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The Effect of Ethanol Associated with Universal Adhesive on Resin Composite Adhesion to Different Dentin Depths: A Long-Term *In Vitro* Study

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ABSTRACT

To evaluate the ethanol wet-bonding protocol with a universal adhesive in etch-and-rinse and self-etch modes on microleakage and microtensile bond strength (μTBS) of resin composite to different dentin depth. Molars were distributed into groups according to protocol and dentin depth: universal adhesive (C - control), ethanol + universal adhesive (E), and phosphoric acid etch + ethanol + universal adhesive (PA+E). All protocols were applied to dentin at superficial, middle and deep depths. The specimens ($n = 10$) were submitted to microleakage and μTBS tests. Half of specimens were submitted to thermocycling (10,000 cycles; 5°C/55°C). Data were submitted to Spearman correlation (μTBS x microleakage) and three-way ANOVA (protocol, aging and depth), followed by Bonferroni post hoc test (α=0.05). PA+E group showed highest μTBS and lowest microleakage mean values in 24h. All groups presented similar μTBS mean values after thermocycling. The deep dentin showed less stable μTBS results. PA+E group presented highest microleakage mean values after thermocycling. Spearman's correlation showed a strong correlation between microleakage and μTBS. The PA+E group improved immediate adhesion and E group promoted a more stable μTBS in the long-term adhesion of universal adhesive. The aging for all protocols jeopardized the stability of the hybrid layer. The ethanol wet bonding technique associated with universal adhesive has enhanced the immediate result of the resin composite adhesion.

INTRODUCTION

Dentistry has seen ongoing changes in adhesive protocols in order to improve dentin bonding effectiveness.[1] However, using dentin as a bonding substrate is a challenge because of its complex composition.[2] The weakness of the adhesive bond to dentin causes several problems, such as restoration debonding, secondary caries, and microleakage.[3] Studies have shown that the main causes of failures at the adhesive interface are hydrolysis of resin bond and enzymatic hydrolysis of collagen fibers.[1-4]

Some alternative agents are used to improve the durability of the bond to dentin, such as chlorhexidine, glutaraldehyde, and primers.[5] However, the literature has shown some concerns about the cytotoxicity of these agents.[2] To overcome the question of cytotoxicity, other protocols such as ethanol wet-bonding (EWB), have been developed as alternatives to improve the adhesive bond strength to dentin. The EWB protocol proposes that it enables better penetration of the adhesive (hydrophobic monomers) into the collagen fiber network, thereby improving the formation of the hydrophobic hybrid layer.[6] Furthermore, the ethanol reduces the water content on the hybrid layer, and as consequence, create a less hydrophilic hybrid layer, reducing the probability of hydrolysis of the adhesive interface.[6,7]

With the increasing simplification of the number of materials used for adhesion to different substrates and less sensitive procedures, universal adhesives have been widely used in Dentistry.[3,4] Universal adhesives can be applied on enamel and dentin in conventional mode (etch-and-rinse), selective etching mode (phosphoric acid on enamel and self-etch on dentin), and self-etch mode on both enamel and dentin.[3,4] The 10-methacryloyloxydecyl dihydrogen phosphate (10-MDP) phosphate monomer can chemically bond to dentin by calcium chelation, creating a stable bond to dental substrates.[8]

Moreover, it is important to understand the play of water in the adhesive process, since the water is responsible for the long-term hydrolysis of the bonded interface (microleakage). The resin monomers are broken down into smaller molecules by the action of ions arising from the ionization of water.[2,6,9] The universal adhesives have a higher number of hydrophilic monomers, thus creating a hybrid layer that is more susceptible to hydrolysis.[3,4,7,9]

Several protocols regarding resin-dentin bond procedures are currently studied, but there are several controversies in the literature about these protocols. Some studies have been reported higher adhesive bond strength to dentin using EWB,[6,10,11] but not necessarily an improvement in infiltration,[11] since the microtensile bond strength (μTBS) of resin composite to dentin is material dependent.[12] On other hand, others studies showed that EWB was unable to increase the immediate bond strength of adhesives to dentin,[13,14] but it promoted stable bond strength values (long-term aging),[15,16] showing that EWB can indeed prevent long-term degradation of adhesive interface.

Furthermore, the effects on the long-term adhesion of universal adhesives using the EWB protocol in etch-and-rinse and self-etch modes remains unclear. In addition to that, several studies evaluate the adhesion of resin materials to dentin without considering the influence of dentinal morphology (dentin depth), which becomes more complex according to its location (dentin depth) in the cavity.[2,8,17]

Therefore, the aim of this study was to evaluate the effectiveness of the EWB protocol with a universal adhesive in etch-and-rinse and self-etch modes on the immediate and long-term microleakage and microtensile bond strength (μTBS) of resin composite to different dentin depths. The hypotheses tested would be that (i) the EWB protocol and (ii) dentin depth would have influence on μTBS and microleakage, and (iii) the long-term would affect the stability of the μTBS.

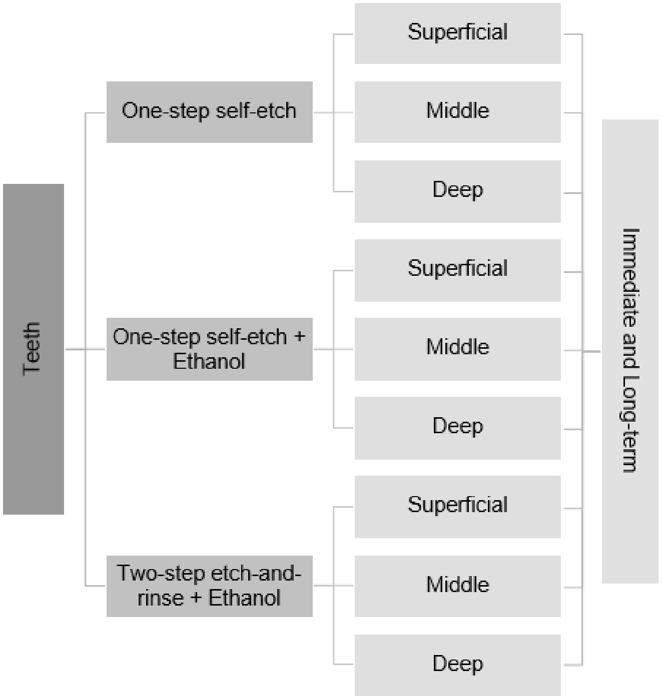
MATERIALS AND METHODS

SPECIMEN PREPARATION

This *in vitro* study involved a 3x3x2 factorial design. The factors were the type of the adhesive application protocol (three levels: one-step self-etch, one-step self-etch + ethanol and two-step etch-and-rinse + ethanol), dentin depth (three levels: superficial, middle and deep), and aging (two levels: immediate - 24 h and long-term - thermocycling) (Flowchart 1). Sixty molars extracted for therapeutic reasons with complete root formation and no presence of caries or restorations were stored in distilled water at 4ºC for utilization within six months after approval from the Ethics Committee (protocol: 29522820.4.0000.5418).

For all teeth, the superficial enamel was removed and the root was sectioned 2.0 mm above the cement-enamel junction using a low-speed water-cooled diamond saw (Isomet 1000, Buehler, Lake Bluff, IL, USA). The coronal flat dentin surface was ground with wet 600-grit sandpaper (APL-4, Arotec, Cotia, SP, Brazil) for 10 s to create a standardized smear layer.

A class I cavity with 4mm high x 3mm long x 3mm wide measured with a digital caliper (Mitutoyo Corporation, Tokyo, Japan) was prepared by an operator previously calibrated using diamond burs with different granulations (1090, 1090F, 1090FF; KG Sorensen, São Paulo, Brazil).



Flowchart 1: Group division according to the following 3 factors: 1 - Different technique (one step self etch; one step self etch + ethanol; two step etch and rinse + ethanol); 2 - Dentin depth (Superficial; Middle; Deep); 3 - Time (Immediate; Long-term).

After cavity preparation, all specimens were cleaned with water to remove debris and they were randomly divided into three main groups according to adhesive application protocol:

- Control (C): application of phosphoric acid 35% (Ultra-Etch, Ultradent, South Jordan, UT, USA) on enamel for 30 s, water rinse for 30 s, and air dry followed by adhesive system (Scotchbond Universal Adhesive, 3M ESPE, St. Paul, MN, USA) was applied actively for 20s and gently air dry for 5 s.

- Ethanol (E): application of phosphoric acid 35% (Ultra-Etch, Ultradent) on enamel for 30 s, water rinse for 30 s, and air dry. Application of 100% ethanol for 60 s using disposable applicator tips (Microbrush, KG Sorensen, Cotia, SP, Brazil). The adhesive system (Scotchbond Universal Adhesive, 3M ESPE) was applied actively for 20 s and gently air dry for 5 s.

- Phosphoric Acid + Ethanol (PA+E): application of phosphoric acid 35% (Ultra-Etch, Ultradent) on enamel for 30 s and dentin for 15 s, water rinse for 30s, and gently air dry followed by 100% ethanol and adhesive system (Scotchbond Universal Adhesive, 3M ESPE) as described above.

The adhesive system was light-cured for 10s by using a multiple-emission peak light-curing unit (1,000mW/cm², VALO Cordless, Ultradent, South Jordan, UT, USA). The light irradiance was measured using a radiometer (RD-7, ECEL, Ribeirão Preto, SP, Brazil). The cavities were restored with 4.0mm increment of bulk-fill resin composite (Tetric N-Ceram Bulk Fill, Ivoclar Vivadent, Schaan, Liechtenstein). The bulk-fill resin composite was used to simplify cavity filling with a less sensitive restorative technique (e.g. less void between layers) when compared to the incremental technique.[18] The resin composite was light-cured using the same light-curing unit (1,000mW/cm² for 10 s). Half of the teeth were stored in distilled water for 24 h at 37°C (n = 10), and the other half were submitted to thermocycling (n = 10) (*Figure 1*).

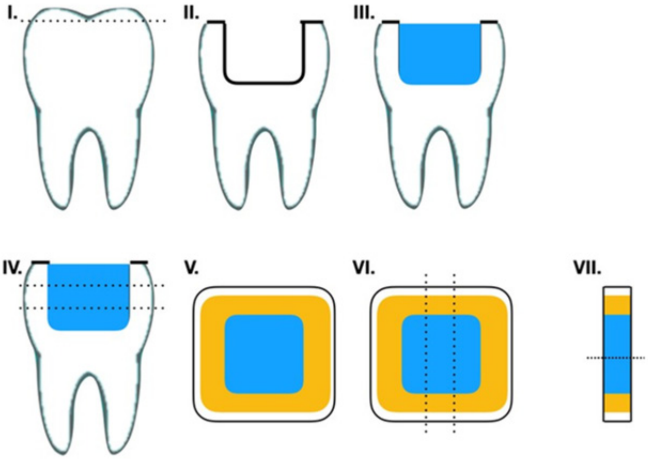


Figure 1: Specimen preparation. I - Dentin exposure. II - Cavity preparation. III - Application of the restorative technique according to protocol of each group tested. IV - Division into dentin depths (superficial, middle and deep). V - Occlusal view after horizontal cut. VI - Longitudinal cut. VII - Stick (μTBS test).

THERMOCYCLING

The teeth were submitted to 10,000 cycles of thermocycling (MSCT-3e, ElQuip, São Carlos, SP, Brazil) between water baths at 5°C and 55°C (dwell time 30 s) for 250 h. Then, the teeth were sectioned into three portions (superficial, middle, deep) with 1 mm thick by a low-speed water-cooled diamond saw (Isomet 1000, Buehler).

MICROTENSILE BOND STRENGTH (μTBS)

After aging in distilled water for 24 h (immediate) and thermocycling (long-term), each dentin depth (superficial, middle and deep) was sectioned to obtain dentin-resin composite rectangular bar-shaped stick (~1 mm^2) using a low-speed water-cooled diamond saw (Isomet 1000, Buehler). The sticks were analyzed in a stereomicroscope (MZ75, Leica Microsystems, Wetzlar, Germany) to verify any defect occurred during the cutting. These sticks were discarded.

The sticks were attached to jig using cyanoacrylate glue (Loctite Super Bonder Power Flex Gel, Henkel, Rocky Hill, NY, USA). The sticks were tested in a universal testing machine (Ez Test, Shimadzu, Kyoto, Japan) at 1.0 mm/min crosshead speed until failure. The bond area (mm^2) was measured using a digital caliper (Mitutoyo Corporation). The μTBS was expressed in MPa following the equation: μTBS = F/A in which F is the force applied during the test (N) and A the specimen bonded area (mm^2).

FRACTURE PATTERN ANALYSIS

The fracture pattern were evaluated using a stereomicroscope (MZ75, Leica Microsystems) with 50x magnification and classified as adhesive (cohesive in adhesive or at adhesive interface), cohesive in dentin, cohesive in resin composite, or mixed.

MICROLEAKAGE

The specimens were immersed in silver nitrate solution (10 g of nitrate crystals in 10mL deionized water to which 28% ammonium hydroxide was added in a dropwise manner) at 37°C, for 24 h, in a dark environment. Sequentially, the specimens were washed in running water for 2 minutes and immersed in developing solution (10.9 g developer powder in 100 mL distilled water) under a fluorescent lamp for 8 h. Subsequently, the specimens were washed with distilled water and immersed in polystyrene resin.

After inclusion, the specimens were polished with wet 600-, 1200-, and 2000-grit sandpapers (APL-4, Arotec) and then felt discs with diamond pastes in decreasing granulations of 3.0-, 0.5-, and 0.25-μm. Between each sandpaper and diamond paste granulation each specimen was placed in an ultrasonic bath for 10 minutes to remove debris. The specimens were dried with absorbent paper and treated with 85% phosphoric acid for 10 s (demineralization), followed by rinsing with distilled water. Specimens were deproteinized using a 2% sodium hypochlorite solution for 10 minutes. The specimens were washed with distilled water and dried at room temperature. Subsequently, each specimen was dehydrated in ethyl alcohol at increasing concentrations of 50%, 75%, 90%, and 100% for 10 minutes per concentration.

The specimens were mounted on aluminum stubs and sputter-coated with a thin layer of carbon (Baltec SCD 050) for observation by SEM under a high vacuum operated at 20kV. The images were captured by backscattered electrons, and recorded for evaluation of the infiltrated area, by using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The total area and infiltrated area were calculated (in µm) at 2,000x magnification and the percentage of infiltration was determined.[19]

STATISTICAL ANALYSIS

The µTBS and microleakage data were assessed by tests for normality (Shapiro-Wilk) and homoscedasticity (Levene). The results were submitted to Pearson correlation (µTBS x microleakage) and three-way ANOVA (adhesive application protocol, aging, and dentin depth) for multiple comparisons followed by Bonferroni *post hoc* test at the significance level of $\alpha=0.05$.

RESULTS

Three-way ANOVA revealed that the three factors studied (adhesive application protocol, dentin depth, and aging) showed significant influence in µTBS ($\rho<0.05$). Furthermore, there was a significant interaction between the three factors studied ($\rho<0.05$).

Table 1 shows that in immediate and long-term groups, in general PA+E group presented the highest µTBS mean values for all dentin depths, except for deep dentin in long-term group ($\rho<0.05$). The superficial dentin showed the highest µTBS mean values for all adhesive application protocols and aging ($\rho<0.05$), except for PA+E and E groups in long-term which presented no statistical difference in µTBS mean values for all dentin depth ($\rho>0.05$). On other hand, the deep dentin showed the lowest µTBS mean values for all adhesive application protocols in immediate group ($\rho<0.05$). The thermocycling decreased significantly the µTBS mean values for all adhesive application protocol and dentin depth ($\rho<0.05$), except for E group in middle dentin ($\rho>0.05$).

The PA+E in both immediate and long-term groups obtained the lowest and the highest microleakage mean values, respectively ($\rho<0.05$). The E group showed the lowest microleakage mean values in immediate and long-term groups ($\rho<0.05$). In immediate aging, PA+E and E groups showed no statistical difference in microleakage mean values ($\rho>0.05$). Generally, deep dentin showed the highest microleakage values for all adhesive application protocols in both the immediate and long-term aging ($\rho<0.05$), except for PA+E group in long-term which presented no statistical difference for all dentin depth ($\rho>0.05$). The thermocycling increased significantly the microleakage for all adhesive application protocol and dentin depth ($\rho<0.05$), except for C group in superficial dentin ($\rho>0.05$). *(Table 2 and Figure 2*).

Pearson›s correlation showed a strong negative correlation between microleakage and µTBS for the superficial ($\rho=-0.609$; $\rho<0.001$), middle ($\rho=-0.598$; $\rho<0.001$) and deep ($\rho=-0.814$; $\rho<0.001$) dentin depths. Fracture pattern analysis predominantly exhibited adhesive failures for all groups *(Figure 3*).

DISCUSSION

The present study showed that the EWB protocol significantly increased the µTBS (PA+E group) and decreased the microleakage (E group) for immediate and long-term aging *(Tables 1 and 2*, respectively). Thus, the first hypothesis was accepted. The second and third hypotheses were also accepted, since

Table 1. Microtensile bond strength mean (±SD) values (MPa) according to adhesive application protocol, dentin depth and aging. Percentage (%) of microtensile bond strength variation between immediate and long-term.

Aging	Dentin Depth	Adhesive Application Protocol		
		C	PA+E	E
Immediate	Superficial	40.64 (8.57)* Aa	45.75 (9.66)* ABa	38.33 (9.75)* Aa
	Middle	33.33 (13.62)*ABb	49.22 (11.50)*Aa	33.80 (7.56) ABb
	Deep	27.60 (4.20)* Bb	40.89 (5.94)* Ba	31.22 (3.92)* Bb
Long-term	Superficial	28.16 (5.00) Aa (-31%)	20.69 (3.63) Aa (-55%)	24.70 (3.17) Aa (-36%)
	Middle	24.46 (4.96) ABa (-27%)	19.16 (1.60) Aa (-61%)	26.80 (3.41) Aa (-21%)
	Deep	21.80 (1.97) Bab (-21%)	18.02 (1.88) Ab (-56%)	23.06 (2.46) Aa (-26%)

Different letters indicate statistically significant differences: lowercase letters for comparison between adhesion protocols for the same dentin depth and aging (rows) and capital letters for comparison between dentin depths for the same adhesion protocol and aging (column). Asterisk indicates differences in aging for each dentin depth within each adhesion protocol ($\rho<0.05$).

the dentin depth influenced on µTBS (*Table 1*) and microleakage (*Table 2*), and the long-term jeopardized the stability of the µTBS (*Table 1*).

The EWB protocol relies on the water present in the substrate being replaced gradually by resin monomers, promoting a more hydrophobic hybrid layer.[9,20,21] The EWB protocol would have the potential to generate a contraction in the diameter of the collagen fibrils, being greater than the contraction of the collagen matrix volume. This situation results in an increase in the interfibrillar spaces, which by consequently it promotes an increase in the efficiency of resin monomers infiltration.[22] Furthermore, the EWB can decrease phase separation, since it reduces the amount of water to avoid unwanted blister and droplet formation of the water residues inside the bonding resin layer, jeopardizing bond strength.[20] Thus, the EWB protocol applied to dentin optimizes the monomeric infiltration rate, improves the sealing of the collagen matrix, reduces the permeability of the polymerized adhesive layer, and minimizes water sorption by polymers, forming more stable and less permeable bonding interface,[20,21,23] corroborating with the results of the present study (*Tables 1 and 2*, respectively).

Table 2. Microleakage mean (±SD) values (%) according to adhesive application protocol, dentin depth and aging.

Aging	Dentin Depth	Adhesive Application Protocol		
		C	PA+E	E
Immediate	Superficial	31.90 (11.08) Ba	25.44 (3.97)* Bb	24.61 (3.12)* Bb
	Middle	34.26 (2.00)* ABa	30.17 (2.08)* Ab	27.83 (1.06)* ABb
	Deep	36.13 (2.25)* Aa	31.27 (2.60)* Ab	29.53 (2.07)* Ab
Long-term	Superficial	35.39 (3.12) Bb	42.20 (1.79) Aa	31.05 (1.65) Bb
	Middle	37.86 (2.80) Bb	43.50 (1.93) Aa	33.85 (3.09) Bc
	Deep	41.06 (3.55) Ab	45.25 (1.13) Aa	38.86 (1.66) Ab

Different letters indicate statistically significant differences: lowercase letters for comparison between adhesion protocols for the same dentin depth and aging (rows) and capital letters for comparison between dentin depths for the same adhesion protocol and aging (column). Asterisk indicates the difference in aging for each dentin depth within each adhesion protocol ($\rho<0.05$).

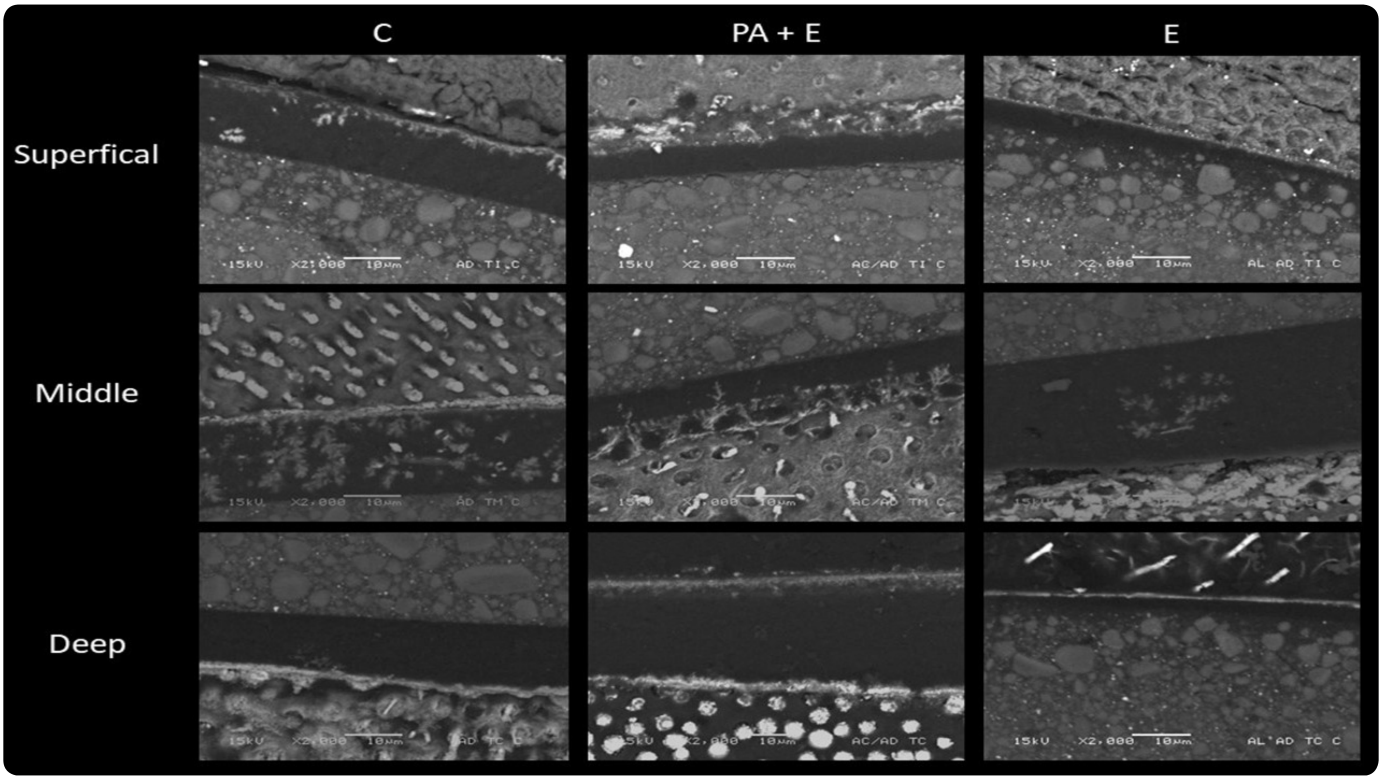


Figure 2: Microleakage representative SEM images.

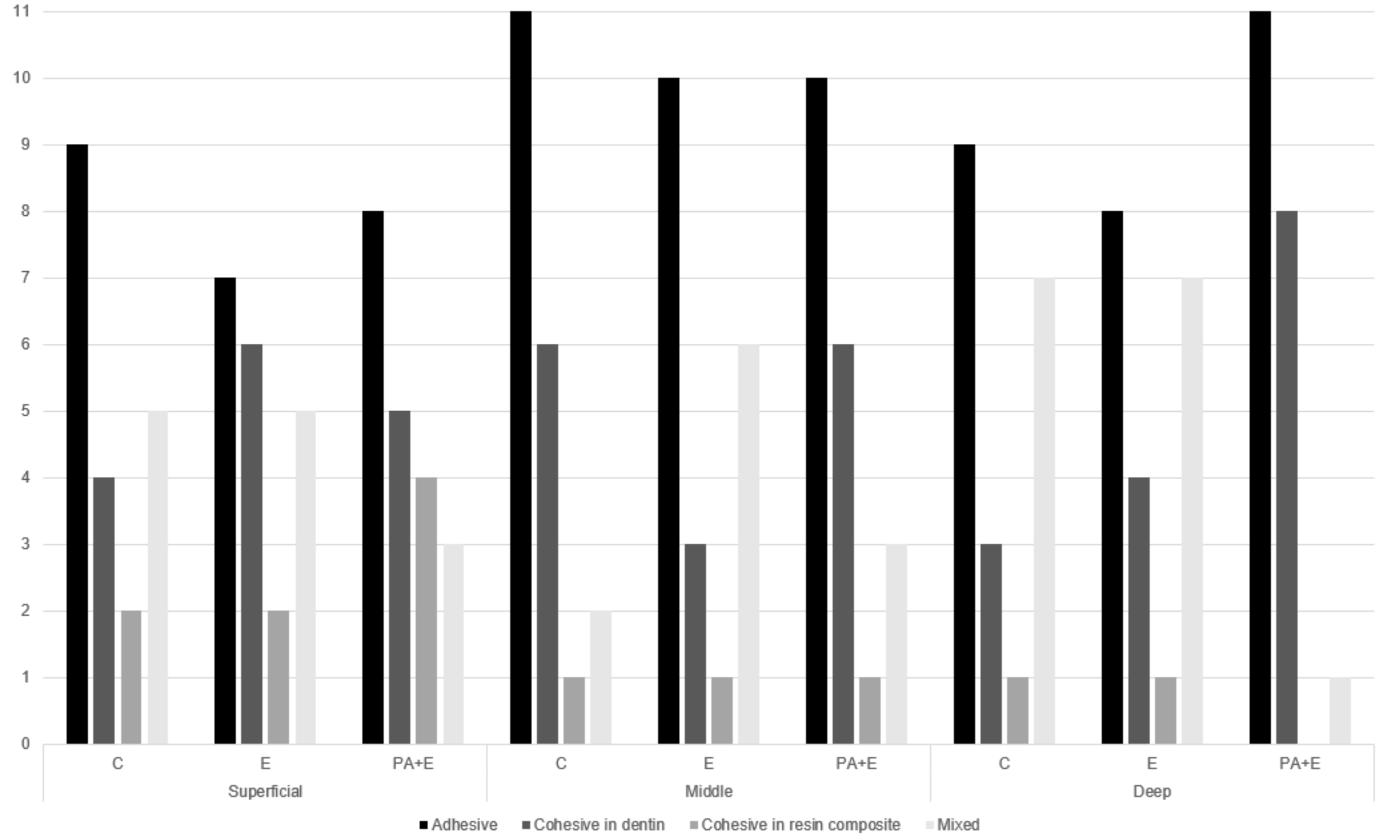


Figure 3: Fracture pattern analysis of all groups.

Several studies show that the non-collagen proteins (such as the matrix metalloproteinases) and remaining water are removed by the ethanol.[20] The resin monomers are dissolved in the ethanol through the interfibrillar field, leading to the molecular immobilization of the proteins after the polymerization process.[24] The enzyme molecules are covalently linked to each other to form a matrix, and this reaction ensures that the binding site does not cover the active (catalytic) site of the enzyme.[24] The reduction of the enzymatic degradation of collagen fibrils will lead to an increase in the hybrid layer stability and durability.[25]

The literature validates the use of the EWB protocol by Hoy's triple solubility parameter theory, which consists of polar forces (δp) used to predict if the material can re-expand the dentin, hydrogen bonding forces (δh), dispersive forces (δd) and total cohesive (δt) forces.[26] A solvent and a resin monomer need to blend with a hydrogen force higher than 14.8 $(Jcm^3)^{½}$ to re-expand a collapsed dentin.[26-28] Ethanol has 20.0 $(Jcm^3)^{½}$. Therefore, it can break hydrogen bonds and promote a re-expansion of the collapsed dentin.[20]

For the microleakage evaluation, the EWB protocol (PA+E and E groups) showed the lowest mean values, except for PA+E in long-term group. This result can be attributed to the poor infiltration of resin monomers into the rich collagen area of the demineralized dentin. The phosphoric acid etching on dentin leaves gaps in the hybrid layer where water (thermocycling test) can infiltrate, producing hydrolysis of the exposed collagen not protected by resin monomer.[29] Furthermore, the microleakage might be influenced by external stress produced during thermocycling, which causes thermal variation and create gaps. This scenario may hinder the mechanical properties of the resin-based materials by creating openings and deforming the tooth, jeopardizing the microleakage for PA+E in long-term group *(Table 2)*. It is important to emphasize that the ethanol used with universal adhesive in self-etch mode (E group) promotes less incorporation of water during the polymerization and as consequence a less hydrophilic hybrid layer.[30,31] Also, hydrophobic monomers (universal adhesive) will perform better with ethanol than water.[32] This may explain why only in PA+E group there was an increase in the microleakage in long-term evaluation *(Table 2)*.

As mentioned previously, the water plays an important role in the bonding process and it is responsible for the ionization process of the resin monomers. The resin-based materials have a high percentage of ionic resin monomers in its composition, which will lead to the osmotic imbibition of fluids from the tubules of dentin.[20,33] This would explain the results of μTBS *(Table 1)* and microleakage *(Table 2)* for all groups in long-term evaluation. Moreover, the more dentinal tubules present, the more fluid in the tissue, and the greater the probability of hydrolysis. Hydrolysis reduces the μTBS and increases the microleakage,[20,33] corroborating with the results of the present study, since the deep dentin showed the worst μTBS and microleakage results *(Tables 1 and 2*, respectively). In this study, there was a strong negative correlation between microleakage and μTBS for all dentin depths, which indicates that the higher the microleakage rate, the lower the μTBS mean value.

Several factors such as polymerization shrinkage and technique, adhesive system, coefficient of thermal expansion, and quality of substrate (enamel or dentin) are reported to influence microleakage.[34] Combining microleakage measurement with bond strength test may provide useful information on the durability of the adhesive interface in resin composite restoration. Furthermore, the μTBS testing can reveal valuable clinical information and if an aging test is performed, then the durability of adhesion can be measured,[35] since a condition for predictable bonding is to avoid microleakage.[36] The dentin has high absorbing capability due to water content. The thermocycling results in high temperature, water evaporation, and smear layer removal[37] and causes thermal variation and external stress, creating gaps[29] which result in lower μTBS and higher microleakage *(Tables 1 and 2*).

The fracture pattern analysis revealed more adhesive failures for all groups, regardless of the adhesive application protocol and dentin depth tested. This finding is similar to other studies, which reported decreased μTBS with increased adhesive failure after thermocycling. The thermocycling test promotes degradation of adhesive interface with aging.[38,39]

This study showed that EWB protocol combined with universal adhesive could significantly improve the μTBS and microleakage mainly in superficial and middle dentin depths. However, further studies, regarding its association to other adhesive systems and also the different times of application are still needed to indicate the best clinical protocol. It is important to consider that ethanol can jeopardize the action of the hydrophobic monomers of the adhesive, but it also can negatively affect the hydrophilic monomers if it reduces the water content in excess. Therefore, it is important to find an equilibrium between those two parts, to improve the hydrophobic part without compromising the hydrophilic part of the universal adhesive. Moreover, In vivo studies are necessary to evaluate the clinical significance of these findings and to associate them with other clinical factors related with longevity of the resin composite restorations not evaluated in the present study. So far, unfortunately the EWB protocol is unable to replicate $^{\text{in vivo}}$ the benefits observed *in vitro*[40].

CONCLUSION

The EWB protocol can improve the immediate adhesion, since the PA+E group improved the μTBS and E group promoted a more stable μTBS in the long-term and showed the lowest microleakage values. The long-term jeopardized the μTBS and microleakage for all groups and the superficial and middle dentin depths showed the best μTBS and microleakage results.

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