Determination of the relationship between mechanical properties, ultrastructural changes and intrafibrillar bond formation in corneal UVA/riboflavin cross-linking treatment for keratoconus

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Abstract

Purpose

UVA/riboflavin induced corneal cross-linking is a common therapeutic approach used to prevent progression in keratoconus. However, there is limited understanding of how changes to the mechanical behavior in cross-linked corneas relate to changes in the corneal ultrastructure. The aim of this study was to determine these relationships and, by so doing, provide a reference against which future modifications to cross-linking approaches may be compared.

Methods

Porcine corneas were treated following the “Dresden” protocol, the current gold standard for clinical treatment, consisting of dropwise application of 0.1% riboflavin in 20% dextran followed by 30 minutes of UVA irradiation. The effect of crosslinking was assessed using uniaxial tensile testing, transmission electron microscopy, and Fourier Transform Infrared Spectroscopy with results compared against corneas treated with each of the treatment solution components individually

Results

Data revealed that UVA/riboflavin cross-linked corneas displayed 28±17% increase in the material tangent modulus compared with dextran treatment alone, and altered collagen architecture within the first 300 μm of stromal depth consisting of 5% increase in the thickness of collagen fibrils, no significant changes to interfibrillar spacing, and an 8% to 12% decrease in number of fibrils per unit area. Fourier Transform Infrared Spectroscopy confirmed formation of interfibrillar bonds ($p=0.012$) induced by UVA mediated cross-linking.

Conclusions

Our data support a model wherein collagen fibril diameter and structural density are fundamental parameters in defining tissue stiffening following UVA/riboflavin corneal cross-linking, and provide benchmarks against which modifications to the Dresden cross-linking protocol can be evaluated.

Precis

This paper provides the connection between ultrastructural and mechanical properties changes induced by the current gold standard UVA/riboflavin cross-linking protocol. It

provides a benchmark against which future protocol modifications can be evaluated.

Introduction

Keratoconus is a corneal thinning disease which results in a focally reduced corneal radius of curvature, abnormal wavefront aberrations, and a localised reduction in corneal thickness and stiffness, these aberrations lead to a decline in visual function which ultimately may require corneal transplantation[1]. Corneal cross-linking with riboflavin and UVA is an established treatment for progressive keratoconus and currently is the only therapeutic approach that is capable of significantly altering disease progression[2] available for many years in Europe, this treatment option has been recently approved by the US Food and Drugs Administration for use in United States[2].. The so-called “Dresden” or “standard” protocol is the current standard approach for corneal cross-linking for keratoconus; consisting of 30 minutes exposure to 3mW fluence (5,4 mJ/cm2) following 30 mins soaking in 0.1% riboflavin [3].

The Dresden protocol involves topical dosing of the cornea, following epithelial removal, with 0.1% riboflavin in 20% dextran for 30 minutes, then exposure to ultraviolet light over a 30 minute period. This rationale behind this approach is that photo-polymerisation, in the presence of the photosensitizer riboflavin, leads to creation of chemical bonds between

77 substrates within the corneal stroma, including between collagens and proteoglycans, and
78 other stromal proteins[3-5]. The effects of these induced cross-links have been variously
79 reported as increases in tissue stiffness, resistance to enzymatic digestion, changes to
80 ultrastructure and altered swelling behaviour[6-9]. However, these studies have generally
81 evaluated the overall effects of full cross-linking protocol without separating the contribution
82 of individual aspects of the treatment and there are some differences in outcome
83 reported[5,6,10,11].
84
85 Mechanical properties of biological tissues are largely dependent upon the intertwining of
86 collagen fibrils, linked lamina layers and interfibrillar spacing[12-14]. Therefore, the efficacy of
87 the Dresden corneal cross-linking protocol is believed to be dependent on changes to the
88 mechanical properties of the tissue induced through modifying the characteristics of collagen
89 fibrils within the cornea, and the induction of intrafibrillar bonds, with the overall level of
90 effect being dependent on treatment depth[15,16]. Previous studies have attempted to evaluate
91 mechanical properties and the effective cross-linking penetration depth by specifically
92 examining anterior and posterior corneal layers [9,17,18]. These studies demonstrated that the

93 collagen cross-linking effect is predominantly located within anterior stroma but did not
94 specify the parameters that may be involved in determining and defining the amount of the
95 tissue stiffening induced. Indeed, the relationship between the mechanical behaviour of
96 cross-linked cornea and its ultrastructure is poorly explored, and the specific contribution of
97 the dextran within the riboflavin solution has not been reported[18,19].
98
99 numerous modifications to the Dresden protocol are being trialed in clinical settings therefore
100 defined knowledge of the effect of the current treatment regime is needed to provide a
101 benchmark against which these modifications can be compared and moreover for rationale
102 design of alternative approaches. The present study aimed to improve this understanding by
103 systematically investigating the role of dextran and the effect of riboflavin and UVA
104 cross-linking in inducing mechanical and ultrastructural changes in the porcine cornea.
105

Materials and methods

Cross-linking Procedure and Experimental Design

Fresh porcine eyes were collected from an abattoir, washed with phosphate-buffered saline (PBS) (Sigma, Dorset, United Kingdom), the central corneas excised and the corneal epithelium removed. To control for inter-animal variation, each cornea was cut into two segments in a superior-inferior fashion with one half being used as the test sample and the second for the control treatment (Fig. 1).

The treatment groups were assigned into groups as follows: Group 1 (PBS vs. PBS): 6 corneas; each half cornea was topically treated with PBS in 3 minute intervals for 1 hour. used to examine the intrinsic differences between the two segments of each porcine cornea. Group 2 (PBS vs. Riboflavin + PBS): 6 corneas; each corneal segment was topically treated in 3-minute intervals for 1 hour with either PBS or 0.1% riboflavin (Sigma) prepared in PBS. Group 3 (PBS vs. Riboflavin + Dextran): As for Group 2, except that the riboflavin was prepared in 20% dextran (Sigma). Group 4 (Dextran vs. Riboflavin + Dextran + UVA): 10 corneas; one half treated following the conventional Dresden protocol[3], the anterior surface

of the corneas were treated with 5ml of 0.1% riboflavin in 20% dextran every 3-minutes intervals for 30 minutes, followed by UVA (370nm) illumination at 3 mW/cm^2 (Opto XLink, Mehra Eyetech Pvt Ltd, Delhi, India) for a further 30 minutes. Topical dosing of riboflavin with dextran drops was continued every 3 minutes manner during the UVA irradiation (for full cross-linking details following the standard convention, see table 1)[20]. Cross-linked corneas were compared to fellow segments treated with 20% dextran only.

Following treatment, the corneal tissues were dissected for mechanical testing and for ultrastructural analysis (Fig 1).

Tensile Testing

Corneal stiffness was examined using a uniaxial tensile tester, Instron 3366 machine (Instron Engineering Corp., MA, USA), equipped with a 10N load cell. Two strips (3mm width x 6mm length) cut in the superior-inferior direction at the central cornea were inserted vertically into custom designed clamps and the protocol set to apply a maximum loading stress of 0.125 MPa, slightly above the stress expected under an intraocular pressure of 80

mmHg[21]. A constant extension of 1 mm/min was applied and the corresponding stress (applied force divided by cross-sectional area) and strain (extension over original length) measured continuously. Five conditioning cycles[21], with four-minute recovery periods between each two cycles, were performed. The tangent modulus (Et), the gradient of a tangent of the stress-strain behavior pattern, was calculated at different stress levels to derive the overall stiffness of the tissue[22,23].

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed as described previously[6]. Briefly, the specimens were isolated from the central regions of cornea (Fig 1) and fixed overnight with 2.5% glutaraldehyde (TAAB Laboratories Equipment Ltd, Reading, UK) in 0.1% tannic acid. Thereafter, specimens were dissected into 1mm x 2mm blocks and incubated with 4% osmium (TAAB), followed by serial dehydration through an acetone gradient (30%, 50%, 70%, 90%, 100%)(Sigma). Specimens were then infiltrated and embedded in medium resin (TAAB) and ultrathin 70 nm-thickness sections cut using a diamond knife microtome and collected onto 200 mesh copper grids (Ted Pella, Inc., CA, USA). Sections were examined

using a Tecnai G2 spirit BioTWIN Transmission electron microscope (FEI Company, OR, USA) operated at 120kV and 60k-fold magnification with a CCD camera. Tissues were sampled at five depth intervals from top of the anterior stroma 0-50μm, 80-150μm, 200-250μm, 300-350μm, 400-450μm.

Collagen fibrils in longitudinal, frontal and oblique profiles were observed in TEM images; only those in frontal profiles were used for quantitative analysis with analyses performed using Fiji software (National Institutes of Health, Bethesda, MA, USA). Ultrastructural parameters evaluated were; i) mean diameter of collagen fibrils ii) interfibrillar spacing and iii) number of fibrils per unit area. For i) circular spots in frontal profiles were isolated, exported and diameters measured. ii) The calculation of interfibrillar spacing was generated from equation (1) while assuming that the collagen fibrils were evenly distributed.

$$R + D = \sqrt{A/N} \quad (1)$$

Here R was defined as interfibrillar spacing (nm), D as mean diameter of collagen fibrils (nm), A as the area of selected zone of measurement (nm^2), N as the number of fibrils (circular spots) within the selected zone. iii) fibrils per unit area was determined by randomly

localising a window of fixed unit size (300 x 300 dpi) and the number of circular spots counted.

The distribution curve of collagen fibrils was assessed with a bespoke code using MATLAB 2016b (The MathWorks Inc., Natick, MA, USA). Original images were converted to binary using adaptive thresholding and a custom designed collagen fibril detection system, using the Circular Hough Transform (CHT) based algorithm [24,25], was established to analyse the radius of circular objects in frontal profiles of TEM images. Distribution curves were generated by plotting the frequency in 2nm increments against fibril radius.

Fourier Transform Infrared (FTIR) Absorption Spectroscopy

Fresh porcine corneas were treated with either PBS, 0.1% riboflavin in 20% dextran or the full Dresden protocol (6 per group), lyophilized for 3 days, then FTIR spectra measured using a Nicolet 6700 FTIR spectrometer (Thermo Scientific, MA, USA) with a attenuated total reflection (ATR) module. 64 accumulative scans were taken with a resolution of 4 cm^{-1} between 800 cm^{-1} to 4000 cm^{-1}. Data was collected using OMNIC software (Thermo Scientific, MA, USA). Analyses of FTIR spectra of each condition were obtained with a

combination of four spectral intervals [ν(C=O) absorption of amide I (1,680–1,630 cm^{-1}), $\delta(NH_2)$ absorptions of amide II (1,570–1,515 cm^{-1}), ν(C–N) absorptions of amide III (1,350–1,200 cm^{-1}), and ν(C–O) absorptions of carbohydrate moieties (1,150–1,000 cm^{-1})]. Area under the curve at each interval was analyzed using KnowItAll® ID Expert™ (Bio-Rad, CA, USA).

Statistical Analysis

Results for mechanical, ultrastructural and FTIR spectra analyses are presented as means ± standard deviation (SD) and statistical significance calculated using one-way analysis of variance (ANOVA) with Turkey's HSD post-hoc tests. The Mann-Whitney U test (two-tailed) was used for analyzing the statistical difference of the distribution curves, and significance differences accepted where $p < 0.05$.

Results

UVA/riboflavin treatment and dextran mediated dehydration both contribute to mechanical property changes in corneal cross-linking.

In order to isolate the effect of the individual components of UVA/riboflavin crosslinking procedure, uniaxial tensile experiments and transmission electron microscopy were conducted on a set of porcine split into paired comparison groups. To account for inter-animal variability, each cornea was cut in two and one half treated with the treatment and compared against the control (Fig 1), tensile measurements and ultrastructural analyses were performed on the same cornea). Comparison groups were: PBS vs. PBS, riboflavin in PBS vs. PBS, riboflavin in 20% dextran vs. PBS and riboflavin in 20% dextran+UVA vs. 20% dextran (Fig 1).

The tangent modulus (Et) versus stress (σ) for each corneal strip was determined and the overall stiffening effect indicated by the ratio of the tangent modulus ($Et_{experimental}$ /$Et_{control}$). Comparisons concentrated on tangent modulus ratios at a stress of 0.03MPa, which is equivalent to a physiological intraocular pressure (IOP) of around 25mmHg[21]. As expected,

215 no significant differences were observed where both corneal segments were treated
216 identically with PBS, confirming the validity of our intra-eye control system ($Et_{\text{experimental PBS}}$
217 vs. $Et_{\text{control PBS}}$: 1.36±0.32 vs. 1.43±0.30 at 0.03MPa, respectively, p = 0.075, Figs 2A and 2B,
218 S1 Table). Riboflavin in PBS treatment also caused no stiffening (riboflavin in PBS
219 1.45±0.15 vs. PBS 1.38±0.17, p = 0.448, Figs 2C, S1 Table). However, the riboflavin in 20%
220 dextran group displayed a 13±9% tangent modulus increase compared to their internal PBS
221 control (riboflavin in dextran: 1.52±0.17 vs. PBS 1.34±0.18, p = 0.011, Figs 2D, S1 Table).
222 Comparing the effect of the full Dresden crosslinking protocol to the effect of dextran
223 treatment alone, we observed a 28±17% increase in tangent modulus in the Dresden treated
224 corneal segments (riboflavin in dextran + UVA 2.09±0.17 vs. dextran 1.62±0.18 p =
225 5.67E-05, Figs 2E, S1 Table).
226
227 The differences observed with dextran alone were somewhat surprising however, upon
228 comparing the tissue thicknesses following treatments, we observed statistically significant
229 reduced thicknesses in the dextran treated samples compared with their PBS controls,
230 indicative of dehydration (mean thickness riboflavin in dextran 0.79±0.04 mm, PBS

231 1.05±0.39 mm, p = $1.39x10^{-6}$)(Fig 2F and S1 Table). Correcting the tangent modulus
232 readings with these thickness measurements removed the apparent dextran effects, whereas
233 residual stiffening effect was still observed in the Dresden protocol treated samples after
234 correction (Fig 2G). Together these data demonstrate that dextran treatment alone causes
235 dehydration and therefore an apparently increased stiffening, whereas UVA exposed
236 riboflavin treated corneas exhibit further increased stiffening beyond that caused by the
237 dextran treatment alone.
238
239 **UVA/riboflavin treatment causes depth dependent changes to collagen fibril**
240 **ultrastructure.**
241 Next we measured changes to the collagen ultrastructure of specimens, measured at five
242 different depth intervals using TEM (S1 Fig, Fig. 2A; representative images at 80-150mm)
243 and the obtained images used to determine collagen fibril diameter (Fig 2B), interfibrillar
244 spacing and fibril number per unit area (density)(S2 Table). The overall morphologies of the
245 collagen ultrastructure did not change following PBS or riboflavin in PBS treatment in the
246 absence of UV (S1 Fig, S2 Table). However, the dextran treatment led to significantly thinner

247 collagen fibrils (Fig 3A), as well as reduced interfibrillar spacing and denser packing at all
248 depth intervals compared to its PBS control (S1 Fig). As dextran also causes loss of tissue
249 hydration we used a mathematical method to correct for dehydration effects, in order to
250 identify the true structural changes[26]. The relationship between thickness (T) and hydration
251 (H) was modelled using the equation T = 0.2*e^(0.33*H) which has been shown to be
252 effective for these types of calculation[26]. The reduction in thickness followed an exponential
253 decrease in tissue hydration, which we used to calculate the hydration state of the tissues and
254 therefore calculate swelling factors at each depth interval (S3 Table). Analysis of fibril
255 diameter at each depth interval revealed that although, in the uncorrected data, dextran
256 treatment caused a reduction in the frequency of large collagen fibrils compared to PBS
257 treatment (Figs 3B; 80-150mm, S2 Table and S3 Table), when correction for swelling factors
258 was included, no significant differences were detected (Fig 3B and S2 Fig), indicating that
259 dextran treatment alone has no effect on collagen fibril diameter.
260
261 In analyzing the effect of the Dresden UVA/riboflavin crosslinking protocol on corneal
262 ultrastructure, both segments within our intra-eye comparisons were treated with dextran

263 therefore any residual difference between the internal controlled comparison groups reflected
264 a true effect of the crosslinking procedure. Analysis revealed a small but statistically
265 significant increase in collagen fibril diameter in the cross-linked group at 80-150μm (5±2%,
266 $p<0.01$) and 200-250μm depths (6±3%, $p<0.01$)) with no differences observed at any other
267 depth intervals (Figs 4C and 4D, S2 Fig). No statistical differences in interfibrillar spacing
268 between treatments were observed at any depths (Fig 4E), however the Dresden protocol
269 caused a decrease in the number of fibrils per unit area in the anterior 250μm of the tissue
270 (0-50μm 6±5%, $p<0.05$; 80-150μm 11±7%, $p<0.01$; 200-250μm 11±5%, $p<0.01$) (F5 and S2
271 Table). Together these data indicate that the UVA crosslinking procedure creates relatively
272 small, depth-localized changes to the collagen ultrastructure.
273
274 **Individual ultrastructural measurements are insufficient to predict mechanical changes.**
275 As we had performed the tangent modulus and ultrastructural measurements upon the same
276 eye (Fig 1), we were able to directly compare the values obtained (Fig 5). Note comparisons
277 were made between mechanical outcomes measured at 0.03MPa stress and structural
278 parameters were determined from tissues in relaxed states. Collagen fibril diameter at

80-150μm, intrafibril spacing increases, and collagen fibril density decreases each displayed correlation with tangent modulus increases across the test population (diameter; r^2 =0.23, Fig. 5A, spacing r^2= 0.39, Fig. 5B, density r^2=0.52, Fig. 5C). When percentage change on an individual eye basis was plotted, the none of the individual ultrastructural parameters were independently indicative of the overall tissue stiffness (Fig. 5D). However, when considered in combination, the ultrastructural measurements performed better, with a positive correlation of 0.177 (collagen fibril diameter + fibril density - interfibrillar spacing, Fig. 5E).

Fourier Transform Infrared Spectroscopy Reveal the Formation of New Amide Bonds in Cross-linked Corneas

To assess the detailed chemical reactions and the conversion of chemical bonds within the cornea tissue after UVA/riboflavin cross-linking, Fourier Transform Infrared Spectroscopy measurements were performed on corneas treated with either PBS, riboflavin+dextran or riboflavin+dextran+UVA (Fig 6A and B). The relevant characteristic bands were; amide I C=O stretching vibration (1,680–1,630 cm^{-1}), amide II NH_2 bending vibration (1570-1515 cm^{-1}), amide III C-N stretching vibration (1,350–1,200 cm^{-1}), and C-O bond stretching

295 vibration (1,150–1,000 cm^{-1}). The area under each band was calculated, and the deformation
296 vibrations of CH_2 (1,485–1,360 cm^{-1}) used as an internal standard to determine the intensity
297 ratios (Fig 6C, S4 Table). These analyses revealed significant increases in the C-O stretch
298 peak, decreased C-N stretch and increased NH_2 deformation following crosslinking (Fig
299 6C). Plotting the ratio of C-N stretch to NH_2 deformation suggests that the decrease
300 intensities of amide II infrared absorption bands are likely to be accompanied by an increase
301 in the formation of amide III bonds (riboflavin+dextran+UVA 2.5±0.5 compared to PBS
302 1.7±0.3, and riboflavin+dextran 1.8±0.2 Fig 6D, S4 Table).
303

Discussion

In this study, we have characterized how corneal mechanical properties relate to ultrastructural changes following Dresden protocol treatment, and identified the contribution of the different components of the protocol to observed effects. Specifically, our data demonstrate that the increases in corneal stiffness measured following Dresden protocol treatment arise from combination of dextran-mediated dehydration along with UVA/riboflavin induced new bond formation and depth-dependent increases in collagen fibril diameter.

Tissue stiffness is considered as a combination of the internal geometry of the tissue and the properties of the material itself. Therefore, although our data indicate that almost half of the measured stiffening effect of the Dresden protocol comes from the dextran component of the protocol, these effects can be explained by dehydration effects. However, we did observe an increase in C-O stretching force in the riboflavin in dextran treated corneas compared with controls indicating that dextran does itself induce collagen changes. A potential explanation for these new bonds is that the dextran induced dehydration increases the swelling pressure of tissue and therefore the resistance pressure of proteoglycan matrix[26,27], giving rise to

320 intermolecular forces. These dextran and dehydration effects could help explain some of the
321 apparently contradictory reports in the literature [5,6,10,11]. Interestingly tissue hydration states
322 have been reported to affect the efficacy of cross-linking treatment[27], therefore, hydration
323 states and the osmolarity of the riboflavin solutions could be important factors to consider in
324 protocol modifications.

325

326 Our FTIR data revealed significant changes in C-O stretching force and conversion rate of
327 amide bonds following the full Dresden protocol. Lysine-based cross-links following
328 UVA/riboflavin cross-linking have previously been postulated but not been found
329 chemically[3,28] but it has been proposed that cross-links form through endogenous carbonyl
330 groups including imidazole formation[29]. Our data support a model where the increased
331 swelling pressure and the involvement of endogenous carbonyls (allysine) leads to the new
332 bond formation[22]. This leads to a broader mechanism where UVA/riboflavin induced
333 intra-fibril bonds. presenting as thickened collagen fibrils and less dense overall structure and
334 drive the increased tissue mechanical strength and resistance to dehydration (Fig. 7).

335

336 It should be noted, however, that the ultrastructural changes we measured do not fully
337 account for the mechanical effects suggesting that collagen fibril diameter and spacing are
338 not the only aspects of stromal biology affected by UVA/riboflavin crosslinking. Effects
339 upon other stromal proteins and particularly the interactions of collagen with proteoglycans,
340 could be contributing to the stiffening. Proteoglycans within the corneal stroma have been
341 proposed to play a pivotal role in regulating the fibril-fibril spacing and
342 hydration-dehydration properties[30], however, the dehydration induced decrease in interfibril
343 spacing has prevented us from being able to determine the sole effect of cross-linking on
344 proteoglycans within our experimental system.

345

346 Understanding the stabilization mechanism of UVA/riboflavin cross-linking is clinically
347 relevant when evaluating modifications to the Dresden protocol and developing optimal
348 protocols or new keratoconus treatments. This study establishes the standards in terms of
349 mechanical, chemical and structure/biological changes induced by the Dresden protocol and
350 therefore provides the baseline against which modifications can be judged.

351

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449 **Figure legends**

450 **Fig 1. Experimental scheme.** Each cornea was cut into two segments in a superior-inferior
451 fashion. The right-hand segment was used as an experimental segment, while the left-hand
452 segment was used as its corresponding control. Four experimental groups were designed,
453 Group 1: PBS vs. PBS; Group 2: PBS vs. riboflavin+PBS; Group 3: PBS vs.
454 riboflavin+dextran; Group 4: dextran vs. riboflavin+dextran+UVA. After each treatment,
455 specimens were dissected and processed for tensile testing (green hollow rectangle) and TEM
456 (red hollow rectangle). S: superior; I: inferior.

457

458 **Fig 2. The tangent modulus (Et) versus stress (σ) behaviour and ratio of tangent**
459 **modulus of paired samples.** (A) The tangent modulus versus stress behavior of right-hand
460 corneal flaps versus left-hand flaps from 6 are plotted. Both flaps were identically treated by
461 soaking in PBS. The ratio of tangent modulus between (B) PBS versus PBS (n=6), (C)
462 riboflavin in PBS versus PBS (n=6), (D) riboflavin in 20% dextran versus PBS (n=6_, (E)
463 riboflavin in 20% dextran+UVA exposure versus 20% dextran only (n=10). Values from
464 each individual cornea pair tested are indicated by grey lines. Average stiffening ratio ±

standard deviation are indicated by the bold line and error bars. The red dash line represented the value of 1; i.e. no difference between paired samples. Gray shaded region in A-E plots represents 0.03MPa. (F) average thickness measurement of each comparison group after treatment. (G) the stiffening ratio at 0.03MPa with and without correction for tissue thickness changes. Values in F and G denote mean ± standard deviation (SD). * p value of < 0.05, ** p<0.01.

Fig 3. Transmission electron microscopy (TEM) images of porcine corneas imaged at depth of 80-150μm (A) Representation TEM images of PBS, riboflavin in dextran, and dextran only and riboflavin/dextran/UVA cross-linked corneas. Bar 500nm.An area of 300 x 300 dpi (yellow dash-square) is shown at higher magnification in (B) with example of the measurement of the area and density of collagen fibrils.

Fig 4. Measurement of ultrastructural parameters and collagen fibril distribution at different depth intervals. (A) Mean collagen fibril diameters of PBS and riboflavin in dextran at each depth interval. (B) Collagen fibril diameter distributions plotted as either

uncorrected values from of riboflavin in dextran group (top panel, black filled squares), or corrected for dehydration (top panel, gray squares) or its corresponding PBS control (middle panel, blue filled squares). Traces are shown overlaid in bottom panels. Grey filled background added to aid visualization. (C) Collagen fibril diameter distribution curve of cross-linking group (green boxes) and its dextran control (black boxes) at each depth interval. (D) Relative collagen fibril diameter, (E) relative interfibrillar spacing, (F) relative collagen density of cross-linking group relative to its dextran group at each depth interval. Values are plotted as mean±SD from n=6 (A and B) or n=10 (C, D, E and F). Asterisks denote significant differences from control groups with * $p < 0.05$, ** $p<0.01$.

Fig 5. Correlation between ultrastructural parameters and tangent modulus. Tangent modulus at 0.03MPa versus (A) collagen fibril diameter, (B) interfibrillar spacing and (C) collagen fibril density. Black boxes; dextran treated eyes, green boxes; Riboflavin+UVA+Dextran treated eyes, (D) percentage change in tangent modulus versus percentage change in each ultrastructural parameter. Each box represents the measurements from one eye for either fibril diameter (red), interfirbil distance (orange) or fibril density

(yellow). (E) Percentage change in tangent modulus plotted against the cumulative effects of percentage change in fibril diameter+interfibril spacing-fibril density. Each box represents one eye. Green line; linear line of best fit. Red dotted line; 100% correlation.

Fig 6. Absorption FTIR spectra of porcine corneas following cross-linking treatments. (A) Representative FTIR spectra of corneas treated with PBS (bottom, gray), riboflavin+dextran (middle, black) or riboflavin+dextran+UVA (top, green). (B) Regions where the characteristic bands are located shown at higher magnification and regions of interest indicated: Amide I (1,680–1,630 cm^{-1}), Amide II (1570-1515 cm^{-1}), Amide III (1,350–1,200 cm^{-1}), and CO absorption band (1,150–1,000 cm^{-1}). (C) Relative intensity ratio of each characteristic band and (D) conversion rate of amide II to amide III.

Fig 7. Proposed model of UVA/riboflavin cross-linking with the presence of dextran. The black solid circle, blue solid line, green solid line and red solid line represent the collagen molecules, proteoglycans, original cross-links formed between collagen molecules and additional cross-links induced by UVA/riboflavin cross-linking treatment, respectively.

513

514 **Supplementary Information Legends.**

515 S1 Fig.Transmission electron microscopy (TEM) images of porcine corneas imaged at
516 different depth intervals following PBS, dextran and/or UVA/riboflavin treatment.
517 Representative TEM images of PBS (A), Riboflavin+PBS (B), Riboflavin+Dextran (C), or
518 Riboflavin+Dextran+UVA (D) and their corresponding controls at five depth intervals of
519 0-50μm, 80-150μm, 200-250μm, 300-350μm, 400-450μm.

520

521 S2 Fig. Collagen radius distribution of each treatment group at each depth intervals. The
522 distribution of control segments (blue); (A) PBS, (D) PBS, (G) PBS, and (K) Dextran, and
523 the experimental segments (orange) (B) PBS, (E) Riboflaivin+PBS, (H) Ribflavin+Dextran,
524 and (L) Riboflaivn+Dextran+UVA. Dashed lines represent trend curves. The overlapped
525 curves (C, F, I, J, M) were used for detecting the shifting of distribution in radius between
526 control and experimental segments. The data were obtained at five depth intervals of 0-50μm,
527 80-150μm, 200-250μm, 300-350μm, 400-450μm. p value of < 0.05 is indicated by an
528 asterisk compared with its internal control.

S1 Table. Summary of the average thickness and stiffening ratio at 0.03MPa of each group before and after thickness correction. Values denote mean ± standard deviation (SD). *p* value of < 0.05 is indicated by an asterisk compared with its control. Red asterisks indicate increase compared with control, green represents decrease.

S2 Table. Mean diameter, interfibrillar spacing, and density of collagen fibrils of corneal segments at different depth intervals following PBS, dextran and/or UVA/riboflavin treatment. Values denote mean ± standard deviation (SD). *p* value of < 0.05 is indicated by an asterisk compared with its control. Red asterisks indicate increase compared with control, green represents decrease.

S3 Table. Summary of area ratio corresponding to indicatged characteristic bands in FTIR spectra. Values denote mean ± standard deviation (SD). *p* value of < 0.05 is indicated by an asterisk compared with its control. Red asterisks indicate increase compared with control, green represents decrease.

S4 Table. Parameters (decreased percentage, hydration loss percentage, remaining hydration percentage and swelling factor) used in correction of the collagen fibril radius distribution curves.