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Role Of Different Collagen Cross-Linking Agents Like Proanthocyanidin, Riboflavin And White Tea On The Shear Bond Strength To Dentin- An In-Vitro Study.

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ABSTRACT

OBJECTIVES: Dentin surface pre-treatment with collagen stabilizing agents prior to bonding procedures increases the bond strength.The current study evaluated the effect of dentin surface pre-treatment with collagen cross-linking agents on the shear bond strength to dentin.

MATERIALS & METHODS: Forty premolars were decoronated 2 mm above cemento-enamel junction and restored with composite. Samples were randomly divided into four groups with n=10 in each group - Group I: (Control)no prior dentin surface treatment and Group II, Group III, Group IV: Dentin pre-treated with 3.75% Proanthocyanidin (PA), 1 % Riboflavin,10 % White tea respectively. Thermocycling was done to simulate the clinical scenario which was followed by shear bond strength evaluation on Universal Testing Machine. The load to failure was recorded individually. The mean shear bond strength was statistically analysed with One-wayANOVA and Students 't' test was used to obtain multiple comparison between the groups.

RESULTS: The highest mean shear bond strength value 42.91 MPa was recorded in Group III (Riboflavin) when compared to Group II(PA) -33.43 MPa, Group I (control) -28.03 MPa and Group IV (white tea)- 19.93 MPa.

CONCLUSIONS: Dentin surface pre-treatmentby the application of Riboflavin and PA enhanced collagen stabilization and increased the shear bond strength to dentin when compared to the control and white tea.

KEYWORDS: Collagen cross linking agents;Collagen stabilization; Proanthocyanidin; Riboflavin;Shear bond strength; White tea

INTRODUCTION:

Adhesive dentistry deals with adhesion of resins to enamel and dentin. To counteract the polymerization shrinkage of composite resin, to obtain adequate retention and function, it is necessary to achieve the idealbondstrength [1]. It was Fusayama in the year 1979 who introduced thetotal etch concept of etching enamel and dentin which increased the bond strength to dentin.Dentin contains 70% inorganic materials and20% organic materials. The organic matrix is reported to becomposed of 90% fibrillar type I collagen and 10%are non-collagen proteins such as phosphoproteins andproteoglycans.Greater the inorganic content superior is the adhesion [2]. The mechanical properties ofdentin collagenand the bond strength can be enhanced by collagen cross-linking agents.Subsequent application of bonding agent into thedemineralised collagen rich dentin leads to the formation ofhybrid layer which is an important factor in dentin adhesion. The biodegradation rate of the resin-dentine interface is reduced by the application of exogenous collagen cross-linkingagents that ensures the longevity period of the restoration. Collagen cross linking agents are of various types that includes both natural (proanthocyanidin, genipin, chitosan, propolis, green tea, white tea) and synthetic agents like (glutaraldehyde,transglutaminase, carbodiimides, formaldehyde, epoxycompounds and others) [3].

Proanthocyanidins (PAs) are polyphenolic bioflavonoids obtained naturally in plant metabolites that leads to stable hydrogen bonding and generate non -biodegradable collagen matrix [4]. PAs transforms soluble collagen to insoluble collagen by cross-linking and exhibit Matrix Metalloproteinase (MMP) inhibition.

Riboflavin (Vitamin B_2) is an essential dietary supplement found in certain foodsand dairy products. When photoactivated by ultraviolet (UV) light it causes the degradation of week intrinsic collagen cross-links generating free radicals with a maximum absorption peak of 270, 366 and 445 nm [5].These free radicals generate the formation ofstrong covalent bonds between adjacent collagen molecules. There is also an observed reduction of amino acids like histidine and tyrosine during cross-linking which leads to the formation of ditryosine (dimer of tyrosine), that constitutes a possible mechanism in riboflavin induced collagen cross-linking [4].

White tea is obtained from a plant (Camellia sinensis). It is rich in catechins, a category of polyphenols that includes epigallocatechin gallate (EGCG), epicatechin gallate, epigallocatechin, epicatechin. Catechins possess antiproliferative, apoptotic and antioxidant properties hence are

being widely used for cancer treatment. It also exhibits profound inhibitory activity on collagenases that degrade the organic matrix [6].Dentin surface pre-treatment with white tea, is known to inhibit proteolytic degradation, prevents MMP release and enhances the mechanical properties of collagen, thereby producing collagen stabilization[7].

Shear bond strength is commonly used for evaluating the effectiveness of restorative resin bond to the dentin and highlights the strength of the bonded interface[8].

Hence, the aim of this study was to evaluate the effect of collagen stabilizing agents like PA, Riboflavin and White Tea on the shear bond strength to dentin. The null hypothesis was that there will be no difference in the shear bond strength of resin composite with or without the application of collagen stabilizing agents.

MATERIALS AND METHODS:

Preparation of the test solutions:

To prepare 3.75% concentrated solution of PA, grape seed extract (La Nutraceuticals, New Delhi, India)commercially available as capsules were cut open and 3.75g of grape seed powder was accurately weighted. 3.75 % PA solution (Group II) was obtained by dissolving the measured powder in 100 ml of de-ionized water.

To obtain 1% Riboflavin solution, 1g of riboflavin-5 phosphate powder (Zenith Nutricorp, USA) obtained from the capsule was dissolvedin 100 ml of distilled water. Light activation of riboflavin was prevented by storing the solution in light proof tubes at room temperature before application on the prepared dentin specimens.

To prepare 10% white tea solution, 50gms of white tea powder (United Nilgiri Tea Estates Co. Ltd, Korakundah, India) was finely crushed, centrifuged, dissolved in 500ml of distilled water and filtered.

Evaluation of Shear Bond Strength:

Forty single rooted, non carious human mandibular premolarsfreshly extracted for orthodontic purpose without cracks or previous restorations, were selected. All the teeth samples were immersed in 10% formalin solution for sterilization and stored in distilled water until usage. Decoronation was done 2 mm above the cemento-enamel junction under copious water cooling with a flexible diamond disc. Sectioning was done from the proximal surface of the teeth. 37%

phosphoric acid gel (3MESPE, USA) etchant was applied to the dentin samples for 15 sec, washed, blot dried with absorbent paper pads and divided into 4 experimental groups.

Group I(n=10)-Etching + Rinsing + Bonding agent + Composite (control)

Group II(n=10)-Etching + Rinsing + Pre-treatment with PA for 30 sec + Rinsing + Bonding agent + Composite

Group III(n=10)**–** Etching + Rinsing + Pre-treatment with Riboflavin for 2 min + Lightcuring + Bonding agent + Composite

Group IV(n=10)**–** Etching + Rinsing + Pre-treatment with White tea for 10 min + Bonding agent + Composite

Following the dentin surface pre-treatment, total etch adhesive (Adper Single Bond -2, 3M ESPE, USA) was applied and light cured for 20 secs to infiltrate the etched dentin. Using a cylindrical teflon mould, nanohybrid composite (Filtek Z 350, 3M ESPE, USA) restoration was done (height 2 mm x diameter 3.5 mm). The advantage of this total etch technique is that it leads to highest enamel and dentin bond strength [9].

Sample preparation was completed followed by thermocycling procedure to simulate the thermal fluctuations occurring in the clinical oral conditions [10]. Thermocycling was done for 500 cycles at 5°C and 55°C with the dwell time of 30 sec and transfer time of 5 to10 sec. Then the samples were stored in distilled water at 37°C. In order to simulate the clinical scenario wax was coated on the root of all the samples and embedded in an acrylic resin on a split mould. The wax was then 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 testing using Universal Testing Machine (Instron, UK). The load was applied at 45^0 angulations to the bonded interface with a cross head speed of 0.5 mm / minute until fracture occurred. The load to failure was recorded, the values were tabulated and statistically analyzed.

Statistical Analysis:

Statistical Package for Social Sciences (SPSS) software (version 17) (SPSS Inc, Chicago, U.S.A) was used. The mean shear bond strength was determined with One-way Analysis of Variance (ANOVA) and Students 't' test was used to obtain multiple comparison between the experimental groups.

RESULTS:

The mean shear bond strength values for Groups I, II, III & IV were 28.3; 33.43; 42.91 and 19.93 MPa respectively. Table A & Figure 1- represents the mean shear bond strength values.According to the results obtained, the highest mean shear bond strength value was recorded in group III (Riboflavin) and the lowest value was recorded in group IV (White tea).

Table A - Mean values of the experimental groups

GROUPS	SAMPLE SIZE (n)	MEAN (MPa)	STANDARD DEVIATION (SD)	STANDARD ERROR OF MEAN (SEM)
I- Control	10	28.0380	11.62514	3.67619
II- Proanthocyanidin	10	33.4370	11.43820	3.61708
III- Riboflavin	10	42.9190	15.87412	5.01984
IV- White tea	10	19.9330	9.85471	3.11633

Table B - Intergroup Comparison between the experimental groups using Students 't' test

GROUPS	CONTROL	PROANTHOCYANIDIN	RIBOFLAVIN	WHITE TEA
CONTROL	-	0.309	0.028*	0.110
PROANTHOCYANIDIN	0.309	-	0.143	0.011*
RIBOFLAVIN	0.028*	0.143	-	0.001*
WHITE TEA	0.110	0.011*	0.001*	-

Probability value (P value) ≤ 0.05 is statistically significant;

*indicates statistically significant

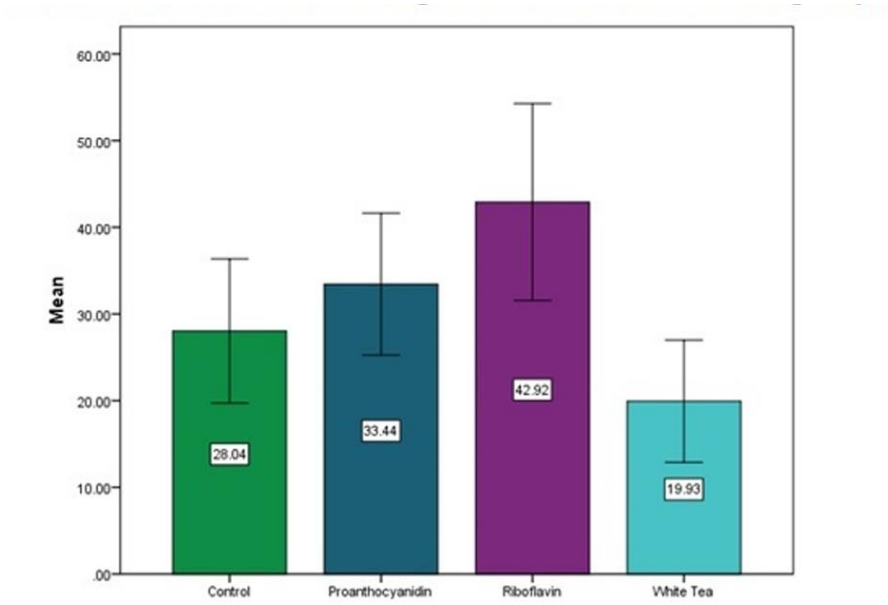

Figure 1 – Mean shear bond strength values between all the groups

Multiple comparisons of mean shear bond strength values between the experimental groups were tabulated. When the probability value (P value) was less than 0.05 it was considered to be statistically significant. There was no significant difference between group I and II (p=0.309); group I and IV (p=0.110); group II and III (p=0.143); Whereas there was statistically significant difference between group II and IV (p=0.011) ; group III and group IV(p=0.001); group I and group III (p=0.028). The results proved that pre-treatment with Riboflavin and PA increased the bond strength compared to white tea and control group.

Table Brepresents the inter-group comparison between the experimental groups using Students 't' test.

DISCUSSION:

Hybrid layer is composed of collagen fibrils and adhesive resins that form an interlocked entanglement, providing the basis of bonding between the bulk adhesive and the underlying intact dentin. It is well accepted that bond strength and durability rely on the quality of the hybrid layer rather than its thickness [11].Pre-treatment of dentin collagen with exogenous collagen cross-linking agents, prior to the application of the bonding agent, results in mechanical and biological collagen stabilization in the hybrid layer by intermolecular cross-linking [12]; improves the properties of the resin/ dentin interface and increases the bond strengthby the creation of a mechanically and enzymatically resistant collagen scaffold[7]. Not many studies are available comparing the

effect of dentin surface pre-treatment with collagen stabilizing agents like PA, riboflavin and white tea. Hence this study, determined the effect of pre-treatment with these collagen cross-linking agents on the shear bond strength to dentin.

PA was used for dentin surface pre-treatment in the current study due to the following reasons- being naturally obtained from plants it is non-toxic and possesses excellent biocompatibility,antibacterial and antioxidant properties. PAs not only inhibits bacterial proteases and host- derived MMPs, as well as enables collagen biosynthesis by binding to proline-rich proteins, such as collagen and facilitates the enzyme proline hydroxylase activity that ensures long term collagen stabilization [10].Riboflavin was used in the present study mainly due to its ability to generate free radicals such as O_2& O_2- when photo-activated. These free radicals produce stable and strong intermolecular covalent bond formation thus cross-linking the dentin collagen [4]. Riboflavin solution with a concentration of 1% was used, as higher concentration can produce yellowish dentin discoloration, directly affecting the esthetics of the restoration [13]. White tea was used for dentin surface pre-treatment as it inhibits MMPs and prevents the enzymatic degradation of the collagen matrix since it contains catechins like EGCG [6].

In the present study the stability of the resin- dentin bonded interface of the pre-treated dentin collagen was evaluated using shear bond strength analysis which helps to predict the clinical performance of the adhesive systems used and indirectly enables to correlate to the long term stabilization of the cross-linked collagen matrix network [8].

The pre-treatment time of the three test solutions namely PA, Riboflavin and white tea varied which was 30 secs, 2 min & 10 min respectively. PA solution was applied for only 30 secs so as suite the regular clinical scenario and it was rinsed immediately to prevent dentin discoloration [14].Moreover, PA can inhibit resin polymerization due to its free radical scavenging effect, hence it was applied only for 30 secs and rinsed immediately [15].Riboflavin was applied for 2 min so as ensure sufficient collagen aggregation by the loss of tyrosine and histidine residues which prolongs the durability of the adhesion and photo-activated by visible blue light to induce the free radical formation[16]. White tea solution was applied to the etched dentin for 10 min as longer pre-treatment time period was needed to obtain the beneficial effects of EGCG such as higher bond strength and longevity of adhesive-dentin bond. Longer the contact time greater is the collagen stabilization [17].

All the prepared samples were subjected to thermo-cycling regimens before the shear bond strength evaluation so as simulate the thermal fluctuations and aging of the restoration that occurs

in the oral environment. According to ISO standardization, the temperature varied from 5^0 – 50^0 C, depending upon the temperature changes in the oral environment [18].

According to the results obtained, the highest mean shear bond strength value was recorded for Group III (Riboflavin), followed by Group II (PA) and Group I (control) which was 42.91 MPa; 33.43 MPa & 28.30 MPa respectively. The lowest value was obtained for Group IV (White tea) - 19.93 MPa. There was a statistically significant difference between Group III & IV and in between Group III & I, clearly indicating that pre-treatment with Riboflavin, increased the shear bond strength compared to White tea and the control group.

UV light activated Riboflavin significantly showed increased shear bond strength due to the following mechanisms:

- It generates free radicals – active singlet oxygen species ($O_2$and O_2-) by breaking down weak intrinsic collagen cross-links, producingstable inter-molecular covalent bond formation in the dentin matrix. Chemical covalent bond formation occurs between the amine groups (N-H) of glycines in one chain with carbonyl groups (C=O) of proline and hydroxyproline in the adjacent chains [13].
- It allows deeper resin monomer infiltration by expanding the collagen matrix and creating many coarse upright collagen fibrils, resulting in a more thicker, stiffer and stable collagen network.
- Enhances the structural integrity of the hybrid layer and stabilizes the adhesive interface by the formation of peptide bonds between adjacent collagen chains.
- The hydroxyl groups present in Riboflavin binds to proline and lysine present in the collagen matrix, promoting the formation of new collagen cross-links, imparting rigidity and stability [16].
- Riboflavin causes collagen aggregation by the loss of tyrosine and histidine residues in Type I dentin collagen, leading to the formation of dityrosin (dimer of tyrosine) thereby increasing the overall stiffness of the dentin collagen matrix [19].
- Reduces the nano-leakage at the resin-dentin interface [20].
- Inhibits and inactivates MMPs, reduces osteoclast derived telopeptidase activity thus preventing collagen (enzymatic or hydrolytic) degradation [18].

In the present study there was also a statistically significant difference between Group II & IV, which proved that pre-treatment with PA increased the shear bond strength compared to White tea. However there was no significant difference between Riboflavin and PA. Application of PA also

had a higher bond strength value due to the following mechanisms : Being natural bio-flavonoids theypromote collagen cross-linking by covalent, ionic, hydrophobic interactions and hydrogen bonding between the protein amide carbonyl and the phenolic hydroxyl present in PA and proteins[8]; PA binds to proline rich proteins in the collagen and triggers the activity of the enzyme proline hydroxylase which is essential for collagen biosynthesis [18].The resistance to enzymatic degradation, by inhibiting bacterial proteases and host-derived MMPs is a crucial property of PA treated dentin matrix, leading to long term stability [3,21]. PAs building blocks: catechin and EGCG are potent collagenase inhibitors that inhibits the biodegradation of unprotected collagen fibrils [22]. The combined cross-linking potential and anti-collagenolytic effects of PA is beneficial in preventing the degradation of dentin collagen within the hybrid layer and increases the density of the collagen network [20]. PA may indirectly interfere with protease production and activation by modulating host immune responses [23].Thus dentin surface pre-treatment with PAincreased the shear bond strength by improving the elastic modulus of dentin and nano-hardness at the resin-dentin interface [15].

The results showed that white tea recorded the lowest mean shear bond strength value (19.93 MPa) compared to all the experimental groups. The reason may be due to the presence of EGCGs which possesses anti-oxidant property and neutralizes the effect of free radicals, thus preventing covalent bond formation in the dentin collagen [24]. Although white tea possess inhibitory effect against MMPs, rinsing was not done after pre-treatment on the etched dentin surface in this study, that reduced the collagen cross-linking and decreased the shear bond strength compared to the other groups.

The results obtained confirmed that pre-treatment with collagen stabilizing agents, produced cross-linking of the dentin collagen and increased the shear bond strength, thus the null hypothesis proposed was rejected. However in-vitro laboratory investigations regarding collagen stabilization cannot entirely correlate with the placement of any restoration in the clinical scenario. Thus further in-vivo clinical trialswith a large sample size may be necessary to determine the long-term performance of the restoration.

CONCLUSION:

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

- Riboflavin showed the highest mean shear bond strength value when compared to the other experimental groups.

- Application of Riboflavin and PA improved the shear bond strength to dentin when compared to the control and white tea.
- No significant difference in shear bond strength was found between PA and Riboflavin.
- Dentin surface pre-treatment with white tea, showed lowest mean shear bond strength value indicating its reduced efficacy in collagen stabilization.

The present study was an in vitro study conducted on extracted human mandibular premolars for orthodontic treatment. Approval was obtained from Sree Balaji Dental College and Hospital Ethical Committee, Bharath institute of Higher Education & Research (protocol Reference no. SBDCH / IEC / 01 / 2018 / 28).

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