Evaluation of the effect of collagen stabilizing agents like chitosan and proanthocyanidin on the shear bond strength to dentin and microleakage of resin composite at enamel and cemental walls: An in vitro study

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Abstract

Objectives: The objective is to evaluate the effect of collagen stabilizing agents-chitosan and proanthocyanidin (PA) on the shear bond strength to dentin and microleakage of resin composite at enamel and cemental walls.

Materials and Methods: Thirty premolars were decoronated 2 mm above cemento-enamel junction and restored with composite resin. Teeth were then randomly divided into three groups: Group I - Control, Group II - Pre-treatment with chitosan, and Group III - Pre-treatment with PA. Samples were then subjected to thermocycling for 500 cycles at 5°C and 55°C with the dwell time of 30 s and transfer time of 5–10 s. Then, the samples were subjected to shear bond strength evaluation on Universal testing machine. Shear load was applied until failure occurred. The load to failure was recorded individually and statistical analysis was done. Microleakage was determined by methylene blue dye penetration method and subjected to stereomicroscopic evaluation. Statistical analysis was carried out using Mann–Whitney test and Chi-square test.

Results: Group II specimens produced the highest median shear bond strength and group I showed the least. In addition, Group I, Group II, and Group III showed no statistically significant difference in microleakage.

Conclusions: Application of Chitosan and PA improved the shear bond strength to dentin as compared to the control. However, no significant difference in shear bond strength and microleakage was found between them.

Keywords: Chitosan; microleakage; proanthocyanidin; shear bond strength

INTRODUCTION

Adhesive dentistry is a rapidly evolving field of dentistry. Adhesive/dentin interface is porous and behaves as a permeable membrane allowing elution of un-reacted monomers, water sorption, polymer swelling, resin hydrolysis, and also enzymatic activity that degrades the exposed Type I collagen fibrils located at the bottom of the hybrid layer.[1,2] The two main patterns of degradation within the hybrid layer: loss of resin from the interfibrillar spaces and degradation of the collagen[3] suggests the need of an innovative treatments that focused not only on the stability of the resin components at the interface but also...
on the stabilization of dentin organic content in the hybrid layer.

Many attempts have been made to achieve adequate and predictable adhesion of resin composite to tooth structure. As the stability of the restoration, high bond strength and less microleakage are the main objectives of reliable bonding.[4]

The stabilization of dentin collagen with biocompatible cross-linking agents increases the mechanical properties and decreases the enzymatic degradation by matrix metalloproteinase inhibitors (MMP-I) which is an important clinical step to improve the dentin bond strength. The application of exogenous cross-linking agents alters the structure of collagen fibrils and improves their degradation resistance as well as stabilization.[5,6] A more resistant and insoluble collagen provides a stable substrate for dental adhesive restorations.

Synthetic collagen cross linking agents such as glutaraldehyde, formaldehyde, carbodimide, epoxy compounds, and others induce exogenous cross-links but exhibits disadvantages such as high cytotoxicity and incompatible mechanical properties. A naturally occurring cross-linking agent like proanthocyanidins (PAs) has overcome most of the drawbacks encountered with the synthetic cross-linking agents, and have been successfully used in the pretreatment of biological tissues to improve their mechanical properties.[6]

Chitosan is a hydrophilic biopolymer (2-amino-2-deoxy-β-d-glucopyranose) with a large number of free hydroxyl and amino groups that have the capability to form crosslinks with other reactive molecules. The free reactive groups present in the chitosan interact with collagen to form chemical bonds. They possess numerous properties because of the presence of free reactive groups and similarity to the extracellular matrix components such as glycosaminoglycans (GAGs).[5]

PAs are a class of bioflavonoids and natural metabolites available in fruits, vegetables, nut, seeds, flowers, and barks. They are natural collagen cross linkers and MMP inhibitors. They possess antibacterial and antioxidant properties that interact with proteins to stabilize and increase the Type I collagen cross-linkage by promoting hydrogen bond formation between protein amide carbonyl and the phenolic hydroxyl.[7]

Hence, the aim of this study was to evaluate the effect of collagen stabilizing agents namely, chitosan and PA on the shear bond strength to dentin and microleakage of resin composite with and without application of collagen stabilizing agents.

**MATERIALS AND METHODS**

**Preparation of chitosan**

Chitosan solution (Panvo Organics Ltd, Chennai, India) with a degree of acetylation >85% was prepared by dissolving 12 mg in 1 ml of 1% acetic acid (Sisco Research Laboratories-SRL, Mumbai, India) using a magnetic stirrer (Remi Laboratory Equipments, Mumbai, India) for 2 h at the rate of 50 rpm at room temperature to bring to a concentration of 1.2%.

**Preparation of proanthocyanidin**

The PA solution was prepared by adding 3.75 g of grape seed extract (La Nutraceuticals, New Delhi, India) in 100 ml of de-ionized water and mixed thoroughly to obtain a concentration of 3.75%.

Collagen stabilizing agents were tested for:

I. Shear bond strength

II. Microleakage.

**Shear bond strength test**

Thirty non carious, freshly extracted premolars without cracks or previous restorations, extracted for orthodontic purpose were used in this study. All samples were immersed in 10% formalin for 2 weeks for sterilization and stored in distilled water until it was used.

Sectioning was done with flexible diamond disc at 2 mm above the cementoenamel junction from the proximal surface under copious water cooling. All samples were etched for 15 s with 37% phosphoric acid (3M ESPE, USA), followed by rinsing with water and blot dried with absorbent paper pads. Samples were then divided into three experimental groups:

- **Group I** (n = 10) - Etching + Pre-treatment with Chitosan + Bonding agent + Composite
- **Group II** (n = 10) - Etching + Pre-treatment with Proanthocyanidins + Bonding agent + Composite
- **Group III** (n = 10) - Etching + Pre-treatment with chitosan or PA respectively for 30 s, washed for 20 s and blot dried with absorbent pads. In all Groups, bonding agent (Adper Single Bond-2, 3M ESPE, USA) was applied and light cured for 30 s and nanocomposite (Filtek Z350, 3M ESPE, USA) restoration was done to height-2 mm and diameter-3.5 mm using cylindrical Teflon mold and light cured for 30 s. Light intensity was checked with radiometer before curing. The samples were stored in distilled water at 37°C and subjected to thermocycling for 500 cycles at 5°C and 55°C with the dwell time of 30 s and transfer time of 5–10 s. Wax was coated on the root of all the samples and embedded in acrylic resin on a split mold. The wax was then

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removed from the resin block and was filled with light body elastomeric impression material to simulate the periodontal ligament. Then, the samples were subjected to shear bond strength evaluation on Universal Testing Machine (Instron, UK). Shear load was applied in a direction of 45° angle to the bonded interface at a cross head speed of 0.5 mm/min until failure occurred. The load to failure was recorded individually and the mean values are tabulated [Table 1].

Statistical analysis was done using Statistical Package for Social Sciences (SPSS) software (version 17) (SPSS Inc., Chicago, U.S.A). Comparisons of mean shear bond strength between the Groups was done using Kruskal–Wallis nonparametric test [Table 1]. Multiple comparisons of Median shear bond strength between the experimental groups was done using Mann–Whitney test \(P < 0.01\) was considered to be statistically significant [Table 2].

**Microleakage**

Thirty freshly extracted premolars, stored in distilled water at room temperature without decay, cracks or previous restoration were used. Samples were immersed in 10% formalin for 2 weeks for sterilization. Class V cavities (width-3 mm mesiodistally; 2 mm occluso-gingivally and depth – 1.5 mm) were prepared using cylindrical diamond bur mounted on a high-speed handpiece. The teeth were then randomly divided into three groups:

- Group I - control
- Group II - pretreated with chitosan
- Group III - pretreated with PA after acid etching

In all the Groups, the same protocol used for shear bond strength testing was followed by restoration of the class V cavity with the same nanocomposite. Thermocycling procedure was done and the root apices of the restored teeth were sealed with sticky wax to avoid dye penetration from the root canal. The surface of the tooth samples were coated with two layers of nail varnish up to within 1 mm of the bonded interface. The samples were then immersed in methylene blue dye solution for 24 h. Samples were washed under tap water for 30 s and then sectioned buccolingually through its mesiodistal center with slow speed diamond disc under copious water cooling. The obtained sections were kept moist until the samples were observed under the stereomicroscope (Optika, Italy, Europe) at 20× magnification to evaluate microleakage. Dye penetration was examined and scored separately for enamel and cemental wall on a 0–3 ordinal scale given by Moosavi et al., in 2013 [Table 3].

**RESULTS**

**For shear bond strength testing**

Mean, median, and standard deviations of shear bond strength values are shown in Table 1. The highest mean shear bond strength value was recorded for Group II (chitosan) (29.69 Mpa), whereas the lowest value was recorded for Group I (control) (17.40 Mpa). Multiple comparisons of median shear bond strength between Group I, Group II, and Group III results showed the significant difference between Group I and II \(P = 0.002\) and Group I and Group III \(P = 0.035\). There was no significant difference between Group II and Group III \(P = 0.39\) [Table 2].

**For microleakage**

**In enamel**

i. Group I - 20% of the samples-no microleakage, 60% - dye penetration up to 1/2 depth of the cavity, 20% - dye penetration more than 1/2 depth of the cavity and no sample (0%) had dye penetration extending to the axial wall of the cavity

ii. Group II - 40% - no microleakage, 20% - dye penetration up to 1/2 depth of the cavity, 10% - dye penetration more than 1/2 depth of the cavity, and 30% - dye penetration extending to the axial wall of the cavity

iii. Group III - 30% - no microleakage, 50% - dye penetration up to 1/2 depth of the cavity, 20% - dye penetration more than 1/2 depth of the cavity and no sample 0% - dye penetration extending to the axial wall of the cavity

iv. As compared to Group II - Group I and Group III had no dye penetration that extends to the axial wall of the cavity but there was no statistical difference among the experimental groups according to Chi-square test \(P = 0.17\) [Table 4 and Figure 1].

**In cementum**

Group I - 10% - dye penetration up to 1/2 depth of the cavity, 40% - dye penetration more than 1/2 depth of the
cavity, and 50% - dye penetration extending to the axial wall of the cavity.

Group II - 20% - no microleakage, 50% - dye penetration up to 1/2 depth of the cavity, 20% - dye penetration more than 1/2 depth of the cavity and 10% - dye penetration extending to the axial wall of the cavity.

Group III - 50% - dye penetration up to 1/2 depth of the cavity, 30% - dye penetration more than 1/2 depth of the cavity, and 20% - dye penetration extending to the axial wall of the cavity [Figure 2].

As compared to Group I and Group III, Group II had 20% of no marginal leakage but the difference was not significant ($P = 0.09$). Statistical results for microleakage scores for cementum are shown in Table 5.

### DISCUSSION

Many challenges are faced in bonding to dentin, when compared to the enamel which includes the following:

- Difference in the substrate composition
- The creation of the hybrid layer (that requires the demineralization of dentin collagen matrix to enable the infiltration of resin monomers)
- Improper infiltration of resin and incomplete conversion of monomer
- Susceptibility of the partially exposed collagen in the hybrid layer to hydrolytic and enzymatic breakdown leading to interfacial collagen degradation
- Matrix metalloproteinases (MMPs) in the dentin that get exposed while acid etching have the ability to degrade Type 1 collagen. Strong MMP 2 and MMP 9 activities were identified at the base of the hybrid layer
- Bacterial collagenases degrades collagen by hydrolyzing the peptide bond on the amino-terminal side of glycine (–X-Gly-Pro).

Exogenous collagen cross-linkage can be formed to enhance the mechanical properties by crosslinking of collagen which increases the number of inter- and intra-molecular bond has been reported in the dental literature. Hence, the use of different collagen cross-linking agents helps in improving the mechanical properties of collagen. Dentin surface pre-treatment by these agents prior to the bonding procedures aids in increasing the bond strength values.

Several naturally occurring cross-linking agents such as Genipin and PAs have overcome some of the drawbacks encountered with synthetic cross-linking agents. PAs are proline-rich proteins that binds to collagen and enables collagen biosynthesis. Apart from its natural occurring

| Enamel scoring | Group I, n (%) | Group II, n (%) | Group III, n (%) | $P$ |
|----------------|---------------|----------------|------------------|-----|
| 0              | 2 (20)        | 4 (40)         | 3 (30)           | 0.17|
| 1              | 6 (60)        | 2 (20)         | 5 (50)           |     |
| 2              | 2 (20)        | 1 (10)         | 2 (20)           |     |
| 3              | 0             | 3 (30)         | 0                |     |

Figure 1: Stereomicroscopic images for microleakage evaluation at the enamel wall (a) No dye penetration; (b) Dye penetration up to ½ the depth of the cavity; (c) Dye penetration more than ½ the depth of the cavity; (d) Dye penetration extending to the axial wall of the cavity

Figure 2: Stereomicroscopic images for microleakage evaluation at the cementum wall (a) No dye penetration; (b) Dye penetration up to ½ the depth of the cavity; (c) Dye penetration more than ½ the depth of the cavity; (d) Dye penetration extending to the axial wall of the cavity
origin, it shows good biocompatibility, faster reaction rate than genipin and also, their building blocks catechin and epigallocatechin gallate, have been identified as potent collagenase inhibitors. Genipin has the disadvantage of discoloration so it is not used widely. Dentin pretreatment with chitosan, improved the mechanical properties and resistance to enzymatic degradation. The intermolecular interaction between positive and negative charged units facilitates the cross-linking between chitosan and collagen. Hence, the present study, evaluated the effect of pretreatment of dentin, before acid etching with collagen stabilizing agents such as Chitosan and PA on the shear bond strength to dentin and microleakage of resin composite at enamel and cemental wall.

Bond strength measurement is one of the most effective methods in characterizing commercial dentin bonding products. They are tested in tension or in shear. Shear bond strength test is a simple evaluation procedure which is used to determine the adhesion of dental adhesives. The performance of adhesive systems and possible correlation with clinical issues can be predicted using in vitro bond strength test. Hence, shear bond strength testing was done with a universal testing machine for evaluating the stability of the adhesive/restorative interface.

Gap formation and microleakage due to thermally induces stresses at the interface occurs because the coefficients of thermal expansion between the restorative materials and natural tooth structure are incompatible. In vitro methods to study the microleakage includes compressed air, neutron activation, electrochemical, fluid filtration, and dye penetration tests. Due to its simplicity, quick action, ability to reproduce and visualized its depth of penetration, dye penetration tests are commonly used. Methylene blue dye was used for assessment of microleakage in this study, since it can diffuse easily through the interface, detected easily and does not get absorbed by dentinal matrix apatite crystals. It can pass through microscopic interfaces easily as it has molecular size 1.2 nm in diameter. The dye penetration was determined after sectioning the specimens and viewing under magnifying aid. The immersion times of the specimens in this dye, ranges from 1 h to 2 weeks in several studies, seems to have no influence on the microleakage results, so the samples were immersed for 24 h.

To simulate the aging of the restorations at body temperature, the samples were subjected to thermocycling procedure before shear bond and microleakage testing to simulate the temperature changes in the oral cavity that may take place during the service life of a restoration. The use of different temperatures was done according to ISO standardization (5°C-55°C) that allows for a better comparison between studies.

According to the results obtained for shear bond strength testing, in this study, the highest median shear bond strength value was recorded for Group II Chitosan (22.43 Mpa) whereas the lowest value was recorded for Group I Control (17.83 Mpa). The shear bond strength value for Group III PA was 22.04 Mpa. Multiple comparisons of median shear bond strength between Group I, II, and III showed the significant difference in between Group I and II ($P = 0.002$); Group I and Group III ($P = 0.035$). Whereas there was no statistical significant difference between Group II and Group III ($P = 0.39$). The results proved that pre-treating with Chitosan and PA increased the bond strength than the control.

Chitosan group showed a slightly higher median shear bond strength value when compared to PA, due to the fact that it possess a large number of free hydroxyl and amino groups which have the ability to form cross-links with other reactive molecules and form chemical bonds with collagen by interaction with the free reactive groups in chitosan. Collagen of the dentin that is incorporated and cross-linked by chitosan resists degradation. Chitosan biopolymers are structurally similar to extracellular matrix materials thereby enables collagen construction. Shrestha et al. in 2011 hypothesized that photodynamic cross-linking of dentin collagen by the incorporation of carboxymethyl-chitosan (CMCS) reinforced collagen stabilization by increasing the number of amine reaction sites, resulting in the formation of ionic complexes between CMCS and collagen thus enhancing collagen cross-linking. The interaction between chitosan and collagen in solution is mainly mediated by the intermolecular interactions between positively and negatively charged units.

The increase in bond strength in PA group in the current study was in accordance with the findings of various authors who showed that the application of PA containing grape seed extract to deep dentin significantly improved the shear bond strength values of composite to dentin. This is due to the greater number of collagen cross-links which enhanced the stability of collagen. The presence of four monomer molecules (catechin, ent-catechin, epicatechin, and ent-epicatechin) and different types of inter flavonoid bonds form the unique characteristics of PAs. By the hydroxylation of proline which is an essential step of the collagen biosynthesis, PAs are able to stabilize and increase the cross-linkage of type I collagen fibrils. It also induces

| Table 5: Comparison of microleakage at cemental wall (Chi-square test) |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Cementum scoring            | Group I, n (%)              | Group II, n (%)             | Group III, n (%)            |
| 0                           | 0                           | 2 (20)                      | 0                           |
| 1                           | 1 (10)                      | 5 (50)                      | 5 (50)                      |
| 2                           | 4 (40)                      | 2 (20)                      | 3 (30)                      |
| 3                           | 5 (50)                      | 1 (10)                      | 2 (20)                      |

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exogenous cross-links in dentin matrix which increases the density of collagen network, reduces the swelling ratio of demineralized dentin, decreasing the absorption of collagenase, thus the matrix resistance against enzymatic degradation is improved.\[6\]

According to the results obtained for microleakage testing, in the present study, there was no statistical significance difference among the three experimental groups in the enamel (\(p = 0.17\)) and cemental wall (\(p = 0.09\)). The reason for slightly less microleakage in chitosan pre-treatment group may be due to presence of smart hydrogels which in response to external physicochemical factors like temperature undergoes a reversible discontinuous volume phase change. This would deny the tendency for microleakage of resin composite. Due to presence of large number of free hydroxyl and amino groups, chitosan has the ability to form a microfibrillar and nanofibrillar network with superior mechanical properties including higher resistance to collagen degradation.\[19\] The organic component of demineralized dentin which is composed of collagen and GAGs forms an electrostatic interactions with the chitosan, which increases the stability of the hybrid layer. At the interface of systems containing chitosan, reduction in nanoleakage could be observed due to the chemical and physical interaction with the dentin substrate, hence establishing a coronal seal.\[20\]

The null hypothesis tested in this study that there is no difference in shear bond strength was rejected as pretreatment with collagen stabilizing agents improved the shear bond strength whereas the null hypothesis was accepted for microleakage as there was no difference with and without pretreatment with collagen stabilizing agents.

It is difficult to entirely correlate laboratory findings with the clinical behavior of restorations. The major limitation in the current study was the short pretreatment time with the collagen stabilizing agents. Longer time is required to establish stable collagen cross-links.\[21\] However, the short pretreatment period of 30 s was applied in the present study to suite the clinical scenario. The best evidence would be achieved with further in vitro and in vivo studies with larger sample size and long-term clinical performance.

**CONCLUSIONS**

Within the limitations of this in vitro study, it can be concluded that the:

- Application of Chitosan and PA improved the shear bond strength to dentin as compared to the control
- No significant difference in shear bond strength was found between Chitosan and PA.

In enamel and cemental wall, application of Chitosan and PA showed no improvement in microleakage.

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**Conflicts of interest**
There are no conflicts of interest.

**REFERENCES**

1. Malacarne J, Carvalho RM, de Goes MF, Svizero N, Pashley DH, Tay FR, et al. Water sorption/solubility of dental adhesive resins. Dent Mater 2006;22:973-80.
2. Pashley DH, Tay FR, Yiu C, Hashimoto M, Breschi L, Carvalho RM, et al. Collagen degradation by host-derived enzymes during aging. J Dent Res 2004;83:216-21.
3. Bedran-Russo AK, Castellan CS, Shinohara MS, Hassan L, Antunes A. Characterization of biomodified dentin matrices for potential preventive and reparative therapies. Acta Biomater 2011;7:1735-41.
4. Chanthachaimongkol S, Sirivimol Srisawasdi DD. Microtensile bond strength of self-adhesive resin composite to dentin (Doctoral dissertation, Chulalongkorn University). CU Dent J. 2015;38:21-34.
5. Kishen A, Shrestha S, Shrestha A, Cheng C, Goh C. Characterizing the collagen stabilizing effect of crosslinked chitosan nanoparticles against collagenase degradation. Dent Mater 2016;32:968-77.
6. Nagpal R, Singh P, Singh S, Tyagi SP. Proanthocyanidin: A natural dentin biomodifier in adhesive dentistry. J Restor Dent 2016;4:1-6.
7. Liu Y, Wang Y. Proanthocyanidins’ efficacy in stabilizing dentin collagen against enzymatic degradation: MALDI-TOF and FTIR analyses. J Dent 2013;41:535-42.
8. Sabatini C, Pashley DH. Mechanisms regulating the degradation of dentin matrices by endogenous dentin proteases and their role in dental adhesion. A review. Am J Dent 2014;27:203-14.
9. Carlinho MR, Tay FR, Donnelly AM, Agee KA, Tjäderhane L, Mazzoni A, et al. Host-derived loss of dentin matrix stiffness associated with solubilization of collagen. J Biomed Mater Res B: Appl Biomater. 2009;90:373-80.
10. Watanabe K. Collagenolytic proteases from bacteria. Appl Microbiol Biotechnol 2004;63:520-8.
11. Srinivasulu S, Vidhya S, Sujatha M, Mahalaxmi S. Shear bond strength of composite to deep dentin after treatment with two different collagen cross-linking agents at varying time intervals. Oper Dent 2012;37:485-91.
12. Nagaoa H, Nagaoka H, Walter R, Boushell LW, Miguez PA, Burton A, et al. Characterization of genipin-modified dentin collagen. Biomed Res Int 2014;2014:702821.
13. Shrestha A, Friedman S, Kishen A. Photodynamically crosslinked and chitosan-incorporated dentin collagen. J Dent Res 2011;90:1346-51.
14. Kerby RE, Knobloch LA, Clelland N, Lilley H, Seghi R. Microtensile bond strengths of one-step and self-etching adhesive systems. Oper Dent 2005;30:195-200.
15. Versluis A, Douglas WH, Sakaguchi RL. Thermal expansion coefficient of dental composites measured with strain gauges. Dent Mater 1996;12:290-4.
16. Joseph A, Santosh L, Hegde J, Panchajanya S, George R. Microleakage evaluation of Silorane-based composite and methacrylate-based composite in class II box preparations using two different layering techniques: An in vitro study. Indian J Dent Res 2013;24:148.
17. Khoroushi M, Ehteshami A. Marginal microleakage of cervical composite resin restorations bonded using etch-and-rinse and self-etch adhesives: Two dimensional vs. three dimensional methods. Restor Dent Endod 2016;41:83-90.
18. Ölmez A, Öztaş N, Bilici S. Microleakage of resin composite restorations.
with glass-ceramic inserts. Quintessence Int 1996;29:725-99.
19. Gajjela RS, Satish RK, Sajjan GS, Varma KM, Rambabu T, Vijaya Lakshmi BH. Comparative evaluation of chlorhexidine, grape seed extract, riboflavin/chitosan modification on microtensile bond strength of composite resin to dentin after polymerase chain reaction thermocycling: An in vitro study. J Conserv Dent 2017;20:120-4.

20. Diolosà M, Donati I, Turco G, Cadenaro M, Di Lenarda R, Breschi L, et al. Use of methacrylate-modified chitosan to increase the durability of dentine bonding systems. Biomacromolecules 2014;15:4606-13.

21. Castellan CS, Bedran-Russo AK, Karol S, Pereira PN. Long-term stability of dentin matrix following treatment with various natural collagen cross-linkers. J Mech Behav Biomed Mater 2011;4:1343-50.