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Photochemical activation increases the porcine corneal stiffness and resistance to collagenase digestion

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ABSTRACT

In this study, we explore the effect of photochemical activation induced corneal cross-linking, utilizing Rose Bengal (RB) and 532 nm green light irradiation (RB-PCL), on porcine corneal biomechanical rigidity and the biochemical resistance against collagenase digestion. A protocol with a wavelength of 532 nm and illumination intensity of 0.4W/cm² for 250 s to deliver a dose of 100 J/cm² was chosen. Using confocal microscopy, we demonstrated that the diffusion depth of RB into porcine cornea was approximately 150 µm and mostly localized in anterior stroma 25 min followed by RB application. After photochemical cross-linking, an increase in tensile strength (by average 200%) and Young's modulus (by average 200%) in porcine corneas was observed. The corneal buttons treated by RB-PCL showed doubling of collagenase digestion time from 10.8 \pm 3.1 days in the blank group to 19.7 \pm 6.2 days in the RB-PCL group, indicating increased resistance to enzymatic digestion. In conclusion, Collagen cross-linking by RB-PCL increased both the biomechanical stiffness and the biochemical resistance against collagenase digestion in porcine corneas, therefore to allow stabilizing and solidifier the cornea. The advantages and disadvantages of RB-PCL versus UVA/riboflavin cross-linking technique (UV-CXL) are fully explored. Due to the nature of minimal penetration of RB into corneal stroma, the RB-PCL method could potentially be used in patients with corneal thickness less than 400 µm where UV-CXL is limited.

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1. Introduction

Corneal ectasia, dilation or distention of cornea, represents a group of disorders with inherent corneal weakness and instability leading to protrusion, astigmatism, substantial distortion of vision, and potentially even perforation (Tomkins and Garzozi, 2008). Keratoconus is the primary cause of corneal ectasia and the prevalence in general population is 50-200 per 100 000 (Randleman et al., 2003). It's a condition characterized by breakdown of the corneal stroma resulting in a reduction in biomechanical strength and biochemical resistance. This leads to asymmetric protrusion of cornea rather than its normal gradual curve. In addition to

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http://dx.doi.org/10.1016/j.exer.2014.04.008 0014-4835/© 2014 Elsevier Ltd. All rights reserved. keratoconus, corneal ectasia can come from refractive eye surgery, specifically LASIK (Randleman et al., 2003, 2008). Traditional treatment for keratoconus includes spectacles, rigid gas permeable contact lenses, which only achieves refractive correction purposes rather to stop the progression of the disease (Dana et al., 1992; McMonnies, 2005). Further progression of the disease requires intra-stromal implants (Colin et al., 2000), mini asymmetric radial keratotomy. Approximately 20% of keratoconus patients eventually need a corneal transplantation (Dhaliwal and Kaufman, 2009; Rabinowitz, 1998; Tuft et al., 1994) and the shortage of donor corneas is currently a critical issue. However, these invasive treatments are not only costly but also associated with several complications and risks. A minimal-invasive treatment option that addresses the underlying pathogenesis of the disease is necessary.

In the last decade, corneal collagen cross-linking (CXL) technique involving the use of riboflavin photo-reactive dyes in conjunction with ultraviolet (UV) irradiation has been successfully implemented in clinical trials (Ashar and Vadavalli, 2010;

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Wollensak et al., 2003a). The riboflavin causes new bonds to form across adjacent collagen strands in the stromal of the cornea, which restores some of the mechanical strength of the corneal tissue therefore to slow or arrest the progressive thinning of the cornea (Wollensak et al., 2003b, 2004c). However, some drawbacks are associated with this technique including a long procedure time and cytotoxicity to keratocytes (Kruger et al., 2011; Wollensak et al., 2004a, 2003a; 2004b).

Therefore we explored an alternative light-activated tissue bonding technique, named photochemical tissue bonding (PTB), as a potential treatment option for progressive keratoconus or other corneal ectasia disorders. PTB, as a photochemical activation method, has been developed for sealing wounds in cornea and many other tissues, utilizing Rose Bengal (RB) as a light-activated dye and low dose 532 nm green light irradiation (Chan et al., 2002; Kamegaya et al., 2005; Mulroy et al., 2000). After absorbing light, RB initiates chemical reactions that lead to the formation of covalent cross-links between collagen molecules (Wachter et al., 2003). To investigate the effects of photochemical activation induced corneal cross-linking (RB-PCL) treatment on biomechanical and biochemistry properties of corneas, we evaluated porcine corneal biomechanical rigidity and biochemical resistance against enzymatic digestion by collagenase. Corneal rigidity was assessed using Young's modulus, known as the elastic modulus, a measure of the stiffness of an elastic material. Tensile test was conducted on corneal strips to generate a stress-strain curve, and Young's modulus was determined experimentally. It has been suggested that collagenase contributes to the break-down of the collagen cross-linkages in the stroma (Critchfield et al., 1988). Therefore, collagenase digestion was performed on photochemical crosslinking treated and control porcine corneas.

2. Materials and methods

2.1. Reagents and instruments

0.1% (w/v) Rose Bengal (RB, C₂₀H₂Cl₄I₄Na₂O₅) was made with RB (95%, w/v) (Sigma–Aldrich Co., St. Louis, MO) and Phosphate Buffer Solution (PBS) as a photosensitizing dye for RB-PCL.0.1% (w/v) Collagenase type 2 (Worthington Biochemical Co., Lakewood, NJ, USA) was diluted to 0.02% (w/v) with PBS, for enzymatic digesting corneas. Nd:YAG 532 nm green light (LRS-0532-PFH-01000-05) was provided by Green light glow Technologies (West Toronto, Canada). The green light output power was calibrated using Field master power meter (Coherent Inc., CA, USA). Zeiss Confocal Scanning Microscope (Zeiss LSM 710 NLO, Carl Zeiss Microscopy Co. Germany) was used for detecting the diffusion depth of RB into corneal stroma. Dynamic Mechanical Analyzer (DMA Q800, TA Instruments, New Castle DE, USA) was used for the stress–strain measurements of corneal strips.

2.2. Eye specimens

Fresh porcine cadaver eyes with intact epithelia and clear corneas were obtained from the local abattoir within 5–6 h postmortem. The eyes were de-epithelialized mechanically with a blunt hockey knife. The diffusion depth of RB into corneal stroma was investigated using confocal microscopy on selected eyes. Eighty eyes were randomly divided into two groups. One is for stress–strain measurements (n = 36) and another is for collagenase digestion (n = 44). The removal of corneal epithelial layer is to increase penetration of the RB into the stroma. For each of the two groups eyes were further divided into four sub-groups (n = 9 each for stress–strain measurements, n = 11 each for collagenase digestion): RB-PCL, green light only, RB only and blank. The central

corneal thickness of each eye was determined with ultrasound pachymetry (PacScan 300 AP, Sonomed, Inc., USA).

2.3. Confocal microscopy

0.1% (w/v) RB was applied to the de-epithelial corneas for four times, once every five minutes, and then allowed to absorb for 10 min in a dark and moist containers in 37 °C water bath. After removing excess RB by PBS rinse three times, a 5 mm by 5 mm disc of central cornea was taken and placed in optical cutting temperature compound using TBS Tissue Freezing Medium (TBS Tissue Freezing Medium, Triangle Biomedical Sciences, USA). Frozen tissue was cut vertically with 8 µm thickness using Leica CM 1850 (Leica Microsystems Nussloch GmbH, Germany). Section was then dried on slides, rinsed and cover-slipped. The RB fluorescence was excited at 559 nm and emission detected at 575-620 nm Zeiss Confocal Scanning Microscope. The RB intensity profile was determined by averaging the signal along 25 lines drawn perpendicular to the stroma surface using Image] software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsbweb.nih.gov/ij/index. html).

2.4. Photochemical cross linking procedure

For the RB-PCL group, the photochemical cross-linking treatment was carried out as followings: 0.1% (w/v) RB was applied in droplet to the corneal surface for four times, once every five minutes, and then allowed to be absorbed for 10 min. During this time, we maintained porcine eyes at dark and moist containers, which were kept in 37 °C water bath. This is to maintain the cornea at the physiological condition and avoid RB degradation upon exposure to the light. Immediately after RB saturation, the eyes were removed from the water bath and excess dye was removed by blotting. The cornea was then exposed to 532 nm green light provided by Nd:YAG green light. The green light was placed on the center area above the cornea. The light reached vertically to the corneal surface with a circular shape (12 mm diameter). A fluence of 100 J/cm^2 was delivered using an irradiance of 0.4 W/cm² for 250 s (The irradiation parameters above were chosen according to the literature review and preliminary experiments)(Cherfan et al., 2013; Gu et al., 2011; Wang et al., 2011; Yao et al., 2010). The power of each light source was measured with a power meter.

The other three control groups were set up as the green light only, the RB only and the blank. For the corneas in the green light group, 0.9% sodium chloride solution was applied to the corneal surface with subsequent application of green light irradiance. The corneas in the RB group were saturated by 0.1% RB without subsequent green light treatment, whereas 0.9% sodium chloride solution was applied to the corneas of the blank group.

2.5. Measurement of corneal surface temperature before and after RB-PCL treatment

A non-contact infrared thermometer (Rycom RC001, Rycom Electron Technology Ltd, Guangzhou, China) was used to measure the corneal surface temperature for all RB-PCL treated corneas. Temperature was measured three times per cornea at room temperature of 25 $^{\circ}$ C with humidity of 45%.

2.6. Stress-strain measurements

There were 36 eyes for stress-strain measurements. After treatments, under the surgical microscopy, a corneal strip of

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2.9. Statistics

5.0 mm width and 14.0 mm length with 1.0 mm sclera on both ends was cut in a superior-inferior fashion at the 12 o'clock position of the cornea, which was easily identified by its oval shape. The corneal strips were clamped vertically at a distance of 8.0 mm between the tension clamps of the Dynamic Mechanical Analyzer (DMA) for the stress-strain measurements (The temperature and humidity inside the DMA were kept at 37 °C and 45%)(Fig. 1). To minimize hydration of specimen and limit the solution influence on the tensile behavior, the corneal strips were exposed to air during the experiment. A pre-stress of 5×10^3 Pa (1 Pa = 1 N/m²) or a force of 20 mN was used to introduce the physiological stress range. Then the strain increased linearly with a velocity of 1.5 mm min⁻¹ by up to 9 mm (1 mm/8 mm = 12.5%) or by 12.5% (Wollensak et al., 2003b). The stress was measured accordingly. The stress-strain values were fitted by an exponential function $\sigma = A \exp(B \times \varepsilon)$ using the 1st Opt software (7D-Soft High Technology Inc). Young's modulus (E) was calculated for 0%, 2%,4%, 6%, 8% and 10% strain as the gradient of the stress-strain graph $[E = d\sigma/d\varepsilon = A \times B]$ $\exp(B \times \varepsilon)$; σ : strain, ε : stress, A and B: constant].

2.7. Collagenase digestion

For the 44 eyes in the collagenase type 2 digestion group, 10 mm central corneal buttons were trephined and incubated with 0.02% (w/v) collagenase in phosphate buffer saline in a condition of 37 °C, 5% CO² and 95% humidity. Collagenase solutions were changed every other day. The extent of the corneal digestion was monitored daily. Complete digestion was defined, as there was no visible specimen piece in a container. The time it took for complete digestion for each corneal button was recorded.

2.8. Histology

Forty-eight hours after collagenase digestion, randomly selected corneal buttons from different groups were fixed in 4% paraformaldehyde formalin and embedded in paraffin. 4 µm thick paraffin sections were stained with hematoxylin/eosin (HE) and evaluated using a light microscope (CX41, Olympus, Japan).



Fig. 1. Stress-strain measurement of corneal strip. The corneal strip between the tension clamps (arrows) of the Dynamic Mechanical Analyzer (DMA800) for the stress-strain measurement.

Data were summarized as mean \pm standard deviation. The statistical analysis of the results on the central corneal thickness, the stress of the corneal strips, Young's modulus, the digestion times and corneal surface temperatures were performed by oneway analysis of variance (ANOVA) using SPSS 13.0 (SPSS Inc., USA); P < 0.05 was considered to be significant.

3. Results

3.1. Central corneal thickness

The mean central corneal thickness of all the porcine eyes was $806 \pm 52 \,\mu\text{m}$. There was no significant difference among the eight groups (P > 0.05 each).

3.2. Diffusion depth of Rose Bengal into the cornea stroma

To investigate the depth of photochemical activation induced corneal cross-linking, we used confocal microscopy to determine the distance that RB diffuses into the stroma 25 min after RB application. Along the lines perpendicular to the stroma surface, the profile of RB intensity showed that the majority of RB fluorescence was approximately localized within 180 µm from the corneal surface using a threshold of 10% of the maximum intensity value. Correcting this value for the increase in thickness due to freezing (to 980 µm from 806 µm), indicates that RB actually diffuses approximately 150 µm into the porcine stroma. The results showed that RB localized near anterior stroma (Fig. 2A, B).

3.3. Corneal surface temperatures before and after RB-PCL treatment

The average measured temperature on the porcine corneal surface was 34.80 \pm 0.34 °C before the RB-PCL and 36.91 \pm 0.78 °C after the RB-PCL (n = 20, P < 0.05). RB-PCL treatment increased the surface temperature by 2.11 \pm 0.85 °C.

3.4. Stress-strain curve and Young's modulus

The stress-strain measurement was performed in air within a humidity and temperature-controlled chamber. The droplet application of RB solution, rather than immersing corneal strips into RB solution, minimizes hydration of specimen and limits the solution influence on the tensile behavior. Stress values and calculated Young's modulus of corneas for different strains were described in Table 1. The measured stress-strain curves are typical of a bioviscoelastic solid, and strain increases exponentially with stress (Fig. 3A). At 2% strain, the stress was 12.71 ± 2.31 kPa in the RB-PCL group, which was significantly different from the blank group (P < 0.05). Although the differences were not statistically significant (P > 0.05 each) compared with the RB group and the green light group at 2% strain, RB-PCL treated corneas showed rapid increase at 4%, 6%, 8% and 10% strain with stresses of 33.52 ± 6.42 kPa, 75.98 \pm 25.64 kPa, 141.57 \pm 55.75 kPa and 223.14 \pm 93.31 kPa, respectively (Table 1), which differed significantly (P < 0.05 each) from the other three groups.

To calculate Young's modulus, the stress-strain values were fitted with an exponential function $\sigma = A \exp(B \times \varepsilon)$. The first derivation of this function in a definite strain is Young's modulus $E = d\sigma/d\varepsilon = A \times B \exp(B \times \varepsilon)$. For the RB-PCL treated corneas at 0%, 2%, 4%, 6%, 8% and 10% strain, the Young's modulus were $0.47 \pm 0.12 \times 10^{6}$ Pa, $0.72 \pm 0.22 \times 10^{6}$ Pa, $1.18 \pm 0.43 \times 10^{6}$ Pa, $2.00\pm0.93\times10^{6}$ Pa, $3.44\pm1.96\times10^{6}$ Pa and $5.95\pm4.16\times10^{6}$ Pa,

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Fig. 2. Diffusion depth of RB into porcine corneal stroma. A. Frozen section of porcine cornea stained on anterior surface with fluorescence of RB (red), Scale bar: 100 μm; B. RB fluorescence intensity as a function of distance from stroma anterior surface; measured on frozen section shown in (A).

respectively (Fig. 3B). At 10% strain, the treated corneas showed the most substantial increase in Young's modulus with 206%, 273% and 191% increment comparing with the blank group, the RB group and the green light group. Compared with other three groups, the RB-PCL group showed statistically significant difference (P < 0.05 each) at all strains. In contrast, there were no statistically significant difference among the blank group, the RB group and the green light group (P > 0.05 each). The increased biomechanical stiffness was significant in photochemical activation treated corneas. Neither green light nor RB dye was effective independently.

In Fig. 4, corneal strips were held horizontally (4A) and vertically (4B). RB-PCL corneal strips (Right) showed preserved corneal curvature in comparison with the control corneal strips (blank group) (Left) due to the increase in bending stiffness by collagen cross-linking. Both corneal strips have the same dimensions.

3.5. Enzymatic digestion

In the collagenase solution, the posterior curvature of the corneal buttons was lost first in RB-PCL groups after two days, in other three groups after one day of digestion, while the anterior curvature of the cornea remained visible during the first couple of days of digestion. Significant swelling was observed in other three groups while it remained less prominent in RB-PCL group. The resistance of cornea against enzymatic digestion was determined by the time it took for compete digestion. Button diameters were not used due to swelling and deformation of the specimen. Complete digestion was found in the RB-PCL group after 12–29 days, in the other three groups after 6–19 days. The average time needed

for complete digestion is 19.7 \pm 6.2 days in the RB-PCL group, 11.8 \pm 3.7 days in the green light group, 11.3 \pm 2.4 days in the RB group and 10.8 \pm 3.1 days in the blank group. Comparing the RB-PCL group with the other three groups, the differences in collage-nase digestion were remarkable and statistically significant (*P* < 0.05 each). Whereas, no significant difference was observed among the green light group, the RB group and the blank group (*P* > 0.05 each).

3.6. Histology

Forty-eight hours after enzymatic digestion, corneal buttons was subjected to histology examination. In Fig. 5, digestion of posterior portion in the blank control group (5A) was more obvious with loss of parallel collagen fiber array, compared with the cross-linked RB-PCL group (5B). The anterior stroma remained relatively intact at this time.

4. Discussion

In the present study, we demonstrated significant increases of tensile strength and modulus by a factor of 2 at various stress level in porcine corneas after RB-PCL treatment (see Table 1). Porcine eyes with mean central corneal thickness of 806 μ m were treated with RB and irradiated with 532 nm green light (0.4 W/cm²) for 250 s. Rose Bengal, a photosensitizer, can be activated by absorbing photons from the 532 nm green light. Upon excitation, RB converts to its triplet state and then transfers the energy to tissue oxygen to form reactive oxygen species (ROS). ROS reacts with surrounding

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Stress values and calculated Young's modulus for different strains.

| | Blank group | RB group | Green light group | RB-PCL group |
|---|-----------------------------------|----------------------------|-----------------------------------|------------------------------------|
| Stress at 0% (10 ³ Pa) | $\textbf{4.46} \pm \textbf{2.71}$ | 5.25 ± 3.78 | $\textbf{4.49} \pm \textbf{1.78}$ | 4.96 ± 1.54 |
| Young's modulus at 0% (10 ⁶ Pa) | $0.23\pm0.10^{\rm c}$ | $0.27\pm0.16^{\rm b}$ | 0.32 ± 0.18^{a} | $0.47\pm0.12^{a,b,c}$ |
| Stress at 2% (10 ³ Pa) | $8.80 \pm \mathbf{2.82^c}$ | 10.38 ± 5.81 | 10.84 ± 4.26 | $12.71 \pm 2.31^{\circ}$ |
| Young's modulus at 2% (10 ⁶ Pa) | $0.36\pm0.18^{\circ}$ | $0.38\pm0.20^{\rm b}$ | $0.46\pm0.23^{\rm a}$ | $0.72\pm0.22^{a,b,c}$ |
| Stress at 4% (10 ³ Pa) | 18.91 ± 6.84^{c} | 22.31 ± 10.69^{b} | 24.67 ± 9.38^a | $33.52 \pm 6.42^{a,b,c}$ |
| Young's modulus at 4% (10 ⁶ Pa) | $0.59\pm0.35^{\circ}$ | $0.58\pm0.31^{\rm b}$ | $0.73\pm0.36^{\rm a}$ | $1.18\pm0.43^{\rm a,b,c}$ |
| Stress at 6% (10 ³ Pa) | $39.56 \pm 19.74^{\circ}$ | 41.39 ± 20.99^{b} | 48.67 ± 20.86^a | $75.98 \pm 25.64^{a,b,}$ |
| Young's modulus at 6% (10 ⁶ Pa) | $0.99\pm0.68^{\rm c}$ | $0.89\pm0.51^{\rm b}$ | 1.15 ± 0.62^{a} | $2.00 \pm 0.93^{a,b,c}$ |
| Stress at 8% (10 ³ Pa) | $70.17\pm42.17^{\circ}$ | 67.09 ± 35.69^{b} | 86.75 ± 42.33^{a} | $141.57 \pm 55.75^{\mathrm{a,b,}}$ |
| Young's modulus at 8% (10 ⁶ Pa) | $1.68 \pm 1.35^{\circ}$ | $1.38\pm0.83^{\rm b}$ | 1.90 ± 1.13^{a} | $3.44 \pm 1.96^{a,b,c}$ |
| Stress at 10% (10 ³ Pa) | $110.40 \pm 74.26^{\circ}$ | $102.73 \pm 58.58^{\rm b}$ | 134.74 ± 72.76^{a} | $223.14 \pm 93.31^{a,b,}$ |
| Young's modulus at 10% (10 ⁶ Pa) | $2.89 \pm 2.60^{\rm c}$ | $2.18 \pm 1.46^{\rm b}$ | 3.11 ± 2.12^{a} | $5.95 \pm 4.16^{a,b,c}$ |

^a Indicates P < 0.05 compared RB-PCL group with green light only group.

^b Indicates P < 0.05 compared RB-PCL group with RB only group.

 $^{\rm c}$ Indicates P < 0.05 compared RB-PCL group with blank control group.

molecules such as amino acids, forming covalent collagen crosslinks (Wachter et al., 2003). Using scanning electron microscopy (SEM) analysis, the study of Chan et al. (2008, 2007) and Chan and So (2005) revealed that the photochemical cross-linked collagen gel showed fine microstructure with interconnected Nano sized fibers forming micron-sized pores, whereas the uncross-linked control had macro-porous structures with sheet-like structures. The results provided the molecular basis of observed increased biomechanical rigidity after collagen cross-linking in porcine corneas.

Increase of corneal stiffness becomes the most informative parameter for clinical application and has been widely evaluated in many crosslinking studies (Kling et al., 2010; Wollensak et al., 2003b). The effect of RB-PCL treatment induced corneal stiffness was also studied in rabbit eyes by Cherfan et al. (2013). Although their study protocol differs in several ways from our protocol including sample choices, RB application time, light exposure and dosage delivered, both studies reached the same conclusion of increased corneal stiffness, which independently confirmed the effect of RB-PCL method. We chose porcine eyes with mean central corneal thickness of 800 μ m, while Cherfan used rabbit eyes with mean central corneal thickness of 400 μ m. The average central corneal thickness of human eyes is around 550 μ m. Thus the study results from the two different species are valuable for further application in human.

Increased collagenase and proteolytic activity have been indicated to play a key role in the breakdown of some collagen crosslinkages in the corneal stroma, which ultimately results in biomechanical weakness and instability (Abalain et al., 2000; Zhou et al., 1998). Therefore, we investigated the effect of RB-PCL on biochemical resistance against collagenase digestion. This is the first time to report a doubling of collagenase digestion time from 10.8 ± 3.1 days in the control group to 19.7 ± 6.2 days in the RB-PCL treated corneas using RB-PCL technique. During the course of digestion, the anterior curvature of the cornea was more durable than the posterior cornea, which is consistent with confocal microscopy results that the RB penetration, therefore collagen crosslinkage, occurred mostly near anterior stroma. According to the histology examination at 48 h after enzymatic digestion, the blank group demonstrated more obvious digestion than the cross-linked RB-PCL group did. This is likely due to the increased resistance to enzymatic digestion after the cross linkage. The increase in biomechanical stiffness and resistance against collagenase digestion in porcine corneas were depended on both RB and light absorption. Neither RB nor light irradiation was effective independently. Our data suggests potential applications in treatment of corneal ectasia disorders such as ulcers or melting processes.

In comparison with RB-PCL method, other cross-linking technique, such as riboflavin and UVA light (UV-CXL) with a wavelength of 370 nm and illumination intensity of 3 mW/cm² for 30 min, is a well-established approach in the treatment of corneal ectasia diseases. However, one disadvantage of the UV-CXL procedure is the long treatment time of 1 h including a soaking time of 30 min for the riboflavin solution and an illumination time of 30 min for the UV light. Whereas cross-linking method using RB and green light was rapid with 250 s irradiation, which could increase patient's comfort and the doctor's patient throughput. To investigate whether a shorter UV-CXL procedure could achieve the same increase in the mechanical and biochemical stability of stromal tissue, it has been demonstrated that an equivalent stiffness increase could be achieved using higher UV irradiation intensity and shorter irradiation time (Schumacher et al., 2011; Wernli et al., 2013). The damage mechanism from the UV light not only depends on the irradiation dosage, but also the irradiation intensity and time. The safety of the ocular, especially the endothelium susceptibility, at a well above standard irradiation intensity is a concern and warrants further investigation (Schumacher et al., 2011; Wernli et al., 2013). In addition to the long treatment time, another limitation for riboflavin/UVA treatment is the minimal thickness of 400 µm required for the treated cornea. With a corneal thickness of less than 400 µm, corneal endothelium or deeper structures will be damaged using this standard procedure (Wollensak et al., 2003c, 2003d). However, progressive corneal thinning often leads to a remaining stromal thickness of less than 400 µm in advanced keratoconus (Wollensak et al., 2003c). For these patients, riboflavin/UVA treatment is impossible. Recently, Hafezi et al. have tried to apply hypoosmolar riboflavin solution to thin cornea using UV-CXL, however, a minimal thickness of 330 µm is still desired (Hafezi, 2011; Hafezi et al., 2009).

Given the limitations of this, albeit widely accepted, UV-CXL technique, we explored an alternative cross-linking approach using Rose Bengal (RB) with a wavelength of 532 nm and irradiation dose of 100 J/cm² (irradiation of 0.4 J/cm² for 250 s) in porcine cornea. RB is a large molecule (molar mass of 973 g/mol) in comparison with riboflavin (molar mass of 376 g/mol). The diffusion coefficient of riboflavin is $D = 6 \times 10^{-7} \text{ cm}^2/\text{s}$ and of Rose Bengal it is $D = 5 \times 10^{-8}$ cm²/s, which means the diffusion of Rose Bengal in the cornea is much lower because the depth can be calculated by an exponential function. Herein, we demonstrated that the diffusion depth of RB into porcine cornea is approximately 150 µm and mostly localized in anterior stroma by using confocal microscopy. Studies from Cherfan et al. using RB-PCL showed the consistent result that RB was concentrated in the anterior corneal stroma of rabbit (approximately 100 µm from corneal surface)(Cherfan et al., 2013). We think the short distance that RB diffuses into the corneal

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Fig. 3. Stress–strain curve and Young's modulus A. Stress–strain curve of porcine corneas; B. Young's modulus–strain curve of porcine corneas.

stroma is actually an advantage. Firstly, it poses less concern in terms of keratocytes phototoxicity since the chemical reactions associated with free radical generation was limited in the region containing RB to absorb the green light. Riboflavin diffuses deeper into the corneal stroma and generates free radical once irradiated with UV-light, which initiates toxicity at a deeper layer if this is beyond the cytotoxic threshold of the keratocytes. Cherfan et al. observed loss of rabbit keratocytes with riboflavin/UVA treatment at standard 5.4 J/cm² irradiation dose (Cherfan et al., 2013). However, no loss of cells was detected using RB-PCL at either 100 or 200 J/cm. Secondly, RB does not penetrate through the corneal stroma; therefore the endothelium phototoxicity is less likely. Lastly and the most importantly, RB-PCL could be an alternative treatment for patients with corneal thickness less than 400 μ m since both the collagen cross-links formed and phototoxicity are limited to the penetration depth of RB, which is near anterior stroma.

Despite the short distance that RB diffuses, the observed corneal stromal stiffness is substantial. In our experiments, we have observed an increase in tensile strength by 1.77-2.01 fold and Young's modulus by 2.00–2.05 fold in treated porcine corneas at 4%, 6% and 8% strain using RB-PCL. Studies from Wollensak et al. (2003b) reported stiffness increase of a factor of 1.66-1.75, and Young's modulus of a factor of 1.75-2.03 in porcine corneas at 4%, 6% and 8% strain using UV-CXL. This is comparable to our data. In addition, we showed a significant increase of enzymatic digestion time from 10.8 \pm 3.1 days in the blank group to 19.7 \pm 6.2 days in the RB-PCL group. Spoerl et al. studied the effect of UV-CXL on the resistance to enzymatic digestion in porcine corneas and reported the prolonged collagenase digestion time from 6 days in the untreated control corneas to 14 days in UV-CXL treated corneas, which are comparable to our results (Spoerl et al., 2004). These findings indicated that the similar efficacy was achieved using either UV-CXL or RB-PCL.

Rose Bengal is a halogenated fluorescein derivative and used with FDA approval as a diagnostic agent to stain damaged conjunctive and corneal cells (Lansche, 1965). Tropical delivery of Hydrophilic formulations (\leq 1% RB) did not result any significant acute toxicity in skin (Wachter et al., 2003). Rose Bengal has an absorbance range of 500–590 nm with a maximum at 549 nm. Therefore, light at wavelength close to 549 nm is the most effective at induction of photochemical crosslinking with RB and increases the sample stiffness (Son et al., 2010). Irradiation of 532 nm green light used here (0.40 W/cm²) is not high enough to cause thermal



Fig. 4. General image of corneal strips after RB-PCL treatment and the blank control. A. The corneal strips held horizontally, Scale bar: 2 mm; B. The corneal strips held vertically. The corneal strip after RB-PCL treatment (Right in Fig. 5A and B) preserved the corneal curvature due to the increase in bending stiffness by RB-PCL, comparing with the blank group (Left in Fig. 5A and B). Scale bar: 5 mm.

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Fig. 5. Corneal structures after collagenase digestion. 48 h after collagenase digestion, the control cornea (A) showed loosely arranged posterior collagen fibers (arrows) in comparison with the RB-PCL treated cornea (B). The anterior stroma remained relatively intact at this time in RB-PCL treated cornea. Scale bar: 50 µm.

cytotoxicity as observed during laser tissue welding, which typically uses irradiation of 10–50 W/cm² (Rossi et al., 2007; Simhon et al., 2007; Talmor et al., 2001). In addition, the exposure of cornea to laser in our study was rapid with 250 s, which causes less concern for thermal damage. A number of studies have support the short-term safety of the RB-PCL protocol with a wavelength of 532 nm and irradiation dose of 100 J/cm². Gu et al. (2011) indicated that the corneal defect with surgically created limbal stem cell deficiency was well resurfaced at 3 and 28 days after attachment of human amniotic membrane pre-cultured with limbal stem cells to corneas by photochemical tissue bonding using the same irradiance, suggesting that photochemical activation is not phototoxic to the cells. Studies showed that RB-PCL induced thermal damage, dehiscence, local tissue reaction to photochemical activation, and infection were absent in mini-pig or rabbit skin and fresh rabbit cornea as assessed by histology (Cherfan et al., 2013; Kamegaya et al., 2005; Yao et al., 2010). Though in principle these findings can be extended to cells in others tissues treated with photochemical activation, more direct safety studies in cornea are warranted.

In conclusion, the cross-linking technology, which combines the photosensitizer Rose Bengal and 532 nm green light irradiation, increased both the biomechanical stiffness and the biochemical resistance against collagenase digestion in porcine corneas, therefore to allow stabilizing and solidifier the cornea. Photochemical cross-linking is simple, faster, minimally invasive, and the degree of crosslinking can be controlled by amount of light delivered. We understand RB-PCL technique is still at its infancy in comparison with more advanced and well-established UV-CXL technique. The long-term effects of RB-PCL on corneal stability and ocular safety require more through studies and investigation. We believe each technique has its own advantages and disadvantages. Due to the nature of minimal penetration of RB into corneal stroma, the RB-PCL method could potentially be used in patients with corneal thickness less than 400 µm where UV-CXL is limited.

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