Mechanical and Chemical Changes in the Adhesive-Dentin Interface after Remineralization

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Purpose: To evaluate the remineralization effects of Bioglass 45S5 (BAG) on dentin composition, adhesive-dentin bond strength, as well as interface and diffusion zone thickness.

Materials and Methods: Dentin specimens were assigned to a control group (CG), in which the adhesive was applied following the manufacturer's instructions, and a remineralized group (RG), in which remineralization treatment was carried out by rubbing a remineralization solution (0.015 g of BAG with 1.35 ml of distilled water) on the etched dentin surface for 30 s before applying the adhesive. For bioactive analysis (n = 10), control and remineralized dentin were investigated using micro-Raman spectroscopy (mRS) and scanning electron microscopy (SEM). Stick specimens prepared with a three-step etch-and-rinse adhesive were submitted to a microtensile bond strength (μTBS) test (n = 10) after 24 h (24 h) and eight months (8 m). Micro-RS 3D-maps (n = 10) characterized the adhesive-dentin interface composition and diffusion zone thickness, and SEM images (n = 10) evaluated interface thickness. Data were analyzed using Student's t-test or two-way ANOVA and Tukey-Kramer's post-hoc test (α = 0.05).

Results: Remineralization treatment increased the mineral content of dentin. Mean μTBSs were statistically different at 24 h, with RG higher than CG; however, this difference was not significant at 8 m. When the adhesive was applied on remineralized dentin, its penetration was reduced, its physical interaction with phosphate was improved, and its degree of conversion increased. The diffusion zone in the CG did not differ from that of the RG, and interface thickness values of the CG did not differ from that of the RG.

Conclusion: Remineralization treatment promoted mineral growth on the dentin surface, improved the interaction of dentin with adhesive monomers, and consequently resulted in higher immediate bond strengths.

Keywords: adhesive-dentin interface, bioglass, bond strength, dentin remineralization, etch-and-rinse adhesives, Raman spectroscopy.

J Adhes Dent 2020; 22: 297–309.
doi: 10.3290/j.jad.a44553

Submitted for publication: 03.05.18; accepted for publication: 18.12.19

Adhesive-dentin bond longevity is still a challenge in clinical restorative dentistry. When compared to amalgam restorations, composite resin fillings have twice the failure rate and half the clinical lifetime.[29] Three-step etch-and-rinse adhesives are considered the gold standard due to their higher mechanical properties and better stability over time.[8,52] The hybrid layer created by etch-and-rinse adhesives depends on the etching step, which is known to promote both cleaning of the prepared surface (smear layer removal) and superficial dentin demineralization.[9] As a consequence, the collagen matrix is exposed for infiltration of the adhesive monomers.[3] Current literature shows that the ideal hybrid layer, a continuous 3D polymer-collagen network, is not achieved because monomers cannot diffuse into the 5–10 μm of demineralized dentin tissue, leaving collagen fibrils unprotected.[31,43] Loss of integrity of the adhesive-dentin interface has been attributed to hybrid layer failure caused by both water sorption-induced hydrolysis of the hydrophilic adhesive resin components[17] and collagen fibril degradation through endogenous metalloproteinase matrices (MMPs) activated by acidic conditioning.[32]

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Self-etch adhesives were developed to eliminate the problems resulting from the acid-conditioning step of etch-and-rinse adhesives.[44] The use of mild self-etching acidic monomers is known to reduce dentin demineralization, thereby avoiding a low diffusion rate of adhesive monomers, although a deficient infiltration area is still found at the base of the hybrid layer.[43] Considering that the mechanical properties of three-step etch-and-rinse adhesives are superior to those of self-etch adhesives,[8] some clinical strategies have been developed to protect collagen fibrils and improve the stability of the hybrid layer created by etch-and-rinse adhesives. Chlorhexidine is the most frequently used MMP inhibitor,[15] because it minimizes immediate degradation of the hybrid layer when applied on the dentin surface after acid etching.[5,6] However, a beneficial outcome has not been reproducible after longer periods of aging.[24] Collagen fibrils can also be protected from degradation by using collagen cross-linkers during adhesive restorative procedures.[1] Although both methods focus on collagen integrity, the literature suggests that hybrid layer hydrolysis is not completely eliminated when using those techniques, because they are unable to remove the intrafibrillar water in collagen.[20,35,36,38] The ethanol-wet bonding technique was developed to chemically dehydrate the demineralized collagen matrix; however, it has been demonstrated that ethanol is incapable of completely replacing the intrafibrillar residual water with resin monomers.[36]

Dentin remineralization has been described as an effective strategy for increasing the durability of adhesive-dentin bonds,[4,19,22] because it dehydrates collagen fibrils by replacing the residual water in hybrid layers with apatite crystallites[23,36] and restores the protective function of collagen by creating inter- and intrafibrillar apatite.[21,33] Different techniques after and during bonding,[45,47] as well as remineralization agents, have been used to promote adhesive-dentin remineralization.[34,48,50] Bioglass 45S5 (BAG) is a biocompatible calcium/sodium phosphate-phyllosilicate (45 wt% SiO_2, 24.5 wt% Na_2O, 24.5 wt% CaO, 6 wt% P_2O_2)[16] that presents a high bioactivity index ($^IB = 12.5$).[7] Its remineralization process involves the exchange of ions (Na^+, Ca^{2+}, PO_4^{3-}, F^-) between the BAG silicate network and the surrounding fluid. This process induces calcium phosphate (CaP) precipitation and its subsequent crystallization into hydroxyapatite on the mineral tissue surface,[37] producing electrostatic, ionic and/or hydrogen bonding between the demineralized collagen and BAG silanols.[27]

Dentin remineralization at the adhesive-dentin interface is well established in the literature; however, no study has evaluated structural dentin modifications, dentin bond strength, and the differences in the adhesive interface/diffusion zone after remineralization treatment. Therefore, the purpose of this study was to investigate how a dentin remineralization technique performed during bonding and using a BAG solution affects dentin chemical compounds, as well as its adhesive characteristics. The null hypotheses tested were: 1. remineralization treatment would not modify dentin chemical composition; 2. dentin microtensile bond strength (μTBS) would not be altered after remineralization treatment; 3. dentin μTBS would not change after 8 months of specimen storage; 4. adhesive interface and diffusion zone thickness would change when the adhesive is applied on remineralized dentin.

MATERIAL AND METHODS

Upon approval from the Research Ethics Committee of State University of Maringa (Research protocol: 50615715.1.0000.0104), 36 previously extracted healthy human molars were selected for use in this study. A flat, mid-coronal dentin disk of 5 mm diameter was prepared from each tooth using a low-speed diamond saw (Diamond Wheel 012" fine, South Bay Technology; San Clemente, CA, USA) under water cooling.

Micro-Raman Spectroscopy and SEM Bioactive Analysis

Specimen preparation and remineralization treatment

Bioactive analysis (n = 10) was performed to investigate the mineral and organic composition of the control, demineralized, and remineralized dentin surfaces (Fig 1). This was done by measuring the micro-Raman spectra (mRS) on the same dentin disks before any treatment (control dentin [CD]), after acid conditioning (etched dentin [ED]), and after remineralization with BAG, in which the analysis was performed after 24-h storage in artificial saliva (remineralized dentin [RD]). Specimens were etched with 35% phosphoric acid for 10 s, followed by a copious water rinse for 1 min. After acid conditioning, remineralization treatment was carried out to induce new mineral formation, using a solution of 0.015 g of Bioglass 45S5, particle size 3 μm (Vitreous Materials Laboratory [LaMaV]; São Carlos, Brazil) diluted in 1.35 ml of distilled water.[46] Ten μl of this solution was applied to the moistened dentin surface using amicropipette (Monocanal VVCS-10, Digipet; Curitiba, PR, Brazil), and the solution was slightly rubbed on the dentin for 30 s using a microbrush. The surface was then washed with deionized water for 15 s and air dried for 2 s. Remineralized specimens were adapted to fit in a plastic device and immersed in 25 ml of artificial saliva[7] in a 37°C water bath for 24 h. The specimens were rinsed with distilled water for 30 s and air dried before acquiring the spectra.

Micro-Raman spectra acquisition and data analysis

Micro-Raman spectra were obtained using a confocal Raman microscope (Senterra Bruker Optik; Ettingen, Germany). The samples were excited by a 785-nm laser source, with 100 mW output, which was focused on the treated and untreated specimen surfaces with a 20X magnification lens. Spectra were recorded in the spectral range of 800 to 1800 cm^{-1} and the spatial resolution was 3 to 5 cm^{-1}. The integration time of the detector was 3 s, and each curve was an average of 60 spectra. Reference points were determined on the specimen surface to enable gathering the mRS spectra at the same position before and

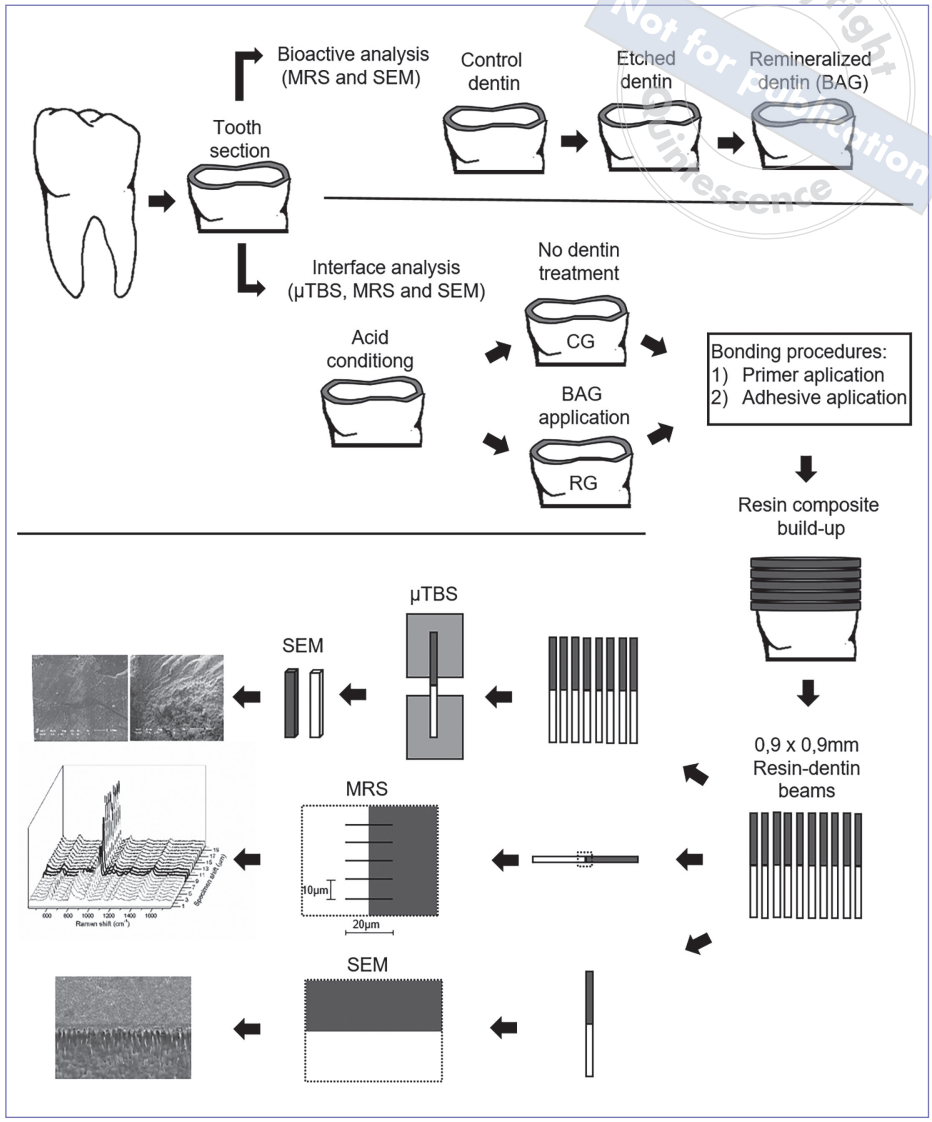

Fig 1 Study design and specimen preparation.

after treatment, as well as during the long-term analyses. Four spectra at different positions were obtained for each measurement, and the final result for each specimen was calculated by the average of these four spectra.

The spectra were baseline-corrected, and the integral areas of the dentin, organic (collagen phenyl group: 1003 cm^{-1}; amide I: 1650 cm^{-1}; amide III: 1245 cm^{-1}) and mineral compounds (ν1 phosphate symmetric stretch: 961 cm^{-1}), were calculated. Then, the dentin structural modifications were characterized using the following ratios: 1. relative presence of mineral, mineral-to-matrix ratio (961 cm^{-1}:1003 cm^{-1});[18,41] 2. organization of collagen (1667 cm^{-1}:1246 cm^{-1}); dentin amide I to amide III (1667 cm^{-1}:1246 cm^{-1}).[47]

Ratio data were statistically analyzed (R statistical software, R Foundation for Statistical Computing, Vienna, Austria) using the Shapiro-Wilk normality test, one-way ANOVA, and Tukey-Kramer post-hoc tests ($p < 0.05$).

Scanning electron microscopy

Two additional specimens of control, etched, and etched-remineralized dentin disks were prepared for SEM analysis using the same specimen preparation and remineralisation treatment described above, to investigate the surface changes of demineralized dentin after immediate remineralization treatment. The dentin disks were sputter-coated with a gold-palladium alloy (Ion Revestidor; IC-50, Shimadzu Biotech; Kyoto, Japan) and observed with SEM (Superscan SS-550, Shimadzu) at a magnification of 1000X.

Microtensile Bond Strength Test, Micro-Raman Spectroscopy 3D Chemical Mapping, and SEM Adhesive-Dentin Interface Analysis

Specimen preparation, dentin bonding, and remineralization treatment

Twenty flat, mid-coronal, 5-mm-thick dentin disks were wet polished using 600-grit SiC paper (Carborundum Abrasives,

Saint-Gobain; Paris, France) for 1 min to remove any remaining enamel and to create a flat surface with a homogeneous and standard smear layer. The specimens were then divided into two groups (n = 10): control group (CG) and remineralized group (RG) (Fig 1).

As described above, dentin disks were treated with 35% phosphoric acid for 10 s and rinsed with water for 1 min. After acid conditioning, CG specimens were treated with primer and adhesive following the manufacturer's instructions, while RG specimens were submitted to remineralization. The remineralization treatment was carried out for the RG specimens using the BAG solution[46] applied with a micropipette (Monocanal VVCS-10, Digipet) and rubbed on the dentin surface with a microbrush for 30 s. The surface was then washed with deionized water for 15 s and air dried for 2 s.

Bonding procedures were performed using a 3-step etch-and-rinse adhesive (Adper Scotchbond Multi-Purpose, 3M Oral Care; St Paul, MN, USa). Priming and bonding agents were used according to the manufacturer's instructions and light cured for 20 s at 1000 mW/cm^2 with a blue LED unit (Translux Power Blue; Heraeus Kulzer; Hanau, Germany). The specimens were immediately restored using 5 layers of 1-mm-thick composite resin (Filtek Z250; 3M Oral Care). Each layer was light cured for 20 s at 1000 mW/cm^2, followed by final curing for 60 s. Specimens were stored in artificial saliva[7] for 24 h at 37°C before testing.

Microtensile bond strength test and SEM failure mode analysis

Each specimen was sectioned perpendicular to the adhesive interface to obtain bonded sticks with cross-sectional areas of approximately 0.9 mm^2. The exact width of each stick was measured with a digital caliper (Zaas Precision, Amatools; Piracicaba, Brazil). Peripheral sticks with residual enamel were excluded from the study, and two sticks per group were set apart for the interface analysis with mRS and SEM; therefore, 16 bonded sticks were obtained per tooth. Half of them were tested after 24 h of storage in artificial saliva, and the remaining half after 8 months of storage in artificial saliva at 37°C.

Restored dentin sticks were attached to the grips of a testing jig with cyanoacrylate glue (Super Bonder, Henkel Loctite; Düsseldorf, Germany) and were tested in a universal test machine (EZ Test; Shimadzu) at 0.5 mm/min until failure. The maximum load at fracture (N) and the cross-sectional area of each failed stick were used to calculate μTBS in MPa. The μTBS was then computed for each tooth by averaging the values of the eight restored dentin sticks from that tooth. The bond strength results were statistically analyzed with the Shapiro-Wilk normality test and a repeated-measures ANOVA and Tukey-Kramer post-hoc test for pairwise comparisons ($\alpha = 0.05$) using R i386 3.0.2 software (R Foundation for Statistical Computing; Vienna, Austria). Pre-test failures were assigned a value of 0 MPa for statistical analysis.

The fractured surfaces of the tested sticks were placed on aluminum disks, sputter-coated with a gold-palladium alloy (Ion Revestidor; IC-50, Shimadzu) and observed with SEM (Superscan SS-550, Shimadzu) at a magnification of 100X. Failure patterns were classified as: adhesive, if the fracture site was located between the adhesive and dentin; mixed, when the fracture involved different regions, such as the adhesive-dentin interface, dentin and composite resin; cohesive in dentin; and cohesive in composite resin.

Micro-Raman spectroscopy 3D chemical mapping

The bonded interface of one untested stick of each tooth from both groups (n = 10) was analyzed using mRS. Before measurements, specimens were only washed with distilled water in an ultrasonic bath for 10 min. Chemical mapping of the interface was performed using a confocal Raman microscope (Senterra Bruker Optik). The spectra were excited using a 785-nm laser source and recorded in the spectral range of 450 to 1800 cm^{-1}. The laser beam (100 mW) was focused on the specimen with a 100X magnification lens. Spatial resolution was from 3 to 5 cm^{-1} and the detector integration time was 3 s. Each final curve resulted from the mean of 60 spectra. Interface line mapping was obtained starting in the composite region and proceeding across each adhesive-dentin specimen. Raman spectra were acquired in steps of 1 μm, using the x-y-z stage from five lines of 20 μm length, with a 10 μm space between the lines, resulting in 100 scanning spectra per specimen. To avoid dehydration of the specimens, gauze soaked in distilled water was kept in contact with the dentin during mRS data collection.

Micro-Raman peaks at 961 cm^{-1} (ν1 phosphate symmetric stretch; representative of the phosphate in the dentin) and at approximately 1113 cm^{-1} (νC—O—C; representative of the carbon chain in the resin monomer present in the adhesive) were used to identify the first spectrum of the interface (mixed spectrum with characteristic bands of dentin and/or adhesive). Modifications on the adhesive-dentin interface spectrum due to remineralization treatment were investigated by intensity and intensity ratio analysis as follows:

1. The relative presence of mineral (961 cm^{-1}) and mineral-to-matrix ratio (961 cm^{-1}:1003 cm^{-1}), with the 961 cm^{-1} band attributed to the -ν1 symmetric stretch of the phosphate and that at 1003 cm^{-1} to the aromatic ring of phenylalanine residues in the collagen.[18,41]
2. Organic-to-matrix mineral intensity ratio (1450 cm^{-1}: 961 cm^{-1}),[54] with the 1450 cm^{-1} band representing the dentin organic CH-group and the 961 cm^{-1} band the dentin mineral component, the phosphate ν1 symmetric stretch.
3. Organization of collagen (1667 cm^{-1}:1246 cm^{-1}), from the intensity ratio between dentin amide I and amide III (1667 cm^{-1}:1246 cm^{-1}).[47]
4. Physical interaction through the ratio 961 cm^{-1}: 1458 cm^{-1}, with the 961 cm^{-1} band representing the mineral component in dentin, the ν1 phosphate symmetric stretch, and that at 1458 cm^{-1} representing the CH_2 group of the methacrylate monomers (deformation δ of CH_2).[56]
5. Degree of adhesive conversion (DC) (1637 cm^{-1}: 1605 cm^{-1}), with the 1637 cm^{-1} peak associated with the C = C band of methacrylate and the 1605 cm^{-1} peak with the C-C from the phenyl of the adhesive monomer.[55]

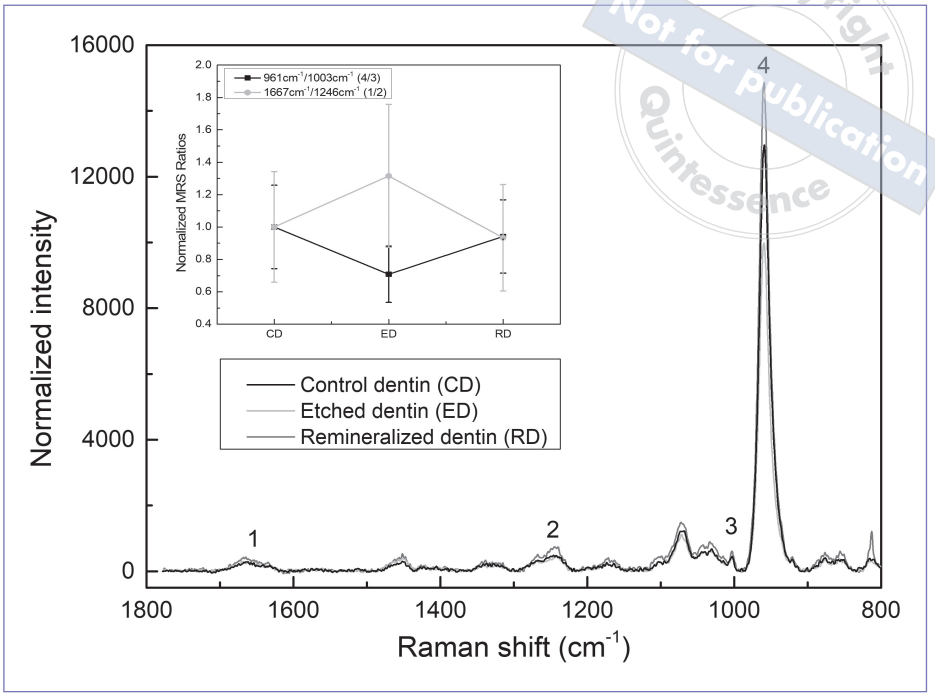

Fig 2 Normalized micro-Raman spectra of control, etched, and remineralized dentin. The mR spectrum shift at around 961 cm^{-1} is attributed to the ν1 phosphate symmetric stretch. The reduction of this band intensity is related to mineral loss, and its increase indicates mineral increase. The inset shows the variation of the mRS ratios of the relative presence of mineral (961 cm^{-1}:1003 cm^{-1}) and organisation of collagen (1667 cm^{-1}: 1246 cm^{-1}) according to dentin treatment. An improvement of dentin mineral compounds after remineralization treatment can be observed.

6. Adhesive/bis-GMA penetration (1667 cm^{-1}), with the 1113 cm^{-1} peak associated with the C-O-C from the adhesive (1113 cm^{-1}) and the 1667 cm^{-1} peak with that of amide I.[11,53]

The ratios of the first interface spectra were submitted to statistical analysis using the Shapiro-Wilk and Student's t-test ($p < 0.05$) (R i386 3.0.2 software, R Foundation for Statistical Computing). Statistical differences were identified by evaluating the effect of remineralization treatment through the comparison of CG and RG.

Micro-Raman spectroscopy has been used as an alternative method to traditional morphological imaging techniques for examining the adhesive interface.[10] The different Raman bands serve as fingerprints for each material analyzed. By observing the different intensities of the spectral bands, the transitional region between the adhesive materials and dental substrates can be established. Diffusion zone thickness was obtained by sigmoidal Boltzmann fitting applied on Raman band peak intensities centred at 1113 cm^{-1} (νC—O—C). This is representative of the carbon chain in the resin monomer present in the adhesive,[42] and reflects its penetration into dentin. This analytical fitting procedure is described in detail in a previous publication.[10] Boltzmann fitting was applied on each of the five-line scans from each specimen, and the final diffusion zone depth was the average for each tooth.

SEM analysis of adhesive-dentin interface

One untested stick per tooth was also prepared for SEM analysis (Quanta FEG 250, FEI; Hillsboro, OR, USA) (n = 10). These additional specimens were embedded in thermoactivated acrylic resin (JET colorless classic; São Paulo, Brazil) in a metallographic filler (PRE-30mi Embossing Press, AROTEC; Cotia, Brazil). Each section was polished with wet abrasive SiC papers (#400, #600, #1200, #2000 and #4000, Carborundum Abrasives), gently decalcified with 37% phosphoric acid for 30 s, deproteinized with 2% NaOCl solution for 1 min, ultrasonicated in 96% ethanol for 2 min, air dried, and sputter-coated with gold/palladium. Intact adhesive-dentin interfaces were observed at 2000X and 4000X magnification to evaluate resin tags and hybrid layer formation, in addition to measuring the interface dimensions. SEM images were processed and analyzed using image software (UTHSCSA Image tool, University of Texas; San Antonio, TX, USA); interface thickness was calculated in μm using the distance tool from 10 regions along the bonded interface of each specimen. The final interface thickness was obtained from the average of these 10 measurements.

Interface thickness measurements (SEM images) and diffusion zone values (mRS Boltzmann adjustment) were checked for normality using the Shapiro-Wilk test and then compared using two-way ANOVA test followed by Tukey's test (R i386 3.0.2 software). The statistical significance was set at $p < 0.05$.

RESULTS

Micro-Raman and SEM Bioactive Analysis

Taking into account that mRS results are relative, mRS spectral findings were always presented as ratio analysis;

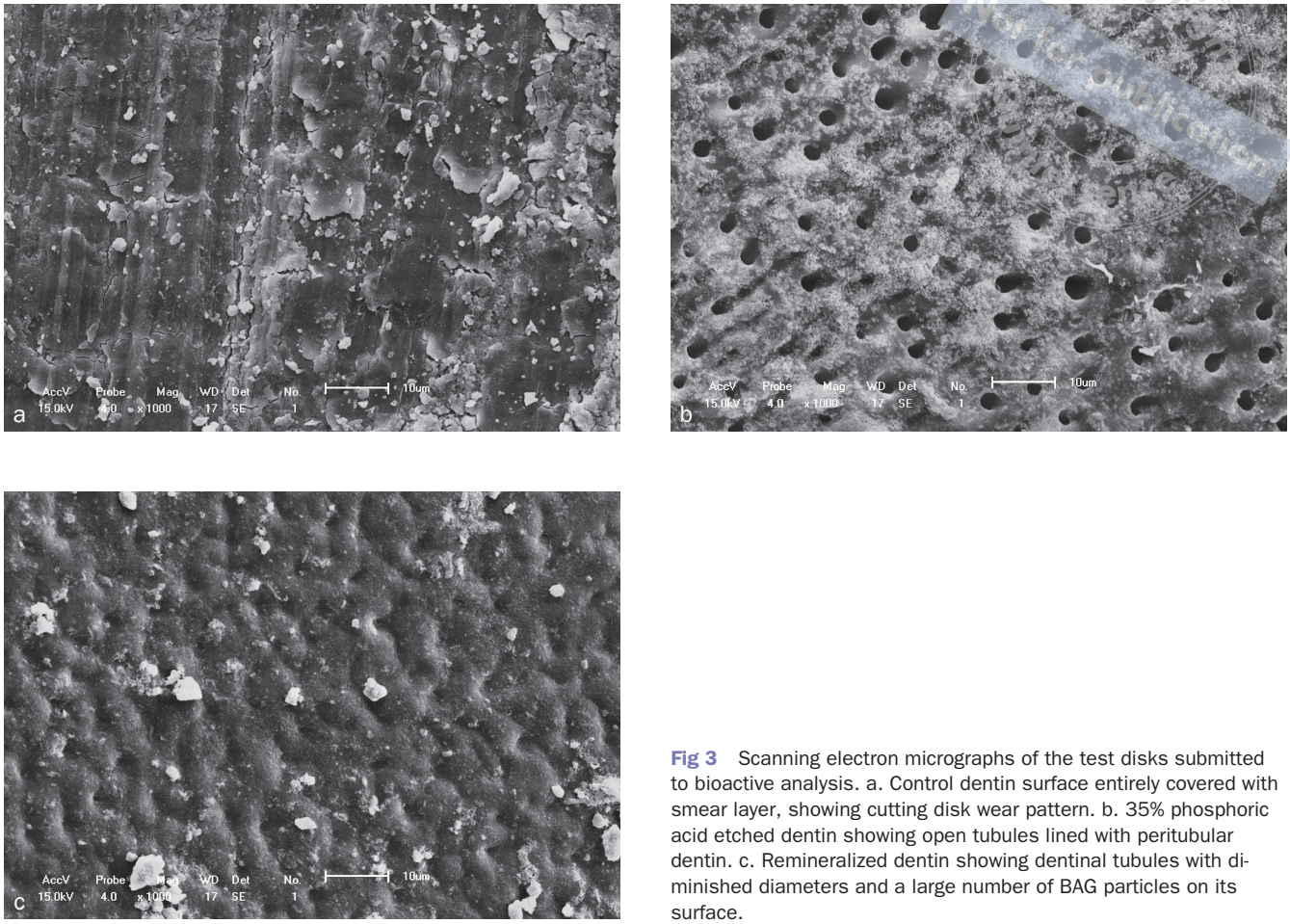

Fig 3 Scanning electron micrographs of the test disks submitted to bioactive analysis. a. Control dentin surface entirely covered with smear layer, showing cutting disk wear pattern. b. 35% phosphoric acid etched dentin showing open tubules lined with peritubular dentin. c. Remineralized dentin showing dentinal tubules with diminished diameters and a large number of BAG particles on its surface.

mRS spectra and ratios obtained from the bioactive analysis are illustrated in Fig 2. The acid conditioning step significantly reduced ($p = 0.00$) the relative presence of mineral (961 cm^{-1}:1003 cm^{-1}) in the control dentin, varying from 17.7 ± 2.3 to 12.5 ± 1.5, and demonstrated that its organic compounds (1667 cm^{-1}:1246 cm^{-1}) changed significantly ($p = 0.00$) from 0.6 ± 0.1 to 0.8 ± 0.1. Remineralization treatment significantly improved ($p = 0.00$) the mineral content of dentin, restoring the mineral-to-matrix ratio (961 cm^{-1}:1003 cm^{-1}) from 17.7 ± 2.3 to 16.7 ± 1.9, and consequently significantly reducing ($p = 0.00$) the number of organic compounds (1667 cm^{-1}:1246 cm^{-1}) on the dentin surface after acid conditioning, changing from 0.8 ± 0.1 to 0.56 ± 0.10.

SEM images confirmed the findings of the mRS bioactive analysis (Fig 3). The control dentin surface presented wear from the cutting disk and was entirely covered with smear layer (Fig 3a). Acid conditioning removed the smear layer, and open tubules lined with peritubular dentin were verified in the demineralized dentin (Fig 3b). BAG remineralization treatment induced mineral formation, as shown by the reduction of dentinal tubule diameters (Fig 3c). BAG particles were observed as bright crystals in the remineralized dentin surface (Fig 3c).

μTBS Test and SEM Failure Mode Analysis

Microtensile bond strength results are presented in Table 1. Remineralization treatment increased dentin bond strengths of the specimens stored for 24 h (RG-24h) ($p = 0.01$). However, after 8-month storage in artificial saliva, no significant difference was found between the control (CG-8m) and remineralized (RG-8m) groups ($p = 0.63$). SEM analysis of the fractured surfaces revealed that most failures in both groups were at the adhesive interface (Table 1). The failure types are illustrated in Fig 4.

Micro-Raman Mapping and Chemical Analysis

Interface 3D chemical mapping is presented in Fig 5. Three major components were identified in the mapping spectra: dentin, adhesive, and adhesive-dentin interface. The peak-ratio analysis (Table 2) revealed that remineralization treatment resulted in significant modifications in the spectra showing the composition of the interface. BAG application promoted an increase in the relative mineral concentration ($p = 0.036$), taking into account the dentin composition, a reduction in the prevalence of organic matrix mineral content ($p = 0.002$), and an increase in the amide I peak inten-

Table 1 Mean and standard deviation (SD) of dentin μTBS (MPa), adhesive diffusion zone (mRS) (μm), and interface thickness (SEM) (μm)

Group, timepoint tested	μTBS mean ± SD in MPa (number of premature failures and number of tested sticks) Failur modes: adhesive/ mixed/cohesive in dentin/ cohesive in composite resin	Adhesive diffusion zone (mRS) in μm, mean ± SD	Interface thickness (SEM) in μm, mean ± SD
CG, 24 h	33.9 ± 6.8 ^{A}		
	(5/75)	3.0 ± 0.6 ^{ab}	3.5 ± 0.6 ^{a}
	49/43/6/2		
RG, 24 h	45.3 ± 8.4 ^{B}	2.5 ± 0.8 ^{b}	3.0 ± 0.6 ^{ab}
	(0/80)		
	54/35/10/1		
CG, 8 m	32.7 ± 7.3 ^{A}		
	(7/73)		
	40/40/15/5		
RG, 8 m	36.8 ± 8.2 ^{A}		
	(6/74)		
	45/44/10/1		

Different superscript capital letters within the 1st column indicate a statistically significant difference ($p < 0.05$) according to repeated-measures ANOVA and Tukey-Kramer's post-hoc test. Different superscript lowercase letters indicate statistically significant differences ($p < 0.05$) within the 2nd and 3rd columns according to 2-way ANOVA and Tukey's tests.

sity ($p = 0.016$). When the adhesive was applied to remineralized dentin, the physical interaction of methacrylate monomers improved ($p = 0.003$), its DC increased ($p = 0.033$), and bis-GMA monomer penetration decreased ($p = 0.001$).

Adhesive Diffusion Zone (mRS) and Interface (SEM) Thickness Comparison

Statistical analysis demonstrated no significant difference between adhesive diffusion zones of RG and CG ($p = 0.68$) or among the interface thicknesses of RG or CG ($p = 0.74$). In addition, there was no significant difference between those two methods to evaluate the adhesive interface ($p = 0.92$).

SEM analysis of intact composite-dentin interfaces (Fig 7) showed differences in the hybrid layer of CG and RG. The interfaces of the remineralized specimens presented a more continuous hybrid layer, in which a majority of the dentin tubular spaces filled with adhesive tags (Figs 7c and 7d). The bioactive glass was observed as bright crystals within the hybrid layer and resin tags (Figs 7c and 7d).

DISCUSSION

Remineralization with a BAG solution increased the inorganic compounds of the dentin and decreased the organic components exposed at the dentin surface. Due to this increase in mineral content, BAG treatment promoted reduced adhesive monomer penetration into dentin. However, remineralization treatment did not significantly change the adhesive diffusion zone or the thickness of the adhesive interface. In addition, remineralization treatment enhanced the interaction of adhesive monomers with dentin mineral compounds, increased adhesvie monomer DC, and consequently improved the immediate dentin bond strength to a three-step etch-and-rinse adhesive.

As a consequence of dentin acid conditioning, mineral components around organic molecules decreased, leaving the collagen fibrils surrounded by water. When this residual water is removed inappropriately, the collagen fibrils collapse and make adhesive infiltration a difficult process, exposing these fibrils to degradation and resulting in damage of the hybrid layer integrity.[11] The first hypothesis of this study was rejected, because mRS and SEM proved the re-

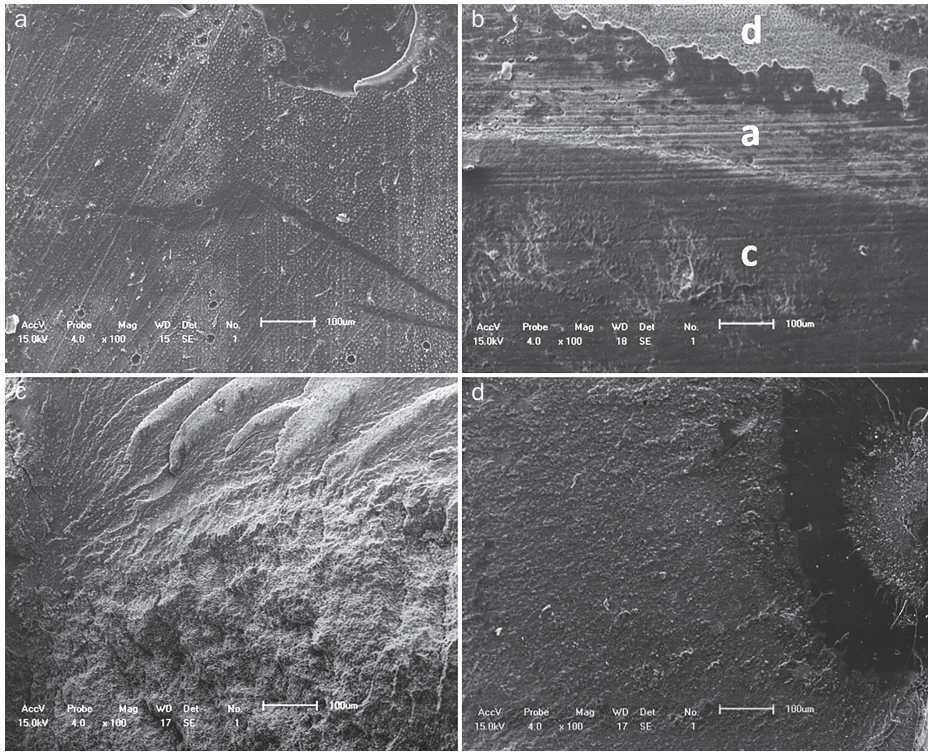

Fig 4 SEM failure analysis of fractured specimens (100X). a. Adhesive failure along the dentin surface in the CG. b. Mixed failure in the RG, displaying adhesive layer (a), dentin surface (d), and cohesive failure in composite resin (c). c. Cohesive failure within dentin in the RG. d. Cohesive failure within composite resin in the CG.

mineralization effect of dentin treated with BAG. Minerals precipitated into the previously partially demineralized dentin surface, promoting an increase in phosphate ion concentration and the apparent relative mineral concentration (Fig 2).[7,13] Changes in dentin organic compounds play an important role in the remineralization process.[26,40,41,45] The mRS band of amide I is considered the best spectral region for studying protein conformation and secondary structure,[18] and the amide I:amide III ratio is used to analyze the structure of collagen.[47] The bioactive analysis revealed that the amide I:III ratio was restored to its initial value after remineralization (Fig 2); therefore, the dentin remineralization effect was also verified by the modifications in the mRS spectral region of amides.

The second null hypothesis was rejected, because the remineralization treatment promoted an improvement in immediate dentin μTBS. This reinforcement at the adhesive-dentin interface could be a consequence of mineral deposition, because the dentin bond strength is directly affected by the degree and quality of its mineral content.[2] Nevertheless, the third null hypothesis must be partially rejected: although the μTBS of control dentin did not change after 8-month storage, the μTBS of remineralized dentin decreased in the long-term analysis. This was the first remineralization study to use a BAG solution as an additional bonding step. BAG has been used in immersion solutions, mixed with adhesive agents, and used as a dental air-abrasion powder. Microtensile studies[14,33,49] revealed that dentin treatment with experimental primers and adhesives containing biomimetic analogs or microfillers (Portland cement, BAG, Ca/P) did not change μTBS in the 24-h analysis, but provided durable adhesive-dentin bonds in the long term. The higher immediate μTBS results obtained in this study indicate that BAG components are more reactive when they are not associated with other chemical molecules. Conversely, this high reactivity of BAG could also be responsible for the decrease in μTBS of the remineralized group after 8 months of storage, since BAG's hydrophilic characteristics were likely increased by the large contact area of the bonded stick interface with artificial saliva. Although the mean μTBS of the remineralized group did not present long-term stability, its final value was statistically similar to that of the control group.

Dentin acid conditioning is another variable that seems to affect the μTBS of adhesive-dentin remineralized interfaces. Even after remineralization, dentin bond strength was significantly lower for the control group when demineralization was performed over a prolonged period[14] or different or stronger acids were used than those applied in the etch-and-rinse protocol.[49] When BAG was applied through air abrasion as a dentin pretreatment before placing resin-modified glass-ionomer cement restorations, its retention on the dentin surface improved the bond durability after six months of storage.[40] Thus, the active application of BAG solution on dentin might have increased the contact of the bioactive particles with the dentin tissue, therefore improving the immediate μTBS in this study.

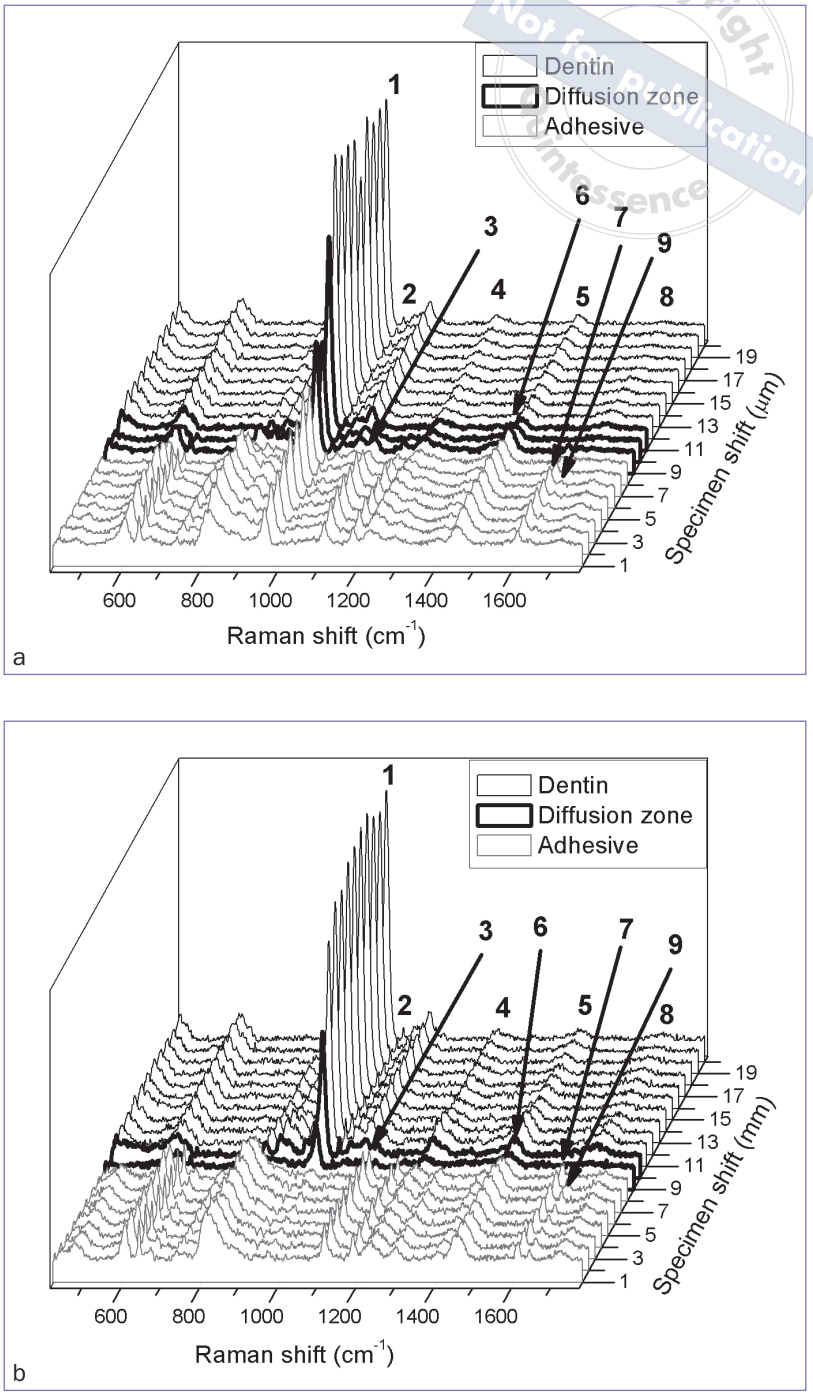

Fig 5 Micro-Raman 3D-mapping spectra acquired with a 1-μm step across the adhesive-dentin interface of control (a) and remineralized groups (b). A numbered line represents each measurement on the z-axis (specimen shift), and the first spectrum was obtained from the adhesive. The simultaneous increase in the phosphate peak (961 cm^{-1}) and a decrease in the adhesive peak (1113 cm^{-1}) suggest the beginning of the hybrid layer. The grey lines represent the adhesive spectra; the bold black lines represent the adhesive-dentin interface spectra; the thin black lines represent the dentin spectra. The numbers indicate the Raman peaks used for the chemical analysis of spectra (1) 961 cm^{-1}; (2) 1003 cm^{-1}; (3) 1113 cm^{-1}; (4) 1246 cm^{-1}; (5) 1450 cm^{-1}; (6) 1458 cm^{-1}; (7) 1608 cm^{-1}; (8) 1637 cm^{-1}; (9) 1667 cm^{-1}.

Although remineralization was performed before applying the adhesive, it enhanced monomer bonding to dentin and increased monomer polymerization. The micromechanical retention of etch-and-rinse adhesives is improved when methacrylate monomers and/or carboxylic esters chemically interact with etched inorganic dentin compounds.[52] The physical interaction ratio (961 cm^{-1}:1458 cm^{-1}) was higher when the BAG solution was applied on etched dentin, demonstrating that methacrylate monomers are more susceptible to interacting with dentin when mineral compounds are present in greater quantity. The results of this study corroborate with those of Toledano et al,[47] because the adhesive DC increased due to dentin remineralization. The mineral formation seems to increase the monomer polymerization ratio as a consequence of its dehydration effect: removing residual water from the hybrid layer increases the ability of adhesive to form C=C bonds. The adhesive monomer-to-polymer conversion is related to the

Table 2 Interface chemical analysis showing means ± SD of mRS ratios (arbitrary units) calculated for the first interface spectra of CG and RG

	mRS ratios					
Group	961:1003	1450:961	1667:1246	961:1458	1637:1605	1113:1667
CG	5.16 ± 1.11[a]	0.43 ± 0.13[c]	0.42 ± 0.10[e]	2.98 ± 0.88[g]	0.84 ± 0.30[i]	2.81 ± 1.16[k]
RG	6.86 ± 2.03[b]	0.25 ± 0.09[d]	0.52 ± 0.04[f]	5.12 ± 1.63[h]	1.19 ± 0.37[j]	1.21 ± 0.51[l]
p-value	0.036	0.002	0.016	0.003	0.033	0.001

mRS chemical ratios of relative mineral concentration (961 cm^{-1}/1003 cm^{-1}); organic matrix mineral content (1450 cm^{-1}:961 cm^{-1}); amide I to amide III (1667 cm^{-1}:1246 cm^{-1}); physical interaction ratio (961 cm^{-1}:1458 cm^{-1}); adhesive DC (1637 cm^{-1}:1605 cm^{-1}); and adhesive bis-GMA monomer penetration (1113 cm-1:1667 cm^{-1}). Different superscript letters indicate statistically significant differences ($p < 0.05$) between the interface chemical ratios of CG and RG according to Student's t-test.

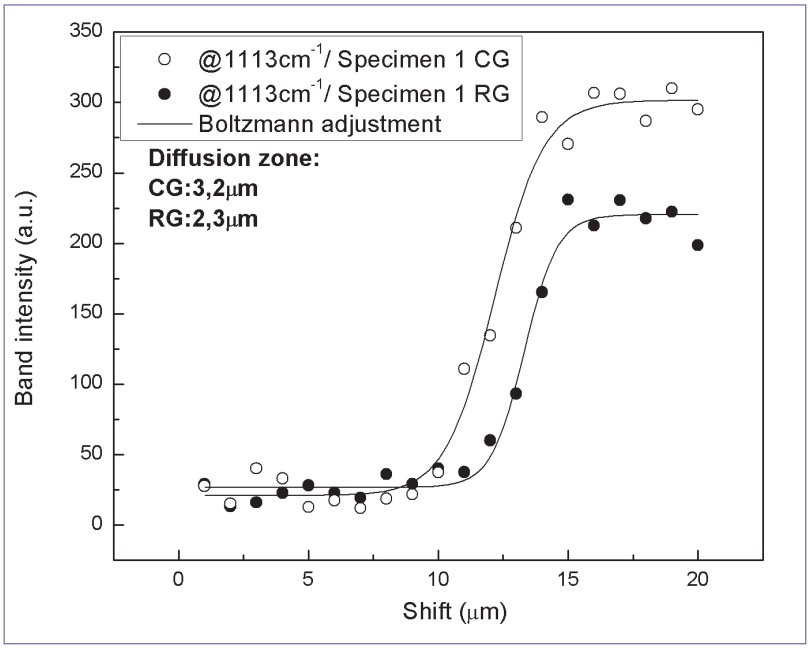

Fig 6 Representative plot of the diffusion zone of the control and remineralized groups. Raman mapping spectra at 1113 cm^{-1} (νC—O—C) were plotted according to the specimen shift position and fitted to the Boltzmann function.

adhesive bond's chemical composition.[12] In the present study, the adhesive's DC was obtained inside the hybrid layer. According to Navarra et al,[25] this is beneficial, because the DC polymerization reaction can be measured considering both monomer conversion and the interaction of the adhesive with the dentin tissue.[25] When BAG particles were added to resin composites, their DC decreased, because the polymerization reaction was affected by the changes in particle size, morphology, and opacity, as well as by more severe light attenuation.[26] Nevertheless, other studies have demonstrated that the inclusion of BAG particles in the resin composite structure increased the DC, because it improved monomer blending and extended the cationic polymerization of the uncured monomers.[30,39]

Even though remineralization increased the amount of mineral matrix on the dentin surface, it did not significantly decrease the adhesive diffusion zone or the adhesive-dentin interface thickness, leading to the acceptance of the fourth hypothesis. The current literature contains conflicting information regarding the remineralization effect on the thickness of the adhesive-dentin interface. In one study, remineralization was described to facilitate and increase the penetration of adhesive monomers, as it prevented dentin collagen collapse.[18] In contrast, another study concluded that mineral accumulation promoted dentin tubule sealing, thereby decreasing the interface thickness.[45] Other studies reported that hybrid layer thickness did not change after dentin remineralization because mineral deposits occurred around dentinal tubular orifices and between collagen fibrils, resulting in a less porous hybrid layer, thus corroborating the present study's results.[34,36] Boltzmann analysis is a chemical method to estimate the adhesive diffusion zone into dentin; it is measured using the sigmoidal behavior of a specific adhesive-monomer mRS band at

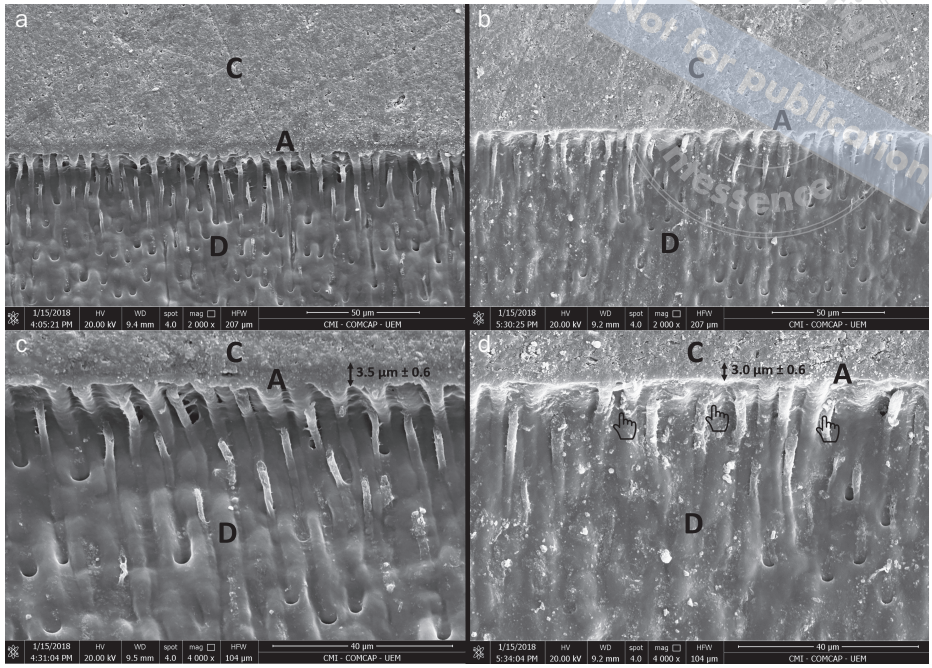

Fig 7 SEM images showing a representative area of the adhesive-dentin interface of the CG (a and b) and the RG (c and d) (2000X and 4000X). The double arrow indicates mean interface thickness. After three-step etch-and-rinse adhesive application, dentin was demineralized and subsequently infiltrated by monomer resin. Resin tags and an adhesive/hybrid zone are identifiable. Gaps can be observed between dentin tubules and adhesive tags (a and b) in some regions of the CG interface. Dentin tubules were not completely infiltrated by adhesive. In the RG, the hybrid layer is more continuous and dentin tubules seem to be better infiltrated by the adhesive (c and d). BAG particles (pointers) appear as bright crystals (d). d = intact dentin; a = adhesive layer; c = composite resin.

the interface region,[18] whereas scanning electron microscopy is a quantitative imaging method that uses images of the adhesive-dentin bond to determine interfacial thickness. The Boltzmann analysis and SEM yielded similar thicknesses, demonstrating that mRS is a useful tool to estimate interface thickness.

Hybridization efficacy is not related to the interface thickness. but is attributed to its infiltration characteristics and bond quality.[51] Therefore, incomplete permeation of adhesive monomers into demineralized dentin enables hybrid layer degradation.[3] Thus, mineral formation resulting from remineralization treatment is an alternative to eliminate residual water on etched dentin, enhancing the adhesive-dentin bonds. Although remineralization treatment did not increase the hybrid layer thickness, it promoted a decrease in the adhesive penetration ratio. This ratio is calculated by subtracting the mRS band intensity of bis-GMA adhesive monomer (1113 cm^{-1}) from that of amide I (1667 cm^{-1}). Mineral deposition improves the bond between hydroxyapatite and collagen, which leads to an increase in mineral compounds and to the reduction of the organic matrix mineral content available on the dentin surface. mRS revealed changes in the structural composition of collagen, ie, a substitution of amide III for amide I. Therefore, the increase in amide I intensity also promoted the reduction of bis-GMA penetration ratios.[47]

Although several studies have measured the amount of resin and/or adhesive penetration into dentin by using the absolute intensity of monomer Raman bands,[10,43,48,49,50] there are some concerns regarding mRS of the interfaces. Three-dimensional mapping spectra are usually larger than the hybrid layer, and therefore result in mixed spectra that can artificially detect a decreased monomer infiltration area. To minimize the effect of this limitation, the hybrid layer thickness and position were obtained by the sigmoidal Boltzmann fitting of the Raman carbon-chain peak for the adhesive.[10,42] In addition, the absolute intensity of the Raman band can be affected by factors such as the position of focus, depth of detection, and fluorescence of biological components.[53] These technical interferences were decreased by the use of Raman band ratio analysis to investigate the modifications on adhesive-dentin interfacial spectra.

In vitro studies may be limited in terms of simulating clinical conditions. When extracted dentin specimens are used, the loss of connection between dentin and pulp tissues might affect organic dentin tissue compounds and their link with mineral compounds. Thus, the remineralization chemical dynamics present in this study might be different if vital teeth were analyzed. Considering that saliva compounds are readily available for remineralizing dental tissue, the use of artificial saliva may also be a limitation in a remineralization study. Nevertheless, both control and experimental groups were immersed in saliva and the remineralization effect was observed only in the remineralized specimens. Taking into account that the BAG solution is hydrophilic and promotes remineralization through ion exchange with the surrounding fluid, the storage of restored dentin sticks, instead of restored dentin blocks, may be a technique limitation, because it increases the interfacial area of contact with saliva, and may corroborate the decreased RG-8m µTBS.

CONCLUSION

The BAG solution applied on etched dentin before bonding promoted immediate dentin remineralization, as demonstrated by mineral increase on the dentin surface. This new mineral growth modified the dentin composition and improved its bonding with an etch-and-rinse adhesive, because monomer interaction with phosphate increased and the degree of conversion was enhanced. Consequently, dentin remineralization resulted in higher immediate adhesive-dentin bond strength. Further studies are in progress on how to improve the stability of adhesive-dentin remineralized specimens.

ACKNOWLEDGMENTS

This study was supported in part by Comissão de Aperfeiçoamento de Pessoal do Nível Superior (CAPES) Foundation Brazil (Programa de Doutorado Sanduíche no Exterior PDSE:88881.134022/2016-01).

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Clinical relevance: Dentin remineralization treatment induces an immediate increase in mineral content. Remineralization treatment performed prior to the application of a 3-step etch-and-rinse adhesive increases the inorganic proportion of dentin, enhances monomer polymerization, and improves the immediate adhesive-dentin bond strength.