8-2020

Effect of Dentin Biomodification Delivered by Experimental Acidic and Neutral Primers on Resin Adhesion

Lívia Tosi Trevelin
University of Illinois at Chicago

Yvette Alania
Marquette University

Mathew Mathew
University of Illinois at Chicago

Rasika S. Phansalkar
University of Illinois at Chicago

Shao-Nong Chen
University of Illinois at Chicago

See next page for additional authors

Journal of Dentistry, Vol. 99 (August 2020): 103354. DOI.

Effect of Dentin Biomodification Delivered by Experimental Acidic and Neutral Primers on Resin Adhesion

Lívia Tosi Trevelin
Department of Restorative Dentistry, College of Dentistry, University of Illinois at Chicago, Chicago, IL
Department of Operative Dentistry, College of Dentistry, University São Caetano do Sul, USCS, SP, Brazil
Yvette Alania
Department of Restorative Dentistry, College of Dentistry, University of Illinois at Chicago, Chicago, IL
Mathew Mathew
Department of Restorative Dentistry, College of Dentistry, University of Illinois at Chicago, Chicago, IL
Rasika Phansalkar
Program for Collaborative Research in the Pharmaceutical Sciences and Department of Pharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago, Chicago, IL
Shao-Nong Chen
Program for Collaborative Research in the Pharmaceutical Sciences and Department of Pharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago, Chicago, IL
Guido F. Pauli

Program for Collaborative Research in the Pharmaceutical Sciences and Department of Pharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago, Chicago, IL

Ana K. Bedran-Russo

Department of Restorative Dentistry, College of Dentistry, University of Illinois at Chicago, Chicago, IL

Abstract

Objectives

Proanthocyanidins (PACs) are biocompounds mimicking native collagen cross-links. The effective and practical delivery of any biocompound is pivotal for clinical usage. The aim was to investigate the dentin biomodification and effective formation of dentin–resin biointerfaces of two highly bioactive PAC-rich extracts, *Vitis vinifera (Vv)* and *Camellia sinensis (Cs*), delivered using neutral (NP) or acidic (AP) rinse-out primer approaches.

Methods

The depth of dentin demineralization (optical profilometry), dentin biomodification (apparent modulus of elasticity, collagen auto-fluorescence) and properties of dentin–resin interfaces (microtensile bond strength - μTBS, and micro-permeability) were investigated. NP consisted of either 15% *Vv* or *Cs* applied for 60 s after surface etching; while AP contained 15% *Vv* or *Cs* in either 35% glycolic acid or tartaric acid applied for 30 s or 60 s. Data were analyzed using ANOVA and post-hoc tests ($\alpha = 0.05$).

Results

The depth of demineralization was statistically higher when applied for 60 s, regardless of rinse-out primer approach ($p < 0.001$). Compared to the AP strategy, NP exhibited statistically higher apparent modulus of elasticity, regardless of PAC extract ($p < 0.001$). Highest μTBS were obtained for NP_{Vv}, which were statistically similar to AP_{GAVv}, when applied for 60 s ($p < 0.001$); both resulted in a dramatic decrease of the interfacial permeability. NP_{Cs} group showed the lowest μTBS ($p < 0.001$).

Conclusions

A combination of high bond strength and low micro-permeability can be accomplished using glycolic acid with the mid- and high-PAC oligomer enriched extract (*Vv*). *Cs extract* containing mostly catechins and dimeric PACs, was found unsuitable for resin-dentin adhesion despite exhibiting high initial dentin biomodification.

Clinical significance

This study provides a new conceptual delivery of PAC-mediated dentin biomodification and conservative dentin surface etching using rinse-out primers. The strategy requires a specific combination of PAC source, α-hydroxy acid, and application time.

Keywords

Alpha-hydroxy acids, Collagen, Dentin, Mechanical properties, Proanthocyanidins, Optical profilometry, Bond strength

1. Introduction

Collagen molecules are the elementary structural unit of the dentin extracellular matrix (ECM). Collagen fibrils are formed by spontaneous staggering of collagen molecules that are stabilized by post-translational modifications via enzymatic inter- and intra-molecular cross-links [1]. Collagen cross-linking provides the basis of stability and strength of collagen fibrils and the viscoelasticity of tissues [2]. The composition and architecture of the dentin ECM play a key role in adhesive restorative procedures and longevity of adhesive restorations [3,4]. A biomimetic approach has shown promising results in adhesive dentistry applications by mediating exogenous covalent and non-covalent cross-linking [2]; contributing to both, the mechanical reinforcement and reduced biodegradability of the dentin ECM [2].

Specifically, plant-derived proanthocyanidins (PACs) exhibit remarkable dentin-specific biomodification potency [2,5]. Their favorable biological responses can further expand PAC applications into therapies of the dentin–pulp complex [6]. However, the biosynthetic nature and vast chemodiversity of PACs require a strict selection of their source, particularly for unrefined preparations such as plant extracts. PACs comprise eight possible flavan-3-ol monomeric units that undergo distinct polymerization to form an array of compounds with unique 3D structural diversity, which can elicit distinct responses of ECM and cells [1,5,6]. Particularly, the presence of galloyl moieties and the degree of polymerization of PACs compounds determine dentin biomodification potency and stability [1,7]. Thus, sourcing, extraction, and preparation of PACs are fundamental for their dentin bioactivity and effectiveness at the dentin–resin interface. In addition to the extraction/fractionation of PACs, yielding more well-defined mixtures or even isolated compounds the effective delivery is a current limitation of their use as biomaterial [2,4,5,[7], [8], [9]].

The effective and practical delivery of any biomaterial is critical for translation into clinical use. Combining current clinical steps is attractive as the vast majority of dental adhesive systems still rely on surface preparation (etching and/or priming) and application of resin adhesive intermediate layer. Conditioning of dentin and enamel with α-hydroxy acids (AHAs) yields similar resin bond strength as standard phosphoric acid etching [4,10,11], with the advantage of producing thinner hybrid layers and being biocompatible. In addition, the pH of the AHAs (pH ∼1.2) favors the bioactivity of PACs [11].

This study comprehensively investigated the potential for dentin biomodification and effective formation of dentin–resin biointerfaces of two highly bioactive PAC extracts delivered using two rinse-out primer approaches. Theses approaches employed two enriched PAC extracts, one from *Vitis vinifera* (*Vv*) contain a broad range of oligomeric PACs, another from the chemically less diverse extract from *Camellia sinensis* (*Cs*), and two AHAs (tartaric and glycolic acids). The null hypothesis tested was that the PAC delivery strategies using as neutral or acidic rinse-out primers would not have a significant effect on the mechanical enhancement to the dentin ECM, etching pattern/depth of dentin, and the dentin–resin adhesion.

2. Materials and methods

2.1. Sourcing of materials and preparation of rinse-out neutral (NP) and acidic primers (AP)

An enriched extract containing a broad range of oligomeric PACs was prepared from a crude extract of *Vitis vinifera* (*Vv*) seeds by a 2-phase partitioning procedure resulting in the exclusion of most of the higher order oligomeric and polymeric proanthocyanidins, as described previously [12]. The enriched extract containing a narrow range of monomeric/dimeric PACs from leaves of *Camellia sinensis (Cs*) was obtained commercially (Sunphenon® 90D, Taiyo International, Inc, Minneapolis, MN, USA).

The selected AHAs, glycolic (GA) and tartaric (TA) acids, were purchased from Sigma-Aldrich (St. Louis, MO, USA); and prepared in liquid formulation at % concentration (GA: pH 1.4 and TA: pH 1.1) [1,4].

Neutral Primers (NP) contained 15% (w/v) of *Vv* or 15% (w/v) of *Cs* in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer at pH 7.2. Acidic Primers (AP) were prepared by mixing either 15% (w/v) *Vv* or 15% (w/v) of *Cs* in either 35% GA or 35% TA; the acidic primers were not pH adjusted (Table 1).

Table 1. Rinse-out primer formulations, dentin–resin adhesion protocols, and experimental group nomenclature.

Delivery	**Primers formulation**		**Dentin-resin adhesion protocols**			**Groups**
	α-hydroxy acid (35 w/v %)	**Proanthocyanidins (15 w/v %)**	**Etch**	**Primer**	**Bonding**	
Neutral primers (NP)	–	*Vitis vinifera* seed (*Vv*) or *Camellia sinensis* leaf (*Cs*)	*Step 1* 15 s GA or TA, 15 s rinse	*Step 2* 60 s application, 15 s rinse	*Step 3* Application of 2 coats, air-dry 10 s, Light-cure 40 s	NP_{GaVv} NP_{TaVv} NP_{GaCs} NP_{TaCs}
Acidic primers (AP)	Glycolic (GA) or Tartaric (TA)	*Vitis vinifera* seed (*Vv*) or *Camellia sinensis* leaf (*Cs)*	*Step 1* 30 s or 60 s application, 15 s rinse		*Step 2* Application of 2 coats, air-dry 10 s, Light-cure 40 s	AP_{GaVv} AP_{TaVv} AP_{GaCs} AP_{TaCs}

2.2. Surface demineralization depth – optical profilometry microscopy

Dentin specimens (n = 3; dimensions, 4 × 4 × 2 mm) from mid-coronal dentin of extracted human molars (Institutional Review Board protocol #2011-0312) were gloss polished wet (EcoMet 3000, Buehler, Lake Bluff, IL, USA) and protected with one layer of acid-resistant nail polish leaving an exposed central window (dimensions, 3 × 3 mm). The dentin surfaces were treated with acidic or neutral rinse-out primers for 30 s and 60 s (Table 1). The nail polish was removed, and the surfaces scanned with an optical profilometer (Nano Contour GT-K, Bruker, Fitchburg, WI, USA). The demineralization depth (ΔZ) was calculated by subtracting the average height of the etched area from the average height of the untreated area [11]. Intragroup variability was assessed using Levene's test and found to be homogenous ($p = 0.36$). Thus, the ΔZ data were analyzed statistically using two-way ANOVA followed by Tukey's post hoc test ($\alpha = 0.05$).

2.3. Bulk mechanical properties of dentin matrix – apparent modulus of elasticity

The dentin biomodification potency of acidic and neutral rinse-out primers was assessed by mechanical measurements of the dentin matrix. Dentin beams (n = 10; dimensions 0.5 × 1.7 × 6 mm) were prepared and fully demineralized in 10% phosphoric acid for 5 h. Specimens were tested in a 3-point bending fixture as previously described [13] using a 1 N load cell mounted on a universal testing machine (EZ Graph, Shimadzu, Kyoto, Japan) at a crosshead speed of 0.5 mm/min. All measurements were carried out with specimens fully immersed in distilled water. The apparent modulus of elasticity was determined at different time points, including baseline and the cumulative exposures to neutral and acidic rinse-out primers for 30 s, 60 s, 5 min, and 10 min. Intragroup variability was assessed using Levene's test and found to be homogenous for times points ($p = 0.10$). Thus, the ΔZ data were analyzed statistically with ANOVA repeated measurements and Tukey's post hoc test ($\alpha = 0.05$).

2.4. Resin–dentin adhesive properties

Standard micro-tensile bond strength (μTBS) and micro-permeability methods were carried out to determine the adhesive properties of the dentin–resin interface. Human molars had the occlusal enamel removed, and dentin surface polished with silicon carbide abrasive papers (180, 320, and 600-grit; Buehler, Lake Bluff, IL, USA). The specimens were randomly divided into 12 groups (n = 5), including 4 NP and 8 AP protocols (Table 1). The rinse-out acidic primer (AP) strategy (Table 1) consisted of the following steps: application of acidic primers for 30 or 60 s and surface rinsing for 15 s. The conventional rinse-out neutral primer (NP) strategy (Table 1) consisted of the following procedure: dentin surface etching for 15 s (35% GA or TA), surface rinsing for 15 s, application of neutral primer for 60 s (15% w/v *Vv* or *Cs*), and surface rinsing for 15 s. After application of NP or AP, a uniform protocol was used to apply the adhesive resin as follows: blot-drying of the surface; application of two layers of experimental adhesive; evaporation of excess solvent; light curing for 40 s (intensity of 700 mW/cm^2; Optilux 501, Kerr, Brea, CA, USA); incremental placement and polymerization of a resin composite (A2 body, Filtek Supreme; 3M ESPE, St. Paul, MN, USA). The specimens were immediately immersed in simulated body fluid (SBF) at 37 °C. After 24 h, specimens were sectioned into resin-dentin specimens with a cross-sectional area of 0.8 × 0.8 mm^2 and assigned to either interfacial bond strength or micro-permeability evaluations.

The SBF consisted of 5 mM HEPES, 2.5 mM $CaCl_2$, 0.05 mM $ZnCl_2$, and 0.3 mM NaN_3 [14]. The experimental adhesive resin blend was formulated with Bisphenol A glycidyl methacrylate (Bis-GMA),

triethylene glycol dimethacrylate (TEGDMA), camphorquinone, ethyl-4-dimethylamino benzoate, 6% of hydroxyethyl methacrylate (HEMA), and 40 wt.% of ethanol [4].

2.4.1. Resin–dentin interfacial bond strength

Five specimens from each tooth were fixed with cyanoacrylate (Super Glue gel Loctite; Henkel Corporation, Rocky Hill, Connecticut, USA) to a jig, which was mounted on a microtensile tester (Bisco, Schaumburg, IL, USA) and subjected to tensile forces at a crosshead speed of 1 mm/min. Microtensile bond strength (μTBS) was calculated by dividing the peak force (N) by the cross-sectional area of the failed interface (mm^2). Intragroup variability was assessed using Levene's test and found to be homogeneously distributed ($p = 0.253$). Data were statistically analyzed using two-way ANOVA, followed by Tukey´s post hoc test ($\alpha = 0.05$).

2.4.2. Interfacial micro-permeability and collagen auto-fluorescence

The micro-permeability of the adhesive interface and the collagen auto-fluorescence was assessed as previously described [15]. Briefly, the resin-dentin beams were embedded in epoxy resin and gloss polished with SiC abrasive papers. Specimens were immersed in 0.1 M rhodamine B solution (pH 7.2, RITC/Rhodamine B; Sigma) for 1 h, rinsed, and immediately observed under confocal laser scanning microscopy (CLSM 710; Zeiss). Parameters were chosen to allow acquisition of simultaneous appearance of collagen (488 nm wavelength, green fluorescence) and interfacial micro-permeability (514 nm wavelength, red fluorescence). Four images were obtained from each resin-dentin beam ($n = 10$) and processed in expert mode using the profile tool in the CLSM software. Data were presented as fluorescence emission intensity (FEI) for each wavelength. Intragroup variability was assessed using Levene's test and found to be not homogeneously distributed for red and green fluorescence ($p < 0.001$). Data were then analyzed statistically using three-way ANOVA, followed by Games-Howell post hoc test ($\alpha = 0.05$).

3. Results

3.1. Demineralization depth

Representative images of the dentin surface demineralization depth are shown in Fig. 1A. Higher primer application time (60 s) resulted in significantly increased demineralization depth, regardless of the rinse-out primer approach ($p < 0.001$, Fig. 1B). However, no significant interactions occurred between studied factors (rinse-out primers vs. time of application, $p = 0.775$), nor did significant differences arise from the two rinse-out primers approaches ($p = 0.191$).

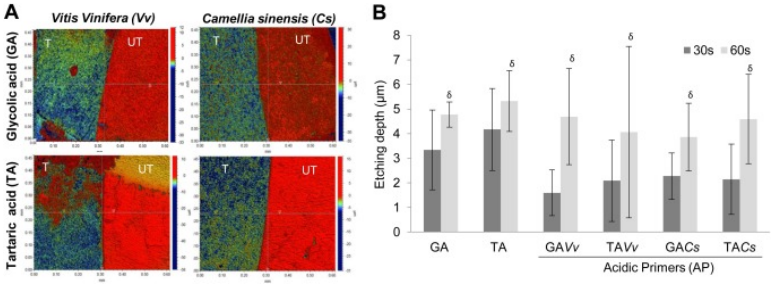

Fig. 1. Results of the dentin etching depth of acidic primers (AP), glycolic (GA) and tartaric acid (TA) by digital profilometry. **(A)** Representative digital profilometry images of the depth of dentin demineralization treated with AP applied for 60 s. **(B)** Results of the demineralization depth (ΔZ, μm) of dentin surface for 30 s and 60 s

application times. Symbol (δ) depicts statistically significant difference between application times ($p < 0.05$) *Vv: Vitis vinifera, Cs:* C*amellia sinensis*, GA: glycolic acid, TA: tartaric acid. T: treated surface, UT: untreated surface.

3.2. Biomechanical analysis – apparent modulus of elasticity

Results of the apparent modulus of elasticity are presented in Table 2. There was statistically significant interaction between factors (rinse-out primers vs. application time, $p = 0.001$) and significant differences within each factor ($p < 0.001$). All time points showed a significant increase in the apparent modulus of elasticity of all AP and NP primers, when compared to baseline ($p < 0.001$). Statistically significant differences ($p < 0.05$) were also observed between NP and AP primers after 10 min of cumulative treatment as follows: $NP_{Vv} = NP_{Cs} > AP_{TAVv}$ AP_{GAVv}, AP_{TACs}, AP_{GACs}. The highest increase was observed for groups treated with neutral primers, regardless of the PAC extract (*Vv* or *Cs*, $p < 0.001$).

Table 2. Results of the apparent modulus of elasticity (Mean ± SD) of dentin matrices evaluated using the 3-point bending method following neutral (NP) and acid (AP) primer treatment and cumulative application times. Vv: *Vitis vinifera*, Cs: *Camellia sinensis*, GA: glycolic acid, TA: tartaric acid. Different upper- and lower-case superscript letters represent statistically significant differences among groups ($p < 0.05$) in each row and column, respectively.

Application mode	**Groups**	**Apparent modulus of elasticity (MPa)**				
		Baseline	**30s**	**60s**	**5 min**	**10 min**
Neutral primer (NP)	$\boldsymbol{NP_{Vv}}$	3.44 ± 0.70 C,a	13.92 ± 5.98 B,a	17.41 ± 4.77 B,a	33.03 ± 5.70 A,a	39.50 ± 5.10 A,a
	$\boldsymbol{NP_{Cs}}$	4.10 ± 1.45 C,a	12.47 ± 3.83 B,a	17.74 ± 5.51 B,a	27.77 ± 7.35 A,a	35.53 ± 8.18 A,a
Acidic primer (AP)	$\boldsymbol{AP_{GaVv}}$	3.80 ± 0.96 B,a	8.21 ± 1.50 AB,a	11.40 ± 3.18 AB,ab	19.32 ± 4.87 A,b	22.62 ± 5.64 A,b
	$\boldsymbol{AP_{TaVv}}$	3.52 ± 1.24 B,a	6.82 ± 2.33 B,ab	9.06 ± 2.96 AB,b	16.62 ± 4.82 A,b	23.71 ± 9.82 A,b
	$\boldsymbol{AP_{GaCs}}$	2.63 ± 0.59 B,a	5.67 ± 1.44 B,b	6.90 ± 2.03 AB,b	12.11 ± 2.58 A,b	17.03 ± 2.02 A,b
	$\boldsymbol{AP_{TaCs}}$	3.23 ± 1.54 B,a	5.67 ± 2.68 B,b	7.36 ± 3.41 AB,b	11.50 ± 5.08 A,b	22.23 ± 10.10 A,b

3.3. Resin–dentin adhesive properties

Table 3 presents the results of μTBS, interfacial micro-permeability and interfacial collagen auto-fluorescence. Interaction between factors showed statistical significance (rinse-out primers vs. time of application, $p = 0.034$), and significant differences within time of application (30 s vs. 60 s, $p = 0.012$) and rinse-out primers approaches (NP vs. AP, $p < 0.001$).

Table 3. Results of dentin bond strength (Mean ± SD), micro-permeability and collagen auto-fluorescence of resin-dentin interfaces prepared using neutral and acidic rinse-out primers enriched with 15% *Vitis vinifera* (Vv) or *Camellia sinensis* (Cs) extract with different application times. GA: glycolic acid; TA: tartaric acid. Microtensile bond strength and collagen auto-fluorescence results: same letters in each column indicate lack of significant differences ($p < 0.05$). Micro-permeability results: same upper- and lower-case letters indicate lack of significant differences ($p < 0.05$) among primer formulation and application times, respectively (based on pooled means).

Application mode	**Groups**	**Application time (s)**	**Microtensile bond strength (MPa)**	**Fluorescence emission intensity (FEI, x10)**	
				Micro-permeability (red)	**Collagen auto-fluorescence (green)**
Neutral primers (NP)	$\boldsymbol{NP_{GaVv}}$	T 60 s	65.6 ± 8.3 A	17.2 ± 7.6 A,b	78.6 ± 13.6 A
	$\boldsymbol{NP_{TaVv}}$	T 60 s	62.0 ± 11.2 A	17.2 ± 12.2 A,b	43.4 ± 21.2 B
	$\boldsymbol{NP_{GaCs}}$	T 60 s	21.8 ± 1.9 DE	65.2 ± 17.6 B,b	19.7 ± 15.4 B
	$\boldsymbol{NP_{TaCs}}$	T 60 s	18.1 ± 7.2 E	18.1 ± 9.8 AB,b	22.9 ± 10.2 B
Acidic primers (AP)	$\boldsymbol{AP_{GaVv}}$	T 30 s	40.1 ± 7.4 BC	57.0 ± 9.9 A,a	12.7 ± 9.9 B
	$\boldsymbol{AP_{GaVv}}$	T 60 s	60.8 ± 7.0 A	40.0 ± 2.6 A,a	20.2 ± 14.1 B
	$\boldsymbol{AP_{TaVv}}$	T 30 s	43.6 ± 8.8 BC	77.2 ± 7.8 A,a	9.3 ± 7.8 B
	$\boldsymbol{AP_{TaVv}}$	T 60 s	46.1 ± 5.4 B	48.1 ± 7.3 A,a	18.5 ± 12.8 B
	$\boldsymbol{AP_{GaCs}}$	T 30 s	27.9 ± 14.4 CD	138.2 ± 17.8 B,a	17.4 ± 7.5 B
	$\boldsymbol{AP_{GaCs}}$	T 60 s	29.4 ± 7.5 CD	99.1 ± 11.7 B,a	28.9 ± 20.1 B
	$\boldsymbol{AP_{TaCs}}$	T 30 s	29.6 ± 7.7 CD	86.9 ± 19.4 AB,a	10.9 ± 4.7 B
	$\boldsymbol{AP_{TaCs}}$	T 60 s	35.0 ± 13.2 C	100.5 ± 19.3 AB,a	12.7 ± 2.7 B

The results of μTBS are shown in Table 3. The neutral primer enriched with *Vv* exhibited the highest values of μTBS ($p < 0.001$), which were statistically similar to $AP_{GAVvT60s}$ ($p > 0.05$). Acidic primers enriched with *Vv* displayed intermediate values of μTBS, regardless of the AHA (GA or TA and the time of application, 30 s or 60 s). Overall, all acidic primers enriched with *Cs* exhibited a poor performance of μTBS when compared to acidic primers of *Vv*. Likewise, the neutral primers enriched with *Cs* showed statistically lower μTBS when compared to neutral primers enriched with *Vv* ($p < 0.001$).

Fluorescence emission intensity (FEI) values of collagen cross-linking and rhodamine-B filled porosities (micro-permeability) at the resin-dentin interfaces are shown in Table 3; representative images are shown in Fig. 2. Analysis of micro-permeability data revealed statistically significant differences among

rinse-out primers ($p < 0.001$), time of application ($p < 0.001$), and no significant interactions between factors (rinse-out primers vs. time of application, $p = 0.401$). The application times of acidic primers (30 and 60 s) resulted in statistically higher micro-permeability when compared to neutral primers at 60 s ($p < 0.05$). Primers using GA and *Cs* showed statistically higher micro-permeability values at the resin-dentin interfaces when compared to primers using *Vv* ($p < 0.001$). Primers using TA and Cs presented intermediate pooled mean values with no signficant difference to the other primers ($p > 0.05$). Analysis of the collagen auto-fluorescence data revealed statistically significant differences among rinse-out primers ($p = 0.009$), time of application ($p < 0.001$), and interactions between these factors ($p < 0.001$). The emission intensity of the collagen auto-fluorescence (cross-linking) at the resin-dentin interface was the highest for the neutral primer NP_{GAVv} ($p < 0.001$), and no statistical significant differences were observed among the other groups (Table 3).

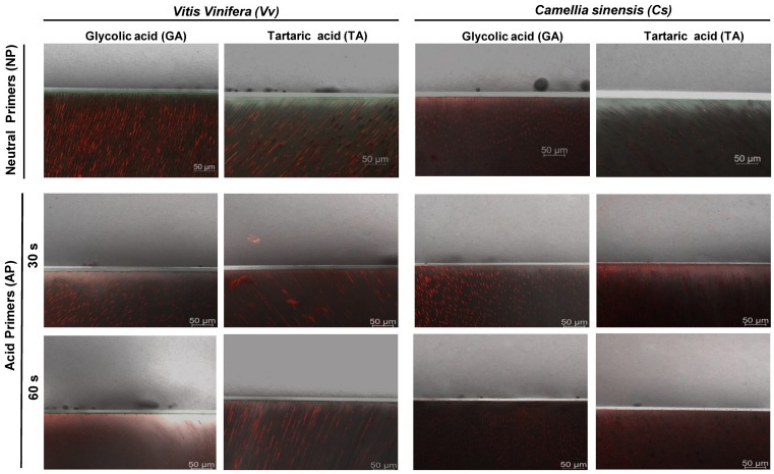

Fig. 2. Representative confocal laser scanning microscopy overlapping images of the resin-dentin interfaces show collagen auto-fluorescence (green) and interfacial micro-permeability (red) of experimental groups of neutral **(A)** and acidic **(B)** primers. GA: glycolic acid; TA: tartaric acid. Magnification set at 50 µm scale bar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

4. Discussion

Dentin biomodification strategies have exploited the mediation of exogenous collagen cross-links as a mechanism to improve the mechanical stability and reduce the biodegradation rates of collagen by multi-interaction with dentin matrix components. In the present study, the mechanical properties of dentin and outcomes of the resin-dentin adhesion were affected differently, depending on the source of PACs and their delivery mode (neutral vs. acidic primers), thereby rejecting the study null hypothesis.

The AHAs were explored as etchants not only for their milder acidity and biocompatibility as compared to phosphoric acid (PA), but also because PACs precipitate in PA solution, particularly at higher concentrations (data not shown). GA and TA effectively demineralize the dentin as demonstrated here and elsewhere [4,11]. Herein, the depth of dentin demineralization by GA and TA were time-dependent (Fig. 1), where increasing acid exposure statistically increased the demineralization effect of both acids. While the same trend applied to both acidic primers, containing *Vv* and *Cs*, the etching

ability of GA and TA in the AP formulation was reduced by approximately 45% when used for 30 s. The presence of PACs and the pH of the acidic primer (higher than GA and TA alone) reduced the surface demineralization efficiency of AHA. In addition, as known calcium-binding agents [16], PACs can bind to the mineral phase of dentin, thus hampering the ability of GA and TA to diffuse in the tissue and remove hydroxyapatite crystals. Accordingly, the acidic primers need longer application time to achieve similar demineralization depth as GA and TA.

All investigated rinse-out primers reinforced the dentin matrix. Thus, all delivery strategies of PACs showed potential to interact with the dentin matrix, specifically, mediation of collagen cross-linking, thereby enhancing the mechanical strength of the dentin matrix (Table 2). The dentin matrix treated with neutral primers showed higher modulus of elasticity than those treated with acidic primers, regardless of the source of PACs (*Vv* and *Cs*). After 10 min of cumulative treatment, neutral primers increased the stiffness of dentin by 12.1 and 9.3 fold for NP_{Vv} and NP_{Cs}, respectively (Table 2) . The low pH (3 or less) of acidic primers decreased the adsorption of PACs into the collagen, plausibly due to the protein swelling phenomenon [17] that limits interaction parameters (hydrogen bond and hydrophobicity). On the other hand, primers with neutral pH optimize PACs interactions with the dentin matrix due to the isoelectric properties of type I collagen (pH 7–8).

The pH ~1.1 of acidic primers containing GA or TA were effective carriers of *Vv* or *Cs*. The acidic primer formulations exhibited favorable potential for dentin biomodification (Table 2), but statistically lower values than neutral primers. Acidic primers of *Vv* elicited rapid effects; however, after 10 min exposure time there were no differences among acidic primers. The *Vv* was a potent source inducing an immediate (30 s treatment) enhancement of the apparent modulus of elasticity, which agrees with a previous study [18].

In addition to the abundance and degree of polymerization of the PAC compounds present, their interflavan linkages are known to define the structure-activity relationships [7]. This provided a rationale for investigating two distinct enriched PAC extracts; the *Vv* extract contains medium size oligomeric proanthocyanidins [4,12], mainly with B-type interflavan linkages [12]; in contrast, *Cs* contains mainly galloylated monomeric catechins and dimeric PACs [19,20] with A-type interflavan linkages [20]. Despite the distinct composition between *Cs* and *Vv*, both enriched extracts elicited similar increases to the modulus of elasticity of the dentin matrix. Thus, no apparent function of the distinct polyphenol compositions in the enriched extracts could be drawn regarding their ability to interact with dentin and increase the immediate modulus of elasticity of dentin matrices. However, the interflavan linkages and the presence of galloylated compounds can negatively affect the long-term stability of dentin biomodification [7]. The long-term stability was not investigated in this study.

In contrast, the resin-dentin adhesive interface was dramatically affected by the source of PACs, formulation of the primer (NP and AP), and primer application time (30 s vs 60 s). The NP_{Vv} exhibited both high μTBS values and excellent sealing ability, regardless of the etchant used (GA and TA); while NP_{Cs} resulted in suboptimal bond strength (assumed here to be below 35 MPa mean value), irrespective of primer composition when compared to *Vv*. Hence, the bond strength mean values of acidic primers were significantly affected by the source of PACs and application time (Table 3). Similarly, the interfacial micro-permeability and the collagen auto-fluorescence were also differently affected by the source of PACs and application time.

The primer AP_{GAVv}, when applied for 60 s, provided high and similar bond strength values as NP_{GAVv} and NP_{TAVv}. These three groups reached an overall better performance, with high μTBS, high collagen auto-fluorescence emission (an indicator of exogenous collagen cross-linking), and low interfacial micro-permeability at the hybrid layer (Fig. 2). In addition to increased mechanical properties of the anchoring dentin collagen, *Vv* changes the physical properties of dentin, resulting in increased surface hydrophobicity [8,21]. Consequently, the bound water is displaced, promoting further resin infiltration and reduction of interfacial micro-permeability [4,22]. The current findings of NP_{Vv} support those reported in prior work with the same enriched PAC extract [4]. The present outcomes also show that the association of etching and priming (acidic primer) applied for 60 s yielded statistically similar outcomes to designated neutral primer strategy. Only for AP_{GAVv}, a 30 s application resulted in lower TBS than with 60 s application, probably due to insufficient demineralization depth and dentin biomodification. Notably, AP_{TAVv} applied for either timeframe of 30 s or 60 s resulted in similar bond strength performance.

However, the acidic primer AP_{Vv} approach did not achieve high-quality sealing to dentin, as demonstrated by the higher intensity of red fluorescence at the resin-dentin interface when compared to neutral primer NP_{Vv} (Table 3). Conversely, rinse-out primers formulated with *Cs* yielded the lowest resin-dentin bond strength and highest interfacial micro-permeability, showing a porous hybrid layer formation and poor adhesion. The negative effect of *Cs* on the resin adhesion properties occurred regardless of the primer mode (neutral or acidic primers) and etchant (GA and TA). Interfaces treated with *Cs* primers also exhibited lower collagen fluorescence intensity, indicating limited formation of exogenous collagen-cross-linking. These findings may explain the low μTBS values, and suggest an incompatibility between the phytochemistry of PACs found in *Cs* and the dental resin chemistry, especially as the dentin biomodification potency of *Cs* is similar to *Vv* (i.e. similar values of apparent modulus of elasticity of the dentin matrix). It should be noted that EGCg, a major constituent of *Cs* (>45%), is known to reduce the degree of polymerization of dental resins in concentrations above 2% [23]. While this study did not incorporate PACs into the adhesive, their presence at the dentin matrix may still have impaired the ability of the resin adhesive to polymerize within the hybrid layer, resulting in increased porosities and ineffective resin adhesion. This assumption deserves further investigation. Therefore, under the investigated strategies, the catechin monomer-enriched extract from *Cs* has to be considered an unsuitable PAC source in terms of dentin–resin adhesion. These findings also highlight the need for properly reporting the sourcing and exact phytochemical composition of PAC-rich biomaterials, and avoiding generalization of the term PACs in the context of dental applications, and elsewhere.

5. Conclusion

This study provides a new conceptual delivery of collagen cross-linkers to dentin via the rinse-out acidic primer (AP) strategy. This approach relies on the simultaneous conservative demineralization of dentin and biomodification of the dentin extracellular matrix, resulting in resin-dentin interfaces of high bond strength and low micro-permeability. Among the investigated formulations, the combination of glycolic acid (GA) with a mid- and high-oligomeric PAC enriched extract from *Vitis vinifera* (*Vv*) resulted in high bond strength and low micro-permeability. Monomeric and dimeric catechins/PACs from *Camellia*

sinensis (*Cs*) were found unsuitable for resin-dentin adhesion, despite their significant initial increase in the modulus of elasticity of the dentin matrix.

Declaration of Competing Interest

The authors report no conflict of interest.

Acknowledgments

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

References

[1] C.M. Vidal, W. Zhu, S. Manohar, B. Aydin, T.A. Keiderling, P.B. Messersmith, A.K. Bedran-Russo. **Collagen-collagen interactions mediated by plant-derived proanthocyanidins: a spectroscopic and atomic force microscopy study.** *Acta Biomater.,* 1 (2015), pp. 110-118, 10.1016/j.actbio.2016.05.026

[2] A.K. Bedran-Russo, C.S. Castellan, M.S. Shinohara, L. Hassan, A. Antunes. **Characterization of biomodified dentin matrices for potential preventive and reparative therapies.** *Acta Biomater.,* 7 (2011), pp. 1735-1741, 10.1016/j.actbio.2010.12.013

[3] L.E. Bertassoni. **Dentin on the nanoscale: hierarchical organization, mechanical behavior and bioinspired engineering.** *Dent. Mater.,* 33 (2017), pp. 637-649, 10.1016/j.dental.2017.03.008

[4] A.A. Leme-Kraus, B. Aydin, C.M. Vidal, R.M. Phansalkar, J.W. Nam, J. McAlpine, G.F. Pauli, S. Chen, A.K. Bed ran-Russo. **Biostability of the proanthocyanidins–dentin complex and adhesion studies.** *J. Dent. Res.,* 96 (2017), pp. 406-412, 10.1177/0022034516680586

[5] C.S. Castellan, A.K. Bedran-Russo, S. Karol, P.N.R. Pereira. **Long-term stability of dentin matrix following treatment with various natural collagen cross-linkers.** *J. Mech. Behav. Biomed. Mater.,* 4 (2011), pp. 1343-1350, 10.1016/j.jmbbm.2011.05.003

[6] D. Kulakowski, A.A. Leme-Kraus, J.W. Nam, J. McAlpine, S.N. Chen, G.F. Pauli, S. Ravindran, A.K. Bedran-Russo. **Oligomeric proanthocyanidins released from dentin induce regeneration dental pulp cell response.** *Acta Biomater.,* 55 (2017), pp. 262-270, 10.1016/j.actbio.2017.03.051

[7] B. Aydin, A.A. Leme-Kraus, C.M.P. Vidal, T.R. Aguiar, R.S. Phansalkar, J.W. Nam, J.B. McAlpine, S.N. Chen, G.F. Pauli, A.K. Bedran-Russo. **Evidence to the role of interflavan linkages and galloylation of proanthocyanidins at sustaining long-term dentin biomodification.** *Dent. Mater.,* 35 (2019), pp. 328-334, 10.1016/j.dental.2018.11.029

[8] A.A. Leme-Kraus, C.M. Vidal, L.S. Hassan, A.K. Bedran-Russo. **Potential role of surface wettability on the long-term stability of dentin bonds after surface biomodification.** *J. Biomech.,* 48 (2015), pp. 2067-2071, 10.1016/j.jbiomech.2015.03.016

[9]J. Zhou, A. Chiba, D.L. Scheffel, J. Hebling, K. Agee, J. Tagami, J. Tan, D. Abuelenain, M.A. Nawareg, A. H. Hassan, L. Breschi, F.R. Tay, D.H. Pashley. **Cross-linked dry bonding: A new etch-and-rinse technique.** *Dent. Mater.,* 32 (2016), pp. 1124-1132, 10.1016/j.dental.2016.06.014

[10] D. Cecchin, A.P. Farina, C. Vidal, A.K. Bedran-Russo. **A novel enamel and dentin etching protocol using α-hydroxy glycolic acid: surface property, etching pattern, and bond strength studies.** *Oper. Dent.,* 43 (2018), pp. 101-110, 10.2341/16-136-L

[11] L.T. Trevelin, J. Villanueva, C.A. Zamperini, M.T. Mathew, A.B. Matos, A.K. Bedran-Russo. **Investigation of five α-hydroxy acids for enamel and dentin etching: demineralization depth, resin adhesion and dentin enzymatic activity.** *Dent. Mater.,* 35 (2019), pp. 900-908, 10.1016/j.dental.2019.03.005

[12]R.S. Phansalkar, J.W. Nam, S.N. Chen, J.B. McAlpine, J.G. Napolitano, A. Leme, C.M. Vidal, T. Aguiar, A.K. Bedran-Russo, G.F. Pauli. **A galloylated dimeric proanthocyanidin from grape seed exhibits dentin biomodification potential.** *Fitoterapia*., 101 (2015), pp. 169-178, 10.1016/j.fitote.2014.12.006

[13] A.K. Bedran-Russo, D.H. Pashley, K. Agee, J.L. Drummond, K.J. Miescke. **Changes in stiffness of demineralized dentin following application of collagen crosslinkers.** *J. Biomed. Mater. Res. B Appl. Biomater.,* 86 (2008), pp. 330-334, 10.1002/jbm.b.31022

[14] A. Tezvergil-Mutluay, K.A. Agee, T. Hoshika, M. Carrilho, L. Breschi, L. Tjäderhane, Y. Nishitani, R.M. Carvalho , S. Looney, F.R. Tay, D.H. Pashley. **The requirement of zinc and calcium ions for functional MMP activity in demineralized dentin matrices.** *Dent. Mater.,* 26 (2010), pp. 1059-1067, 10.1016/j.dental.2010.07.006

[15] B. Aydin, L.S. Hassan, G. Viana, A.K. Bedran-Russo. **Assessing collagen and micro-permeability at the proanthocyanidins–treated resin-dentin interfaces.** *J. Adhes. Dent.,* 18 (2016), pp. 529-534, 10.3290/j.jad.a37359

[16] Q. Xie, A.K. Bedran-Russo, C.D. Wu. **In vitro remineralization effects of grape seed extract on artificial root caries.** *J. Dent.,* 36 (2008), pp. 900-906, 10.1016/j.jdent.2008.07.011

[17] C.S. Ku, M. Sathishkumar, S.P. Mun. **Binding affinity of proanthocyanidin from waste Pinus radiata bark onto proline-rich bovine achilles tendon collagen type I.** *Chemosphere*., 67 (2007), pp. 1618-1627, 10.1016/j.chemosphere.2006.11.037

[18]T.R. Aguiar, C.M.P. Vidal, R.S. Phansalkar, I. Todorova, J.G. Napolitano, J.B. McAlpine, S.N. Chen, G.F . Pauli, A.K. Bedran-Russo. **Dentin biomodification potential depends on polyphenol source.** *J. Dent. Res.,* 93 (2014), pp. 417-422, 10.1177/0022034514523783

[19] R.J. Moore, K.G. Jackson, A.M. Minihane. **Green tea (*Camellia sinensis*) catechins and vascular function.** *Br. J. Nutr.,* 102 (2009), pp. 1790-1802, 10.1017/S0007114509991218

[20]C.M. Vidal, T.R. Aguiar, R. Phansalkar, J.B. McAlpine, J.G. Napolitano, S.N. Chen, L.S. Araújo, G.F. Pauli, A. Bedran-Russo. **Galloyl moieties enhance the dentin biomodification potential of plant-derived catechins.** *Acta Biomater.,* 10 (2014), pp. 3288-3294, 10.1016/j.actbio.2014.03.036

[21] L. He, C. Mu, J. Shi, Q. Zhang, B. Shi, W. Lin. **Modification of collagen with a natural cross-linker, procyanidin.** *Int. J. Biol. Macromol.,* 48 (2011), pp. 354-359, 10.1016/j.ijbiomac.2010.12.012

[22] N.N. Fathima, M. Baias, B. Blumich, T. Ramasami. **Structure and dynamics of water in native and tanned collagen fibers: effect of crosslinking.** *Int. J. Biol. Macromol.,* 47 (2010), pp. 590-596, 10.1016/j.ijbiomac.2010.08.003

[23] S. Pallan. **M.V. Furtado Araujo, R. Cilli, A. Prakki, Mechanical properties and characteristics of developmental copolymers incorporating catechin or chlorhexidine.** *Dent. Mater.,* 28 (2012), pp. 687-694, 10.1016/j.dental.2012.03.003