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Are universal adhesives in etch-and-rinse mode better than old 2-step etch-and-rinse adhesives? One-year evaluation of bonding properties to dentin

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ABSTRACT

Objectives: This study compared the bonding properties of dentin of three 2-step etch-and-rinse adhesives (2-ERAs) to those of three universal adhesives (UAs) applied with an etch-and-rinse strategy (ER), immediately and after 1 year of water storage.

Methods: Sixty caries-free molars were divided into 6 groups according to the adhesive systems used ($n = 10$). The 2-ERA systems included were: 1) Adper Single Bond 2 (SB), 2) Tetric N-Bond (TB), and 3) Ambar (AM); and the UAs systems were: 4) Single Bond Universal (SBU) 5) Tetric N-Bond Universal (TBU), and 6) Ambar Universal (AMU). The occlusal third of each tooth was removed and the adhesives were applied. After the composite build-up, specimens were sectioned and tested for microtensile bond strength (μTBS) and nanoleakage (NL) immediately and after 1 year of water storage. In situ degree of conversion (DC) was only evaluated in the immediate time. For water sorption (WS), solubility (SO), and mass change (MC) tests, 48 disk-shaped specimens were prepared ($n = 8$) and assessed according to ISO 4049:2009.

Results: UAs showed higher μTBS and lower NL values than 2-ERAs did after 1 year of water storage ($p = 0.001$). Regarding DC, 2-ERAs showed higher DC values than UAs ($p = 0.001$). Regarding WS, 2-ERAs showed higher WS values than those of UAs ($p = 0.00001$), except for AM and AMU. Lower WS was observed for AM than for other 2-ERAs ($p = 0.00001$).

Conclusion: The use of UAs applied with the ER strategy seems to be a more effective technique for maintaining adhesion to dentin substrate than 2-ERAS.

Clinical significance: This study may support clinicians in selecting the most adequate adhesive system to be used in ER strategy in dentin, demonstrating that UAs were more effective, considering the longevity of the resin restorations.

1. Introduction

Resin-based dental restorations are one of the most common procedures in daily dental practice [1]. This is due to several advantages of resin composite when associated with adhesive systems, such as resin's intrinsic potential to match the natural tooth color, its ability to bond to tooth tissues, its reduction of necessary sound tooth removal, and its low cost as compared to indirect materials [2,3]. However, the main reasons

Abbreviations: 2-ERAs:, 2-step etch-and-rinse adhesives; UAs:, Universal adhesives; SB:, Adper Single Bond 2; SBU:, Single Bond Universal; AM:, Ambar; AMU:, Ambar Universal; TB:, Tetric N-Bond; TBU:, Tetric N-Bond Universal; μTBS:, Microtensile bond strength; NL:, Nanoleakage; WS:, Water sorption; SO:, Water solubility; DC:, In situ degree of conversion; MC:, mass change; ER:, Etch-and-rinse; SE:, Self-etch; 10-MDP, 10-methacryloyloxydecyl dihydrogen phosphate; Ca:, calcium.

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for the clinical failure of direct resin composite restorations in posterior teeth over time are secondary caries and restoration fractures [4,5]. Therefore, in efforts to improve resin composite restorations' clinical success and the adhesive interface's durability, constant evolution in the adhesive dentistry field has led to a large variety of adhesive systems on the market [3,6].

Currently, dental adhesives are generally classified into etch-and-rinse (ER) and self-etch (SE) systems. Moreover, the primer and bonding material can be combined or separated, resulting in 3 or 2 steps of adhesive [7]. For ER adhesives (ERAs), a preliminary phosphoric acid must be used before monomer infiltration, to dissolve the smear layer and the superficial mineral components of dentin [8]. However, despite the strong performance of the ER strategy on enamel substrate [9], in dentin, the bond depends on the infiltration of monomers into the demineralized tissue, replacing the dissolved hydroxyapatite crystals and surrounding collagen fibrils [10,11]. Unfortunately, an incomplete formation of a collagen/resin structure can occur due to the insufficient infiltration or protection of collagen fibrils by resin monomers, increasing bonding's susceptibility to water sorption as well as to attack by host-derived proteolytic enzymes and compromising the durability of the restoration [11,12].

On other hand, the SE adhesive systems omit the pre-etching, due to the presence of acidic monomers that partially demineralize the substrate and, at the same time, infiltrate the adhesive in the demineralized collagen mesh, decreasing the number of empty spaces in the adhesive layer [13,14].

Along the same line of simplification, universal adhesives (UAs) were introduced as a more versatile clinical option [15]. This versatility enables UAs' use in ER, SE, or selective enamel etching strategies for direct restorations, according to the clinical situation or preference of the operator [16]. Such broad application was made possible by several manufacturers' addition of acidic functional monomers, such as 10-methacryloyloxydecyl dihydrogen phosphate (10-MDP), to provide chemical bonding to hard tooth tissue [17].

According to several studies, the presence of 10-MDP in the composition of UAs can incur chemical interactions through ionic bonds established by the phosphate radical present in the 10-MDP that reacts with the hydroxyapatite, forming monomer-Calcium (Ca) salts that are stable to degradation [18]. Given that 10-MDP's mechanism of chemical bonding to dentin occurs due to interaction with Ca, there is no reason to apply phosphoric acid before adhesive application, since all Ca in the demineralized dentin is removed after etching with phosphoric acid [19].

This rationale seems to suggest that UAs applied in SE systems would have better long-term results than UAs applied in ER mode would [20,21]. However, the evaluation of different adhesive strategies (ER or SE) for UAs has produced controversial results [22]. It is worth mentioning that a recently published systematic review of in vitro studies showed no significant difference between the two adhesive strategies' effects on the immediate and long-term bond strength of UAs [23].

Although the use of phosphoric acid before the application of UAs is not well understood, some authors have identified a potential chemical interaction between MDP and demineralized dentin. For instance, Hidari et al. compared an MDP-containing UA to an experimental MDP-free adhesive from the same manufacturer, both associated with a phosphoric acid application. The results showed higher bond strength values for the former than for the MDP-free UA [24]. This result led the authors of the present study to speculate that a certain interaction might occur between exposed collagen fibrils and MDP [25]. However, Hidari et al. [24] did not compare the UA against the previous generation of ERAs.

Thus, the aim of the present study was to evaluate the bonding properties of the previous generation of 2-step ERAs (2-ERAs) alongside those of the new generation of UA (applied with an ER strategy) from the same manufacturer. The microtensile bond strength (μTBS) and nanoleakage (NL) to the resin-dentin interface were evaluated immediately and after 1 year of water storage. Furthermore, the *in situ* degree of conversion (DC), water solubility (SO), water sorption (WS), and mass change (MC) were evaluated immediately. The null hypotheses to be tested were that the μTBS, NL, DC, SO, WS, and MC of UA in ER mode would not differ from those of the 2-ERAs.

2. Materials and methods

2.1. Experimental design

Three 2-ERAs (Adper Single Bond 2, SB, 3M Oral Care, St. Paul, MN, USA, also known as Adper Scotchbond 2 in some countries; Tetric N-Bond, TB, Ivoclar Vivadent, Schaan, Liechtenstein; and Ambar, AM, FGM, Joinville, SC, Brazil) and three UAs (Single Bond Universal, SBU; 3M Oral Care, St. Paul, MN, USA, also known as Scotchbond Universal in some countries; Tetric N-Bond Universal, TBU, also known as Adhese Universal in some countries; Ivoclar Vivadent, Schaan, Liechtenstein; and Ambar Universal, AMU; FGM, Joinville, SC, Brazil) applied in the ERs strategy were evaluated in this study. The properties evaluated were resin-dentin microtensile bond strength (μTBS) and nanoleakage (NL), both evaluated immediately and after 1 year of water storage. *In situ* degree of conversion on the resin-dentin interface was only evaluated at the immediate time. Water sorption, solubility, and mass change were evaluated after 7-day water storage. (Fig. 1) The batch number, composition, and application mode of the six adhesives tested are listed in Table 1.

2.2. Teeth preparation

Sixty caries-free extracted human third molars were used in the present study (n = 10 for each adhesive system). The teeth were collected after the patient's informed consent. The University Ethics Committee of the State University of Ponta Grossa, PR, Brazil approved this study under protocol number 2.321.442. Teeth were disinfected in 0.5% chloramine for one week [26], stored in distilled water, and used within 3 months after extraction. A flat dentin surface was exposed on each tooth after wet-grinding the occlusal enamel with 180-grit SiC paper. The enamel-free, exposed dentin surfaces were further polished with 600-grit silicon-carbide paper for 60 s to standardize the smear layer.

All adhesives were applied in ER strategy, as per the manufacturer's instructions (Table 1). Briefly, the dentin surface was acid-etched with phosphoric acid for 15 s, water rinsed for 15 s, and dried with absorbent paper keeping the dentin surface slightly wet. After the bonding procedures, resin composite blocks (A3.5; Opallis, FGM, Joinville, SC, Brazil) were buildup on the bonded surfaces in 3 increments of 1.0 mm of thickness each and then individually light activated for 40 s with a LED light source at 1,160 mW/cm^2 (Bluephase N, Ivoclar Vivadent, Schaan, Liechtenstein). The irradiance of the light curing unit was measured using an accurate radiometer (Bluephase Meter II, Ivoclar Vivadent, Schaan, Liechtenstein). A single operator carried out all bonding procedures in an environment with controlled temperature and humidity. Ten teeth were randomly assigned for each experimental group.

After storage of the bonded teeth in distilled water at 37 °C for 24 h, teeth were longitudinally sectioned in both "x'' and "y'' directions across the bonded interface with a diamond saw in a cutting machine (Isomet 1000; Buehler, Lake Bluff, USA), under water cooling at 300 rpm to obtain resin-dentin bonded sticks with a cross-sectional area of approximately 0.8 mm^2. The number of premature failures per tooth during specimen preparation was recorded. The cross-sectional area of each resin-dentin bonded stick was measured with the digital caliper to the nearest 0.01 mm and recorded for subsequent calculation of the microtensile bond strength values (Absolute Digimatic, Mitutoyo, Tokyo, Japan). The resin-dentin bonded sticks were randomly divided as follows: 1) three resin-dentin bonded sticks per tooth and from each

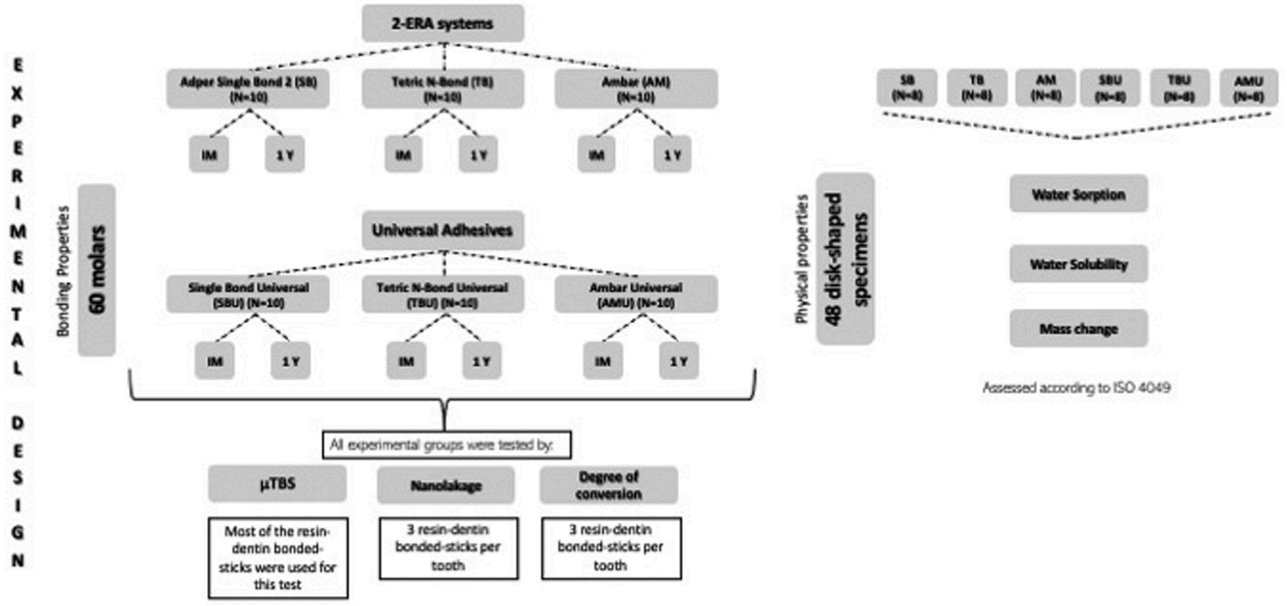


Fig. 1. Representative image of the experimental design (ERA: etch-and-rinse adhesives; IM: immediate; 1Y: 1 year; µTBS: microtensile bond strength test.

experimental condition group were evaluated for immediate *in situ* conversion of degree within the resin-dentin adhesive interface; 2) three resin-dentin bonded sticks per tooth and from each experimental condition group were tested after 24 h or 12 months of water storage for nanoleakage, and 3) the rest of the resin-dentin bonded sticks were tested after 24 h or 12 months of water storage for microtensile bond strength test. The distilled water was changed monthly.

2.3. Microtensile bond strength testing

Each resin-dentin bonded stick was attached to a modified device (Odeme, Luzerna, SC, Brazil) for a microtensile bond strength test with cyanoacrylate resin (IC-Gel, bSi Inc., Atascadero, CA, USA) and subjected to a tensile force in a universal testing machine (Kratos, São Paulo, SP, Brazil) at 0.5 mm/min. The failure mode was evaluated under an optical microscope (SZH-131, Olympus; Tokyo, Japan) at 40x and classified as cohesive in dentin (failure exclusive within cohesive dentin – CD); cohesive in resin (failure exclusive within cohesive resin – CR); adhesive (failure at resin/dentin interface – A), or mixed (failure at resin/dentin interface that included the cohesive failure of the neighboring substrates, M). The number of premature failures (PF) was recorded, but it was not included in the average mean bond strength.

2.4. Nanoleakage evaluation (NL)

All resin-dentin bonded sticks were coated with two layers of nail varnish applied up to within 1mmof the bonded interfaces. The specimens were re-hydrated in distilled water for 10 min prior to immersion in the tracer solution for 24 h. Ammoniacal silver nitrate was prepared according to the protocol previously described by Tay et al. [27]. All resin-dentin bonded sticks selected for this test were immersed in 50 wt % ammoniacal silver nitrate solution in total darkness for 24 h, rinsed thoroughly in distilled water, and immersed in the photo-developing solution for 8 h under fluorescent light to reduce silver ions into metallic silver grains within voids along the bonded interface. Specimens were mounted on aluminum stubs, polished with 1000-, 1500-, 2000- and 2500-grit SiC paper and 1 and 0.25 µm diamond paste (Buehler Ltd., Lake Bluff, IL, USA). Then, they were ultrasonically cleaned, air dried and gold sputter coated (MED 010, Balzers Union, Balzers, Liechtenstein). The interfaces were observed in a scanning electron microscope in the backscattered mode at 15 kV (VEGA 3 TESCAN, Shimadzu, Tokyo, Japan).

In order to standardize image acquisition, three pictures were taken of each specimen. The first picture was taken in the center of the resin-dentin slice. The other two pictures were taken 0.3 mm to the left and right of the first one. A technician who was blinded to the experimental conditions under evaluation took them all. The relative percentage of NL within the adhesive and hybrid layer areas was measured in all pictures using the public domain Image J software, a Java-based image processing software package developed at the National Institutes of Health (NIH) [28].

2.5. In situ degree of conversion within the adhesive interface

All resin-dentin bonded sticks selected for this test were wet polished using 1500, 2000, 2500, and 4000-grit SiC paper for 30 s each. The specimens were ultrasonically cleaned for 10 min and positioned into micro-Raman equipment. The in situ degree of conversion measurements were performed in a micro-Raman spectrometer (Bruker Optik GmbH, Ettlingen, Baden-Württemberg, Germany). The micro-Raman spectrometer was first calibrated for zero and then for coefficient values using a silicon specimen. Specimens were analyzed using the following micro-Raman parameters: 20-mW Neon laser with 532-nm wavelength, a spatial resolution of ≈ 3 µm, spectral resolution ≈ 5 cm^{-1}, accumulation time of 30 s with 6 co-additions, and magnification of 100x (Olympus UK, London, UK) to beam diameter of ≈ 1 µm. The spectra were taken at the resin-dentin bonded interface, in the middle of the hybrid layer within the intertubular dentin. Care was taken to select an area between two dentin tubules. The measurement was taking at three different sites for each specimen and the values were averaged for statistical purposes. Spectra of uncured adhesives were taken as reference. Post-processing of spectra was performed using the dedicated Opus Spectroscopy Software version 6.5 (Bruker Optik GmbH, Ettlingen, Baden-Württemberg, Germany). The ratio of the double-bond content of monomer to polymer in the adhesive was calculated according to the following formula: DC (%) = $(1 - R_{cured}/R_{uncured})$ x 100, where R is the ratio of aliphatic and aromatic peak areas at 1639 cm^{-1} and 1609 cm^{-1} in cured and uncured adhesives [29,30].

Table 1
Adhesive Systems, composition and application mode.

Adhesive (Manufacturer)	Batch number	Composition	Application mode
Adper Single Bond 2 (SB, 3M Oral Care, MN, St. Paul, USA)	#N893302	Scotchbond Universal Etchant: 34% phosphoric acid; Bis-GMA, HEMA, glycerol 1,3-dimethacrylate, UDMA, ethyl alcohol, water, silane treated silica, copolymer of acrylic and itaconic acids	1 Apply etchant for 15 s; 2 Rinse for 15 s; 3 Air dry 5 s; 4 Apply 2-3 coats of theadhesive with a microbrush and rub it in for 15 s. 5 Direct a gentle stream of air over the liquid for about 10 s until it no longer moves and the solvent has evaporated completely; 6 Light cure for 10 s;
Single Bond Universal (SBU, 3M Oral Care, MN, St. Paul, USA)	#71201B	Scotchbond Universal Etchant: 34% phosphoric acid; Adhesive: 10- MDP, HEMA, dimethacrylate resins, Vitrebond copolymer acid polyalkenoic, filler, ethanol, water, initiators, silane	1 Apply etchant for 15 s; 2 Rinse for 15 s; 3 Air dry 5 s; 4 Apply the adhesive to the entire preparation with a microbrush and rub it in for 20 s. If necessary, rewet the disposable applicator during treatment; 5 Direct a gentle stream of air over the liquid for about 10 s until it no longer moves and the solvent has evaporated completely; Light cure for 10 s;
Tetric N-Bond (TB, Ivoclar Vivadent, Schaan, Liechtenstein)	#X16310	Total Etch: 37% phosphoric acid; Adhesive: Phosphoric acid acrylate, HEMA, Bis-GMA, urethane dimethacrylate, ethanol, film-forming agent, catalysts and stabilizers	1 Apply etchant for 15 s; 2 Rinse for 15 s; 3 Air dry 5 s; 4 Apply one coat of the adhesive with a microbrush and rub it in for 10 s 5 Direct a gentle stream of air over the liquid for about 10 s until it no longer moves and the solvent has evaporated completely; 6 Light cure for 10 s;
Tetric N-Bond Universal (TBU, Ivoclar Vivadent, Schaan, Liechtenstein)	#W15553	Total Etch: 37% phosphoric acid; Adhesive: Methacrylates monomers (10-MDP), ethanol, water, highly dispersed silicon dioxide, initiators and stabilizers	1 Apply etchant for 15 s; 2 Rinse for 15 s; 3 Air dry 5 s; Apply one coat of adhesive to the entire preparation with a microbrush and rub it in for 20 s. 1 Direct a gentle stream of air over the liquid for about 10 s until it no longer moves and the solvent has evaporated completely Light cure for 10 s
Ambar (AM, FGM, Joinville, SC, Brazil)	#28618	Condac 37% Adhesive: UDMA, HEMA, and other hydrophilic methacrylate monomers, acid methacrylate monomers, ethanol, silanated silica, photoinitiators, coinitiators, and stabilizers	1 Apply etchant for 15 s; 2 Rinse for 15 s; 3 Air dry 5 s; 4 Apply the adhesive with a microbrush and rub it in for 10 s. 5 Direct a gentle stream of air over the liquid for about 10 s until it no longer moves and the solvent has evaporated completely; 6 Light cure for for 10 s;
Ambar Universal (AMU, FGM, Joinville, SC, Brazil)	#260917	Condac 37% Adhesive 10-MDP, methacrylic monomers, photoinitiators, coinitiators, stabilizers, silica nanoparticles and ethanol	1 Apply etchant for 15 s; 2 Rinse for 15 s; 3 Air dry 5 s; 4 Apply the adhesive to the entire preparation with a microbrush and rub it in for 20 s. If necessary, rewet the disposable applicator during treatment 5 Direct a gentle stream of air over the liquid for about 10 s until it no longer moves and the solvent has evaporated completely Light cure for 10 s

Bis-GMA, bisphenol A diglycidyl ether methacrylate; HEMA, 2-hydroxyethyl methacrylate; UDMA, diurethane dimethacrylate; 10-MDP, 10-methacryloyloxydecyl dihydrogen phosphate.

2.6. Water sorption (WS), water solubility (SO), and mass change (MC)

A total of forty-eight disk-shaped specimens were prepared (n = 8 for each adhesive system). For specimen preparation, it was used a stainless-steel matrix (6.0 mm, diameter x 1.0 mm, thickness), that was positioned over a glass slide that was previously covered with a thin Mylar strip. As the matrix consisted in a disk-shaped hole, a white background was used in order to standardize the reflection/absorption of light emitted by the light-curing procedure.

The adhesives were directly dispensed within the matrix and immediately covered with a Mylar strip that was manually pressed with another 1.0 mm-thickness glass slide. Then, the adhesives were photo-activated for 10 s (according to manufacturer instructions) with a LED light curing unit (Bluephase N Ivoclar Vivadent, Schaan, Liechtenstein) at 1,160 mW/cm^2, placing the light tip in contact with the glass slide. The irradiance of the light curing unit was measured as previously described. The specimens were then removed from the matrix and, if necessary, the edges were carefully regularized with 600-grit SiC paper.

Water sorption and solubility of all adhesives were determined

according to ISO 4049:2009 specification [31] with modifications on dimensions and photoactivation time of the disk-shaped specimens, as described before. [32] The polymerized specimens were stored individually in eppendorf tubes at 37°C in a desiccator containing silica gel. The disks were weighed after a 24 h interval in an analytical balance until a constant mass (m_1) was obtained (when the mass loss was not greater than 0.1 mg in 24 h). The diameter and thickness of the disks to the nearest 0.01 mm were measured with a digital caliper to calculate the exact volume of the specimens.

Then, they were individually immersed into Eppendorf tubes containing 1.0 mL of distilled water at 37°C. After 7 days of water storage, disks were removed, washed in a glass container with distilled water, blotted away surface water with absorbent paper for 45 s, waved in the air for 15 s, and then immediately weighed again (m_2). Care was taken to register the weight m_2 exactly 1 minute after removal from the water. Next, specimens were dried in the desiccator at 37°C, as previously described, and weighed daily until a dried constant mass was obtained (m_3).

Water sorption and solubility were calculated over the 7 days of water immersion using the following formula:

$$WS = m_2 - m_3/V$$

$$SO = m_1 - \ m_3/V$$

where m_1 is the mass of the specimen before immersion in water (in μg); m_2 is the mass of the specimen after immersion in water (in μg); m_3 is the mass of the specimen after desiccation (in μg); V is the volume of each specimen (in mm^3).

Additionally, the percentage of mass change (MC) of the specimens was calculated for identifying if water uptake or elution of adhesive components was predominant after 7 days of water immersion, according to the following formula:

$$MC = (m_2 - - m_1) \times 100\%/m_1$$

2.7. Statistical analysis

For microtensile bond strength and nanoleakage, the experimental unit was the tooth with half being tested immediately and the other half after 1 year of water storage. The μTBS and nanoleakage values of all resin-dentin bonded specimens from the same hemi-tooth were averaged for statistical purposes. After confirming the normality of the data distribution and the equality of the variances (data not shown), the microtensile bond strength (MPa) and nanoleakage (%) data were subjected to a two-way (adhesive generation vs. time) repeated measures ANOVA and Tukey's test. The repeated measure was the tooth at the two-evaluation period. Data for fracture pattern mode were subjected to Chi-square and Fisher exact test and data for *in situ* degree of conversion (%), water sorption (μg/mm^3), water solubility (μg/mm^3), and mass change (%) were subjected to a one-way ANOVA (adhesive generation). Tukey's post hoc test was used for pair-wise comparisons ($\alpha = 0.05$) using the Statistica for Windows software (StatSoft, Tulsa, OK, USA).

3. Results

3.1. Microtensile bond strength (μTBS)

The frequency of each fracture pattern mode, as well as the number of specimens with premature failures, are shown in Table 2. The most frequent mode of failure in the present study were adhesive or adhesive/mixed failures with 89.5% of all specimens, following for premature failure (6.9%) and cohesive failures in the study (3.5% of all specimens). When submitted to statistical analysis, all the groups showed similar results in terms of failure mode ($p > 0.32$).

The cross-product interaction was significant (Table 3; $p = 0.001$). It was observed significantly lower μTBS values after 12 months of water storage when compared with the immediate time for all adhesives evaluated (Table 3; $p = 0.001$). However, UAs showed higher μTBS values when compared to 2-ERAs, regardless of evaluation time (Table 3; $p = 0.001$), with exception of SB vs SBU in the immediate time and for AM/AMU in the immediate time and after 12 months of water storage (Table 3; $p > 0.05$).

Table 2
Number of specimens (%) according to fracture mode (*).

Adhesives	Time	Fracture patten Adhesive or mixed fracture mode	Cohesive	Premature Failure
SB	Immediate	104 (84.6)	7 (5.7)	12 (9.7)
	1 year	92 (83.7)	7 (6.3)	10 (9.0)
SBU	Immediate	110 (94.1)	0 (0)	12 (5.9)
	1 year	89 (86.4)	4 (3.9)	10 (9.7)
TB	Immediate	92 (85.9)	5 (4.8)	10 (9.3)
	1 year	85 (86.7)	4 (4.1)	9 (9.2)
TBU	Immediate	105 (91.4)	5 (4.3)	5 (4.3)
	1 year	98 (94.2)	2 (2.0)	4 (3.8)
AM	Immediate	100 (98)	0 (0)	12 (2.0)
	1 year	90 (85.7)	7 (6.7)	8 (7.6)
AMU	Immediate	102 (93.6)	0 (0)	7 (6.4)
	1 year	91 (90)	4 (4.1)	6 (5.9)

(*) No significant differences between each pair group was found (Chi-square/Fisher exact test, $p > 0.05$).

Table 3
Microtensile bond strength (MPa), silver nitrate uptake (%) and in situ degree of conversion (%) values of all adhesives immediate and 1 year of water storage (*).

Adhesives	Time	Microtensile bond strength (MPa)	Nanoleakage (%)	In situ degree of conversion (%)
SB	Immediate	50.4 ± 3.1 B	13.2 ± 3.7 b	83.2 ± 3.9 ^{a}
	1 year	37.2 ± 3.1 D	24.4 ± 4.5 d	-
SBU	Immediate	54.9 ± 3.9 A,B	6.9 ± 1.5 a	69.9 ± 3.9 ^{b,c}
	1 year	44.4 ± 2.9 C	10.2 ± 4.0 a,b	-
TB	Immediate	53.2 ± 4.3 A,B	21.0 ± 4.1 c	76.2 ± 4.1 ^{a,b}
	1 year	31.3 ± 3.6 E	32.3 ± 4.0 e	-
TBU	Immediate	58.0 ± 5.5 A	9.3 ± 2.9 a,b	67.2 ± 3.8 ^{c}
	1 year	42.0 ± 2.7 C,D	14.2 ± 2.8 b	-
AM	Immediate	49.2 ± 2.1 B	14.2 ± 3.2 b	81.3 ± 5.1 ^{a}
	1 year	42.1 ± 4.8 C,D	26.7 ± 3.1 d	-
AMU	Immediate	54.7 ± 4.1 A,B	6.1 ± 1.4 a	73.1 ± 4.2 ^{b}
	1 year	46.2 ± 3.6 C	10.2 ± 3.0 a,b	-

(*) Different capital, lowercase and superscript letters mean statistically significant differences for each test (columns) (Tukey test, $p < 0.05$).

3.2. Nanoleakage evaluation (NL)

The cross-product interaction was significant (Table 3; $p = 0.0001$). It was observed significantly higher nanoleakage values after 12 months of water storage when compared with the immediate time for 2-ERAs (Table 3; $p = 0.0001$). On the other side, for all UAs tested, no significant increase in the nanoleakage values was observed after 12 months of water storage when compared with immediate time (Fig. 2; Table 3; $p > 0.05$).

3.3. In situ degree of conversion (DC)

The cross-product interaction was significant (Table 3; $p = 0.001$). For all comparisons, 2-ER adhesives showed significantly higher DC values than UAs (Table 3; $p = 0.001$). No significant difference was observed among 2-ER adhesives (Table 3; $p > 0.05$). However, when UAs were compared, AMU showed higher DC when compared to TBU (Table 3; $p = 0.001$).

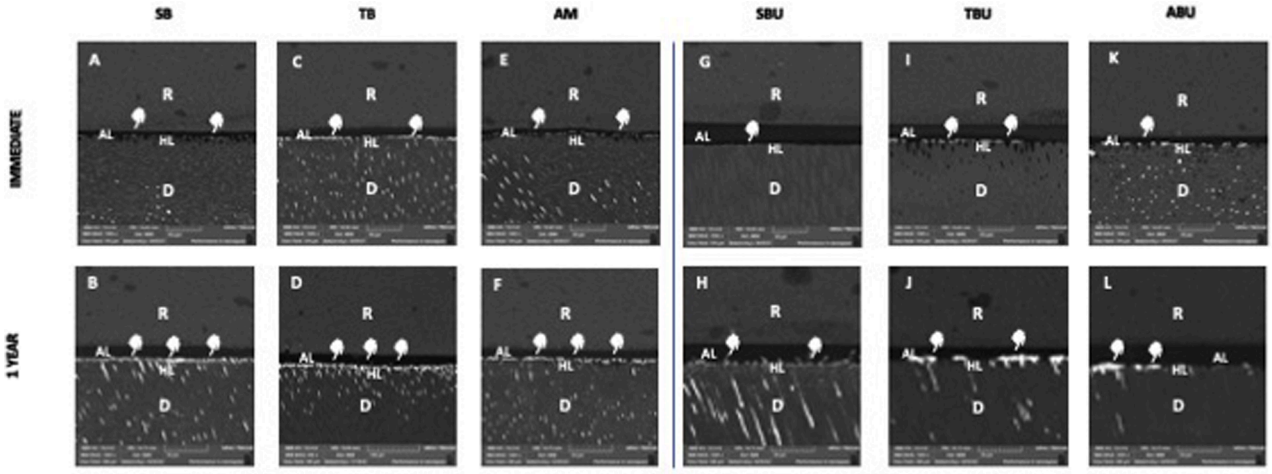


Fig. 2. Representative backscatter scanning electron microscope micrographs of adhesive interface for all experimental groups (1.0 kx). Silver nitrate deposits were detected in all groups, mainly in the hybrid layer (white hands). Usually, 2-ERA's SB (A-B), TB (C-D), and AM (E-F) exhibited more areas with silver nitrate infiltration than UA's SBU (G-H), TBU (I-J) and ABU (K-L) at both evaluation times. Overall, silver nitrate infiltration increased significantly for the 2-ERA's adhesives SB (B), TB (D) and AM (F) compared to the UA's SBU (H), TBU (J) and ABU (L) after one year of water storage.

3.4. Water sorption, water solubility, and mass change

The one-way ANOVA was significant for all properties (Table 4; $p < 0.0002$). Regarding the WS, 2-ER adhesives showed higher WS values than UAs (Table 4; $p = 0.00001$), except for AM/AMU. Also, lower WS was observed for AM/AMU when compared to all adhesives evaluated (Table 4; $p = 0.00001$). Usually, TB/TBU showed lower WS when compared to the SB/SBU (Table 4; $p = 0.00001$).

Regarding SO, 2-ERAs showed lower SO values than UAs (Table 4; $p = 0.0002$), except for SB/SBU. Lower SO values were observed for TB and AM when compared to SB (Table 4; $p = 0.0002$). Also, lower SO values were observed for AMU when compared to TBU and SBU (Table 4; $p = 0.0002$).

In relation to mass change, 2-ERAs showed higher mass change values than UAs (Table 4; $p = 0.0001$). Lower mass change values were observed for SB and AM when compared to TB (Table 4; $p = 0.0001$). No significant mass change was observed when all UAs were compared (Table 4; $p = 0.0001$).

4. Discussion

As previously described, ER necessitates the use of a preliminary phosphoric acid [8]. The phosphoric acid removes the smear layer and its appended smear plugs. Additionally, this step induces a decalcification of 5-10 μm of superficial dentin, removing hydroxyapatite and leaving some amount of collagen fibrils filled by dentinal fluid and water from rinsing the acid [11]. This porous structure should be infiltrated by a hydrophilic solution of resinous monomers and solvent to form the hybrid layer [7,33]. Observe that, at the time of immediate testing, all adhesives and their commercial counterparts tested showed similar μTBS results, indicating that all adhesives achieve some degree of infiltration of resinous monomers into the demineralized dentin, replacing the dissolved hydroxyapatite crystals and surrounding collagen fibrils [3,7].

It is important to mention that, in the present study the premature failure specimens were not added in the μTBS results. Despite the correct handling of these failures on the μTBS test is still up for debate [34,35], it is generally accepted that premature failures cannot just be omitted as this will cause a bias to higher μTBS [36–39]. Even though the exclusion of premature/pretesting failures could be interpreted as a loss of statistical power for bond strength results, this analysis was performed according to previous literature [36–39] which reports that including or not the premature failures into the statistical analysis will not mean any significant difference when lower percentage of premature failure was observed, as well as in the present study. Despite the exact value that mean a "lower percentage" is still unclear, a recent study consider that if less than 10% of the total specimens prepared were premature failure, these specimens should not be included in the final bond strength values [9] as well as in the present study. Worth adding that, when a study considers the tooth as the experimental unit of the study, i.e., the final bond strength values were the result of the average of all the specimens evaluated in each tooth. The loss of specimens due to pretest failures should be consider less important in terms of statistical analysis because the intra-tooth variability is higher than the inter-tooth variability for μTBS [40].

Interestingly, despite no significant difference being observed in terms of μTBS values for each pair of adhesives from the same manufacturer, a significant difference favoring ERAs was observed when DC was evaluated. Due to the presence of MDP, water is necessary within the adhesive solution for the ionization of functional groups [41] as water is more difficult to evaporate than other organic solvents [42], the remaining water could be responsible for the lower DC of UAs. However, for all UAs, the manufacturer's recommendation indicates application for 20 seconds, instead of the 10-15 seconds recommended for their commercial counterparts. This increase in the application time probably improves the chemical interaction between the 10-MDP and the dentin and, consequently, to guarantee adequate immediate μTBS results [43,44].

Regarding to DC, it is well known that inferior DC could be related to poor mechanical properties [45], as bond strength values are directly related to the mechanical properties of the adhesive layer [29]. Therefore, μTBS values are expected to differ among the adhesives evaluated. Unfortunately, there is no consensus regarding the correlation between

Table 4
Water sorption, solubility and mass change values of simplified adhesives after 7-day water storage (mean ± standard deviation) (*).

Adhesives	Water sorption (μg/mm^3)	Solubility (μg/mm^3)	Mass change (%)
SB	182.8 ± 7.9 E	181.6 ± 23.2 c	0.3 ± 2.0 ^{b}
SBU	140.8 ± 12.0 C	201.7 ± 36.2 c	-5.3 ± 3.1 ^{a}
TB	154.0 ± 1.9 D	93.8 ± 9.9 a	5.2 ± 0.7 ^{c}
TBU	117.9 ± 5.7 B	185.5 ± 29.9 c	-5.7 ± 2.1 ^{a}
AM	101.7 ± 2.8 A	82.2 ± 8.8 a	1.7 ± 0.9 ^{b}
AMU	106.3 ± 9.0 A	147.7 ± 16.9 b	-3.5 ± 1.8 ^{a}

(*) Different capital, lowercase and superscript letters mean statistically significant differences for each test (columns) (Tukey test, $p < 0.05$).

DC and μTBS values [46–48]. However, in the present study, higher DC values (> 67%) were observed for all adhesives evaluated, and it is in accordance with the literature [29,48,49].

On the other side, lower NL was observed for UAs than for ERAs in the immediate test. One theory attributes the presence of NL within the adhesive and hybrid layers to the incomplete penetration of monomers into demineralized dentin [50]. This could be directly related to the presence of MDP in the composition of UAs. MDP is the most-used functional monomer in dentin adhesives, which can superficially demineralize the substrate [42] and the infiltration in the demineralized collagen mash can be more uniform, and a less amount of empty spaces between the hybrid layer is achieved in comparison to the use of phosphoric acid in the ER approach. [51] Additionally, MDP interacts chemically with the Ca in hydroxyapatite [52] and this concept involves nano-layering of the MDP-Ca salt's self-assembly and has drawn increasing attention in the last decade [53]. Unfortunately, none of the adhesives evaluated guarantee a nanoleakage free adhesive interface, even with the present of MDP in UAs. This could be related to the higher amount of Ca is removed after etching dentin, when phosphoric acid is applied [54,55].

Fortunately, the role of MDP and MDP-Ca salt in bonding to fibrils collagen within the hybrid layer has recently been elucidated. When the dentin is conditioned, the collagen exposed needs to be adequately protected. When an MDP-containing adhesive, such as a UA, is applied under demineralized dentin, it incurs an interaction between the MDP and the collagen [25,56]. MDP possesses a dihydrogen phosphate group and a long aliphatic chain, considered a hydrophobic chain [57], that interact through the hydrophobic aliphatic moiety in MDP and on the collagen's surface [25,58]. Also, strong bonding between collagen and MDP was observed via hydrogen bonds [56,59].

Moreover, Jin et al. [56] showed the formation of irregular deposits and decreases in open tubules after the application of MDP in demineralized dentin using SEM and, according to the EDS maps, the main component of these deposits is MDP-Ca salt. Based on these findings, the authors suggested that MDP-Ca salt refills the water-rich, resin-sparse regions within the hybrid layer to prevent the invasion of exogenous proteases and MMPs, providing mechanical protection for the underlying demineralized collagen. [56].

Some authors believe that the long and relatively hydrophobic spacer chains of 10-MDP indirectly reduce the surface area of exposed collagen fibers by removing water from the local microenvironment [25, 58]. All these chemical interactions between MDP-containing adhesives and collagen fibrils help us explain the lower presence of NL in the former when compared to that of their commercial counterparts' ERs, in agreement with Shen et al [60].

Unfortunately, for all adhesives tested, μTBS values decreased significantly after 1 year of water storage. Although the exact mechanism that causes the degradation of the hybrid layer is not yet completely understood, it is well established that the hybrid layer degrades by hydrolysis and leaching of the hydrophilic resinous monomers from the interfibrillar spaces and by the disorganization and solubilization of collagen fibrils [7,33].

For enhanced dentin infiltration, 2-ERAs and UAs are composed of hydrophobic resin monomers homogeneously mixed with hydrophilic monomers and solvents, since dentin is a naturally moist substrate [3]. However, in the first stage, the presence of hydrophilic resinous monomers can absorb water and chemicals from the environment, leading to the biodegradation of the hybrid layer [33,61]. Observe that 2 of the 3 UAs evaluated showed lower water sorption than ERAs did. This can be explained by the fact that, in the second stage of degradation, the presence of water softens the polymer by swelling the network and facilitating the release of unreacted resinous monomers trapped in the polymer network into their surroundings through nanovoids in the material [32,62]. Interestingly, despite UAs' lower water sorption in comparison to that of ERAs, UAs showed higher solubility and negative mass change than ERAs did. This means that, while for ERAs the water remains untapped in the adhesive layer and increases the mass change, for UAs all the water sorption causes the solubility of unreacted monomers, leading to negative mass change. [45] A closer view regarding DC revealed the observation of lower DC for all UAs than for ERAs. This means that UAs contain more unreacted monomers than ERAs do. These unreacted monomers are more easily leached for the adhesive layer, which could result in higher solubility and negative mass change for UAs, as was previously observed [32,62]. Future studies are needed to confirm this hypothesis. However, all these results regarding WS, SO and mass change requiring the rejection of the part of the null hypothesis.

However, upon a comparison of the 1-year results of commercial counterparts' adhesives, higher μTBS values were observed for UAs than for ERAs, indicating that UAs' adhesion continues to outperform ERAs'. These results are similar to those observed by Sezinando et al. and Hidari et al [24,41]. This seems to have also been confirmed by the observation of NL results. While a significant degradation was observed for all ERAs after 1 year of water storage, no significant difference was observed between the μTBS,NL and DC values observed immediately and for μTBS and NL measured after 1 year of water storage for all UAs evaluated, requiring the rejection of the other part of the null hypothesis.

The second mechanism of degradation is related to the disorganization and solubilization of collagen fibrils [7,33]. Due to an incomplete infiltration of resin monomers, collagen fibrils that are unprotected by resin monomers can be present. This collagen mesh is vulnerable to attack by host-derived proteolytic enzymes and thereby becomes more susceptible to degradation [33,61].

As previously described, deposits of MDP-Ca salts formed may have the functions of refilling the water-rich, resin-sparse regions within the hybrid layer, providing mechanical protection for the underlying demineralized collagen and improved resistance to exogenous enzymes [56]. Some studies have shown that the MDP monomer and MDP-Ca salt formed can inhibit enzymatic activity [60]. Therefore, the lower NL values observed for UAs after 1 year of water storage should be attributed to the fact that presence of MDP when applied in demineralized dentin preserves the hybrid layer by enhancing the resistance of dentin collagen to enzymatic degradation by interacting with collagen and forming MDP-Ca salt as a protective covering upon demineralized dentin. Furthermore, MDP-Ca salt exhibits a direct inhibition effect against MMPs [56].

It is worth mentioning that, despite the focus of the present study was compared counterparts ERAs with and without MDP, several other components presented in the adhesive composition should be co-responsible for the better results of UAs. Observe that, despite all UAs tested are ethanol-based and presented a mild or ultra-mild pH, SBU and TBU contain an additional monomer (copolymer of acid polyalkenoic and methacrylated carboxylic acid polymer, respectively) for improve the interaction with dentin [63,64]. Unfortunately, based on the results of the present study, this second monomer in SBU and TBU did not have any significant impact in the bonding to dentin when compared to AMU.

Actually, the exact composition of each adhesive is proprietary information and it's difficult to compare all the components present in each one. Indeed, it has been accepted that the new UAs available in the market contain a much better balance of hydrophilic and hydrophobic monomers, an improvement on the concentration of solvents, new initiator, and co-initiators, that could explain their good results in terms of bonding and mechanical properties [65]. However, future studies need to be done to evaluate the effect of these other hypotheses on the bonding performance to dentin.

It is worth mentioning that, despite the increase in the bonding performance of UAs when applied in dentin in comparison to that of the old 2-step ERAs, the role of MDP is not conclusively established, mainly because some studies have shown that nano-layering is rarely observed at the resin–dentin interface formed by commercial MDP-containing adhesives [66,67]. Actually, a few studies evaluating the role of MDP in collagen fibril chemical bonding applied an ethanol-based, MDP-containing experimental primer [56,68], while other studies only

evaluated a single MDP-containing commercial UA [56,60]. Therefore, future studies must evaluate the role of different MDP-containing commercial UAs in the chemical bonding of collagen fibrils.

The result of the present study partially confirms that the presence of MDP in UAs seems to play a fundamental role in chemical bonding to dentin after the application of phosphoric acid. This finding helps us to understand the superior clinical performance of UAs when applied in ER mode rather than with the SE approach [69–71].

5. Conclusion

When compared to the previous generation of adhesives, the use of UA applied with the ER strategy seems to be a more effective technique of maintaining adhesion to dentin substrate.

CRediT authorship contribution statement

M. Wendlinger: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **C. Pomacóndor-Hernández:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **K. Pintado-Palomino:** Methodology, Investigation, Writing – original draft. **G.D. Cochinski:** Methodology, Investigation, Writing – original draft. **A.D. Loguercio:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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