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Bonding Effectiveness at Resin Dentin Interface following Cross linking Treatment

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Abstract

Objective: To evaluate the effect of pre-treatment with different collagen cross-linkers in a clinically relevant time period on the immediate and delayed bond strength of two different etch and rinse adhesives to dentin.

Materials and methods: Flat coronal dentin surfaces were prepared in 132 extracted human molars. Teeth were randomly divided into six groups according to three different surface pre-treatments: (i)no pretreatment (ii)3% riboflavin pretreatment (iii) 2M EDC pretreatment and two adhesive systems (Prime & Bond NT and Optibond Solo Plus) used. Composite cylinder build ups were done in all the samples. Ten samples from each group were subjected to immediate shear bond strength (SBS) evaluation and ten samples were stored for delayed (nine months) SBS evaluation. Additionally, two samples per group were subjected to scanning electron microscopic analysis for observation of resin-dentin interface.

Results: Treatment of acid etched dentin with 2M EDC or 3% riboflavin for one minutebefore adhesive application resulted in significantly higher SBS values of both the etch and rinse adhesives when compared to the control groups after nine months storage.

Conclusion: Dentin pretreatment with both the collagen cross-linkers resulted in significantly higher resin-dentin bond strength at nine months for both the adhesives tested.

Keywords: Shear strength, Riboflavin,1-ethyl-3-(3-Dimethylaminopropyl)Carbodimide

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Introduction

Dental adhesion has made remarkable progress recently as a result of major advances in dentin bonding technology[1] which has led to an increase in the clinical longevity of dental composite restorations. Bonding to dentin is achieved by hybrid layer formation. The chemical and mechanical stability of the collagen fibrils within the hybrid layer is important for durable bonding.[2] However, the use of simplified adhesives and larger amounts of hydrophilic monomers, results in a hybrid layer which is more porous. Additionally, the phosphoric acid etchant is regarded to have an aggressive effect on the dentin surface, nearly denuding the collagen. The lack of resin protection and presence of water makes the demineralized collagen fibrils at the bottom of the hybrid layer vulnerable to hydrolytic breakdown by host-derived proteases.[3]The degradation of collagen fibrils is considered to be a likely mechanism for bond deterioration.[4]

In 2004, Pashley et al.[5] proposed that the poor durability of resin-dentin bonds was due to the presence of endogenous MMPs in the dentin matrix. Matrix metalloproteinases (MMPs) or matrixins are a family of 20 host derived proteolytic enzymes, a class of zinc- and calcium-dependent endopeptidases that are trapped within the mineralized dentin matrix during tooth development.[6,7] MMPs are synthesized and mostly secreted as inactive proenzymes (zymogens) and get exposed and activated by acidic agents during adhesive bonding procedures.

These activated MMPs can slowly hydrolyze the collagen fibrils in the hybrid layer that anchors resin composites to the underlying mineralized dentin, thus decreasing the longevity of bonded restorations.

One of the approaches for stabilizing the resin-dentin interface involves use of cross-linking agents which can be divided into chemical methods, wherein different cross-linking solutions such as glutaraldehyde, formaldehyde, transglutaminase, EDC, genepin, and proanthocyanidin are used or a physical method (also called photo-oxidative) that uses light exposure, especially ultraviolet radiation (riboflavin activation with blue or UV light).[8] Covalent cross-links produced with exogenous cross-linkers (e.g. glutaraldehyde, grape seed extract and EDCs) inactivate the active sites of dentin proteases by reducing the molecular mobility of the active site or by changing negatively charged ionized carboxyl groups into positively charged amides.[9]

EDC is a synthetic cross linking agent which when applied directly to demineralized dentin has been found to improve the bond strength and structural integrity of the resin/dentin interface over time by preventing the enzymatic and/or hydrolytic degradation, through the formation of inter- and intra-molecular crosslinks.[10,11] Of the current crosslinkers, EDC, has some attractive qualities, including very low cytotoxicity, and an ability to preserve dentin bond strength within clinically acceptable treatment times.[9]Mazzoni et al.[12] recently reported promising results on the use of an EDC conditioning treatment in stabilizing dentin bonds. Various other studies have also reported beneficial effect of EDC pretreatment on the durability of resin dentin bond.[13-15]

Riboflavin activated by ultraviolet A (UVA), is a crosslinker, introduced as a new treatment for keratoconus and has proven to promote collagen type I crosslinking and increase the biomechanical strength of the human cornea by 300%. Riboflavin is an appropriate candidate for crosslinking dentin collagen due to its biocompatibility and its ability to produce free radicals when photo-activated with spectral range from UV to visible light. These free radicals, or so-called reactive oxygen species such as O2 and O2-, are released when riboflavin is photo-activated and light is absorbed, forming covalent crosslinks between adjacent collagen molecules.[16]

Recently, Cova et al.[8] concluded that UVA activated riboflavin increased the immediate bond strength to dentin, stabilized the adhesive interface, and inhibited dentin matrix metalloproteinases, thereby increasing durability of resin/dentin bonds. Fawzy et al.[16] also investigated the effect of photo-activated riboflavin on dentin collagen using UVA and blue light and reported that both increased the biodegradation resistance, the mechanical properties, and the stability of the dentin matrix. Chiang et al.[17] concluded that pretreatment with riboflavin enhances resin-dentin bond possibly through enhancing the stiffness. Daood et al.[4] conducted a study using experimental adhesive-system, modified with different concentrations of riboflavin and concluded that the incorporation of riboflavin in the experimental etch-and-rinse adhesive improved the immediate bond strength and bond durability after nine months storage. Although the use of UVA has proven effective as a photoactivation method of riboflavin for collagen crosslinking, the safety issues regarding the use of UVA and its practicality for dental use should be considered. Conventional blue-light halogen-lamp curing units might be a possible alternative for UVA light sources to activate riboflavin owing to its ready availability and its ease and safe use in dentistry.[16]

Various authors have used EDC and riboflavin in different concentrations, with different light activation sources for riboflavin and with different application techniques. However, the effect observed on a specific adhesive cannot be generalized to other adhesives as well. No general consensus exists in the literature regarding the use of these cross-linking agents in dentin bonding. Therefore, the aim of this study was to evaluate and compare the shear bond strength of two simplified etch and rinse adhesives to dentin after pretreatment with riboflavin or EDC at 24 hours and 9 months storage period. Null hypothesis tested was that there is no significant difference in the shear bond strength values of both the adhesives at 24 hours and 9 months storage period regardless of the surface pretreatment used.

Materials and Methods

One hundred and thirty-two freshly extracted non-carious, human molars were used in this study. The teeth were examined under stereomicroscope (SZX10, Olympus, Tokyo, Japan) and teeth free of caries, cracks, or any developmental defects were included. Teeth were cleaned and stored in 0.5% chloramine T trihydrate (Sigma Aldrich, Bangalore, KA, India) for no more than 3 months. Tooth crowns were flattened occlusally using a low-speed diamond saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) under water irrigation to expose superficial dentin. A standardized smear layer was created with 600 grit silicon-carbide (SiC) paper. The samples were embedded in an autopolymerizing resin at the level of cementoenamel junction with long axis perpendicular to the acrylic resin surface.

Experimental solutions

All chemicals (Sigma-Aldrich) were of analytical grade and were used without further purification. All the required concentrations were prepared by diluting the extract in distilled water (pH 7.4): 2M EDC-hydrochloric acid (HCl) solution was prepared by dissolving 1.92 mg EDC into 5 mL distilled water; 3% riboflavin 5 phosphate solution was prepared by dissolving 3g of riboflavin 5 phosphate (Sigma Aldrich) in 100 ml distilled water. The prepared diluted solutions were filtered with paper filter No. 6 (Whatman, London, UK).

Adhesive procedures

An adhesive tape with a 2.5 mm central hole was positioned on the flat dentin surface to demarcate the pre-treatment and bonding area. All the samples were subjected to acid etching procedure with Scotchbond Universal etchant (3M ESPE Dental Products, St. Paul, MN, USA) for 15 seconds followed by rinsing with water for 10 seconds and lastly blot dried. Then the following treatments were done on acid etched dentin surface with corresponding pre-treatment solutions (groups 2a, 2b and 3a, 3b) with a microbrush for 60 seconds except for the control groups(Group 1a and 1b).

Groups:

Group 1a: No crosslinker was applied to dentin surface and Prime & Bond NT (Dentsply) adhesive was used following the manufacturer's instruction.

Group 2a: Etched dentin surface was treated with 3% riboflavin solution for 60 seconds, gently air-dried for 5 seconds, photo-activated with blue light for 60 seconds. Then, dentin bonding was done using Prime & Bond NT adhesive.

Group 3a: Etched dentin surface was treated with 2M EDC solution for 60seconds. Excess solution was gently blotted with filter paper. This was followed by dentin bonding with Prime & Bond NT adhesive.

Group 1b: No crosslinker was applied to dentin surface and Optibond Solo Plus (Kerr Dental) adhesive was used following the manufacturer's instructions.

Group 2b: Pretreatment was done as described in group 2a. This was followed by application of Optibond Solo Plus adhesive.

Group 3b: Pretreatment was done as described in group 3a. Dentin bonding was done using Optibond Solo Plus adhesive.

Transparent plastic tubes (TYGON laboratory tubing, Saint Gobain, Akron, OH, USA) with 2.5 mm diameter and 2 mm height were placed perpendicular to the previously etched, pre-treated and bonded dentin surface. A nanohybrid resin composite (Filtek Z350 XT, Body Shade

A1; 3M ESPE Dental Products) was filled into the precut tubes. Each bonded specimen was light-cured for 20 seconds using quartz-tungsten-halogen (QTH) light curing unit (Spectrum 800, Dentsply Caulk, Milford, DE, USA) at a light intensity of 600 mW/cm2. The plastic tubes were gently cut and carefully removed with a number 11 surgical blade after polymerization

Storage of samples before testing

Half of the specimens (immediate testing group) were then stored in distilled water at 37°C for 24 hours for completion of polymerization before immediate testing and scanning electron microscopic (SEM) analysis. The remaining half samples from each group (delayed testing group) were stored in artificial saliva (Wet Mouth, ICPA Heath Products Ltd.) at 37°C in an incubator for 9 months before SBS and SEM evaluation. 0.2 percent sodium azide (pH 7.31) was further added to prevent bacterial growth. Storage solution was changed every 2 weeks.

Determination of SBS

SBS was determined using a universal testing machine (Instron, ADMET, Enkay Enterprises, New Delhi, DL, India) using the corresponding computer software. The specimens were placed and stabilized by the jig, while a straight knife-edge rod (2.0 mm) was applied at the tooth-restoration interface at a crosshead speed of 1 mm/min until fracture occurred. SBS in MPa was calculated by dividing the peak force (N) by the cross-sectional area of the failed interface (mm2), measured by a digital caliper.

Fractographical analysis

The mode of failure of all 20 samples from each group was determined by observation under a stereomicroscope (SZX10, Olympus) at 10x magnification and classified into adhesive (A), mixed (M), or cohesive (C) failures in either dentin or resin.

SEM evaluation

Two samples per group (1 for immediate testing subgroup and 1 for delayed testing subgroup) were used for SEM (EVO18 Special Edition, Carl Zeiss, Jena, Germany) evaluation. The restored samples were sectioned mesiodistally and polished with a series of increasingly finer SiC abrasive papers up to 1200 grit and highly polished with a diamond paste. Acid-base treatment (6N HCl for 30 seconds followed by 4% NaOCl for 10 minutes) was done, and the samples were dehydrated in ascending ethanol concentrations (50%, 75%, and 95% for 20 minutes each and 100% for 1 hour) and then transferred to a critical point dryer for 30 minutes. The specimens were then gold sputter coated and the resin-dentin interface was examined under SEM.

Statistical analysis

Data was normally distributed as tested using the Shaperio-Wilk W test (p-value was more than 0.05) in SPSS Version 21.N-way analysis of variance (ANOVA) (for comparing more than two groups) with Post Hoc Tukeys test was used. Level of significance was set at 0.05.

Results

SBS

A three way-factor Analysis of Variance was conducted to evaluate the effect of collagen cross-linker treated with Optibond Solo Plus and Prime & Bond NT on the immediate and delayed bond strength. Mean SBS values and standard deviation of all the groups are presented in Table 1. When used as control, there was no significant difference in the SBS values of two adhesives either at 24hrs or at 9 months. Both the EDC treated groups showed the highest 24hrs shear bond strength values amongst all the groups, which was significantly higher than the control group. 3% RF-modified specimens also showed higher SBS values at 24hrs when compared to the control group but the difference was not statistically significant. Storing the samples for 9 months in artificial saliva significantly decreased the shear bond strength within each of the control and the cross-linker treated groups. However, when compared to the control group, the shear bond strength values of the cross-linker treated groups were significantly higher at 9-months

Group No.	Groups	Immediate	Delayed	P value	Results of two way anova	P value
		MEAN	MEAN		Main effect	
1a	Control-a	42 (11.02) A*,a	30.4 (3.26) A*,b	0.007*	Corrected model	0.00*
1b	Control-b	41.36 (11.16) A*, a	30.1 (3.64) A*, b	0.010*	intercept	0.00*
2a	Riboflavin-a	51.7 (7.62) A,a	44.8 (6.58) B*,b	0.049*	Adhesive effect	0.00*
2b	Riboflavin-b	50.1 (7.48) A,a	43.5 (5.33) B*,b	0.045*	Duration effect	0.00*
3a	EDC-a	55.4 (7.12) B*,a	46.2 (4.94) B*,b	0.005*	Adhesive * duration effect	0.333
3b	EDC-b	52.9 (6.10) B*, a	45.5 (4.37) B*,b	0.008*		
ONE WAY ANOVA	P VALUE	0.003*	0.00*			

A,A (B, B) - no statistical significance
A*,A* (B*, B*)- no statistical significance
A*, A – no statistical significance
A*,B*- statistical significance
a,a - no statistical significance
a,b- statistical significance

Fractographical analysis

The effect of different dentin pre-treatment and different time intervals on the distribution of failure pattern were compared using the $\chi 2$ test and results are summarized in Figure 1. Most of the failures encountered were mixed in all the groups tested immediately with no statistically significant difference (p=0.586). At 9 months, an increase in the number of adhesive failures was observed in all the groups with greater increase in the control group than the experimental groups which still continued to show predominantly mixed failures. However, the difference was not statistically significant (p=0.210). There was no statistically significant difference in the failure modes of a group at two different time periods

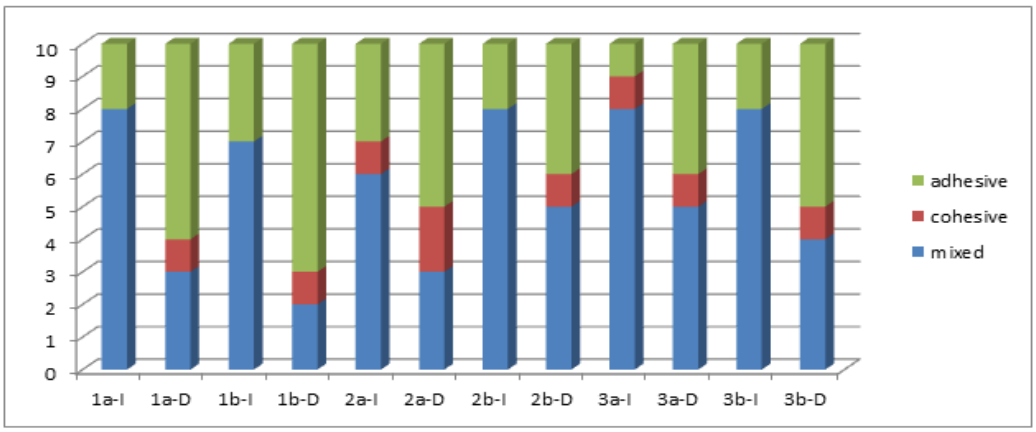

Fig 1: Failure patterns of all the groups according to the dentin pre-treatments and time intervals

SEM

Figure 2(A) and 3(A) summarizes the resin- dentin interfaces of the control groups at 24 hoursdepicting good adaptation. Figure 2(B) and 3(B) summarizes the resin-dentin interfaces of the control groups at 9 months showing gap at the interface with poor adaptation. Figures 4(A) to 7(A) summarizes the resin- dentin interfaces after different surface treatments viewed 24 hours after bonding. Perfect interfacial adaptation with absence of gap was observed. Figures 4(B) to 7(B) shows interface of dentin and resin with the same surface treatments viewed after nine months of storage in artificial saliva. There was good adaptation in the samples pretreated with riboflavin and EDC.

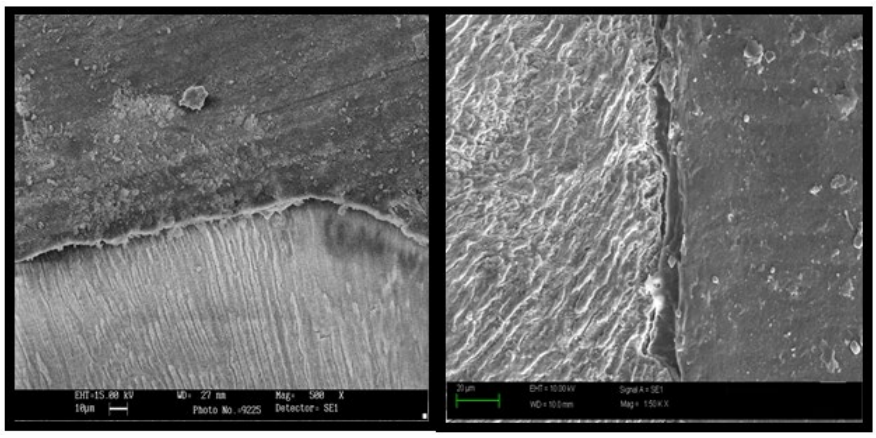

Fig. 2: Scanning electron micrograph of control group 1a at 24 hrs (A): a good interfacial adaptation can be seen; at nine months (B): Poor seal can be seen.

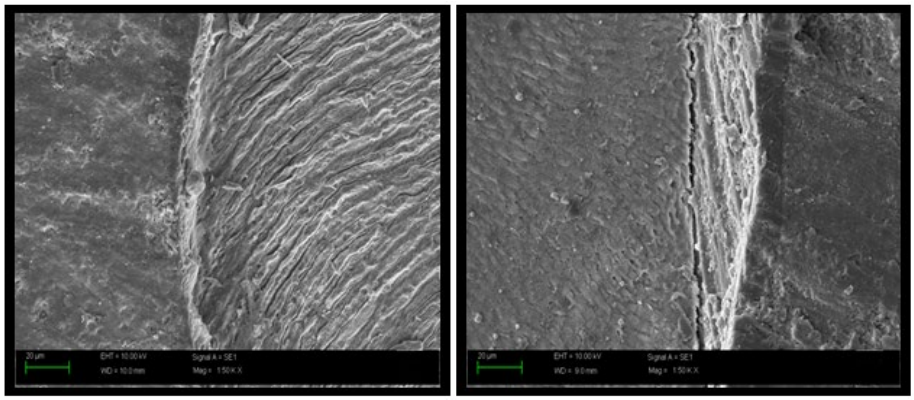

Fig. 3: Scanning electron micrograph of control group 1b at 24 hrs (A): a good interfacial adaptation can be seen; at nine months (B): Poor seal can be seen.

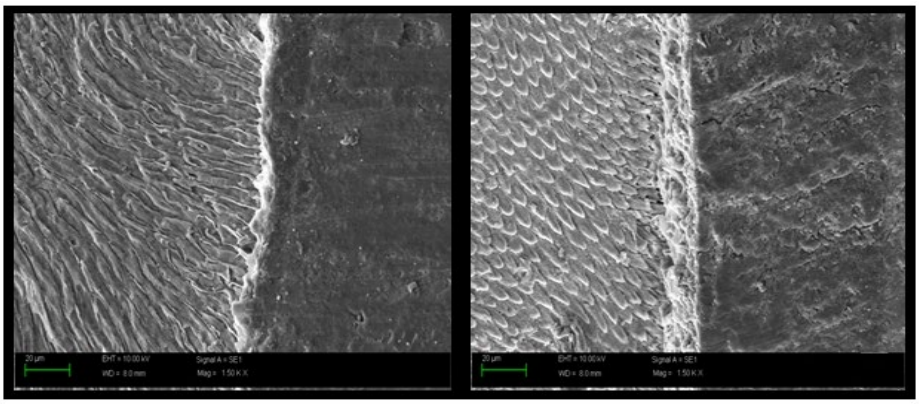

Fig. 4: Scanning electron micrograph (A): good interfacial adaptation can be seen in riboflavin group 2a at 24 hrs; (B): at nine months

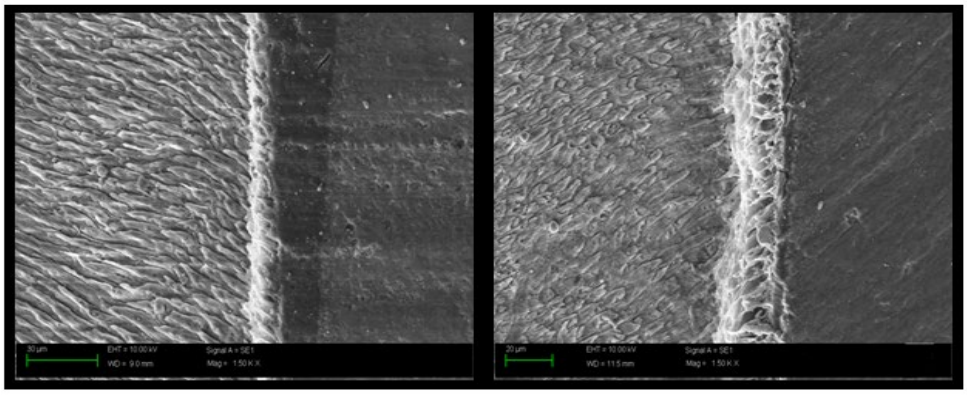

Fig. 5: Scanning electron micrograph (A): good interfacial adaptation can be seen in riboflavin group 2b at 24 hrs; (B): at nine months

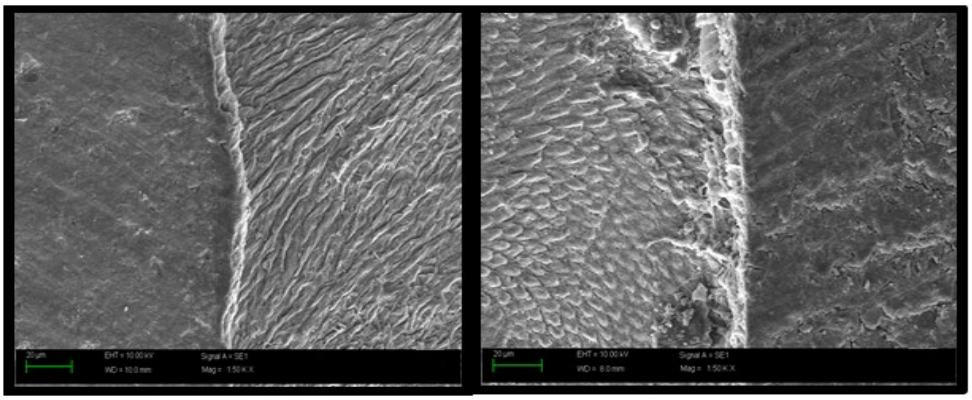

Fig. 6: Scanning electron micrograph good interfacial adaptation can be seen in EDC group 3a (A): at 24 hrs; (B): at nine months.

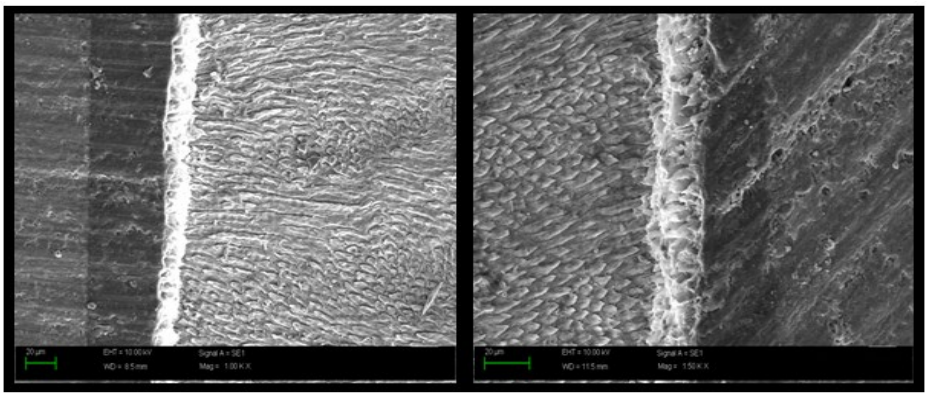

Fig. 7: Scanning electron micrograph (A): good interfacial adaptation can be seen in EDC group 3b at 24 hrs; (B): at nine months

Discussion

The strengthening of exposed collagen fibrils after acid etching by cross linking agents to increase the mechanical properties and decrease the enzymatic degradation may be an important application in restorative dentistry. Several synthetic and natural chemicals have the ability to increase the number of covalent inter and intramolecular collagen crosslinks and are able to affect its properties. It is well known that the stiffness of the collagen matrix decreases from 18000 MPa in the mineralized state to 1–3 MPa in the demineralized state. This low elasticity modulus permits more rotational and lateral movements of the adjacent collagen peptides, bringing them within reach of the active site of the MMPs, which is a likely phenomenon for dentin collagen degradation. The induction of exogenous collagen crosslinks has been proposed as a mechanism for improving the mechanical stability and reducing the collagen biodegradation rate.[4]

However, when selecting a suitable crosslinking agent and/or the photo-activation method, a number of clinical and safety aspects should be considered such as ease of use clinically, acceptable application and activation time, biocompatibility and safety issues. The safety of using UVA to activate riboflavin is a concern when considering its clinical applicability. To overcome the use of UVA, visible blue light emitted from tungsten/halogen lamp curing units, which is regularly used in dental clinics, can also be considered as a possible replacement of UVA light sources. Riboflavin absorbs a wide spectral range from UV to visible spectrum with three maximum absorption peaks at 270, 366 and 445 nm.[18-21] Although the riboflavin spectral absorption is much higher at 270 nm, owing to the safety precautions related to UVB, it is not recommended for clinical applications. Riboflavin has close spectral absorption peaks at 366 nm (UVA) and 445 nm wavelengths (visible blue light). Fawzy et al.[16] used blue light for the photoactivation of riboflavin and found an increase in the bond strength. They compared one-step photo-activation method in which the riboflavin and the bonding agent were photoactivated in the same step and two-step photo-activation method in which the riboflavin and the bonding agent were photoactivated separately and concluded that one-step photo-activation method was less effective in enhancing and maintaining the μTBS after short-term water storage when compared with two-step photo-activation method even when blue light was used as a light source for both activation methods. These findings could be due to both riboflavin and the photo-initiator system in the dentin bonding agent competing in absorbing the blue light. Consequently, it could be assumed that more the riboflavin content in the demineralized dentin matrix,

the lower is the degree of polymerization of the bonding resin as riboflavin could perform as a shield against incident light. Therefore, in this study two-step photo-activation method with blue light was used.
In the current study, use of 3% riboflavin activated with blue light resulted in significantly higher bond strength of both the adhesives at nine months compared to the control. Our results are in agreement with various other studies which reported improved long term resin dentin bonds with riboflavin pretreatment. Fawzy et al.[22] using MRS (Micro-Raman spectroscopy) and hydroxyproline(HYP) release clarified the crosslinking effect of riboflavin on dentin collagen matrix. They reported that the stability of the sub-fibrillar triple-helical structure was achieved by the formation of peptide bonds between adjacent collagen chains. Furthermore, collagen type-I triple-helical domains have an amino acid sequence (primary structure) that, in addition to glycines, is rich in hydroxyproline and proline, which impart rigidity and stability to the collagen triple-helical domain. They stated that the lower HYP liberation found with riboflavin crosslinking might indicate the higher collagen content and resistance of the demineralized dentin matrix to bacterial collagenase-mediated collagen degradation. 3% riboflavin concentration in the collagen matrix could lead to higher light absorption, generating more free-radicals and reactive oxygen species, mainly through the type-I pathway of photosensitized oxidation. This could explain the improved durability found with 3% riboflavin. A higher concentration of photo-initiator is desired for more efficient crosslinking.
In accordance with this study, Venigalla et al.[23] reported increased microtensile bond strength values of cross-linked groups (riboflavin and EDC) when compared to control group. Even after 6 months storage, cross-linked groups showed significantly higher values compared to initial bond strength values of control group.
In our study, use of 2M EDC for 60 seconds resulted in significant increase in the immediate (24 hr) bond strength value in both the adhesives when compared to the control group. At nine months also, EDC treated group had significantly higher bond strength compared to the control. EDC is a synthetic cross-linking agent which when applied directly to demineralized dentin improves the bond strength and structural integrity of the resin/dentin interface over time by preventing the enzymatic and/or hydrolytic degradation, through the formation of inter and intra molecular crosslinks.[24] It is the most stable cyanamide isomer and produces cross-links which are very stable. EDC is known as a zero-length cross-linking agent, [25] due to its ability to cross-link peptides to one another without introducing additional linking groups. It contains a functional group (RN=C=NR) and is capable of forming covalent peptide bonds between proteins nonspecifically by activation of free carboxyl group of glutamic acid and aspartic acid present in protein molecules.[26] It results in the formation of O-acylisourea intermediate that reacts with the epsilon amino group of lysine or hydroxylysine in an adjacent polypeptide chain to form a stable, covalent amide bond. Improved resistance to collagenase challenge and increased mechanical properties of collagen-based materials have been reported following treatment with EDC.[27] EDC causes cross-linking to occur in dentin collagen as well as in dentin matrix-bound MMPs. It is found that when collagen cross-linkers are applied on demineralized dentin, cross-linking occurs more rapidly in MMPs as compared to collagen. This could be due to better accessibility of carboxyl and amino groups in MMPs than in collagen.[28] Thus, it becomes clear that EDC is a potent MMPI and its MMP inhibition effect is much quicker than its cross-linking effect. This might be the reason for immediate increase in bond strength besides long-term preservation of bond strength, in case of EDC treated groups as observed in the current study. Our results are supported by various studies which reported improvement in resin-dentin bond durability with EDC pre-treatment.
Scheffel et al.[29] evaluated the effect of EDC on elastic modulus (E), MMPs activity, HYP release and thermal denaturation temperature of demineralized dentin collagen. All cross-linking agents decreased MMP activity and HYP release and increased thermal degradation temperature (TDT). It was concluded that with the application time of 60 seconds, EDC significantly increased the elastic modulus at 1M or 2M concentrations. Therefore, we used a concentration of 2M and application period of 60seconds in our study.
Scheffel et al.[30] also found that 1M and 2M EDC application increased the collagen modulus of elasticity and decreased MMP activity compared to negative control group and no differences were identified among EDC groups. Our findings are also in agreement with Mazzoni and others, who found that EDC application for 1 min on demineralized dentin produced significant MMP inactivation and preserved bond strength over time.[15] Cadreno et al.[31] found that 1M EDC is capable of improving other properties of dentin collagen such as, higher thermal denaturation temperature which is considered an indirect indicator of a more resistant and highly cross-linked collagen network.
Angeloni et al.[32] also found pre-treatment with EDC containing conditioner on etched dentin inhibited MMP-2 and MMP-9 and preserved the resin-dentin bond strength of etch and rinse adhesives over six months. Scheffel et al.[33] reported that using 0.5mol/l EDC for 30 or 60 seconds, resulted in bond strength values which were significantly higher than the control group at 6months and 12 months interval. Tezvergil et al.[13] concluded that 0.3 M EDC when applied, even a short pre-treatment (i.e., 1 min) of acid etched dentin matrix is sufficient to inactivate endogenous protease activity of dentin without significantly stiffening the collagen. Angeloni et al.[14] using 0.3 M EDC for 1 min demonstrated that the use of EDC containing conditioner on etched dentin surfaces before application of etch and rinse adhesives contribute to stabilize the adhesive interface over time.
Within the limitations of this study it can be concluded that collagen cross-linking appears to be a clinically feasible approach for improving the strength and durability of resin-dentin bonds. More studies are required to evaluate the effect of riboflavin and EDC on bonding to caries affected dentin. Riboflavin interaction with adhesive monomer polymerization and the potential of photo-activation/photo-polymerization in one-step adhesives, with dental blue-light as a more practical and safer alternative to UVA, needs to be investigated. Moreover, optimization of the experimental parameters, including riboflavin concentrations and application times/technique, photoactivation times, and power/densities, is required to achieve desired results. Clinical application protocol for collagen cross-linking needs to be optimized and in vivo studies are required to further support this concept before recommending it in routine clinical practice.

Conclusion

The two different etch and rinse adhesives (Prime & Bond NT and Optibond Solo Plus) depicted similar bond strength values both at 24 hours and at 9 months. However, use of riboflavin and EDC as collagen cross linker resulted in significantly higher dentin shear bond strength of both the etch and rinse adhesives at 9 months. While EDC pretreatment significantly increased the shear bond strength of both the adhesives even at 24 hrs. Thus, it may be concluded that dentin biomodification using collagen cross-linkers appears to be a promising approach to improve the resin-dentin bond durability and thus to enhance the clinical longevity of adhesive composite restorations.

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