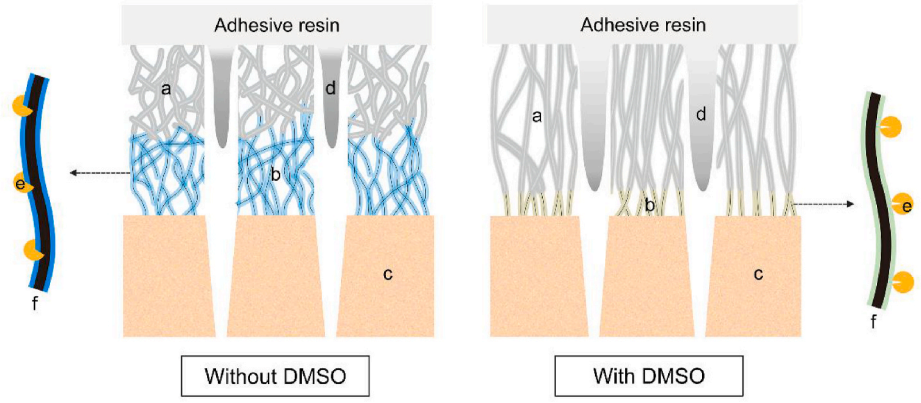

Fig. 3. Mechanism of DMSO's function on hybrid layer. DMSO dissociates collagen fibrils into a sparser network. Thus, spaces occupied by resin monomer through bonding procedure are increased, and less unprotected collagen fibers are exposed. Collagen fibers covered with bound water are colored with blue without the application of DMSO; collagen fibers covered with less associated DMSO/H_2O mixture are colored with green after the application of DMSO. Hydrophilic and hydrophobic monomers are dissolved due to DMSO's amphiphilic nature, thereby reducing phase separation and enhancing adhesive infiltration. With its affinity to collagen fibrils, DMSO blocks binding of MMPs with their substrate to hinder collagenolytic activity. a: Hybrid layer, b: Exposed collagen network, c: Mineralized dentin, d: Resin tags, e: MMPs, f: Collagen fibril. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

extracellular matrix [69], among which the collagenolytic MMP-8, gelatinolytic MMP-2, and MMP-9 play important roles in collagen degradation [70,71]. These collagenases can be activated through acid-etching and lead to degradation of dentin collagen fibrils [72], which threaten the long-term stability of resin–dentin bonds [73,74]. In vitro zymographic analysis shows that DMSO with the concentration above 5% significantly inhibited the gelatinolytic activities of MMP-2 and MMP-9 [33,51]. The collagen breakdown of wet and dry dentin is significantly reduced over 66% after the application of 50% DMSO pretreatment [51] and reduced fluorescence in the hybrid layer when DMSO pretreated dentin surface prior to bonding [38,41,50,51]. The possible mechanism of DMSO to inhibit MMPs is that DMSO blocks binding sites of MMPs with their substrate (dentin collagen) to hinder the interaction between them **(Fig. 3)** [75,76]. Different from other polar solvents, DMSO can dissolve most of the common proteins rapidly [77]. Via its solubilizing power, it interacts dynamically with protein peptides and provides collagen matrix reliable supports for enzyme stripping to reduce the degradation of collagen fibrils **(Fig. 3)** [33]. Another possible explanation is that in high DMSO-water concentrations, enzymes could exert a stronger affinity to DMSO than to pure water. With the increase in the solvent concentration and diminish of the substrate hydration, less polar proteins tend to bind more DMSO. Bonds between DMSO and protein hydrophobic moieties may unfold its tertiary structure and result in denaturation [78].

In recent years, natural plant extracts, including baicalein, quercetin, naringenin, and epigallocatechin-3-gallate (EGCG), have exhibited cross-linking effects on collagen fibrils and inhibitory effects on MMP activity to preserve dentin matrix [79–82]. Their antibacterial capability on biofilm formation of cariogenic bacteria to prevent secondary caries is also introduced [83–85]. However, the low solubility of such organic compounds in water limits their biological functions [86]. DMSO could compensate for this limitation due to its outstanding performance to solvate organic molecules and act as an infiltration facilitator to enhance the bioactivities of these natural extracts. A recent study that applied EGCG in 50% DMSO aqueous solution to obtain 1% EGCG (a much higher concentration than in water or other solution) revealed that the combined application of DMSO and EGCG significantly improved hybrid layer integrity and exhibited antibacterial activity [50]. Mussel-inspired molecule dopamine methacrylamide dissolved in DMSO at a concentration of 1.0 mM showed its superiority to preserve resin–dentin bond durability over time [49]. As a universal organic solvent and an outstanding infiltration facilitator, DMSO not only dissolves natural extracts to maximize their biological activity and penetration ability, but also exerts a synergistic effect with them in MMP inhibition to protect dentin collagen [50,87].

Despite its outstanding performance in promoting dentin bonding, DMSO as a primer or pretreatment agent has not appeared in dental market because the cytotoxicity of DMSO is one of the main concerns that limit its clinical application [88]. Results pertaining to the cytotoxicity of DMSO remain controversial despite that it has been widely used in cell cryopreservation. Apoptosis in human cell lines was induced by DMSO at a concentration of >10% (v/v) owing to plasma membrane pore formation [89]. Even at a concentration of 0.01%, DMSO can significantly disrupt the attachment of human embryonic stem cells and affect cell viability in a dose-dependent pattern [90]. However, no evident impact on cell viability, adhesion, and death was observed when DMSO with a concentration of up to 1 mM (0.008%) was in direct contact with odontoblasts [91]. According to the FDA guidance for industry, DMSO is graded Class 3 solvent, which is the same as acetone and ethanol (http://www.fda.gov/RegulatoryInformation/Guidances/ucm128223.htm). Whereas, unlike acetone and ethanol, DMSO has remarkable penetration potential and significantly enhances cell membrane permeability [92]. Previous studies reveal that the penetration of resin monomers might affect pulp cell metabolism and induce cytotoxic effects on dental pulp tissues [93,94]. Thus, the use of DMSO in resin–dentin bonding may increase the potential toxicity of resin monomers through pulp tissues [88,95]. However, no randomized controlled trial can fully mimic the indirect contact with odontoblasts when DMSO and adhesive monomers are applied on dentin surface. Experts insisted that in vitro and in vivo cytotoxicity tests on DMSO with different adhesive systems are definitely needed.

Thus far, only one registered clinical trial in the Cochrane Library is carried out. No laboratory test on bond strength can directly predict the clinical efficacy of the adhesives given the divergence in bond strength recorded for a certain type of adhesive or a prime agent [96]. Although thermocycling and water storage are regarded as the most frequently used approaches, the in vivo oral environment is relatively complicated, and the challenges include degradation by enzymes, mechanical loading, and pulpal pressure [97,98]. Optimistic results from in vitro studies may be obtained, and clinical investigation of the bond durability by DMSO treatment under complex oral environment is highly suggested. Notably, the GRADE approach is commonly used to assess the level of evidence for systematic reviews [99]. However, its criteria (i.e. study design, risk of bias, inconsistency, imprecision, and indirectness and magnitude of effect) are more related to randomized controlled clinical trials. It is difficult to make objective judgement on inconsistency, imprecision, and indirectness and magnitude of effect of in vitro studies on dental materials included in this systematic review. Therefore, the GRADE was not adopted in our review to assess the quality of evidence. Instead, the modified CONSORT checklist of items carried out by Faggion was selected for reporting in vitro studies of dental materials because it is a pilot proposal primarily suited to the reporting of

experiments with extracted human teeth [43,44]. Moreover, the modified Cochrane risk of bias tool was used to evaluate the risk of bias [45,46]. However, few laboratory studies include this necessary information for quality assessment considering random sequence generation, group allocation concealment and blinding [100]. The included studies only mentioned that the specimens were randomly allocated into the control and experimental groups, but no details about the random sequence generation and allocation concealment were provided. Whereas, information concerning the blinding of the specimen preparation and outcome assessment were not clear. Future work on DMSO application in dental material study design should be carried out more carefully, and reporting of preclinical in vitro studies should be more statistically rigorous.

Meta-analysis was not performed because of the great heterogeneity in the study designs among the included studies, such as variation in adhesive systems, DMSO concentration and moisture condition of dentin surface, ageing methods, and duration, significantly affecting the bond strength test results. For this reason, conducting interstudy comparisons was considered inappropriate [101]. Therefore, only the bond strength values of every study group in each study were reported to show the intra-study comparison regarding the extent of the effect of DMSO application on the bond strength values.

5. Conclusion and prospect

Preliminary research findings indicate that DMSO has the ability to rearrange the demineralized collagen structure, suppress the collagenolytic activity of MMPs, and enhance the penetration of resin monomers when applied on demineralized dentin. Evidence proves the promotion of DMSO wet bonding on the long-term dentin bonding stability of E&R system (but not in a dose-dependent level). However, its effect on SE system remains controversial and requires more evidence to prove. DMSO can act as an outstanding solvent for incorporating natural plant extracts to obtain a synergistic effect to optimize dentin bonding. The capability to improve dentin bonding on either wet or dry condition makes DMSO more user-friendly in clinical practice. It may also enlighten the concept of “wet bonding” technique from surface moisture to biomodification of dentin. Further investigations are expected to explore the possible interactions between DMSO and adhesive monomers (or dentin collagen) to obtain the long-term stability of the resin–dentin bonding.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijadhadh.2021.103037.

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