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Original Article

Bond strength of a resin-based composite to aged Biodentine using different adhesive strategies

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Abstract

Aim: The objective of the present study was to evaluate the microshear bond strength (µSBS) of resin composite to Biodentine, which is a bioactive dentin substitute after aging periods using different adhesive systems.

Material and Methods: One hundred and eighty cylindrical shaped blocks with a cavity of 5-mm in diameter and 2-mm in depth were fabricated from a self-cured acrylic. The Biodentine was mixed and loaded into the cavities. The prepared specimens were divided into 3 groups in accordance with aging periods (12-min; 24-h; 1-week). After the aging, each group was allocated to 6 subgroups: subgroup 1, no adhesive (control); subgroup 2, two-step etch-and-rinse adhesive; subgroup 3, two-step self-etch adhesive; subgroup 4, one-step self-etch adhesive; subgroup 5, universal adhesive in self-etch mode; subgroup 6, universal adhesive in etch-and-rinse mode. After adhesive application, resin composite cylinder was applied over Biodentine surface to assess µSBS. The µSBS was evaluated using a µSBS tester and failure modes were at 30× magnification. Statistical analyses were performed using two-way ANOVA and Tukey tests.

Results: Biodentine shows lower µSBS at the 12-min. There were no considerable differences between 24-h and 1-week aging periods. Universal adhesive exhibited the highest bond strength values.

Conclusion: Twenty-four hour waiting for the final restoration after the Biodentine placement could be useful to obtain better bond strength. In addition, highest µSBS values were detected for the universal adhesive irrespective to the application mode.

Keywords: Biodentine;dental composites; dental adhesives; self-etch adhesives; microshear bond strength

INTRODUCTION

The main goal of the restorative treatment is the protection and preservation of healthy dental tissues. For this purpose, protective liners and bases are placed under the restorative material, in some cases (1). The most popular materials are calcium hydroxide-based and calcium oxide-based ones. Unfortunately, these are materials that weakening the restoration by dissolving over time and causing necrotic layer formation by raising the pH (2). Mineral trioxide aggregate (MTA), a bioceramics material, which has gained tremendous attention recently (3), has advantages, such as regenerative characteristics (4), setting in humid environments (5), low solubility after setting (6), induction of reparative dentin formation (5). However, MTA has a number of drawbacks, such as long setting time, high solubility during setting, difficulty in use, and discoloration after application (7). Moreover, etch-and-rinse procedures cannot be performed due to the probable dislodgement of unset MTA or adverse effects on the physical properties of the MTA (8,9) because of its high solubility during the setting. Therefore, the completion of the final restoration is extended to the next session.

Recently, new calcium silicate-based cement, Biodentine, was introduced to overcome the shortcomings of MTA. Biodentine is a bioactive dentin substitute, which contains calcium silicates as the main component and calcium carbonate as a filler (10). The superiorities of Biodentine over MTA are its short setting time (12 min), ease of use and better physical properties (11) that provide completion of final restoration in a single appointment.

A previous study indicated a notable improvement of the bond strength between Biodentine and dentin substrate from 2-day to 1-week aging periods (12). They concluded that this finding can be related to the setting mechanism of Biodentine, which may continue for more than a

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week. The manufacturer of the Biodentine recommends restorative treatments 12 min after the mixing, which allows completion of the final restoration in the same appointment (13). However, some studies have reported that the surface roughness (Ra) of this material varies between 11.53 nm and 18.4 nm depending on the time elapsed after mixing (12-min, 45-min, and 24-h) (14,15), and increased surface hardness over time (16). Jang et al. (17) indicated 15-min of setting time for Biodentine, whereas Grech et al. (18) indicated as 45 minutes, which differs from the time recommended by the manufacturer.

There are still controversies about the optimal setting time of the Biodentine before the completion of the final restoration. Some researchers recommend performing the final restoration after at least 2 weeks, which allows fully maturation of Biodentine (19). While others suggest the possibility of the final restoration in the same appointment (20). Besides, there is also still no consensus on the usage of adhesive material and its application mode. In some studies, the etch-and-rinse mode was reported to be superior to self-etch mode (20,21), vice versa (22,23). On the contrary, the application mode was reported to be irrelevant (19,24). Therefore, the aim of this in vitro study was to investigate the microshear bond strength of Biodentine to a resin-based composite at different aging periods and with different adhesive strategies. The null hypotheses were as follows: (1) the aging period would not influence the bond strength, and (2) the adhesive strategy would not influence the bond strength.

MATERIAL and METHODS

The materials used in the present study with application strategy are presented in Table 1. A total of 180 acrylic cylinders with a central cavity (5 mm in diameter and 2 mm in depth) were prepared for the present study.

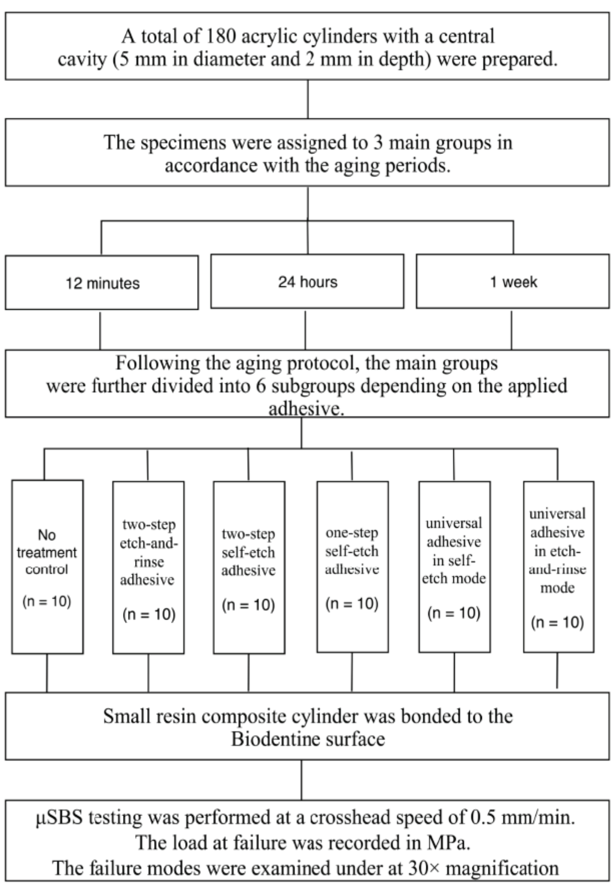
Table 1. Details and application protocols of materials used in the study.

Material	Type	Composition	Application
Biodentine® (Septodont, Saint Maur des Fosses, France)	Calcium silicate cement	Powder: Tricalcium silicate, dicalcium silicate, calcium carbonate and oxide, iron oxide and zirconium oxide. Liquid: Calcium chloride and hydrosoluble polymer.	Five doses liquid and premeasured powder mixed for 30 s with a amalgamator.
Filtek Flowable Restorative (3M ESPE, St. Paul, MN, USA)	Flowable resin-based composite	Bis-GMA, TEG-DMA, Bis-EMA, functionalized dimethacrylate polymer, silica and zirconia nanofiller.	Light-cured for 20 s.
Adper Single Bond 2 (3M ESPE, St. Paul, MN, USA)	Two-step etch-and-rinse adhesive	Bis-GMA, HEMA, dimethacrylates, polyalkenoic acid copolymer, initiator, 3-8% water, ethanol.	- Apply etchant for 15 s. - Scrub for 30 s. - Air-thin. - Light-cure
Clearfil SE Bond (Kuraray Noritake Dental Inc., Okayama, Japan)	Two-step self-etch adhesive		- Apply primer for 20 s. - Dry with air for 5 s - Apply bond for 10 s. - Apply air gently. - Light-cure for 10 s.
Adper Easy One (3M ESPE, St. Paul, MN, USA)	One-step self-etch adhesive	HEMA, Bis-GMA, Methacrylated phosphoric ester, 1,6-hexanediol dimethacrilate, methacrylate functionalized polyalkenoic, Finely dispersed bonded silica filler, ethanol, water, initiators, stabilizers. pH: 0.8-1	- Apply for 20 s. - Dry with air for 5 s. - Light cure for 10 s.
Single Bond Universal (3M ESPE, St. Paul, MN, USA)	Universal adhesive	HEMA, MDP, dimethacrylate resins, VitrebondTM copolymer, silane, filler, ethanol, water, initiators. pH: 2.7	- Apply etchant for 15 s (For etch-and-rinse mode). - Apply the adhesive and rub it in for 20 s. - Gently air dry for 5 s. - Light cure for 10 s.

Abbreviations: HEMA, 2-hydroxyethyl methacrylate; MDP, 10-methacryloyloxydecyl dihydrogen phosphate; Bis-EMA, ethoxylated bisphenol-A dimethacrylate; Bis-GMA, bisphenol A glycidyl methacrylate

A calcium silicate cement material (Biodentine; Septodont, Saint-Maur-des-Fossés, France) was mixed in accordance with the manufacturers' recommendations and then loaded into the prepared cavities. The specimens were assigned to 3 main groups in accordance with the aging periods: 12 minutes, 24 hours, and 1 week.

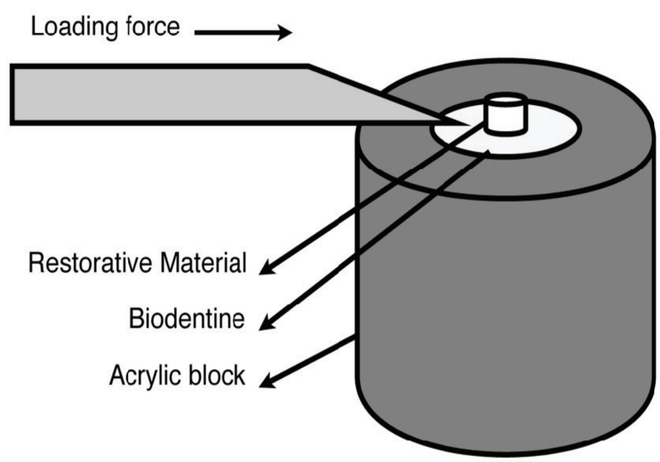
The prepared samples were kept at 37°C with 100% relative humidity for the aging protocol. Following the aging protocol, the main groups were further divided into 6 subgroups depending on the applied adhesive: subgroup 1, no adhesive application (control); subgroup 2, two-step etch-and-rinse adhesive application (SB2; Adper Single Bond 2; 3M ESPE, St. Paul, MN, USA); subgroup 3, two-step self-etch adhesive application (CSE; Clearfil SE Bond; Kuraray Noritake Dental Inc., Okayama, Japan); subgroup 4, one-step self-etch adhesive application (AEO; Adper Easy One; 3M ESPE, St. Paul, MN, USA); subgroup 5, universal adhesive (SBU; Single Bond Universal; 3M ESPE, St. Paul, MN, USA) application in self-etch mode; subgroup 6, application of SBU in etch-and-rinse mode. Study design is depicted in Figure 1.



Figur 1. Study design

All adhesives were applied over the Biodentine surface in accordance to the manufacturers' recommendations and then light-cured using a light emitting diode (LED) curing unit (Valo Grand; Ultradent, South Jordan, UT, USA). The light intensity was controlled before each specimen with a curing radiometer (Benlioglu radiometer, Benlioglu Dental, Ankara, Turkey). Following this, small transparent microtubules were carefully cut from the polyvinyl tube with 1-mm of inner diameter and 0.5-mm height with parallel ends. Each microtubule was adjusted over the Biodentine surface and carefully filled with resin-based composite (Filtek Ultimate Flowable Restorative, 3M ESPE, St. Paul, MN, USA). In this way, a small resin composite cylinder was bonded to the Biodentine surface. Light curing of the resin composite was performed using LED for 20 s. Polymerized samples were kept in 100% relative humidity at 37°C for 24 hours.

A shear force was performed to the adhesive interface using a microshear bond strength (μSBS) testing device (MOD Dental, Esetron Smart Robotechnologies, Ankara, Turkey) at a crosshead speed of 0.5 mm/minute (Figure 2) (25). The load at failure was recorded in MPa. The failure modes were observed under a stereomicroscope at 30× magnification (Olympus SZ61, Munster, Germany). Failure types were classified as adhesive failure (A), cohesive failure (CBD; failure within Biodentine or CRBC; failure within resin-based composite), or mixed failure (M).



Figur 2. Schematic illustration of the shear bond strength test set-up

The sample size was calculated to be 10 samples in each subgroup considering a confidence interval of 0.95, an effect size of 0.395, an alpha of 0.05. The effect size of 0.395 was estimated according to the previous study in which they considered $\alpha = 0.05$, $\beta = 0.8$ and 80% power of the study (25). The power of the present study was determined as 0.95% using G*Power (Heinrich Heine University Dusseldorf, Germany). Data for μSBS were analyzed using a two-way analysis of variance (ANOVA) to investigate two factors: (1) the adhesive strategy and (2) aging period on the μSBS. Pairwise analyses were tested using the Tukey HSD test. The statistical analysis software (SPSS; SPSS Inc., Chicago, IL, USA) was used and the level of significance was set as 5%.

RESULTS

Mean µSBS values are listed in Table 2. Two-way ANOVA indicated that the adhesive strategy ($F = 147.009$, $p < 0.001$) and aging period ($F = 53.075$, $p < 0.001$) exhibited significant influence on µSBS values (Table 4). In addition, significant interactions between those factors observed ($p < 0.001$). According to the pairwise analysis, the adhesive application effectively improved µSBS values compared to the control group, irrespective of the aging period ($p < 0.05$). However, the improvement obtained for universal adhesive in both etch-and-rinse and self-etch modes were significantly higher than other adhesives. The µSBS values of the etch-and-rinse and self-etch modes of the universal adhesive application were not considerably different ($p > 0.05$). No statistically significant differences were detected among the aging periods (12-min, 24-h, and 1-week) for the control group and CSE.

The bond strength values for different aging periods varied according to the adhesive material. Lowest bond strength value was obtained for SB2 and AEO at 12 min (7.30 ± 1.43 and 7.37 ± 1.27), whereas the highest one obtained for etch-and-rinse and self-etch modes of SBU at 24-h (18.82 ± 3.00 and 19.13 ± 3.25). 24-h aging period promoted slightly higher µSBS values than other periods. However, there is no significant difference between 24-h and 1-week aging periods for all adhesives. Aging for more than 12 minutes following the application of Biodentine resulted in better bond strength values for universal adhesives in both etch-rinse and self-etch modes ($p < 0.05$). According to the ANOVA conducted to analyze the data for each time period, a statistically significant difference was found among the adhesive systems (Table 3). The results of SBS from the lowest to the highest were as follows: Control < SB2 = CSE = AEO < SBU-SE = SBU-TE.

In addition, Table 2 presents the failure modes of the study groups. Cohesive failures were detected only in Biodentine not within the composite according to the stereomicroscope analysis. The highest amount of pretest and cohesive failures was observed for the control group. It is noteworthy; the cohesive failure rates were higher than the other failure modes in all subgroups.

Table 2. Mean microshear bond strength (µSBS) values (MPa), standard deviation (SD) and failure mode distribution

Adhesive	Aging Duration	µSBS (MPa)	A	M	C_{BD}	C_{RBC}	P
Control (No-Adhesive)	12 minutes	1.64 ± 0.35 ^{a}	4	0	3	-	3
	24 hours	2.00 ± 0.32 ^{a}	6	0	2	-	2
	1 week	1.82 ± 0.29 ^{a}	5	0	3	-	2
Single Bond	12 minutes	7.30 ± 1.43 ^{a}	4	2	4	-	0
	24 hours	12.16 ± 1.59 ^{b}	3	1	5	-	1
	1 week	11.36 ± 2.68 ^{b}	3	1	5	-	1
Clearfil SE Bond	12 minutes	9.97 ± 1.43 ^{a}	5	0	5	-	0
	24 hours	12.41 ± 2.44 ^{a}	2	2	5	-	1
	1 week	11.33 ± 1.86 ^{a}	4	1	4	-	1
Adper Easy One	12 minutes	7.37 ± 1.27 ^{a}	3	1	4	-	2
	24 hours	11.18 ± 1.53 ^{b}	4	1	5	-	0
	1 week	10.18 ± 1.46 ^{ba}	4	2	3	-	1
Single Bond Universal (Self- Etch Mode)	12 minutes	12.58 ± 1.64 ^{a}	3	2	4	-	1
	24 hours	19.13 ± 3.25 ^{b}	3	1	6	-	0
	1 week	16.59 ± 2.56 ^{b}	4	2	4	-	0
Single Bond Universal (Etch-and-Rinse Mode)	12 minutes	11.54 ± 1.41 ^{a}	4	1	5	-	1
	24 hours	18.82 ± 3.00 ^{b}	3	1	6	-	0
	1 week	17.78 ± 3.39 ^{b}	3	1	6	-	0

A, adhesive failure; CBD, cohesive failure within Biodentine; CRBC, cohesive failure within the resin-based composite; M, mixed failure; P, pretest failure. Means followed by same lower-case letters (among adhesive groups) are not significantly different

Table 3. Mean microshear bond strength (μSBS) values (MPa) and standard deviation (SD) by aging duration

	12 m	24 h	1 week
Control (No-Adhesive)	1.64 ± 0.35 [A]	2.00 ± 0.32 [A]	1.82 ± 0.29 [A]
Single Bond	7.30 ± 1.43 [B]	12.16 ± 1.59 [B]	11.36 ± 2.68 [B]
Clearfil SE Bond	9.97 ± 1.43 [BC]	12.41 ± 2.44 [B]	11.33 ± 1.86 [B]
Adper Easy One	7.37 ± 1.27 [B]	11.18 ± 1.53 [B]	10.18 ± 1.46 [B]
Single Bond Universal (Self- Etch Mode)	12.58 ± 1.64 [C]	19.13 ± 3.25 [C]	16.59 ± 2.56 [C]
Single Bond Universal (Etch-and-Rinse Mode)	11.54 ± 1.41 [C]	18.82 ± 3.00 [C]	17.78 ± 3.39 [C]

Mean values represented with similar uppercase letters are not significantly different ($p > 0.05$)

Table 4. Influence of adhesive application and aging duration on μSBS according to the two-way ANOVA

	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4348.949[a]	17	255.821	53.282	.000
Intercept	19098.935	1	19098.935	3977.911	.000
adhesive	3529.123	5	705.825	147.009	.000
aging	509.649	2	254.824	53.075	.000
adhesive×aging	178.652	10	17.865	3.721	.000
Error	710.585	148	4.801		
Total	25611.303	166			
Corrected Total	5059.534	165			

a. R Squared = .873 (Adjusted R Squared = .858)

DISCUSSION

The bond strength between calcium silicate cement and resin composite covering it is crucial for the longevity of the restoration (19,22). In this in vitro study, the effect of several adhesive strategies on the μSBS of resin composite to aged Biodentine substrates was investigated. The control group, which did not receive any adhesive application yielded the lowest μSBS values and caused more pretest failures. In addition, two-way ANOVA indicated that both the aging period and adhesive strategy influence the μSBS. Therefore, the first hypothesis – that the adhesive strategy would not influence the μSBS compared to the control group – was rejected. On the other hand, pairwise analyses showed that the impact of the aging period on the μSBS is adhesive strategy dependent. Significant differences among the aging periods were only detected in some of the adhesive subgroups. Thus, the second hypothesis – that the aging period would not influence the μSBS – was partially accepted.

In the present study, approximately 2 MPa of μSBS value was observed for the control group, which is similar to the previous studies (26,27). Mean μSBS values varied between 7.30 and 19.13 MPa after adhesive application. The lowest μSBS was observed for SB2 and AEO at 12 min (7.30 and 7.37), while the highest was observed for etch-and-rinse and self-etch modes of SBU at 24-h (18.82 and 19.13). There are not many studies on the bond strength between Biodentine and resin composites in the dental literature. Odabas et al. (22) investigated the bond strength between a resin composite and Biodentine at two periods (12-min and 24-h) and detected favorable bond strength values in the 24-h groups. Similar results were obtained from our data. The highest μSBS were detected in 24-h of aging groups. However, these improvements did not make a significant difference only for CSE. Therefore, it can be concluded that a 24-h postponement is necessary for the completion of the final restoration and that a 1-week postponement would not be beneficial. Many studies have reported a significant increase in the bonding strength of the composite resin to Biodentine after waiting longer than 12 minutes. Aksoy and Unal (24) aged the calcium silicate cement for 12-min, 24-, 48-, 72- and 96-h periods and found a considerable difference between 12-min and other aging periods. They also indicated that 24-h aging would be sufficient since no significant increase was observed after the 24-h, which is supporting our data. Bachoo et al. (28) reported that the crystallization of calcium silicate hydrate gel texture lasted for two more weeks, in which Biodentine reached only a superficial hardness at 12-min. However, no significant difference was observed between 24 hours and 2 weeks, in another study (29).

Self-etch adhesives have been categorized in accordance with their pH as: ultra-mild (pH > 2.5), mild (pH ≈ 2), intermediately strong (1 < pH < 2), strong (pH ≤ 1) (30). Strong self-etch adhesives provide more dissolution depth on dentin and dentin-like materials (30). The self-etch adhesives used in this study categorized as: ultra-mild, Single Bond Universal (2.7); mild, Clearfil SE Bond (2.1); and strong, Adper Easy One (0.8 - 1). It is known that resin composite applied to the Biodentine surface at 12-min with AEO had the lowest μSBS (7.60 MPa). This could be explained by AEO's acidity, which poses an aggressive character. However, the bond strength of AEO applied Biodentine improved at the end of 24-h and 1-week of aging, which may be related to the increased surface hardness of the material over time.

CSE showed a lower μSBS to the fresh Biodentine compared to the universal adhesive. CSE may have influenced the superficial gel-like amorphous structure (8), calcium silicate hydrate gel (5), and the freshly formed crystalline structure (5,30), which could delay further hydration of the cement at the superficial layers of Biodentine as the acidic primer is more aggressive (pH = 2.1) (31) than the Single Bond Universal (pH = 2.7). Nekoofar et al. (27) investigated the μSBS of CSE and a universal adhesive (All Bond Universal; Bisco Inc, Schaumburg, IL, USA) to the surface of Biodentine, and similarly reported that the universal adhesive provides better bond strength. All Bond Universal, which is an ultra-mild universal adhesive with a pH of 3.1 yields a similar bond strength as the ultra-mild universal adhesive used in our study. Accordingly, it can be suggested that adhesives with aggressive acidity would damage the Biodentine surface and would adversely affect the bond strength.

Both MTA and Biodentine materials have similar compositions containing tricalcium silicate. Moreover, because of similar hydration, low pH, such as orthophosphoric acid etching, can adversely affect the microstructure of the Biodentine by inhibiting the hydration of the tricalcium silicate. Kayahan et al. (9) suggested that mild acid etching for a short duration of time may protect crystalline structures, which promotes sufficient micromechanical interlocking.

Colak et al. (23) compared two self-etch adhesives with different functional monomers and pHs (Clearfil S3 Bond and Adper Prompt L-Pop), and they stated that the mild self-etch adhesive (Clearfil S3 Bond, pH = 2.7) provided a more convenient bond strength than the strong one (Adper Prompt L-Pop, pH = 0.8-1). Furthermore, the authors also suggested that the 10-methacryloyloxydecyl dihydrogen phosphate (MDP) functional monomer of Clearfil S3 Bond could influence the bond strength since it is capable of forming chemical bonds to calcium ions of the Biodentine, which creates chemical adhesion and promotes micromechanical attachment. However, they did not record statistically significant differences in SBS values at different aging periods. This result suggests the presence and the type of the functional monomer in the adhesives have a considerable influence on the bond strength than the acid etching or demineralization capability of the adhesive used. It should also be emphasized that the Single Bond Universal used in this study, which presented the highest μSBS, contains water and ethanol as the solvent. This may favor better wettability of Biodentine.

In the current study, adhesive application mode either etch-and-rinse or self-etch did not significantly influence the bond strength. This finding also coincides with previous studies (19,32), who found that etching mode does not markedly affect the bond strength. Hashem et al. (19) concluded that the difference between application modes can be eliminated by the surface porosity of the Biodentine. Similarly to our results, Aksoy and Unal (24) investigated several adhesives and found lower SBS values in the 12-min aging period. They also concluded that no significant differences were found between etch-and-rinse or self-etch application modes. Moreover, the Biodentine may inhibit the effect of application mode by buffering their acidity with its alkaline properties.

The findings of this study also indicated that the bond strength of the two-step self-etch adhesive and universal adhesive to 1-week aged the Biodentine substrates were lower than those measured after 24-h. This can be attributed to the setting reaction of the Biodentine continues over time, which results in decreased porosity and improved crystallinity by the hardening of the porous calcium silicate hydrate (5,33). Thus, increased hardness could adversely affect the influence of mild or ultra-mild self-etch adhesive systems on the Biodentine surface. However, no significant differences were detected for the μSBS of the etch-and-rinse adhesive system to 24-h and 1-week aged the Biodentine. On the other hand, in this study, etch-and-rinse adhesive (SB2) had higher μSBS values on 1-week aged Biodentine in comparison with the two-step self-etch adhesive, which is ultra-mild (CSE). It can be suggested that the phosphoric acid etchant may create more evident and retentive porosities on Biodentine surface that promote micromechanical interlocking in comparison to the demineralization provided by ultra-mild CSE. Differently, Nekoofar et al. (27) suggested that the maturation of the Biodentine was complete after one week since they observed no significant differences between 1-week and 1-month aged groups in etch-and-rinse mode.

In the current study, the main type of failure was presented as a cohesive failure in Biodentine, which is similar with the previous studies (24,34). In general, cohesive failure is a result of the bond strength being higher than the internal strength of the substrate or the low internal strength (35).

Biodentine is a material with poor physical properties following the initial mixing, and becomes physically sufficient with the ongoing Si-O polymerization (28). These initial low physical characteristics may explain the high rate of pretest failure encountered in the control group. Naoum et al. (36) showed that the shrinkage stress at the surface of Biodentine during polymerization of different adhesives ranged from 2.89 to 3.49 MPa. This stress may be the reason for the low bond strength and high cohesive breaks occurring in 12-min groups. Ha et al. (34) concluded that resin composite over freshly mixed Biodentine may cause cracks and fractures, especially if Biodentine applied in a thin layer, thus they suggested that covering the Biodentine with a provisional restoration and completing the final restoration in a second session would be a better approach.

There are a limited number of studies analyzing failure type of composite to the Biodentine (25,37-40). Studies without adhesive failure have been reported 72-h after the placement of the Biodentine (39). However, those findings are in contrast to those obtained in the present study. Several studies reported varying adhesive failure rates ranging between 40% to 80% 24-h after the Biodentine placement (39,40). Carretero et al. (25) evaluated the bond strength of composite resin to the Biodentine and indicated increasing cohesive failure rates with increasing aging period in line with the present study.

Since the present investigation was conducted in laboratory conditions, the main limitation of the present study is that the outcomes cannot directly interpolate to the clinical conditions. Thus, in vivo studies are necessary for better understanding. Furthermore, in order to fully understand the bonding mechanism to the Biodentine, studies are needed to investigate the surface alterations caused by adhesive strategies on the substrate.

CONCLUSION

Considering the limitations of the present in vitro study, 24-h of aging period is sufficient for adequate bond strength values to Biodentine. The 1-week aging period did not produce a significant increase. According to the findings, universal adhesive application yields better bond strength compared to other adhesive approaches.

Competing interests: The authors declare that they have no competing interest.

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Ethical approval: This study was approved by the Institutional Ethics Committee and conducted in compliance with the ethical principles according to the Declaration of Helsinki.

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