ABSTRACT

Aim: The aim of this study was to comparatively assess the gelatinolytic activity of matrix metalloproteinases (MMPs) and Cysteine Cathepsins (CCs) in the adhesive interface using etch and rinse adhesive at different time intervals using zymographic technique.

Methodology: Twenty freshly extracted non-carious human third molars were used in this study. Occlusal surfaces were ground flat and 1mm thick horizontal dentin slabs were obtained from each tooth using a diamond disc. The dentin surface was polished with 600-grit silicon-carbide paper. Five out of 20 samples were directly pulverized. In the remaining fifteen samples, the dentin was etched and adhesive was applied and light cured according to the manufacturer’s instructions. A 1mm thick flowable composite was build up and light cured. Bonded specimens were cut vertically into 3 to 4 dentin slabs by means of diamond disc to expose the adhesive/dentin interfaces. These were then ground down to 500 µm thick resin-dentin interface using a hard tissue microtome. These sections were then pulverised into powder. Following this, every five samples were subjected to zymographic analysis after 1 day, 7 days and 21 days.

Results: Zymograms showed clear, thicker bands on all three isoforms in the etched samples compared to control samples at 1st and 7th day intervals and became inactive at 21st day for all three isoforms. MMP 9 activity was relatively higher when compared to CCs and MMP 2.

Conclusion: Etch and rinse adhesive activated MMPs and CCs within the hybrid layer that remained active till 7th day and no gelatinolytic activity was found on 21st day and MMPs are more active compared to CCs and MMP-2.

Key words: Cysteine cathepsin, etch-and-rinse adhesives, hybrid layer, matrix metalloproteinase, zymography
Recent studies have identified CCs, papain-like endopeptidases, from dentinal tubules of intact dentin. They were also found to be present more abundantly in carious dentin. Restorations are almost always done on carious teeth as it can be safely assumed that higher levels of active CCs are also present in affected dentin. These activated CCs in turn are shown to also activate matrix-bound MMPs.

Although the activation of MMPs is implicated in hybrid layer degradation, only a few studies have shown the presence of MMPs within the hybrid layer. Similarly, direct evidence of the activity of CCs in endogenous dentin hybrid layer is lacking. Moreover, no studies have demonstrated the activity of gelatinases at various time intervals.

Hence, the aim of this study was to assess the gelatinolytic activity of MMPs and CCs in the adhesive interface using etch-and-rinse adhesive at different time intervals using zymographic technique.

**METHODOLOGY**

The methodology for sample preparation was adapted from Mazzoni et al. 2012. Twenty freshly extracted noncarious human third molars were used in this study. Occlusal surfaces were ground flat to expose the underlying dentin. One millimeter thick horizontal dentin slabs were obtained from each tooth using a diamond disc after removing the enamel rim. The dentin surface was polished with 600-grit silicon carbide paper. Five out of twenty samples were directly pulverized.

In the remaining 15 samples, the dentin was etched for 15 s with 37% phosphoric acid gel (3M ESPE, USA) and rinsed with water for 30 s. After removal of excess water, adhesive (Adper Single Bond, 3M ESPE, USA) was applied onto the moist dentin and light cured according to the manufacturer’s instructions. One millimeter thick flowable composite was added to the dentin specimens and light cured for 20 s with quartz-tungsten-halogen light. All the samples were stored in artificial saliva.

Bonded specimens were cut vertically into 3–4 dentin slabs by means of diamond disc to expose the adhesive/dentin interfaces. These were further ground down to 500 μm thick sections using a hard tissue microtome. These sections were then pulverized into powder. The adhesive was removed from this powder with 1 ml of acetone, centrifuged (20,800 g for 20 min), resuspended in acetone, and recentlyrifuged twice for proper removal of the resin. The dentin powder is then subjected to protein extraction followed by zymographic analysis after 1, 7, and 21 days for enzymatic activities.

**Dentinal protein extraction**

The pulverized dentin powder was suspended in extraction buffer (50 mM Tris-HCl, pH 6.0, containing 5 mM CaCl₂, 100 mM NaCl, 0.1% Triton X-100, 0.1 mM ZnCl₂, 0.02% NaN₃) and ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail (Sigma Chemical, St. Louis, MO, USA). The mix was ultrasonicated at 40 W output (Sonicator Ultrasonic Liquid Processor Model W-385, Heat Systems-Ultrasonic Inc., Farmingdale, NY, USA) or 3 bursts of 10 s each at 4°C. The vials were centrifuged at 18,000 rpm for 30 min at 4°C, and the supernatants were collected. All the proteins present in the supernatants were precipitated at 4°C by the addition of powdered ammonium sulfate (w/v) to achieve a final concentration of 85% with pH 7.0. The precipitate was collected by centrifugation at 24,000 rpm for 30 min at 4°C, redisolved in a 10-fold dilution in extraction buffer, dialyzed through a 30-kDa membrane against extraction buffer overnight and stored at 4°C until analyzed.

**Zymographic analysis**

Dentin proteins were subjected to electrophoresis under nonreducing conditions on 7.5% sodium dodecyl sulfate-polyacrylamide gels copolymerized with 2 g/L gelatin from porcine skin (Sigma Chemical). After electrophoresis, gels were washed in 2.5% Triton X-100 with agitation and then incubated for at least 24 h at 37°C in enzyme incubation buffer (50 mM Tris-HCl, pH 7.5, containing 5 mM CaCl₂, 100 mM NaCl, 0.01% Triton X-100, 0.1 mM ZnCl₂, 0.2% brij nonionic detergent, and 0.002% NaN₃). Negative control zymograms were incubated in the presence of 5 mM EDTA and 2 mM 1 and 10-phenanthroline for specific inhibition studies. Activation of gelatinase pro formas was achieved with 2 mM p-aminophenylmercuric acetate at 37°C for 1 h and zymographic gels were stained in 0.2% Coomassie Brilliant Blue R-250 and destained. Wet gelatin zymograms were scanned at 600 nm by means of a densitometer equipped with an image analyzer. The active enzymes degrade the gelatin and the gelatinolytic activity of the enzymes then appear as cleared sharp bands over a blue background [Figure 1].

**RESULTS**

Zymograms of gelatinolytic activity are shown in the Figure 2a-d. Three main forms of gelatinases were identified by zymography in all the samples analyzed. The enzymes migrated with apparent molecular mass of 72, 92, and 50 kDa, characteristic of latent MMP-9, -2, and cathepsins-B, respectively, clear bands as shown in Figure 2a. After acid etching, zymograms showed clear, thicker bands on 1 and 7 days’ time intervals [Figure 2b and c]. Densitometric analysis showed that there was an increase in the band thickness from 1 to 7 days for all three isoforms. MMP-9 activity was relatively more when compared to CCs-B and MMP-2. All three isoforms became inactive at 21st day [Figure 2d].

**DISCUSSION**

Degradation of dentin matrix components within hybrid layer has been correlated with the activity of host-derived...
proteases, such as MMPs and CCs. Current research efforts are focused on the preservation of the collagen matrix using MMP inhibitors in an attempt to achieve durable dentin bonding.\textsuperscript{[11-13]} Considering the key role of MMPs and CCs in interfacial degradation over time, the evaluation of the duration of activity of these proteolytic enzymes and their inhibition, may be an effective approach for the improvement of bond durability.

Gelatin zymography is mainly used for the detection of gelatinase activities by the degradation of their preferential substrate and by their molecular weight. Although it is one of the most simple and easiest methods, it is an extremely sensitive technique.\textsuperscript{[14]} The innovative approach of this study was to compare the activities of CCs with MMP-2 and -9 selectively at various time intervals in the resin-dentin interface by gelatin zymography.

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**Figure 1:** Diagrammatic representation of methodology

**Figure 2:** Zymographic analysis of matrix metalloproteinases and cysteine cathepsins. (a) Control group shows thin band (mild gelatinolytic activity). (b and c) Clear, thicker bands on 1 and 7 days' time interval (increased gelatinolytic activity). (d) No bands on 21st day (no gelatinolytic activity)
The control samples of nontreated dentin showed very mild gelatinolytic activity at all time intervals. On 1st day, the gelatinolytic activity of MMP-2, -9, and CCs were higher compared to the control group. This activity further increased on 7th day interval compared to 1st day. The ability of etch-and-rinse adhesive to increase the gelatinolytic activities of host-derived dentinal MMPs is consistent with the previous findings.[5,15] Moreover, the determination of protease activity at various time intervals showed that MMPs are relatively more active than the CCs at 1st and 7th day as depicted by the bandwidth in Figure 2b and c. The acidic resin monomers activate latent forms of MMPs (pro-MMPs) through the cysteine switch mechanism which exposes the catalytic domains of these enzymes.[16] The increase in MMP-2 and -9 activities could also be related to the loss of inhibitory activity of tissue inhibitors of metalloproteinases (TIMPs). TIMPs are specific endogenous inhibitors of dentin matrix that participate in controlling the local activities of MMPs in tissues.[17] TIMP-1 binds to pro-MMP-9 and TIMP-2 and -4 bind to pro-MMP-2. These natural inhibitors have shorter shelf life than MMPs; hence, prolonged interruption of the MMP-TIMP interaction, such as cessation of dentinal fluid flow and acidic environments of caries, may prevent replenishment of TIMPs.[18] Similarly, acid etchants and acidic monomers in the dentin-bonding agents may play a role in the activation of MMP-2 and -9 by removing TIMP-1and -2. Mazzoni et al.[3] showed that 37% phosphoric acid reduced collagenolytic activity by 65% while Tay et al.[19] found 15% increase in collagenolytic activity with self-etch adhesives, suggesting that organic acids may play a more active role in activating MMPs.

CC-B, one of the most prevalent cathepsins, is expressed by mature human odontoblasts and pulp tissue. Our study proved that etch-and-rinse system activates not only MMPs but also CCs. CCs activity has been demonstrated not only in intact dentin[9] but found to be approximately 10-fold higher in carious dentin.[10] Acidic pH activates CCs which in turn may act in three ways: Either activating pro-MMPs, degrading TIMP inhibiting MMP, or both resulting in active MMPs and functional activity after neutralization of pH. CCs are known to have mainly peptidyl-dipeptidase and carboxypeptidase activity at an optimum pH of around 7.4. This may be the reason for increased MMP activity compared to CCs at acidic pH.[20]

Cathepsin B has a flexible loop that partially occludes the active site aperture of mature enzyme and carries pH sensitive arginine and histidine residues which modulates the gelatinolytic activity. Cathepsin B directly cleaves and inactivates MMP-TIMP-1 and TIMP-2, changing the balance between MMPs and their inhibitors. In addition, MMPs are shown to activate procathepsin B.[21,22] This could probably represent the potential MMP-cathepsin interactions in the degradation of the hybrid layer collagen. The gelatinolytic activity was found inactive on 21st day for all the gelatinases. Thus, this study showed that the activation of MMPs and CCs last only for 3 weeks. This result questions the role of etchants and acidic monomers on the long-term degradation of collagen.

**CONCLUSION**

Etch-and-rinse adhesives activate MMPs and CCs within the hybrid layer that remained active till 7th day and became inactive on 21st day and MMPs are more active compared to CCs. Future research works have to be carried out on the rate and effect of various organic and inorganic acids used in dentin bonding agents on MMPs and CCs on long-term survival of hybrid layer.

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**Conflicts of interest**

There are no conflicts of interest.

**REFERENCES**

1. Van Meerbeek B, Eick JD, Robinson SJ. Epoxy-embedded versus nonembedded TEM examination of the resin-dentin interface. J Biomed Mater Res 1997;35:191-7.
2. Mazzoni A, Nascimento FD, Carrilho M, Tersario I, Papa V, Tjäderhane L, et al. MMP activity in the hybrid layer detected with in situ zymography. J Dent Res 2012;91:467-72.
3. Breschi L, Mazzoni A, Ruggeri A, Cadenaro M, Di Lenarda R, De Stefano Dorigo E. Dental adhesion review: Aging and stability of the bonded interface. Dent Mater 2008;24:90-101.
4. Liu Y, Tjäderhane L, Breschi L, Mazzoni A, Li N, Mao J, et al. Limitations in bonding to dentin and experimental strategies to prevent bond degradation. J Dent Res 2011;90:953-68.
5. Mazzoni A, Pashley DH, Nishitani Y, Scaffa P, Carrilho M, Tjäderhane L, et al. Reactivation of inactivated endogenous proteolytic activities in phosphoric acid-etch dentine by etch-and-rinse adhesives. Biomaterials 2006;27:4470-6.
6. Osorio R, Yamauti M, Osorio E, Ruiz-Requena ME, Pashley D, Tay F, et al. Effect of dentin etching and chlorhexidine application on metalloproteinase-mediated collagen degradation. Eur J Oral Sci 2011;119:79-85.
7. Moon PC, Weaver J, Brooks CN. Review of matrix metalloproteinases' effect on the hybrid dentin bond layer stability and chlorhexidine clinical use to prevent bond failure. Open Dent J 2010;4:147-52.
8. Mazzoni A, Tay FR, Papa S, Mazzotti G, Lenarda RD, Pashley DH, et al. Zymographic analysis and characterization of MMP-2 and -9 forms in human sound dentin. J Dent Res 2007;86:436-40.
9. Tersario IL, Geraldeli S, Minciotti CL, Nascimento FD, Pääkkönen V, Martins MT, et al. Cysteine cathepsins in human dentin-pulp complex. J Endod 2010;36:475-81.
10. Nascimento FD, Minciotti CL, Geraldeli S, Carrilho MR, Pashley DH, Tay FR, et al. Cysteine cathepsins in human carious dentin. J Dent Res 2011;90:506-11.
11. Carrilho MR, Geraldeli S, Tay E, de Goes MF, Carvalho RM, Tjäderhane L, et al. In vivo preservation of the hybrid layer by chlorhexidine. J Dent Res 2007;86:529-33.
12. Zhang SC, Kern M. The role of host-derived dentinal matrix metalloproteinases in reducing dentin bonding of resin adhesives. Int J Oral Sci 2009;1:163-76.
13. Breschi L, Martin P, Mazzoni A, Ruggeri A, Cadenaro M, Di Lenarda R, et al. Use of specific MMP inhibitor (galardin) for preservation of hybrid layer. Dent Mater 2010;26:571-8.
14. Wilder CL, Park KY, Keegan PM, Platt MO. Manipulating substrate and pH in zymography protocols selectively distinguishes cathepsins K, L, S, and V activity in cells and tissues. Arch Biochem Biophys 2011;516:52-7.

15. Mazzoni A, Scaffa P, Carrilho M, Tjäderhane L, Di Lenarda R, Polimeni A, et al. Effects of etch-and-rinse and self-etch adhesives on dentin MMP-2 and MMP-9. J Dent Res 2013;92:82-6.

16. Tallant C, Marrero A, Gomis-Rúth FX. Matrix metalloproteinases: Fold and function of their catalytic domains. Biochim Biophys Acta 2010;1803:20-8.

17. Brew K, Dinakarpandian D, Nagase H. Tissue inhibitors of metalloproteinases: Evolution, structure and function. Biochim Biophys Acta 2000;1477:267-83.

18. Hebling J, Pashley DH, Tjäderhane L, Tay FR. Chlorhexidine arrests subclinical degradation of dentin hybrid layers in vivo. J Dent Res 2005;84:741-6.

19. Tay FR, Pashley DH, Loushine RJ, Weller RN, Monticelli F, Osorio R. Self-etching adhesives increase collagenolytic activity in radicular dentin. J Endod 2006;32:862-8.

20. Tjäderhane L, Nascimento FD, Breschi L, Mazzoni A, Tersariol IL, Geraldeli S, et al. Optimizing dentin bond durability: Control of collagen degradation by matrix metalloproteinases and cysteine cathepsins. Dent Mater 2013;29:116-35.

21. Cox SW, Eley BM, Kiili M, Asikainen A, Tervahartiala T, Sorsa T. Collagen degradation by interleukin-1beta-stimulated gingival fibroblasts is accompanied by release and activation of multiple matrix metalloproteinases and cysteine proteinases. Oral Dis 2006;12:34-40.

22. Hara K, Kominami E, Katunuma N. Effect of proteinase inhibitors on intracellular processing of cathepsin B, H and L in rat macrophages. FEBS Lett 1988;231:229-31.