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Influence of Different Application Methods on the Bonding Effectiveness of Universal Adhesives to Dentin in the Early Phase

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Purpose: To investigate the changes in the dentin bond strengths of universal adhesives during the early phase and evaluate the effect of a double-layer adhesive application on the performance of the dentin bond.

Materials and Methods: Three universal adhesives and a two-step self-etch adhesive were employed to ascertain the shear bond strengths (SBS) of specimens to bovine dentin with the use of the etch-and-rinse or self-etch mode. The specimens were further divided into two groups based on adhesive application in a single or a double layer. The bonded specimens were stored in distilled water at 37°C for 5 min or 1, 6, 12, or 24 h prior to SBS measurement and the adhesives' Knoop hardness number (KHN).

Results: All the adhesives showed increased SBS with prolonged storage periods regardless of the adhesive layer (single or double) or etching mode. Most universal adhesives in the double adhesive layer groups showed significantly higher SBS than single adhesive layer groups for the same storage period. All the adhesives also showed increased KHN with increased storage period.

Conclusion: The SBS and KHN values of the adhesives increased with increasing storage duration over a 24-h period. Double adhesive layer application mediated increased dentin bond strength in the early phase.

Keywords: dentin bond strength, double-layer application, universal adhesive, early bond effectiveness, SEM analysis.

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Universal adhesives have been developed based on previous generations, such as single-step or self-etch (SE) adhesives; therefore, they are generally similar with regard to the adhesive components, particularly as concerns the single-bottle adhesives.[4,20] To date, various types of functional monomers have been used in different two- or single-step SE adhesives, with phosphoric-acid ester, carboxylic acid, and alcohol functional monomers. Among these functional monomers, 10-methacryloyloxydecyl dihydrogen phosphate (MDP) is one of the most effective for chemical bonding to hydroxyapatite (HAp).[13] Most universal adhesives also employ MDP as a functional monomer. Functional monomers, in conjunction with water, demineralize the tooth tissues and, at the same time, establish a chemical bond with

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hydroxyapatite (HAp).[38] In single-step SE adhesives, certain amounts of water and solvents are required to ionize the functional monomers and facilitate resin-monomer penetration into the demineralized tissue.[38] Moreover, the applied adhesive must be polymerized sufficiently in order to create a stable interface between the resin composites and tooth substrates. In terms of clinical use, universal adhesives are different from the previous generation of single-step SE adhesives,[29] as they can be used in any mode (etch-and-rinse [ER], SE, or selective etch), depending on clinical factors such as the location, size, depth, and enamel:dentin ratio of the cavity.[34]

The bonding mechanism of each adhesive is distinguished not only by the etching protocol but also by the number of steps necessary for bonding.[22,38] Previous studies proposed that the bonding mechanisms of universal adhesives to enamel and dentin in SE mode are similar to those of conventional single-step SE adhesives.[5,20,34] On the other hand, previous SEM investigations of morphological features of resin-dentin interfaces suggested that the dentin bonding mechanism of universal adhesives in ER mode may be different from those of three or two-step ER adhesives, because universal adhesives clearly created a reaction layer between the hybrid layer and intact dentin.[16,35] In addition, ER mode to dentin is not commonly used in SE adhesives. It is likely that the dentin bonding mechanism of universal adhesives may also be different when compared to the previous generation of single-step self-etch adhesives.

The different bonding mechanisms of different adhesives may also influence bond effectiveness in the early phase, ie, immediately after placement of composite restorations, as well as bond durability. Although many laboratory studies have been conducted after 24 h or long-term water storage, few results are available for the bond effectiveness in the early phase, which we define as within 24 h.[12,14,31] The internal and external forces generated immediately after placing a composite restoration may affect the bond effectiveness of all adhesives.[7,25] Polymerization shrinkage of the composite following light irradiation generates contraction stress in the vicinity of the composite-tooth interface. Furthermore, conducting finishing and polishing procedures immediately after composite restoration placement might generate external forces on the restorations, resulting in increased interfacial gap formation.[14] Although laboratory investigations have demonstrated that the bond strength during the early phase is lower than that after 24 h of water storage, the values tend to increase over time due to post-polymerization effects following light irradiation.[3,12,14,27,28,31] Hirokane et al[12] revealed that the enamel bond strength of universal adhesives during the early phase increased with prolonged storage time; moreover, they demonstrated an increase in the mechanical properties of the employed composite material over time. To improve the initial bond performance and long-term durability of single-step SE adhesives, adding a hydrophobic layer, applying a double layer of adhesive, or blowing with hot air have been reported to be effective.[1,8,21,23,24,30,37] In particular, applying a double layer is easy in the clinic; it has proven effective in enhancing the enamel and dentin bond durability of universal adhesives in terms of fatigue stress.[8] In addition, the double-layer application of universal adhesives might increase the enamel bond strength during the early phase.[9] However, this raises the question of whether double-layer application is also effective in enhancing dentin bond quality in the early phase.

It is probable that an adhesive layer (AL) after light irradiation in the early phase may increase its mechanical properties over time due to post-polymerization effects, as for resin-based restoration materials. However, in the case of universal adhesives, the presence of unreacted functional monomer and water, as well as solvent remnants in the cured AL might reduce the degree of conversion. In particular, the dentin bond effectiveness is likely to be more affected, as dentin is a water-rich tissue compared with enamel. However, information on the relationship between the dentin bond strength of universal adhesives within 24 h of water storage and the degree of polymerization of the cured AL over time is scarce.

This study aimed to determine the changes in the dentin bond strengths of universal adhesives during the early phase and explain the relationship between the post-polymerization effects in the cured AL and the dentin bond strength. Because the surface hardness of a composite material is thought to be correlated with its mechanical properties, abrasion resistance, and degree of conversion, we measured the surface hardness as a proxy for the mechanical properties of the cured AL.[6,11,26] Furthermore, we aimed to determine whether a double-layer application improved the dentin bond performance. The null hypotheses to be tested were: 1. the early dentin bond performance of universal adhesives would not change in the course of the test period; 2. the mechanical properties of the cured AL of the universal adhesive as measured by the hardness test would not be associated with the dentin bond strength; and 3. the double-layer application technique would not increase the dentin bond strength of the universal adhesive.

MATERIALS AND METHODS

Materials Used

Table 1 presents the materials used in this study. Three universal adhesives, namely, Clearfil Universal Bond Quick (CUB, Kuraray Noritake; Tokyo, Japan), G-Premio Bond (GPB, GC; Tokyo, Japan), and Scotchbond Universal (SUB, 3M Oral Care; St Paul, MN, USA), were used. Clearfil SE Bond 2 (CSE, Kuraray Noritake), a two-step SE adhesive, was used for comparison. The Ultra-Etch (Ultradent; South Jordan, UT, USA) phosphoric-acid etchant and Clearfil AP-X (Kuraray Noritake) composite were also employed.

Specimen Preparation

Bovine incisor dentin was used as a substitute for human dentin.[40] Approximately two-thirds of the apical root structure of each tooth was removed using a diamond-impregnated disk employing a low-speed saw (Isomet Low-Speed Saw, Buehler; Lake Bluff, IL, USA). The labial surfaces were subjected to mechanical grinding and polishing (Ecomet 4

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Table 1 Materials used in this study

Code	Adhesive (Lot No)	Main component	pH	Manufacturer
CUB	Clearfil Universal Bond Quick (9T0050)	Bis-GMA, MDP, HEMA, hydrophilic amide monomer, filler, ethanol, water, NaF, photo initiators, chemical polymerization, accelerator, silane coupling agent, others	2.3	Kuraray Noritake; Tokyo, Japan
GPB	G-Premio Bond (4G0011)	MDP, 4-MET, MEPS, BHT, acetone, dimethacrylate resins, initiators, filler, water	1.5	GC; Tokyo, Japan
SUB	Scotchbond Universal (41256)	MDP, HEMA, dimethacrylate resins, Vitrebond copolymer, filler, ethanol, water, initiators, silane	2.7	3M Oral Care: St Paul, MN, USA
CSE	Clearfil SE Bond 2 (Primer: 5852494) (Adhesive: 5847004)	Primer: MDP, HEMA, water, initiators Adhesive: MDP, HEMA, bis-GMA, initiators, microfiller	2.0 (Primer)	Kuraray Noritake
Pre-etching agent		**Main component**		**Manufacturer**
Ultra-Etch (G017)		35% phosphoric acid		Ultradent; South Jordan, UT, USA
Resin composite		**Main component**		**Manufacturer**
Clearfil AP-X (380094)		Bis-GMA, TEG-DMA, silane barium glass filler, silane silica filler, silanated colloidal silica, CQ, pigments, others		Kuraray Noritake

Bis-GMA: 2,2-bis[4-(2-hydroxy-3- methacryloyloxypropoxy) phenyl] propane; MDP: 10-methacryloyloxydecyl dihydrogen phosphate; HEMA: 2-hydroxyethyl methacrylate, 4-MET: 4-methacryloyloxyethyl trimellitate; MEPS: methacryloyloxyalkyl thiophosphate methylmethacrylate; BHT: butylated hydroxytoluene; TEG-DMA: triethyleneglycol dimethacrylate, CQ: dl-camphorquinone.

Grinder Polisher, Buehler) using wet 180-grit silicon carbide (SiC) paper to create a flat dentin surface. Subsequently, the prepared tooth was mounted in a self-curing acrylic resin (Tray Resin II, Shofu; Kyoto, Japan) leaving the flattened dentin exposed. The dentin bond surfaces were polished using 240-grit SiC paper, followed by 320-grit SiC paper (Fuji Star Type DDC, Sankyo Rikagaku; Saitama, Japan), under both running water and dry conditions.

Adhesive Application Protocol

The adhesive application protocols are presented in Table 2. A total of 15 specimens were utilized in each test group to measure the shear bond strength (SBS) to dentin in the SE mode (without phosphoric acid pre-etching) or ER mode (phosphoric acid pre-etching for 15 s). The adhesives were applied (single- or double-layer application groups) to the dentin surfaces in accordance with the manufacturers' instructions. For the two-step SE adhesive CSE, the adhesive was applied the second time without priming, followed by exposure to light irradiation using a visible-light curing unit (Optilux 501, SDS Kerr; Orange, CA, USA). The light irradiance (600 mW/cm^2) of the curing unit was monitored using a dental radiometer (Model 100, Kerr).

SBS Tests

Following adhesive application to the dentin bonding sites, the specimens were clamped in a jig (Ultradent Bonding clamp, Ultradent Products). Resin composite disks were formed using plastic molds (internal diameter, 2.38 mm; height, 2.00 mm; Bonding Mold Insert, Ultradent) placed on the dentin surfaces, followed by light irradiation for 30 s. The molds were removed, and prior to SBS testing, the bonded specimens were stored in distilled water at 37°C for 5 min or 1, 6, 12, or 24 h. In this study, we defined the early stage as within 24 h of applying the resin composite restoration, and measured the values at 5 min and 1, 6, and 12 h.

The SBS to dentin was measured using the notched-edge SBS test according to ISO 29022 after every storage period.[15] A SBS Test Kit (Ultradent) was employed for this purpose, and the specimens were loaded to failure at a crosshead speed of 1.0 mm/min in a universal testing machine (Type 5500R, Instron; Canton, MA, USA). The SBSs (MPa) were calculated as the peak load at failure divided by the bonded surface area. The bonded dentin surfaces and resin composite disks were observed under an optical microscope (SZH-131, Olympus; Tokyo, Japan) at a magnification of 10X after testing to determine the bond failure mode. The failure modes were classified based on the percentage of the substrate area of the debonded specimen. If more than 80% of the adherent area was occupied by adhesive, composite, or dentin, the failure mode was classified as adhesive, cohesive failure in composite, or cohesive failure in dentin, respectively. Other failure patterns, such as partially adhesive and partially cohesive, were classified as mixed.

Knoop Hardness Number of the Tested Adhesives

From each group, 12 flat dentin specimens were prepared as described for the bond strength test. To define the bonding area and the AL thickness, a piece of adhesive tape with a hole (internal diameter, 6 mm; thickness, 300 µm)

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Table 2 Application protocol for pre-etching and the tested adhesives

Etching mode	Pre-etching protocol
SE (self-etch)	Phosphoric acid pre-etching was not performed.
ER (etch&rinse)	Dentin surface was phosphoric acid etched for 15 s. Etched surface was rinsed with water for 15 s (three-way dental syringe) and air dried.
Adhesive	Adhesive application protocol
CUB	Single layer: Adhesive was applied to air-dried dentin surface for 10 s and then medium air pressure was applied over the liquid adhesive for 5 s or until the adhesive no longer moved and the solvent had completely evaporated. Light irradiated for 10 s.
	Double layer: The above application procedure was performed twice, applying the second coat of adhesive immediately after completing light irradiation.
GPB	Single layer: Adhesive was applied to air-dried dentin surface and immediately a strong stream of air applied over the liquid adhesive for 5 s or until the adhesive no longer moved and the solvent had completely evaporated. Light irradiated for 10 s.
	Double layer: The above application procedure was performed twice, applying the second coat of adhesive immediately after completing light irradiation.
SUB	Single layer: Adhesive was applied to air-dried dentin surface (not desiccated) with rubbing motion for 20 s and then medium air pressure applied to surface for 5 s. Light irradiated for 10 s.
	Double layer: The above application procedure was performed twice, applying the second coat of adhesive immediately after completing light irradiation.
CSE	Single layer: Primer was applied to air-dried dentin surfaces for 20 s followed by medium air pressure for 5s. Adhesive was then applied to primed surfaces and was air thinned gently. Adhesive was light irradiated for 10 s.
	Double layer: The primer was applied once, followed by two applications of adhesive, as above. Applying the second coat of adhesive began immediately after completing light irradiation.

was attached to the dentin surface, and the adhesives were applied in a single layer in SE mode according to the manufacturers' instructions, apart from GPB. Mild air blowing was applied to the adhesive to maintain the standardized adhesive thickness of GPB. The AL was covered with a piece of transparent matrix tape (Matrix Tape and Dispenser; 3M Oral Care) and light irradiated for 10 s. Following the removal of the matrix tape, the specimens were stored at 100% humidity and 37°C for 5 min or 1, 6, 12, or 24 h prior to the measurement of the Knoop hardness number (KHN). The KHN was obtained from the indentation following the application of a 98.07-mN load for 5 s using a microhardness tester (HMV-2; Shimadzu; Kyoto, Japan). The distance between the indentations was approximately 0.5 mm. Three measurements per specimen were obtained from various locations, and the mean values were calculated.

Scanning Electron Microscopy (SEM) Observations

Representative composite-dentin interfaces from each group and fracture site on the composite side following SBS testing were observed via field-emission SEM (ERA-8800FE, Elionix; Tokyo, Japan). To examine the morphological features of the composite-dentin interface, bonded specimens with single- or double-layer application were embedded in epoxy resin (Epon 812, Nisshin EM; Tokyo, Japan) and longitudinally sectioned using a low-speed saw (Isomet Low-Speed Saw, Buehler). Then, the sectioned surfaces were polished to a high gloss using abrasive disks (Fuji Star Type DDC), followed by the use of diamond pastes down to a particle size of 0.25 µm (DP-Paste, Struers; Ballerup, Denmark). All the SEM specimens were dehydrated in ascending grades of tert-butyl alcohol and were then transferred from the final 100% bath to a freeze dryer (Model ID-3, Elionix) for 30 min. Subsequently, the specimens were subjected to argon-ion beam etching (EIS-200ER, Elionix) for 40 s, with the ion beam (accelerating voltage, 1.0 kV; ion current density, 0.4 mA/cm^2) directed perpendicular to the polished surfaces. Finally, the fracture sites and composite-dentin interface specimens were coated in a vacuum evaporator (Quick Coater, Type SC-701, Sanyu Electron; Tokyo, Japan) with a thin gold film to increase the conductivity of the specimens. The SEM observations were performed at an operating voltage of 10 kV.

Statistical Analysis

A statistical power analysis (G Power calculator) indicated that at least 12 specimens were required to effectively measure SBS in each cured AL group, and 10 specimens were required for the KHN test. The parameters employed were $f = 0.25$, $\alpha = 0.05$, $\beta = 0.2$, and power = 0.8. Thus, we performed the experiment using 15 specimens for the SBS and 12 specimens for KHN tests. Post-hoc power tests were performed on the gathered data using a statistical software system (Sigma Plot Version 13, Systat Software; Chicago, IL, USA). These tests indicated that the sample size was adequate.

The SBS data for each group were tested for homogeneity of variance (Bartlett's test) and normal distribution (Kolmogorov-Smirnov test). Three-way ANOVA, followed by Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$), was

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Table 3 Influence of adhesive application on early dentin bond strength (SE mode)

	5 min		1 h		6 h		12 h		24 h	
	Single layer	Double layer	Single layer	Double layer	Single layer	Double layer	Single layer	Double layer	Single layer	Double layer
CUB	16.5 (1.7)^{bE} [49.4%]	23.6 (2.2)^{cC} [70.7%]	19.7 (1.2)^{cD} [59.0%]	23.5 (3.0)^{cC} [70.4%]	22.3 (4.2)^{cCD} [66.8%]	30.4 (3.3)^{cB} [91.0%]	26.1 (3.1)^{cC} [78.1%]	31.1 (3.4)^{cB} [93.1%]	33.4 (2.8)^{cB} [100%]	37.0 (2.2)bA [110.8%]
GPB	17.6 (1.9)^{bE} [57.7%]	22.4 (1.0)^{cD} [73.4%]	23.9 (2.6)^{bD} [78.4%]	27.4 (3.2)^{bBC} [89.8%]	25.0 (3.9)^{bcCD} [82.0%]	28.4 (1.4)^{cBC} [93.1%]	28.2 (3.2)^{bcBC} [92.5%]	30.4 (1.4)^{cAB} [99.7%]	30.5 (4.1)^{cAB} [100%]	33.3 (3.5)^{cA} [109.2%]
SUB	16.2 (2.0)^{bD} [43.0%]	28.4 (1.9)^{bB} [75.3%]	20.0 (3.0)^{cC} [53.1%]	30.1 (4.1)^{bB} [79.8%]	27.4 (4.7)^{bB} [72.7%]	36.6 (2.5)^{bA} [97.1%]	31.6 (4.5)^{bB} [83.8%]	37.6 (3.4)^{bA} [99.7%]	37.7 (5.8)^{bA} [100%]	39.3 (2.9)^{bA} [104.2%]
CSE	24.2 (4.3)^{aE} [51.6%]	34.2 (1.5)^{aD} [72.9%]	26.9 (2.9)^{aE} [57.3%]	35.3 (3.1)^{aD} [75.3%]	37.4 (2.1)^{aD} [79.7%]	43.0 (3.0)^{aC} [91.7%]	43.9 (3.8)^{aBC} [93.2%]	44.3 (3.8)^{aBC} [94.5%]	46.9 (2.2)^{aAB} [100%]	49.1 (2.8)^{aA} [104.7%]

n = 15, mean (SD) in MPa. Same superscript lower-case letters in columns indicate no difference at the 5% significance level. Same superscript upper-case letters in rows indicate no difference at the 5% significance level. Values in parentheses indicate standard deviation. Percentage values in brackets indicate strength relative to single-layer strength at 24 h.

Table 4 Influence of adhesive application on early dentin bond strength (ER mode)

	5 min		1 h		6 h		12 h		24 h	
	Single layer	Double layer	Single layer	Double layer	Single layer	Double layer	Single layer	Double layer	Single layer	Double layer
CUB	16.1 (3.5)^{bE} [48.1%]	22.3 (3.6)^{cCD} [66.6%]	16.9 (3.4)^{cE} [50.4%]	25.7 (2.9)^{bC} [76.7%]	21.8 (2.9)^{cD} [65.1%]	29.7 (2.6)^{cB} [88.7%]	25.2 (3.0)^{cC} [75.2%]	30.7 (5.9)^{cB} [91.6%]	33.5 (3.7)^{bAB} [100%]	36.0 (2.5)^{cA} [107.5%]
GPB	19.4 (1.0)^{bE} [65.8%]	23.8 (1.5)^{cD} [80.7%]	22.4 (2.4)^{bD} [75.9%]	27.4 (2.5)^{bBC} [92.9%]	24.6 (3.2)^{bcCD} [83.4%]	27.8 (3.1)^{cB} [94.2%]	28.9 (2.6)^{bB} [98.0%]	29.1 (1.5)^{cB} [98.6%]	29.5 (3.1)^{cB} [100%]	33.0 (2.4)^{dA} [111.9%]
SUB	17.1 (3.0)^{bG} [47.8%]	28.1 (3.3)^{bE} [78.5%]	22.8 (1.6)^{bF} [63.7%]	32.6 (1.6)^{aCD} [91.1%]	27.5 (3.0)^{bE} [76.8%]	35.6 (3.9)^{bBC} [99.4%]	30.9 (2.9)^{bDE} [86.3%]	38.1 (4.2)^{bAB} [106.4%]	35.8 (4.0)^{bBC} [100%]	40.0 (2.3)^{bA} [111.7%]
CSE	25.8 (3.1)^{aE} [63.4%]	32.2 (3.6)^{aD} [79.1%]	28.6 (3.5)^{aE} [70.3%]	33.2 (3.3)^{aD} [81.6%]	33.2 (3.0)^{aD} [81.6%]	41.8 (2.9)^{aB} [102.7%]	37.9 (3.0)^{aC} [93.1%]	42.9 (3.0)^{aB} [105.4%]	40.7 (3.0)^{aBC} [100%]	45.1 (1.6)^{aA} [110.8%]

n = 15, mean (SD) in MPa. Same superscript lower-case letters in columns indicate no difference at the 5% significance level. Same superscript upper-case letters in rows indicate no difference at the 5% significance level. Values in parentheses indicate standard deviation. Percentage values in brackets indicate strength relative to single-layer strength at 24 h.

employed for data analysis. The SBS data in the SE and ER modes were separately analyzed. The analysis factors included the application technique, storage time, and adhesive that were employed. One-way ANOVA followed by Tukey's HSD test ($\alpha = 0.05$) was applied for comparisons within data subsets.

To analyze the KHN data, one-way ANOVA followed by Tukey's HSD test ($\alpha = 0.05$) was used. To reveal the relationship between SBS and KHN over time, linear regression analysis was performed for each adhesive. Statistical analysis was performed using Sigma Plot software (v 11.0, SPSS; Chicago, IL, USA).

RESULTS

SBS

Tables 3 and 4 present the SBS results. Three-way ANOVA revealed that the adhesive application technique, storage period, and adhesive significantly influenced the SBS ($p < 0.001$), regardless of the etching mode. Moreover, all the pairwise interactions between the factors were significant ($p < 0.05$) in both etching modes. However, the three-way interaction between adhesive application technique, storage period, and adhesive in the ER mode was not significant ($p = 0.370$).

For SE mode (Table 3), a similar trend was observed for all adhesives: the SBS increased with prolonged storage, regardless of whether the adhesive was applied in a single or double layer. The SBS of the single-layer application in the 24-h storage group for each tested adhesive was determined to be 100%. The SBSs ranged from 49.4% to 110.8% in CUB, 57.7% to 109.2% in GPB, 43.0% to 104.2% in SUB, and 51.6% to 104.7% in CSE (Fig 1). The double-layer application groups exhibited higher SBS than did the single-layer application groups under similar storage conditions. In particular, all the adhesives in the 5-min and 1-h groups following double-layer application exhibited significantly higher SBS compared with those following a single-layer application. The two-step SE adhesive CSE exhibited significantly higher SBS than did the universal adhesives, regardless of the storage period or adhesive application.

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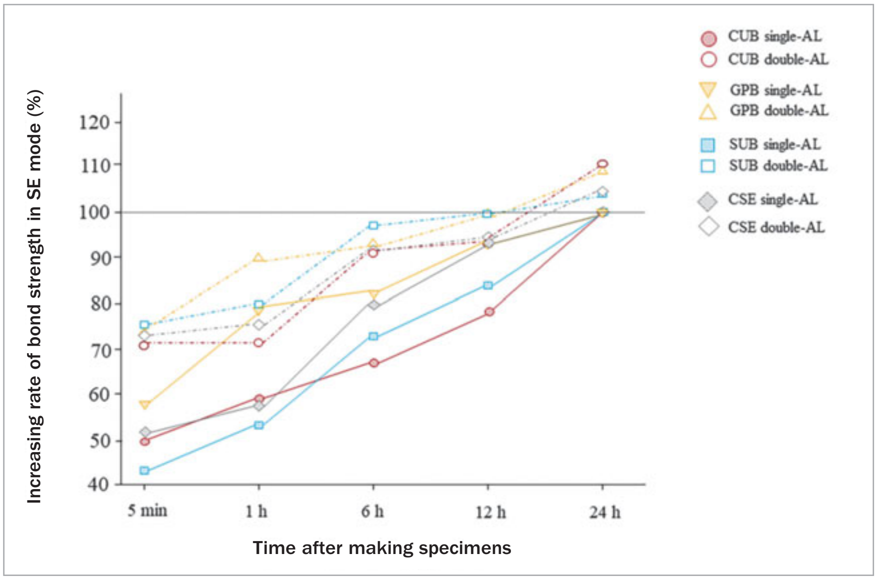

Fig 1 Changes in shear bond strength (%) in the self-etch mode. CUB: Clearfil Universal Bond Quick; GPB: G-Premio Bond; SUB: Scotchbond Universal; CSE: Clearfil SE Bond; AL: adhesive layer.

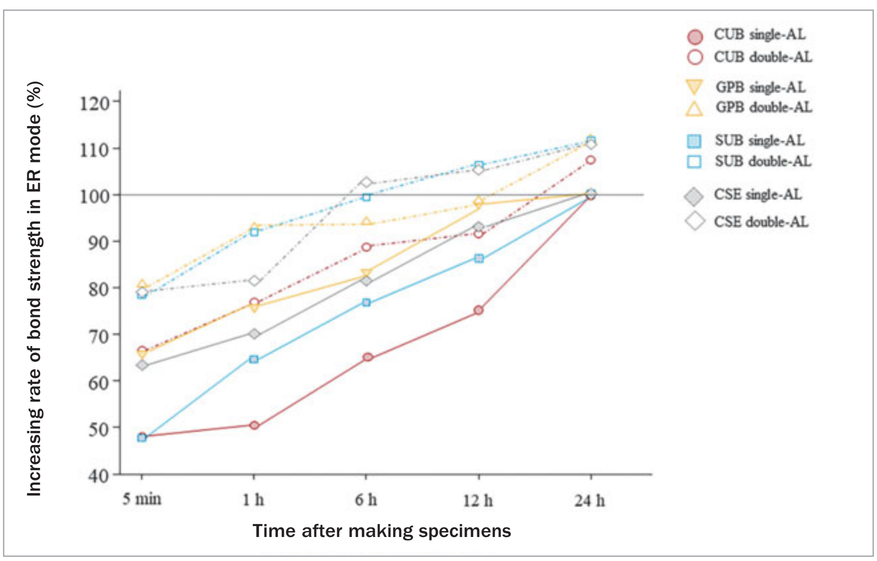

Fig 2 Changes in shear bond strength (%) in the etch-and-rinse mode. CUB: Clearfil Universal Bond Quick; GPB: G-Premio Bond; SUB: Scotchbond Universal; CSE: Clearfil SE Bond; AL: adhesive layer.

Table 5 Changes in the KHN of the adhesives over time

	5 min	1 h	6 h	12 h	24 h
CUB	0.5 (0.1)^{cE} [2.8%]	2.3 (0.6)^{cD} [13.1%]	6.4 (0.7)^{bC} [36.4%]	11.6 (0.8)^{bB} [65.9%]	17.6 (1.8)^{bA} [100%]
GPB	0.4 (0.1)^{cD} [4.2%]	0.6 (0.1)^{dD} [6.3%]	2.7 (0.2)^{dC} [28.1%]	5.2 (0.3)^{dB} [54.2%]	9.6 (1.1)^{cA} [100%]
SUB	1.7 (0.2)^{bE} [9.4%]	4.3 (0.5)^{bD} [23.9%]	6.5 (0.6)^{bC} [36.1%]	10.1 (0.5)^{cB} [56.1%]	18.0 (1.3)^{bA} [100%]
CSE	18.1 (0.4)^{aD} [70.7%]	19.9 (1.2)^{aC} [77.7%]	22.6 (0.6)^{aB} [88.3%]	25.5 (0.5)^{aA} [99.6%]	25.6 (0.5)^{aA} [100%]

Percentage values in brackets indicate KHN relative to values at 24 h. Same superscript lower-case letters in columns indicate no difference at the 5% significance level. Same superscript upper-case letter in rows indicate no difference at the 5% significance level.

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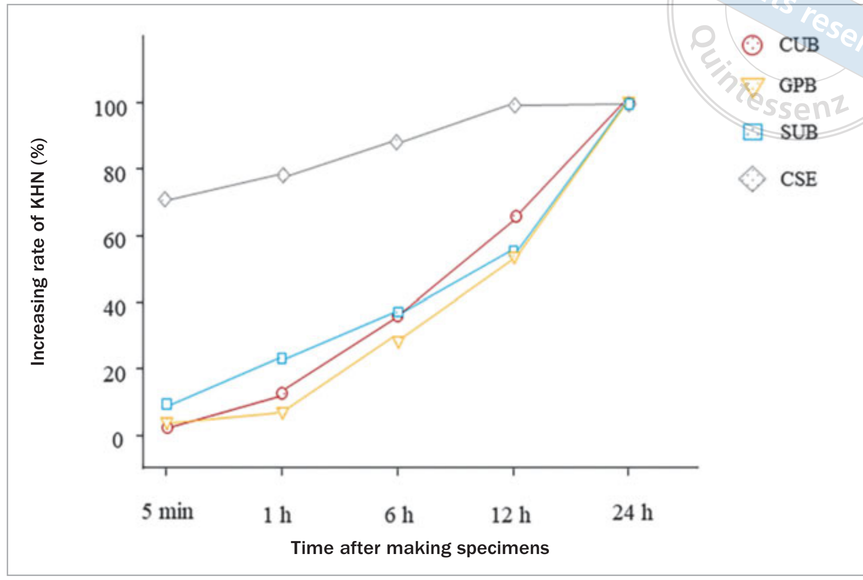
Fig 3 Changes in KHN (%) of the cured adhesive layer. CUB: Clearfil Universal Bond Quick; GPB: G-Premio Bond; SUB: Scotchbond Universal; CSE: Clearfil SE Bond.



For ER mode (Table 4), the SBS of all adhesives increased with prolonged storage, regardless of the adhesive application method. After defining the SBS of the single-layer application in the 24-h storage group for each tested adhesive as 100%, the SBSs in the CUB, GPB, SUB, and CSE groups ranged from 48.1% to 107.5%, 65.8% to 111.9%, 47.8% to111.7%, and 63.4% to 110.8%, respectively (Fig 2). The double-layer application groups exhibited higher SBSs than did the single-layer application groups under similar storage conditions, as also observed in the SE mode. Moreover, the double-layer application groups demonstrated significantly higher SBSs than did the single-layer application groups at all storage periods, except for those at 12 h in GPB and 24 h in CUB. The two-step SE adhesive CSE exhibited significantly higher SBSs compared with the universal adhesives, regardless of the storage time or application technique, as observed in the SE mode.

Failure Mode

For each adhesive, the predominant failure mode was adhesive in both the SE and ER modes in the single-layer application groups. However, the mixed failure and cohesive failure proportions in dentin increased with lengthening of the storage period in the double-layer application groups, except for the GPB specimens. In the case of the two-step SE adhesive, CSE, the mixed and cohesive failure proportions in dentin were higher compared with those of the universal adhesives, and in both SE and ER modes, these proportions increased with prolonged storage periods.

KHN of Adhesives

The changes in the KHN of the tested adhesives over time are presented in Table 5. The tested adhesives exhibited an increase in the KHN with prolonged storage. After defining the KHN in the 24-h storage group for each tested adhesive

Table 6 Linear regression analysis between SBS and KHN over time with single application in SE mode

	R	R_f^2	SEE	p-value	Regression equations
CUB	0.991	0.976	1.006	0.001	16.52 + 0.922*KHN
GPB	0.866	0.666	2.836	0.058	20.93 + 1.111*KHN
SUB	0.959	0.893	2.842	0.010	15.91 + 1.314*KHN
CSE	0.990	0.974	1.637	0.001	-30.79 + 2.983*KHN

R: correlation coefficient; R_f^2: adjusted determination coefficient;SEE: standard error of estimate; explanatory variable: SBS; response variable: KHN.

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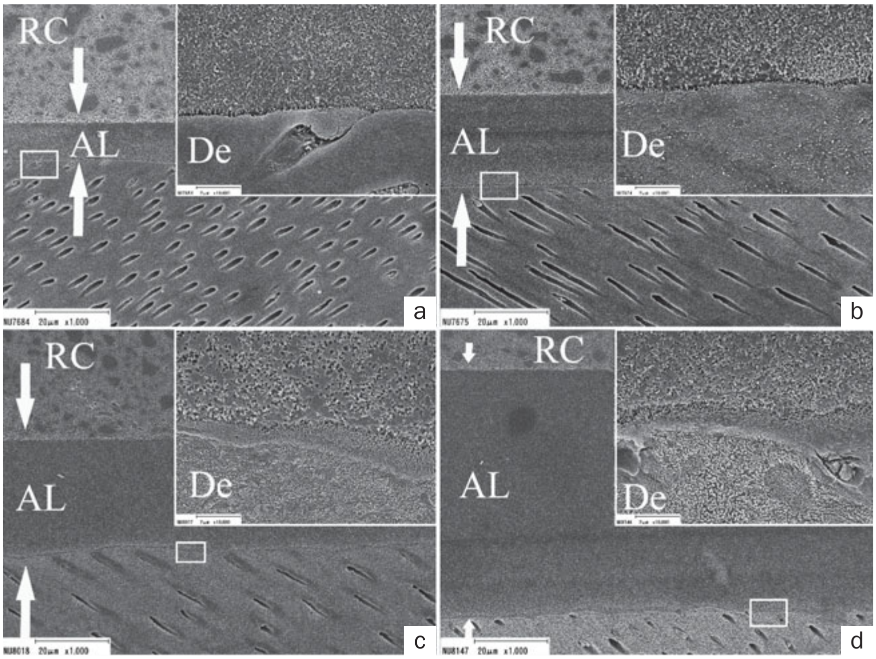

Fig 4 Representative SEM images of the composite-dentin interface of SUB and CSE in the SE mode. a. SUB with single-layer application in SE mode at magnifications of 1000X and 20,000X; b. SUB with double-layer application in SE mode at magnifications of 1000X and 20,000X; c. CSE with single-layer application in SE mode at magnifications of 1000X and 20,000X. RC: resin composite; AL: adhesive layer; De: dentin; d. CSE with double-layer application in ER mode at magnifications of 1000X and 20,000X.

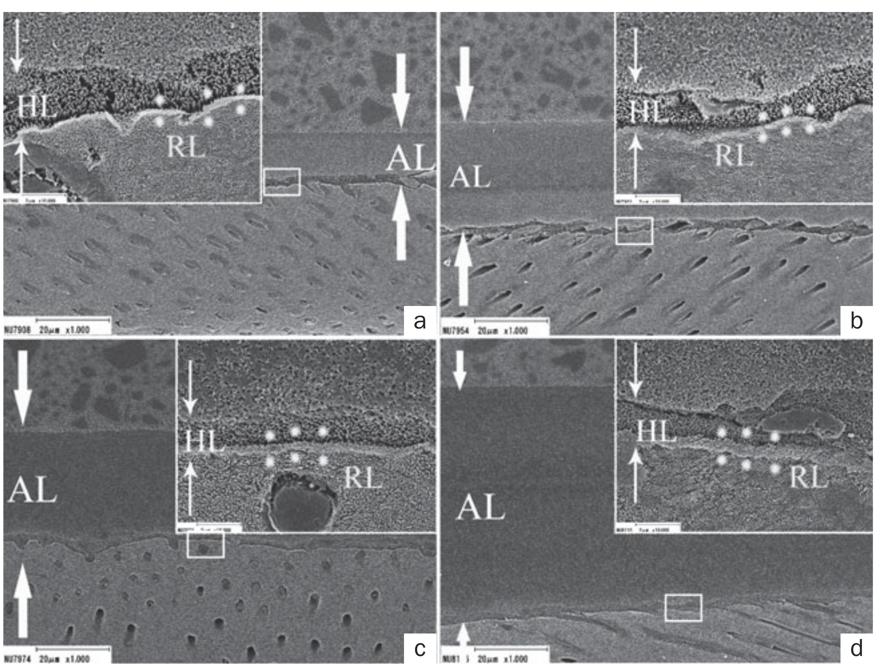

Fig 5 Representative SEM images of the composite-dentin interface of SUB and CSE in ER mode. a. SUB with single-layer application in ER mode at magnifications of 1000X and 20,000X; b. SUB with double-layer application in ER mode at magnifications of 1000X and 20,000X. C. CSE with single-layer application in ER mode at magnifications of 1000X and 20,000X; d. CSE with double-layer application in ER mode at magnifications of 1000X and 20,000X; AL: adhesive layer; HL: hybrid layer; RL: reaction layer.

as 100%, the values ranged from 2.8% to 65.9% in CUB, 4.2% to 54.2% in GPB, 9.4% to 56.1% in SUB, and 70.7% to 99.6% in CSE (Fig 3). The universal adhesives exhibited significantly lower KHN at the 12-h storage period than with the 24-h storage period. However, in the case of CSE, no significant difference in KHN was observed between the 12-h and 24-h storage groups. Furthermore, the CSE group exhibited significantly higher KHN compared with the other universal adhesives, regardless of the storage time.

Linear Regression Analysis Between SBS and KHN over Time

The results of linear regression analysis are presented in Table 6. The correlation coefficient (R) for the adhesives ranged from 0.866 to 0.991, and all the adhesives showed a strong correlation between SBS and KHN over time. The adjusted determination coefficient (R_f^2) of the adhesives ranged from 0.666 to 0.976. Although the SBS of GPB was found to have no significant linear relationship with KHN

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($p = 0.058$), the SBSs of the other adhesives showed a significant linear relationship with KHN ($p < 0.05$).

SEM Observations

Figures 4 and 5 depict representative SEM images of composite-dentin interfaces following argon-ion etching. Although AL thickness was similar between the SE and ER modes for each adhesive, it differed between the single-layer and double-layer application groups. The universal adhesives exhibited similar AL thicknesses of approximately 10 µm in the single-layer application groups and 20 µm in the double-layer application groups (Figs 4a, 4b, 5a, 5b). Conversely, the CSE AL thickness was 30–40 µm in the single-layer application group and 50–60 µm in the double-layer application group (Figs 4c, 4d, 5c, 5d).

At the composite-dentin interface, all the adhesives exhibited excellent adaptation between the dentin substrate and adhesive, regardless of the adhesive application or etching mode. The ultrastructure in the vicinity of the interfaces differed between the SE and ER modes. Approximately 1–2 µm of the hybrid layer (indicated by yellow arrows) was observed in the ER mode (Fig 5), but not the SE mode. Moreover, a thin, high-density reaction layer (indicated by the blue stars) was observed below the hybrid layer in the ER mode for all the tested adhesives (Fig 5).

Representative SEM images of the resin side of the debonded specimens are presented in Figs 6 and 7. The failure pattern was dependent on the adhesive employed, application method, storage period, and etching mode. Numerous voids were observed at a lower magnification in both the SE and ER modes in the single-layer application and 5-min storage group (Figs 6a and 6b). Conversely, cracks and cleavages in the adhesive were clearly observed at a higher magnification in both etching modes in the double-layer application and 5-min storage group (Figs 6c and 6d). Cracks, cleavages, and attached dentin fragments on the adhesive were observed in both etching modes in the single-layer application groups that were stored for 24 h (Figs 6e and 6f). Conversely, in the double-layer application groups, obvious cohesive failure was observed in dentin in the SE mode, and clear evidence of resin tags was observed in the ER mode (Figs 6 g and 6h).

Although the failure patterns of the two-step SE adhesive CSE exhibited a similar trend to those of the universal adhesives, the voids on the adhesive were not observed in any of the groups. In particular, complicated failure patterns with cracks, cleavages, and clear evidence of dentin fragments were observed in the double-layer application groups after both 5-min and 24-h storage periods (Figs 7c,7d, 7 g, and 7h) and in the single-layer application groups at the 24-h storage period, regardless of the etching mode (Figs 7e and 7f). Furthermore, a clear evidence of resin tags was observed in all groups in the ER mode.

DISCUSSION

In this study, we focused on the performance of the dentin bond of universal adhesives during the early phase based on bond strength and KHN of the cured ALs at five different time points within a 24-h period. Furthermore, the influence of a double-layer application for all the materials on the effectiveness of the dentin bond was investigated. The storage period was found to significantly influence the SBS, and all the adhesives exhibited an increase in the bond strength with an increase in the storage time in both etching modes (Tables 3 and 4). Therefore, the first null hypothesis, which stated that the dentin bond performance of universal adhesives in the early phase would not change over time, was rejected. This result was in agreement with previous investigations on the dentin bond performance of SE adhesives.[12,14,31] In addition, a study on the enamel bond performance of universal adhesives during the early phase reported similar findings, in which the bond strength increased with storage time.[12]

Enhancement of the physical properties of the interface materials, such as the AL and restorative materials, due to the post-polymerization reaction is considered to be one of the reasons for the increase in the bond strength over time.[2,19,22] Regarding this phenomenon in composites, Hirokane et al[12] reported an approximately 140% increase in flexural strength and 175% increase in elastic modulus in the 24-h storage group when compared to those in the 5-min group. Therefore, the post-polymerization reaction of resin composites may be related to the increased bond strength over time during the early phase. A similar reaction may transpire in the cured AL[18] and may be more significant due to the presence of two interfaces with the tooth substrate and cured composite. Therefore, in order to understand the relationship between the mechanical properties of the AL and bond strength, we determined the KHN of the cured adhesive layer in the early phase at the same time points when the bond strength test was performed.

In general, tests for flexural, tensile, and compressive strengths are widely conducted to evaluate the mechanical properties of resin-based materials. However, evaluation of the mechanical properties of the cured AL using these experimental methods requires specimens with thicknesses in the millimeter range and measurable stiffness, which are time consuming to prepare and clinically unrealistic. In addition, it is impossible to measure the change in mechanical properties over time using the same specimen. Universal adhesives contain a large amount of water and solvent, which may cause specimen deformation when removed from a mold. Conversely, microhardness tests can be employed for measurement within a limited area, and the same specimen can be utilized at different measuring times.[11] Moreover, microhardness values have been strongly correlated with the degree of conversion of the resin composite.[6,11] Although there are some limitations to measuring a cured adhesive layer with KHN testing, KHN testing can be performed within the constraints of the protocol of the present study, and the testing procedure is close to real bonding procedures. From the SEM observation, the thickness of the cured layers in the case of the universal adhesives was approximately 10 µm, and that of the CSE was approximately 30–40 µm. Conversely, the thickness of the cured AL for

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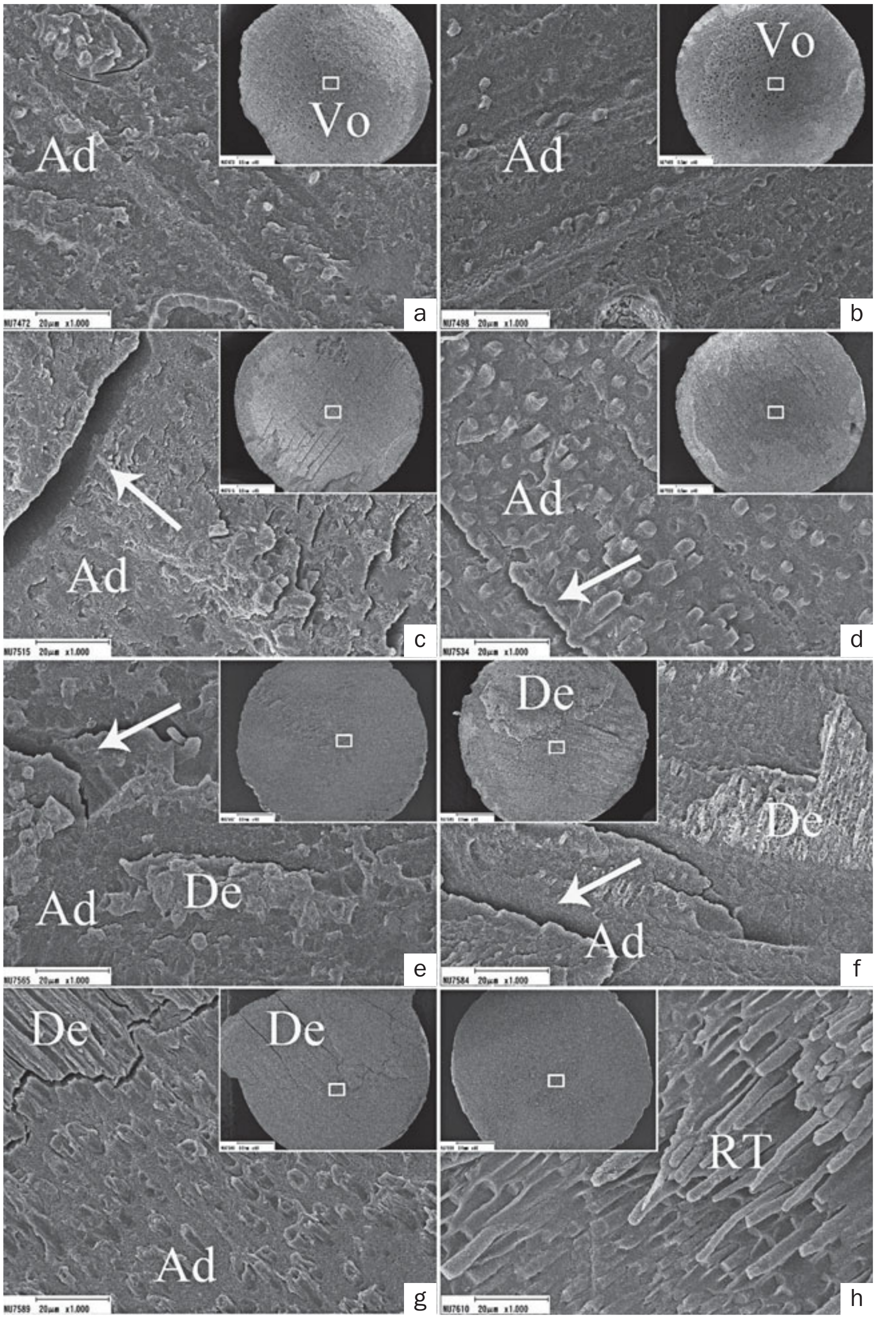

Fig 6 Representative debonded failure sites in SUB. a. Single-layer application in SE mode at 5 min (40X and 1000X); b. double-layer application in SE mode at 5 min (40X and 1000X); c. single-layer application in SE mode at 24 h (40X and 1000X); d. double-layer application in SE mode at 24 h (40X and 1000X); e. single-layer application in ER mode at 5 min (40X and 1000X); f. double-layer application in ER mode at 5 min (40X and 1000X); g. single-layer application in ER mode at 24 h (40X and 1000X); h. double-layer application in ER mode at 24 h (40X and 1000X). Ad: adhesive; De: dentin; RT: resin tag; Vo: void. The arrows indicate cracks and cleavages.

KHN testing was approximately 300 µm. Although this is not a negligible difference between the in vitro conditions and the conditions of composite restorations in clinical situations, a cured adhesive layer at least 300 µm thick was needed to avoid results reflecting the background dentin hardness. Furthermore, the GPB manufacturers must include air blowing with a strong stream of air in their instruction manual. In this study, air blowing on GPB was performed in a similar manner to that for the other adhesives to maintain a constant AL thickness.

The tested adhesives exhibited an increase in KHN with prolonged storage. In addition, in the linear regression analysis between SBS and KHN, all the adhesives showed a strong correlation between SBS and KHN and had a rather high adjusted determination coefficient. Therefore, the second null hypothesis, which states that the mechanical properties of the cured AL of universal adhesives measured through KHN would not be associated with dentin bond strength, was rejected. Takahashi et al[33] found a strong positive correlation between the dentin microtensile

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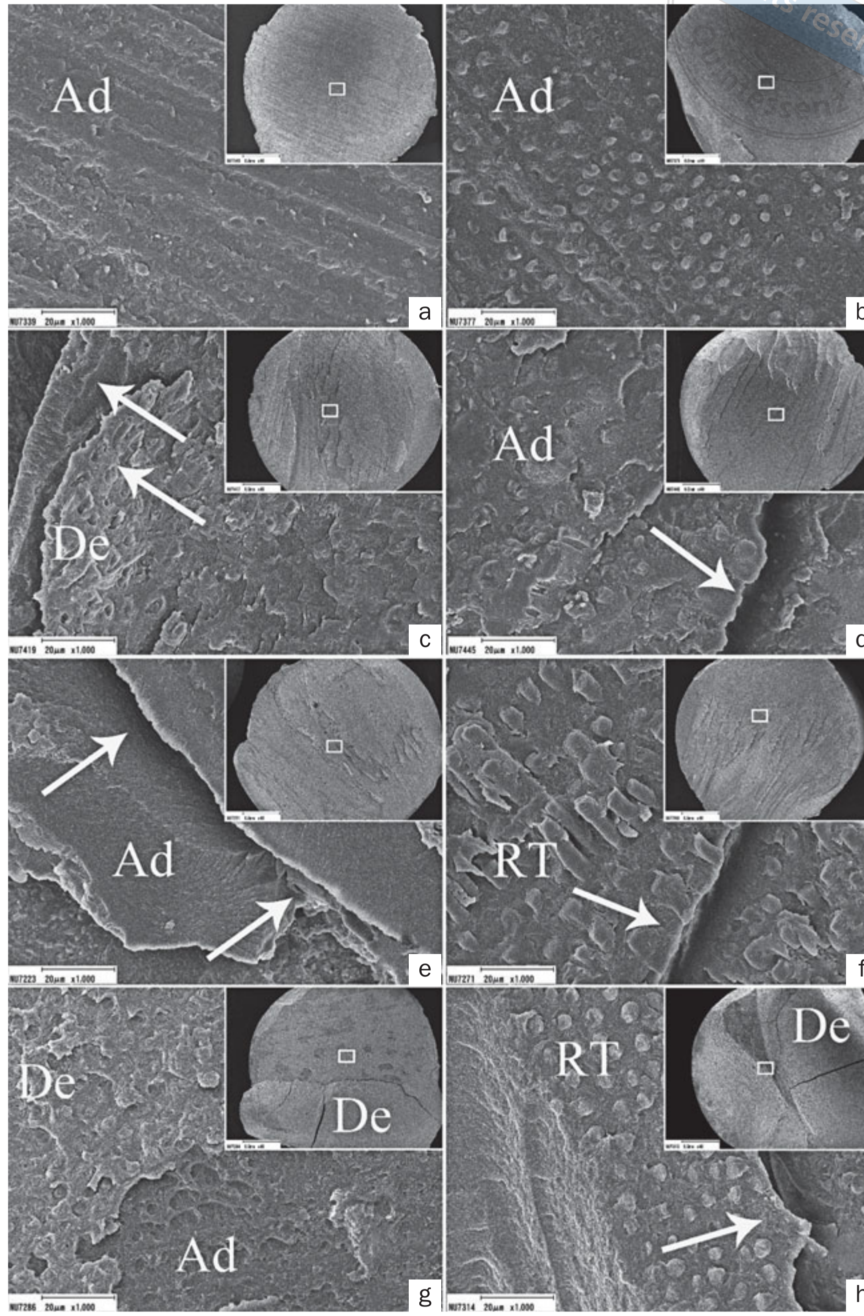

Fig 7 Representative debonded failure sites in CSE. a. Single-layer application in SE mode at 5 min (magnification 40X and 1000X); b. double-layer application in SE mode at 5 min (40X and 1000X). c. single-layer application in SE mode at 24 h (40X and 1000X). d. double-layer application in SE mode at 24 h (40X and 1000X); e. single-layer application in ER mode at 5 min (40X and 1000X); f. double-layer application in ER mode at 5 min (40X and 1000X); g. single-layer application in ER mode at 24 h (40X and 1000X); h. double-layer application in ER mode at 24 h (40X and 1000X). Ad: adhesive; De: dentin; RT: resin tag. The arrows indicate cracks and cleavages.

bond strengths (μTBS) of two-step SE adhesives and the ultimate microtensile strength of the cured adhesives. Furthermore, Hass et al[10] reported that the ultimate tensile strengths of single-step SE adhesives are correlated with their dentin bond strengths. Taken together, these results and the findings of the present study indicate that dentin bond strength is closely related to the mechanical properties of the cured AL; thus, the enhancement of these mechanical properties may increase the bond strength over time during the early phase. The trend in the increase in KHN was different between the universal adhesives and CSE. Although the KHN of the universal adhesives at 5 min did not reach even 10% of that at 24 h, the KHN of CSE at 5 min reached 70% of that at 24 h. In addition, the KHN of the CSE was significantly higher than those of the other universal adhesives, regardless of the storage time. Suh et al[32] indicated that the SE adhesives take a relatively long time to achieve an adequate degree of conversion, due to their nature. Single-step universal adhesives contain water and solvents to ionize the functional monomers and to fa-

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cilitate resin monomer infiltration.[38] The low-pH environment and remaining water and solvent in the cured AL might hinder the post-curing effect in universal adhesives and delay its hardening when compared with CSE.

Another purpose of the present study was to investigate the effect of the double-layer application technique of universal adhesives on the dentin bond strength during the early phase. Most of the adhesives exhibited significantly higher SBS when applied as a double layer, compared with those in the single-layer application groups, regardless of the etching mode (Figs 1 and 2). Therefore, the third null hypothesis, which states that double-layer application would not increase the early dentin bond strength of universal adhesives (Tables 3 and 4), was rejected. This result was in agreement with prior studies, which investigated the effectiveness of a double layer or hydrophobic layer application of SE adhesives.[4,8] From the SEM images of the composite-dentin interfaces, it was determined that although the thickness of the cured adhesive was different between the single- and double-layer application groups, the morphological features in the vicinity of the interface between the dentin substrate and AL were not different. In particular, the possibility of a chemical interaction evident in the reaction layer in the ER mode (Fig 5) did not seem to cause any difference in the thickness and density between the single- and double-layer application groups.[16,35] Thus, the primary factor in the effectiveness of a double-layer application may be associated with an increase in the thickness of the cured adhesive. The stress distribution at the interface might be better due to the increase in the size of the plastic zone and improved elasticity.[36,39] The SEM images of the failure sites of the debonded specimens may support this inference; complicated failure patterns with cracks, cleavages, and attached dentin fragments were more clearly observed in the double-layer application groups than in the single-layer application groups in both etching modes. Conversely, Lodovici et al[17] reported that double-layer application did not reduce the damage caused by thermal and mechanical cycling in the three-step ER adhesive and two-step SE adhesive. Excessive AL thickness may serve as a scaffold for degradation because of thermal changes and mechanical stress. The double ALs in their study were approximately 4–10 times thicker than those of the present study. Hashimoto et al[9] investigated the influence of consecutive coats of ER adhesives on the dentin bond strength with the use of the µ-TBS test. They demonstrated that the bond strengths increased with each coating up to four coats, and no increase was observed after more than four coats. Therefore, an optimum thickness for the AL of each adhesive may be likely; further investigations are necessary to evaluate the optimal thickness for this technique.

Although most of the previous studies on add-on adhesive effects have determined the bond strength after a 24-h storage period, the current study aimed to evaluate the bond strength at five time points within a 24-h period (Figs 1 and 2). The specimens in the 5-min groups exhibited a higher rate of increase in dentin bond strength compared with those of the other storage periods in both etching modes. In clinical situations, the bond effectiveness of an adhesive may weaken in the early phase because of the internal and external forces that arise following placement of the composite restoration. Internal forces from contraction stress are thought to occur immediately following light irradiation of the composite restoration. External forces may be generated due to the removal of the matrix and the finishing and polishing procedures during the early phase. Therefore, double-layer application for universal adhesives may have additional clinical benefits, as it may enable the composite-dentin interface to withstand the internal and external forces during the early phase following placement of the composite restoration.

CONCLUSION

The results of this study indicated that the adhesive application technique, storage period, and adhesive employed significantly influenced the dentin bond strength, regardless of the etching mode. The SBSs of all the adhesives increased over the 24-h storage period in both the adhesive application techniques and etching modes. The double-layer application technique is effective in initially increasing the dentin bond strengths of universal adhesives, and could therefore potentially improve the stability of the composite-dentin interface in the early phase after placing composite restoration.

ACKNOWLEDGEMENTS

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Clinical relevance: For universal adhesives, double-layer application may be helpful to enhance the stability of the composite-dentin interface in the early phase after placement of composite restorations.