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ABSTRACT

Matrix metalloproteinases (MMPs) cause collagen degradation in hybrid layers created by dentin adhesives. This *in vitro* study evaluated the feasibility of using a cross-linking agent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), to inactivate soluble rhMMP-9, as an example of dentin MMPs, and matrix-bound dentin proteases. The inhibitory effects of 5 EDC concentrations (0.01-0.3 M) and 5 incubation times (1-30 min) on soluble rhMMP-9 were screened with an MMP assay kit. The same EDC concentrations were used to evaluate their inhibitory effects on endogenous proteinases from completely demineralized dentin beams that were incubated in simulated body fluid for 30 days. Decreases in modulus of elasticity (E) and dry mass of the beams, and increases in hydroxyproline content of hydrolysates derived from the incubation medium were used as indirect measures of matrix collagen hydrolysis. All EDC concentrations and pre-treatment times inactivated MMP-9 by 98% to 100% ($p < 0.05$) compared with non-cross-linked controls. Dentin beams incubated in 0.3 M EDC showed only a 9% decrease in E (45% decrease in control), a 3.6% to 5% loss of dry mass (18% loss in control), and significantly less solubilized hydroxyproline when compared with the control without EDC cross-linking ($p < 0.05$). It is concluded that EDC application for 1 min may be a clinically relevant and effective means for inactivating soluble rhMMP-9 and matrix-bound dentin proteinases if further studies demonstrate that EDC is not toxic to pulpal tissues.

KEY WORDS: carbodiimide, dentin, hydroxyproline, matrix metalloproteinase, soluble collagen, degradation..

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Carbodiimide Cross-linking Inactivates Soluble and Matrix-bound MMPs, *in vitro*

INTRODUCTION

Etch-and-rinse dentin adhesives rely predominantly on micromechanical retention for bonding resins to dentin. Acid-etching of dentin exposes matrix metalloproteinases (MMPs) which are inactive in mineralized dentin and bone (Collins *et al.*, 2002; Breschi *et al.*, 2010). These enzymes are zinc- and calcium-dependent hydrolases that add water across specific peptide linkages in collagen peptides (Nagase *et al.*, 2006). Upon activation by mildly acidic adhesive resin components (Mazzoni *et al.*, 2006; Tay *et al.*, 2006), MMPs (MMP-2, -8, -9) (Martin-De Las Heras, 2000; Sulkala *et al.*, 2002, 2007; Mazzoni *et al.*, 2006) cause progressive degradation of exposed collagen fibrils.

Chlorhexidine has been used as a non-specific MMP inhibitor (Gendron *et al.*, 1999) to prevent degradation of hybrid layers (Hebling *et al.*, 2005; Carrilho *et al.*, 2007; WW Brackett *et al.*, 2007; MG Brackett *et al.*, 2009). However, chlorhexidine is water-soluble and may leach out of hybrid layers, compromising its long-term anti-MMP effectiveness (Ricci *et al.*, 2010). An entirely different approach is to treat the acid-etched dentin containing activated matrix-bound MMPs with cross-linking agents that inactivate the catalytic site of proteases (Liu *et al.*, 2011). Carbodiimides have been used as alternative cross-linking agents to glutaraldehyde, since they contain no potentially cytotoxic aldehyde residuals (Huang *et al.*, 1990; Petite *et al.*, 1995). They non-specifically cross-link all proteins by activating the carboxylic acid groups of glutamic and aspartic acids to form an O-acylisourea intermediate. The latter reacts with the ϵ -amino groups of lysine or hydroxylysine to form an amide cross-link, leaving urea as the terminal by-product. Carbodiimides are examples of zero-length cross-linking agents wherein peptides are cross-linked to one another without introducing additional linking groups. Previous research utilized EDC to increase the durability of resin-dentin bonds by increasing the mechanical properties of the collagen matrix (Bedran-Russo *et al.*, 2010); however, the 1 to 4 hrs required for that procedure is clinically unacceptable.

Calero *et al.* (2002) compared the effectiveness of glutaraldehyde and EDC in inactivating MMPs in porcine pericardium after chloroform-methanol

extraction of lipids. In that study, 0.3 M EDC coupled with 0.05 M N-hydroxysuccinimide inhibited both MMP-2 and MMP-9 by 80%, whereas glutaraldehyde inhibited MMP-2 only by less than 25%. Thus, the objective of the present study was to evaluate the feasibility of using EDC to inactivate soluble rhMMP-9 as a representative dentin MMP, and in inactivating the endogenous collagenolytic activity of demineralized dentin. The null hypotheses tested were: (1) that EDC does not inactivate soluble rhMMP-9, (2) that EDC does not inactivate matrix-bound MMPs, and (3) that EDC does not inactivate matrix-bound MMPs within 1 min of treatment.

MATERIALS & METHODS

Inactivation of Soluble rhMMP-9

Purified rhMMP-9 and a generic colorimetric MMP assay kit (Sensolyte) were obtained from AnaSpec Inc. (Freemont, CA, USA). To evaluate the effect of EDC incubation time on inactivation of a representative soluble MMP, we dissolved EDC in pH 7 assay buffer to reach a final concentration of 0.3 M. Two different experiments were performed. The first was to determine the concentration of EDC required to inactivate rhMMP-9 completely. The enzyme was pre-incubated in each EDC concentration for 20 min prior to the assay of its activity. The second experiment was to determine how rapidly 0.3 M EDC could inactivate rhMMP-9.

To evaluate the effect of EDC concentration on MMP-9 inactivation, we diluted 0.3 M EDC with de-ionized water to 0.01, 0.02, 0.05, or 0.1 M. After pre-incubation of EDC with rhMMP-9 for 20 min, additional assay buffer and substrate were added (see Appendix). A proprietary MMP kit inhibitor (GM6001) from the assay kit was used as the control. Details of the experiments can be found in the Appendix.

Since the normality and homoscedasticity assumptions of the data were valid, % inactivation was analyzed by two-way ANOVA (concentration vs. time) and Tukey multiple-comparison tests at $\alpha = 0.05$.

Matrix-bound MMPs in Demineralized Dentin

Sixty extracted human third molars were obtained from 18- to 21-year-old patients under a protocol approved by the Georgia Health Sciences University. The teeth were stored at 4°C in 0.9% NaCl containing 0.02% NaN₃ to prevent bacterial growth. The enamel and superficial dentin of each tooth were removed by means of an Isomet saw (Buehler Ltd., Lake Bluff, IL, USA) under water cooling. Dentin beams with dimensions 6 x 2 x 1 mm were sectioned from the mid-coronal dentin (60 beams).

The beams were completely demineralized in 10 wt% H₃PO₄ (pH 1) for 18 hrs at 25°C. Digital radiography was used to confirm the absence of residual minerals. The initial modulus of elasticity of each demineralized beam was determined with three-point bending. Ten beams were assigned to each of 6 groups (N = 10) so that the mean initial modulus of elasticity of each group was statistically similar. Five EDC concentrations (0.01, 0.02, 0.05, 0.1, or 0.3 M) were evaluated as previously described. Demineralized dentin beams from each experimental

group were dipped into an EDC solution of the designated concentration for 1 min and blot-dried and then dropped into 1 mL of medium. The control group consisted of dentin beams without EDC pre-treatment. Each beam was in 1 mL of a calcium- and zinc-containing simulated body fluid (SBF) in labeled polypropylene tubes. This diluted the EDC concentration in the final medium below cross-linking values. The SBF contained 5 mM HEPES, 2.5 mM CaCl₂·H₂O, 0.05 mM ZnCl₂, and 0.3 mM NaN₃ (pH 7.4). The sealed tubes were incubated in a shaker-water bath (60 cycles/min) at 37°C for 30 days.

Indirect Assessment of Matrix-bound MMP Activity

Change in Modulus of Elasticity over Time

Each dentin beam was placed over a miniature three-point bending device with a span length of 2.5 mm (Carrilho *et al.*, 2009; Tezvergil-Mutluay *et al.*, 2010, 2011). A universal testing machine with a 1 N load cell (Transducer Techniques, Temecula, CA, USA) was used to load the beams to 10% strain while immersed in de-ionized water with a displacement rate of 0.5 mm/min. After maximum displacement, the load was returned immediately to 0% stress without further holding to prevent creep of the demineralized collagen matrix. Following initial testing, each beam was returned to its designated polypropylene tube. After 30 days of incubation, the beams were rinsed free of salts for 10 min with running distilled water and re-tested with three-point bending under the same conditions.

Stress-strain curves were prepared from the load-displacement data. The modulus of elasticity (E; in MPa) of each specimen was calculated as the slope of the linear portion of the stress-strain curve (see Appendix). We analyzed the data by a two-factor repeated-measures analysis of variance to examine the effects of EDC concentrations (*i.e.*, control – 0.01 M, 0.02 M, 0.05 M, 0.1 M, 0.3 M EDC) and the repeated-factor storage time (*i.e.*, baseline vs. 30 days) and the interaction of these two factors on the modulus of elasticity. All pair-wise multiple comparisons were performed by the Tukey test. Statistical significance was set at $\alpha = 0.05$.

Loss of Dry Mass over Time

Loss of dry mass after the 30-day incubation period was used as an indirect measure of MMP-induced hydrolysis of the collagenous dentin matrix (Carrilho *et al.*, 2009; see Appendix). Since the normality and homoscedasticity assumptions of the data were violated, loss of dry mass from the demineralized beams was analyzed with Kruskal-Wallis one-way ANOVA and Dunn's multiple-comparison tests at $\alpha = 0.05$.

Solubilized Collagen Peptides

Demineralization of the mineralized collagen matrix exposes and activates endogenous MMPs, even though they remain bound to the collagen (Dayan *et al.*, 1983; Martin-De Las Heras *et al.*, 2000). We determined the third index of matrix degradation by measuring the amount of solubilized collagen peptides over the 30-day incubation period (Carrilho *et al.*, 2009). A hydroxyproline assay (Jamall *et al.*, 1981; see Appendix) was used for estimation of the percentage of degraded collagen. For

Figure 1. Effect of EDC concentration and preincubation time on activation of rhMMP-9. **(A)** The mean percentage inhibition of rhMMP-9 by different EDC concentrations when pre-incubated for 20 min ($N = 5$). The control consisted of the GM6001 MMP inhibitor from the assay kit. Vertical lines represent standard deviations. **(B)** Bar chart comparing the effect of incubation time on the inhibition of rhMMP-9 by 0.3M EDC. Values are means and standard deviations of the percentage inhibition ($N = 5$). Groups with the same letters on top of the bars are not statistically different ($p > 0.05$).

each specimen, the solubilized collagen was expressed as μg of hydroxyproline/mg of the dry mass of demineralized dentin before incubation. The data were evaluated by Kruskal-Wallis one-way ANOVA and Dunn's multiple-comparison tests at $\alpha = 0.05$.

RESULTS

Inactivation of Soluble MMP-9

The effect of incubation time of 0.3 M EDC on inhibition of the enzyme activity of soluble rhMMP-9 varied from 98% to 101% (Fig. 1B). There was no significant difference in the inactivation of rhMMP-9 between 1 min and 30 min of incubation ($p > 0.05$) (Fig. 1B). The inactivation effects of 20 min of pre-incubation of the 5 EDC concentrations on rhMMP-9 varied between 97% and 100% (Fig. 1A). Significant differences were observed among the 6 groups ($p = 0.004$); however, there were no statistically significant differences among the 5 EDC concentrations in inhibiting rhMMP-9 ($p > 0.05$).

Inactivation of Endogenous Matrix-bound Proteases

The initial mean E of the demineralized dentin beams was 2.9 ± 0.7 MPa (Fig. 2). After 30 days of incubation, the control specimens in the simulated body fluid (SBF) lost 46% of their initial stiffness (Fig. 2). Dentin beams pre-treated with 0.3 M EDC lost only 9% of stiffness, whereas the 0.01 M EDC pre-treated beams lost 30% of their stiffness. EDC concentration had a

Figure 2. Initial and 30-day modulus of elasticity of completely demineralized dentin beams pre-treated with various concentrations of EDC ($N = 10$). The group incubated in simulated body fluid (SBF) without EDC pre-treatment served as the control. Groups identified by different letters are significantly different ($p < 0.05$).

Figure 3. Loss of dry mass from demineralized dentin beams pre-treated with different concentrations of EDC after 30 days of incubation. The group incubated in simulated body fluid (SBF) without EDC pre-treatment served as the control ($N = 10$). Groups with the same letter designations are not statistically significant ($p > 0.05$).

significant effect on the E of the experimental beams ($p < 0.05$). There were significant interactions between EDC concentrations and time ($p < 0.05$).

Loss of dry mass (Fig. 3) of experimental beams over the 30-day incubation period showed significant differences from the SBF controls. Dentin beams incubated with EDC showed 3.5% to 5% decrease in dry mass, compared with an 18% decrease in the SBF control ($p < 0.05$). Among the EDC groups, a slight dose-response was observed; however, the difference was not significant ($p > 0.05$), except for the lowest EDC concentration (0.01 M; $p < 0.05$). The group with the highest EDC concentration (0.3 M) showed a dry mass loss of 3.6%, whereas the 0.01 M pre-treated group lost around 5%.

Similar to the loss of dry mass and elastic modulus measurements, an EDC dose-response was observed with the solubilization of collagen peptides (Fig. 4). Control beams stored in the SBF medium liberated $9.4 \mu\text{g}$ hydroxyproline/mg dentin, whereas beams pre-treated with various concentrations of EDC liberated between 0.74 and $1.5 \mu\text{g}$ hydroxyproline/mg dentin. Beams pre-treated with the highest EDC concentrations (0.1 and 0.3 M) showed lower degrees ($p < 0.05$) of collagen solubilization compared with the beams pre-treated with the lowest (*i.e.*, 0.01 M) EDC concentrations.

DISCUSSION

Carbodiimide has previously been investigated as a cross-linking agent to increase the mechanical properties of the dentin matrix (Bedran-Russo *et al.*, 2010). However, a 1- to 4-hour application time was required for the mechanical properties of the collagen matrix to increase, making it impossible for use in clinical applications (Bedran-Russo *et al.*, 2010). The results of the present study indicate that even a short pre-treatment (*i.e.*, 1 min) of acid-etched dentin matrix is sufficient to inactivate endogenous protease activity of dentin without significantly stiffening the collagen.

Since the rhMMP-9 inactivation potential of non-specific EDC was similar to or higher than that of the specific MMP inhibitor GM6001, the first null hypothesis—that EDC does not inhibit soluble rhMMP-9—was rejected. The results of the soluble rhMMP-9 assay showed that even at 0.01 M, EDC successfully inhibited rhMMP-9 by over 99%. This is in line with previous work which showed a large decrease in MMP-9 activity following treatment with 0.1 M EDC for 4 hrs (Calero *et al.*, 2002).

Based on the rhMMP-9 results, a 1-minute pre-treatment time was selected for inactivation of matrix-bound protease activity in completely demineralized dentin. Previous work has shown that endogenous proteases in dentin matrices slowly lower the modulus of elasticity of incubation time as collagen peptides are slowly solubilized (Carrilho *et al.*, 2009; Tezvergil-Mutluay *et al.*, 2010, 2011). When the decreases in stiffness of various groups were compared, the highest residual stiffness was obtained in the group treated with 0.3 M EDC, whereas the lowest stiffness was observed with the control (46% drop), where no pre-treatment was used. This suggests that the endogenous protease activities in the EDC pre-treated dentin beams were much lower than those in the control beams. The same trend was observed with the evaluation of dry mass and the hydroxyproline assay, which are indirect measures of solubilization of collagen from the dentin matrix. The loss of dry mass in the control group was around 5 times more than in the EDC pre-treated groups. Likewise, the control group released 5 to 6 times more hydroxyproline compared with the EDC pre-treated groups. This requires rejection of the second null hypothesis, that EDC does not inactivate matrix-bound proteases. We speculate that the EDC cross-links peptide chains in MMPs and cysteine cathepsins (Tersariol *et al.*, 2010; Nascimento *et al.*, 2011) of the dentin matrix. Cross-linking decreases the molecular mobility of the catalytic sites in these enzymes, which are critical for their function (Liu *et al.*, 2011). This cross-linking is done very rapidly in acid-etched dentin that is 30 vol% collagen and 70 vol% water (Pashley *et al.*, 2011). The carboxyl and amino groups in collagen may not be as accessible as those in MMPs (Orgel *et al.*, 2006; Perumal *et al.*, 2008), thereby permitting more rapid cross-linking of MMPs than of collagen.

There are potential advantages of inactivating the proteolytic enzymes of the dentin matrix (*i.e.*, MMPs and cathepsins) by using EDC instead of using non-specific MMP inhibitors such as chlorhexidine, or specific MMP inhibitors like Galardin (Breschi *et al.*, 2010). To be effective, enzyme inhibitors must

Figure 4. Hydroxyproline content of the medium hydrolysate derived from EDC pre-treated demineralized dentin and the untreated control in simulated body fluid (SBF) after the 30-day incubation period (N = 10). For each specimen, the solubilized collagen from the demineralized dentin beam was expressed as µg of hydroxyproline/mg of the dry mass of the demineralized dentin. Groups with the same letter designations are not statistically different ($p > 0.05$).

remain firmly bound to the enzyme. While specific MMP inhibitors such as Galardin can inhibit MMPs at micromolar concentrations (Galardy *et al.*, 1994), chlorhexidine requires millimolar concentrations, because collagen is not saturated by chlorhexidine until it reaches 30 mM (Kim *et al.*, 2010). High concentrations of chlorhexidine (*e.g.*, 2 wt%) can interfere with conversion of monomers to polymers (Hiraishi *et al.*, 2010). Water-soluble inhibitors may slowly leach from resin-dentin interfaces over time. Recent studies showed that chlorhexidine inhibition of resin-dentin bonds was effective for 9 to 12 mos, after which it begins to lose its effectiveness (Ricci *et al.*, 2010; Sadek *et al.*, 2010). We speculate that functional stresses on hybrid layers may cause them to compress 0.6% under function and then rebound (Wood *et al.*, 2008), which may allow unbound water within the hybrid layers to elute residual chlorhexidine from the acid-etched collagen matrix.

Within the limits of this *in vitro* study, it may be concluded that EDC can inactivate matrix-bound dentin proteinases in demineralized dentin matrices with a 1-minute application time. This result requires rejection of the third null hypothesis. Once cross-linked, these covalent bonds may be stable and therefore contribute to improved durability of resin-dentin bonds (Bedran-Russo *et al.*, 2010) if newly synthesized odontoblast MMPs (Lehmann *et al.*, 2009) in dentinal fluid cannot diffuse around resin tags in dentinal tubules bonded to dentin to reach the hybrid layer. This speculation needs to be tested by longitudinal *in vivo* studies and *in vitro* bond strength studies on the use of EDC to stabilize resin-bonded dentin, if EDC can be shown to be non-toxic to pulpal soft tissues. The use of EDC on acid-etched dentin would not prevent hydrolysis of the resin polymer component of the hybrid layer.

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REFERENCES

- Bedran-Russo AK, Vidal CM, Dos Santos PH, Castellan CS (2010). Long-term effect of carbodiimide on dentin matrix and resin-dentin bonds. *J Biomed Mater Res Part B: Appl Biomater* 94:250-255.
- Brackett MG, Tay FR, Brackett WW, Dib A, Dipp FA, Mai S, *et al.* (2009). *In vivo* chlorhexidine stabilization of hybrid layers of an acetone-based dentin adhesive. *Oper Dent* 34:379-383.
- Brackett WW, Tay FR, Brackett MG, Dib A, Sword RJ, Pashley DH (2007). The effect of chlorhexidine on dentin hybrid layers *in vivo*. *Oper Dent* 32:107-111.
- Breschi L, Martin P, Mazzoni A, Nato F, Carrilho M, Tjäderhane L, *et al.* (2010). Use of a specific MMP inhibitor (Galardin) for preservation of hybrid layer. *Dent Mater* 26:571-578.
- Calero P, Jorge-Herrero E, Turnay J, Olmo N, Lopez de Silanes I, Lizarbe MA, *et al.* (2002). Gelatinases in soft tissue biomaterials. Analysis of different cross-linking agents. *Biomaterials* 23:3473-3478.
- Carrilho MR, Geraldeli S, Tay FR, de Goes MF, Carvalho RM, Tjäderhane L, *et al.* (2007). *In vivo* preservation of the hybrid layer by chlorhexidine. *J Dent Res* 86:529-533.
- Carrilho MR, Tay FR, Donnelly AM, Agee KA, Tjäderhane L, Mazzoni A, *et al.* (2009). Host-derived loss of dentin stiffness associated with solubilization of collagen. *J Biomed Mater Res Part B: Appl Biomater* 90:373-380.
- Collins MJ, Nielsen-March CM, Hiller J, Smith CI, Roberts JP, Prigodich RV, *et al.* (2002). The survival of organic matter in bone: a review. *Archaeometry* 44:383-394.
- Dayan D, Binderman I, Mechanic GL (1983). A preliminary study of activation of collagenase in carious human dentin matrix. *Arch Oral Biol* 28:185-187.
- Galaray RE, Cassabonne ME, Giese C, Gilbert JH, Lapiere F, Lopez H, *et al.* (1994). Low molecular weight inhibitors in corneal ulceration. *Ann NY Acad Sci* 732:315-323.
- Gendron R, Grenier D, Sorsa T, Mayrand D (1999). Inhibition of the activities of matrix metalloproteinases 2, 8 and 9 by chlorhexidine. *Clin Diagn Lab Immunol* 6:437-439.
- Hebling J, Pashley DH, Tjäderhane L, Tay FR (2005). Chlorhexidine arrests subclinical degradation of dentin hybrid layers *in vivo*. *J Dent Res* 84:741-746; *erratum in J Dent Res* 85:384, 2006.
- Hiraishi N, Yiu CK, King NM, Tay FR (2010). Effect of chlorhexidine incorporation into a self-etching primer on dentin bond strength of a luting cement. *J Dent* 38:496-502.
- Huang LL, Cheung DT, Nimni ME (1990). Biochemical changes and cytotoxicity associated with the degradation of polymeric glutaraldehyde derived cross-links. *J Biomed Mater Res* 24:1185-1201.
- Jamall IS, Finelli VN, Quettee SS (1981). A simple method to determine nanogram levels of 4-hydroxyproline in biological tissues. *Anal Biochem* 112:70-75.
- Kim J, Uchiyama T, Carrilho M, Agee KA, Mazzoni A, Breschi L, *et al.* (2010). Chlorhexidine binding to mineralized versus demineralized dentin. *Dent Mater* 26:771-778.
- Lehmann N, Debret R, Roméas A, Magloire H, Degrange M, Bleicher F, *et al.* (2009). Self-etching increases matrix metalloproteinase expression in the dentin-pulp complex. *J Dent Res* 88:77-82.
- Liu Y, Tjäderhane L, Breschi L, Mazzoni A, Li N, Mao J, *et al.* (2011). Limitations in bonding to dentin and experimental strategies to prevent bond degradation. *J Dent Res* 90:953-968.
- Martin-De Las Heras S, Valenzuela A, Overall CM (2000). The matrix metalloproteinase gelatinase A in human dentine. *Arch Oral Biol* 45:757-765.
- Mazzoni A, Pashley DH, Nishitani Y, Breschi L, Tjäderhane L, Toledano M, *et al.* (2006). Reactivation of inactivated endogenous proteolytic activities in phosphoric acid-etched dentin by etch-and-rinse adhesives. *Biomaterials* 27:4470-4476.
- Nagase H, Visse R, Murphy G (2006). Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc Res* 69:562-573.
- Nascimento FD, Minciotti S, Geraldeli S, Carrilho MR, Pashley DH, Tay FR, *et al.* (2011). Cysteine cathepsins in human carious dentin. *J Dent Res* 90:506-511.
- Orgel JP, Irving TC, Miller A, Wess TJ (2006). Microfibrillar structure of type I collagen *in situ*. *Proc Natl Acad Sci USA* 103:9001-9005.
- Pashley DH, Tay FR, Breschi L, Tjäderhane L, Carvalho RM, Carrilho M, *et al.* (2011). State-of-the-art of etch-and-rinse adhesives. *Dent Mater* 27:1-16.
- Perumal S, Antipova O, Orgel JP (2008). Collagen fibril architecture, domain organization, and triple-helical conformation govern its proteolysis. *Proc Natl Acad Sci USA* 105:2824-2829.
- Petite H, Duval JL, Frei V, Abdul-Malak N, Sigot-Luizard MF, Herbage D (1995). Cytocompatibility of calf pericardium treated by glutaraldehyde and by acyl azide methods in an organotypic culture model. *Biomaterials* 16:1003-1008.
- Ricci HA, Sanabe ME, de Souza Costa CA, Pashley DH, Hebling J (2010). Chlorhexidine increases the longevity of *in vivo* resin-dentin bonds. *Eur J Oral Sci* 118:414-416.
- Sadek FT, Braga RR, Muench A, Liu Y, Pashley DH, Tay FR (2010). Ethanol wet-bonding challenges current anti-degradation strategy. *J Dent Res* 89:1499-1504.
- Sulkala M, Larmas M, Sorsa T, Salo T, Tjäderhane L (2002). The localization of matrix metalloproteinase-20 (MMP-20, enamelysin) in mature human teeth. *J Dent Res* 81:603-607.
- Sulkala M, Tervahartiala T, Sorsa T, Tjäderhane L (2007). Matrix metalloproteinase 8 (MMP-8) is the major collagenase in human dentin. *Arch Oral Biol* 52:121-127.
- Tay FR, Pashley DH, Loushine RJ, Weller RN, Monticelli F, Osorio R (2006). Self-etching adhesives increase collagenolytic activity in radicular dentin. *J Endod* 32:862-868.
- Tersariol IL, Geraldeli S, Minciotti CL, Nascimento RD, Pääkkönen V, Martins MT, *et al.* (2010). Cysteine cathepsins in human dentin-pulp complex. *J Endod* 36:475-481.
- Tezvergil-Mutluay A, Agee KA, Hoshika T, Tay FR, Pashley DH (2010). The inhibitory effect of polyvinylphosphonic acid on functional MMP activities in human demineralized dentin. *Acta Biomater* 26:4136-4142.
- Tezvergil-Mutluay A, Agee KA, Uchiyama T, Imazato S, Mutluay MM, Cadenaro M, *et al.* (2011). The inhibitory effects of quaternary ammonium methacrylates on soluble and matrix-bound MMPs. *J Dent Res* 90:535-540.
- Wood JD, Sobolewski P, Thakur V, Arola D, Nazari A, Tay FR, *et al.* (2008). Measurement of microstrain across resin-dentin interfaces using microscopic moiré interferometry. *Dent Mater* 24:859-866.