Ultrastructural and Mechanical Effects of Collagen Cross-linking Treatment on Human Corneo-scleral Tissues

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ABSTRACT

The aim of this study was to quantitatively examine the immediate effects of a photooxidative collagen cross-linking (CxL) treatment with photosensitizer riboflavin (RF) and 370 nm UVA light in in vitro rabbit dermal collagen fibrils. These effects would be measured at the corneo-scleral (CS) stroma, 20 8 × 2 mm CS strips, based on clinical corneal CxL procedures on the morphological and mechanical response level using histology and AFM analyses. Five parameters including the density, area, adhesion force, and stiffness of CS tissues before and after RFUVA catalyzed collagen CxL treatment were investigated. The RFUVA catalyzed collagen CxL treatment led to an increase in the area and density of both corneal (>108%) and scleral (>118%) stromal collagens. Furthermore, RF-UVA catalyzed collagen CxL treatment led to an increased biomechanical response of CS; 125% for corneal stiffness and 108% for scleral stiffness, and 124% for corneal adhesion force and 122% for scleral adhesion force, $p > 0.05$. The collagen CxL treatment through RF-sensitized photoreaction may cause morphological and biomechanical property changes in the collagen fibril network of the cornea as well as the sclera, due to the stromal edema and interfibrillar spacing narrowing.

Keywords: Photooxidative collagen CxL treatment, riboflavin and UVA light, corneo-scleral collagens, biomechinical property

1 INTRODUCTION

Collagen cross-linking (CxL) techniques using the photosensitizer riboflavin (RF) and ultraviolet A (UVA) light with a 370 nm wave length were first introduced for the management of progressive keratoconus [1,2]. The basic principle of this therapeutic method is that the RFUVA-catalyzed corneal collagen CxL reaction achieves additional covalent binding between collagen molecules, leading to an increase in corneal stiffness and enhanced resistance [3]. High success rates in clinical trials on keratoconic corneas suggest the possibility of an efficient treatment against myopic progression. Several studies [2,4,5] have reported the effect of collagen CxL treatment through RF-sensitized UVA photoreaction on the sclera. However, there are no reports on ultrastructural and biomechanical effects of RFUVA-catalyzed collagen CxL treatment on both the cornea and sclera, simultaneously. Therefore, this study quantitatively examined and compared the immediate structural and biomechanical response of the collagen CxL treatment with photosensitizer RF and 370 nm UVA irradiation on *in vitro* human corneo-sclera (CS) using three investigation methods: histology and AFM assessments.

2 MATERIALS AND METHODS

2.1 Sample Preparation

Four human CS tissues from males who were 35 ± 7 years old were collected at the Eye Bank of the Kyung Hee University Medical Center. Informed consent for the use of human tissue for research was obtained from the Eye Bank. Serologic tests of the donor tissues were negative for hepatitis, syphilis and human immune deficiency virus. The tissue was preserved for three months in 90% ethanol for sterilization after donation. Before the photooxidative collagen CxL treatment, five 8 × 2 mm strips that included the cornea and sclera were dissected sagitally from donor tissue using a scalpel. All strips were then removed from the ethanol and irrigated with BSS at room temperature.

2.2 RFUVA-Catalyzed Collagen CxL Treatment

Half of the prepared strips (n = 10) were immediately fixed in a 4% buffered paraformaldehyde solution for histology (n = 5) or immersed in BSS solution for AFM (n = 5) for one day, and the other half (n = 10) were used in the following procedure. A 0.1% RF photosensitizer solution (10 mg RF-5-phosphate in 10 ml 20% dextran-T-500) was instilled into the CS strips for 10 min before UVA irradiation. A 370 nm UVA irradiation was applied using a double UVA source (UV-XTM Illumination System, IROC AG, Zurich, Switzerland), with a surface irradiance of 4.2 mW/cm^2 at a distance of 3 cm from the strips for 30 min. The collagen CxLed strips were irrigated with BSS, and fixed in a 4% buffered paraformaldehyde solution for histology (n = 5) or immersed in BSS solution for AFM (n = 5) for one day.

2.3 Histological Analysis

After fixation, the specimens were embedded in paraffin.

They were sectioned on the sagittal plane and stained with Masson's trichrome (MT) to semi-quantify the collagen components and interfibrillar spacing. A histological assessment of CS was examined by two professional pathologists, blinded to experimental group assignment

2.4 AFM Analysis

The specimens were scanned under BSS (property measurement) and dehydrated (imaging) conditions. In BSS conditions, two areas of each strip were scanned using a NANOstation II (Surface Imaging Systems, Herzogenrath, Germany) in contact mode. The force-distance curve measurement was performed to measure the adhesion force and stiffness of human CS using reflex-coated silicon cantilevers. After taking biomechanical measurements under BSS conditions, the CS strips were fixed in a 4% buffered paraformaldehyde solution for one day. Two areas of each strip were also scanned using a NANOS N8 NEOS (Bruker, Herzogenrath, Germany) in non-contact mode. AFM tapping mode CS images were scanned using a silicon cantilever with an integral pyramidal shaped tip, in air at 35% relative humidity, with a resolution of 256 × 256 pixels, a scan speed of 0.8 line/sec, and a size of 5 × 5 μm^2 (SICONG, Santa Clara, CA, USA).

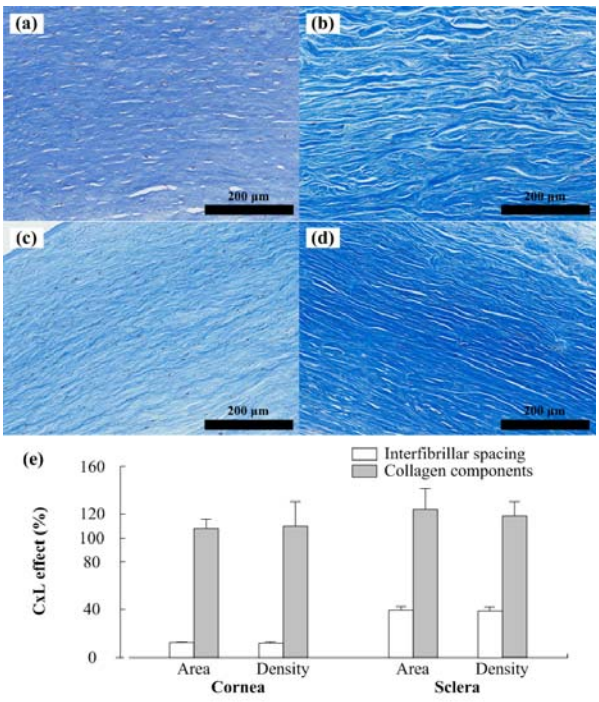

Figure 1: Representative histological images and structural responses of human corneal and scleral stromal tissues before and after collagen CxL treatment through RF-sensitized UVA irradiation (MT, 400x). The collagen CxLed cornea (c) and sclera (d) show the stromal edema and narrowing of interfibrillar spacing compared to the normal cornea (a) and sclera (b).

2.5 Statistics

The density, area, adhesion force, and stiffness of CS stromal tissues before and after photooxidative collagen CxL treatment were expressed as mean ± standard deviation. Statistical analysis was performed to compare the structural and biomechanical changes in collagen fibrils of CxLed CS stromal tissues with the control using a two-tailed Student's t-test. P-values < 0.05 were considered significant.

3 RESULTS

Fig. 1 shows a representative result of a histopathology examination of human CS tissues before and after collagen CxL treatment with RF and UVA light. The normal corneal (Fig. 1a) and scleral stroma (Fig. 1b) showed the collagen lamellae and interfibrillar spacing. The photooxidative collagen CxLed corneal (Fig. 1c) and scleral stroma (Fig. 1d) showed mild inflammatory infiltration, stromal swelling, and narrowing of interfibrillar spacing. Density and area of collagen fibrils increased by 108/110% for the cornea and by 123/118% for sclera. Density and area of interfibrillar spacing decreased by 87/88% for the cornea, and density and area of interfibrillar spacing both decreased by 61% for the sclera (Fig. 1e). These changes were primarily in the scleral stroma; the interfibrillar spacing was significantly different for both density and area ($p < 0.0001$). However, the collagen fibrils were not significantly different ($p < 0.3447$ for density and $p < 0.0529$ for area).

Fig. 2 shows representative AFM tapping mode topographical images (5 × 5 μm^2) and biomechanical properties of human CS stromal surfaces before and after RFUVA-catalyzed collagen CxL treatment. AFM tapping mode topographical images showed that typical normal corneal (Fig. 2a) and scleral (Fig. 2b) surfaces have a regular parallel arrangement of collagen fibrils with clear axial periodicity. Corneal diameters were smaller than scleral diameters. However, the corneal (Fig. 2c) and scleral (Fig. 2d) surfaces after collagen CxL treatments through RF-sensitized photoreaction showed an irregular parallel arrangement of collagen fibrils with increased diameters. These changes were more pronounced in the sclera than in the cornea. Corneas in the control group had an adhesion force of 10.91 ± 4.70 nN (n = 100) and a stiffness of 9.37 ± 1.55 nN/μm (n = 100). Scleras in the control group had an adhesion force of 14.32±3.61 nN (n = 100) and a stiffness of 30.63 ± 2.49 nN/μm (n = 100). There was a significant difference ($p < 0.0001$) in the biomechanical properties of cornea and sclera. The CS collagen CxL treatments with RFUVA catalysis led to a significant increase in biomechanical responses in both the cornea and sclera. There was an increase of 124% (13.52 ± 4.54 nN, $p = 0.0216$, n = 100) and 125% (11.71 ± 4.09 nN/μm, $p = 0.0025$, n = 100) in corneal adhesion force and stiffness, respectively. There was an increase of 122% (17.42 ± 5.42 nN, $p = 0.0068$, n = 100) and 108% (33.17 ± 3.33 nN/μm, $p = 0.0006$, n = 100) in scleral adhesion force and stiffness, respectively.

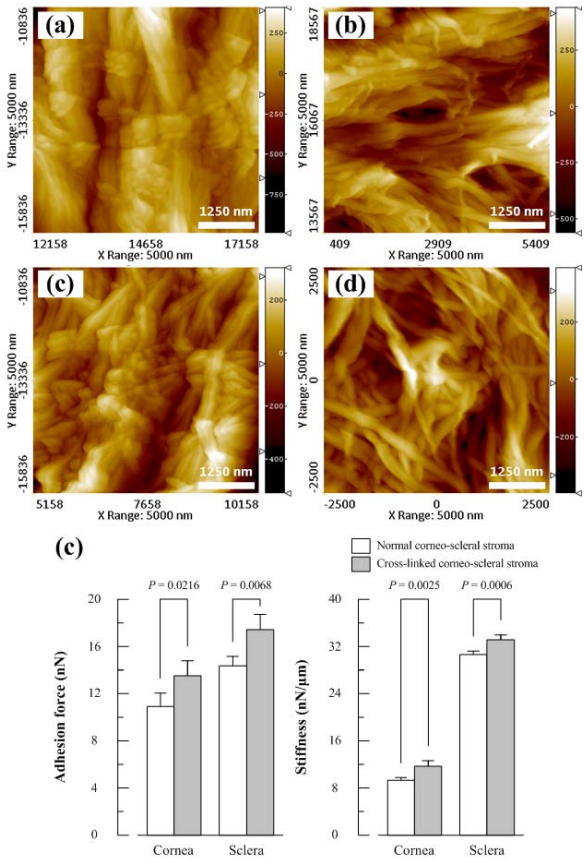

Figure 2: Representative AFM tapping mode topographical images and responses of biomechanical properties for normal and collagen CxLed CS stromal surfaces. Typical normal corneal (a) and scleral (b) surfaces showed a regular parallel arrangement of collagen fibrils with clear axial periodicity. The corneal diameters (a, c) are smaller than the scleral diameters (b, d). Overall, the collagen CxL treatment through RF-sensitized photoreaction leads to an increase in biomechanical response of human CS.

4 DISCUSSION

Most studies about the effect of collagen CxL treatment are restricted to a single tissue: corneal tissue for keratoconus treatment or scleral tissue for myopia treatment. However, this study quantitatively examined the immediate effects of collagen CxL treatment through RF-sensitized UVA photoreaction on the morphology and biomechanical properties of collagen fibrils in human corneal tissues as well as scleral tissues. This study provides a novel method to examine and compare immediate effects of RFUVA-catalyzed CS collagen CxL treatment, through changes in collagen fibrils using three investigation assessments. There were two main findings in this study: i) The collagen CxL treatment through RF-sensitized UVA irradiation led to an increase in the area and density of CS stromal collagens, and this change was more pronounced in the scleral stroma than in the corneal stroma, although this increase was not statistically significant in either tissue. ii) The collagen CxL treatment through RF-sensitized UVA irradiation led to a significant increase in the CS biomechanical responses, and this change was more pronounced in the cornea.

Clinically, the collagen CxL treatment with the photosensitizer RF and 370 nm UVA irradiation is used to prevent further corneal stromal thinning associated with keratoconus by increasing corneal rigidity [6,7]. Corneal collagen CxL is a minimally invasive procedure in which a chemical agent is applied to the residual cornea after epithelium removal. This chemical agent initiates the formation of new molecular bonds between the collagen fibrils and lamellae, either by itself or when exposed to UVA light. New bonds likely increase the mechanical strength of the cornea because they can physically link individual collagen fibrils and adjacent lamellae of the corneal stroma. Raiskup-Wolf et al. [6] revealed that patients who have had progressive keratoconus for more than three years show long-term stabilization and improvement after collagen CxL. They reported that a decrease in astigmatism and corneal curvature as well as topographical homogenization of the cornea lead to improvements in vision after the RFUVA-catalyzed collagen CxL treatment. McCall et al. [8] explained the mechanism of collagen CxL with RFUVA, in which the RF-sensitized UVA irradiation generates free radicals and so-called reactive oxygen species (ROS) like superoxide anion (O_2-), hydroxyl radical (•OH) or hydrogen peroxide (H_2O_2) mainly via the so-called type-I pathway of photosensitized oxidation. Wittig-Silva et al. [7] reported that randomized controlled trials show a temporary stabilization of all treated eyes after collagen CxL. Most studies about the collagen CxL treatment of keratoconic progression suggest that the collagen CxL treatment through RF-sensitized UVA irradiation induces an increase in corneal rigidity. These results are consistent with the three findings of this study. The RFUVA-catalyzed collagen CxL treatment led to structural and biomechanical improvements in corneal tissue. This finding suggests that the collagen CxL treatment through RF-sensitized UVA irradiation is an effective treatment against progressive keratoconic cornea.

Stromal thinning and the biomechanical processes of progressive myopia are similar to progressive keratoconus, suggesting the possibility of a novel therapy against progressive myopia. Myopia is one of the most prevalent ocular disorders characterized by a mismatch between the power and axial length of the eye due to scleral thinning and localized ectasia of the posterior sclera [2]. Various clinical treatments, including pharmaceutical agents, progressive addition lenses, rigid gas-permeable (RGP) contact lenses, orthokeratotic lens, and scleral reinforcement surgery, have been proposed to reduce myopic progression, but they did not achieve outstanding results in clinical trials.

The effect of scleral collagen CxL treatment through RF-sensitized photoreaction has been reported in several studies [2]. RFUVA-catalyzed collagen CxL treatment leads to a significant increase in the biomechanical rigidity

efficiency of scleral stromal collagens, which may prevent myopic progression. However, the studies provided no information about the chemical bonding and structural changes in the scleral stromal collagens. A previous study [5] revealed that RFUVA-catalyzed collagen CxL treatment led to changes in the molecular structure and chemical composition of sclera through Raman spectroscopy, but did not provide clear qualitative information about the structural effect of collagen CxL treatment on the sclera through histology and AFM. This study showed that the corneal collagen CxL treatment through RF-sensitized UVA irradiation leads to immediate structural and biomechanical rigidity of the weakened or thinned corneas. This finding also suggests that the collagen CxL treatment through RF-sensitized UVA irradiation is an effective treatment against progressive myopic sclera. As a result, the interaction of the collagen catalyzed by RFUVA from a human cornea or sclera leads to formation of stable CxLs by increasing the number of CxLed collagens. This phenomenon can be identified by an increase in the area and density of CS stromal collagens and the CS adhesion force and stiffness leading to thicker CS tissues. Interestingly, under the experimental conditions in this study, the collagen CxL treatment leads to remarkable structural changes in the sclera and stiffness changes in the cornea.

In this study, the collagen fibrils of CS stroma were examined using three investigation methods. Conventional histological analysis was used to examine structural response of RFUVA-catalyzed CS collagen CxL treatment on human tissues. Novel AFM analysis was used to examine the adhesion force and stiffness of the CS collagen CxL treatment with the photosensitizer RF and 370 nm UVA irradiation. Histology and AFM assessments provided satisfactory results as expected. In particular, AFM analysis with the force-distance curve measurement is reliable and provides promising results.

5 CONCLUSIONS

This study compared the immediate quantitative effects of collagen CxL treatments with photosensitizer RF and 370 nm UVA irradiation on human CS tissues through structural and biomechanical changes in stromal collagen components. The results suggest that although there was not a dramatic improvement in CS tissues, the collagen CxL technique leads to an increase in the density of human CS tissues. This technique can be used for the treatment of progressive keratoconus in the cornea as well as progressive myopia in the sclera. Long-term collagen CxL treatment of keratoconic and myopic progression is certain to dramatically improve weakened CS tissues.

There are some limitations to this study, including the small sample size and the fact that examinations were only made immediately following treatment and not over time. In addition, no biological information was collected. Therefore, further studies are required to evaluate the long-term effects of various clinically-based applications and analyses in the collagen CxL treatment through RF-sensitized UVA irradiation.

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