Published in final edited form as:

Dent Mater. 2014 July ; 30(7): 752–758. doi:10.1016/j.dental.2014.04.006.

Inhibition of endogenous human dentin MMPs by Gluma

Camila Sabatini^{1,*}, Débora L.S. Scheffel², Régis H. Scheffel², Kelli A. Agee³, Katelyn Rouch³, Masahiro Takahashi⁴, Lorenzo Breschi^{5,6}, Annalisa Mazzoni⁷, Leo Tjäderhane⁸, Franklin R. Tay⁹, and David H. Pashley^{3,10}

¹Department of Restorative Dentistry, School of Dental Medicine, University at Buffalo, Buffalo, NY, USA ²Department of Orthodontics and Pediatric Dentistry, Universidade Estadual Paulista-UNESP, Araraquara Dental School, Araraquara, São Paulo, Brazil ³Department of Oral Biology, College of Dental Medicine, Georgia Regents University, Augusta, GA, USA ⁴Department of Cariology and Operative Dentistry, Tokyo Medical and Dental University, Tokyo, Japan ⁵Department of Biomedicine, University of Trieste, Trieste, Italy ⁶IGM-CNR, Unit of Bologna c/o IOR, Bologna, Italy ⁷Department of SAU & FAL, University of Bologna, Bologna, Italy ⁸Institute of Dentistry, University of Oulu and Oulu University Hospital, Oulu, Finland ⁹Department of Endodontics, College of Dental Medicine, Georgia Regents University, Augusta, GA, USA ¹⁰Highly Cited Investigator of King Abdulaziz University School of Dentistry, Jeddah, Saudi Arabia

Abstract

Objective—The objective of this study was to determine if Gluma dentin desensitizer (5.0% glutaraldehyde and 35% HEMA in water) can inhibit the endogenous MMPs of dentin matrices in 60 sec. and to evaluate its effect on dentin matrix stiffness and dry mass weight.

Methods—Dentin beams of 2×1×6 mm were obtained from extracted human third molars coronal dentin. To measure the influence of Gluma treatment time on total MMP activity of dentin, beams were dipped in 37% phosphoric acid (PA) for 15 sec. and rinsed in water. The acid-etched beams were then dipped in Gluma for 5, 15, 30 or 60 sec., rinsed in water and incubated into SensoLyte generic MMP substrate (AnaSpec, Inc.) for 60 min. Controls were dipped in water for 60 sec. Additional beams of 1×1×6 mm were completely demineralized in 37% PA for 18 h, rinsed and used to evaluate changes on the dry weight and modulus of elasticity (E) after 60 sec. of Gluma treatment followed by incubation in simulated body fluid buffer for zero, one or four weeks. E was measured by 3-pt flexure.

Results—Gluma treatment inhibited total MMP activity of acid-etched dentin by 44, 50, 84, 86 % after 5, 15, 30 or 60 sec. of exposure, respectively. All completely demineralized dentin beams lost stiffness after one and four weeks, with no significant differences between the control and Gluma-treated dentin. Gluma treatment for 60 sec. yielded significantly less dry mass loss than the control after four weeks.

*Corresponding author: Camila Sabatini, DDS, MS, Assistant Professor, SUNY at Buffalo, School of Dental Medicine, Department of Restorative Dentistry, 3435 Main Street, 215 Squire Hall, Buffalo, NY 14214 USA, Phone: +1 (716) 829-2862, Fax: +1 (716) 829-2440, cs252@buffalo.edu.

Significance—The use of Gluma may contribute to the preservation of adhesive interfaces by its cross-linking and inhibitory properties of endogenous dentin MMPs.

Keywords

Degradation; dentin collagen; glutaraldehyde; matrix metalloproteinase; mechanical properties; proteolytic activity

INTRODUCTION

Although resin-enamel bond strengths are known to be stable over time [1], resin-dentin bonds are not very stable, falling 30–40% in six months and 60–70% in one year [2, 3] *in vitro*. The premature degradation of hybrid layers is known to be the consequence of a myriad of factors, including incomplete resin monomer infiltration of demineralized dentin and elution of unpolymerized monomers, both of which yield areas of poorly infiltrated collagen fibrils at the bottom of the hybrid layer that are prone to the attack by endogenous proteases. Armstrong *et al.* was the first to demonstrate that collagen fibrils in hybrid layers disintegrated over time as bond strengths fell over five years [4]. That led Pashley *et al.* to demonstrate that dentin contained endogenous proteases that could cleave collagen [5]. The presence of matrix metalloproteinase-2 (MMP-2) in dentin matrix was reported in 2000 by Martin-De Las Heras *et al.* [6]. This was confirmed by Mazzoni *et al.* and expanded to include the presence of MMP-9 [7]. Sulkala *et al.* reported that dentin matrix also contained MMP-8, a true collagenase [8]. In recent years, substantial work has been conducted to evaluate the role of host-derived proteases in the degradation of incompletely infiltrated collagen fibrils, as well as the mechanisms to regulate enzyme-mediated collagenolysis, such as the use of MMP inhibitors or cross-linking agents in dentin. Gendron *et al.* reported that chlorhexidine (CHX), a potent antimicrobial agent, was a nonspecific inhibitor of MMP-2, 8 and 9 [9]. Scaffa *et al.* reported that CHX also inhibited dentin cathepsins [10]. Breschi *et al.* showed that a specific MMP-inhibitor (galardin) could prolong the durability of resin-dentin bonds [11]. Many other dentin MMP inhibitors have also been described [12]. Unfortunately, CHX and other proposed inhibitors do not co-polymerize with dental resins and can slowly leach out of hybrid layers within 1–2 years [13] allowing proteases to resume collagen cleavage. This may explain recent observations by Sadek *et al.* who showed preservation of the bonds with the use of CHX after nine months but not after 18 months [14].

Efforts are currently being directed towards creating hybrid layers with sustained anti-proteolytic potential. A proposed strategy to reduce collagen degradation within hybrid layers is to increase the extent of cross-linking of the demineralized matrix prior to adhesive application. One of the main advantages of using cross-linking agents is their ability to cross-link both active and allosteric enzyme sites of MMPs, as well as collagen molecules, preventing thus collagenases from binding to the collagen binding sites. Moreover, cross-linking agents can cross-link all MMPs, cysteine cathepsins and any other enzymes in the matrix simultaneously, precluding the need to treat dentin with separate enzyme inhibitors. By covalently cross-linking proteases to matrix components, they can be permanently

inactivated. Several agents, including carbodiimide, glutaraldehyde, proanthocyanidin are currently being investigated for their cross-linking and anti-proteolytic properties [15, 16].

Gluma is the brand name of a primer containing 5.0% glutaraldehyde, 35% hydroxyethylmethacrylate (HEMA) and 60% water, that was used in a three-step adhesive system that is no longer marketed [17]. It was shown that this primer could prevent post-operative sensitivity if applied to crown preparations before the castings were cemented [18]. Similar results were obtained by Dondi dall'Orologia and Malferrai [19] and Davidson and Suzuki [20]. However, the mechanism responsible for the desensitization of dentin remained elusive until the study by Schüpbach *et al.* [21]. In that study, buccal cusps of human molars scheduled for extraction were flattened into dentin *in vivo* and then treated with EDTA, 5.0% glutaraldehyde or 35% HEMA or the combination of 5.0% glutaraldehyde and 35% HEMA in water. The teeth were then extracted and stained with fluorescein dye and the dentin examined by confocal laser scanning microscopy. The authors observed multiple intratubular septa in the lumens of the tubules of exposed dentin treated with 5.0% glutaraldehyde and HEMA, but not in dentinal tubules treated with either 5.0% glutaraldehyde alone or 35% HEMA alone. They suggested that glutaraldehyde reacts with plasma proteins in dentin [22] to precipitate them. Later work showed that HEMA reacts with the precipitate to form insoluble polymeric plugs in the tubules [23]. In addition to its desensitizing properties, glutaraldehyde is a potent antimicrobial and cross-linking agent known to improve the resistance of uncross-linked or mildly cross-linked collagen matrices to enzymatic degradation by collagenases [24, 25]. By improving dentin's mechanical properties, it is expected that glutaraldehyde can also contribute to improving the resistance to degradation of the bonds.

Recently, a simple model has been proposed to evaluate the rate of degradation of demineralized dentin matrices free of resin [26]. Beams of coronal dentin are completely demineralized in 10% phosphoric acid, treated with a given MMP inhibitor or cross-linking agent, and then incubated in complete media for 30-day period. Changes in the modulus of elasticity, as determined by 3-point flexure, and dry mass loss before and after 30 days of incubation in complete media at 37°C can be measured. If the MMPs are active, they will slowly solubilize collagen peptides resulting in the loss of dentin matrix stiffness and dry mass, thus providing indirect evidence of proteolytic activity.

The purpose of this work was to determine if Gluma can inactivate endogenous MMPs in acid-etched human dentin within 60 seconds of topical treatment, and to evaluate the effect of Gluma on dentin matrix stiffness and dry mass weight of demineralized dentin beams. The null hypotheses were that the 5.0% glutaraldehyde in Gluma cannot inactivate MMPs in acid-etched human dentin in 60 seconds, and that Gluma treatment of demineralized dentin beams has no effect on the matrix stiffness or loss of dry weight.

MATERIALS AND METHODS

Thirty extracted non-carious human third molars were obtained from 18–21 year-old patients with informed consent under a protocol approved by the Georgia Regents University and stored frozen until required. After removal of the roots, mid-coronal dentin

was exposed by removing enamel and superficial dentin using an Isomet saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) under water cooling. One 1 mm-thick dentin disk was obtained from each tooth. Then, fifty dentin beams of 2×1×6 mm and twenty beams of 1×1×6 mm were sectioned from the dentin disks.

Dentin MMP Activity

Because soluble MMPs extracted from dentin matrices or purchased rhMMPs are more susceptible to inhibitors or inactivators than matrix-bound MMPs [27], we elected to study dentin MMP activity while they were matrix-bound. To determine the total MMP activity, fifty beams of 2×1×6 mm were etched with 37% phosphoric acid (PA) for 15 sec. and then rinsed in deionized water (DW) for another 15 sec. The etched-beams were treated either with DW for 60 sec. or Gluma (Gluma Desensitizer Liquid, Heraeus Kulzer GmgH, Lot# 010205, Hanau, Germany) for 5, 15, 30 or 60 sec. Each group contained ten specimens. Then the beams were immediately placed in 300 µl of a generic MMP substrate (Sensolyte Generic MMP colorimetric assay kit - catalog No. 72095, AnaSpec Inc., Lot# 131-029, Fremont, CA, USA) for 1 h at 25°C in a 96-well plate. After 1 h, the beams were removed from the wells and the total MMP activity was spectrophotometrically determined by measuring the absorbance of each of the wells at 412 nm in a plate reader (Synergy HT microplate reader, BioTek Instruments, Winooski, VT, USA) against blanks. Ten individual values were averaged to obtain a mean value for each subgroup. All chemicals were purchased from Sigma/Aldrich Chemical Co. and used as received.

The generic MMP assay uses a proprietary thiopeptide to assay MMP-1, 2, 3, 7, 8, 9, 12, 13 and 14. Thus, the kit measured the total endogenous MMP activity of dentin. A standard curve of absorbance of the substrate vs. rh MMP-9 activity (ng) was constructed to permit expression of total MMP activity in MMP-9 equivalents. The rhMMP-9 was activated using trypsin at a final concentration of 10 µg/ml, pH 7.4 at 37°C for 2 h. Then the trypsin was inactivated by addition of trypsin inhibitor at a final concentration of 100 µg/ml. As the ability of Gluma to inactivate MMPs was the same, regardless of whether we used the 412 nm absorbance values or converted it to MMP-9 equivalents, we elected to publish the absorbance values.

Elastic Modulus and Mass Loss

Twenty beams of 1×1×6 mm were completely demineralized in 10 wt% liquid PA (pH 1.0) for 18 h at 25°C under constant stirring and rinsed with DW for 2 h at 4°C. Before demineralization, the initial modulus of elasticity of each mineralized beam was determined, by three-point flexure, to be between 16–18,000 MPa (16–18 GPa). After complete demineralization, the modulus of elasticity fell to 2.0–2.8 MPa indicating complete demineralization. An aluminum testing jig with a 2.5 mm support span was fixed to the bottom of a Petri glass dish. Specimens were tested under compression, while immersed in distilled water, by means of a testing machine (Vitrodyne V1000, Liveco Inc., Burlington, VT, USA) with a 100 g load cell, at a cross-head speed of 1 mm/min. Load displacement curves were converted to stress-strain curves, and the apparent modulus of elasticity was calculated at 10% strain. The beams were then allocated into two groups (n=10) with

statistically similar mean initial elastic modulus. The following formula was used to calculate the elastic modulus:

$$E = \frac{mL^3}{4bd^3}$$

where m = slope (N/mm); L = support span (mm); d = thickness of beam (mm); b = width of beam (mm). Because specimen displacement was estimated from cross-head displacement, and the specimens thickness was not one-sixteenth of the length (ASTM D 790-03) [28], the calculated elastic moduli were only approximate but very reproducible.

To determine initial dry mass, the beams were dried in a desiccator containing calcium sulfate for 48 h and weighed to a constant dry weight in a microanalytical balance (XP6 Microbalance, Mettler Toledo, Columbus, OH). That is, the specimens were weighed four times, initial wet weight at time zero, dry weight at 24 h, 36 h and 48 h. Consistency of the last two weights indicates a constant dry mass. The beams were rehydrated in DW for 1 h and treated with either Gluma Desensitizer Liquid or DW (Control) for 60 sec. and stored in 500 ul of simulated body fluid (SBF, composition in mM/L: KCl, 13; KSCN, 2; Na₂SO₄·10H₂O, 2.4; HEPES, 5; sCaCl₂·2H₂O, 1.5; NaHCO₃, 7.5; ZnCl₂, 0.02, 0.02% sodium azide) at 37°C for up to four weeks. After weeks one and four, the same procedures described above were repeated to re-determine the elastic modulus and dry mass of the beams.

Statistical Analysis

A one-way ANOVA and post-hoc Student-Newman Keuls test were used to evaluate the effect of Gluma application time on the percentage inhibition of total MMP activity since the data was normally distributed. Kruskal-Wallis test followed by Dunn's multiple comparisons test were used for evaluation of the effect of Gluma application time on the absorbance values since these values were not normally distributed. Two-way repeated measures ANOVA and a pairwise multiple comparisons Holm-Sidak test were used to determine the effect of Gluma treatment and application time on the modulus of elasticity and dry mass loss. A significance level of p<0.05 was used for all tests. All statistical analysis was performed with SigmaStat version v.3.11 (San Jose, CA).

RESULTS

Dentin MMP Activity

Gluma applied for 5 or 15 sec. inhibited the total MMP activity by 44.1% and 50.1%, respectively (Figure 1A). Gluma treatment for 30 or 60 sec. inhibited the total MMP activity by 83.9% and 85.9%, respectively. The 5 and 15 sec. values were both significantly higher than the control (p<0.05) and were not different from each other. The 30 and 60 sec. values represented significantly greater MMP inhibition than the 5 and 15 sec. values (p<0.001), but were not significantly different from each other. The absorbance values are shown in Figure 1B. The greatest absorbance values, suggestive of more MMP activity, were shown for the control group (0.523). Lower absorbance values were seen for the Gluma treatment

groups with values of 0.292, 0.261, 0.100 and 0.074 for 5, 15, 30 and 60 sec., respectively. This indicates that there was a time-dependent inhibition of total MMP activity by Gluma. Figure 2 shows the absorbance vs. amount of rhMMP-9 (ng/well). Increasing amounts of rhMMP-9 were added to the SensoLyte substrate, which gave increasing absorbance of colored product, demonstrating that the colorimetric method is sensitive to changes in enzymatic activity. The control (uninhibited) absorbance value in Figure 1.B was 0.523 at 412 nm. Insertion of this absorbance value (x) in the regression equation of Figure 2 shows that absorbance was equivalent to 6.2 ng of MMP-9 per well.

Elastic Modulus and Dry Mass Loss

Two-way repeated measures ANOVA demonstrated a significant effect of both variables, treatment and time in the specimens' elastic modulus with $p=0.048$ and $p<0.001$, respectively. There was no significant interaction between the variables. Multiple comparisons revealed that although the elastic modulus varied significantly among the testing times (baseline, one week and four weeks), for both control and Gluma treatment groups ($p<0.05$), no significant differences were observed between the control and Gluma treatment groups at any of the testing periods (Figure 3).

Two-way repeated measures ANOVA of the changes in total dry mass revealed a significant effect of both treatment and time ($p=0.035$ and $p<0.001$). There was no significant interaction between the variables. Multiple comparisons demonstrated significant differences in the percentage of total dry mass loss between one and four weeks for both the control and Gluma groups ($p<0.05$) (Figure 4). Even though the percentage of total dry mass loss of the control group did not appear to be significantly different from that of the Gluma group at one week, significant differences between the control and Gluma groups were evidenced at 4 weeks ($p<0.05$).

DISCUSSION

The present study determined whether Gluma could inactivate endogenous MMPs in acid-etched human dentin within 60 seconds of treatment, and the possible changes to the matrix stiffness and dry mass of demineralized dentin beams treated with Gluma over a 30-day period. Our results require rejection of the first part of the null hypotheses since a significant inhibition of dentin MMP activity was shown when Gluma was applied for as little as 5 sec. that increased with treatment time up to 60 sec. The second part of the null hypothesis was only partially rejected. Although Gluma treatment showed no effect on the matrix stiffness, significantly less loss of dry weight was demonstrated for the Gluma-treated beams after four weeks of storage. These results confirm those from previous studies, which have shown that glutaraldehyde improved the resistance of uncross-linked or mildly cross-linked collagen matrices to degradation by collagenases [24, 25].

Gluma (Heraeus Kulzer GmH, Hanau, Germany) has been used since 1991 as a dentin desensitizer [18]. The mechanism remained elusive until Schüpbach *et al.* showed that topical application *in vivo* of glutaraldehyde / hydroxyethylmethacrylate (HEMA) combination products resulted in a series of horizontal partitions within the lumens of exposed dentinal tubules [21]. More carefully designed spectrophotometric studies *in vitro*

[23] revealed that the glutaraldehyde in Gluma reacts with plasma proteins like albumin to form protein precipitates, that then react with HEMA to form a mixture of polyHEMA copolymerized with glutaraldehyde-cross-linked albumin [29]. These precipitates occlude open dentinal tubules beneath the surface, which interferes with the hydrodynamics of dentinal fluid, thereby preventing dentin sensitivity. It remains unclear how long these precipitates stay in dentinal tubules. Dentinal fluid and saliva contain esterases [30] that could attack the ester and peptide bonds in these intratubular precipitates [31]. Saliva also contains matrix metalloproteinases (MMPs) [32] and kallikreins [33] that could attack collagen and plasminogen, respectively. If these enzymes attacked the Gluma-created precipitates, their desensitizing activity could be lost if the intratubular precipitates were destroyed.

Glutaraldehyde is known to cross-link all proteins it comes into contact with within minutes [34]. While glutaraldehyde cross-links albumin in dentinal fluid [23], it could also cross-link any esterases, kallikreins or MMPs in dentinal fluid, perhaps inactivating their enzymatic activity. However, it has been shown that dentinal fluid is slowly and continually replaced by fresh dentinal fluid, which forms as a transudate from pulpal capillaries [35]. Thus, dentin treatment with Gluma would only be able to transiently inactivate enzymes in dentinal fluid.

Relative to the use of protease inhibitors alone, which can slowly leach out of hybrid layers [13], the use of cross-linking agents with anti-proteolytic properties may offer longer anti-proteolytic benefits. Our group has shown that the protein cross-linking agent, carbodiimide, can inactivate endogenous dentin proteases within 60 sec. when it is applied to acid-etched dentin [16]. This was also shown to increase the durability of resin-dentin bonds *in vitro* [36]. In the present study, we demonstrated that the 5.0% glutaraldehyde in Gluma can also inactivate 86% the endogenous MMPs in dentin after 60 sec. of topical treatment of acid-etched dentin. This can increase the stability of resin-dentin bonds *in vivo* because dentin does not turn over, unlike bone [37]. It is well accepted that acid etching activates pro-forms of MMPs and cathepsins that are bound to dentin collagen [5], allowing them to slowly hydrolyze the collagen to which they are bound. Once exposed, if proteases that were activated by acid etching are inactivated by glutaraldehyde cross-linking, and then covered with a hydrophobic adhesive resin, the collagen fibrils in the dentin hybrid layers should remain stable over time. Lehmann *et al.* reported that treating human teeth *in vivo* with acidic self-etching adhesives produced synthesis of new MMPs in the pulp that could be detected in dentinal tubules in cultured tooth slices [38]. However, it is unlikely that any newly synthesized MMPs could reach the hybrid layer because in resin-bonded dentin, a resin tag blocks each tubule. These tags are hybridized with the surrounding hybrid layer that would prevent diffusion of these relatively large proteins (ca. 70–80 kDa).

The results of the present study demonstrate that, in addition to its antimicrobial benefits, glutaraldehyde treatment of acid-etched dentin for 60 sec. can effectively inactivate most MMP activity in demineralized dentin. Clinically, this indicates that 60 sec. is long enough to inactivate endogenous MMPs in acid-etched dentin, which has been demineralized only to a depth of 8–10 μm . Sixty-seconds treatment was also enough time to reduce the loss of dry mass of completely demineralized dentin beams, which has been used by our group to

quantitate the total protease activity in demineralized dentin by 17–21%. The loss of dry mass is a measure of how much insoluble collagen has been solubilized by endogenous dentin proteases [16, 26]. However, 60 sec. was not enough time to increase the stiffness of completely demineralized dentin beams 1000 μm thick indicating that Gluma was not able to diffuse 500 μm into each side of the demineralized dentin beams in 60 sec. Nonetheless, clinically relevant demineralized zones of dentin are only 8–10 μm and not 500 μm deep. Contrary to these results, a different study by our group recently demonstrated an increased modulus of elasticity following treatment of acid-etched dentin with 5.0% glutaraldehyde for one minute [39]. Our findings confirmed the effectiveness of glutaraldehyde as a cross-linking agent previously demonstrated by other studies [24, 25, 40]. Other reports have shown that glutaraldehyde cross-linked collagen significantly increased its resistance to enzymatic degradation [41, 42]. The mechanism of glutaraldehyde cross-linking is known to be dependent on the formation of covalent bonds between the amino groups of proteins and the aldehyde groups of glutaraldehyde. By improving dentin's mechanical properties, it is expected that glutaraldehyde can also contribute to improving the resistance to bond degradation [40].

The use of glutaraldehyde / HEMA combination products offers promise since benefits can be derived from each of its active components. Hydroxyethyl methacrylate, a water-soluble monomer present in most current hydrophilic dental adhesives, is also known to facilitate the reaction with dentin collagen due to its ester and hydroxyl groups, as well as its hydrophilic nature [43]. Recent studies have shown strong inhibitory properties of MMP-2 [44] and MMP-9 [45] with the use of monomeric HEMA. However, the frequently reported fall in the microtensile bond strength of HEMA-containing adhesives [4, 46] indicates that the MMP-inhibitory effects of HEMA are lost when it is copolymerized with other adhesive monomers [45]. Nonetheless, because of the water content in this mixture, these products also serve as rewetting agents expanding the demineralized collagen network and increasing its surface energy; all of which facilitate the diffusion of the hydrophilic resin monomer into the etched dentin, improving the resin-dentin bonds [47].

Whether glutaraldehyde-induced cross-linking of dentin collagen can contribute to the long-term integrity of the hybrid layer needs to be further investigated. The favorable results shown in the present study indicate that further work should be undertaken to evaluate whether the use of glutaraldehyde / hydroxyethylmethacrylate (HEMA) combination products can yield more durable resin-dentin bonds over a 6–12 months period or longer.

CONCLUSION

Within the limitations of this *in vitro* study, it may be concluded that Gluma Desensitizing solution is a potent inhibitor of MMP activity in dentin matrices. Application of the 5.0% glutaraldehyde/35% HEMA mixture for 5, 15, 30 or 60 sec. inactivated the total MMP activity by 44, 50, 84 and 86% respectively. Glutaraldehyde can thus inactivate matrix-bound dentin proteinases in demineralized dentin matrices almost entirely within 30 or 60 sec. of exposure. Although Gluma treatment for 60 sec. showed no effect on the relative dentin matrix stiffness, significantly less loss of dry weight was observed after four weeks. This indicates that once cross-linked, these covalent bonds may be stable, preventing

collagen solubilization and consequently, contributing to an improved durability of resin-dentin bonds.

Acknowledgments

The authors wish to thank Mrs. Michelle Barnes for her outstanding secretarial support. The study was partially supported by grant R01DE015306 from the National Institute of Dental and Craniofacial Research (NIDCR) to DHP (PI).

References

1. Loguercio AD, Moura SK, Pellizzaro A, Dal-Bianco K, Patzlaff RT, Grande RH, Reis A. Durability of enamel bonding using two-step self-etch systems on ground and unground enamel. *Oper Dent*. 2008; 33:79–88. [PubMed: 18335737]
2. Shono Y, Terashita M, Shimada J, Kozono Y, Carvalho RM, Russell CM, Pashley DH. Durability of resin-dentin bonds. *J Adhes Dent*. 1999; 1:211–218. [PubMed: 11725669]
3. Hashimoto M, Ohno H, Sano H, Kaga M, Oguchi H. In vitro degradation of resin-dentin bonds analyzed by microtensile bond test, scanning and transmission electron microscopy. *Biomaterials*. 2003; 24:3795–3803. [PubMed: 12818552]
4. Armstrong SR, Vargas MA, Chung I, Pashley DH, Campbell JA, Laffoon JE, Qian F. Resin-dentin interfacial ultrastructure and microtensile dentin bond strength after five-year water storage. *Oper Dent*. 2004; 29:705–712. [PubMed: 15646228]
5. Pashley DH, Tay FR, Yiu C, Hashimoto M, Breschi L, Carvalho RM, Ito S. Collagen degradation by host-derived enzymes during aging. *J Dent Res*. 2004; 83:216–221. [PubMed: 14981122]
6. Martin-De Las Heras S, Valenzuela A, Overall CM. The matrix metalloproteinase gelatinase A in human dentine. *Arch Oral Biol*. 2000; 45:757–765. [PubMed: 10869489]
7. Mazzoni A, Mannello F, Tay FR, Tonti GAM, Papa S, Mazzotti G, Di Lenarda R, Pashley DH, Breschi L. Zymographic Analysis and Characterization of MMP-2 and -9 Forms in Human Sound Dentin. *J Dent Res*. 2007; 86:436–440. [PubMed: 17452564]
8. Sulkala M, Tervahartiala T, Sorsa T, Larmas M, Salo T, Tjäderhane L. Matrix metalloproteinase-8 (MMP-8) is the major collagenase in human dentin. *Arch Oral Biol*. 2007; 52:121–127. [PubMed: 17045563]
9. Gendron R, Grenier D, Sorsa T, Mayrand D. Inhibition of the activities of matrix metalloproteinases 2, 8, and 9 by chlorhexidine. *Clin Diagn Lab Immunol*. 1999; 6:437–439. [PubMed: 10225852]
10. 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]
11. Breschi L, Martin P, Mazzoni A, Nato F, Carrilho M, Tjäderhane L, Visintini E, Cadenaro M, Tay FR, Dorigo EDS, Pashley DH. Use of a specific MMP-inhibitor (galardin) for preservation of hybrid layer. *Dent Mater*. 2010; 26:571–578. [PubMed: 20299089]
12. Carrilho MR. Can exogenous protease inhibitors control dentin matrix degradation? *J Dent Res*. 2012; 91:1099–1102. [PubMed: 23023764]
13. Ricci HA, Sanabe ME, de Souza Costa CA, Pashley DH, Hebling J. Chlorhexidine increases the longevity of in vivo resin-dentin bonds. *Eur J Oral Sci*. 2010; 118:411–416. [PubMed: 20662916]
14. Sadek FT, Braga RR, Muench A, Liu Y, Pashley DH, Tay FR. Ethanol wet-bonding challenges current anti-degradation strategy. *J Dent Res*. 2010; 89:1499–1504. [PubMed: 20940353]
15. Bedran-Russo AK, Pereira PN, Duarte WR, Drummond JL, Yamauchi M. Application of crosslinkers to dentin collagen enhances the ultimate tensile strength. *J Biomed Mater Res B*. 2007; 80:268–272.
16. Tezvergil-Mutluay A, Mutluay MM, Agee KA, Seseogullari-Dirihan R, Hoshika T, Cadenaro M, Breschi L, Vallittu P, Tay FR, Pashley DH. Carbodiimide cross-linking inactivates soluble and matrix-bound MMPs, in vitro. *J Dent Res*. 2012; 91:192–196. [PubMed: 22058118]
17. Asmussen E, Uno S. Adhesion of restorative resins to dentin: chemical and physicochemical aspects. *Oper Dent*. 1992; 17(Suppl 5):68–74. [PubMed: 1470555]

18. Felton DA, Bergenholtz G, Kanoy BE. Evaluation of the desensitizing effect of Gluma Dentin Bond on teeth prepared for complete-coverage restorations. *Int J Prosthodont.* 1991; 4:292–298. [PubMed: 1810321]
19. Dondi dall'Orologio G, Malferrari S. Desensitizing effects of Gluma and Gluma 2000 on hypersensitive dentin. *Am J Dent.* 1993; 6:283–286. [PubMed: 7880476]
20. Davidson DF, Suzuki M. The Gluma bonding system: a clinical evaluation of its various components for the treatment of hypersensitive root dentin. *J Can Dent Assoc.* 1997; 63:38–41. [PubMed: 9009533]
21. Schupbach P, Lutz F, Finger WJ. Closing of dentinal tubules by Gluma desensitizer. *Eur J Oral Sci.* 1997; 105:414–421. [PubMed: 9395102]
22. Maita E, Simpson MD, Tao L, Pashley DH. Fluid and protein flux across the pulpodentine complex of the dog in vivo. *Arch Oral Biol.* 1991; 36:103–110. [PubMed: 2059160]
23. Qin C, Xu J, Zhang Y. Spectroscopic investigation of the function of aqueous 2-hydroxyethylmethacrylate/glutaraldehyde solution as a dentin desensitizer. *Eur J Oral Sci.* 2006; 114:354–359. [PubMed: 16911108]
24. Avila MY, Navia JL. Effect of genipin collagen crosslinking on porcine corneas. *J Cataract Refract Surg.* 2010; 36:659–664. [PubMed: 20362860]
25. Ma DH, Lai JY, Cheng HY, Tsai CC, Yeh LK. Carbodiimide cross-linked amniotic membranes for cultivation of limbal epithelial cells. *Biomaterials.* 2010; 31:6647–6658. [PubMed: 20541801]
26. Carrilho MR, Tay FR, Donnelly AM, Agee KA, Tjäderhane L, Mazzoni A, Breschi L, Foulger S, Pashley DH. Host-derived loss of dentin matrix stiffness associated with solubilization of collagen. *J Biomed Mater Res B.* 2009; 90B:373–380.
27. Kim J, Uchiyama T, Carrilho M, Agee KA, Mazzoni A, Breschi L, Carvalho RM, Tjaderhane L, Looney S, Wimmer C, Tezvergil-Mutluay A, Tay FR, Pashley DH. Chlorhexidine binding to mineralized versus demineralized dentin powder. *Dent Mater.* 2010; 26:771–778. [PubMed: 20472280]
28. ASTM D-790-03. Standard Test Methods for Flexural Properties of Unreinforced and Reinforced Plastics and Electrical Insulating Materials, American Standards Testing Methods, 2007.
29. Munksgaard EC. Amine-induced polymerization of aqueous HEMA/aldehyde during action as a dentin bonding agent. *J Dent Res.* 1990; 69:1236–1239. [PubMed: 2113067]
30. Lin BA, Jaffer F, Duff MD, Tang YW, Santerre JP. Identifying enzyme activities within human saliva which are relevant to dental resin composite biodegradation. *Biomaterials.* 2005; 26:4259–4264. [PubMed: 15683649]
31. Finer Y, Santerre JP. Salivary esterase activity and its association with the biodegradation of dental composites. *J Dent Res.* 2004; 83:22–26. [PubMed: 14691108]
32. Ingman T, Tervahartiala T, Ding Y, Tschesche H, Haerian A, Kinane DF, Kontinen YT, Sorsa T. Matrix metalloproteinases and their inhibitors in gingival crevicular fluid and saliva of periodontitis patients. *J Clin Periodontol.* 1996; 23:1127–1132. [PubMed: 8997658]
33. Hernandez CC, Donadi EA, Reis ML. Kallikreins and kininogens in saliva and plasma of patients presenting with rheumatoid arthritis. *Scand J Rheumatol.* 2002; 31:38–40. [PubMed: 11924648]
34. Kiernan JA. Formaldehyde, formalin, paraformaldehyde and glutaraldehyde: What they are and what they do. *Microscopy Today.* 2000; 00:8–12.
35. Pashley DH. Smear layer: overview of structure and function. *Proc Finn Dent Soc.* 1992; 88 (Suppl 1):215–224. [PubMed: 1508877]
36. Mazzoni A, Angeloni V, Apolonio FM, Scotti N, Tjäderhane L, Tezvergil-Mutluay A, Di Lenarda R, Tay FR, Pashley DH, Breschi L. Effect of carbodiimide (EDC) on the bond stability of etch-and-rinse adhesive systems. *Dent Mater.* 2013; 29:1040–1047. [PubMed: 23916318]
37. Tjäderhane L, Nascimento FD, Breschi L, Mazzoni A, Tersariol IL, 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.* 2013; 29:116–135. [PubMed: 22901826]
38. Lehmann N, Debret R, Romeas A, Magloire H, Degrange M, Bleicher F, Sommer P, Seux D. Self-etching increases matrix metalloproteinase expression in the dentin-pulp complex. *J Dent Res.* 2009; 88:77–82. [PubMed: 19131322]

39. Scheffel DL, Hebling J, Scheffel RH, Agee KA, Cadenaro M, Turco G, Breschi L, Mazzoni A, De Souza Costa CA, Pashley DH. Stabilization of dentin matrix after cross-linking treatments, in vitro. *Dent Mater*. In press.
40. Bedran-Russo AK, Pashley DH, Agee K, Drummond JL, Miescke KJ. Changes in stiffness of demineralized dentin following application of collagen crosslinkers. *J Biomed Mater Res B*. 2008; 86:330–334.
41. Walter R, Miguez PA, Arnold RR, Pereira PN, Duarte WR, Yamauchi M. Effects of natural cross-linkers on the stability of dentin collagen and the inhibition of root caries in vitro. *Caries Res*. 2008; 42:263–268. [PubMed: 18523385]
42. Xu C, Wang Y. Cross-linked demineralized dentin maintains its mechanical stability when challenged by bacterial collagenase. *J Biomed Mater Res B*. 2011; 96:242–248.
43. Xu J, Stangel I, Butler IS, Gilson DF. An FT-Raman spectroscopic investigation of dentin and collagen surfaces modified by 2-hydroxyethylmethacrylate. *J Dent Res*. 1997; 76:596–601. [PubMed: 9042083]
44. Carvalho RV, Ogliari FA, De Souza AP, Silva AF, Petzhold CL, Line SRP, Piva E, Etges A. 2-Hydroxyethyl methacrylate as an inhibitor of matrix metalloproteinase-2. *Eur J Oral Sci*. 2009; 117:64–67. [PubMed: 19196320]
45. Tezvergil-Mutluay A, Agee KA, Hoshika T, Uchiyama T, Tjäderhane L, Breschi L, Mazzoni A, Thompson JM, McCracken CE, Looney SW, Tay FR, Pashley DH. Inhibition of MMPs by alcohols. *Dent Mater*. 2011; 27:926–933. [PubMed: 21676453]
46. Okuda M, Pereira PN, Nakajima M, Tagami J. Relationship between nanoleakage and long-term durability of dentin bonds. *Oper Dent*. 2001; 26:482–490. [PubMed: 11551013]
47. Perdigao J, Van Meerbeek B, Lopes MM, Ambrose WW. The effect of a re-wetting agent on dentin bonding. *Dent Mater*. 1999; 15:282–295. [PubMed: 10551097]

Figure 1.

Figure 1A - Percent inhibition of total MMP activity by 5.0% glutaraldehyde (Gluma, Heraeus Kulzer) with increasing exposure times (5, 15, 30 and 60 sec.) using the SensoLyte

Generic MMP assay kit, relative to an untreated control with 100% of total MMP activity. Bars represent the mean values; brackets indicate the SD values. Groups identified by different letters are significantly different (Student-Newman Keuls; $p < 0.05$). **1B** - Absorbance values (412 nm) of demineralized beams untreated (control) or treated with 5.0% glutaraldehyde (Gluma, Heraeus Kulzer) for 5, 15, 30 and 60 sec. using the SensoLyte Generic MMP assay kit. Bars represent the mean values; brackets indicate the SD values. Groups identified by different letters are significantly different (Dunn's test; $p < 0.05$). N=10.

Figure 2.

Increase in absorbance at 412 nm as a function of increasing concentrations of rhMMP-9 when incubated with the SensoLyte Generic MMP assay kit.

Figure 3.

Modulus of elasticity (MPa) of completely demineralized human dentin beams at different testing times (Baseline, one week and four weeks) after incubation in simulated body fluid (SBF). Control beams were pre-incubated in deionized water for 60 sec. and then incubated in SBF. Gluma beams were pre-treated with Gluma for 60 sec. and then incubated in SBF. Bars represent the mean values; brackets indicate the SD values. Groups identified by different letters are significantly different (Holm-Sidak; $p < 0.05$).

Figure 4.

Percent total dry mass loss of completely demineralized human dentin beams after one week and four weeks of incubation in simulated body fluid (SBF). Control beams were pre-incubated in deionized water for 60 sec. and then incubated in SBF. Gluma beams were pre-treated with Gluma for 60 sec. and then incubated in SBF. Bars represent the mean values; brackets indicate the SD values. Groups identified by different letters are significantly different (Holm-Sidak; $p < 0.05$).