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Experimental primers containing synthetic and natural compounds reduce enzymatic activity at the dentin–adhesive interface under cyclic loading

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ABSTRACT

Objective. To evaluate the effect of experimental primers (chlorhexidine, enriched mixture of proanthocyanidins, and doxycycline) on the adhesive properties and gelatinolytic activity at dentin–resin interfaces of occlusal Class I restorations.

Methods. The inactivation of enzymes by the experimental primers was assessed by fluorescence assay and gelatin zymography. To assess the adhesive properties, occlusal Class I cavities were prepared in sound human molars, etched with phosphoric acid and restored with one of the primers and an etch-and-rinse adhesive system (Adper Single Bond Plus—3M ESPE). After the restorative procedures, specimens were divided into two subgroups ($n = 6$) consisting of storage in incubation buffer or axial cyclic loading at 50 N and 1,000,000 cycles. Then, the specimens were sectioned and slices were assigned to *in situ* zymography assay and microtensile bond strength (TBS) test.

Results. Fluorescence assay and gelatin zymography revealed that the experimental primers inactivated rMMPs. *In situ* zymography (2-way ANOVA, Tukey, $p < 0.05$) showed that cyclic loading increased the gelatinolytic activity at the resin–dentin interface and the experimental primers decreased the gelatinolytic activity at the adhesive interface. The experimental primers had no significant effects on dentin–adhesive bond strengths with or without cyclic loading (2-way ANOVA, $p > 0.05$).

Significance. The use of experimental primers impaired the enzymatic activity at the dentin–adhesive interface after cyclic loading and the activity of rMMPs. Cyclic loading did not have a significant effect on the bond strength.

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1. Introduction

Chemical and technical advances in adhesive materials/techniques have improved the performance of resin composite restorations [1–3]. However, there are still challenges that must be overcome, mostly those associated with the stability of dentin–resin composite interfaces [4,5]. The dentin matrix degradation [6] along with the breakdown of hydrophilic contemporary etch-and-rinse adhesives are believed to be the two major causes of suboptimal adhesive interface over time [7]. Non-collagenous components, such as endogenous enzymes play key biological role as mediators of cellular interactions in the maturation and mineralization of dentin [3,4] and also in the *in vitro* breakdown of organic dentin matrix in intimate contact with adhesive interfaces. Specifically a group of endogenous zinc/calcium-dependent matrix metalloproteinases (MMPs) degrades extracellular matrix components including collagen in its native and denatured forms [4,8].

Poorly resin infiltrated collagen fibrils [2,8,9] are susceptible to enzymatic degradation mediated by endogenous proteases [1,8]. Such proteases are activated during surface conditioning as shown by high enzymatic activities at the bottom of the hybrid layer [1,10]. The most well investigated synthetic agent to successfully inactivate endogenous proteases at the dentin–adhesive interface is chlorhexidine digluconate (CHX) [5,7,11–13]. Other synthetic inactivators of endogenous proteases with fewer outcomes include tetracycline [4,7], carbodiimide [14,15] and galardin [7,16]. Doxycycline (DOXY) is a tetracycline semi-synthetic analog, which is considered the most potent and non-selective MMPs inactivating agent among tetracyclines [7,17]. Encapsulation and sustained short term effect of DOXY from a nanotube-modified dentin adhesive has recently shown promising outcomes [18].

The use of plant derived compounds to preserve the dentin–adhesive interface is an attractive and potent alternative to synthetic agents [3,7,8]. Proanthocyanidins (PAC) are known antioxidant and collagen cross-linking agent with vast biological and functional activities [3]. Certain grape seed extracts (GSE) are main sources of PAC [19] shown to enhance the mechanical properties and reduce biodegradation rates of demineralized dentin [3,20] by multi-interaction with dentin matrix components, including type I collagen [3], proteoglycans [3,14] and endogenous proteases [3,21,22]. Isolation of highly bioactive compounds of GSE has recently shown promising results [20] for future design of a standardized clinical intervention material.

In this context, the use of protease inactivators in the demineralized dentin as a pretreatment before resin infiltration appears to be a logical approach for extending the longevity of resin composite restorations [3–5,7]. The inactivation of MMPs by experimental primers may increase the functional stability of dentin–adhesive interfaces [23]. However the effectiveness of such primers under simulated oral conditions is still not well known. The aim of this study was to evaluate the effect of different experimental primers on the enzymatic activity and adhesive properties of dentin–resin interfaces from occlusal Class I restorations under simulated cyclic loading. The null hypotheses tested were that (1) there would be no difference among the anti-proteolytic action of the experimental primers on MMPs activities and on gelatinolytic activity at the hybrid layer (2) there would be no difference in the dentin–adhesive bond strength, regardless of the use of experimental primers and simulated cyclic loading.

2. Material and methods

2.1. *Preparation of experimental primers*

Three experimental primers were prepared as follow: (i) oligomeric proanthocyanidin enriched grape seed extract (e-GSE) obtained by a solvent partitioning protocol previously published [20] and prepared at 15% w/v concentration in buffer solution (20 mM HEPES pH 7.4); (ii) primer of Doxycycline Hydrochloride (DOXY – Fisher Scientific – New Jersey, NJ, USA) at 3% w/v in buffer solution; (iii) Chlorhexidine digluconate (CHX) primer prepared by dilution of stock solution (20% CHX, Sigma; St. Louis, MO, USA) to 0.2% CHX in distilled water. HEPES buffer solution was used as negative control primer. The primers were freshly prepared and the pH adjusted to 7.2 using NaOH at room temperature.

2.2. *rMMP-2 activity—fluorescence assay*

The gelatinolytic activity of rMMP-2 (Human MMP-2, recombinant, 10 μg/mL, AnaSpec, Fremont, CA, USA) incubated with the experimental primers was assayed according to the protocol described by Tay et al. [24], using EnzChek Gelatinolytic/Collagenolytic Assay Kit (D-12054, Molecular Probes, Eugene, OR, USA). Primers concentrations were 0.2% CHX, 0.65% e-GSE and 3% DOXY. Enzyme activation with 4-aminophenylmercuric acetate (APMA) was done previously for one hour at 37 °C [17]. The fluorescent cleavage products were read in a 96-well fluorescent plate reader (Victor X5, PerkinElmer, Waltham, MA, USA), operated with an absorption maxima at 495 nm and fluorescence emission maxima at 515 nm. Fluorescence measurements were taken at 0 (baseline), 1 h and 2 h incubation at 37 °C and data were expressed in percentage of enzyme inactivation. All analyses were carried out in triplicate and included positive control gelatinase standards as well as reagent blanks.

2.3. *Gelatinolytic activity of rMMP-2 and -9—zymography assay*

The rMMP-2 (AnaSpec) and -9 (Human MMP-9, recombinant, catalytic domain, AnaSpec) enzymes incubated with experimental primers for 1 h at 37 °C were subjected to electrophoresis under non-reducing conditions in 10% SDS-polyacrylamide gels copolymerized with 0.1% gelatin from porcine skin (Sigma–Aldrich, St. Louis, MO, USA), as previously described [25]. Activation of gelatinase proforms was done with 2 mM of APMA at 37 °C for 1 h. After electrophoresis, gels were washed in 2% Triton-X 100 (Sigma–Aldrich, St. Louis, MO, EUA) with agitation and then incubated for 24 h at 37 °C in enzyme incubation buffer (Tris–HCl 50 mM, $CaCl_2$ 5 mM and $ZnCl_2$ 1 μM). Negative control zymogram was incubated in the same buffer with presence of 2 mM 1,10-phenanthroline.