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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/cm^2 for 250 s to deliver a dose of 100 J/cm^2 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 ± 3.1 days in the blank group to 19.7 ± 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.

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 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 cross-linking treated and control porcine corneas.

2. Materials and methods

2.1. Reagents and instruments

0.1% (w/v) Rose Bengal (RB, $C_{20}H_2Cl_4I_4Na_2O_5$) 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 post-mortem. 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 ImageJ 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/cm^2 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 °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