DENTAL MATERIALS 35 (2019) 900–908

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.intl.elsevierhealth.com/journals/dema

Investigation of five α-hydroxy acids for enamel and dentin etching: Demineralization depth, resin adhesion and dentin enzymatic activity

Lívia Tosi Trevelin[a,b], *Jose Villanueva*[a], *Camila A. Zamperini*[a], *Mathew T. Mathew*[a], *Adriana Bona Matos*[b], *Ana K. Bedran-Russo*[a,*]

[a] *Department of Restorative Dentistry, College of Dentistry, University of Illinois at Chicago, UIC, Chicago, IL, USA*
[b] *Department of Operative Dentistry, College of Dentistry, University of São Paulo, USP, São Paulo, SP, Brazil*

ARTICLE INFO

Article history:
Received 5 December 2018
Received in revised form
11 March 2019
Accepted 15 March 2019

Keywords:
Enamel
Dentin
α-hydroxy acids
Surface etchants
Resin adhesion
Enzymatic activity
Optical profilometry
Micro-permeability

ABSTRACT

Objectives. Surface conditioning of enamel and dentin is a key step during adhesive restorative procedures and strategies. The aim of this study was to investigate the effectiveness of five α-hydroxy-acids (AHAs) as enamel and dentin surface etchants.

Methods. Enamel and dentin specimens were prepared from human molars to determine the depth of demineralization by optical profilometry (Δz), the resin bond strength to enamel and dentin (μTBS), the micro-permeability of dentin–resin interfaces, and the gelatinolytic activity of dentin matrix induced by AHAs [glycolic (GA), lactic (LA), citric (CA), malic (MI) and tartaric (TA)] and controls [phosphoric (PA) and maleic (MA)]. All acids were prepared at 35% concentration. Adhesion studies employed Adper Single Bond Plus bonding system. Data were individually processed and analyzed by ANOVA, post-hoc tests and Pearson correlations ($\alpha = 0.05$).

Results. AHA exhibited statistically lower depth of demineralization of enamel and dentin (average 4 fold) than controls ($p < 0.001$). In enamel, MA and PA etching resulted in higher μTBS than AHA groups ($p < 0.001$). In dentin, GA, TA, CI and LA etching resulted in statistically similar μTBS than PA ($p < 0.05$). The hybrid-layer (HL) thickness and interfacial micro-permeability intensity were statistically lower for AHA groups ($p < 0.05$). A significant positive correlation was observed between the intensity of micro-permeability and the thickness of HL ($p < 0.05$). AHA etchants elicited lower dentin enzymatic activity than controls ($p < 0.05$).

Significance. AHAs effectively etched enamel and dentin surfaces. In particular, GA and TA resulted in suitable μTBS and sealing ability as well as induced less gelatinolytic activity in dentin than PA and MA.

* *Corresponding author at*: University of Illinois at Chicago, College of Dentistry, 801 S Paulina St. room 531, Chicago, IL, 60612, USA.
E-mail address: bedran@uic.edu (A.K. Bedran-Russo).

https://doi.org/10.1016/j.dental.2019.03.005
0109-5641/

1. Introduction

Surface conditioning is a prerequisite step for the micro-mechanical interlocking of dental resins to enamel and dentin. In the etch-and-rinse adhesion strategy, a separate acid etching step dissolves the mineral phase and exposes a collagen dense dentin matrix. Dental resin monomers then infiltrate to form resin tags and a hybrid layer [1]. Phosphoric acid (PA) (30–40%) is the conditioner of choice for enamel and dentin. Within a short application time, PA increases the surface area, wettability and roughness [2]. The etching pattern associated with the low water content of PA-treated enamel favors resin penetration resulting in stable adhesion [3,4]. On the contrary, the high water (~20%v) and organic content (~30%v) [5,6] accompanying by increased tissue permeability, present an immediate challenge for the infiltration and polymerization of dental resins and the long-term stability of the dentin–resin bonds.

Ideally, the intra and inter-fibrillar space of the dentin matrix must be completely filled by resin [1,7]. However, suboptimal infiltration of resin monomers within a 5-μm [8] PA-etched dentin leaves exposed type I collagen fibrils that are susceptible to degradation [8,9]. Hence, the acidity of PA triggers endogenous enzymes to breakdown the exposed collagen fibrils, a key component of the dentin matrix for anchoring dental resins [9,10].

The depth of dentin demineralization does not correlate with bonding effectiveness [3]. Instead, it may induce structural changes to collagen; [2] and increase the micro and nano-porosity at the adhesive interface [8,9,11]. Alternatives to PA have included other acids [12–16] and chelating agents [17,18]. Recently, glycolic acid (GA) etching resulted in similar resin adhesion of various dental adhesive systems to enamel and dentin when compared to PA [19,20]. The α-hydroxy acids (AHAs) contains a carboxylic acid moiety with an adjacent hydroxy group in α position. AHAs are weak organic acids extensively used in cosmetic formulations to induce skin turnover and to prevent skin disorders [21].

Surface conditioning that induces minimal disruption to the extracellular matrix while effectively etching enamel and dentin may aid in the stability of the dentin–adhesive interfaces. Based on their pH and pKa in solution, herein we investigated the effectiveness of selective AHAs on the demineralization depth, resin adhesion to enamel and dentin and endogenous enzymatic activity of dentin. The specific research hypotheses were (a) AHA etching would yield similar depth of demineralization in enamel and dentin substrate than controls (b) AHA etching would create a thinner and less permeable dentin–resin interfaces when compared to controls and, (c) AHAs would induce less endogenous enzymatic activity when compared to controls. To test these hypotheses, we comprehensively assessed the dentin and enamel and their adhesive interfaces using optical profilometry, micro-tensile bond strength (μTBS), interfacial fluorescence micro-permeability, and dentin matrix gelatinolytic activity essays.

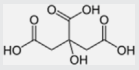
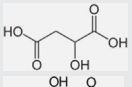
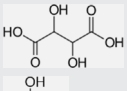
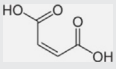
2. Materials and methods

2.1. *Teeth and experimental acids*

De-identified extracted sound human molars were selected, cleaned and kept frozen (−20 °C) until use (approved by the University of Illinois at Chicago IRB #2011-0312). In general, studies of enamel utilized polished buccal and lingual surfaces, while polished occlusal surfaces were prepared for studies on dentin. Pulverized coronal dentin was prepared for gelatinolytic assay.

The selected AHAs, control acids, and their final pH and pKA are shown in Table 1. The reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), and prepared to a final concentration of 35% in distilled water. The pH values of the seven etching solutions were measured at room temperature (22–25 °C) using a pH meter (Model 8603, Mettler Toledo, Inc., Columbus, Ohio, USA).

Table 1 – Description of pH and pKa, molecular weight, chemical formulas and structures of the experimental surface etchants acids.

Surface etchant (Acids)	pH&(pKa)	Molecular weight(m.w.)	Chemical formula	Chemical structure
Glycolic	1.4 (3.83)	76.05	$C_2H_4O_3$	
Lactic	1.4 (3.86)	90.08	$C_3H_6O_3$	
Citric	1.24 (3.13)	192.12	$C_6H_8O_7$	
Malic	1.44 (3.4)	134.08	$C_4H_6O_5$	
Tartaric	1.2 (2.98)	150.08	$C_4H_6O_6$	
Phosphoric	0.3 (2.14)	97.99	H_3PO_4	
Maleic	0.76 (1.92)	116.10	$C_4H_4O_4$	

2.2. Surface demineralization depth - optical profilometry

A total of eleven (11) enamel specimens from the buccal and lingual surfaces and 11 dentin specimens from occlusal dentin surface were sectioned into dimensions of 4 × 4 × 2 mm using a diamond saw under water irrigation (Isomet 1000, Buehler, Lake Bluff, IL, USA). The enamel and dentin fragments were divided into seven groups (n = 8), according to the experimental etchants (Table 1). The embedded fragments in epoxy resin were polished wet (EcoMet 3000, Buehler, Lake Bluff, IL, USA) with abrasive paper (#400, 600, 800 and 1200 grit). The specimens were cleaned ultrasonically in deionized water for 15 min to remove residual polishing material and their surfaces were protected with one layer of nail varnish, except for a 3 × 3 mm central area. Acids were applied on the surfaces of enamel and dentin for 15 s and thoroughly rinsed with distilled water for 30 s. The nail polish layer was removed and the specimens scanned in an optical profilometer (Nano Contour GT-K, Bruker, Cheryl Pkwy, Fitchburg, WI). The scanned area encompassed the etched and non-etched surfaces (previously protected by nail polish). The demineralization depth (ΔZ) was calculated by subtracting the average height of the etched area from the average height of the untreated area (Vision64 imaging software, Bruker Optical Profilometer). Intragroup variability was assessed using Levene's test and found to not meet homogeinity assumption (p = 0.001). Thus, data (ΔZ) were statistically analyzed using one-way ANOVA followed by Games-Howell post hoc test (α = 0.05).

2.3. Studies of resin adhesion to enamel and dentin

A total of 98 teeth were used for adhesion studies. Occlusal dentin (49 specimens) and buccal enamel (49 specimens) surfaces were prepared to produce dental adhesive interfaces. The crowns were sectioned using a low-speed diamond saw (Isomet 1000, Buehler Ltd.) and further polished with 180 and 320 grit abrasive paper to expose middle dentin. The enamel specimens were obtained by first sectioning the crown with a low-speed diamond saw and then wet polishing the buccal with 320 grit abrasive paper to obtain a flat enamel surface. Both dentin and enamel received a final polish with # 600 grit abrasive paper to standardize the smear layer. The dentin and enamel specimens were randomly divided into 7 experimental groups (n = 7), according to the etchants (Table 1). After the etching procedures, two consecutive adhesive drops of Adper Single Bond (batch #N848811; 3M ESPE; St. Paul, MN, USA) were applied on the surface, the excess solvent was evaporated, and the surface light-cured for 40 s (intensity of 700 mW/cm2; Optilux 501, Kerr Corp., Orange, CA, USA). A 4-mm resin composite (Z250- Filtek batch # 1,511,900,505; 3M ESPE; St. Paul, MN, USA) was built-up in four increments of 1-mm thick. Each increment was polymerized for 40 s. The specimens were stored in simulated body fluid (SBF) at 37 °C for 24 h prior to serial sectioning into specimens with an adhesive interface area of approximately 0.8 × 0.8 mm^2. The SBF consisted of 5 mM HEPES, 2.5 mM $CaCl_2$, 0.05 mM $ZnCl_2$, and 0.3 mM NaN_3 [22].

2.3.1. Strength of adhesion - microtensile bond strength

A total of seven (7) dentin–resin specimens were selected per tooth. The specimens were mounted in a jig and tested in a tensile testing instrument (Bisco, Schaumburg, USA) at a crosshead speed of 1.0 mm/min. The bond strengths were calculated by dividing the peak load by the adhesive interface cross-sectional area. Intragroup variability was assessed using Levene's test and found to be homogenously distributed for enamel and dentin (p = 0.53 and p = 0.26, respectively). Data were then statistically analyzed using one-way ANOVA, followed by Scheffe's post hoc test (α = 0.05).

2.3.2. Interfacial micro-permeability and thickness of hybrid layer - Fluorescence microscopy

The micro-permeability of the adhesive interface was determined by detection of infiltrated fluorescent dye. Two (2) resin–dentin specimens per tooth (n = 7) were randomly selected, embedded in epoxy resin, and polished with SiC abrasive papers (#320, 600, 800, and 1200 grit). Specimens were immersed in freshly prepared 0.1 mM rhodamine-B solution (pH 7.2, RITC/ Rhodamine-B; Sigma) for 1 h, rinsed for 1 min with deionized water. The fluorescence intensity of infiltrated rhodamine-B was analyzed under a fluorescence microscope (DMI6000 B, Leica), using red emission and DIC (differential interference contrast) channels. Images were taken from each beam (0.8 × 0.8 mm) at ×10 magnification. The fluorescent emission intensity (FEI) of infiltrated rhodamine-B and the HL thickness were assessed by a calibrated examiner blind to the groups/acids. The values of interfacial micro-permeability were obtained using a parallel line profile traced along the adhesive interface [23] by an image analysis software (Image J 1.48p, National Institutes of Health, USA). The HL thickness was measured in 5 different sites along the length of the resin–dentin interface using Image J tools. The FEI and the HL thickness data variability was assessed using Levene's test (p = 0.007 and p = 0.193, respectively). Data were statistically analyzed using one-way ANOVA, followed by Games–Howell or Scheffe's post hoc tests (α = 0.05). In addition, correlations between the thickness of HL, micro-permeability and gelatinolytic activity was carried out using Pearson correlation coefficient at a significance level of α = 0.05.

2.4. Endogenous proteases gelatinolytic activity

Eighteen (18) molars with complete root formation had the enamel, pulp tissue and cementum removed. The dentin remnants were cryo-pulverized (CryoMill, Retsch GmbH, Haan, Germany) into a fine powder with average size particles of 0.5 μm. The dentin powder was demineralized in 5% formic acid [24] in a ratio of 1:5 for 14 days at 4 °C under constant stirring. The dentin powder was rinsed 5 times with distilled water, centrifuged (12,000 rpm, 5 min, at 4 °C) and lyophilized. The lyophilized dentin was divided into 7 groups according to the etchants (Table 1). Aliquots of dentin powders (5 mg) were etched with experimental acids (25 ml) for 1 min, the reaction was stopped by raising the pH 7.0 with 4 M NaOH, and then centrifuged at 10,000 rpm for 2 min. The supernatant was discarded and the acid-etched dentin powder was re-suspended in distilled water and centrifuged for

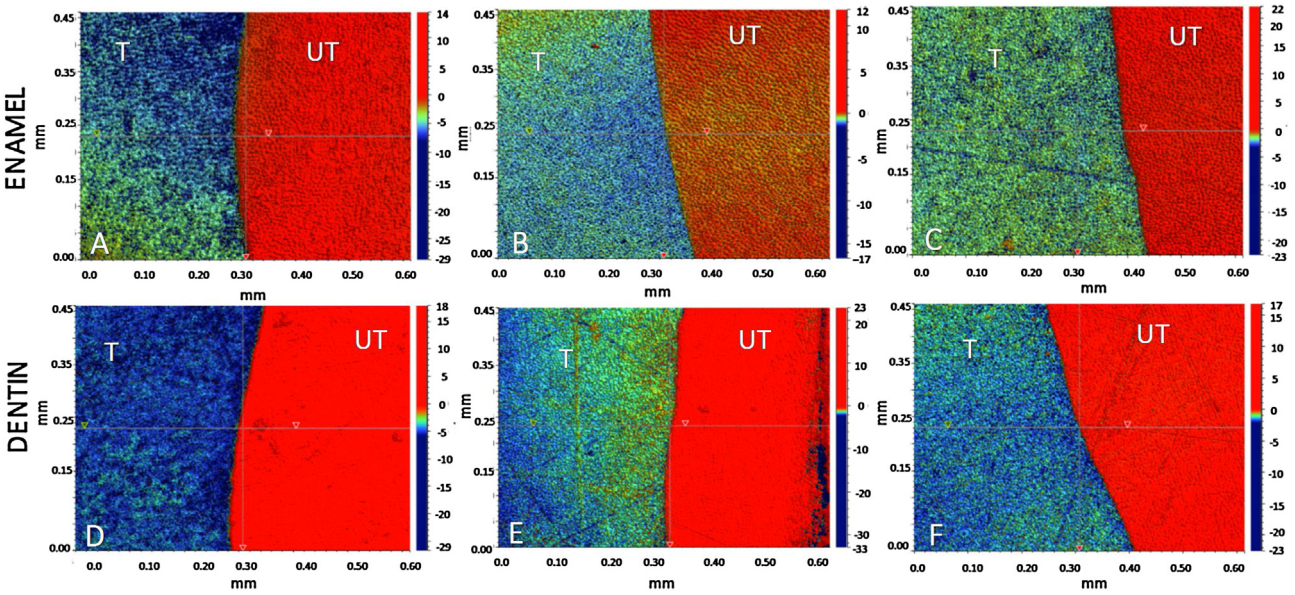


Fig. 1 – Representative 2D images of enamel and dentin surfaces etched for 15 s with experimental acids. (A) enamel etched with phosphoric acid, (B) enamel etched with glycolic acid, (C) enamel etched with citric acid, (D) dentin etched with maleic acid, (E) dentin etched with lactic acid, and (F) dentin etched with tartaric acid. Right insets depicts color mapping of the surface topographies in μm, areas of distinct colors have different topographies (depth of demineralization). Red color corresponds to high values of topography and blue color corresponds to low values of topography (μm). UT: untreated surface; T: etched surface (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

1 min. The rinse procedure was repeated 3 times. Then, the etched powder was re-suspended in reaction buffer (0.5 M Tris–HCL, 1.5 M NaCL, 50 mM $CaCl_2$, 2 Mm Sodium Azide, pH 7.6). A total of 10 μL of suspended power (dentin powder 0.5 mg/10 μl in reaction buffer) were incubated with 20 μl of DQ gelatin EnzChek Gelatinolytic/Collagenolytic Assay Kit (D-12054, Molecular Probes, Eugene, OR, USA) and completed with buffer reaction to reach 200 μl final volume in each well. Negative controls were incubated in the presence of 5 mM EDTA [25] and stop solution (Sensolyte 520, Activity Assay Kit, Anaspec, USA). The plates were incubated at 37 °C and the fluorescent cleavage products were read in a 96-well fluorescent plate reader (Victor X5, Perkin Elmer, Waltham, MA, USA), operated with absorption maxima at 495 nm. The relative fluorescence units (RFU) were recorded continuously at 535 nm every hour for 48 h. All analyses were carried-out in quadruplicate whereas gelatinase standards as well as reagent blanks were carried out in triplicate. The RFU was calculated by subtracting the background fluorescence of the dentin powder and the mean background fluorescence of the reagent blanks. After 25 h of incubation, a plateau in the gelatinolytic activity was reached for all acids, thus statistical analyzes were carried out using the first 24 h. Gelatinolytic activity data variability was assessed using Levene's test ($p = 0.364$). Therefore, the data were statistically analyzed until 24 h using two-way ANOVA repeated measures and Tukey's post-hoc test ($\alpha = 0.05$).

3. Results

3.1. *Surface demineralization depth*

Representative 2D profiles of enamel and dentin surface demineralization are shown in Fig. 1. The average demineralization depth (Δz) of enamel by AHA ranged from 0.69 to 1.20 μm for malic and glycolic groups, respectively. No significant differences were observed between AHA groups ($p < 0.05$). However, a significantly greater Δz average was observed for control groups (phosphoric: 3.94 μm and maleic: 4.37 μm).

In dentin, the average Δz ranged from 1.20 to 1.43 μm for tartaric and glycolic, respectively. AHA groups showed intermediate demineralization with no statistical differences among them and with the phosphoric acid group. The average Δz (6.17 μm) of maleic acid was significantly higher than all other groups ($p < 0.05$).

3.2. *Resin adhesion studies*

3.2.1. *Strength of adhesion*

The bond strength results are shown in Table 2. In enamel, phosphoric resulted in statistically higher bond strength values when compared to all groups ($p < 0.001$), except maleic group ($p < 0.05$). Among the AHA groups, the lactic group

Table 2 – Results [mean and (standard deviations)] of demineralization depth, microtensile bond strength, interfacial micro-permeability and hybrid layer thickness. Superscript letters next to means depict statsitically significant differences among groups ($p < 0.05$), in each column and tooth substrate (enamel and dentin).

Substrate	Surface etchant (Acids)	Demineralization depth (μm)	Bond strength (MPa)	Interfacial micro-permeablity (FEI)	Hybrid layer thickness (μm)
Enamel	Glycolic (GA)	1.20 ^{B} (0.95)	28.66 ^{B} (4.35)	–	–
	Lactic (LA)	0.99 ^{B} (0.53)	21.32 ^{C} (2.39)	–	–
	Citric (CA)	0.93 ^{B} (0.66)	24.60 ^{B,C} (2.26)	–	–
	Malic (MI)	0.69 ^{B} (0.67)	24.80 ^{B,C} (4.03)	–	–
	Tartaric (TA)	1.09 ^{B} (0.44)	26.31 ^{B,C} (4.54)	–	–
	Phosphoric (PA)	3.94 ^{A} (1.16)	35.25 ^{A} (2.3)	–	–
	Maleic (MA)	4.37 ^{A} (1.55)	29.83 ^{A,B} (2.89)	–	–
Dentin	Glycolic (GA)	1.43 ^{B} (0.71)	53.39 ^{A} (8.24)	13.81 ^{B} (5.09)	2.98 ^{B} (0.76)
	Lactic (LA)	1.27 ^{B} (0.9)	51.28 ^{A} (15.01)	22.58 ^{A,B} (14.06)	3.64 ^{AB} (0.83)
	Citric (CA)	1.09 ^{B} (0.45)	51.98 ^{A} (9.8)	16.59 ^{A,B} (5.17)	3.04 ^{B} (0.46)
	Malic (MI)	1.31 ^{B} (0.62)	50.30 ^{B} (3.6)	22.21 ^{A,B} (8.13)	2.90 ^{B} (0.44)
	Tartaric (TA)	1.20 ^{B} (0.46)	52.50 ^{A} (7.02)	16.18 ^{A,B} (5.24)	3.18 ^{B} (0.57)
	Phosphoric (PA)	4.03 ^{A,B} (2.11)	60.49 ^{A} (4.43)	29.10 ^{A} (13.71)	3.89 ^{A} (0.99)
	Maleic (MA)	6.17 ^{A} (3.15)	51.06 ^{B} (3.56)	25.45 ^{A,B} (5.34)	3.90 ^{A} (0.9)

showed the lowest values of bond strength with significant differences only to glycolic ($p < 0.001$), maleic ($p < 0.001$) and phosphoric ($p = 0.004$) groups. Citric, malic, tartaric and glycolic acids showed statistically similar enamel bond strength values ($p > 0.05$). In dentin, all AHAs showed similar values of μTBS with no significant differences among groups ($p < 0.05$). Lactic, glycolic, citric and tartaric acids were not significantly different than controls (phosphoric and maleic acids, $p > 0.05$). Surface etching with malic and maleic acids resulted in the lowest values of μTBS, which were significantly lower than phosphoric acid ($p < 0.05$).

3.2.2. *Interfacial micro-permeability and hybrid layer thickness*

The results of micro-permeability and hybrid layer thickness are summarized in Table 2. Phosphoric acid group exhibited the highest fluorescent intensity at the adhesive interface, with statistically significant differences to glycolic and tartaric groups ($p < 0.001$). Maleic showed statistically higher values of permeability than glycolic acid ($p < 0.001$). The lowest micro-permeability at the resin–dentin interface was observed for the glycolic group, although no significant differences were observed between AHA groups ($p < 0.05$). Representative fluorescence images of resin–dentin interfaces of experimental groups are found in Supplemental material (Appendix A).

The thickness of the hybrid layer was significantly affected by the etchant solutions (Table 2). Surface etching with phosphoric, maleic and lactic acids produced thicker HL. The control groups (phosphoric and maleic) formed HLs which were statistically thicker than glycolic, tartaric, citric, and malic groups ($p = 0.005$). Lactic acid produced a HL statistically higher than glycolic and malic groups ($p = 0.005$). The results showed a statistically significant correlation between micro-permeability intensity and HL thickness ($p = 0.005$; Fig. 2A). A positive correlation was observed between the increase in micro-permeability levels with thicker HLs, particularly for phosphoric and maleic groups. Glycolic exhibited the lowest micro-permeability rates and thinner HL when compared to the other groups, the lower fluorescence intensity was strongly correlated with the reduced thickness of the HL ($r = 0.76$)

3.3. *Gelatinolytic activity*

There was a significant interaction between studied factors (acids vs. incubation time, $p < 0.001$). There were also significant differences among acids ($p < 0.001$) and incubation time ($p < 0.001$). All groups depicted similar values of RFU until 16 h of incubation ($p < 0.05$). At 20 h of incubation, the gelatinolytic activity reached the maximum activity (Supplementary data – Appendix B). Within 20 h incubation (Fig. 3), phosphoric and maleic exhibited higher values of RFU with statistically significant differences to AHA groups ($p < 0.05$). Furthermore, citric showed an intense peak of RFU, which differed statistically from glycolic and lactic groups ($p < 0.001$). In contrast, there was no statistical difference among glycolic, tartaric, lactic and malic groups ($p > 0.05$).

After 24 h of incubation, maleic group resulted in statistically higher enzymatic activity as compared to glycolic, lactic and tartaric groups ($p < 0.05$). The phosphoric group showed statistically higher gelatinolytic activity when compared to lactic and glycolic ($p < 0.05$). No statistical difference was observed among AHA groups ($p < 0.05$). No fluorescence was detected in negative controls, i.e., both, EDTA-treated and specimens incubated with standard non-fluorescent gelatin (stop solution) (data not shown). The results showed statistically significant negative correlation between gelatinolytic activity and pH of etchants solutions ($p < 0.05$; Fig. 2B), where acidic etchants (lower pH value) exhibited higher rates of enzymatic activity.

4. Discussion

The dissolution of hydroxyapatite crystals is a prerequisite for dentin hybrid layer formation, often achieved by a separate step of acid etching. The current study investigated alternative

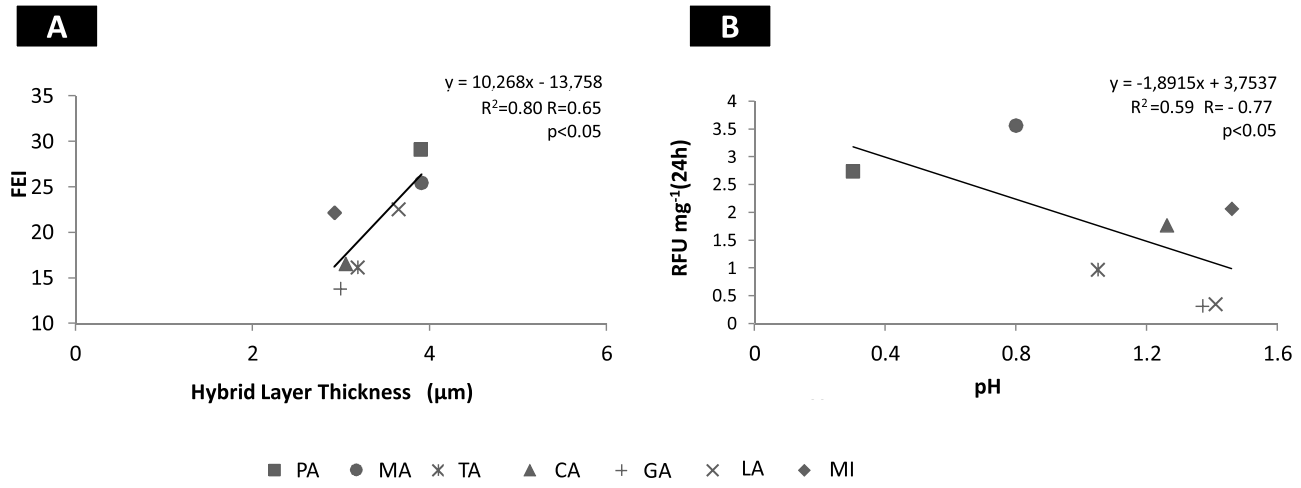


Fig. 2 – Results of the correlations between (A) Micro-permeability intensity (FEI) vs. hybrid layer thickness and (B) Gelatinolytic activity (RFU) vs. pH of the acids. A significant positive correlation was found between micro-permeability with thickness of the hybrid layer (A). There is a significant negative correlation between pH and gelatinolytic activity (B). PA: phosphoric acid; TA: tartaric acid; GA: glycolic acid; MI: malic acid; MA: maleic acid; LA: lactic acid; CI: Citric acid; FEI: Fluorescent Emission Intensity; RFU: Relative fluorescence units.

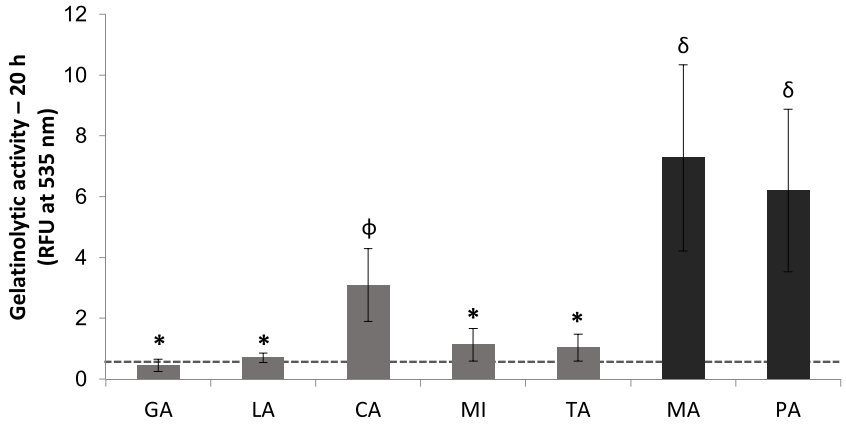


Fig. 3 – Results of the gelatinolytic activity of demineralized dentin treated with α-hydroxy acids and controls (phosphoric and maleic acids) for 1 min after 20 h incubation. Different symbols indicate statistically significant differences among groups ($p < 0.05$). Dashed line indicates values of demineralized dentin powder (formic acid). GA: glycolic acid; LA: lactic acid; CA: citric acid, MI: malic acid; TA: tartaric acid; MA: maleic acid; PA: phosphoric acid; RFU: relative fluorescence units.

approaches to etch dentin and enamel using AHAs to provide similar etching depths, produce similar bond strength, reduce interfacial micro-permeability interfaces, and reduce the gelatinolytic activity of dentin when compared to controls (phosphoric acid and maleic acid).

The surface etching pattern of AHAs resulted in shallower depths of demineralization of enamel and dentin when compared to control groups. Thus, the first null hypothesis was rejected. The mild acidity of AHA groups dissolved the hydroxyapatite crystals resulting in an average demineralization depth of approximately 1 μm and 1.26 μm deep in enamel and dentin, respectively. In contrast, phosphoric and maleic (controls), with the lowest pH values (pH < 1.0), led to four-fold deeper demineralization depth in both substrates, as clearly demonstrated by optical profilometry (Table 2). Interestingly, the control groups had large standard deviations, resulting in a lack of statistical differences to the glycolic and tartaric group in dentin substrates.

In enamel, the higher etching pattern of phosphoric and maleic resulted in high bond strength values. This is largely attributed to the readily dissolution of hydroxyapatite rods and deep micromechanical anchorage of resin tags [3]. Based on the same concept, the shallow demineralization pattern promoted by AHA groups in the enamel substrate supports lower resin bond efficiency. Although glycolic and maleic were not statistically different, they produced contrasting depth of demineralization.

The etching approach works less favorably in the dentin substrate. Our results clearly indicate that the high etching depth of maleic acid did not result in high bond strength. The aggressive etching of phosphoric and maleic, expose mineral free collagen network, which cannot be completely

hybridized [26,27] even with hydrophilic adhesives [11]. As a result, bound and unbound water fill into this zone, creating weaker resin–dentin interfaces likely vulnerable to hydrolytic breakdown over time. These features are distinctly supported by the high interfacial micro-permeability found for the control groups (Table 2). On the other hand, glycolic, tartaric, citric and lactic acids reached adequate bond strength values, which were statistically similar to gold standard phosphoric acid. Despite similar dentin bond strength to phosphoric acid, the glycolic and tartaric acids resulted in significantly lower micro-permeability. It is worth noting that this study used one commercial adhesive system and outcomes may vary based on the chemistry of different adhesive systems.

As expected, the thickness of the hybrid layer and demineralized zones depends on the pH/acidity of the solution [28,29]. Indeed, etch and rinse adhesives produce thick hybrid layers; [30] although excessive acid demineralization produced by control groups resulted in significantly thicker HLs when compared to glycolic, tartaric, citric and malic groups. Also, a significant correlation was observed between the thickness of the HL and the interfacial micro-permeability (Fig. 2A). For instance, glycolic acid formed a thinner HL, which positively correlated with increased sealing ability of resin–dentin interfaces (low values of micro-permeability). In theory, the degradation of dentin matrix at the interface may decrease with increases infiltration of resin monomers [8]. Interestingly, the lactic group produced statistically similar HL thickness than control groups and high micro-permeability at the resin–dentin interfaces. The findings support the positive correlation between thickness HL and the increase in dentin interfacial micro-permeability (Fig. 2 A). Thus, the second hypothesis that AHA etchants would create thinner and less permeable dentin–resin interfaces when compared to the control groups is partially rejected.

Herein it was observed that the HL thickness does not affect the dentin bond strength. Overall, lactic, glycolic, tartaric, citric and phosphoric acid etching resulted in varying thicknesses of HL while exhibiting statistically similar bond strength values. However, the HL thickness has been associated with resin–dentin degradation; [31] thus, it is speculated that high permeability of thick hybrid layer produced by phosphoric, maleic, and lactic would be more prone to both hydrolysis and enzymatic breakdown when compared to those formed by glycolic, tartaric, and citric acids. The suboptimal resin infiltration/encapsulation of the collagen fibrils in those groups makes the hybrid layer especially vulnerable to enzymatic degradation over time [32–35].

While the experimental acids belong to the AHA family, the mechanisms of tooth demineralization may be different. AHAs, as well as maleic acid, are organic acids containing at least a carboxylic acid which could be absorbed and bonded to hydroxyapatite by ionic interactions. These phenomena is explained by the adhesion-decalcification concept proposed by Yoshida et al. [15], involving two steps: (a) first, acid anions adsorb on the mineral surface to form calcium–anion complex, mainly determined by the pKa of the acids (Table 1); and (b) second, the acid anions will either remain attached to the biomineral surfaces with only limited dissolution or the calcium–anion complex will debond and promoted the decalcification process. The ability of a carboxylic acid to decalcify hydroxyapatite depends on the dissolution rate of the calcium–anion complexes into solution [15,36]. Noticeably, the dissociation constant for maleic and tartaric acids are the smallest among the organic acids investigated. At equal concentration, maleic and tartaric acids, in aqueous solution, are present in more dissociated form, which means more acidic (Table 1).

Besides the anion-adsorption scenario, the molecular size may be an ancillary factor influencing the diffusion behavior, given the same concentration of acids [36], as the molecular diffusion rate is roughly inversely proportional to its size (Table 1) [36,37]. Glycolic has the smallest molecular weight amongst all the AHAs, which might facilitate its diffusion rate along the tissue and demineralization. Glycolic acid has two carbon atoms: one carbon atom in the carboxyl group and the other in the hydroxy group (Table 1). Glycolic is extremely hydrophilic [38,39]. Therefore, the chemical structure of glycolic may favor superior demineralization profile in both substrates, especially in enamel substrate when compared to all other AHAs. Overall, the mechanism of action of AHA is not fully known [21], but the hard dental tissue demineralization with AHA was effective and less aggressive than demineralization performed by control groups.

Besides the different degrees of acid interaction with hydroxyapatite, it is well established that the use of acidic components uncovers the dentin matrix and activates proforms of latent endogenous dentin proteases (MMPs) [40,41]. This is precisely what the findings show; there was significant differences among acids treatment as well as the incubation times (Fig. 3)Overall, the results of this study support the tested hypothesis that etchants solutions are sufficiently acidic to activate gelatinolytic activity in dentin powder, particularly by the control acids (phosphoric and maleic). The maximum fluorescent cleavage products representing higher gelatin degradation was observed at 20 h of incubation, for all groups. The control groups (phosphoric and maleic) depicted the highest enzymatic activity while glycolic had the lowest level of gelatinolytic activation of demineralized dentin powder. Several studies have demonstrated that activation and functionality of dentin MMPs are dependent on pH [42–45]. Indeed, our results are consistent with previous findings, since control groups exhibited the lowest pH value and the maximum enzymatic activity. Although, the pH is not the only factor which determines the level of the enzymatic activity. In addition, we have shown that enzymatic activity of the dentin matrix varies as a function of the chemical structure of acid solution and therefore, their interaction with hydroxyapatite-crystals. Within AHA groups, malic group exhibited both high pH value as well as an intense enzymatic activity. We attribute this result to the chemical structure of the malic acid; which consists of a dicarboxylic acid group in contrast to the carboxylic group found in all other investigated AHAs (Table 1).

Among the control groups, phosphoric exhibited the lowest enzymatic activity and pH, although not statistically significant. An interesting observation was that phosphoric acid reduced the inherent dentin gelatinolytic activity until 14 h. It was shown that higher concentrations of phosphoric (35%–40%), exhibiting a pH as low as 0.4, elicit transient MMPs inactivation [43,45,46]. In contrast, maleic displayed an average pH of 0.8 and kept the highest enzymatic activity of

dentin powder along the 24 h incubation period. Thus, there is accumulating evidence that the chemical structure of acid solution and the correspondent pH have key roles in the gelatinolytic activity of dentin matrix. Here, a strong negative linear correlation observed between increase in fluorescent activity of demineralized dentin powder and the corresponding pH value (Fig. 2B), further demonstrates that MMPs will activate in a large variety of acidic conditions. Thus, the third study hypothesis, that AHAs will exhibit lower endogenous enzymatic activity when compared to control groups, was accepted. The findings highlight the importance of dentin treatment surface with less aggressive and more biocompatible acid solution. It is important to note that the immersion of dentin powder in acidic etchants for 1 min overestimates the clinical recommendation of 15 s [46].

In summary, overall AHAs, and more specifically glycolic and tartaric, exhibited a lower depth of demineralization, lower host-derived enzymatic activation, similar dentin bond strength, and lower interfacial micro-permeability when compared to control acids. None of the AHAs yielded similar enamel bond strength to phosphoric acid. Collectively, the research outcomes indicate that the use of AHAs, with short time of application, may be favorable for predictable and stable dentin bonds. The investigation will be extended to study the actual long-term outcomes.

5. Conclusion

Within the limitations of an in vitro study, it can be concluded that all investigated AHAs demineralize enamel and dentin, within an average 4-fold reduction in demineralization depth than controls (phosphoric and maleic), resulting in lower enamel bond strength than control acids. Dentin etching with glycolic and tartaric resulted in dentin bond strengths comparable to those of phosphoric acid, with the advantage of decreasing the interfacial micro-permeability and inducing the less gelatinolytic activity of dentin matrix.

Declaration of interest/Acknowledgments

This work was supported by the National Institutes of Health [DE021040], and FAPESP/Brazil [2015/12651-1, 2016/18118-6].

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.dental.2019.03.005.

REFERENCES

[1] Pashley DH, Tay FR, Breschi L, Tjaderhane L, Carvalho RM, Carrilho M, et al. State of the art etch-and-rinse adhesives. Dent Mater 2011;27:1–16.
[2] Zafar MS, Ahmed N. The effects of acid etching time on surface mechanical properties of dental hard tissues. Dent Mater J 2015;34:315–20.
[3] Carvalho RM, Manso AP, Geraldeli S, Tay FR, Pashley DH. Durability of bonds and clinical success of adhesive restorations. Dent Mater 2012;28:72–86.
[4] Mahn E, Rousson V, Heintze S. Meta-analysis of the influence of bonding parameters on the clinical outcome of tooth-colored cervical restorations. J Adhes Dent 2015;17:391–403.
[5] Marshall Jr GW, Marshall SJ, Kinney JH, Balooch M. The dentin substrate structure and properties related to bonding. J Dent 1997;25:441–58.
[6] Orrego S, Xu H, Arola D. Degradation in the fatigue crack growth resistance of human dentin by lactic acid. Mater Sci Eng C Mater Biol Appl 2017;73:716–25.
[7] Van Meerbeek B, De Munck J, Yoshida Y. Adhesion to enamel and dentin: current status and future challenges. Oper Dent 2003;28:215–35.
[8] Frassetto A, Breschi L, Turco G, Marchesi G, Di Lenarda R, Tay FR, et al. Mechanisms of degradation of the hybrid layer in adhesive dentistry and therapeutic agents to improve bond durability — a literature review. Dent Mater 2016;32:41–53.
[9] Porto ICCM, Nascimento TG, Oliveira JMS, Freitas PH, Haimeur A, França R. Use of polyphenols as a strategy to prevent degradation in the dentin-resin interface. Eur J Oral Sci 2018;126:146–58.
[10] Tjaderhane L, Nascimento FD, Breschi L, Mazzoni A, Tersariol ILS, Geraldeli S, et al. Optimizing dentin bond durability: control of collagen degradation by matrix metalloproteinases and cysteine cathepsins. Dent Mater 2013;29:116–35.
[11] Li B, Zhu X, Ma L, Wang F, Liu X, Yang X, et al. Selective demineralization of dentine intrafibrillar minerals — a potential method to eliminate water-wet bonding in the etch-and-rinse technique. J Dent 2016;52:55–62.
[12] Hermsen RJ, Vrijhoef MMA. Loss of enamel due to etching with phosphoric or maleic acid. Dent Mater 1993;9:332–6.
[13] Chan AR, Titley KC, Chernecky R, Smith DC. A short- and long-term shear bond strength study using acids of varying dilutions on bovine dentine. J Dent 1997;25:145–52.
[14] Tay FR, Gwinnett JA, Wei SHY. Micromorphological spectrum of acid-conditioned dentin following the application of a water-based adhesive. Dent Mater 1998;14:329–38.
[15] Yoshida Y, Van Meerbeek B, Nakayama Y, Yoshioka M, Snauwaert J, Abe Y, et al. Adhesion to and decalcification of hydroxyapatite by carboxylic acids. J Dent Res 2001;80:1565–9.
[16] Breschi L, Gobbi P, Mazzotti G, Falconi M, Ellis TH, Stangel I. High resolution SEM evaluation of dentin etched with maleic and citric acid. Dent Mater 2002;18:26–35.
[17] Imbery TA, Kennedy M, Janus C, Moon PC. Evaluating EDTA as a substitute for phosphoric acid-etching of enamel and dentin. Gen Dent 2012;60:55–61.
[18] Cecchin D, Farina AP, Bedran-Russo AK. The effect of endodontic substances and naturally reducing agents on the bond strength of epoxy resin-based sealer to root dentin. J Conserv Dent 2017;20:302–6.
[19] Cecchin D, Farina AP, Vidal C, Bedran-Russo AK. A novel enamel and dentin etching protocol using α-hydroxy glycolic acid: surface property, etching pattern, and bond strength studies. Oper Dent 2018;43:101–10.
[20] Leme-Kraus AA, Aydin B, Vidal CM, Phansalkar RM, Nam JW, McAlpine J, et al. Biostability of the proanthocyanidins-dentin complex and adhesion studies. J Dent Res 2017;96:406–12.
[21] Tang SC, Yang JH. Dual effects of alpha-hydroxy acids on the skin. Molecules 2018;23(4).
[22] Tezvergil-Mutluay A, Agee KA, Hoshika T, Carrilho M, Breschi L, Tjaderhane L, et al. The requirement of zinc and

calcium ions for functional MMP activity in demineralized dentin matrices. Dent Mater 2010;26:1059–67.
[23] Aydin B, Hassan LS, Viana G, Bedran-Russo AK. Assessing collagen and micro permeability at the proanthocyanidin-treated resin-dentin interface. J Adhes Dent 2016;18:529–34.
[24] Egan CA, Hector MP, Bishop MA. On the pulpal nerve supply in primary human teeth: evidence for the innervation of primary dentine. Int J Paediatr Dent 1999;9:57–66.
[25] Mazzoni A, Apolonio FM, Saboia VP, Santi S, Angeloni V, Checchi V, et al. Carbodiimide inactivation of MMPs and effect on dentin bonding. J Dent Res 2014;93:263–8.
[26] Sauro A, Watson TF, Mannocci F, Miyake K, Tay FR, Pashley DH. Two-photonlaser confocal microscopy of micropermeability of resin–dentin bonds madewith water or ethanol wet bonding. Biomed Mater Res B Appl Biomater 2009;90:327–37.
[27] Hashimoto M, Ohno H, Endo K, Kaga M, Sano H, Oguchi H. The effect of hybrid layer thickness on bond strength: demineralized dentin zone of the hybrid layer. Dent Mater 2000;16:406–11.
[28] Nurrohman H, Nikaido T, Takagaki T, Sadr A, Ichinose S, Tagami J. Apatite crystal protection against acid-attackbeneath resin-dentin interface with four adhesives: TEM and crystallography evidence. Dent Mater 2012;28:89–98.
[29] Skupien JA, Susin AH, Angst PD, Anesi R, Machado P, Bortolotto T, et al. Micromorphological effects and the thickness of the hybrid layer — a comparison of currentadhesive systems. J Adhes Dent 2010;12:435–42.
[30] Van Landuyt KL, De Munck J, Mine A, Cardoso MV, Peumans M, Van Meerbeek B. Filler debonding & subhybrid-layer failures in self-etch adhesives. J Dent Res 2010;89:1045–50.
[31] Suzuki M, Kato H, Wakumoto S. Vibrational analysis by Raman spectroscopy of the interface between dental adhesive resin and dentin. J Dent Res 1991;70:1092–7.
[32] Wang Y, Spencer P. Hybridization efficiency of the adhesive dentin interface with wet bonding. J Dent Res 2003;82:141–5.
[33] Spencer P, Wang Y, Katz JL. Identification of collagen encapsulation at the dentin/adhesive interface. J Adhes Dent 2004;6:91–5.
[34] Spencer P, Wang Y, Bohaty B. Interfacial chemistry of moisture-aged class II composite restorations. J Biomed Mater Res B Appl Biomater 2006;77:234–40.
[35] De Munck J, Van Meerbeek B, Yoshida Y, Inoue S, Vargas M, Suzuki K, et al. Four-year water degradation of total-etch adhesives bonded to dentin. J Dent Res 2003;82:136–40.
[36] Chien YC, Burwell AK, Saeki K, Fernandez-Martinez A, Pugach MK, Nonomura G, et al. Distinct decalcification process of dentin by different cariogenic organic acids: kinetics, ultrastructure and mechanical properties. Arch Oral Biol 2016;63:93–105.
[37] Brogioli D, Vailati A. Diffusive mass transfer by nonequilibrium fluctuations: fick's law revisited. Phys Rev E Stat Nonlin Soft Matter Phys 2001;63(1 Pt. 1), 012105.
[38] Landau M. Chemical peels. Clin Dermatol 2008;26:200–8.
[39] Roberts WE. Chemical peeling in ethnic/dark skin. Dermatol Ther 2004;17:196–205.
[40] Mazzoni A, Mannello F, Tay FR, Tonti GAM, Papa S, Mazzotti G, et al. Zymographic analysis and characterization ofMMP-2 and -9 forms in human sound dentin. J Dent Res 2007;86:436–40.
[41] Breschi L, Mazzoni A, Nato F, Carrilho M, Visintini E, Tjäderhane L, et al. Chlorhexidine stabilizes the adhesive interface: a 2-year in vitro study. Dent Mater 2010;26:320–5.
[42] Tjaderhane L, Larjava H, Sorsa T, Uitto VJ, Larmas M, Salo T. The activation and function of host matrix metalloproteinases in dentin matrix breakdown in caries lesions. J Dent Res 1998;77:1622–9.
[43] Mazzoni A, Pashley DH, Nishitani Y, Breschi L, Mannello F, Tjäderhane L, et al. Reactivation of inactivated endogenous proteolytic activities in phosphoric acid- etched dentin by etch-and–rinse adhesives. Biomaterials 2006;27:4470–6.
[44] Nishitani Y, Yoshiyama M, Wadgaonkar M, Breschi L, Mannello F, Mazzoni A, et al. Activation of gelatinolytic/collagenolytic activity in dentin by self-etching adhesives. Eur J Oral Sci 2006;114:160–6.
[45] DeVito-Moraes AG, Francci C, Vidal CM, Scaffa PM, Nesadal D, Yamasaki LC, et al. Phosphoric acid concentration affects dentinal MMPs activity. J Dent 2016;53:30–7.
[46] Pashley DH, Tay FR, Yiu C, Hashimoto M, Breschi L, Carvalho RM, et al. Collagen degradation by host-derived enzymes during aging. J Dent Res 2004;83:216–21.