Evaluation of the effect of natural versus synthetic matrix metalloproteinase silencers in preservation of dentin collagen and long-term bond strength of total etch adhesive

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Abstract

Aim: This study investigated the effect of various synthetic (galardin [Gal] and benzalkonium chloride [BAC]) and natural agents (hesperidin [HES] and epigallocatechin gallate) on the stability of dentin collagen matrix to resist collagenase degradation and improve long-term microtensile bond strength.

Materials and Methods: Ten sound-impacted third molars were collected and manual removal of pulp, periodontal ligament, cementum, and enamel was done. Remaining dentin fragments were pulverized under liquid nitrogen to obtain dentin powder. 2 mg aliquot of dentin powder was allocated to each of the test solutions and subjected to hydroxyproline assay. Another 60 sound human third molars were collected and occlusal enamel was ground flat to reach dentinoenamel junction. Class I cavities were prepared in dentin, followed by etching using 37% phosphoric acid for 15 s. Samples were then subjected to surface treatment with different agents for 60 s, followed by application of Optibond S and restoration with P 60 composite resin. Samples of all groups except control were subject to thermocycling. Samples were sectioned to 1 mm thick slabs which were subject to universal testing machine to determine ultimate tensile strength. One-way analysis of variance and Bonferroni post hoc test with a significance level of P < 0.05 were used to analyze data.

Results: HES resulted in maximum resistance to collagen degradation, followed by epigallocatechin gallate (EGCG), Gal, and BAC with a significant difference among the groups. Samples of Gal group showed the highest microtensile bond strength values, followed by HES, EGCG, BAC with a significant difference between the groups except HES and EGCG where the difference was nonsignificant.

Conclusion: The use of matrix metalloproteinase silencers could improve the mechanical properties of collagen and resist enzymatic degradation, leading to an improved long-term intimate restoration.

Keywords: Benzalkonium chloride; collagen degradation; epigallocatechin gallate; galardin; hesperidin; matrix metalloproteinase; matrix metalloproteinases silencer; microtensile bond strength

INTRODUCTION

The aim of inserting an adhesive restoration is ideally to sustain a tight and sealed adaptation between the restorative material and tooth. This perfectly ideal intimate attachment is difficult to achieve, as dentin

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contains a significant amount of water and organic material. This led to the development of different bonding strategies, i.e., self-etch and total etch.

Total etch bonding systems involve the removal of the smear layer using phosphoric acid, followed by the application of a primer and an adhesive in two different steps or in the same step. Acid etching facilitates the penetration of adhesive systems into inter- and intratubular dentin resulting in the formation of hybrid layer and resin tags. The creation of a hybrid layer has been considered the most efficient adhesion mechanism of recent dentin bonding agents. The drawback of total etch systems includes over drying and over etching due to which some part of the demineralized collagen layer is left uninfiltred by adhesive, thus compromising bonding.

The previous researches have stated that decreased resin monomer penetration in acid-etched dentin and subsequent resin leaching from hydrolytically unstable polymeric hydrogels in the hybrid layers makes the collagen fibrils unprotected and vulnerable to degradation by endogenous matrix metalloproteinases (MMPs) and cathepsins. Therefore, in an attempt to stabilize the organic matrix, two strategies can be incorporated. First, to inhibit the proteolytic activity and enzymatic degradation of the organic matrix by the MMPs by introducing the exogenous MMP silencers/inhibitors. Second, to improvise the strength of the collagen fibrils interaction using cross linkers that help in preserving the collagen by masking their active sites of cleavage and making it resistant to enzymatic degradation. Hence, utilization of exogenous MMP silencers/inhibitors, such as chlorhexidine (CHX), galdarin (Gal), and flavonols may be an effective strategy to improvise the longevity of adhesive restorations. The longevity of bonded restorations can be tested by evaluating the extent to resist collagen degradation and by estimating mechanical preservation of aged hybrid layer simply by evaluating long-term microtensile bond strength.

Although various studies have been done to evaluate the effects of cross linkers and MMP inhibitors on dentin collagen, literature shows very little evidence regarding the use of total etch adhesive and comparative evaluation between synthetic and natural MMP silencers in the preservation of dentin collagen and their effects on microtensile bond strength.

Therefore, the purpose of this study was to comparatively evaluate natural MMP silencers (hesperidin [HES] and epigallocatechin gallate [EGCG]) and synthetic MMP silencers (Gal and benzalkonium chloride [BAC]) in the preservation of dentin collagen and to comparatively evaluate the performance of total etch adhesives after surface treatment with these natural and synthetic MMP silencers. The null hypothesis tested was that there is no effect of MMP silencers on collagen degradation and long-term microtensile bond strength of total etch adhesive.

**MATERIALS AND METHODS**

**Part A Evaluation of collagen degradation**

**Specimen preparation**

Ten sound-impacted human third molars, freshly extracted and free of caries or any hypoplastic defects were selected for this study. Teeth were carefully examined under magnification (×30) using operating microscope (St Louis, United States) to rule out any preexisting cracks, fractures, and craze lines. After the collection of teeth specimens, manual removal of pulp, periodontal ligament, cementum, and enamel was done. The remaining dentinal fragments were pulverized under liquid N\textsubscript{2} in a cryogenic mill, and dentin powder was obtained. Dentin powder was demineralized with 0.5% ethylenediaminetetraacetic acid (pH – 7.4) for 10 days at 4°C and then extensively washed with distilled water and lyophilized.

Grouping of samples was done depending on the test solutions used with six samples per group:

- **Group 1**: Negative control - No test solution was used, samples were untreated with collagenase
- **Group 2**: Positive control - No test solution was used, samples were treated with collagenase
- **Group 3**: 0.5% Gal test solution used (Water solution, Sigma Aldrich, USA)
- **Group 4**: 0.5% BAC test solution used (Ava chemicals, Thane, India)
- **Group 5**: 0.5% HES test solution used (Swanson, New Jersey, United States)
- **Group 6**: 0.5% EGC test solution used (Vistas, Bangalore, India)

Each test solution was prepared by mixing 500 mg of MMP silencer in 100 mL of distilled water.

2 mg aliquot of demineralized dentin powder was allocated to each of six samples of all groups. All groups except 1 and 2 were incubated in 2 ml of their respective test solutions for 12 h at 37°C with stirring. After incubation, the groups were centrifuged and extensively washed with distilled water three times. The residues of all groups except 1 were digested with 2 ml of bacterial collagenase derived from clostridium histolyticum (7.5 U/1 ml) for 24 h in artificial saliva with stirring at 37°C.

**Determination of degraded collagen**

The amount of collagen degradation was determined by estimating the amount of hydroxyproline in the supernatants obtained from centrifugation (it was assumed that 12.5% of collagen is hydroxyproline). The solution aliquot was
hydrolyzed with 2 N sodium hydroxide by autoclaving at 120°C for 20 min. 0.056M Chloramine-T (450 µl) was added to the hydrolyzate to allow oxidation for 25 min at room temperature. This was followed by the addition of Ehrlich’s aldehyde reagent (500 µl) for hydroxyproline assay. When chromophore was developed, the absorbance of each specimen was read at 550 nm using a spectrophotometer and converted to concentration of hydroxyproline.

Part B Evaluation of long-term microtensile bond strength of total etch adhesive
Sixty sound human third molars, freshly extracted and free of caries or any hypoplastic defects were chosen for the study. The occlusal enamel was ground flat using a model trimmer under running water so as to reach the dentinocenamel junction. Class 1 cavities (4 mm long, 4 mm wide, and 2 mm deep) were prepared in dentin using a straight fissure bur (837 L.12) and air rotor handpiece under the copious air-water spray. At this time, the specimens that showed visible pulp exposure were excluded from this study.

Samples were divided into six main groups of ten teeth each according to the type of agent used for surface treatment and with or without thermocycling. Samples that were subjected to thermocycling were submitted to 10,000 cycles of thermocycling, with temperature changing from 5°C to 55°C, with a dwell time of 15 s and an interval time 10 s each.

- Group 1: No surface treatment was done, not subjected to thermocycling
- Group 2: No surface treatment was done, subjected to thermocycling
- Group 3: Surface treatment was done with 0.5% Gal, subjected to thermocycling
- Group 4: Surface treatment was done with 0.5% BAC, subjected to thermocycling
- Group 5: Surface treatment was done with 0.5% HES, subjected to thermocycling
- Group 6: Surface treatment was done with 0.5% EGCG, subjected to thermocycling.

Samples of all groups were etched with 37% phosphoric acid for 15 s, rinsed with water for 30 s. Samples were then subjected to surface treatment with different agents for 60 s depending on their group, followed by the application of OptiBond S (Kerr, Orange CA, USA). The cavities were restored with P-60 resin composite (3M ESPE, St Paul, USA) using the incremental method. Additional 2 mm thick buildup of composite was done over the restored cavity in increments. The samples of all groups were stored in moist conditions for 24 h at 37°C and then samples of all groups except Group 1 were subjected to thermocycling (Hitachi, Japan).

The restored samples of all groups were serially sectioned with the help of diamond disks in a straight handpiece, creating approximately 1 mm thick slabs, under copious water irrigation. The specimens were hand trimmed with the help of diamond points to obtain 0.9 mm thick resin dentin sticks which were checked with a digital caliper. Two sticks were obtained from each sample. A custom-made metallic jig (Millard Metallic Jig, India) was used for holding the samples to determine the ultimate bond strength with Universal Testing Machine (Instron, USA). The samples were held in such a manner that the junction of composite and tooth interface will face toward the chisel load applicator. The microtensile bond strength was evaluated at a crosshead speed of 0.5 mm/min until debonding at the dentin adhesive interface occurred.

Statistical analysis
The data obtained were subjected to statistical analysis using SPSS Version 15.0 Software (IL, Chicago, USA). One-way ANOVA test was applied to check the significant difference between the groups and repeated measures ANOVA test to check the significance within the group. The Bonferroni post hoc test was used for analysis for comparison of means. $P = 0.05$ was considered as statistically significant level.

RESULTS
The mean hydroxyproline values obtained in Part A (estimation of collagen degradation) are shown in Table 1. Maximum hydroxyproline values were seen in Group 3 (Gal) followed by Group 4 (BAC) and Group 5 (HES) with statistically significant differences between the groups. This was followed by Group 6 (EGCG), Group 5 (HES), and Group 1 (negative control) with a statistically insignificant difference between Group 5 and Group 1.

Microtensile bond strength values obtained in Part B of the study are shown in Table 2. Maximum microtensile bond strength was seen in Group 1 (negative control), followed by Group 5 (HES), Group 6 (EGCG), Group 4 (BAC), and least was seen in Group 2 (positive control). However, differences between Group 1 with Group 3 and that of Group 5 with Group 6 were not statistically significant.

Table 1: Mean collagen degradation values of different groups with intergroup comparison

| Groups | Mean±SD |
|--------|---------|
| Positive control ($n=6$) | 32.93±2.53 |
| Negative control ($n=6$) | 2.22±0.66a |
| GAL ($n=6$) | 4.53±1.05 |
| BAC ($n=6$) | 10.57±1.77 |
| HES ($n=6$) | 3.58±1.76a |
| EGCG ($n=6$) | 4.20±1.76 |

$P<0.05$ was considered statistically significant. Same superscript along the column denotes statistically insignificant difference. EGCG: Epigallocatechin gallate, HES: Hesperidin, BAC: Benzalkonium chloride, Gal: Galardin, SD: Standard deviation.
The decrease in bond strength is mainly due to two factors which are related to the collagen network of dentin and its constituent MMPs. First, the collagen fibers exposed by the etching process may not be completely infiltrated by subsequently applied adhesive bonding agents and in consequence, a thin layer of exposed, but noninfiltrated collagen remains. Second, this noninfiltrated collagen contains active MMPs that may degrade collagen by hydrolysis. Sulkala et al. [9] confirmed the localization of MMP 2, 8, 9, 20 in mineralized human dentin in an inactive state. The detrimental role of MMPs in dentin bonding has been documented. [10] Hence, adjunctive collagen pretreatment strategies have been proposed to improve dentin adhesion, via the use of agents that maintain the stability of fibrillar collagen toward enzymatic degradation. These agents include the use of substances that are considered to be inhibitors of MMPs and collagen cross linkers. The use of MMP inhibitors either before the application of the adhesive or as a component of the adhesive is a promising approach. [5,11] CHX, a potent nonspecific MMP inhibitor, has been reported to arrest degradation of the hybrid layer. [12] Cross-linkers such as tannic acid have been found not only to lower the rate of enzymatic degradation of collagen but also to increase the mechanical properties of dentin. [13]

In this study, we evaluated the effect of different MMP silencers (HES and epigallocatechin gallate [natural] and BAC and Gal [synthetic]) in the preservation of dentin collagen and on the long-term bond strength.

Degradation of collagen was determined by estimating hydroxyproline, an amino acid characteristic of collagen. Hydroxyproline, a major component of collagen, comprises around 12.5% of its amino acid composition. Due to its highly restricted distribution in collagen, the hydroxyproline content accurately reflects the amount of collagen in the sample. In the present study, microtensile bond strength of the samples was analyzed. Microtensile bond testing is based on the idea that better understanding of the interface bond strength can be obtained from multiple specimens obtained from a tooth. [14]

The samples tested were artificially aged by thermocycling to assess the longevity and comparatively evaluate the change in long-term bond strength of the pretreated samples and the control. The previous studies have shown that clinical application of MMP inhibitors for 1 min to the etched dentin after rinsing off the acid and before applying the dentin bonding primer and resin is able to stop significant in vivo degradation of bond strength from MMPs for at least 14 months. [15]

The present study describes the concentration used for the application of the MMP silencers to be 0.5% for 60 s as high concentration of MMP silencer may cause oversaturation of the substrate with rapid release of the silencer in excess. [5,6,16] Total etch adhesives were selected as higher levels of MMP2 and MMP 9 activity has been demonstrated for etch and rinse compared to self-etching adhesives and therefore, decrease in bond strength can be better analyzed. [17]

The restored specimens were serially sectioned to create approximately 1 mm thick slabs. Sano et al. have reported that there is an inverse relationship between bond strength and bond area: the smaller the area, the greater is the bond strength. [18]

The negative control (Group 1) resulted in significantly least hydroxyproline release in collagen degradation when compared to other groups as it had no test solution and no collagenase to degrade the collagen fibers. Hence, it resisted maximum collagen digestion.

This group also exhibited the maximum tensile bond strength when compared with all other groups as the samples of this group had not been subjected to any kind of artificial aging, and the immediate bond strength was evaluated. This is in accordance with many previous studies, where the negative control group resisted maximum collagen degradation as compared to other groups [8] and reported the highest tensile bond strength. [17,19,20]

The positive control (Group 2) in collagen degradation resulted in significantly maximum hydroxyproline release when compared with other groups as this group had no test solution (MMP silencer) but was treated with collagenase. The collagenase in this untreated solution digested and hydrolyzed the collagen fibers and released the hydroxyproline content. Our results confirm those from previous studies, which have shown that untreated dentin collagen was almost completely digested by bacterial collagenase, but the treated groups showed significantly less collagen digestion. [8,16,21,22]

The positive control group resulted in a significant reduction in bond strength as compared to all other groups. This was because the samples of this group had

| Group                        | Mean ± SD     |
|------------------------------|--------------|
| Negative control (n=10)      | 41.20 ± 3.15*|
| Positive control (n=10)      | 24.80 ± 2.73 |
| GAL (n=10)                   | 39.87 ± 3.09*|
| BAC (n=10)                   | 29.34 ± 3.06 |
| HES (n=10)                   | 35.74 ± 3.41*|
| ECGC (n=10)                  | 34.02 ± 3.51*|

*P<0.05 was considered statistically significant. Same superscript along the column denotes statistically insignificant difference. ECGC: Epigallocatechin gallate, HES: Hesperidin, BAC: Benzalkonium chloride, Gal: Galardin, SD: Standard deviation
undergone thermocycling. Thermal stresses generate mechanical stresses by virtue of differences in coefficient of thermal expansion which might have resulted in bond failure at tooth restoration interface and thus reduction in bond strength. The results of our study are in accordance with a previous study that showed a significant reduction in microtensile bond strength of a total etch adhesive after 10,000 thermocycles.\(^{[19]}\)

HES (Group 5) treated samples resulted in the highest resistance to collagen degradation exhibiting the minimum release of hydroxyproline, which was nonsignificant to the negative control group but significantly less than all other groups, and also resulted in second highest bond strength after Gal (Group 3). The least collagen degradation shown by HES as compared to other treated groups and also significantly improved the bond strength after thermocycling can be attributed by the presence of phenyl hydroxyl groups that can form bridge-type hydrogen bonds with the side chains of hydroxyl, carboxyl, amino, or amide groups of the collagen molecules. The formation of these hydrogen bonds is the reason for the stability of HES collagen interaction.

This is in accordance with another study that reported treatment with HPN may resist collagenase degradation and arrest demineralization.\(^{[6]}\)

ECGC (Group 6) treated samples resulted in the second lowest collagen digestion and also significantly improved the bond strength when compared to positive control (Group 2), Gal (Group 3), and BAC (Group 4). The resistance to collagen degradation was because of the existing hydrogen bonding and hydrophobic interactions with thecollagenases which changes its structure and consequently reducing its enzymatic activity.\(^{[23]}\) The improved mechanical integrity was due to the MMP inhibitory effects of ECGC.\(^{[24]}\)

ECGG and HES inhibited the expression and activity of MMPs by altering or masking the catalytic sites of MMP molecules.\(^{[24]}\) In addition to this, they exhibit cross linking ability by hydrogen bonding and hydrophobic interactions with collagenases. This combined cross-linking and anticollagenolytic effects of HES and ECGG resulted in maximum resistance against collagen degradation and in turn better mechanical integrity of these protected collagen fibers which significantly improved the long-term bond strength when compared to other groups. This is in accordance with previous study that reported 0.5% ECGG increased bond strength of etch and rinse adhesive after aging.\(^{[25]}\)

HES resulted in significantly more resistance against collagen degradation than ECGC because the amphiphilic property of HPN may be different from that of ECGC. This predominant hydrophobic feature of HES may enhance its association to collagen, resulting in increased resistance to biodegradation of the collagen matrix. This is in accordance with study by Hiraishi et al. who investigated the effect of various plant-derived agents (HES, proanthocyanidin, ECGG, and genipin) on the stability of dentin collagen matrix to resist collagen degradation and concluded that HES resisted maximum collagen degradation compared to all other groups.\(^{[16]}\) Similarly, Jain et al. reported highest pushout bond strength of fiber postulated to radicular dentin with 10% HES.\(^{[26]}\)

Gal (Group 3) treated samples resulted in significant less collagen digestion than positive control (Group 2) and BAC (Group 4). Gal resulted in significantly highest bond strength when compared to all other groups and nonsignificant to the negative control group. The reason for the less digestion to collagen may be the hydroxamate structure of GAL which chelates the zinc ion located in the catalytic domain of MMPs and causing inhibition of active MMPs. The collagen-like backbone of GAL facilitates its binding to the active site of MMPs which makes the antiproteolytic activity effective against endogenous MMPs. This is in accordance with Breschi et al. who evaluated the contribution of Gal against the proteolytic activity of dentinal MMPs and resulted in improved resistance against collagen degradation.

Host-derived MMPs 2, 3, 8, 9, 13, and 20 have been found within the mineralized dentin matrix which are responsible for the degradation of extracellular matrix components.\(^{[2,9,10]}\) And Gal selectively and specifically inhibits active MMP-2, -3, -8, and 9 which accounts for its highest bond strength as compared to all aged and treated groups and almost restored the original bond strength present before aging. This is in accordance with another study where pretreatment with Gal when compared to CHX, ECGG, and ferrous sulfate resulted in increased bond strength over time.\(^{[17]}\) There is no study done till date comparing Gal to BAC or HES. Hence, the results of this study cannot be contradicted or corroborated.

BAC (Group 4) pretreated samples resulted in significantly improved resistance to collagen degradation and improved bond strength, when compared to the positive control. The possible explanation is that quaternary ammonium compounds such as BAC are cationic with one positive charge. Following its application to acid-etched dentin, BAC binds strongly to demineralized dentin, via cationic-anionic reaction involving electrostatic attraction between the protonated amine group of BAC with carboxylic and hydroxyl groups of collagen. This interaction is likely to change the 3-D configuration of collagen fibrils and stabilize the collagen structure.

This is in accordance with another study where BAC concentrations >0.5% produced 100% inhibition of soluble
endogenous MMPs and inhibited matrix-bound MMPs.[21] Hence, BAC may, therefore, be useful in enhancing the stability of bonded restorations because of its anti-MMP property.

In the present study, the bond strength and collagen resistance of BAC treated samples was inferior when compared to other groups; this might be because of the fact that BAC is a broad spectrum and nonspecific inhibitor of MMP and also, because of its water-soluble nature, it leaches out of the hybrid layer leaving a limited viable amount present which further limits its proteolytic effects.

This is in accordance with previous study that evaluated the resin–dentin bond stability of experimental adhesive blends containing BAC.[28] Inferior results were seen with 0.5% BAC when applied separately.

One of the limitations of this study was that the combined effect of thermocycling and mechanical loading on resin dentin bond strength was not tested in this study. Further evaluation regarding the performance of these MMP silencers in the clinical scenario is required to substantiate the findings of our study. The biocompatibility of tested natural agents should be also confirmed before considering their clinical application.

CONCLUSION

Within the limitations of the study, it can be concluded that the use of natural and synthetic MMP silencers inhibited collagen degradation of demineralized dentin samples and increased long-term microtensile bond strength.

Further studies are still needed to evaluate their biocompatibility and long-term clinical performance.

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Conflicts of interest
There are no conflicts of interest.

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