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Original

UVA-activated riboflavin improves the strength of human dentin

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Abstract: The aim of this study was to evaluate the effects of UVA-activated riboflavin (UVA-RF) on the mechanical properties of non-demineralized human dentin. Dentin specimens obtained from 20 teeth were randomly divided into the following four groups: group 1 (control): no treatment, group 2 (low UVA-RF): specimens were exposed to UVA-RF for 10 min, group 3 (medium UVA-RF): specimens were exposed to UVA-RF for 30 min, and group 4 (high UVA-RF): specimens were exposed to UVA-RF for 60 min. Three-point flexural test and Raman spectroscopic analyses were performed. The mean flexural strengths (MPa) were 129.96, 128.96, 144.21, and 147.54, and the mean elastic modulus (GPa) were 8.59, 8.38, 10.21, and 9.87 for groups 1 to 4, respectively. Raman spectra showed chemical modifications of dental collagen under medium and high UVA-RF treatment. We conclude that medium and high UVA-RF increases the strength of non-demineralized human dentin by collagen crosslinking.
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Keywords: ultraviolet; riboflavin; dentin; collagen; mechanics.

Introduction

Tooth fracture is still a major problem in the dental clinic. A previous study that was assessing the outcome of a preventive dental treatment over a 30 year period, (1) reported that in a population with high standards of oral hygiene, fracture accounted for 62% of all tooth loss, while only 12% was caused by progressive periodontitis and caries. Tooth fracture is frequently observed in dental practices, particularly in devitalized teeth. Dentin accounts for a major volume of the tooth structure, and is responsible for maintaining the integrity of the tooth. Thus, effective methods for improving the strength of the dentin could help to prevent tooth fracture.

Dentin is a complex mineralized tissue arranged in a three-dimensional framework. Its principal function is to resist mechanical forces and fractures. Dentin is comprised of 45% minerals, 33% organic components, and 22% water by volume. Most of the organic matrix is composed of type I collagen which contributes considerably to its biomechanical properties, and improving its mechanical properties may help in preventing dentin fractures.

Crosslinking (increasing collagen crosslinks), is a means of improving the mechanical properties and molecular stability of collagen-based tissues, such as dentin (2,3). The basic methods for modification of the number of collagen crosslinks can be divided into two types: chemical methods, where various cross-linking solutions such as glutaraldehyde are used (3), and physical methods, using high intensity ultraviolet radiation (2). However, the toxic effects of chemical agents limit their application, and the biological safety of high intensity ultraviolet radiation is still uncertain. In addition to the efficiency of collagen crosslinking approaches, their biocompatibility and clinical applicability must be considered for clinical applications.

Riboflavin is non-toxic and can produce free radicals, such as singlet oxygen that are capable of crosslinking collagen through a photo-oxidation pathway (4), with maximum absorption peaks at 266-nm, 373-nm, and 445-nm wavelengths (5). UVA-activated riboflavin

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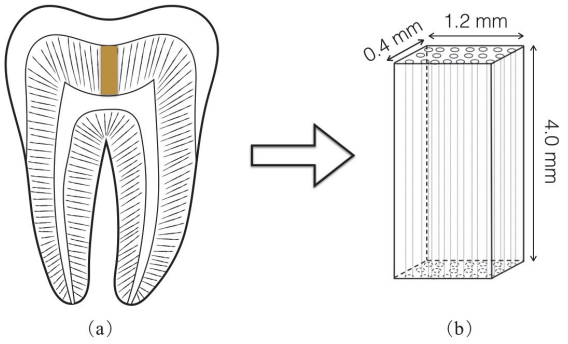

Fig. 1 Schematic sequence illustrating the procedure used to obtain the dentin specimens. Each tooth was cut longitudinally to yield 1.2-mm sections. Beam-shaped dentin specimens were obtained from the coronal central portions of the molars (a). Dentinal tubules in the specimens were organized to run parallel to the loading surface along the specimen length, and the specimens were approximately 0.4 × 1.2 × 4.0 mm in size (b).

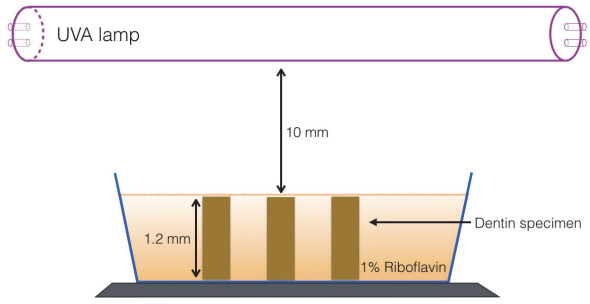

Fig. 2 Schematic illustration of UVA-RF treatment. Testing specimens were immersed in 1% RF solution and photo-activated with UVA (368 nm) at 6 mW/cm^2, using a UVA lamp irradiation unit. The distance from the UVA light source to the dentin beam was 10 mm.

(UVA-RF) has been considered to be a successful treatment strategy for progressive keratoconus because of its consistent stabilizing effect in corneal collagen crosslinking (4). Recently, UVA-RF has been shown to enhance the mechanical properties and mechanical stability of dentin collagen matrix (6,7). However, these studies focused on completely demineralized dentin collagen. To our knowledge, there are no reports regarding the effects of UVA-RF on the mechanical properties of non-demineralized dentin.

Because the collagen network plays an important role in regulating the mechanical strength of dentin, we investigated whether UVA-RF could significantly strengthen the mechanical properties of non-demineralized dentin by promoting chemical modifications of the collagen. The purpose of this study was to investigate the effects of UVA-RF on the flexural strength and elastic modulus of non-demineralized bulk dentin. The null hypothesis tested was that UVA-RF did not affect either of these properties.

Materials and Methods

Sample preparation

In total, 20 non-carious third molars were collected from patients, after obtaining informed consent. The study protocol was reviewed and approved by the Ethics Committee for Human Studies, Peking University, Beijing, China (IRB-2012060). The patients were aged between 18 and 30 years, and required extractions in connection with other dental treatments. The teeth were stored in 0.9% sodium chloride containing 0.02% sodium azide at 4°C for no more than 1 month. The coronal central portions of these molars were then sectioned into beam-shaped dentin specimens, using a low-speed diamond saw (Isomet 1000; Buehler Ltd., Lake Bluff, IL, USA) under water cooling. The specimens measured approximately 0.4 × 1.2 × 4.0 mm. Dentinal tubule orientation in the specimens was arranged to run parallel to the loading surfaces (Fig. 1). Five to eight specimens were collected from each tooth, and the final sample contained 128 dentin bars. After ultrasonic cleaning for 10 min, the specimens were stored in distilled water until UVA-RF treatment.

Treatment of dentin specimen with UVA-RF

The dentin specimens were randomly assigned into four groups:

group 1 (Control): Specimens were stored in distilled water until testing.

group 2 (Low UVA-RF): Specimens were exposed to UVA-RF for 10 min.

group 3 (Medium UVA-RF): Specimens were exposed to UVA-RF for 30 min.

group 4 (High UVA-RF): Specimens were exposed to UVA-RF for 60 min, during which the RF solution was changed after 30 min.

A 1% RF solution was prepared by dissolving riboflavin-5-phosphate (Sigma-Aldrich, St. Louis, MO, USA) in distilled water, and was then stored in lightproof tubes to avoid light-activation before use. Specimens from groups 2 to 4 were immersed in 1% RF solution, and photo-activated with UVA (368 nm), in a UVA lamp irradiation unit (TL Actinic BL; Philips, Hamburg, Germany), at 6 mW/cm^2 for 10, 30, or 60 min, respectively. The distance from the UVA light source to the dentin was 10 mm. The specimens were kept immersed

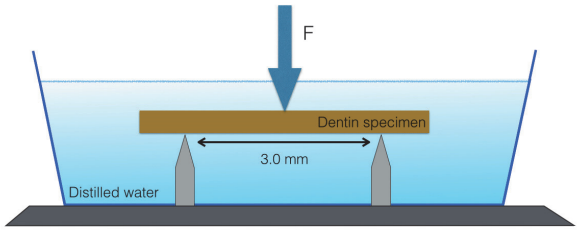

Fig. 3 Schematic diagram of the setup used for the three-point flexural test. Dentin specimens were placed across the lower supports of the test jig and loaded at the mid-point through the loading head and shaft. The span distance between the two metal supports was 3.0 mm.

Table 1 Mechanical properties of dentin specimens under different treatment conditions

Group	Test condition	Number of specimens	Mean flexural strength and standard deviation (MPa)	Mean elastic modulus and standard deviation (GPa)	Mode of fracture
1	No Treatment	30	129.96 (12.66)[a]	8.59 (1.46)[a]	11 / 19[a]
2	Low UVA-RF	30	128.96 (15.02)[a]	8.38 (1.56)[a]	12 / 18[a]
3	Medium UVA-RF	30	144.21 (11.86)[b]	10.21 (1.69)[b]	15 / 15[a]
4	High UVA-RF	30	147.54 (12.59)[b]	9.87 (1.76)[b]	15 / 15[a]

For each row, groups identified by different superscript letters are significantly different ($P < 0.05$).
X / Y of fractural models: X = number of complete fracture specimens, Y = number of incomplete fracture specimens.

in 1% RF solution for the entire exposure time (Fig. 2).

Three-point flexural test

A three-point flexural test of dentin specimens was performed, as described earlier (8). In total, 120 specimens were subjected to flexural testing on a three-point bend testing apparatus and a universal testing machine (EZ-L; Shimadzu, Tokyo, Japan). All specimens were kept immersed in distilled water during experimental procedures. The width and thickness of the dentin specimens were measured using electronic calipers (111N-101; Guanglu, Guilin, Guangxi, China). Each bar was placed across the lower supports of the test jig, and loaded at the mid-point through the loading head and shaft (Fig. 3). The span distance between the two metal supports was 3.0 mm. The loading test machine was run at a crosshead speed of 1 mm/min to failure. Data was recorded on a plotter to give load-displacement curves. The mode of fracture was recorded as complete or incomplete.

The flexural strength (s) was calculated using the following equation:

$$s = (3PL) / (2bd^2),$$

where P (N) is the load at the moment of fracture, L (m) is the support span, and b (m) and d (m) are the width and thickness of the test specimen, respectively.

The slope of the initial straight-line section of the load deflection curve was determined, and the elastic modulus (E) was calculated using the following equation:

$$E = (L^3 m) / (4bd^3),$$

where L (m) is the support span, m (Nm^{-1} of deflection) is the slope of the initial straight-line section of the load deflection curve, and b (m) and d (m) are the width and thickness of the test specimen, respectively.

Data are expressed as means (standard deviations) of flexural strength and elastic modulus. One-way analysis of variance was used for analysis, and Tukey's test was used for *post hoc* comparisons. The distribution of failure modes was analyzed by a Chi-square test. Level of statistical significance was fixed at $\alpha = 0.05$, and all analysis was performed using SPSS 13.0 software (SPSS, Chicago, IL, USA).

Laser Raman spectroscopic analysis

Eight beam-shaped dentin specimens ($n = 2$ for each test group) were demineralized in 0.5 M ethylenediamine tetraacetic acid at a pH of 7.4 for 10 days, and complete demineralization was confirmed using X-ray films.

Micro-Raman spectroscopy measurements were performed, as reported previously (2,9). Raman spectra were measured using laser Raman microscopy (Renishaw inVia; Renishaw Plc, London, UK). The excitation source was a semiconductor laser operating at 785 nm with a nominal power of 100 mW. The laser beam was focused and irradiated onto the specimen with

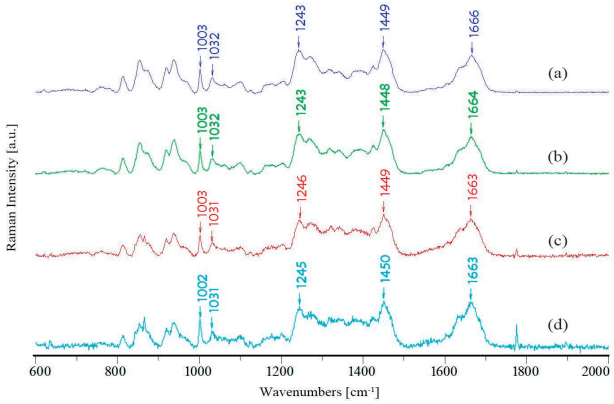

Fig. 4 Raman micro-spectroscopy of the dentin specimens in the four test groups. The specimens were untreated (a), treated with low UVA-RF (b), medium UVA-RF (c), or high UVA-RF (d).

Table 2 Typical Raman modes, positions, and assignments detected in the dentin collagen matrix

Raman shifts	Assignment/vibrational mode	Experimental positions of Raman modes			
		group 1	group 2	group 3	group 4
1,003 (15)	C-C phenyl group	1,003	1,003	1,003	1,002
1,032 (16)	CC pyridine aromatic ring	1,032	1,032	1,031	1,031
1,245 (17)	Amide III/C-N stretch; N-H def; Peptide carbonyl	1,243	1,243	1,246	1,245
1,450 (16)	C-H alkyl group	1,449	1,448	1,449	1,450
1,667 (16)	Amide I/N-H in-plane deformation alpha helix	1,666	1,664	1,663	1,663

an objective lens (5×, NA = 0.3). The area of irradiation was 1.0 × 1.0 μm^2, and the accumulation time was 180 s. The excited Raman scattering light was collected with the same objective lens and guided to the spectrograph, which had a focal length of 500 mm. The Raman spectra obtained was normalized for comparison (2,9). This was done by first reducing the background intensity caused by fluorescence, using an algorithm described previously (10), and then normalizing the spectra to the intensity of the peak at 815 cm^{-1}, corresponding to the backbone vibration of collagen (11). Raman band positions were compared with those reported for dentin (12-14).

Results

The mean values and standard deviations of flexural strength, elastic modulus, and failure modes for dentin beams, as a function of UVA-RF exposure time, are provided in Table 1. Exposure of the dentin bars to low UVA-RF did not alter the physical properties of the dentin, and no statistically significant difference in flexural strength or modulus of elasticity was found between the exposed and control groups. Statistical analysis showed a significant ($P < 0.05$) increase in the flexural strength and elastic modulus after medium and high UVA-RF treatment, compared with the control group. However, there was no significant difference in flexural strength or elastic modulus between the two groups. Moreover, there was no significant difference in failure mode between the four groups.

The Raman spectra of the test dentin specimens in the range of 600-2,000 cm^{-1} are shown in Fig. 4. Regions of the spectra, characterized by the appearance of vibrational modes of collagen, were analyzed and presented in Table 2, along with the reference data. The shifts in Amide I for specimens treated with low, medium, and high UVA-RF were 1,664, 1,663, and 1,663 cm^{-1}, respectively, compared with the control at 1,666 cm^{-1}. A slight increase in wave number was also observed after crosslinking in the Amide III region. The shifts were seen at 1,243, 1,243, 1,246, and 1,245 cm^{-1} for groups 1 to 4, respectively. The Raman band around 1,032 cm^{-1}, which was assigned to a pyridinium ring vibration, showed a slight shift towards 1,031 cm^{-1} for medium and high UVA-RF treatment. The bands at 1,003 cm^{-1} and 1,450 cm^{-1} were assigned to the C-C bond in the phenyl group and the C-H alkyl group of the collagen, respectively. The peaks at 1,003 cm^{-1} and 1,450 cm^{-1} in each group remained unchanged.

Discussion

The results of this study showed that UVA-RF signifi-

cantly increased the flexural strength and elastic modulus of dentin specimens when the treatment time was 30 or 60 min. However, when the treatment time was 10 min, the mechanical properties were unaffected by UVA-RF. Moreover, the 30 and 60 min UVA-RF treatments induced chemical modifications in the dentin collagen, and this could be seen in the Raman spectra. We conclude that medium and high UVA-RF significantly increase the bulk mechanical properties of non-demineralized dentin, likely attributable to increased collagen crosslinking. Thus, the null hypothesis was rejected.

Previous studies have used conventional three-point bending tests (15,16) and nano-indentation tests (17) to measure the mechanical properties of dentin. Although the latter test has high precision, measurements are limited to only submicron surface depths, and the test results may not reflect overall changes in the entire bulk of the dentin beam (16,17). Thus, a three-point flexural test was used to assess the effects of UVA-RF on the overall mechanical properties of the dentin specimens. In this study, medium and high UVA-RF treatments increased the flexural strength and elastic modulus of dentin beams significantly ($P < 0.05$), but low UVA-RF treatment did not. This may be because low UVA-RF was unable to induce the chemical modifications that were seen in the Raman spectra. The RF solution was changed at 30 min in group 4 because of the steady state of equilibrium between the continuous UVA-triggered generation of HO· from RF and the simultaneous ongoing decay of the radical adducts, and the 30% reduction in RF concentration after 30-min illumination (18). However, there were no significant differences ($P > 0.05$) in mechanical properties between the medium and high UVA-RF groups. Exposure of the dentin specimens to medium and high UVA-RF significantly ($P < 0.05$) increased not only their flexural strength but also the elastic modulus. Thus, it seems reasonable to conclude that the effects were exerted not only on the surface of the dentin but throughout the bulk of the specimen.

The failure modes comprised complete fracture, in which the dentin bars exhibited sudden, complete "brittle" fracture on incremental loading; and incomplete fracture, in which the dentin bars appeared to exhibit incomplete "green stick" fracture, as described previously (19). The UVA-RF treated specimens showed a tendency for complete fractures. In the treated group, dentin bars fractured at higher loads and there was an obvious decrease in the deformation of specimens on loading. Therefore, the failure modes were in compliance with increase in elastic modulus and flexural strength of dentin beams caused by UVA-RF. This indicates that UVA-RF improves dentin strength. However, there were no significant differences ($P > 0.05$) in the mode of fracture between the four groups.

The theoretical model of human bone (20), which has a composition similar to that of dentin, shows that an increase in the elastic modulus of the organic matrix leads to increase in the elastic modulus of bone tissue. In the present study, the dentin collagen was recognized to be crosslinked, which could have caused the increase in the elastic modulus of the dentin beam. The Raman spectroscopic analysis confirmed chemical modification of the collagen on exposure to UVA-RF treatment. Upon UVA photo-activation, riboflavin was excited into a triplet state, generating free oxygen radicals and reactive oxygen species [superoxide anions (O^{2-}), hydroxyl radicals (HO·), and hydrogen peroxide (H_2O_2)], which bridged amine groups (N-H) of glycines of one chain with carbonyl groups (C=O) of hydroxyproline and proline in adjacent chains, with no reported side effects (21). Raman spectroscopy showed a strong dependency of certain Raman bands (1,032/1,245/1,667 cm^{-1}) on crosslinking. In the medium and high UVA-RF groups, the amide I and amide III shifts for cross-linked dentin specimens were towards lower and higher frequencies, respectively. This was consistent with previous reports of type-I collagen crosslinking with riboflavin (22). Additionally, the CC pyridine aromatic ring vibration at 1,032 cm^{-1}, known to be due to the 3-hydroxypyridinium ring which is a crosslinking residue in the collagen (23), showed a notable shift at 1,031 cm^{-1} in the medium and high UVA-RF groups. These regions showed spectral changes that resulted from changes to the secondary structure of the polypeptide chain. However, the Raman modes characteristic for C-C phenyl and C-H alkyl groups remained unchanged in all test groups. Thus, the micro-Raman results confirmed the crosslinking effects of medium and high UVA-RF on the dentin collagen matrix.

In the present study, the strength of dentin was increased by medium and high UVA-RF treatment. The most likely reason for significant improvement in the bulk mechanical properties of non-demineralized dentin is the formation of covalent crosslinks in the dentin collagen matrix, which was detected by Raman analysis. Recently, UVA-RF was found to crosslink the collagen matrix, and to increase the mechanical properties of demineralized dentin (7). In this study, the dentin specimens were etched with 37% phosphoric acid to dissolve the mineral components and expose the collagen matrix to UVA-RF directly. Two minutes of treatment was found to be effective. However, in our study, the dentin beam was not

demineralized so as to mimic the clinical situation. Only a longer duration of treatment was found to be effective in improving the strength of dentin beam. This difference could be attributed to the mineral components. However, the influence of UVA-RF on the mineral components of dentin is unknown; therefore, future studies investigating this are required. Moreover, 30-min treatment time was not quite acceptable for clinical use. The application technique of UVA-RF, including UVA intensity and RF concentration, should be optimized to achieve a clinically acceptable time-frame.

The clinical application of this study lies in the possible use of UVA-RF to strengthen dentin to prevent fractures in devitalized teeth. However, the clinically important parameters should be optimized further.

In conclusion, within the limitations of the experiments performed, medium and high UVA-RF treatment increased the mechanical properties of non-demineralized bulk dentin, possibly because of increased crosslinking of the dentin collagen matrix.

Acknowledgments

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