Dimeric Proanthocyanidins on the Stability of Dentin and Adhesive Biointerfaces

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Appendix

Dentin Biostability - Quantification of Hydroxyproline Released in the Media

In order to estimate the long-term collagen solubilization by endogenous proteases, a standard protocol with modifications (Reddy & Enwemeka, 1996; Leme-Kraus et al. 2017) was used to quantify the amount of hydroxyproline (HYP) in the storage media (SBF). The method assumes that the typical amount of hydroxyproline in collagen is ≈15% (Reddy and Enwemeka, 1996), therefore the increased amount of hydroxyproline detected in the media is indicative of more collagen degradation. SBF was collected every 2 wk (n = 15) and pooled into two time-points: 0-6 mo and 7-12 mo. Specimens were lyophilized and small aliquots of 0.01 mL were hydrolyzed in 2 M NaOH at 120 °C for one hour and mixed with 0.056 M Chloramine T reagent for 25 min at room temperature. Next, 1 M Ehrlich's reagent was used to develop color at 60 °C for 40 min. Absorbance was measured at 550 nm in a spectrophotometer (Spectramax Plus, Molecular devices, Sunnyvale, CA, US). Reference values for HYP concentrations (0.5, 1, 2, 3, 4, 5 μg/mL) were used for a linear standard curve (Figure S1). Duplicate measurements of HYP content were averaged for each specimen. The estimated HYP release was normalized by the dry weight of individual specimens at baseline and is reported in μg/mL per mg of dentin matrix. The data were statistically analyzed by two-way ANOVA and Tukey's post hoc test (α=0.05).

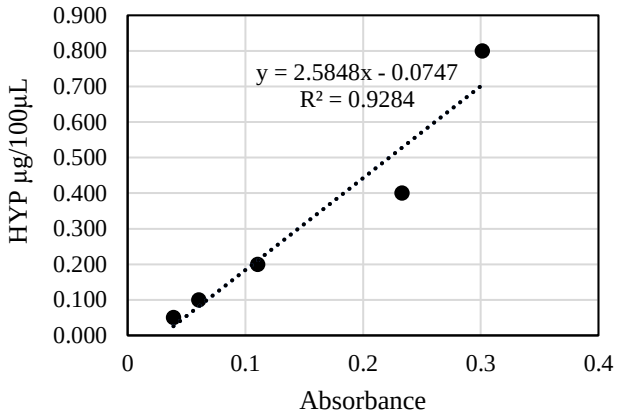

Figure S1. Regression analysis showing the linear relationship of the calibration curve between amounts of hydroxyproline (HYP) detected in the media and absorbance at 550 nm.

Studies of Dentin-Resin Interface

Experimental Resin Composition

The experimental resin was selected after an initial study (Leme-Kraus et al. 2017) showed the bond strength of e-GSE biomodified dentin was not affected by the hydrophilicity of experimental adhesive systems (0%, 6% and 18% wt. HEMA (Hydroxyethyl methacrylate). The composition of the resin H_0 (in wt.%) consists of 41.25% bisphenol A diglycidyl methacrylate (bis-GMA), 18% triethylene glycol dimethacrylate (TEGDMA), 0.15% camphorquinone and 0.6% ethyl-4-dimethylamino benzoate (EDMAB) and 40% ethanol.

Dentin-resin Bioadhesion

The dentin-resin bioadhesive properties of $DIMER_G$, $DIMER_{NG}$ were compared to their precursor, e-GSE. A standard microtensile bond strength test (TBS; Leme-Kraus et al. 2017; Silva Sousa et al. 2016) was carried out using an experimental hydrophobic adhesive resin (H_0) (Leme-

Kraus et al. 2017), and a commercially available resin composite (Filtek™ Supreme Ultra, 3M, St. Paul, MN, US). The occlusal mid-dentin surfaces of third molars (n = 7 per group) were standardized using a 600 grit SiC paper under water irrigation. The dentin was etched with 35% phosphoric acid solution for 15 s, rinsed-out, surface was blotted dry, the respective primer was applied for 1 min ($DIMER_G$, $DIMER_{NG}$ and e-GSE, all prepared at 15% w/v, with pH 7), rinsed-off, blotted-dry and the H_0 adhesive was applied and light-cured for 40 s (Optilux 501, Kerr Dental, Middleton, WI, US). Following, incremental placement of a 5-mm thick resin composite buildup was performed. The same protocol was followed for the control group, except that there was no primer. After 24 h at 37 °C in SBF, interfaces were serially sectioned into 0.8 × 0.8 mm^2 (± 0.1 mm) dentin-resin specimens, which were tested in tensile at crosshead speed of 1 mm/min (microtensile tester, Bisco, Schaumburg, IL, US), at 24 h and after 6- and 12-mo storage in SBF. Data were statistically analyzed using two-way ANOVA and Tukey's post hoc test ($\alpha = 0.05$).

Assessment of the Fracture Pattern

Limited analysis of the fracture pattern of micro-tensile bond strength debonded specimens was done by selecting representative specimens of experimental groups tested 24 h following the bonding procedure. Specimens were mounted in a stub for SEM, sputter-coated with Au-Pd for 2 min and imaged under secondary electron mode in a Hitachi S-3000N Variable Pressure SEM (Hitachi Ltd., Tokyo, Japan).

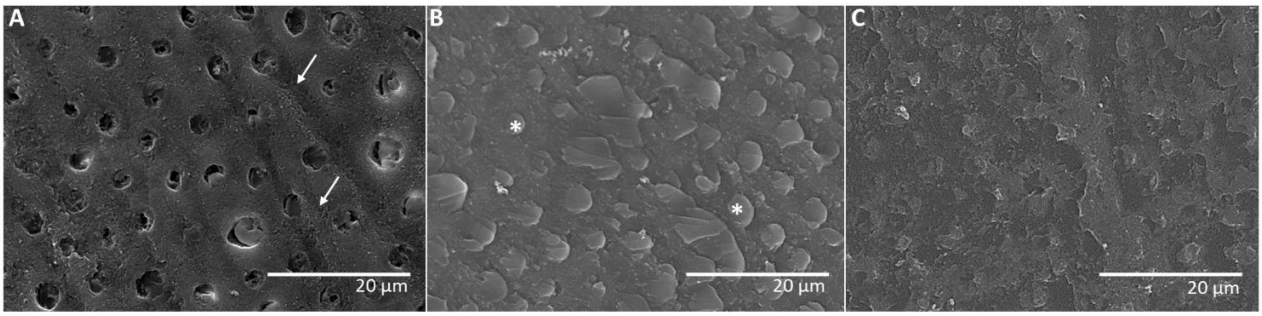

Figure S2. Representative scanning electron microscopy images of the fractured debonded surface following micro-tensile bond strength evaluation. Overall, fracture pattern occurred at the hybrid and adhesive layers, with subtle differences among groups. (**A**) Fractured interfaces treated with $DIMER_G$, which presented the lowest TBS, depict fractures at the bottom of the hybrid layer (HL), porous interfaces and areas of exposed collagen network (arrows), indicating poorer resin infiltration. (**B**) Fractured interfaces treated with $DIMER_{NG}$ show fracture at the HL, presence of fractured tags (*), remnants of adhesive layer, and no apparent collagen exposed. (**C**) Fractured interface treated with e-GSE depicts fracture mainly at the top of the HL, and no apparent exposed collagen.

Study of the Resin Degree of Conversion

Detailed Preparation of the Macro-hybrid Layer Specimens

Macro-hybrid layers (Chiaraputt et al. 2008; Matuda et al. 2016) were prepared for the study of the degree of conversion of the adhesive system at the hybrid layer, as this is the interfacial layer in which PACs would interact with the adhesive resin. Dentin specimens (0.5 × 2.0 × 7.0 mm, n = 5) were demineralized for 5 h in 10% phosphoric acid solution and treated with the respective biomodification primer for 1 h: $DIMER_G$, $DIMER_{NG}$ and e-GSE, all prepared at 15%

w/v, with pH 7; and HEPES buffer (control). The preparation of the macro-hybrid layer specimens followed two steps: (1) each specimen was dehydrated with ascending ethanol/water concentrations (25% for 15 min, 50% for 15min, 75% for 30 min, 95% for 30 min, and 2× in 100% ethanol for 1 h each); and (2) infiltrated with the experimental resin H_0 (50/50 ethanol/resin for 1 h and 100% resin for 1 h) (Matuda et al., 2016). Each specimen was positioned between 2 mylar strips and light-cured for 60 s on top and bottom surfaces (Optilux 501, Kerr Dental, Middleton, WI, US). After polymerization, specimens were polished with SiC paper grits 600, 800 and 1,200 using a water-free silicon (Silicon Oil, Aldrich, St. Louis, MO).

Resin Degree of Conversion

The degree of conversion of the adhesive resin was assessed in a Fourier transformed infra-red spectrometer (FTIR – Nicolet iS50, ThermoFisher Scientific, Waltham, MA) equipped with an attenuated total reflectance (ATR) element. Infra-red spectra was obtained between 4,000 and 600 cm^{-1} of the uncured and cured macro-hybrid specimens and the degree of conversion was calculated using a standard method for calculation of the degree of conversion of methacrylate-based resins through the formula: DC (%) = [1-(R cured/R uncured)] × 100, where R is the ratio between the aliphatic C=C and aromatic C=C (constant) peaks, respectively at 1,638 cm^{-1} and 1,608 cm^{-1} of the cured and uncured macro-hybrid layer specimen (Cadenaro et al. 2009; Malacarne-Zanon et al. 2009). Three spectra were obtained from each specimen. Data were statistically analyzed by one-way ANOVA and Tukey's post hoc ($\alpha = 0.05$).

References

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