Analysis of the Change Induced by Riboflavin and Ultraviolet Light on Corneal Collagen by Infrared Spectrometry

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Abstract

Aim: Corneal collagen cross-linking (CCL) is a procedure that exposes the cornea to ultraviolet light and/or riboflavin to halt the progression of corneal ectatic disease. Currently, most investigations using Fourier-transform infrared spectroscopy (FTIR) of corneal changes following CCL focus on corneal ultrastructure, and not on changes at the molecular level. The aim of this study was to investigate the temporal and spatial separation of corneal collagen linkages that underlie the success of CCL.

Materials and methods: Controlled experimental trial. Pairs of donor globes from five patients ($n=10$) were divided into interventional and control groups. Interventional group corneas ($n=5$) were exposed to riboflavin 0.1% and ultraviolet-A (UVA) light according to the modified Dresden protocol, harvested, cryo-microtomed, and placed on glass slides. Control group corneas ($n=5$) underwent cryo-microtoming without CCL. Molecular changes were imaged using the synchrotron mid-infrared beamline at the Canadian Light Source.

Results: Fourier-transform infrared spectroscopy imaging of total protein, integrated area under the amide I band from 1,700 to 1,600 cm⁻¹, FTIR imaging of collagen triple helix structures, second-derivative intensity as 1,666 cm⁻¹, and FTIR imaging of aggregated proteins, second-derivative intensity as 1,625 cm⁻¹ detected no difference in intramolecular cross-links between the interventional and control corneas. The secondary structure of collagen was neither significantly altered nor was their evidence of aggregation or denaturation within the cornea.

Conclusion: Our data suggest that intramolecular cross-linking does not play a major role in CCL and that it is more likely an increase in intermolecular linkages that accounts for increased corneal strength.

Clinical significance: An increase in intermolecular linkages likely accounts for the increased corneal strength observed following CCL. We hope that these results will guide future work to optimize techniques for CCL.

Keywords: Corneal collagen, Corneal collagen cross-linking, Keratoconus, Riboflavin, Ultraviolet light.


Introduction

Keratoconus is a non-inflammatory, progressive, ectatic disease of the cornea resulting in irregular astigmatism and myopia, affecting 1 in 2,000 Canadians.¹ In the normal cornea, collagen cross-linking (CCL) is an anatomical function of collagen fibrils to establish strong adherence to neighboring fibrils. In keratoconus, the structural integrity of the corneal collagen scaffold is weakened, resulting in the focally reduced radius of curvature, abnormal wavefront aberrations, and a reduction in corneal stiffness and thickness. Ultimately, aberrations can result in significant reductions in best-corrected visual acuity, which may eventually require corneal transplantation.²⁻⁵

Corneal CCL is a procedure whereby the photochemical reaction between ultraviolet-A (UVA) light and a photosensitizer, riboflavin, is used to increase the biomechanical rigidity of the cornea. First introduced by Spörl et al.,⁶ this is only the treatment approach capable of significantly altering disease progression and preventing the need for eventual corneal transplantation.⁵,⁶ The current standard approach to CCL is the Dresden protocol, whereby the cornea is treated with 3 mW/cm² UVA intensity over 30 minutes.⁷ Other accelerated protocols have been developed with purportedly equivalent efficacy.⁸⁻⁹

Although CCL is an approved therapeutic method in Canada, USA, and Europe, and the chemical process of cross-linking is well understood, the exact spatial and temporal mechanism of CCL in situ, following treatment with riboflavin and ultraviolet light, is incompletely understood. Previous studies report treatments of riboflavin and UVA light can alter the molecular scaffolding in the cornea; investigators hypothesize that both intermolecular and intramolecular linkages of collagen fibrils are responsible for increased corneal strength posttreatment.¹⁰⁻¹⁵

The synchrotron mid-infrared beamline using Fourier-transform infrared spectroscopy (FTIR) imaging offers the opportunity for direct molecular imaging in situ at the cellular spatial resolution the changes induced by CCL. Previous laboratory-based investigations have focused on the effects of CCL on the corneal ultrastructure; there is a lack of understanding of the specific molecular changes induced by CCL.³,⁷,¹⁰⁻¹¹,¹⁶⁻¹⁷ The main purpose of this study is to investigate the temporal and spatial separation of the collagen linkages in the cornea that underlie the success of this therapy. We further sought to investigate the ultimate depth at which riboflavin...
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and ultraviolet light treatment influence the rigidity of the corneal structure.

**MATERIALS AND METHODS**

**Human Cornea Collection, Dissection, and Accelerated Cross-linking Procedure**

With approval from the Biomedical Research Ethics Board at the University of Saskatchewan, 10 donor globes were provided by the Lions Eye Bank of Saskatchewan from five different donors. Pairs from each donor were split up into control and intervention groups.

The intervention group underwent an accelerated corneal cross-linking procedure (modified Dresden protocol). The epithelium was removed with spears. VibeX Rapid (Simovision BVBA, Overijse, Belgium), formulation of riboflavin (0.1%) was applied until the exposed stroma was completely covered, followed by applications of one drop every 2 minutes for 10 minutes total. Corneal thickness was measured, and the globes were rinsed with a balanced salt solution. UVA treatment was applied using the KXL system (Simovision BVBA, Overijse, Belgium). Four minutes of irradiation at 30 mW/cm² was applied for a total treatment dose of 7.2 J. The cornea was moistened throughout the irradiation procedure with balanced salt solution (BSS) as required. Corneas were then excised from the globes.

**Sample Preparation**

To avoid the introduction of chemical artifacts that can result in sample preparation of biological samples, all cornea tissue immediately flash frozen through immersion in liquid nitrogen-cooled iso-pentane. 10 μm thick sections of the cornea were cut with a cryo-microtome at −18°C; the sections were melted onto the substrate. Two adjacent serial sections were cut for each sample, one melted onto a CaF₂ substrate for FTIR analysis, the other melted on a regular glass microscope slide for routine hematoxylin and eosin (H&E) histology analysis. The tissue sections were air-dried in an ambient laboratory environment at ambient room temperature (−23.5°C) and analysed using FTIR immediately or stained with H&E immediately (within 24 hours of tissue sectioning).

**Validation of UV-treatment Efficacy**

In order to ensure that corneas were treated during the accelerated cross-linking procedure, a separate experiment of the trypsin digestion of collagen was performed. Tissue sections were cut at 10 and 30 μm and treated with trypsin or a blank buffer for either 2, 10, and 30 minutes. Samples were then rinsed in buffer, fixed with 4% buffered formalin and stained with H&E. The intensity of H&E-stained was used as a relative indicator of protein content, that is, the more pink the tissue the more protein content. The aim was to demonstrate that UV-treated samples contained more protein in the sclera relative to the cornea, in images of thin cornea and thick cornea, respectively.

**Validation of Cross-linking Efficacy—Trypsin Test**

The validation protocol demonstrated that collagen content increased with increased tissue thickness, as evidenced by more intense eosin staining. Increased trypsin incubation time decreased corneal collagen content. Both results taken together support the efficacy of the test itself. The CCL-treated corneas stained more intensely than the control corneas, demonstrating that the collagen in the treated corneas is more resistant to digestion (Fig. 1).

**FTIR Imaging of Total Protein in the Cornea Compared with Sclera**

Fourier-transform infrared spectroscopy imaging demonstrated increased protein in the sclera relative to the cornea, in images of thin cornea and thick cornea, respectively.

**Results**

**Validation of Cross-linking Efficacy**

The validation protocol demonstrated that collagen content increased with increased tissue thickness, as evidenced by more intense eosin staining. Increased trypsin incubation time decreased corneal collagen content. Both results taken together support the efficacy of the test itself. The CCL-treated corneas stained more intensely than the control corneas, demonstrating that the collagen in the treated corneas is more resistant to digestion (Fig. 1).
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In brief, FTIR imaging was not able to detect any differences in the collagen structure between untreated and treated corneas. We used trypsin digestion of collagen and H&E histology to confirm that the UV treatment had worked, that is, the collagen in UV-treated corneas was more resistant to trypsin digestion. These results taken together with the FTIR data suggest that CCL improves corneal strength by causing the formation of crosslinks between collagen fibrils (intermolecular bonds) rather than within the same collagen fibril (intramolecular bonds).

This contrasts with previous investigators who hypothesized that both intermolecular and intramolecular linkages of collagen fibrils are responsible for increased corneal strength post-CCL. Specifically, previous reports have suggested that the formation of dityrosine, a molecule capable of forming intermolecular and intramolecular bonds.
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Intramolecular linkages between collagen fibrils, is formed in CCL. However, prior to the present study, investigators have not shown which types of bonds are specifically formed. Furthermore, data from Wollensak et al. demonstrate that CCL does not increase interlamellar cohesive force; taken together with the present findings, it appears that interfibrillar cohesion is the primary mechanism underlying CCL.

In addition, FTIR data suggest that while cross-links between collagen fibrils are being produced by UV treatment, which serves to strengthen the cornea, alterations in the collagen secondary structure are not occurring. This outcome is promising from a health safety perspective, as alterations in the protein secondary structure could potentially lead to a loss of function. This is consistent with previous reports as well.

It is also worth noting that the FTIR techniques used in the present study are highly sensitive for the detection of aggregated and denatured proteins. Collagen cross-linking treatment has the potential to damage a small amount of the collagen protein. By itself, this small amount of damage would not affect corneal clarity, however, as other intact proteins come into contact with protein aggregates, they too may become aggregated or denatured, ultimately affecting corneal clarity. Work published by Xia et al. suggests that CCL can lead to a wound healing response, described as a complex process involving keratocyte apoptosis, proliferation, and migration. Keratocytes are transformed into myofibroblasts, which are intrinsically involved in stromal remodeling, thus serving as major determinants of corneal transparency following CCL.

The primary limitation of this investigation was the inability to detect changes in intermolecular cross-linking directly. The absence of intramolecular bond changes strongly suggests that intermolecular changes must occur. No gradient in the treatment effect was detected, based on the depth at which treated corneal tissue was sampled, thus we were unable to quantify the depth to which CCL treatment penetrates the cornea.

The X-ray scattering beamline may be able to confirm our hypothesis. X-ray scattering can reveal, at atomic resolution, the distribution of atoms in proteins. The cross-linking process may change the X-ray scattering properties of collagen, enabling us to observe where the cross-linking has occurred.

**Conclusion**

Previous studies hypothesize that both intermolecular and intramolecular linkages of collagen fibrils are responsible for increased corneal strength posttreatment. Our data from H&E staining demonstrate that UV treatment strengthens corneal collagen. Fourier-transform infrared spectroscopy data obtained...
suggest that intramolecular cross-linking does not play a major role in CCL and that it is more likely an increase in intermolecular linkages that accounts for the change in corneal strength. Furthermore, FTIR imaging demonstrates that UV treatment does not significantly affect the secondary structure of collagen, nor does it denature collagen or produce protein aggregates in the tissue.

**Clinical Significance**

Findings of the present investigation highlight that an increase in intermolecular linkages between collagen fibrils likely accounts for the increase in corneal strength seen following CCL. We hope our work will guide future investigations in optimizing techniques of corneal CCL to maximize patient outcomes.

**References**

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