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Special Issue

Evaluation of the Physiological Corneal Intrastromal Riboflavin Concentration and the Corneal Elastic Modulus After Violet Light Irradiation

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Purpose: KeraVio is a corneal crosslinking treatment modality that utilizes violet light (VL)-emitting glasses and topical epithelium-on riboflavin administration. We focus on the new KeraVio protocol without riboflavin. This study aims to quantify the physiological intrastromal concentrations of riboflavin in corneas without riboflavin decreases and evaluate the biomechanics of corneas after VL irradiation.

Methods: Twelve human donor corneas were included in this study and randomly categorized into four groups. The corneas underwent four imbibition techniques (physiological riboflavin without drops, epithelial [epi]-on with 0.05% flavin adenine dinucleotide [FAD], epi-off with FAD, and 0.1% riboflavin epi-off). Corneas in the FAD epi-on, FAD epi-off, and riboflavin epi-off groups were instilled with the respective solution every 2 minutes for 30 minutes. An ex vivo experiment was conducted with 24 porcine corneas arranged into three treatment groups and one control group. Corneas in the KeraVio with FAD epi-on group were treated with VL irradiation at 0.31 mW/cm^2 for 4.8 hours (5.4 J/cm^2) and simultaneously received FAD drops every 30 minutes during the VL irradiation. Corneas in the group with KeraVio without FAD epi-on were only treated with VL irradiation (5.4 J/cm^2).

Results: We identified the original physiological riboflavin of human corneal stroma at a concentration of 0.31 $\pm$ 0.03 µg/g, but its value was approximately 39-fold smaller than that in the 0.1% riboflavin epi-off group. The group with KeraVio without FAD and the standard corneal crosslinking group showed a significant increase in biomechanical stability compared with the controls, whereas the elastic modulus in the treated groups was equivalent.

Conclusions: We preliminarily identified physiological riboflavin in human corneas without adding riboflavin drops. The VL exposure may strengthen the corneal biomechanics without requiring the use of additional riboflavin drops.

Translational Relevance: We preliminarily identified physiological riboflavin in the human cornea without adding riboflavin drops. VL irradiation without riboflavin drops may increase the corneal stiffness using physiological riboflavin.

Introduction

Keratoconus (KC) is a progressive, frequently asymmetric, corneal thinning disorder characterized by changes in the structure and organization of corneal collagen.[1,2] It results in corneal thinning and protrusion, progressive myopia, and irregular astigmatism. KC is a common clinical disorder throughout the world with a reported incidence of approximately 1 per

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2000 persons, and there is no sex or race predilection.[3] It is the most common disorder for corneal transplantation in developed countries.[1] Thus, KC has become the focus of extensive clinical and basic research in ophthalmology. The exact etiology of KC remains unknown, although it may involve both genetic and environmental factors.[4,5]

Corneal crosslinking (CXL) was first introduced by Seiler et al.[6] as a promising technique to slow or stop the progression of KC. In CXL, riboflavin (vitamin B_2) is administered in conjunction with ultraviolet A (UVA; 365 nm). The interaction between riboflavin and UVA forms reactive oxygen species, which form additional covalent bonds between collagen molecules and result in the biomechanical stiffening of the cornea. Since the first clinical study of CXL was published by Wollensak et al.,[6] additional studies have been published to report the safety and efficacy of the treatment in slowing down or halting the progression of KC and other corneal ectatic disorders. Standard Dresden CXL received approval from the U.S. Food and Drug Administration for use in the treatment of progressive corneal ectasia in the United States. However, the current method of CXL requires epithelial removal, which is responsible for most of its major complications, including postoperative pain, vision impairment, and an increased risk of infection.[7–9] To avoid these side effects, transepithelial approaches to load riboflavin into the corneal stroma have been proposed.[10] Transepithelial protocols incorporate supplementary topical medications as corneal penetration enhancers to disrupt the corneal epithelium and improve the corneal penetration of riboflavin. Further assistive techniques include the use of iontophoresis and other wearable adjuncts to facilitate the epithelium-on riboflavin administration. Accelerated protocols have been developed to deliver higher UVA irradiance at a shorter duration to decrease patient discomfort. Currently, CXL is not covered by most health insurance providers in many countries, and information regarding its cost effectiveness is scarce. The high financial cost of treatment and high cost of a CXL system have limited the access of ophthalmologists and patients to this effective new treatment.

Recently, we reported a novel technology—KeraVio—consisting of violet light (VL) irradiation and riboflavin treatment in rabbit and human corneas.[11] KeraVio halted the disease progression in eyes with corneal ectasia, which was similar to the outcome of the CXL technique. KeraVio treatment uses an eyeglass with a 375-nm wavelength VL source that transmits this light to the cornea; the patients wear the glass daily without limitations. According to the international lighting vocabulary of the Commission Internationale de l'Eclairage,[12] the lower limits of visible light wavelengths are defined to be 360 to 400 nm, which overlaps with the upper end of the UVA spectrum.[13] This range is visible as VL, but it is also recognized as UVA. KeraVio allows patients to be ambulatory, which decreases patient discomfort during the procedure with no safety concerns, which is not a common feature of all CXL procedures. However, its efficacy has not been compared with that of CXL. To simplify the treatment procedure, we hypothesized that KeraVio use without adding riboflavin might be effective in preventing disease progression if there was original physiological riboflavin in the human cornea. Subsequently, a clinical trial of KeraVio without riboflavin drops was launched (jRCTs032190267). In the present study, to verify the hypothesis in KeraVio, we identified physiological riboflavin in the corneal stroma and compared the concentrations after applying different commercially available riboflavin formulations. Additionally, we evaluated the corneal stiffness after the VL irradiation without riboflavin drops using the KeraVio procedure.

Methods

Riboflavin Concentration

All animals were treated according to The Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research. Twelve human eye bank corneal buttons with scleral rims were included in the study and randomly categorized into four groups. The mean donor age was 56.3 years (range, 52–61 years). The average death-to-tissue-harvest time was 8.5 hours (range, 5–11 hours). The mean storage time (between eye bank procedures and experiments) was 8.2 days (range, 6–12 days). To quantify the physiological riboflavin in the human cornea, three corneas were washed with saline solution (physiological group). We defined the concentrations of physiological riboflavin as the naturally existing concentration in the cornea. The corneoscleral buttons were mounted on an anterior chamber maintainer (Barron artificial anterior chamber; Katena Products Inc, Denville, NJ) filled with corneal storage medium (Optisol; Chiron Ophthalmics, Irvine, CA) to obtain adequate pressure and stability of the corneal tissue. Three corneas received 0.05% flavin adenine dinucleotide (FAD) (Santen Pharmaceutical Co., Ltd., Osaka, Japan) imbibition via a transepithelial approach.[14,15] The FAD solution was formulated

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with ethylenediaminetetra-acetic acid and hydroxypropyl methylcellulose, but the concentrations of these components were not disclosed. FAD is a coenzyme of riboflavin, and its drops have been used historically as the counter ophthalmic solution in Japan. In the current study, we considered that FAD might be functionally equivalent to riboflavin. For one group, FAD drops were applied to the corneal epithelium every 2 minutes for 30 minutes (FAD epi-on group). Three corneas also received 0.05% FAD drops after the epithelial removal, which consisted of removing the central 10 mm of the corneal epithelium and soaking the stroma with 0.05% FAD every 2 minutes for 30 minutes (FAD epi-off group). Similarly, three corneas underwent the same procedure using 0.1% riboflavin solution in 20% dextran (PESCHKE Meditrade GmbH, Huenenberg, Switzerland) every 2 minutes for 30 minutes (riboflavin epi-off group). After exposure to FAD or riboflavin, the anterior surface of the specimens was washed with 10 mL 0.9% NaCl to remove excess riboflavin. Corneas were excised using a manual trepan of 8-mm diameter (Katena Products Inc) and prepared for the high-performance liquid chromatography analysis as described elsewhere in this article.

High-performance liquid chromatography analysis was subsequently performed to quantify the concentration of riboflavin in each volume of tissue. First, 20% ethanol was used to remove the corneal epithelium from the stroma in the physiological and FAD epi-on groups. Then, corneal stromal samples from individual eyes were frozen at −60 °C to −80 °C until analysis with liquid chromatography and mass spectrometry. The samples were weighed, and a solution of 20:80 methanol:water was added to bring the final concentration to 4 mL/1 g sample. Then, the samples were homogenized (Virsonic 100 ultrasonic homogenizer, Virtis, SP Scientific) and stored frozen until analysis. For the liquid chromatography and mass spectrometry analyses, 50-mLaliquots of the corneal homogenate were mixed with 10 mL of 50:50 acetonitrile:water. Proteins were precipitated in 150 mL of acetonitrile with 100 ng/mL warfarin, after which the samples were centrifuged at 13,000 rpm for 10 minutes. The supernatant was aliquoted into 96-well plates for analysis. The control group consisted of untreated human corneas similarly processed to measure the physiological concentrations of riboflavin. In the FAD groups, we quantified the riboflavin concentrations in the corneal stroma instead of the FAD concentration under identical condition to the other groups. high-performance liquid chromatography was performed using a Shimazu LC-VP (Shimazu, Kyoto, Japan). Mass spectrometry was performed using an Xevo TQ-S spectrometer (Waters Corp).

Corneal Elastic Modulus

Porcine corneas with intact epithelium were randomly sorted into four different treatment groups (total $n = 24$). To evaluate the corneal biomechanical properties without epithelial removal and with no drops, KeraVio without FAD was performed ($n = 6$). VL irradiation (375 nm) was applied using a single VL diode (Nitride Semiconductors Co., Ltd., Tokushima, Japan) with an irradiance of 0.31 mW/cm^2 for 4.8 hours at a distance of 60 cm from the cornea (total energy dose 5.4 J/cm^2). To avoid the cytotoxic threshold of the corneal endothelium, which is 0.36 mW/cm^2, we applied a VL intensity of 0.31 mW/cm^2.[16–18] A collection of samples comprising the KeraVio with FAD group was also prepared ($n = 6$). For this group, during the initial 30 minutes of VL irradiation, 0.05% FAD drops were applied simultaneously to the corneal epithelium every 2 minutes. VL irradiation was administered at a similar dose to that administered to the KeraVio group without FAD (0.31 mW/cm^2 for 4.8 hours). Standard CXL equivalent to the Dresden protocol was performed as described previously ($n = 6$).[6] Before the VL irradiation, the epithelium was removed using a hockey knife, corneas were saturated with 0.1% riboflavin solution in 20% dextran (PESCHKE Meditrade GmbH) every 2 minutes for 30 minutes, and the epithelial-off CXL procedure was performed using the VL diode. Corneas in the standard CXL group were irradiated using a total energy dose of 5.4 J/cm^2 (3 mW/cm^2 for 30 minutes). Corneas that received no treatment with VL irradiation or riboflavin delivery served as the controls ($n = 6$).

Corneas from the four groups were allowed to rest in a wet chamber for 30 minutes after the VL or sham VL treatment. The corneas were harvested en bloc along the sclera. A 2- to 3-mm scleral rim was preserved, and the cornea was attached along a custom-made scale. Then, a 5-mm-wide strip was resected vertically along the cornea. The corneal strips were clamped vertically at a distance of 5 mm between the jaws. To calculate the cross-sectional area of the corneal strip, we used the central corneal thickness in each cornea. After the prepared corneal strip was placed on a computer-controlled electronic universal testing machine (TA. XTplusC Texture Analyser, Stable Micro Systems, Ltd., London, UK), a fixture was used to hold the corneoscleral limbus of the corneal strip for a uniaxial tensile test. For the actual measurement, the

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sample was stretched at a velocity of 1.8 mm/min up to a maximum force of 5 N. The elastic modulus was defined as the ratio of tensile stress (amount of force causing deformation per unit trans-sectional area of corneal strips) to tensile strain (percentage change in the length caused by the stress). For the subsequent statistical analysis, the elastic modulus was consistently evaluated at 10% strain.

Statistical Analysis

Statistical analysis was performed using the Statistical Analysis Software (version 9.4; SAS Institute, Cary, NC). Data are expressed as the median and interquartile range and graphed as a box-whisker plot. The preliminary pilot results to compare the difference in riboflavin concentration and elastic modulus in the four treatment groups were tested by Friedman nonparametric test coupled with Scheffe's multiple comparison test. A P value of less than 0.05 was considered statistically significant.

Results

In human corneas, the riboflavin concentrations in the stroma were 0.31 ± 0.03, 2.19 ± 1.02, 6.82 ± 0.79, and 12.16 ± 1.20 µg/g in the physiological riboflavin, FAD epi-on, FAD epi-off, and riboflavin epi-off groups, respectively. Figure 1 shows the mean riboflavin concentration in the excised corneal stroma in these four groups after riboflavin imbibition. Unexpectedly

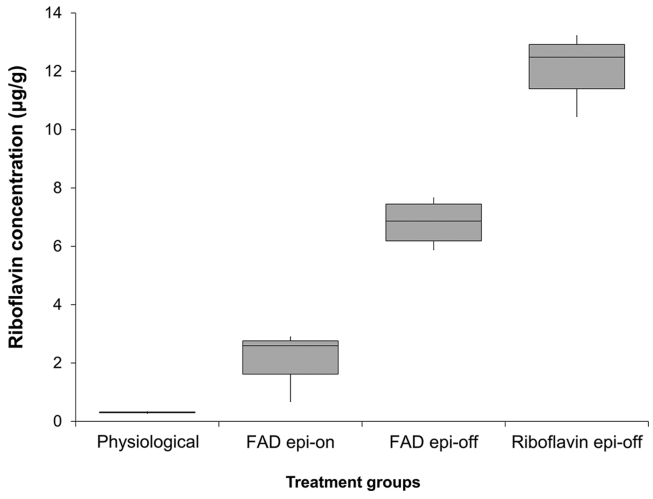

Figure 1. Box plot of the intrastromal riboflavin concentration (µg/g) in each group (physiological, FAD epi-on, FAD epi-off, and riboflavin epi-off). The pilot study results confirm that lower riboflavin concentrations originally existed in the physiological group without adding riboflavin drops, but its concentrations were significantly lower than those in the riboflavin epi-off group.

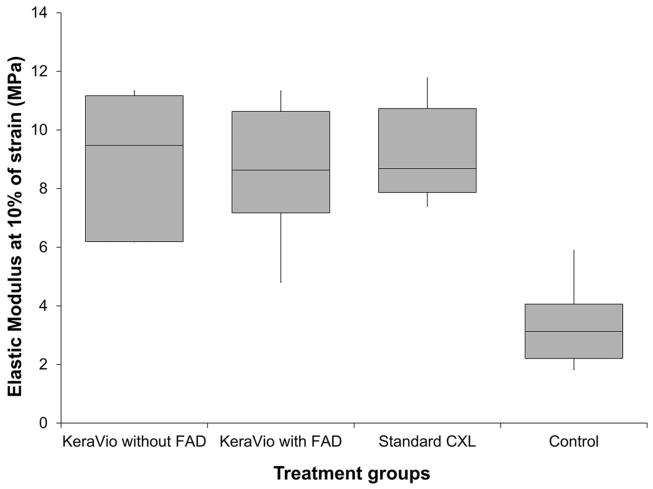

Figure 2. Box plot of the corneal elastic modulus in each group (KeraVio without FAD, KeraVio with FAD, standard CXL, and control). The pilot study results confirm that KeraVio without FAD and standard CXL increased the corneal elastic modulus compared with the control in porcine corneas.

lower riboflavin concentrations were measured in the physiological group. The riboflavin concentration significantly varied among the groups according to the nonparametric Friedman test for the four groups ($P = 0.007$). The P values for the Scheffe multiple comparison between the physiological and riboflavin epi-off groups indicate a significant difference ($P = 0.013$). There was no difference in riboflavin concentration between the physiological and FAD epi-on groups ($P = 0.753$), physiological and FAD epi-off groups ($P = 0.187$), FAD epi-on and FAD epi-off groups ($P = 0.753$), FAD epi-on and riboflavin epi-off groups ($P = 0.187$), and FAD epi-off and riboflavin epi-off groups ($P = 0.753$).

The elastic modulus and percentage strains of the treated corneas at 10% strain were determined for each of the four groups (Fig. 2). The average elastic modulus at 10% strain in the KeraVio without FAD, KeraVio with FAD, standard CXL, and control groups was 8.97 ± 2.39 MPa, 8.53 ± 2.37 MPa, 9.19 ± 1.72 MPa, and 3.37 ± 1.57 MPa, respectively. The elastic modulus at 10% showed a significant difference among the groups according to the nonparametric Friedman test for the four groups ($P = 0.007$). The P values for the Scheffe multiple comparison between the group with KeraVio without FAD and the control groups indicate a significant difference ($P = 0.038$). Additionally, the P values for the Scheffe multiple comparison between the standard CXL and the control groups demonstrate a significant difference ($P = 0.020$). There was no difference in the elastic modulus at 10% between the groups with KeraVio with and without FAD

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($P = 0.849$), the group with KeraVio without FAD and the standard CXL group ($P = 0.997$), the group with KeraVio with FAD and the standard CXL group ($P = 0.741$), and the group with KeraVio with FAD and the control group ($P = 0.256$).

Discussion

After a decade of clinical and experimental studies, it is well-known that a sufficient concentration of riboflavin in the corneal stroma is crucial to obtain a comparable biomechanical effect to corneal CXL.[19,20] Because riboflavin cannot penetrate an intact corneal epithelium owing to its chemical properties, the central corneal epithelium is mechanically debrided in current standard CXL (epi-off) techniques to enable sufficient riboflavin stromal imbibition.[21] However, the epithelial removal induces various side effects, including postoperative pain and visual decline after the procedure. Furthermore, it can predispose patients to serious corneal infections and loss of corneal transparency owing to abnormal corneal stromal scarring processes.[21] Thus, in recent years, much effort has been put into developing new efficient transepithelial riboflavin penetration techniques. We reported a novel technology, KeraVio, which consisted of VL irradiation and riboflavin treatment in human corneas.[11] KeraVio halted disease progression in eyes with corneal ectasia, which was similar to the outcome of the CXL technique. The topical epithelium-on riboflavin administration might have an impact on corneal cross-linking and its concentration of corneal stroma was observed in the current study. The KeraVio treatment uses an eyeglass with a 375-nm wavelength VL source to apply light to the cornea. The patients wore the eyeglass daily without limitations. The KeraVio treatment avoids the complications of CXL surgery and may become another option to treat KC, but its efficacy has not been compared with that of CXL. In the current study, we preliminarily identified physiological riboflavin in the human cornea without adding riboflavin drops. No studies investigated the association between oral riboflavin and physiological riboflavin concentrations in the corneal stroma. Recently, some clinicians reported a possible solution, where high doses of oral riboflavin combined with sunlight could similarly stabilize the cornea to CXL treatment. In a small, unpublished study, participants with KC who took dietary riboflavin and spent time outside achieved corneal stabilization and/or flattening.[22] The strategy might enhance the impact of the proposed natural cross-linking technique. As for oral supplementation, there were no studies investigating the riboflavin concentrations in the corneal stroma after oral administration. In our study, a relatively low intensity of VL irradiation strengthened the corneal stiffness in porcine corneas. In a normal eye, VL is absorbed by the cornea, which contains physiological riboflavin and other photosensitizers, leading to CXL in the cornea. If physiological riboflavin originally exists in the human cornea, VL irradiation may strengthen the corneal stiffness without adding riboflavin drops in the KeraVio technique. To our knowledge, this study is the first to quantify the concentration of physiological corneal riboflavin in humans and compare the corneal elastic modulus between treatments with and without drops. A recent clinical trial of KeraVio without riboflavin drops was launched (jRCTs032190267).

The corneal epithelium is the critical obstacle to the permeation of riboflavin into the corneal stroma; it affects the corneal stiffness because a complete and intact epithelial layer is a tough lipophilic barrier to water-soluble riboflavin.[23] FAD drops (0.05%) were administered every 2 minutes for 30 minutes; the drops contained EDTA, which increases the epithelial permeability and topical medication bioavailability to the corneal stroma.[24] In this study, the FAD epi-on group achieved a certain riboflavin concentration, but no significant difference in its concentration was found compared with the other three groups. The presence of riboflavin in the FAD epi-on group may be attributed to the EDTA and deterioration of donor epithelium over time. In the FAD groups, we focused on the riboflavin concentration in the cornea, not the FAD concentration, in terms of the mechanism of CXL. Comparing the riboflavin concentration between the FAD and control physiological groups, the values in the FAD epi-on and epi-off were slightly higher than those in the control. Thus, FAD drops have a role in riboflavin. In a clinical setting in Japanese ophthalmology, FAD drops are popular over the counter. When we submit KeraVio therapy to regulatory authorities in Japan, FAD drops can easily start a clinical trial using drugs and devices.

In the current study, the KeraVio without FAD and standard CXL groups had a significantly higher elastic modulus at 10% compared with the control group, whereas no significant difference between KeraVio and CXL groups was found. The presented data show the dependence of the change in corneal stiffness on the illumination intensity for a constant irradiation dose of 5.4 J/cm^2 in these treatment groups. Our results indicate that the Bunsen–Roscoe law is applicable to both KeraVio and CXL treatments. The

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Bunsen–Roscoe law describes the photochemical reaction of a material to a certain energy dose. However, Hammer et al.[25] concluded that the stiffening effect of CXL decreases with increasing irradiance and decreased treatment times. Regarding the impact of KeraVio without FAD on the cornea, no significant difference was shown in the corneal elastic modulus at 10% between groups with KeraVio with and without FAD. We suggest that VL irradiation without FAD drops may increase the corneal stiffness using physiological riboflavin. Further study comparing the clinical parameters is required to confirm our findings. To explain the similar mechanical stiffness effects of the group with KeraVio without drops (VL irradiation only) and those with KeraVio with FAD and with standard CXL, we assume that the concentrations of physiological corneal riboflavin in KeraVio without drops may achieve the adequate value. In the previous literature, the threshold concentrations in riboflavin-photosensitized protein crosslinking are unknown. We hypothesize that lower irradiance of VL activates corneal biomechanical properties under sufficient riboflavin concentrations. We are testing the minimum riboflavin concentrations in human cornea by dividing into different concentration groups.

A greater dispersion of the corneal elastic modulus was found in the KeraVio groups than in the control group. Generally, pretreatment tests are characterized by a wide dispersion because of natural variability. In contrast, post-CXL tests are characterized by low to moderate dispersion around the average.[26] The discrepancy may be attributed to the differences in irradiance of VL in the KeraVio procedure (0.31 mW/cm^2 for 4.8 hours). Long-term irradiation of VL on the cornea can induce degradation over time in an ex vivo porcine eye model. In addition, we are evaluating the corneal elastic modulus in the eyes of another control group with natural aging (observing 4.8 hours without drops and VL). We should have involved human corneas in biomechanical testing. However, similar studies used porcine corneas to evaluate the corneal stiffness after treatments.[25,27,28] Thus, the porcine corneas were involved for the biomechanical assessment in the present study.

In conclusion, we report that there is originally a small amount of riboflavin in the human corneal stroma, and the ex vivo corneal stiffness significantly increases after VL irradiation based on physiological riboflavin without drops. Thus, the VL exposure may strengthen the corneal biomechanics without the use of additional riboflavin drops. Whether the change in biomechanical stiffness may be clinically relevant to the KeraVio trial in humans using lower irradiance of VL is currently unknown. If KeraVio without riboflavin drops is effective for halting the disease progression in KC, it may be a novel invasive treatment option for those patients. Further long-term follow-up studies are required to confirm these findings.

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