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To etch or not to etch, Part III: On the hydrophobic-rich content and fatigue strength of universal adhesives after long-term ageing

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ABSTRACT

Objectives: To examine whether dentin-etching extension and/or the hydrophobic-rich content of hybrid layers would affect fatigue strengths of a mild universal adhesive after long-term aging.

Methods: Twin-bonded resin-dentin interfaces were produced by etching sound midcoronal dentin beams with 32 % ortho-phosphoric acid for 15 s (OPA15s), 3 s (OPA3s) or 10 % meta-phosphoric acid for 15 s (MPA15s). Samples were bonded with a mild universal adhesive with or without additional coating using a solvent-free bisGMA-based bonding resin. Self-etch application served as control. Composite buildups were made with a nanofilled composite. Bar-shaped twin-bonded interfaces (0.9 × 0.9 × 12 mm) were aged for two years in artificial saliva at 37 °C and tested under 4-point flexure at quasi-static ($n = 16$) and cyclic loads ($n = 35$) until failure. The stress-life fatigue behavior was evaluated using the staircase method at 4 Hz. Crack initiation and fracture patterns were evaluated by SEM. Cyclic-loaded data was analyzed by Kruskal-Wallis on Ranks ($\alpha = 0.05$).

Results: Significantly higher fatigue life distributions and higher endurance limits were observed for less aggressive etch-and-rinse protocols (OPA3s and MPA15s) after long-term ageing. Hydrophobic-rich coating produced 20–32 % higher endurance limits, prevented micrometer-sized porosities at bonded interfaces, reduced etching-associated variability and lowered crack formation.

Significance

Long-term hydrolysis produces detrimental effects on the fatigue strength of resin-dentin interfaces. The bond-promotion effect of less aggressive etch-and-rinse protocols and the creation of hybrid layers with higher hydrophobic-rich content are critical to extend the durability of mild universal adhesives. Therefore, current oversimplification trends in adhesive dentistry may limit resin-dentin bonding performance.

1. Introduction

Extending the longevity of tooth-bonded interfaces through simplified bonding procedures is a central concern in adhesive dentistry. The staggering number of publications regarding resin-dentin bonding illustrates the continuous effort to better understand and improve such complex, albeit commonly underrated procedure. Bonding methacrylate-based monomers to dentin is a form of *in situ* tissue engineering, in which formed bio-constructs should not deteriorate over time [1]. Only if hybrid layer components were stable, resin-dentin interfaces would successfully resist the numerous oral challenges including mastication loads and hydrolytic degradation. Hence, the stability of dentin-bonded interfaces becomes a key aspect for successful restorative outcomes. Considering simplified approaches to hybridize dentin, the use of universal adhesives has generated a fair amount of debate.

Universal adhesives are the most recent addition (*i.e.*, 2011) to the great variety of bonding resins available in the market. Apart from claiming simplification in bonding to glass-rich (*i.e.*, *via* silane) and glass-poor (*i.e.*, *via* 10-MDP) ceramics, they propose bonding to tooth structures in either self-etch or etch-and-rinse mode. In theory, “multi-mode” applications would confer a unique advantage to clinicians considering more individualized bonding possibilities with a single “all-in-one” adhesive. Different pH-formulated resin blends are available;

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however, mild universal adhesives seem to perform better than more or less aggressive versions [2]. Although the use of universal adhesives in self-etch mode normally results in better *in vitro* outcomes after long-term ageing [2–4], recently published *in vivo* studies (*i.e.,* 18 months to 5 year follow-ups) begin to show lower retention rates, poor marginal adaptation and higher marginal staining for self-etch applications [5–8]. In actual fact, the long-term stability of universal adhesives is still insufficiently proven [9,10] remaining a matter of discussion regarding which application mode produces the best outcome [2,10]. A recent meta-analysis indicate that *in vitro* long-term bonding performance of mild universal adhesives does not seem to depend on application mode [2]; even though, reports may suggest otherwise [10]. The underperformance of self-etch application *in vivo* [5–8] invariably raises concerns about the ability of universal adhesives to perform equally well in both application modes [10]. Such controversies arise partly due to the fact that studies employing quasi-static strengths may only timidly signalize differences between application modes [10–12]. It is reasonable to assume that material dependency and bonding protocols play a bigger role on dentin-bonding effectiveness of universal adhesives than originally thought [4,10–12]. Clearly, it is difficult to stablish an exact relationship between bond-strength findings and the outcomes determined by randomized clinical trials [13]. In this context, fatigue testing can provide valuable insights that are not revealed by measures of static strength alone [14] enabling more realistic assumptions.

Fatigue is now recognized as either the primary mode of failure or a main contributing mechanism in the failure of adhesively bonded restorations [14]. Depending on the magnitude and duration, cyclic stresses across tooth-bonded interfaces can accelerate degradation over time. This is further aggravated by the simultaneous hydrolysis of collagen and methacrylate-based polymers in the oral cavity [15], which invariably result in increased interfacial flaws [16,17]. Creating resin-dentin interfaces less prone to collagen-resin degradation could thereby contribute to higher fatigue strengths over time. The stability of methacrylate-based polymer is strongly related to their ability to interact with water [18–20]. Universal adhesives, however, require a relatively high hydrophilic content to be used as one-step systems [21]. The resultant pitfalls of such high water affinity normally emerge only after long-term function, which may disguise their suboptimal dentin-bonding performance [4,6,9,22,23] in short periods. Several reports indicate that increasing the hydrophobic-rich content of universal adhesives, by additional coating, may improve their long-term bonding performance [6,23] and fatigue strengths [12]. A central question now resides on determining the extent in which collagen degradation and polymer quality interfere in fatigue strengths under more dynamic conditions as those seen in the oral cavity. As such, the association of a fatigue test setup and a long-term ageing regime could help elucidate controversies regarding the use of universal adhesives. Therefore, the aim of this study was to examine whether the extent of dentin demineralization and/or the hydrophobic-rich content of hybrid layers would affect fatigue strengths of a mild universal adhesive after long-term ageing. The central hypothesis evaluated whether ageing resin-dentin bonded interfaces could render more realistic metrics of performance to fatigue setups currently used in adhesive dentistry. The tested null hypotheses were that after long-term ageing: (*i*) different dentin-etching protocols and (*ii*) the hydrophobic-rich content of hybrid layers would have no effect on the bonding performance of a mild universal adhesive.

2. Materials and methods

Fifty-two extracted sound third-molars were obtained with informed consent from patients (age 18–34 years) under a protocol (#23–2003) approved by the University of Oulu, Finland. Teeth were stored at 4 ℃ in 0.9 % NaCl containing 0.02 % NaN_3 used within 1 month after extraction.

2.1. Experimental design and long-term ageing

This study replicated some of the acid-etching protocols previously reported in the Part II series [12]; however, resin-dentin interfaces were aged hydrolytically before cyclic loading. The long-term fatigue strength of a mild universal adhesive (Scotchbond Universal Adhesive: SU, 3 M ESPE; pH 2.7) [2,24] applied under different dentin-etching protocols was assessed in terms of 4-point flexural strength (n = 16/group) and stress life fatigue (n = 35/group). Ageing consisted of storing resin-dentin samples for two years at 37 °C in artificial saliva (pH 7.4) composed by 5 mM HEPES, 2.5 mM $CaCl_2 \cdot H_2O$, 0.05 mM $ZnCl_2$, and 0.3 mM NaN_3 [25]. During the storage period, samples were kept inside watertight containers in a shaking water bath (OLS200, Grant Instruments, UK) at 60 RPM. Artificial saliva was changed biweekly to prevent possible pH changes [26]. Table 1 shows adhesive system compositions, etching agents and application modes. The experimental design was composed of two-study factors: (i) dentin-etching extension and (ii) hydrophobic-rich content of the hybrid layer. Dentin-etching extension was composed by four levels: (i) self-etch application (SE), (ii) 15 s (OPA 15 s) and (iii) 3 s (OPA 3 s) etching with 32% ortho-phosphoric acid and (iv) 10% meta-phosphoric etching for 15 s (MPA 15 s) [27]. Hydrophobic-rich content of the hybrid layer was composed by two levels: (i) the hydrophobic content offered by the tested mild universal adhesive (control) or (ii) presence of an additional bisGMA-based resin coating (Scotchbond Multi-Purpose Adhesive, 3 M ESPE) actively mixed with uncured universal adhesive on the dentin surface. Eight groups were obtained for flexure strength and stress-life fatigue testing.

2.2. TBI resin-dentin beam preparation

Roots were removed 1 mm below the cervical line of sound third molars and discarded. Crown segments were longitudinally sectioned occluso-cervically to produce mesio-distal slabs under water-cooling

Table 1
Adhesive system compositions, etching agents and application modes.

	Composition	~ pH	Application Mode
Scotchbond Universal Adhesive (3 M-ESPE)	MDP phosphate monomer, dimethacrylate resins, HEMA, methacrylate modified polyalkenoic acid copolymer, filler, ethanol, water, initiators and silane	2.7	Self-etch* Etch-and-rinse**
Scotchbond Multi-Purpose Adhesive (3 M-ESPE)	bisGMA, HEMA, dimethacrylates and photoinitiators	-	Coating# (hydrophobic-rich resin; H)
Scotchbond Universal Etchant (3 M-ESPE)	32 % ortho-phosphoric acid, water, synthetic amorphous silica, polyethylene glycol and aluminum oxide	0.1	3 s (OPA 3 s)** 15 s (OPA 15 s)**
Meta-phosphoric acid (Sigma-Aldrich)	10 % meta-phosphoric acid, distilled water	1.3	15 s (MPA 15 s)**

Abbreviations: bisGMA = bisphenol glycidyl methacrylate; TEGDMA = tryethylene glycol dimethacrylate; MDP = methacryloyloxydecyl dihydrogen phosphate; HEMA = 2-hydroxyethyl methacrylate; H = hydrophobic-rich coating; 3 s = dentin etching for 3 s; 15 s = dentin etching for 15 s; OPA = ortho-phosphoric acid, MPA = meta-phosphoric acid.

*Self-etch: active universal adhesive application for 20 s; gentle blow dry for 10 s; light cure for 10 s

* *Etch-and-rise: dentin etching according to experimental groups (OPA 3 s, OPA 15 s or MPA 15 s); water rinse 15 s; blot-drying; active universal adhesive application for 20 s; gentle blow dry for 10 s; light cure for 10 s

#Coating: universal adhesive application according to the experimental group without light curing; active Scotchbond Multi-Purpose Adhesive application for 20 s; gentle blow dry for 5 s; and light cure for 10 s using a slow-speed diamond saw (Isomet, Buehler Ltd, Lake Bluff, IL, USA). Slabs were wet-polished on both sides with 320-grit SiC paper (Buehler-MET II, Buehler; grit size ≈ 36 μm) for 30 s to standardize the dentin surface and smear layer. Bar-shaped dentin beams (roughly 1 × 1 × 8 mm) were then sectioned from slabs avoiding pulp chamber and enamel areas. One beam was obtained from each dentin slab. Dentin beams were randomly selected to produce twin-bonded interface (TBI) specimens in a specially designed stainless steel mold as described by Mutluay et al., [28]. Briefly, dentin beams were placed inside the mold with the tubules oriented nominally parallel to the bonding interface. Bonding was concomitantly performed on both opposing dentin surfaces according to each specific group. After dentin hybridization, a nanofilled composite (Filtek Supreme XTE, 3 M ESPE) was applied in a single increment to fill the mold cavities on both sides of the dentin beam. Light curing was performed for 20 s on both sides using a LED unit (Elipar Deepcure, 3 M ESPE) with output intensity of 1400 mW/cm^2. Bonded sections were carefully released from the mold, inspected for voids, wet-polished with 600- and 1200-grit SiC paper and longitudinally sectioned with a slow-speed diamond saw (Isomet, Buehler Ltd) to produce bar-shaped twin-bonded samples. The cross-sectional area of TBI resin-dentin beams was roughly 0.81 mm^2 (± 0.13). Such cross-sectional area was deliberately chosen to allow comparisons with previous publications [11,12]. A minimum of 50 TBI samples were prepared for each group. Specimens were re-inspected for flaws at the bonded interface using a stereomicroscope (Leica M60, Leica Microsystems) at 40 × magnification and stored in artificial saliva [25] at 37 ℃ for 48 h prior to testing. Samples presenting flaws were discarded and replaced.

2.3. Bonding protocols

Bonding protocols are summarized in Table 1. In more detail, the self-etch application mode (SE) was performed according to the manufacturer's instructions. After smear layer standardization, dentin surfaces were rinsed with air-water blasts for 10 s, blow dried for 5 s to remove superficial moisture. One coat of the universal adhesive was actively applied for 20 s under manual pressure equivalent to approximately 40 g for 20 s, followed by gentle solvent evaporation for 10 s and light curing for 10 s using a LED unit (Elipar Deepcure, 3 M ESPE) at 1400 mW/cm^2. For experimental groups, dentin surfaces were etched with 32 % ortho-phosphoric acid (Scotchbond Universal Etchant, 3 M ESPE, St. Paul, MN, USA) for 3 s (OPA 3 s) or 15 s (OPA 15 s) or with an aqueous 10% meta-phosphoric acid solution (meta-Phosphoric acid, Merck Group, Darmstadt, Germany) for 15 s (MPA 15 s). Etched dentin surfaces were then rinsed for 15 s, blot-dried with absorbent paper following the wet-bonding technique [1]. Similarly to the self-etch group, one coat of the universal adhesive was actively applied under similar manual pressure and the solvent evaporation was gently performed for 10 s. For the groups composed by the additional hydrophobic-rich coating (H), after the active application of the universal adhesive for 20 s and solvent evaporation for 10 s, a bisGMA-based bonding resin (Scotchbond Multipurpose Adhesive, 3 M ESPE) was actively applied on the dentin surface for 20 s. Adhesive thinning was gently performed for 5 s and light-curing for 10 s (Elipar Deepcure, 3 M ESPE). All bonding procedures were carried out by the same pair of calibrated operators.

2.4. Characterization of the fatigue behavior

After long-term ageing, TBI specimens were tested under quasi-static and cyclic flexure using a universal testing system (Electropuls E1000, Instron) with load capacity of 250 N and sensitivity of 0.025%. A blinded operator conducted the 4-point flexural strength and fatigue testing. All experiments were performed with specimens fully immersed in artificial saliva at room temperature. TBI specimens were placed on a 4-point flexural fixture so that the load was applied on the occlusal surface. Quasi-static loading was applied at a rate of 0.05 mm/min. The flexural strength (*FS*) of the beams was calculated using conventional beam theory [29] in terms of the maximum measured load (P) in N and beam geometry (width b, thickness h in mm) according to $FS = 3Pl/bh^2$, where l is the distance from interior and exterior supports ($l = 2$ mm). Sixteen specimens (n = 16) were evaluated per group. Cyclic loading of the TBI specimens was conducted using the same flexure configuration under load control with frequency of 4 Hz and stress ratio (R = ratio of minimum to maximum cyclic load) of 0.1. A minimum of thirty specimens (n = 30) were evaluated per group. The cyclic loading experiments followed the staircase fatigue method beginning at approximately 90% of the determined 4-point flexural strength. Stress amplitudes were identified from the quasi-static loading and followed sequential reductions in the order of approximately 10 % until failure. The process continued until reaching stress amplitudes (MPa) at which specimens did not fail within a minimum of 1.2 × 10^6 cycles. The cyclic stress amplitude was plotted in terms of the number of cycles to failure in log-base format. The data was fit through a non-linear regression with a Basquin-type model, according to equation $\sigma = A(N)^B$, where σ corresponds to the stress amplitude (MPa), A and B are the fatigue-life coefficient and fatigue-life exponent, respectively. The apparent endurance limit was estimated from the models for a fatigue limit defined at 1 × 10^7 cycles [28,30].

2.5. Detection of crack propagation sites

The unfractured-tensile side of TBI specimens, which withstood a minimum of 10^4 loading cycles, were evaluated by scanning electron microscopy (SEM) to identify the origins of failure and potential weak links at resin-dentin bonded interfaces. The fractured composite side of TBI specimens were also evaluated to determine fracture patterns after cyclic loading. The unfractured side of TBI specimens were lightly wet-polished with 1200- and 4000-grit SiC paper and ultrasonically cleaned in water for 60 s. Both fractured and unfractured specimens were dehydrated in a series of ascending ethanol series (50, 70, 80, 90 and 3 × 100 %), fixed in hexamethyldisilazane, mounted on aluminum stubs, sputtered with gold/palladium and analyzed on backscattering mode at 10 kV (Phenom ProX, Phenom-World). SEM micrographs (1000–12,000× magnification) were taken to analyze the entire extension of cyclic-loaded bonded interfaces. A blinded-calibrated operator scanned the entire extension of bonded interfaces to determine the most common crack propagation sites. Considering the fracture mechanics of 4-point bending specimens [31], fracture pattern characterization (250 – 3000× magnification) was based on fractured surfaces below compressions curls.

2.6. Statistical analyses

Data normality and equality of variance were confirmed by Shapiro-Wilk and Levene tests, respectively. Aged 4-point flexural strengths obtained after quasi-static loading measurements (Shapiro-Wilk test = 0.238; Levene test = 0.081) along with immediate flexural strengths obtained from the previously published Part II series [12], were analyzed with three-way ANOVA followed by the Tukey test. Fatigue life distributions were compared using the Kruskal-Wallis One-Way Analysis of Variance on Ranks. Significance levels were set at 5 % ($\alpha = 0.05$). Statistical analyzes were performed on IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Quasi-static 4-point flexural strength

Three-way ANOVA revealed that "dentin-etching extension" ($p < 0.001$; $\eta^2 = 0.414$), "hydrophobic-rich content" ($p < 0.001$; $\eta^2 = 0.112$) and "ageing" ($p < 0.001$; $\eta^2 = 0.511$) had significant effects on 4-point flexural strengths of the tested universal adhesive. No interactions were observed between study factors (*i.e.*, “dentin etching”, “hydrophobic-rich content” or “ageing”). Bar-shaped TBI specimens presented no significant differences regarding resin-dentin cross-sectional areas (0.82 $mm^2 \pm 0.11$) between groups ($p = 0.29$). Aged 4-point flexural bond strengths are reported in Table 2. Ageing significantly reduced flexural bond strengths (roughly 21–45 % reductions) for all groups compared to immediate values reported previously in Part II series [12] ($p < 0.05$) (Fig. 1). No significant differences in flexural strengths were observed between self-etch application (SE) and etching with ortho-phosphoric acid for 15 s (OPA 15 s) or between their coated applications (SE H and OPA 15 s H) ($p > 0.05$). OPA 3 s H produced significantly higher flexural bond strengths than OPA 3 s ($p < 0.05$). No significant differences were identified between MPA 15 s and MPA 15 s H ($p > 0.05$). Fractures involved bonded interfaces without exclusive cohesive fractures in either dentin or composite.

3.2. Fatigue response

The cross-sectional area of TBI specimens were not significantly different between groups ($p = 0.501$). Fatigue life diagrams (S-N curves) are shown in Fig. 2. Basquin-type power law models ($\sigma = A(N)^B$) are listed for each group describing the mean fatigue strength distribution for the different bonding protocols. Pairwise comparisons for the fatigue life distributions according to Kruskal-Wallis One Way Analysis of Variance on Ranks are shown in Table 2. Ageing significantly reduced fatigue life distributions and endurance limits (8 – 33 % reduction) (Table 2) compared to the immediate values reported in Part II series [12]. After ageing, self-etch application and ortho-phosphoric acid etching for 15 s (OPA 15 s) produced specimens that endured the lowest stress ($p < 0.05$) without significant differences from each other ($p > 0.05$). Ortho-phosphoric acid etching for 3 s (OPA 3 s) produced specimens that endured significantly higher stress than the self-etch application ($p < 0.05$) without significant differences compared to OPA 15 s ($p > 0.05$). Meta-phosphoric acid etching for 15 s (MPA 15 s) produced specimens that endured the highest stress for uncoated groups after ageing ($p < 0.05$). Increasing the hydrophobic-rich content significantly increased endured stress after ageing for SE, OPA 15 s and OPA 3 s etching protocols ($p < 0.05$). The biggest impact was observed for coated interfaces bonded in self-etch mode (SE), which presented a 2-fold increase in the endurance limit compared to applying the universal adhesive in the self-etch mode alone. No significant differences in endured fatigue stress occurred between uncoated MPA 15 s and MPA 15 s H after ageing ($p > 0.05$). OPA 15 s H, OPA 3 s H and MPA 15 s H resulted in 38 %, 30 % and 16 % higher endurance limits compared to their uncoated versions, respectively.

3.3. Crack initiation and fracture patterns

Fig. 3 shows representative SEM micrographs of crack-initiation sites at the tensile side of unfractured TBI specimens (A – H) and representative fracture patterns for all groups (A′ – H′). Cracks with different extensions were identified in all samples regardless of bonding protocols. Higher cyclic loading resulted in greater crack formation along the tensile side of aged-TBI specimens. Dentin-etching protocols and coating affected crack-initiation sites in aged resin-dentin interfaces. Uncoated (control) samples presented a “Swiss-cheese” porous aspect showing wider cracks situated along the bulk of the adhesive and hybrid layer. Cohesive cracks were commonly observed extending 5–25 μm into the bulk of the composite. Regardless of dentin-etching protocol, composite filler dislodgment with cracks extending mostly around filler particles were observed along with filler cracking to a lesser extent. Self-etch (SE) samples (Fig. 3 A) presented cracks originating at the hybrid layer extending through the adhesive layer towards the composite. The “Swiss-cheese” porous aspect was more pronounced in areas corresponding to the hybrid layer and its boundaries. SE (Fig. 3 A′) was characterized as mixed failure involving mostly the hybrid layer and composite. OPA 15 s (Fig. 3 B) presented cracks originating at the hybrid layer and extending towards the adhesive-composite boundaries. OPA 15 s (Fig. 3 B′) was characterized as adhesive failure involving mostly the adhesive and hybrid layers. OPA 3 s samples (Fig. 3 C) presented cracks at the hybrid layer surrounding areas, albeit with smaller dimensions compared to SE and OPA 15 s. The “Swiss-cheese” porous aspect was identified along the adhesive and hybrid layers. OPA 3 s (Fig. 3 C′) was characterized as mixed failure involving mostly the hybrid layer and composite. MPA 15 s samples (Fig. 3 D) presented the lowest incidence of cracks at the at the hybrid layer boundaries, albeit the “Swiss-cheese” aspect was observed. MPA 15 s (Fig. 3 D′) was characterized as mixed failure involving mostly the adhesive layer and composite.

Hydrophobic-rich coating (H) reduced the occurrence of the “Swiss-cheese” aspect and the incidence of cohesive cracks at the composite boundaries adjacent to the adhesive layer. In general, coating produced lower crack formation with reduced extension at the adhesive and hybrid layers (Fig. 3 E – H) compared to their respective uncoated counterparts (Fig. 3 A – D). SE H (Fig. 3 E′) was characterized as mostly adhesive failure involving the hybrid layer. OPA 15 s H (Fig. 3 F′) and OPA 3 s H (Fig. 3 G′) were characterized as mixed failure involving

Table 2
Stress-life fatigue response, power law constants and estimated endurance limits for TBI resin-dentin interfaces aged for 2 years in artificial saliva at 37 ℃. Bonded interfaces were produced by a mild universal adhesive (Scotchbond Universal, 3 M ESPE) under different etching conditions followed or not by coating with a hydrophobic-rich bonding resin (Adhesive, Scotchbond Multipurpose, 3 M ESPE).

	Etching protocol	A (MPa)	B	R^2	4-point flexural strength (MPa) *	Endurance limit (MPa) * *	Endurance limit reduction after ageing (%) * **	Pairwise comparison between aged fatigue life distributions#	Significance between 24 h and aged fatigue life distributions##
Control	Self-etch	28.81	-0.082	0.69	24.64 ± 4.73^{d}	7.68	-27.86	D	$p = 0.001$
	OPA 15 s	23.97	-0.039	0.67	27.95 ± 6.48^{cd}	12.79	-32.38	CD	$p < 0.01$
	OPA 3 s	26.01	-0.033	0.75	36.04 ± 6.14^{bc}	15.28	-33.24	BC	$p < 0.01$
	MPA 15 s	33.36	-0.038	0.68	45.01 ± 9.53^{ab}	18.08	-10.63	A	$p = 0.001$
Coating (H)	Self-etch	25.70	-0.03	0.61	36.32 ± 6.51^{bc}	15.85	-20.61	BC	$p < 0.01$
	OPA 15 s	31.86	-0.037	0.6	43.15 ± 8.77^{ab}	17.55	-30.66	AB	$p < 0.01$
	OPA 3 s	33.53	-0.033	0.67	51.79 ± 11.42^{a}	19.70	-20.06	A	$p < 0.01$
	MPA 15 s	34.14	-0.03	0.65	51.20 ± 11.68^{a}	21.05	-8.41	A	$p = 0.02$

R^2 values represent the coefficient of determination for each model. ± represent standard deviations. *Different lower-case letters indicate significant differences for 4-point flexural strength according to the Tukey Test ($p < 0.05$). * *Endurance limits were calculated at 1×10^7 cycles. * **Percentage reductions were calculated considering immediate endurance limits previously reported in Part 2 series [12]. # Different capital letters indicate significant differences in fatigue life distributions of aged specimens according Kruskal-Wallis One Way Analysis of Variance on Ranks ($p < 0.05$). Significance values were adjusted by the Bonferroni correction for multiple tests. ## Different capital letters indicate significant differences in fatigue life distributions of aged specimens according to Kruskal-Wallis on Ranks ($p < 0.05$). 24 h fatigue life distributions were obtained from Part II series [12]. *Abbreviations:* TBI = twin-bonded interfaces; OPA = 32% ortho-phosphoric acid; MPA = 10% meta-phosphoric acid; H = coating using a bisGMA-based bonding resin.

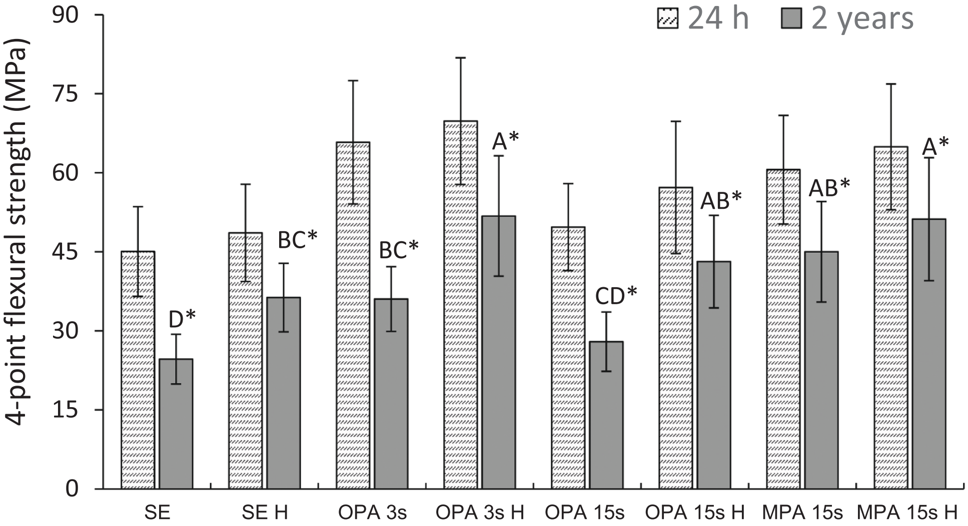

Fig. 1. Quasi-static loading measurements (MPa) for the 4-point bending test of resin-dentin TBI interfaces aged for 2 years in artificial saliva at 37 ℃ (n = 16). A mild universal adhesive (Scotchbond Universal, 3 M ESPE) was bonded to dentin in self-etch mode (SE), after ortho-phosphoric acid etching for 3 s (OPA 3 s) or 15 s (OPA 15 s), meta-phosphoric acid etching for 15 s (MPA 15 s). Hydrophobic coating (H) was performed by adding a solvent-free hydrophobic-rich resin over the uncured universal adhesive. Note that the 24 h results are reported in Part II series [12]. Columns identified by different capital letters represent significant differences between 2-year aged groups according to Tukey's test ($p < 0.05$). Columns identified by an * indicate significant differences between 24 h and 2-year aged groups according to Tukey's test ($p < 0.05$).

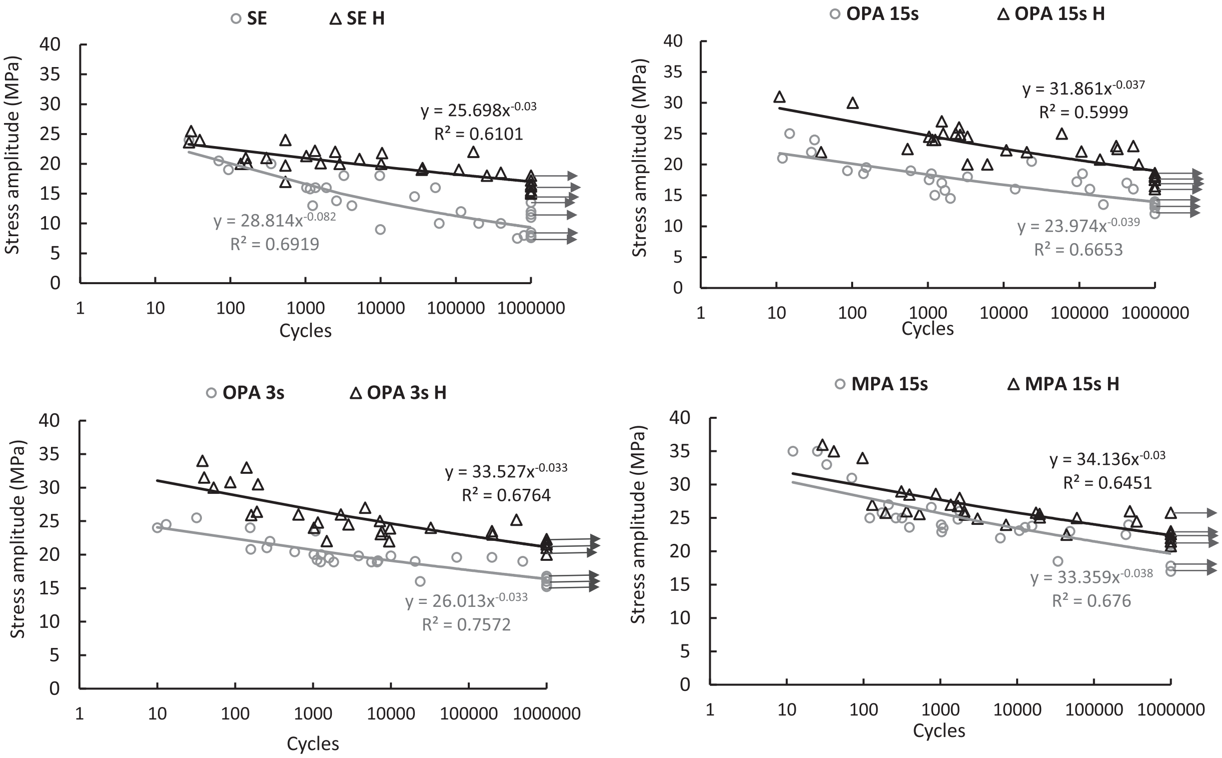

Fig. 2. Fatigue life diagrams (S-N curves) of resin-dentin TBI interfaces (n = 35) aged for 2 years in artificial saliva at 37 ℃. Bonded interfaces were produced by a mild universal adhesive (Scotchbond Universal, 3 M ESPE) bonded to dentin in self-etch mode (SE), after ortho-phosphoric acid etching for 3 s (OPA 3 s) or 15 s (OPA 15 s), meta-phosphoric acid etching for 15 s (MPA 15 s), with or without coating. Coating (H) was performed by adding a solvent-free hydrophobic-rich resin over the uncured universal adhesive. Note that data points with arrows represent those specimens that reached 1.2×10^6 cycles and the test was discontinued. R^2 values represent the coefficient of determination.

mostly the hybrid layer and composite. MPA 15 s H (Fig. 3 H′) was characterized as mixed failure involving mostly the adhesive layer and composite.

4. Discussion

Undoubtedly, conventional fatigue testing of unaged resin-dentin interfaces brings new-valuable perspectives to determine bonding performance [11,12,14]. Nonetheless, additional long-term storage in

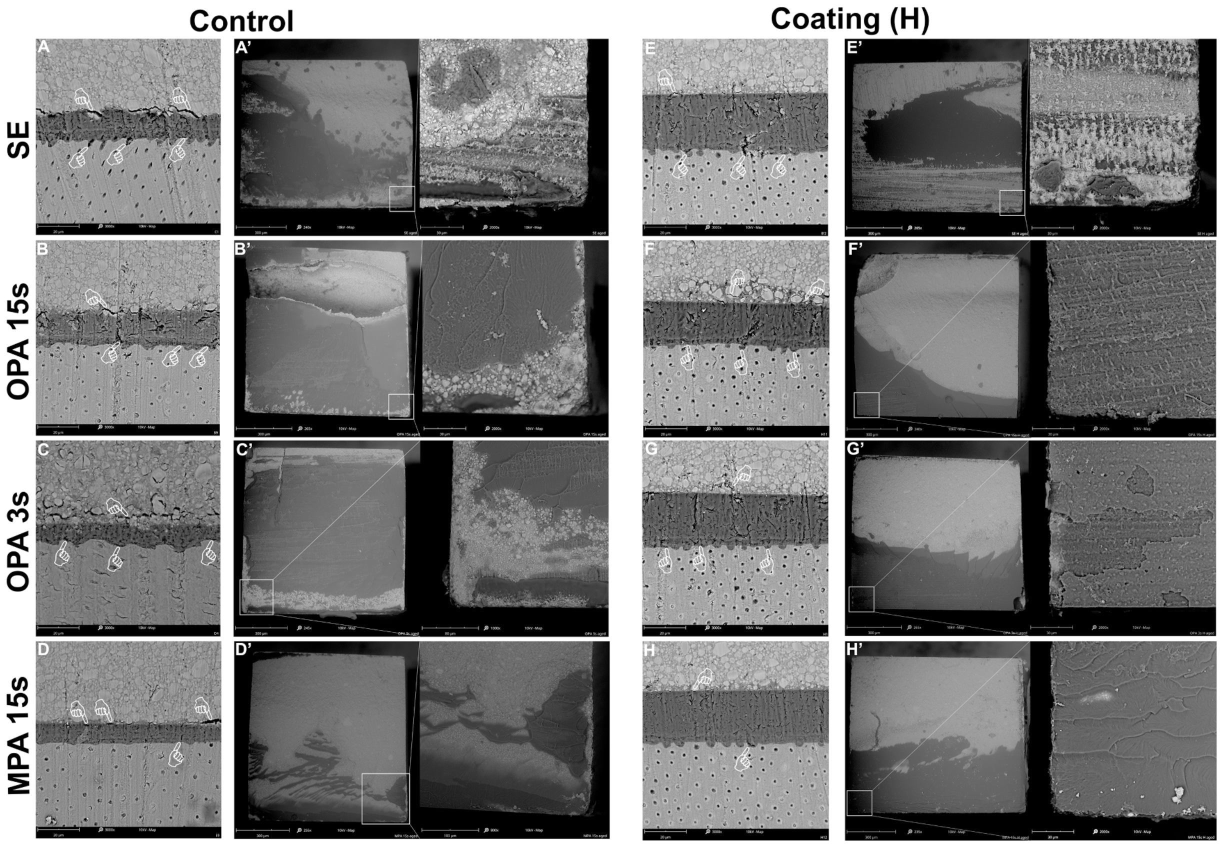

Fig. 3. Representative SEM micrographs showing the profile view of the tensile side of unfractured TBI resin-dentin specimens subjected to a minimum of 10^4 cycles (A – H) and fracture surfaces of the composite side of cyclic-loaded TBI specimens (A′ – H′). Bonding protocols consisted of a mild universal adhesive (Scotchbond Universal, 3 M ESPE) used in self-etch mode (SE), after ortho-phosphoric acid etching for 3 s (OPA 3 s) or 15 s (OPA 15 s), meta-phosphoric acid etching for 15 s (MPA 15 s), with or without coating using a bisGMA-based bonding resin.

artificial saliva exposed differences between bonding protocols that were not initially identified without ageing [12]. Therefore, the hypothesis that ageing resin-dentin bonded interfaces could render more realistic metrics of performance to fatigue setups was accepted. To the best of our knowledge, this is the first study to associate long-term ageing and cyclic loading to determine fatigue life of resin-dentin interfaces following the stress-life (S-N) approach. The rationale was to better understand the extent in which hydrolyzes of interfacial bonding constituents would affect fatigue strengths. Under clinical conditions, tooth-bonded interfaces are simultaneously submitted to masticatory loads and hydrolytic degradation over time. The absence of such degradative physio-chemical factors found *in vivo* in conventional fatigue setups invariably limits *in vitro* bonding assessments of resin-dentin interfaces. Degradation of resin-dentin interfaces is a complex-multifactorial process [31–35]. Storage in artificial saliva at 37 °C attempted to replicate, to some extent, *in vivo* degradation of the collagen-anchoring portion of hybrid layers by dentinal endogenous enzymes (*e.g.* metalloproteinases and cathepsins) [26,32,33] and simultaneously induce hydrolytic degradation of methacrylate-based polymeric chains [20,36,37]. Even though the tested universal adhesive shows signs of interfacial degradation as early as 6 months [16], ageing bonded interfaces for two years extended the potential deleterious effects *in vitro* to better reflect longer clinical service times [38]. Under such ageing conditions, breakdown of the collagen matrix [26,32,33] and methacrylate-based polymeric chains through hydrolyzes [20,36,37] compromised the integrity of hybrid layers increasing weak links and porosities of the tested universal adhesive [16]. SEM analyses of crack-initiation sites revealed the formation of micrometer-sized pores at the adhesive-containing portions of the bonded interface (Fig. 3 A – D) after cyclic loading. This was mainly observed in uncoated samples with lower hydrophobic content. Such flaws existing within or between bonding materials negatively affect bond strengths [39] and, to greater degree, fatigue strengths [14,31]. The inevitable increase of interfacial flaws under clinical function over time is a matter of concern. Storage in artificial saliva produced reductions in flexural strength ranging between − 22 and − 45 % (*i.e.,* immediate values reported previously in Part II series [12]). This is corroborated by previous reports that employed quasi-static loads (*i.e.,* mictotensile testing) showing degradation of resin-dentin interfaces after storage in aqueous media [35]. Reductions in the apparent endurance limits after long-term storage (up to −33 %; Table 2) strongly indicate that hydrolytic degradation of hybrid layers contribute to loss of fatigue strength over time. The ability to estimate such metrics under hydrolytical conditions that resemble those found in the oral cavity conferred more realistic insights to assess long-term resin-dentin bonding performance.

Estimating the apparent endurance limit of bonded interfaces after ageing using non-linear regression ($\sigma = A(N)^B$) was a valuable approach to assess application modes and bonding protocols. The apparent endurance limit, also referred as fatigue limit, correspond to the cyclic stress amplitude below which the interface does not fail or exhibits a tremendously long life [14]. Assuming an average of 500k mastication cycles per year, the calculated apparent limits would correspond to approximately 20 year of clinical service time. Since bonding protocols including a separate etching-step positively affected fatigue strengths after long-term storage in artificial saliva, the first null hypothesis was rejected. The presumption of effectively applying universal adhesives in both application modes for dentin bonding has been recently questioned [10]. This became more evident considering *in vitro* [11,12] and *in vivo* [5–8] findings that may suggest otherwise. The obtained power models of fatigue life distributions also corroborated the underperformance of self-etch application for the tested universal adhesive. Similarly to unaged samples (findings published in Part II series [12]), dentin-etching protocols containing a separate etching step (OPA 15 s, OPA 3 s and MPA 15 s) improved fatigue strengths after ageing. Apparent endurance limits were 66 % to 2.3-fold higher compared to self-etch application after the separate etching step. Clearly, the underperformance of the mild universal adhesive in self-etch mode indicates that modifying the smear layer before adhesive application may greatly improve dentin bonding of at least some universal adhesives after ageing. This may seem controversial at first considering current trends in adhesive dentistry that, with reason, advocate avoiding extensive dentin etching with H_3PO_4 [9]. Interestingly, comparable reductions in apparent endurance limits over time (*i.e.* aged over immediate; Table 2) indicate that the supposed higher stability of universal adhesives in self-etch mode against the conventional etch-and-rinse approach (OPA 15 s) may not necessarily take place. While self-etch application presented reductions in the order of 28 % after ageing, OPA 15 s approached only 32 %. Furthermore, the lower fatigue-life slope (Fig. 2) obtained for SE after ageing (numerically identified by the fatigue coefficient B; Table 2) shows that applying the tested mild-universal adhesive in self-etch mode was not as reliable under cyclic loading as the protocols incorporating a separate etching-step (OPA 15 s, OPA 3 s and MPA15s). Note that monotonic 4-point flexural testing did not identify significant differences between self-etch application and conventional H_3PO_4-ecthing for 15 s (OPA 15 s). This finding reinforces the notion that quasi-static evaluations are not reliable fatigue strength indications of materials in general [14] nor of dentin-bonded interfaces [11,12]. Reduction in the load-carrying capacity of bonded interfaces produced by cyclic stresses must be considered to avoid misleading extrapolations to clinical applications. The obtained results suggest that fatigue testing could help elucidate conflicting findings between *in vitro* [2–4] and *in vivo* [5–8] studies regarding the performance of resin-dentin bonding protocols.

The underperformance of the universal adhesive in self-etch mode after ageing indicates higher interfacial susceptibility to hydrolysis compared to etch-and-rinse protocols. The presence of residual smear layer played an important role on long-term resin-dentin degradation. Etching depths of simplified self-etch adhesives are generally reduced when more clinically relevant smear layers are present [11,12,40]. Considering that the tested universal adhesive is unable to effectively etch through the entire extension of the produced smear layer [11,12], the latter acted as a barrier against adequate monomer diffusion into dentin. Ageing exacerbated such limitations. The inherent deficiency of the tested universal adhesive to interact with underlying dentin [11,12] not only perpetuated overtime, but it was more prone to degradation compared to bonding protocols composed by a separate etching step. This was confirmed not only by lower fatigue strengths, but also by fracture patterns. The exposure of a relatively thick amorphous-residual content at the hybrid layer boundaries, for uncoated and coated samples (Fig. 3 A′ and E′), indicates suboptimal adhesive-dentin interactions. Although stable calcium-phosphate and calcium-carboxylate ions can be formed between functional acidic monomers (*e.g.,* 10-MDP) and the bonded interface [41], their origination within weakly attached smear layer undermines their bonding potential [41]. Hence, the supposed greater stability of universal adhesives in self-etch mode [42] was not confirmed. In fact, self-etch application produced the worst bonding outcomes after ageing. From a fatigue-testing perspective, poorly attached areas acted as interfacial flaws facilitating crack propagation. This reinforces the importance of creating strong-stable interactions between methacrylate-based acidic monomers and the underlying dentin to extend the durability of bonded interfaces [9]. Future studies should evaluate the fatigue strength of universal adhesives with different pH after long-term ageing.

It is important to note that fatigue testing also revealed differences between dentin-etching protocols that were not identified by quasi-static flexural strengths. This corroborates previous findings showing higher discriminative power of fatigue tests compared to monotonic tests [11,12]. After ageing, fatigue strengths varied according to dentin-etching protocols. Even though pairwise comparisons of fatigue distributions (Table 2) identified no significant differences between dentin-etching protocols using 32% H_3PO_4 for 15 s (OPA 15 s) or 3 s (OPA 3 s), endurance limits were 19% higher for the latter. Dentin demineralization produced by longer etching times complicate resin impregnation into overexposed collagen fibrils [43,44]. Such areas composed by unprotected collagen are more susceptible to enzymatic degradation, invariably resulting in higher probability of flaw distribution with ageing. Hence, more conservative demineralization approaches (*i.e.*, OPA 3 s and MPA 15 s) produced higher fatigue strengths than conventional H_3PO_4-ethcing for 15 s. Curiously, MPA 15 s, albeit exposing more collagen than OPA 3 s (OPA 15 s > MPA 15 s > OPA 3 s) [12], produced the highest endurance limit and significantly higher fatigue life distributions. Superior adhesive-dentin interaction for MPA 15 s was also confirmed by fracture patterns showing predominantly cohesive failure of bonding resin (Fig. 3 D′ and H′). Meta-phosphoric acid [HPO_3] produces metaphosphate anions [PO_3^-] in aqueous solutions through the ionization of the single hydroxyl group [-OH] to produce a hydrogen ion [H^+]. PO_3^- is an interesting anion that may increase collagen cross-linking potentially reducing enzymatic breakdown [27] and thus contributed to highest fatigue strengths after ageing. Future studies are required to confirm HPO_3 inhibition of endogenous proteases. Selection of etching protocols that remove smear layer without overexposing collagen is thereby a promising step for successful long-term resin-dentin bonding.

Since the hydrophobic-rich content of hybrid layers significantly affected long-term bonding of the tested mild universal adhesive, the second null hypothesis was rejected. Hydrophobic-rich coating of hybrid layers produced 16 % to 2-fold higher endurance limits. Coating not only produced higher fatigue strengths, but also lowered etching-associated variabilities in bonding performance after ageing. Increasing the hydrophobic-rich content of hybrid layers benefits monomer conversion [21] and likely improves the mechanical strength of its polymeric portion [45]. Additionally, lower water affinity certainly reduced water sorption over time [18]. Reducing water diffusion through polymer matrices also lowers polymer solubility [18] contributing to better stabilization of the methacrylate portion of hybrid layers [46]. Reduction/absence of micrometer-sized pores depicted as the “Swiss-cheese” aspect (Fig. 3 E – H) reinforces the improved polymeric quality of bonded interfaces higher hydrophobic monomeric content. The highest impact of hydrophobic-rich coating occurred when no separate etching was performed (SE H). Since the universal adhesive was left uncured, the application of hydrophobic-rich resin (*i.e.* for additional 20 s) extended the total etching time. Although endurance limits were not comparable to the best performing bonding protocol (MPA 15 s H), hydrophobic-rich coating produced a 2-fold increase when used in self-etch mode. This may be a more user-friendly approach to improve the bonding performance of universal adhesives. Dentin hybridization is a non-homogenous demanding procedure [1,9,41]. Monomers diffusion into the entire collagen extension is not feasible, especially considering more hydrophobic compounds [44]. Providing larger ratios of hydrophobic monomers during hybridization likely facilitated, at least to some extent, collagen impregnation. Determining whether degradation of methacrylate-based or collagen portion of hybrid layers have a stronger effect on interfacial degradation was beyond the scope of this work. Nonetheless, it was evident that increasing the hydrophobic-rich content of hybrid layers created on dentin with lower collagen exposure (OPA 3 s H and MPA 15 s H) produced higher endurance limits after ageing. Controlled dentin etching using H_3PO_4 for 3 s or HPO_3 for 15 s fully removed smear layers without overexposing collagen [12]. Smear layer debris may act as residual contaminants at the bonded interface preventing polymer chains from getting closer during polymerization. As a result, higher degree of conversions may be observed for etch-and-rinse applications of universal adhesives [47]. Considering larger extensions of demineralized collagen, the positive effect of hydrophobic-rich coating was even stronger (OPA 15 s H). The endurance limit of OPA 15 s H was 37 % higher than OPA 15 s. This reinforces the importance of increasing the hydrophobic-rich content of hybrid layers to extend their long-term durability especially for more aggressive dentin etching protocols (*e.g.*, 32 % H_3PO_4 for 15 s). Clearly, the process is far from perfect; however, the substantial improvements justify the additional bonding steps. Future studies should investigate different etching agents combined with universal bonding resins under extended application times after long-term ageing.

5. Conclusion

While conventional fatigue testing of resin-dentin interfaces remains a highly valuable metric of performance in adhesive dentistry, additional hydrolytic ageing renders improved assessments of bonding performance. Increasing the hydrophobic-rich content of hybrid layers considerably reduces the inevitable impairment of fatigue strengths produced by interfacial hydrolysis over time. Long-term bonding efficiency of universal adhesives is strongly associated to the hydrophobic-rich content of hybrid layers and to proper smear layer management. Therefore, current oversimplification trends in bonding protocols seem to compromise the durability of resin-dentin interfaces.

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