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AN IN VITRO COMPARATIVE STUDY ON EFFECT OF CHLORHEXIDINE, GRAPE SEED EXTRACT, RIBOFLAVIN/CHITOSAN ON SHEAR BOND STRENGTH OF COMPOSITE RESIN TO DENTIN

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ABSTRACT

Background: The biggest limitation in the restorative dentistry is the squalor property of the adhesive dentin layer interface which further includes non-organization between the collagen and resin from the inter-fibril space. An important challenge to the dentin bond durability is degradation of collagen from the matrix-bound proteases, namely matrix metalloproteinases (MMPs) and cysteinecathepsins. Pretreatment of the bonding substrate with agents that inhibit the activity of MMPs might improve bond durability. Chlorhexidine (CHX) is reported to be a strong MMP inhibitor. Riboflavin helps in collagen cross-linking by its ability to produce free radicals when photoactivated with spectral range from ultraviolet to visible light.(6) In addition to cross-linking, reinforcement of the collagen can be achieved by incorporating biopolymers such as chitosan that can be cross-linked with collagen fibrils. Recent studies have shown that a PA-based cross-linker agent (grape seed extract) increased the mechanical properties of demineralized dentin matrixand enhanced the resin–dentin bond strength after one hour treatment. **Objective:** The objective of this study was to evaluate the influence of chlorhexidine (CHX), riboflavin/chitosan, grape seed extract (GSE) modification on shear bond strengthof composite resin to dentin after thermocycling. **Methods:** Sixty extracted human mandibular secondpremolars were used and a flat surface was then prepared by removing the occlusal one-third to expose the mid-colonal dentin. The teeth were randomly assigned into four groups - Group A in which self-etch adhesive was applied and Groups B, C, D were pretreated with 2% CHX, 1% riboflavin/chitosan and 6.5% GSE, respectively, before the application of self-etch adhesive. Composite build-ups were constructed and subjected to thermo cycling. The shear bond strength was evaluated using the universal testing machine. Data were analyzed using one-way analysis of variance and Tukey's test. **Results:** The mean shear bond strength values for Group A(control), Group B (CHX), Group C (riboflavin/chitosan) and Group D (GSE) modification) were 22.26, 28.76, 26.57, 24.87 MPa, respectively. A stastically significant difference was found between the shear bond strength of all the groups ($P<0.05$) of 2% CHX, 6.5% GSE, 1% Rivoflabin/chitosan when compared with the control group. **Conclusions:** Pretreatment with CHX, rivoflabin/chitosan and GSE leads to a significant increase in shear bond strength of composite resin to dentin.

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INTRODUCTION

Adhesive restorations reinforce the weakened tooth structure by effectively transmitting and distributing the functional stresses across the bonding interface.(1)

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Adhesion also reduces microleakage at restoration tooth interface resulting in lesser clinical problems such as postoperative sensitivity, marginal staining and recurrent caries all of which otherwise jeopardize the clinical longevity of the restoration.(2) Adhesive technique also allows deteriorating restorations to be repaired and debonded restorations to be replaced with minimum or no additional loss of tooth structure. Achieving efficient and stable bond between composite and dentin still remains a challenge in restorative dentistry.(3)

An important challenge to the dentin bond durability is degradation of collagen from the matrix-bound proteases, namely matrix metalloproteinases (MMPs) and cysteine cathepsins.[4] Pretreatment of the bonding substrate with agents that inhibit the activity of MMPs might improve bond durability.[4] Chlorhexidine (CHX) strongly inhibits theproteolytic activities of MMP-2, -8, and -9.[5] Proanthocyanidin (PA) is a natural collagen crosslinker[6] well known to readily precipitate proline rich proteins (such as collagen) due to hydrogen and covalent bonds[7] Recent studies have shown that a PA-based cross-linker agent (grape seed extract) increased the mechanical properties of demineralized dentin matrix[7,8] and enhanced the resin–dentin bond strength after one hour treatment[9]. In addition to its cross-linking effect, proanthocyanidin has also been shown to inhibit the synthesis of several MMPs from macrophages and inhibit the catalytic activity of MMP-1 and MMP-9.[4] Riboflavin helps in collagen cross-linking by its ability to produce free radicals when photoactivated with spectral range from ultraviolet to visible light.[10] In addition to cross-linking, reinforcement of the collagen can be achieved by incorporating biopolymers such as chitosan that can be cross-linked with collagen fibrils. Incorporation of chitosan improves the biological and mechanical properties of collagen.[11-13] A very recent development in the field of adhesive dentistry, is the introduction of self etching primer adhesive.[14,15] These materials have incorporated all the components of bonding systems (acidic conditioner, hydrophilic primer and hydrophobic adhesive resin) into one bottle and are the first true 'one step agents'. This takes simplification of the bonding procedure a step further ahead. The purpose of this study is to evaluate the effects of using chlorhexidine, Grape seed extract, riboflavin/chitosan modification on shear bond strength of composite resin bonded to dentin with self-etch adhesive after thermocycling.

MATERIALS AND METHODS

SPECIMEN PREPARATION PROCEDURES:

Initial preparation of sample teeth: Teeth included that were extracted in the department of oral and maxilla-facial surgery, Haldia Institute of Dental Sciences and Research for orthodontic and/or periodontal reasons. Following the extraction, teeth were stored in 0.1% (w/v) thymol at room temperature(27˚C) for not more than 1 month.Initial preparation of the teeth involved the removal of any superficial staining, calculus, and adherent soft tissue using an ultrasonic scaler (EMS, Switzerland).

Scetioning of the teeth samples: A flat surface was prepared with diamond disk by removing the occlusal one-third of the tooth crowns to expose the mid-coronal dentin. The dentin surface was polished using silicon carbide paper to create a standardized smear layer.

Mounting of samples: The roots of all the 60 teeth were mounted vertically in a self-cure acrylic resin block of the dimension of 1.5 cm x 1.5 cm up to the level of 2 mm. apical to the CEJ.

PREPARATION OF SOLUTIONS:

2% Chlorhexidine Gluconate solution: 2% Chlorhexidine gluconate solution was collected from Chloro-Hx; Maarc Dental, 2% Chlorhexidine gluconate irrigation solution.

6.5% Grape seed extract solution: 6.5 gram of Grape seed extract in the form of powder (Nutra magik, Sigmek nutrisciences, Delhi, India) was collected from the capsules and dissolved in 100 mL of distilled water.

1% Riboflavin and chitosan solution: One gram of riboflavin (Zenith nutrition, medizen lab Pvt. Ltd. , Bengaluru,India) in the form of powder was collected from the capsules and dissolved in 100 mL of distilled water. One gram of chitosan in the form of powder (Inlife Health Care, Inlife Pharma Pvt. Ltd.,Hydrabad,India) was dissolved in 100 mL of distilled water. Then, chitosan was added to riboflavin at 20% v/v ratio.

SPECIMEN PREPARATION: The teeth were randomly divided into four groups, the control group of fifteen teeth and the experimental groups of forty five teeth.

Group A (n = 15,control): No pretreatment was done on the exposed dentin surface. The self-etch adhesive, 3M Single Bond Universal, 3M ESPE, was used according to the manufacturer's instructions. Adhesive was applied to tooth surface for a total of 20 s and then gently air dried for 5 sec and light cured for 10 sec. Composite build-up was done on flat dentin surface by placing 4 mm thick, 3.5 mm diameter composite resin cylinder in two increments. Each increment being light cured for 20 sec. The experimental specimens (n = 45) were randomly divided into three groups based on the surface treatment of dentin as follows.

Group B (n = 15) 2% CHX solution pretreatment: A composition of 2% CHX solution was applied to the dentin for 30 s, and then dried with absorbent paper point. Then, dentin bonding and composite restoration were done as described in the control group.

Group C (n = 15) 6.5% GSE solution pretreatment: The specimens were pretreated with 6.5% GSE solution for 10 min and rinsed with distilled water and then dried with absorbent paper. Dentin bonding and composite restoration were done as described in the control group.

Group D (n = 15) 1% riboflavin/chitosan pretreatment: Dentin surface was pretreated with 1% riboflavin/chitosan for 5 min and photoactivated by conventional dental blue light-curing unit of output for 20 sec. Then, dentin bonding and composite restoration were done as described in the control group.

PRESERAVATION OF SAMPLES AND THERMOCYLING: The samples were stored in artificial saliva(Wet Mouth,ICPA) for 4 weeks followed by thermocycling at 5°C and 55°C for 1 minute each for 500 cycles on thermocycle machine (Censia, i-therm AI-5742, Bengaluru,India).

.**MEASUREMENT OF SHEAR BOND STRENGTH:** The shear bond strength (SBS) testing of specimens was performed using a Hounsfield H50K Universal testing machine (Hounsfield Test Equipment Ltd., England).The acrylic block of the prepared specimens were placed horizontally on a holder slot that was fixed to the lower arm of the universal testing machine. A knife-edge metal indenter was fixed to the upper arm of a universal testing machine that was set to deliver increasing loads until fracture occurred. The load was applied

perpendicular to the dentin-composite interface junction at a crosshead speed of 1 mm/min. The force required to fracture each tooth was recorded in Newtons. All the specimens were loaded continuously until fracture. The peak force, at the point of breaking of the composite cylinder from the test specimen, was taken as the point of bond failure and recorded in Newtons (N). Shear bond strength valuesin MPa were then calculated by dividing this force by the bonded area of the composite cylinder i.e. 3.5 mm diameter circular area on the dentin and expressed in MPa.\

Shear bond strength (MPa) = Debonding force (N)/ Area(mm^2)

Where area $A=\pi r^2$

r = D/2 3.5mm/2 = 1.75 mm

π = 3.14(constant)

A = 3.14x 1.75x1.75 mm^2= 9.62 mm^2

RESULTS

Statistical analysis The data presented as mean ± standard deviations were calculated using SPSS version 16.0 (SPSS, Chicago, IL, USA) software. One-way analysis of variance was applied to evaluate the shear bond strength values and post hoc multiple comparison tests were conducted using Tukey's test at 5% significance level. The results of this study are shown in Table 1.

Table 1. Means ±standard deviations of shear bond strengths in MPa of different study groups

Group	Pretreatment	Mean±SD
Group A	No pretreatment	22.26 ± 9.56
Group B	CHX	28.76 ± 8.84
Group C	Riboflavin/Chitosan	26.57 ± 8.23
Group D	GSE	24.87± 8.25

Table 2. Post hoc multiple comparisons using Tukey's test

Tukey's multiple comparisons test	Mean Diff.	Significant?	Summary	Adjusted P Value
GROUP A vs. GROUP B	-6.491	Yes	****	<0.0001
GROUP A vs. GROUP C	-4.303	Yes	***	0.0002
GROUP A vs. GROUP D	-2.603	Yes	*	0.0402
GROUP B vs. GROUP C	2.187	No	Ns	0.1101
GROUP B vs. GROUP D	3.888	Yes	***	0.0008
GROUP C vs. GROUP D	1.701	No	Ns	0.2895

The results showed that the mean shear bond strength values (MPa) of Group B (28.76 ± 8.84), C (26.57 ± 8.23), D (24.87± 8.250) were significantly higher than the mean shear bond strength value in the control group (22.26 ± 9.56) ($P < 0.001$). Intergroup comparisons are shown in Table 2. Mean shear bond strength of Group B, C and D showed a difference of -6.491, -4.303 and -2.603 MPa, respectively, when compared with Group A which was statistically significant ($P < 0.05$).

DISCUSSION

The use of composite restorations has transfigured today's dental practice by being able to replace the lost tooth tissue in an invisible and conservative way with immense success.[16] Achieving efficient and stable bond between composite and dentin still remains a challenge in restorative dentistry. The major limitations of dentin as a bonding substrate are its heterogeneous composition and hydrophilic nature.[17] An important challenge to the dentin bond durability is degradation of collagen from the matrix-bound proteases, namely matrix metalloproteinases (MMPs) and cysteine cathepsins. Pretreatment of the bonding substrate with agents that inhibit the activity of MMPs might improve bond durability. Chlorhexidine (CHX) strongly inhibits the proteolytic activities of MMP 2, 8, and 9.[18] Several chemicals, both natural and synthetic, which have the ability to increase the collagen cross-links are used to improve the bond durability. Proanthocyanidins (PAs) are oligomeric flavonoids found in high concentrations in grape seed, pine bark, cranberries, lemon tree bark, and hazelnut tree leaves.[19] Very few studies have been done to find the role of grape seed extract (GSE) in improving the bonding characteristics of dental adhesives. Riboflavin helps in collagen cross-linking by its ability to produce free radicals when photoactivated with spectral range from ultraviolet to visible light.[20] In addition to cross-linking, reinforcement of the collagen can be achieved by incorporating biopolymers such as chitosan that can be cross-linked with collagen fibrils. Incorporation of chitosan improves the biological and mechanical properties of collagen.[21-23] Hence, this study was undertaken to evaluate and compare the effects of pretreatment using CHX, GSE, riboflavin/chitosan modification on shear bond strength (SBS) of composite resin bonded to dentin with self-etch adhesive after thermocycling. It has been postulated that minimum bond strength of 17-20 MPa is needed to resist contraction forces of resin composite materials, for enamel and dentin. Clinical experiences confirm that this bond strength is sufficient for successful retention of resin restoration.[24] Senawongse *et al.*, demonstrated that two self etching systems, One-up bond and Clearfil SE bond demonstrated lower bond strength than the total etch system Single bond.[25] However, Kiremitci *et al.* concluded that self etching adhesive systems produced higher bond strength than conventional total etch systems, especially the all-in-one system, which produced the highest bond strength.[26] Whereas, Sensi *et al.*, stated that self etch and total etch primer showed comparable dentin bond strength.[26]

Self-etching adhesive systems rely on acidic monomers to simultaneously demineralize and infiltrate enamel and dentin. This acidity must be neutralized by the mineral content of the tooth structure, to allow complete polymerization of the adhesive film.[27] With total etch adhesive, smear layer and dissolved mineral are removed during the rinsing step. Because of some questions about residual acidity and the fact that the smear layer is not removed, the issue of long term hydrolytic stability of the self etching adhesive systems still remains unresolved.[27] Most single-step self etch adhesives contain hydroxyethyl methacrylate, which can polymerize in the presence of water to form microporous hydrogel with pore size ranging from 10-100nm.[28] However, self etching adhesives are capable of penetrating the aqueous channels formed between the smear layer particles, widening these channels and interacting at the top of the underlying dentin. These agents offer a simpler clinical application than total etch systems, because they are capable of conditioning the tooth surface and simultaneously preparing it for adhesion.[29] In our study self etch resin adhesive, 3M Single Bond Universal is used as adhesive which bonds methacrylate based restoratives, cement

Figure 1. A. 2% Chlorhexidine, B. 6.5% Grape seed extract, C. 1% Riboflavin/Chitosan

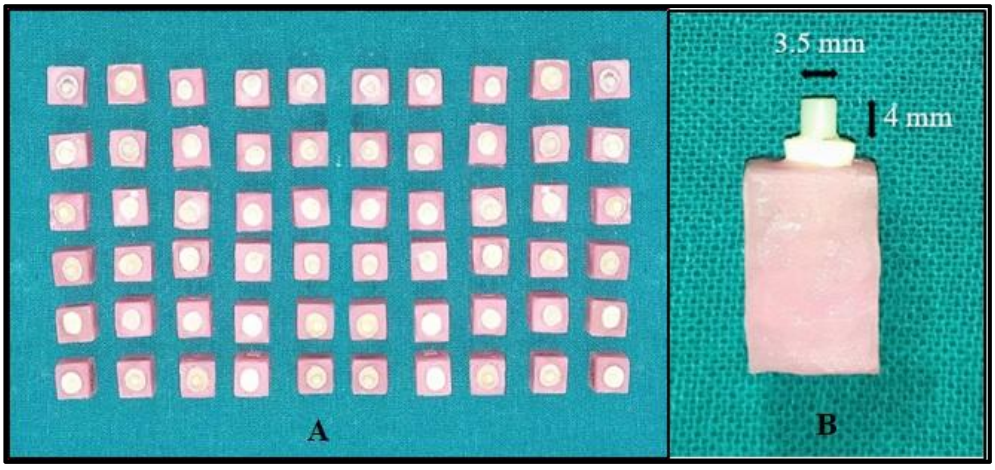

Figure 2. A. Total test samples, B. Final test sample

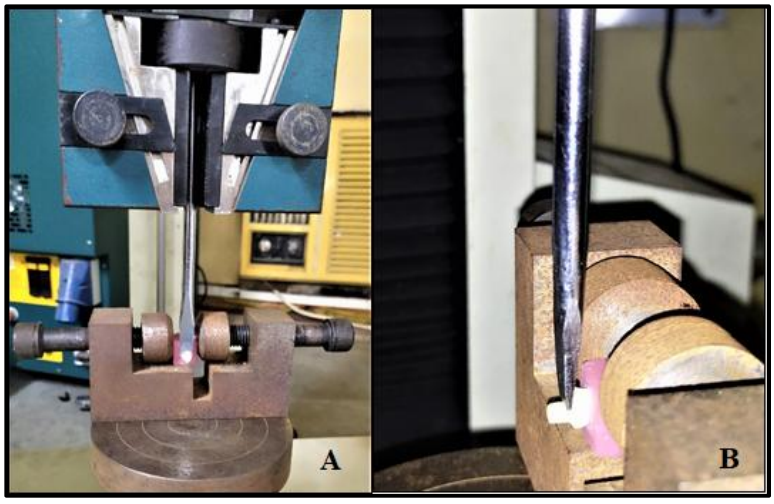

Figure 3. Sample attached to UTM A.Front view, B. Side view

and sealant materials to dentin, enamel, glass ionomer and various indirect restorative substrates (metals, glass ceramics, alumina and zirconia) without an extra primer step. It contains MDP Phosphate Monomer (10, Methacryloyloxydecyl dihydrogen phosphate), Dimethacrylate resins, HEMA (2-hydroxyethyl methacrylate), filler, ethanol, water and silane. Thermal cycling simulates the introduction of hot and cold extremes in the oral cavity and shows the relationship of the linear coefficient of thermal expansion between tooth and restorative materials. Thermal cycling stresses the bond between resin and the tooth and depending on the adhesive system, may affect the bond strength.

As Buonocore stated, the in vitro bond strength tests could include the thermal cycling of the specimens to assess the durability of the bond. Otherwise, in vitro results might not be indicative of the effect of oral moisture conditions on bond strength.[30]Gale and Darvell pointed out to the absence of agreement and standardization between the various thermocycling studies. Different thermocycling regimens are used in in-vitro studies. The main difference lies in the number of thermal cycles used(500, 750, 1500, 2500, 6000 and 10,000cycles).[31] In this study the standard 500 cycles protocol has been used, as given by ISO for in-vitro studies. But whether the thermocycling procedure had any effect on the

shear bond strength of composite resin to normal dentin and pretreated dentin is not considered in this study. In our study CHX, GSE, and riboflavin/chitosan groups showed higher bond strength values compared to the control group. Group B (2% CHX) showed a significantly higher bond strength to dentin compared with Group A (control), Group C (GSE), and Group D(riboflavin/chitosan). It can be due to its MMP-inhibitory properties which prevent the binding of metal ions, such as zinc or calcium, to MMPs, thus inhibiting its catalytic activity.[32] Not only MMPs but also evidence of inhibition of dentinal cysteine cathepsins B, K, and L by CHX had recently been demonstrated.[33] Group C (6.5% GSE) showed significantly higher bond strength to dentin compared with Groups A and D ($P < 0.001$). This is in accordance with the findings of Srinivasulu et al., who showed that the application of 6.5% GSE to deep dentin significantly improved the shear bond strength values of composite to dentin compared with the use of 10% sodium ascorbate.[17] The increase in bond strength may be due to the greater number of collagen cross-links which improved collagen stability. PAs bind to proline-rich proteins, such as collagen, and facilitate the enzyme proline hydroxylase activity, essential for collagen biosynthesis.[7] Castellan et al, showed that when demineralized dentin was treated with PA, it resulted in improved mechanical properties and reduced water absorption due to the formation of dense collagen network. The proposed mechanisms for interaction include covalent, ionic, hydrogen bonding, and hydrophobic interactions.[34]

Group D (1% riboflavin/chitosan) showed higher bond strength to dentin compared with Group A and was statistically significant. Riboflavin is a strong free radical-producing agent when activated by light with maximum absorption peaks at wavelengths of 270, 366 and 445 nm. Although the use of ultraviolet light activation was proven effective as a photoactivation method, the safety issues regarding the use of ultraviolet A (UVA) and its practicality for dental use should be considered. Conventional blue light-curing units might be a possible alternative owing to its ready availability and its safe use in dentistry.[10] The free radicals (O_2 and O_2^-) are released when riboflavin is photoactivated forming covalent cross-links between adjacent collagen molecules. Reduction in histidine and tyrosine during cross-linking and the formation of dityrosine is a possible mechanism in collagen aggregation mediated through riboflavin.[35] Cova et al. showed that riboflavin/UVA pretreatment can also inactivate MMPs, particularly MMP-9 through direct cross-linking. Telopeptidase activity of osteoclast-derived MMP-9, eliminating collagen molecule telopeptides, is considered essential for collagenase activity against insoluble bone collagen. The riboflavin-induced MMP-9 inhibition may be responsible for the increased durability of hybrid layers, through reduced MMP-9 telopeptidase activity.[36] Fawzy et al. showed that modification with riboflavin/ chitosan increased the mechanical properties, enhanced the mechanical stability of demineralized dentin substrates against hydrolytic and/or collagenolytic degradation challenges and decreased hydroxyapatite release with collagenase exposure.[10] When chitosan was added to riboflavin at 20% v/v ratio, significant improvement in bond strength at 24 h and 6 months in distilled water medium was found indicating the positive dual effect on bonding to dentin. The use of riboflavin and chitosan/riboflavin formulations to modify dentin collagen-matrix, with the defined ratios, stabilizes the collagen fibrillar network and enhances resin infiltration and hybrid layer formation.[37] Yi Liu et al. found that different concentrations of PA (0%, 2.5%, 5%, and 10%) hamper the monomer conversion and alters the polymerization kinetics of bis-GMA/HEMA model adhesive, but within acceptable limits.[38] GSE may stain dentin brown and the durability of long-term bond strength need to be examined.[39] The basic principle of photo-oxidative cross-linking is the same as that of photopolymerization, that is, UVA radiation causes the release of reactive oxygen species that can induce the formation of covalent cross-links through oxidation.[40] In cross-linking therapy with riboflavin/UVA, yellow riboflavin works as a photosensitizer that stimulates the formation of reactive oxygen species, and, at the same time, it also acts as a shield from the penetration of UVA.[36] The effects of cross-linking agents on stabilizing dentin matrix degradation have been attributed to their capacity to increase the stiffness of dentin collagen. Nevertheless, the present results strongly indicate that riboflavin/UVA pre-treatment can also inactivate MMPs, particularly MMP-9.[10] However, future research should more fully investigate the role of MMP inhibitors and cross-linking agents on dentinal MMPs.

CONCLUSION

Within the limitations of this study all the pretreatment groups showed increase of shear bond strength while compared to the no pretreatment/control group. 2% CHX pretreatment showed maximum increase in shear bond strength while 1% Riboflabin/Chitosan showed least increase of shear bond strength to composite resin to dentin.

CONFLICT OF INTEREST: There is no confict of interest.

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