Riboflavin as a dentin crosslinking agent: Ultraviolet A versus blue light

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\textbf{Abstract}

Objectives. To investigate the effect of photo-activation of riboflavin either by ultraviolet (UVA) or visible blue light (BL) on the biodegradation resistance, strength of demineralized dentin matrix, bond strength to dentin and resin/dentin interface morphology.

Methods. Dentin beams were demineralized, treated with 0.1% or 1% riboflavin solution for 5 min and photo-activated with UVA or BL for 20 s. The ultimate tensile strength (UTS) and hydroxyproline (HYP) release were assessed after 24 h collagenase challenge. For micro-tensile bond strength (\textmu TBS) testing and resin/dentin interface morphology investigation, dentin was acid-etched, crosslinked with riboflavin and bonded with an etch-and-rinse adhesive system. Riboflavin was photo-activated separately with UVA or BL followed by photo-polymerization of the bonding resin with BL (two-step) or both riboflavin photo-activation and bonding resin photo-polymerization were done in one-step using BL.

Results. Significant improvement in the UTS and biodegradation resistance against collagenase challenge was found when riboflavin was photo-activated either with UVA or BL. However, UVA showed more significant improvement compared to BL. After 4 months of water-storage, both UV and BL two-step photo-activation methods significantly preserved higher values of the \textmu TBS compared to the non-crosslinked control group, where UVA showed significantly higher \textmu TBS than BL.

Significance. Although UVA most effectively activated riboflavin, visible blue light showed to be a promising substitute for UVA as it is clinically more applicable and acceptable, and still managed to increase the biodegradation resistance, enhance the mechanical properties of dentin collagen and improve and maintain the bond strength and interface integrity after short-term water storage.

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1. Introduction

The structural integrity and mechanical properties of collagen fibrils directly affect the quality of the bond strength and its durability [1]. Adhesive resin monomers infiltrate and encapsulate the exposed collagen fibrils to form what is known as the hybrid layer [2]. When there is inadequate infiltration, exposed collagen fibrils are vulnerable for degradation and nano-leakage [3]. Etch-and-rinse dentin adhesives involve the

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application of an acid conditioner, followed by priming and bonding resin monomers to the demineralized dentin collagen fibrils [4]. Resin infiltration and hybrid layer formation is a sensitive procedure due to the delicate nature of dentin collagen fibrils, which can easily collapse due to dehydration interfering with monomer diffusion and consequently affect the quality of the hybrid layer [5–8].

Dentin collagen reinforcement and strengthening through collagen crosslinking might be of importance to improve bond strength and durability and structural integrity of the resin/dentin interface with time against enzymatic and/or hydrolytic degradation through the formation of inter- and intra-molecular crosslinks [9]. Several studies reported the use of grape seed extract, proanthocyanidins, and other crosslinking agents for crosslinking of the dentin collagen [10–12]. However, many variables related to the clinical applicability, suitability and biocompatibility of such compounds in adhesive dentistry are still questionable and need further investigations.

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 [13] and increase the biomechanical strength of the human cornea by 300% [14]. 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 [15–17]. 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 [18]. It has been proposed that the observed reduction in histidine and tyrosine during crosslinking and the formation of dityrosine, dimer of tyrosine, is a possible mechanism in collagen aggregation mediated through riboflavin [19].

Recently, Cova et al. [20] concluded that the effect of UV-activated riboflavin to increase the immediate bond strength to dentin, stabilized adhesive interface, and inhibited dentin matrix metalloproteinases, thereby increasing durability of resin/dentin bonds. Riboflavin is a strong free-radical producing agent when activated by light with maximum absorption peaks at wavelengths of 270, 366 and 445 nm [15,16,21,22]. Although the use of UVA was proven effective as a photo-activation method of riboflavin for collagen crosslinking [17], 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.

The aim of this study is to investigate the effect of photo-activation of riboflavin either by UVA or visible blue light (BL) on: (1) the biodegradation resistance and tensile strength of demineralized dentin matrix and (2) the variations in bond strength and resin/dentin interface morphology with short-term water storage using an etch-and-rinse dentin adhesive system. The null hypotheses tested were: first, pretreatment with photo-activated (UVA or BL) riboflavin would not significantly affect the ultimate tensile strength (UTS), collagenase-mediated biodegradation resistance of demineralized dentin–collagen matrix and the bond strength to dentin. Second, photo-activation of riboflavin either with UVA or BL has no significant difference on UTS, biodegradation resistance and bond strength.

2. Materials and methods

In this study, the effect of riboflavin (RF) photo-activated by UVA or visible blue light (BL) on the strength and biodegradation rate of the demineralized dentin matrix was evaluated by ultimate tensile strength test (UTS) and hydroxyproline (HYP) release rate, respectively. In addition, the variations in the bond strength and resin/dentin interface morphology with short-term water storage were investigated by micro-tensile bond strength (µTBS) and SEM, respectively. Non-carious and non-restored human molars, which were extracted for clinical purposes, were collected and used in this study and approved by the Institutional Review Board of the National University of Singapore. Teeth were stored in 0.2% thymol at 4°C and used within 3 months following extraction.

2.1. Ultimate tensile strength (UTS) testing

The occlusal enamel was removed 1 mm below DEJ using a low speed diamond saw (Buehler, Lake Bluff, IL, USA) under water cooling, and dentin discs of 1.7±0.1 mm in diameter were obtained from mid-coronal dentin by sectioning the cervical and occlusal portions of each crown. The discs were sectioned into 0.5 ±0.1 mm thick beams in the mesio-distal direction and further trimmed by cylindrical bur (#557D, Brasseler, Savannah, GA, USA) to a rectangular dimension of 0.5 mm thickness × 1.7 mm width × 6.5 mm length. The beams were made into hour-glass shaped specimens with a neck area of 0.5 × 0.5 ±0.1 mm at middle, with the use of a cylindrical diamond bur. The beams were then fully demineralized in 10% phosphoric acid for 5 h [23], which was verified by X-ray, and then thoroughly rinsed in distilled water for 5 min at room temperature.

The demineralized beams were immediately divided into three control groups and four experimental groups (n = 13/group) according to the photo-activation/crosslinking method. In the first control group (DW), beams were placed in distilled water for 5 min and no crosslinking or photo-activation was done. The beams of the second control group (DW/BL) were placed in distilled water for 5 min, gently air dried followed by 20 s exposure to a blue-light (BL) tungsten/halogen-lamp curing unit (Curing Light 2500; 3M ESPE, MN, USA) with maximum spectral output range of 470–480 nm and 600 mW/cm² output at a distance of 10 mm from the light source and 7 mm illuminated diameter. One specimen was irradiated separately at each time in air and room temperature in the hydrated moist condition. The third control group (DW/UVA) was exactly treated as the previous group, except UVA (λ = 368 nm) light source (Dymax, BlueWave, 75 UV curing light spot) was used for 20 s at ~10 mW/cm² placed at 10 mm distance from the specimen surface. The UVA was delivered by 70° wave-guide with an illuminating diameter of 7 mm. As for BL, one specimen was irradiated with UVA separately at each time in air and room temperature in the hydrated moist condition. The power outputs of the UVA and BL light sources were frequently monitored and checked.

for the purpose of this study.
Table 1 - Crosslinking and bonding of the dentin specimens.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (DW)</td>
<td>No crosslinking was applied to dentin surface and bonding was done following the manufacturer’s instruction; two thin coats of the bonding agent were applied for 15 s, air-dried for 5 s, and cured for 10 s by blue visible light-curing unit</td>
</tr>
<tr>
<td>Two-step photo-activation 0.1% RF/BL</td>
<td>Dentin surface was crosslinked with 0.1% riboflavin (RF) for 5 min and photo-activated by conventional dental blue light-curing unit (BL) of 600 mW/cm² output for 20 s as previously described. Then, dentin bonding was done as described in the control group (DW)</td>
</tr>
<tr>
<td>0.1% RF/UVA</td>
<td>Dentin surface was crosslinked and bonded as described for group (0.1% RF/BL) except 1% riboflavin was used</td>
</tr>
<tr>
<td>1% RF/BL</td>
<td>Dentin surface was crosslinked and bonded as described for group (0.1% RF/BL) except 1% riboflavin was used</td>
</tr>
<tr>
<td>1% RF/UVA</td>
<td>Dentin surface was crosslinked and bonded as described for group (0.1% RF/UVA) except 1% riboflavin was used</td>
</tr>
<tr>
<td>One-step photo-activation 0.1% RF-Adv/BL</td>
<td>Dentin surface was crosslinked with 0.1% RF for 5 min and then dentin surface was blotted with absorbent paper leaving the surface slightly moist. After that, dentin bonding (Adv) agent was applied as in the control group (DW) followed by photo-activation of the riboflavin and the bonding agent in the same step using conventional blue-light curing unit as described previously</td>
</tr>
<tr>
<td>1% RF-Adv/BL</td>
<td>As in the above group, except 1% riboflavin was used in crosslinking</td>
</tr>
</tbody>
</table>

with an optical power-meter (Newport, USA). The following two experimental groups (0.1% RF/UVA and 0.1% RF/BL) were treated for 5 min with 0.1% solution of riboflavin, gently air dried and photo-activated either by UVA or BL, as previously described. The final two experimental groups (1% RF/UVA and 1% RF/BL) were treated in the same way as the two previous groups respectively, except 1% riboflavin solution was applied. The 0.1% and 1% riboflavin solutions were prepared from riboflavin-5-phosphate (Sigma–Aldrich) dissolved in distilled water and the pH was adjusted at approximately 7. The prepared riboflavin solutions were kept in light-proof test tubes to avoid any light activation of riboflavin before use and applied to dentin specimens (15 μL) at room temperature (21 °C).

The control and experimental groups were then further divided to be stored either for 24 h in distilled water at 37 °C (baseline measurement) or for 24 h in collagenase type I solution at 37 °C. The collagenase solution was prepared by dissolving 100 mg of collagenase type I (Clostridiopeptidase A from Clostridium histolyticum, 125 U/mg, Sigma–Aldrich) in 6 ml of tricine buffer and 3 ml of distilled water. Tricine buffer solution was prepared with 50 mmol/L tricine, 12 mmol/L CaCl₂ and 400 mmol/L NaCl and adjusted to a pH of 7.5. For UTS testing, beams were fixed to a custom-made metallic jig designed for tensile strength measurement by cyanoacrylate adhesive (Zapit, Dental Ventures of America, Corona, CA, USA) and mounted on to a universal testing machine (Instron, 5848 Microtester, USA). Tensile load was applied at the center and parallel to the longitudinal axis of the beam with the direction of the dentinal tubules at a crosshead speed of 1 mm/min through a 50 N load cell until failure. Test was run until failure and the maximum load was divided by the specimen cross-sectional area to calculate the UTS in MPa [9]. All measurements were done in air while the specimens were in hydrated state.

2.2. Collagen-mediated collagen resistance to degradation

The hydroxyproline (HYP) release in the supernatant was measured using an assay kit (BioVision Inc., CA, USA) according to its manufacturer’s instructions. Dentin slabs, with dimensions of 4.5 mm length × 3.5 mm width × 0.5 mm thickness, were prepared from the coronal dentin and demineralized in 10% phosphoric acid for 5 h. The demineralized slabs were rinsed and treated as described previously for UTS testing according to their respective groups (n = 5 slabs/group). All specimens were then exposed for 24 h to 100 μg ml⁻¹ bacterial collagenase type I in tricine buffer. Then, 100 μl aliquot of the supernatant was collected and hydrolyzed in 12 N HCl at 120 °C for 3 h. Next, 10 μl of hydrolyzed specimen aliquots from each group were transferred to a 96-well plate reader (Infinite 200 Tecan, Switzerland) used to measure the absorbance at 560 nm. Standard curves for the quantity of HYP [0–1 μg ml⁻¹/well] were generated. HYP content for each specimen was averaged from quadruplicate measurements of each specimen.

2.3. Dentin bonding and μTBS testing

The occlusal enamel 1 mm below the DEJ was removed using a low-speed diamond saw under water-cooling and grinded with 600 grit-size silicon-carbide papers (Carbimet; Buehler, Lake Bluff, IL, USA) to create a standardized smear layer using a micro-grinder/polisher machine (Phoenix Beta Polisher/Grinder). In this study, one type of etch-and-rinse dentin adhesive system was used according to manufacturer’s instruction (Adper™ Singlebond 2; 3M ESPE). Dentin surfaces were etched with 35% phosphoric acid gel (3M ESPE) for 10 s
and rinsed thoroughly with distilled water. After acid etching, dentin specimens were crosslinked and bonded as described in Table 1. After dentin bonding, a crown of 4 mm height was built up for each tooth by equal increments, cured with a blue-light halogen-lamp (Curing Light 2500; 3M ESPE, MN, USA) for 20 s using a resin-based restorative composite (Filtrek Z350 XT A3; 3M ESPE). The restored teeth were then placed in distilled for 24 h at 37 °C to complete the polymerization reaction. Then, the teeth were sectioned serially in both x- and y-directions across the adhesive interface to obtain resin/dentin beams of approximately 1 mm × 0.9 mm. The prepared beams were then divided to be stored either for 24 h or 4 months in distilled water at 37 °C. Fifteen beams for each group at each storage time were tested. The distilled water was changed weekly for 4 months. After the storage period, beams were fixed to custom-made metallic-jig with cyanoacrylate adhesive mounted to the universal testing machine and stressed to failure using 50 N load cell at cross-head speed of 1 mm/min. The µTBS was calculated by dividing the maximum load to the respective surface area which was confirmed by digital caliper.

2.4. SEM evaluation

Additional resin/dentin slabs were prepared as described in µTBS testing for SEM examination of the resin/dentin interface morphology. The slabs were stored in distilled water for 24 h or 4 months at 37 °C and polished with 600, 800 and 1000 grit-sizes silicon carbide paper discs to produce smooth polished surfaces. The slabs were etched with 35% phosphoric acid gel for 15 s, rinsed for 15 s and dried with compressed oil-free air. The slabs were immersed in 5.25% sodium hypochlorite solution for 20 min and washed thoroughly under running water for 5 min. Then, slabs were post fixed in osmium tetroxide solution, washed twice with PBS solution followed by deionized water for 1 min. Then, they were dehydrated in ascending concentrations of ethanol, starting with 33, 50, 70 and 85% for 15 min each, followed by 95, 100, 100 and 100% ethanol for 10 min each. After the final 100% ethanol treatment, the specimens were dried in a critical point dryer (CPD30 from Leica). The slabs were then mounted to aluminum stubs with conductive tape (double-sided carbon tape) and sputter coated with gold–palladium alloy for 120 s while placed in a vacuum evaporator. Specimens were examined using SEM (XL30 FEG SEM, FEI), operated at an accelerating voltage of 10 kV, and selected representative areas of the resin/dentin interface were photographed at different magnifications.

2.5. Statistical analysis

All data of UTS, HYP release and µTBS tests were expressed as means and standard deviations. Statistical analysis was carried out using SPSS program (Release 15, 2006). Two-way ANOVA was used to test the effect of both dentin crosslinking with riboflavin and photo-activation light source (UVA or BL) on UTS, HYP release and µTBS. Tukey–Kramer multiple-comparison post hoc test was used to compare between the tested groups in terms of UTS, HYP release µTBS for repeated pair-wise comparison. p-Values ≤ 0.05 were considered statistically significant.

3. Results

Two-way ANOVA showed the significant effect (p ≤ 0.001) of either dentin crosslinking and photo-activation light source (UVA or BL) on both UTS and HYP release. In addition, a significant interaction (p ≤ 0.05) was found between the two independent variables (dentin crosslinking and photo-activation source) on the results of the dependent variables (UTS and HYP release). Table 2 shows the means, standard deviation and statistical comparison of the UTS and HYP release between all groups. All crosslinked specimens with different concentrations of RF photo-activated either with UVA or BL showed statistically significant higher UTS values at baseline measurement and lower HYP release after 24 h exposure to collagenase as compared to the control groups. Within the same concentration of riboflavin (0.1% or 1%), photo-activation by UVA significantly increased the UTS compared to photo-activation by BL in both of baseline measurement and after 24 h exposure to collagenase. The same pattern was also found for the HYP release. In contrary, within the same photo-activation source, no difference was found in UTS and HYP release between the two RF concentrations.

The variations in micro-tensile bond strength (µTBS) are shown in Table 3. Two-step photo-activation of riboflavin by UVA significantly enhanced the µTBS at 24 h and 4 months storage in distilled water compared to the control group.
Photo-activation by BL had no significant effect on enhancing the μTBS of both 0.1% and 1% riboflavin crosslinked specimens at 24 h compared to the control group. However, it led to significantly higher μTBS after 4 months storage in distilled water in comparison to the control group. Within the same RF concentration, the type of the photo-activation light source significantly affects the μTBS results at both 24 h and 4 months storage in distilled water. One-step photo-activation of 0.1% RF by BL did not enhance the μTBS in comparison to the control group at both 24 h and 4 months storage in distilled water. However, with one-step photo-activation of 1% RF, a significant decrease in μTBS was found in both 24 h and 4 months storage time. Generally, significantly higher μTBS values were found with two-step compared to one-step photo-activation.

Selected representative SEM images of the resin/dentin interface of specimens crosslinked with riboflavin (0.1% or 1%) and photo-activated with two-step or one-step techniques at 24 h and 4 months storage in distilled water are shown in Figs. 1 and 2, respectively. A uniform hybrid layer with long well-formed resin tags can be observed when the demineralized dentin surfaces were crosslinked with RF and photo-activated with either UVA or BL in two-step after 24 h (Fig. 1A and B) and after 4 months storage in distilled water (Fig. 2A and B). SEM images of 0.1% and 1% RF one-step photo-activated specimens after 24 h (Fig. 1C and D) and after 4 months storage in distilled water (Fig. 2C and D) are presented. Relatively thick hybrid layer can be observed after 24 h in distilled water. However, after 4 months, a more break-down appearance of the hybrid layer and resin tags was distinguishably found with 1% RF one-step photo-activated specimens.

### Table 3 – Means ± standard deviations of micro-tensile bond strength (μTBS) in MPa at 24 h and 4 months storage in distilled water at 37 °C.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Storage conditions</th>
<th>24 h/dH2O</th>
<th>4 M/dH2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control/DW</td>
<td></td>
<td>37.6 ± 7.3a</td>
<td>24.2 ± 6.9ab</td>
</tr>
<tr>
<td>Two-step photo-activation*</td>
<td></td>
<td>0.1% RF/BL</td>
<td>39.8 ± 8.4a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1% RF/UVA</td>
<td>46.8 ± 12.4Ca</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1% RF/BL</td>
<td>42.4 ± 10.9Ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1% RF/UVA</td>
<td>50.2 ± 9.2Ca</td>
</tr>
<tr>
<td>One-step photo-activation**</td>
<td></td>
<td>0.1% RF–Adv/BL</td>
<td>36.4 ± 9.6a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1% RF–Adv/BL</td>
<td>22.8 ± 7.3a</td>
</tr>
</tbody>
</table>

Groups with different superscript uppercase and lowercase letters are statistically significant (Tukey’s test; p ≤ 0.05) in each column and row, respectively.

RF, riboflavin; BL, blue visible light; UVA, ultraviolet light A; Adv, dentin bonding agent (Adper™ Single Bond 2).

* RF was used as a dentin crosslinker and photo-activated either by UVA or BL before dentin bonding agent application.

** RF was applied as a dentin crosslinker followed by bonding agent and both were photo-activated simultaneously by BL.

### 4. Discussion

Through collagen crosslinking it is possible to engineer dentinal collagen fibrils to be more resistant to biodegradation and to preserve the structural integrity and properties of dentin/resin interface over time. Previous studies have investigated the effect of different agents on collagen fibrils crosslinking for dental applications [9,10,20,24] and

![Fig. 1 – Selected SEM images of the resin/dentin interface of two-step and one-step photo-activated riboflavin (RF) crosslinked specimens after 24 h in distilled water. Uniform hybrid layer and long well-formed resin tags can be observed when demineralized dentin collagen was crosslinked with 0.1% and 1% RF and photo-activated with UVA or BL in a separate step prior to dentin bonding agent application and light curing respectively (A and B). More textured, thicker hybrid layer and long well-formed resin tags can be observed in specimen crosslinked with 0.1% RF and photo-activated simultaneously with the dentin bonding agent in one-step by BL (C). Thick, textured hybrid layer and short conical-shaped resin tags are observed in specimen crosslinked with 1% RF and photo-activated with the dentin bonding agent using BL in one-step (D). HL, hybrid layer and RT, resin tags.](image)
non-dental applications [13,14] with promising results. However, a number of clinical and safety aspects should be considered when selecting a suitable crosslinking agent and/or the photo-activation method, such as ease of use clinically, acceptable application and activation time, biocompatibility and safety issues.

The collagen crosslinking effect of photo-activated riboflavin [13,14,17,20] could explain the overall increase in UTS and overall decrease in HYP release found in this study (Table 2) with specimens crosslinked with riboflavin and photo-activated either by UV or BL. In addition, after 4 months of hydrolytic challenge (Table 3), crosslinking with riboflavin photo-activated with two-step method showed another advantage by improving μTBS in both BL and UVA light sources compared to the non-crosslinked control group. Therefore, within the result of this study the first null hypothesis could be rejected.

Characterization of the variation in the mechanical properties, such as UTS, with challenging in collagenase could be considered as indirect evaluation of the effect of photo-activated riboflavin-crosslinking on the resistance of dentin collagen to collagenase-mediated collagen degradation. HYP release could be resulted from collagenase-mediated collagen degradation, from other dentin enzymes such as cysteine and cathepsins [25] and other non-specific collagen-degradation pathways. Although, HYP-assay is non-specific it could indicate the tissue-collagen concentration [26]. Accordingly, the lower HYP release found, in this study, with photo-activated riboflavin-crosslinking might indicate the higher collagen content and the higher resistance of demineralized dentin collagen to bacterial collagenase-mediated collagen degradation. Previous studies reported the significant increase in the UTS and enzymatic degradation resistance of dentin collagen crosslinked with proanthocyanidins from various natural sources [9,10] or glutaraldehyde [27]. However, in the first, the time required to achieve the desired results might not be clinically relevant and with the second, the biocompatibility issue should be considered.

Degradation of both of collagen and/or resin phases at resin/dentin interface increases the water content at the bonded interface leading to collagen degradation and subsequent detrimental effect on the longevity of resin/dentin bond [4]. In addition, dentin contains bound matrix metalloproteinases (MMPs; 2, 3, 8, 9 and 20) and cathepsins [28,29] which have a detrimental effect on bond durability. Therefore, the higher μTBS found in this study with photo-activated riboflavin crosslinking after short-term hydrolytic challenge in distilled water could be explained by the collagen crosslinking and also by the inhibitory effect of photo-activated riboflavin on MMPs activities reported previously [20].

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 [15,16,21,22]. 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 [30]. Riboflavin has close spectral absorption peaks at 366 nm (UVA) and 445 nm wavelengths (visible blue light). In this study, the tungsten/halogen-lamp visible light source used has a maximum spectral output of 470–480 nm with a sudden decrease in the spectral irradiance at wavelengths outside this range, which is not in close match.

Fig. 2 – Selected SEM images of resin/dentin interface of two-step and one-step photo-activated riboflavin (RF) crosslinked specimen after 4 months storage in distilled water. Specimen treated with 0.1% RF prior to bonding agent application and cured either by UVA (A) or BL (B) preserved uniform hybrid layer with long resin tags. A relatively textured hybrid layer can be seen in 0.1% RF one-step photo-activated specimens (C). A more break-down appearance of the hybrid layer and resin tags was found in 1% RF one-step photo-activated specimens (D). HL, hybrid layer and RT, resin tags.
with the maximum absorption peak of riboflavin in the visible spectrum. On the contrary, the used UVA light source has a maximum spectral output of 368 nm which is in close match with 366 nm absorption peak of riboflavin. Accordingly, this difference in the spectral output peaks between the two used light sources might partially explain the superior UTS values and the lower HYP release with collagenase challenges (Table 2) found in this study, when dentin collagen was crosslinked and photo-activated with UVA compared to BL. In addition, the improvement in μTBS with specimens irradiated with UVA after short-term water storage when riboflavin and bonding agents were photo-activated separately in two-step (Table 3) could also be partially attributed to the same reason. Consequently, the UVA light could be more efficiently absorbed by riboflavin to induce collagen crosslinking. Accordingly, within the results of this study the second hypothesis could also be rejected.

One-step photo-activation was done in this study using only BL to induce dual activation effect of both riboflavin and at the same time activate the polymerization reaction of the resin monomers of the dentin bonding agent. However, within the result of this study, one-step photo-activation method showed to be less effective in enhancing and maintaining the μTBS after short-term water storage when compared with two-step photo-activation method even when BL was used as a light source for both activation methods (Table 3). Furthermore, one-step photo-activation with 1% riboflavin showed an adverse effect on μTBS in both immediate and short-term water storage. These findings could be due to both riboflavin [15,16,18] and the photo-initiator system in the dentin bonding agent [31] competing in absorbing the BL. Consequently it could be assumed that with more 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 [18]. This might be supported by the SEM images of the resin/dentin interface of one-step photo-activated specimens, especially when 1% riboflavin was used for crosslinking at both 24 h (Fig. 1C and D) and short-term water storage for 4 months (Fig. 2C and D). However, further studies quantifying the effect of riboflavin on the degree of conversion of the dentin bonding monomers should be considered for future studies.

On the contrary, SEM images of the resin/dentin interface of the one-step photo-activated specimens, the two-step photo-activated specimens using either UVA or BL showed a uniform hybrid layer with well-formed long resin tags for 0.1% and 1% riboflavin at both 24 h (Fig. 1A and B) and 4 months (Fig. 2A and B) water storage. However, it has been reported that neither the thickness of the hybrid layer nor the length of the resin tags necessarily improves the bond strength [32,33].

Physical crosslinking of collagen through irradiation, such as short wavelength UV, was suggested previously as it does not include chemicals which could cause biological harm or interfere with other materials or chemicals in an adverse manner. However, within the results of this study, exposing dentin collagen to UVA or BL without riboflavin as a photo-sensitizer showed to be insufficient in terms of improving the tensile strength and the biodegradation resistance (Table 2). Interestingly, the riboflavin concentration barely showed any difference on the tensile strength and biodegradation resistance on the dentin collagen and on μTBS when photo-activated in two-step within either UVA or BL. Therefore, the combined use of riboflavin followed with photo-activation is essential for efficient crosslinking of dentin collagen.

In accordance with previous studies which showed riboflavin capability to crosslink collagen type I for other medical applications [13,14], this study showed that riboflavin can also efficiently crosslink dentin collagen and enhance the bond strength to dentin when activated with either UVA or visible blue light. In addition, riboflavin photo-activation by UVA clearly showed to be more effective than visible blue light. However, due to its clinical safety, BL photo-activation can be also considered as a good alternative.

5. Conclusion

Within the limitations of this study, it can be concluded that although UVA most effectively activated riboflavin, visible blue light showed to be a promising substitute for UVA as it is clinically more applicable and acceptable, and still managed to increase the biodegradation resistance, enhance the mechanical properties of dentin collagen and improve and maintain the bond strength and interface integrity after short-term water storage. However, further studies are recommended concerning the use of visible light sources with different spectral output peaks, visible light penetration and activation depths in the dentin matrix, and the interaction with matrix metalloproteinases and bonding monomers.

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