Avicenna J Dent Res. 2017 September; 9(3):e13254. doi: 10.5812/ajdr.13254.

Published online 2017 May 30. **Research Article**

The Efficacy of Riboflavin for Collagen Cross-Linking and Optimizing the Bond Strength of an Etch and Rinse Adhesive System to Dentin

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Received 2016 October 06; **Revised** 2017 January 23; **Accepted** 2017 April 03.

Abstract

Background and Objectives: Previous studies have shown that increasing collagen resistance to degradation stabilizes the resin-dentin interface and collagen cross-linkers can prevent the degradation of collagen fibrils as such. This study sought to assess the efficacy of light-activated riboflavin for collagen cross-linking and optimizing the micro tensile bond strength (μTBS) of an etch and rinse adhesive system to dentin.

Methods: Occlusal surfaces of 12 sound premolars were ground in order to expose dentin. The teeth were randomly divided into two groups. Adper single bond was applied on etched dentin and light cured in the control group and Composite was then applied. In the test group, all the steps were done in a similar manner to the control group with the difference that after acid etching, 0.1% riboflavin solution was applied on dentin surface and was activated by blue light irradiation for 2 minutes. After thermocycling, the teeth were sectioned in 2 directions in such a way that 14 resin-dentin sticks with 1 mm2 cross-section were prepared in each group (n = 14). The μTBS of samples was then measured and analyzed with independent t-test. The statistical analyses' significance level was set at $\alpha = 0.05$. Mode of failure was determined under a stereomicroscope at $\times$ 40 magnification. Moreover, the cross-linking effect of light-activated riboflavin solution on type I collagen was assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and resin-dentin interface was photographed under a scanning electron microscope (SEM).

Results: In gel electrophoresis, bands were formed in wells in the test group (and not in the negative control group). T-test found no significant difference in the mean μTBS of the test and the control groups (P = 0.9).

Conclusions: Based on the results, light-activated riboflavin was capable of collagen cross-linking, but its application as a collagen cross-linker on etched dentin had no significant effect on μTBS of Single Bond dentin bonding agent to dentin.

Keywords: Photo-Activation, Riboflavin, Crosslinking, Dentin, Biodegradation, Collagen

1. Background

The durability of hybrid layer depends on the stability of its components such as collagen fibrils and polymeric chains. Collagen fibrils exposed by acid etching which have not been infiltrated by resin cannot resist denaturation (1). These collagen fibrils are more susceptible to creep and degradation due to cyclic fatigue after long-term function (2). On the other hand, matrix metalloproteinases (MMPs) are exposed and activated by acidic agents during the bonding process and since they play a role in the degradation of type I collagen, which is the organic component of the hybrid layer, they can gradually break down collagen fibrils at the resin-dentin interface (3-7).

It appears that increasing the collagen resistance to biodegradation can further stabilize the resin-dentin interface (8-10). Collagen cross-linkers can protect collagen fibrils from degradation and enhance their mechanical and chemical properties (11-13). This is the main objective behind the use of collagen cross-linkers along with adhesives in the bonding process. The advantages offered by collagen cross-linkers when used in conjunction with bonding agents are mainly due to their ability to inhibit MMPs. Some studies have provided evidence regarding the limited activity of MMPs following the use of collagen cross linkers (9, 14, 15).

The currently available approaches to increase collagen cross-linking are divided into two groups of chemical and physical or photo-oxidative methods (9). The currently used chemical cross-linkers have drawbacks such as toxicity, poor control over the degree of cross-linking and instability (16). Although several cross-linkers such as glutaraldehyde and proanthocyanidin have shown efficient therapeutic effects on dentin collagens, they need to be used for long periods of time, which limits their application in the clinical setting (17-20). Cross-linkers must be al-

lowed adequate time clinically to prevent collagen degradation. Moreover, they must have minimal cytotoxicity to protect the resin-dentin interface (2).

It has been reported that the presence of oxygen free radicals is necessary for collagen cross-linking in the photo-oxidative method. Riboflavin (vitamin B2) is among the most potent generators of oxygen free radicals when activated with light (21, 22). UVA-activated riboflavin (RF/UVA) was recently used as a cross-linker in ophthalmology. It has been documented that it increases the cross-linking of type I collagen (23) and stops keratoconus (22). Maximum absorbance peaks of riboflavin as a cross-linker are at 270, 366, and 445 nm wavelengths (24, 25).

Light-activated riboflavin can break weak bonds within collagen fibrils and create oxygen free radicals. Also, new covalent bonds are formed between hydroxyl functional groups of riboflavin and proline or lysine in collagen (26, 27). Collagen cross-linking with riboflavin enhances its mechanical properties and delays its enzymatic degradation (28).

Due to the biocompatibility and cross linking of dentin collagen matrix, this technique may be used as a method of dentin surface preparation or as an optimizer of adhesive systems (29). This study aimed to assess the efficacy of riboflavin activated with light (by a light curing unit) for collagen cross linking and optimizing the bond strength of an etch and rinse adhesive system to dentin. The first null hypothesis was that light-activated riboflavin would have no effect on collagen cross linking. The second null hypothesis was that activated riboflavin would have no effect on bond strength of etch and rinse adhesives to dentin.

2. Methods

This in-vitro experimental study was conducted on 12 sound human premolars without caries, occlusal wear or restorations which had been extracted for orthodontic purposes within the past 3 months. The teeth were immersed in 0.2% thymol solution.

Riboflavin solution was added to 1.5mg/mL bovine collagen (Sigma Aldrich, St. Louis, MO, USA) (30). The cross linking effect of riboflavin solution on type I collagen was assessed using SDS-PAGE; 0.1% riboflavin solution was prepared by dissolving riboflavin-5-phosphate (Sigma Aldrich, St. Louis, MO,USA) in distilled water. In the negative control group, distilled water was added to collagen. In the positive control group, 0.1% riboflavin solution was added to collagen and activated by UVA with 365 nm wavelength (Jenway 6105 U.V/Vis Spectrophotometer, Jenway LTD, Dunmow, Essex, UK).

To prepare samples for SDS-PAGE, the samples were diluted with buffer and boiled at 100°C for five minutes. Next, 40 μgr of each sample was placed in wells of 8% SDS-polyacrylamide gel. Electrophoresis was performed at 150 v voltage and gel was stained with Coomassie blue. After two hours of gentle vibration of gel on a shaker, the dye solution was removed and discoloring solution was added. The standard molecular weight markers (8 - 220 KDa; Sigma-Aldrich, St. Louis, MO, USA) were run in parallel with the samples in gel. The prepared riboflavin solutions were stored in light-resistant test tubes and applied on the dentin surfaces (15 μL) at room temperature (24°C). An LED light curing unit (DemiTM Plus; Demetron Kerr, USA) with alight guide diameter of 8 mm, 450 to 470 nm wavelength and light intensity of 700 mW/cm^2 was used.

2.1. Specimen Preparation

The occlusal enamel of the teeth was trimmed using an orthodontic trimmer (Pars Medical, Tehran, Iran) under water coolant to 1mm below the dentinoenamel junction and continued to expose 5 mm diameter of dentin. To create a standard smear layer, the surface of the teeth was polished with 600-grit moist silicon carbide paper. The teeth were then randomly divided into two groups of six. In the control group, the surface of samples was etched with 35% phosphoric acid for 10 seconds and was then rinsed with distilled water. Two layers of etch and rinse adhesive (Adper Single Bond) was applied on dentin surface for 15 seconds and was gently air sprayed for 5 seconds followed by 10 seconds of light curing with an LED light curing unit.

In light-activated riboflavin group, all steps were performed in a similar manner to the control group with the difference that after acid etching, 0.1% riboflavin solution was applied on dentin surface, gently air dried and activated by light using an LED light curing unit for two minutes. The next steps of adhesive application were similar to those in the control group.

2.2. Micro Tensile Bond Strength Tests

To measure the μTBS, after applying the dentin bonding agent, 2 mm thick increments of composite (Filtek Z250, 3M ESPE, St Paul, MN, USA) were applied on the surface and light cured to do a 4 mm thick composite buildup. The teeth were immersed in distilled water at 37°C for 24 hours to complete polymerization and were then subjected to 5,000 thermal cycles between 5 - 55°C with 15 seconds of dwell time (Nemo Co, Mashhad, IRAN). Using a diamond blade at low speed (Nemo Co, Mashhad, IRAN) under water coolant, the samples were sectioned into resin-dentin bars measuring 1 $\times$ 1 mm and were stored in water at 37°C for three months (31). A total of 14 resin-dentin sticks were prepared in each group (n = 14). The sticks were mounted on a custom-made metal jig of a universal testing machine (Santam T20, Tehran, Iran) with 50 N load-cell

using cyanoacrylate glue. Load was applied at a crosshead speed of 1 mm/minute until failure. Tensile stress at fracture was recorded as the μTBS. The data were analyzed using SPSS version 16 and independent t-test. $P < 0.05$ was considered statistically significant.

2.3. Evaluation of the Mode of Failure and SEM Analysis

To determine the mode of failure, each sample was observed under a stereomicroscope (SZ40, Olympus, Tokyo, Japan) at $\times$ 40 magnification twice. Mode of failure was determined as adhesive, cohesive in dentin, cohesive in composite resin or mixed. The bonding interface of samples were also inspected under a SEM (Leo 1450VP, Zeiss, Wetzlar, Germany). Some specific areas at the resin-dentin interface were photographed at $\times$ 2500 and $\times$ 5000 magnifications.

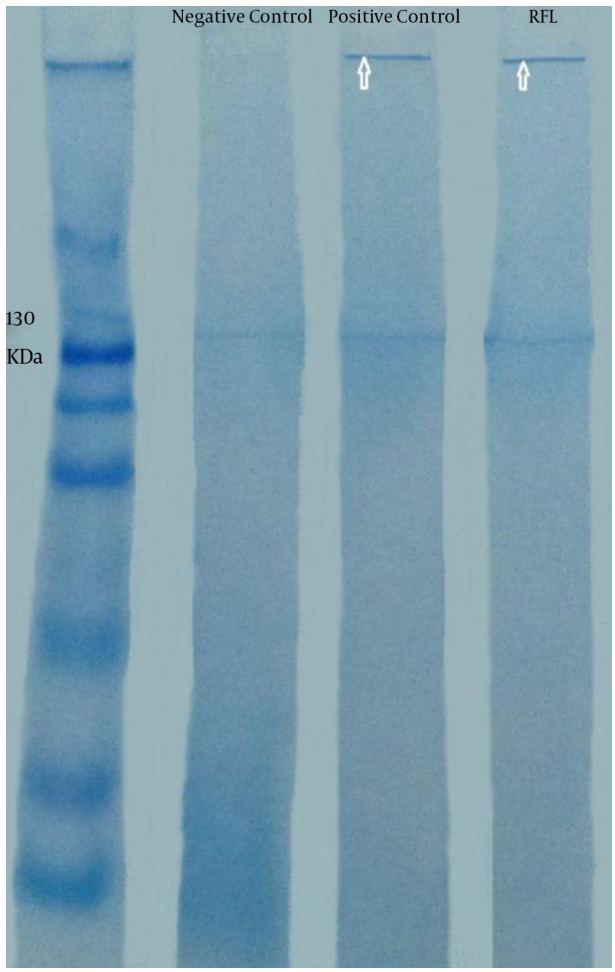
3. Results

In gel electrophoresis analysis, bands were seen at 130 KDa region in all columns, which belonged to α1 and α2 monomers. Except for the negative control group, bands were formed in the wells (location of placement of samples in gel). Moreover, the negative control column, which only included collagen and distilled water, showed weak bands in regions with lower molecular weights (Figure 1).

T-test found no significant difference in the mean μTBS of riboflavin groups ($P = 0.9$). Comparison of the groups and the modes of fracture failure are presented in Table 1.

Figure 2 indicates SEM micrographs of the groups. Hybrid layer in riboflavin group was non-uniform and thicker than that in the control group. Resin tags were longer and conical and had less surface roughness. In the riboflavin group, spherical appendages were seen on resin tags, which was probably due to the slight and incomplete penetration of resin into dentinal tubules.

Figure 1. The results of SDS-PAGE



Arrows indicate high molecular weight residues in wells.

4. Discussion

Previous studies have reported that light-activated riboflavin can efficiently protect the resin-dentin interface by improving the collagen properties and inhibiting MMPs (2, 30, 32).

Based on the result of gel electrophoresis analysis, light-activated riboflavin was effected as a collagen cross linker agent and the first null hypothesis was rejected.

The results revealed that dentin surface treatment with riboflavin caused no significant difference in μTBS compared to the control group ($P > 0.05$). Thus, the second null hypothesis was confirmed.

In contrast to the current results, some previous studies stated that dentin surface preparation with RF/UVA increased the μTBS values immediately and after water storage (9, 30). They attributed this increase in bond strength to the stiffness of dentin collagen matrix (9, 30, 32), upstanding of collagen fibrils and enhanced resin infiltration following cross-linking (30), decreased activity of MMPs due to increased cross linking of dentin collagen (9, 29) and direct cross linking of MMPs (9). The difference between the current results and those of previous studies may be explained as follows:

It has been reported that irradiated light is absorbed by the superficial layers; thus, the cross-linker is not activated to function in deeper areas. This may explain lower efficacy of riboflavin in the current study (33, 34). No significant change in bond strength to dentin may also be at-

Table 1. Means Value (SD) of Micro Tensile Dentin Bond Strength (MPa) and Failure Modes of Study Groups

Group	Surface Pretreatment	Micro Tensile Bond Strength	95% Wald Confidence Interval		P Value[a]	Failure Mode A/M/CR/CD
			Lower	Upper		
Control	No pretratment	12.64 (2.35)	-2.53	2.23	0.90	3/11/0/0
RF/BL	Application and photoactivation of RF% 0.1 (PH = 3)	12.79 (3.64)	-2.55	2.25		8/6/0/0

Abbreviations: A, Adhesive; CD, Cohesive in Dentin; CR, Cohesive in Resin composite; M, Mixed; RF, Riboflavin; SD, Standard Deviation.
[a] Independent T-test, n = 14.

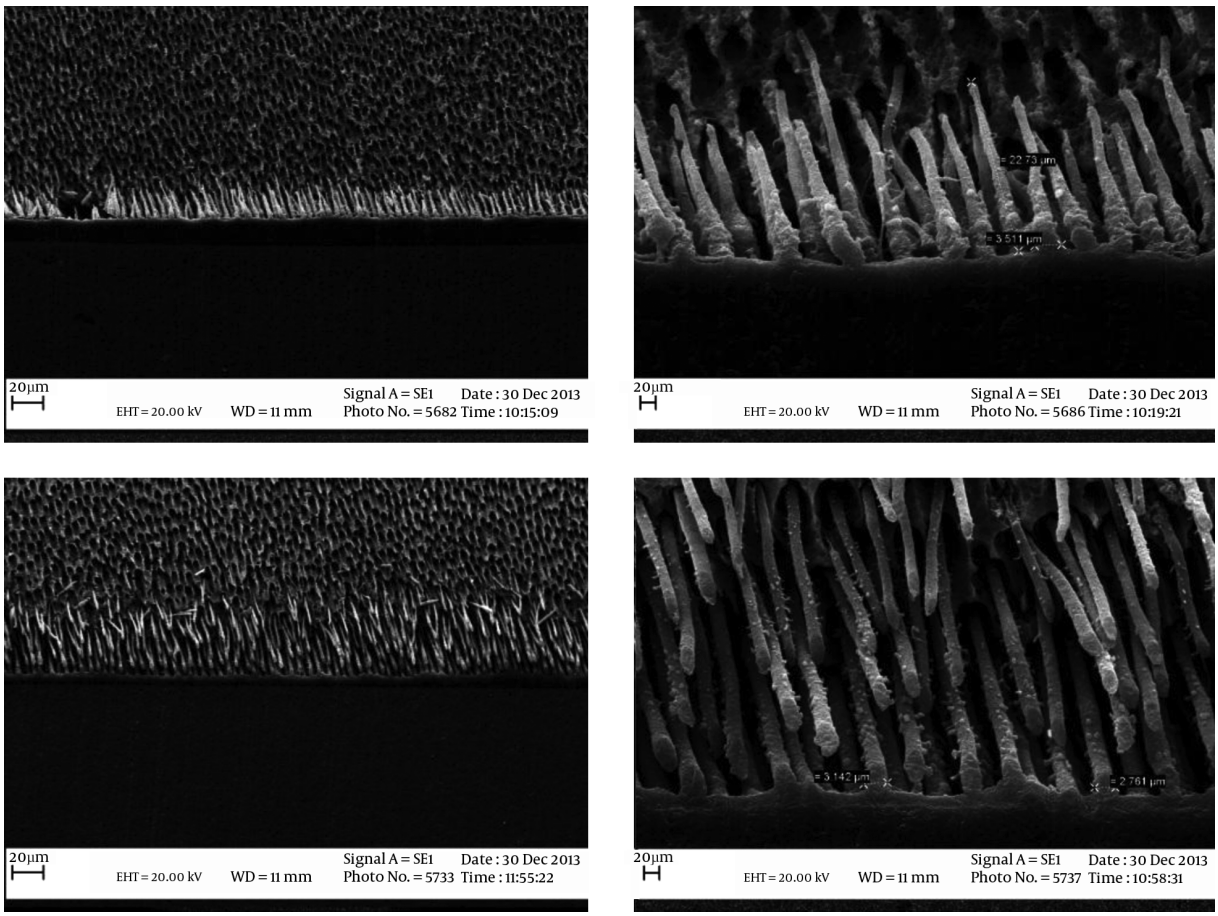


Figure 2. SEM Micrographs Showing the Resin-Dentin Interface Following the Application of Etch and Rinse Adhesive and Light Curing with an LED Light Curing Unit with and Without the Application of Riboflavin; E, Control Group at × 2500 Magnification; F, Control Group at × 5000 Magnification; G, Riboflavin Group at × 2500 Magnification; H, Riboflavin Group at × 5000 Magnification

tributed to the reinforcing effect of hydration by RF/UVA (27, 35), which can adversely affect the formation of an optimized and stable hybrid layer (31).

Extending the application time of Riboflavin and the exposure time of the light activated may generate a better result, but is less practicable for dental use. However, these limited findings may serve as a foundation for future studies of Riboflavin as an adjunctive dentin bonding treatment (30).

Riboflavin can impair the function of adhesive system by water sorption from dentin and the dilution of the primer. Water sorption after polymerization and the release of un-reacted hydrophilic monomers from the hybrid layer can decrease the physical properties of the adhesive layer after 24 hours (36). Moreover, chemical-cross linking does not change the internal stiffness of collagen

molecules (37). Thus, loose collagen fibrils are susceptible to creep and subsequent fracture due to fatigue after long-term function (2).

On the other hand, the use of cross-linkers such as light activated riboflavin can reinforce the collagen smear remaining on the surface (38). Collagen smear on the surface of etched dentin decreases the infiltration of adhesive into the remaining demineralized collagen network. It also forms a layer rich in organic materials with very small resin content on the surface of hybrid layer. Eventually, both of these factors decrease the strength and durability of the hybrid layer as well as the bond to dentin substrate. This finding was in agreement with the results of Hass et al. who reported that riboflavin increased microhardness, indicating that collagen cross-linkers can serve as bio-modifiers and increase the mechanical strength and stability and decrease degradation compared to normal tissue. They added that cross-linkers create a barrier which inhibits further penetration into the tissue. It affects the fixation depth and limits the efficacy for increasing the strength of dental substrate (31).

Further studies are required on the efficacy of cross-linkers for the creation of a stable and optimized hybrid layer and their effects on the strength and durability of bond to dentin.

4.1. Conclusions

Within the limitations of this study, the results showed that light activated riboflavin was capable of collagen cross linking, but its application on etched dentin as a collagen cross linker had no significant effect on μTBS of Single Bond dentin adhesive agent to dentin.

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