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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, the 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 sectioned and sliced specimens 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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Keywords

Matrix metalloproteinases; Cross-linking agent; Dentin; Adhesion; Cyclic loading; In situ zymography; Enzymatic activity; Proanthocyanidins; Chlorhexidine; Doxycycline

Introduction

Chemical and technical advances in adhesive materials/techniques have improved the performance of resin composite restorations [1, 2, 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, 12, 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 analogue, 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, 4, 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 obtained as follow: (i) oligomeric proanthocyanidin enriched grape seed extract (e-GSE) prepared 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. (2006) [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, Perkin Elmer, 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 hr. 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, CaCl₂ 5 mM and ZnCl₂ 1 µM). Negative control zymogram was incubated in the same buffer with presence of 2 mM 1,10-phenanthroline. Then, gels were stained in 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad,

Laboratories Inc, CA, USA), de-stained and scanned by an imaging system Odyssey CLx (LI-COR, NE, USA) with white light exposure. The quantification of gelatinolytic activity was carried out in ImageJ software (NIH, Frederick, MD, USA) and expressed as percentage of inactivation considering MMP-2 and MMP-9 positive control bands as 100%.

2.4. Dental Restorative Procedures and Simulated Cyclic Loading

A total of 48 freshly extracted human sound molars stored at -20°C were selected (University of Illinois at Chicago IRB approved protocol # 2011-0132). Roots were cleaned, embedded in acrylic resin and the occlusal surfaces were ground flat 5 mm above the cemento-enamel junction. Occlusal Class I preparations were done using a #245 carbide bur (Regular Carbide Burs – Dentsply, Des Plaines, IL, USA) in a high-speed water cooled handpiece to final dimensions of 5 mm length, 4 mm width and 3 mm depth.

A total-etch bonding protocol was selected using 35% phosphoric acid (3M ESPE, St. Paul, MN, USA) for 15 seconds, followed by active application of Adper Single Bond Plus (3M ESPE, St. Paul, MN, USA - N561025) on the cavity surfaces for 15 seconds. Then, gentle air jets were applied to remove the excess solvent, and the adhesive was light cured for 20 seconds using a halogen light unit (Optilux 501 – Halogen Curing Light, Kerr Corporation, Orange, CA, USA $G 830\text{ mW/cm}^2$). Experimental primers were used after acid etching step as follow: Control (20 mM HEPES), 15% e-GSE or 3% DOXY 3% primers were applied to the preparations for 60 s, rinsed for 60 s and the excess water removed with absorbent paper (Kimwipes, Kimberly Clark, Irving, TX, USA); or 0.2% CHX primer applied to the preparation for 60 s followed by removal of excess primer with absorbent paper.

The cavity preparations were filled with two 1.5 mm thick horizontal increments of Filtek Supreme Ultra resin composite (3M ESPE, St. Paul, MN, USA - N566267) and each increment was light-cured for 20 s (Optilux 501-Kerr Corp., Orange, CA, USA). The specimens were stored in distilled water at 37°C for 24 h and then divided into two subgroups ($n=6$): storage in incubation buffer (5 mM HEPES, 2.5 mM CaCl_2 , 0.3 mM NaN_3 , 0.05 mM ZnCl_2 pH 7.4) at 37°C for 9 days; or simulated cyclic loading (Coil – Cycler, Proto-tech, at 50 N load, 1.5 Hz frequency, and 1×10^6 cycles for 9 days). After cyclic loading, sections from each tooth were obtained as follows, and assigned to *in situ* zymography assay and microtensile bond strength test.

2.4.1. In situ Zymography at the dentin-resin interface—Restored teeth were cut vertically into 0.6-mm-thick slices to expose the adhesive interfaces using a slow-speed saw under water cooling (Isomet 1000, Buehler, Lake Bluff, IL, USA) and further manually polished (Buehler) to obtain 500- μm -thick specimens. *In situ* zymography was performed with quenched fluorescein-conjugated gelatin as MMPs substrate (EnzChek Gelatinolytic/Collagenolytic Assay Kit, D-12054, Molecular Probes, Eugene, OR, USA). A 1.0 mg/mL stock solution of fluorescein-labeled gelatin was diluted 1:8 in buffer (NaCl 150 mM, CaCl_2 5 mM, Tris-HCl 50 mM, pH 8.0). An 80- μL quantity of the fluorescent gelatin mixture was placed on top of each slab and covered with a coverslip (Microscope Cover Slips, Rochester Scientific CO., INC, Rochester, NY, USA). The hydrolysis of the quenched fluorescein-conjugated gelatin substrate, indicative of endogenous gelatinolytic enzyme activity, was

assessed by examination under a fluorescence microscope in $40\times$ magnification (Leica DMI 6000, Buffalo Grove, IL, USA) after 2 h incubation in humidified chamber at 37°C [26, 27]. Images were acquired along the adhesive interface of all specimens, and then three representative images of each group were selected for software analysis with ImageJ (NIH, Frederick, MD, USA). The intrinsic fluorescence was expressed as relative fluorescence units (RFU). The data did not meet the criteria of equality of variance across groups (Levene's test, $p < 0.001$). The results were statistically compared using ANOVA and Games-Howell post hoc tests ($\alpha = 0.05$).

2.4.2. Dentin-Resin Microtensile Bond Strength (TBS)—The occlusal Class I restorations ($n = 6$) were sectioned perpendicular to the adhesive-dentin interface into dentin-resin specimens (0.8×0.8 mm; 5 per tooth) using a slow-speed diamond saw under water cooling (Buehler – Series 15 LC Diamond, Buehler, Lake Buff, IL, USA). The specimens were fixed with cyanoacrylate glue (Loctite Super Glue – Gel Control, Rocky Hill, CT, USA) to a jig, which was mounted on a microtensile tester machine (Bisco Inc., Schaumburg, IL, USA) and subjected to a tensile force at 1 mm/min. Each tooth was considered as a statistical unit. The data did not meet the assumption of equality of variance across groups (Levene's test, $p < 0.001$). The results were statistically subjected to ANOVA ($\alpha = 0.05$).

3. Results

3.1. rMMP-2 Activity

The use of the e-GSE primer presented complete inactivation (100%) of rMMP-2 in both times tested. DOXY primers presented 100% and 99.6% of rMMP-2 inactivation at 1 h and 2 h, respectively. CHX primer was effective reducing the rMMP-2 activity after 1 h and 2 h, presenting 62.2% and 67%, respectively.

3.2. Gelatinolytic activity of rMMP-2 and -9 - Zymography assay

Lower intensity in bands corresponding to rMMP-9 (92 and 86 kDa) and rMMP-2 (72 and 64 kDa) was observed when the enzymes were incubated with the experimental primers (Fig. 1). The gel densitometry (Fig. 2) showed inactivation rates above 50% for all experimental primers tested. DOXY/rMMP-9 group showed the highest percentage of inactivation (99%), while the CHX/rMMP-2 showed the lowest inactivation (68.85%). The reduction of gelatinolytic activity was higher for rMMP-9 when compared to rMMP-2, regardless of the experimental primer. Complete inhibition by phenanthroline confirmed the nature of the gelatinolytic activity as being from rMMPs (data not shown).

3.3. In situ zymography at the dentin-resin interface

There was statistically significant interaction between factors (experimental primers vs. simulated aging, $p = 0.0012$). There were significant differences among primers ($p = 0.005$) and simulated aging ($p = 0.006$). Under no cyclic loading condition, there were no statistical significant differences among primers ($p > 0.05$) (Fig. 3 - A, C, E and G). The lowest RFU value representing less gelatin degradation was observed in CHX group, though not statistically different from the other primers. Cyclic loading significantly increased the

enzymatic activity at the resin-dentin interface of the control group ($p < 0.001$, Table 1). The three experimental primers decreased the enzymatic activity at the adhesive interface after cyclic loading when compared to control; and no significant differences were observed among the experimental primers ($p > 0.05$, Table 1).

3.4. Dentin-Resin Microtensile Bond Strength (TBS)

The TBS results are presented in Fig. 4. Two-way ANOVA test ($p < 0.05$) showed no statistically significant differences among the experimental primers ($p = 0.637$) or simulated aging ($p = 0.722$). No interaction was found when these two factors were compared ($p = 0.993$).

4. Discussion

It is well known that the dentin-adhesive interface can be degraded by MMPs [4, 5, 8], therefore the use of inactivators of MMPs seems to be a valid strategy to increase the dentin-resin bond durability [3, 4, 15]. Our first null hypothesis was rejected as there were significant differences among the anti-proteolytic actions of experimental primers on recombinant MMPs and enzymatic activity at the dentin-resin interface. Zymography assay findings demonstrated the effectiveness of all experimental primers against rMMP-9 (Figs. 1 and 2). The ability of experimental primers to inactivate rMMP-2 was lower than rMMP-9; where DOXY primer was the most effective and CHX was the least effective. Despite similar functional properties, MMPs -2 and -9 have different sensitivities to the inhibitors [28]. Specific inhibition of MMP-9 may be linked to its hemopexin domain that differs from MMP-2 because of the longer hinge region [1]. These features could explain the differences in results shown by the densitometry analysis of the gelatin zymography (Figs. 1 and 2).

The enzyme inactivation effect of CHX is attributed to the amphipathic origin of its molecule that can bind to several proteins by a chelation mechanism [29]. Through a similar mechanism, it is suggested that CHX prevents binding of ions such as Zn^{2+} and Ca^{2+} to the MMPs structure, thereby inhibiting its catalytic activity [10]. Moreover, CHX presents cationic properties with strong affinity for organic (collagen) and inorganic (hydroxyapatite) structures in dentin, especially when applied after acid etching [30]. Contrary to other studies [1, 12] CHX showed lower inactivation results when compared with the other experimental primers in both gelatin zymography and collagenase/gelatinase assays. One explanation for this behavior is that collagen can compete with MMPs by binding with CHX, which often requires the use of relatively high concentrations of CHX [31]. However the toxicity of this substance may limit the use of high concentrations [32].

The potent effect on enzyme inactivation of e-GSE would be based on the concept that collagen cross-linking agents such as PACs can directly silence the enzymes [7, 33] as well as hide cleavage sites in the collagen due to collagen modifications [21]. The interaction of PACs with MMPs could induce irreversible changes in the catalytic domain, or by allosteric inhibition of other modular domains involved in collagen degradation [34]. Such modifications can result in a decreased ability of the enzymes to degrade the substrate [2], confirmed by recent findings [22, 35]. Like CHX, DOXY directly inactivates collagenases by binding to metal ions such as Zn^{2+} and Ca^{2+} that are required by the enzyme to maintain

its proper conformation and proteolytic activity [36]. DOXY also exhibits direct inactivation of MMPs and expression by its antioxidant properties [36, 37]. Similarly to other studies [36, 38], herein doxycycline impaired proteolytic activity of MMP -2 and -9.

The *in situ* zymography analysis shows that cyclic loading induced significantly higher gelatinolytic activity at the dentin-resin interface that could potentially increase the dentin matrix breakdown. In the current study, after cyclic loading all experimental primers were able to decrease the activity of MMPs at the adhesive interface. As recently described, the mechanical loading exacerbates the damage in regions where hydrolytic degradation was already present at the interface [40]. Hence, increased degradation of collagen with cyclic loading could be reversed by protease inhibitors [39]. Since the dentin matrix is reinforced through the use of enzymes inactivators and cross-linking agents, the deleterious effects caused by the mechanical loading in the adhesive interface may be reduced [40]. Therefore, the use of experimental primers would be a very attractive clinical strategy, particularly within clinically relevant protocols, as investigated in this study.

Higher enzymatic activity at the adhesive interface does not appear to affect the dentin-resin bond strength under the adopted simulated cyclic loading (Fig. 4). Therefore the second hypothesis was accepted. Cyclic loading is an *in vitro* method to simulate aging of adhesive restorations through the application of repeated load cycles [41]. Effectiveness of such strategy is dependent on the applied protocol, materials and cavity configuration [42]. The cavity configuration design induces higher polymerization stress at the interface when compared to flat surfaces, resulting in lower bond strength [43]. In addition, the TBS outcome disagrees with previous studies, which showed immediate increase on the dentin-resin bond strength after dentin treatment with certain bioactive agents [6, 21]. Most studies using bioactive agents have been performed on flat surfaces [1, 15, 18, 21]. Besides, a high standard deviation was observed in TBS results and such values could be explained by the constraints on achieving smooth surfaces for adhesion on an occlusal Class I cavity configuration.

Recently, the modification of the dentin matrix has been extensively studied with extracts rich in proanthocyanidins (PACs) [3, 20]. The effective interaction of PACs and the dentin matrix remarkably increases the dentin matrix's resistance to enzymatic degradation [3, 7, 22, 33, 44] without interfering with the formation of the hybrid layer [6, 21, 35]. The lack of enhanced bond strength by e-GSE may be also due to the use of a high-C factor cavity design, application time and restorative protocol [21]. The immediate effect of DOXY in the dentin-resin interface as protease inhibitor has been recently investigated [13, 18]. Herein, a 3% DOXY primer was prepared to exert an effective MMP inactivation effect at sub-antimicrobial concentration [38]. Similar to other *in vitro* studies, no significant effect was observed on the TBS; but a significant decrease in enzymatic activity supports the efficacy of DOXY as MMPs inhibitor [45]. Concerns with increased risk for development of drug-resistance bacteria warrant studies on tetracyclines analogs to reduce continuous exposure to antibiotics.

In summary, increased enzymatic activity takes place under simulated cyclic loading at the dentin-resin interface of occlusal Class I preparations. The experimental primers

significantly inactivated the enzymatic activity at the adhesive interface by their unique inactivation mechanisms. The enzymatic activity at the interface indicates a dynamic process of degradation that can potentially accelerate the degradation of the dentin matrix components. The decrease in proteolytic activity did not affect the TBS, possibly due to the short term evaluation.

5. Conclusion

The use of experimental primers decreased the enzymatic activity at the dentin-resin interface after cyclic loading and inactivated rMMPs-2 and 9. Increased endogenous MMP activity may be expected at dentin-resin interfaces when in function; even though the cyclic loading appears to not have a significant effect on the short term bond strength. Thus, the experimental primers can reduce *in vitro* the proteolytic activity at the dentin-resin interface.

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References

1. Breschi L, Mazzoni A, Nato F, Carrilho M, Visintini E, Tjäderhane L, Ruggeri A Jr, Tay FR, Dorigo E, Pashley D. Chlorhexidine stabilizes the adhesive interface: a 2-year in vitro study. *Dent Mater.* 2010; 26:320–325. [PubMed: 20045177]
2. Liu Y, Tjäderhane L, Breschi L, Mazzoni A, Li N, Mao J, Pashley DH, Tay FR. Limitations in bonding to dentin and experimental strategies to prevent bond degradation. *J Dent Res.* 2011; 90:953–968. [PubMed: 21220360]
3. Bedran-Russo AK, Pauli GF, Chen SN, McAlpine J, Castellan CS, Phansalkar RS, Aguiar TR, Vidal CMP, Napolitano J, Nam JW, Leme AA. Dentin Biomodification: Strategies, Renewable Resources and Clinical Applications. *Dent Mater.* 2014; 30:62–76. [PubMed: 24309436]
4. Perdigão J, Reis A, Loguercio AD. Dentin adhesion and MMPs: a comprehensive review. *J Esthet Restor Dent.* 2013; 25:219–241. [PubMed: 23910180]
5. Tjäderhane L. Dentin Bonding: Can We Make it Last? *Oper Dent.* 2015; 40:4–18. [PubMed: 25615637]
6. Fang M, Liu R, Xiao Y, Li F, Wang D, Hou R, Chen J. Biomodification to dentin by a natural crosslinker improved the resin–dentin bonds. *J Dent.* 2012; 40:458–466. [PubMed: 22366684]
7. Tjäderhane L, Nascimento FD, Breschi L, Mazzoni A, Tersariol IL, Geraldini S, Tezvergil-Mutluay A, Carrilho M, Carvalho RM, Tay FR, Pashley DH. Strategies to prevent hydrolytic degradation of the hybrid layer—A review. *Dent Mater.* 2013a; 29:999–1011. [PubMed: 23953737]
8. Tjäderhane L, Nascimento FD, Breschi L, Mazzoni A, Tersariol ILS, Geraldini S, Tezvergil-Mutluay A, Carrilho MR, Carvalho RM, Tay FR, Pashley DH. Optimizing dentin bond durability: control of collagen degradation by matrix metalloproteinases and cysteine cathepsins. *Dent Mater.* 2013b; 29:116–135. [PubMed: 22901826]
9. Agee KA, Prakki A, Abu-Haimed T, Naguib GH, Nawareg MA, Tezvergil-Mutluay A, Scheffel DL, Chen C, Jang SS, Hwang H, Brackett M, Grégoire G, Tay FR, Breschi L, Pashley DH. Water distribution in dentin matrices: bound vs. unbound water. *Dent Mater.* 2015; 31:205–216. [PubMed: 25612786]
10. De Munck J, Van den Steen PE, Mine A, Van Landuyt KL, Poitevin A, Opdenakker G, Van Meerbeek B. Inhibition of enzymatic degradation of adhesive-dentin interfaces. *J Dent Res.* 2009; 88:1101–1106. [PubMed: 19861692]

11. Hebling J, Pashley DH, Tjäderhane L, Tay FR. Chlorhexidine arrests subclinical degradation of dentin hybrid layers *in vivo*. J Dent Res. 2005; 84:741–746. [PubMed: 16040733]
12. Scaffa PM, Vidal CM, Barros N, Gesteira TF, Carmona AK, Breschi L, Pashley DH, Tjäderhane L, Tersariol IL, Nascimento FD, Carrilho MR. Chlorhexidine inhibits the activity of dental cysteine cathepsins. J Dent Res. 2012; 91:420–425. [PubMed: 22266526]
13. Stanislawczuk R, Pereira F, Munoz MA, Luque I, Farago PV, Reis A, Loguercio AD. Effects of chlorhexidine-containing adhesives on the durability of resin-dentin interfaces. J Dent. 2014; 42:39–47. [PubMed: 24252801]
14. Bedran-Russo AKB, Vidal CMP, Santos PHD, Castellan CS. Long-term effects of carbodiimide on dentin matrix and resin–dentin bonds. J Biomed Mater Res B Appl Biomater. 2010; 94:250–255. [PubMed: 20524201]
15. Scheffel D, Delgado C, Soares DG, Basso F, Souza Costa CA, Pashley D, Hebling J. Increased Durability of Resin-Dentin Bonds Following Cross-Linking Treatment. Oper Dent. 2015; 40:533–539. [PubMed: 25764044]
16. Zheng P, Zaruba M, Attin T, Wiegand A. Effect of different matrix metalloproteinase inhibitors on microtensile bond strength of an etch-and-rinse and a self-etching adhesive to dentin. Oper Dent. 2015; 40:80–86. [PubMed: 24815915]
17. Sulkala M, Wahlgren J, Larmas M, Sorsa T, Teronen O, Salo T, Tjäderhane L. The effects of MMP inhibitors on human salivary MMP activity and caries progression in rats. J Dent Res. 2001; 80:1545–1549. [PubMed: 11499510]
18. Feitosa SA, Palasuk J, Kamocki K, Geraldini S, Gregory RL, Platt JA, Windsor LJ, Bottino MC. Doxycycline-Encapsulated Nanotube-Modified Dentin Adhesives. J Dent Res. 2014; 93:1270–1276. [PubMed: 25201918]
19. Joshi SS, Kuszynski CA, Bagchi D. The cellular and molecular basis of health benefits of grape seed proanthocyanidin extract. Curr Pharm Biotechnol. 2001; 2:187–200. [PubMed: 11480422]
20. Phansalkar RS, Nam JW, Chen SN, McAlpine JB, Napolitano JG, Leme A, Vidal CMP, Aguiar T, Bedran-Russo AK, Pauli GF. A galloylated dimeric proanthocyanidin from grape seed exhibits dentin biomodification potential. Fitoterapia. 2015; 101:169–178. [PubMed: 25542682]
21. Castellan CS, Bedran-Russo AK, Antunes A, Pereira PN. Effect of dentin biomodification using naturally derived collagen cross-linkers: one-year bond strength study. Inter J Dent. 2013; 9:18010.
22. Seseogullari-Dirihan R, Mutluay MM, Vallittu P, Pashley DH, Tezvergil-Mutluay A. Effect of pretreatment with collagen crosslinkers on dentin protease activity. Dent Mater. 2015; 31:941–947. [PubMed: 26037790]
23. Ricci HA, Sanabe ME, Costa CA, Hebling J. Effect of chlorhexidine on bond strength of two-step etch-and-rinse adhesive systems to dentin of primary and permanent teeth. Am J Dent. 2010; 23:128–132. [PubMed: 20718207]
24. Tay FR, Pashley DH, Loushine RJ, Norman Weller R, Monticelli F, Osorio R. Self-etching adhesives increase collagenolytic activity in radicular dentin. J Endod. 2006; 32:862–868. [PubMed: 16934629]
25. Manello F, Sebastiani M. Zymographic analyses and measurement of matrix metalloproteinase-2 and -9 in nipple aspirate fluids. Clin Chem. 2003; 49:1546–1550. [PubMed: 12928245]
26. Mazzoni A, Nascimento FD, Carrilho M, Tersariol I, Papa V, Tjäderhane L, Di Lenarda R, Tay FR, Pashley DH, Breschi L. MMP Activity in the hybrid layer detected with *in situ* zymography. J Dent Res. 2012; 91:467–472. [PubMed: 22354448]
27. Pessoa JIC, Guimarães GN, Viola NV, Silva WJ, Souza AP, Tjäderhane L, Line SR, Marques MR. *In situ* study of the gelatinase activity in demineralized dentin from rat molar teeth. Acta Histochem. 2013; 115:245–251. [PubMed: 22897943]
28. Xu X, Chen Z, Wang Y, Yamada Y, Steffensen B. Functional basis for the overlap in ligand interactions and substrate specificities of matrix metalloproteinases-9 and -2. Biochem J. 2005; 392:127–134. [PubMed: 16008524]
29. Negrelo Newton AP, Cadena SM, Merlin Rocha ME, Skäre Carnieri EG, Martinelli de Oliveira MB. New data on biological effects of chlorhexidine: Fe²⁺ induced lipid peroxidation and mitochondrial permeability transition. Toxicol Lett. 2004; 151:407–416. [PubMed: 15261985]

30. Iskander M, Elkassas D, Mohsen MA. Effect of two matrix metalloproteinase inhibitors on the color stability of a nanofilled resin composite. *Oper Dent*. 2015; 40:11–20.
31. Carrilho MR, Carvalho RM, Sousa EN, Nicolau J, Breschi L, Mazzoni A, Tjäderhane L, Tay FR, Agee K, Pashley DH. Substantivity of chlorhexidine to human dentin. *Dent Mater*. 2010; 26:779–785. [PubMed: 20472282]
32. Mobarak EH. Effect of chlorhexidine pretreatment on bond strength durability of caries-affected dentin over 2-year aging in artificial saliva and under simulated intrapulpal pressure. *Oper Dent*. 2011; 36:649–660. [PubMed: 21864128]
33. Epasinghe DJ, Yiu CK, Burrow MF, Hiraishi N, Tay FR. The inhibitory effect of proanthocyanidin on soluble and collagen-bound proteases. *J Dent*. 2013; 41:832–839. [PubMed: 23806340]
34. Sela-Passwell N, Rosenblum G, Shoham T, Sagi I. Structural and functional bases for allosteric control of MMP activities: Can it pave the path for selective inhibition? *Biochim Biophys Acta*. 2010; 1803:29–38. [PubMed: 19406173]
35. Cecchin D, Pin LC, Farina AP, Souza M, Vidal CM, Bello YD, Ferraz CC, Bedran-Russo A. Bond Strength between fiber posts and root dentin treated with natural cross-linkers. *J Endod*. 2015; 41:1667–1671. [PubMed: 26259647]
36. Kudalkar MD, Nayak A, Bhat KS, Nayak RN. Effect of *Azadirachta indica* (Neem) and Aloe vera as compared to subantimicrobial dose doxycycline on matrix metalloproteinases MMP-2 and MMP-9: An *in-vitro* study. *Ayu*. 2014; 35:85–89. [PubMed: 25364206]
37. Bartoli MA, Parodi FE, Chu J, Pagano MB, Mao D, Baxter BT, Buckley C, Ennis TL, Thompson RW. Localized administration of doxycycline suppresses aortic dilatation in an experimental mouse model of abdominal aortic aneurysm. *Ann Vasc Surg*. 2006; 20:228–236. [PubMed: 16572291]
38. Mata KM, Tefé-Silva C, Floriano EM, Fernandes CR, Rizzi E, Gerlach RF, Mazzuca MQ, Ramos SG. Interference of doxycycline pretreatment in a model of abdominal aortic aneurysms. *Cardiovascular Pathology*. 2015; 24:110–120. [PubMed: 25466491]
39. Turco G, Frassetto A, Fontanive L, Mazzoni A, Cadenaro M, Di Lenarda R, Tay FR, Pashley DH, Breschi L. Occlusal loading and cross-linking effects on dentin collagen degradation in physiological conditions. *Dent Mater*. 2016; 32:192–199. [PubMed: 26743966]
40. Zhang Z, Beitzel D, Majd H, Mutluay M, Tezvergil-Mutluay A, Tay FR, Pashley DH, Arola D. Effect of carbodiimide on the fatigue crack growth resistance of resin–dentin bonds. *Dent Mater*. 2016; 32:211–222. [PubMed: 26739775]
41. Toledano M, Aguilera FS, Yamauti M, Ruiz-Requena ME, Osorio R. In vitro load-induced dentin collagen-stabilization against MMPs degradation. *J Mech Behav Biomed*. 2013; 27:10–18.
42. Vidal CMP, Pavan S, Briso ALF, Bedran-Russo AK. Effects of three restorative techniques in the bond strength and nanoleakage at gingival wall of Class II restorations subjected to simulated aging. *Clin Oral Invest*. 2013; 17:627–633.
43. Choi KK, Ryu GJ, Choi SM, Lee MJ, Park SJ, Ferracane JL. Effects of cavity configuration on composite restoration. *Oper Dent*. 2004; 29:462–469. [PubMed: 15279488]
44. Macedo GV, Yamauchi M, Bedran-Russo AK. Effects of chemical cross-linkers on caries-affected dentin bonding. *J Dent Res*. 2009; 88:1096–1100. [PubMed: 19892915]
45. Acharya MR, Venitz J, Figg WD, Sparreboom A. Chemically modified tetracyclines as inhibitors of matrix Metalloproteinases. *Drug Resist Updat*. 2004; 7:195–208. [PubMed: 15296861]

Highlights

- The use of different experimental primers on dentin-resin restorations is proposed.
- Experimental primers significantly reduced the enzymatic activity at the dentin-resin interface.
- Proanthocyanidins from enriched-grape seed extracts, chlorhexidine and doxycycline inactivated activity of rMMPs-2 and 9.
- Increased enzymatic activity took place under simulated cyclic loading. Cyclic loading did not have a significant effect on the resin-dentin bond strength.

Figure 1.

Gelatin zymography gel showing gelatinase activity of MMPs-2 and -9 when incubated with experimental primers. Std Lane: Molecular weight standard. Lane 1: MMP-9 isoform. Lane 2: MMP-2 isoform. Lanes 3, 5 and 7: incubation of MMP - 9 with CHX, e-GSE and DOXY respectively. Lanes 4, 6 and 8: incubation of MMP - 2 with CHX, e-GSE and DOXY, respectively.

Figure 2.
Gelatinolytic activity presented by enzymes incubated or not with the experimental primers.

Figure 3.

Representative dentin-resin interface images after 2 h of incubation time with quenched fluorescein-conjugated gelatin substrate. **R** = resin composite, **I** = adhesive interface and **D** = dentin. **A, C, E** and **G**: Images from control group, CHX, GSE and DOXY, respectively, not submitted to the load cycling. **B, D, F** and **H**: Images from control group, CHX, GSE and DOXY, respectively, subjected to cyclic loading.

Figure 4. Results of the dentin-resin microtensile bond strength at the pulpal floor of occlusal Class I restorations.

Table 1

In situ zymography quantification of the enzymatic activity at the resin-dentin interface by the quantification of quenched gelatin fluorescence expressed in relative fluorescence units (RFU).

Gelatinolytic activity at the dentin-resin interface <i>Fluorescence units (RFU)</i>				
Simulated aging	Experimental Primers			
	Control	0.2% CHX	15% e-GSE	3% DOXY
No Cyclic Loading (Control)	1.47 Aa (1.88)	0.00 Aa (0.00)	0.73 Aa (1.40)	0.91 Aa (2.6)
Cyclic Loading	24.45 Ab (11.35)	1.88 Ba (5.64)	3.78 Ba (7.56)	2.83 Ba (7.72)

* Different letters indicate statistically significant differences ($p < 0.05$) in each row (uppercase) and column (lowercase). CHX: chlorhexidine digluconate; e-GSE: oligomeric proanthocyanidins enriched grape seed extract; DOXY: Doxycycline Hydrochloride.