1 **Determination of the relationship between mechanical properties, ultrastructural**

2 **changes and intrafibrillar bond formation in corneal UVA/riboflavin cross-linking**

3 **treatment for keratoconus**

4

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27

28   **Abstract**

29   **Purpose**

30   UVA/riboflavin induced corneal cross-linking is a common therapeutic approach used to

31   prevent progression in keratoconus. However, there is limited understanding of how changes

32   to the mechanical behavior in cross-linked corneas relate to changes in the corneal

33   ultrastructure. The aim of this study was to determine these relationships and, by so doing,

34   provide a reference against which future modifications to cross-linking approaches may be

35   compared.

36   **Methods**

37   Porcine corneas were treated following the "Dresden" protocol, the current gold standard for

38   clinical treatment, consisting of dropwise application of 0.1% riboflavin in 20% dextran

39   followed by 30 minutes of UVA irradiation. The effect of crosslinking was assessed using

40   uniaxial tensile testing, transmission electron microscopy, and Fourier Transform Infrared

41   Spectroscopy with results compared against corneas treated with each of the treatment

42   solution components individually

43   **Results**

44   Data revealed that UVA/riboflavin cross-linked corneas displayed 28±17% increase in the

45   material tangent modulus compared with dextran treatment alone, and altered collagen

46   architecture within the first 300 μm of stromal depth consisting of 5% increase in the

47   thickness of collagen fibrils, no significant changes to interfibrillar spacing, and an 8% to

48   12% decrease in number of fibrils per unit area. Fourier Transform Infrared Spectroscopy

49   confirmed formation of interfibrillar bonds (p=0.012) induced by UVA mediated

50   cross-linking.

51   **Conclusions**

52   Our data support a model wherein collagen fibril diameter and structural density are

53   fundamental parameters in defining tissue stiffening following UVA/riboflavin corneal

54   cross-linking, and provide benchmarks against which modifications to the Dresden

55   cross-linking protocol can be evaluated.

56

57   **Precis**

58   This paper provides the connection between ultrastructural and mechanical properties

59   changes induced by the current gold standard UVA/riboflavin cross-linking protocol. It

60   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

106   **Materials and methods**

107   **Cross-linking Procedure and Experimental Design**

108   Fresh porcine eyes were collected from an abattoir, washed with phosphate-buffered saline

109   (PBS) (Sigma, Dorset, United Kingdom), the central corneas excised and the corneal

110   epithelium removed. To control for inter-animal variation, each cornea was cut into two

111   segments in a superior-inferior fashion with one half being used as the test sample and the

112   second for the control treatment (Fig. 1).

113

114   The treatment groups were assigned into groups as follows: Group 1 (PBS vs. PBS): 6

115   corneas; each half cornea was topically treated with PBS in 3 minute intervals for 1 hour.

116   used to examine the intrinsic differences between the two segments of each porcine cornea.

117   Group 2 (PBS vs. Riboflavin + PBS): 6 corneas; each corneal segment was topically treated

118   in 3-minute intervals for 1 hour with either PBS or 0.1% riboflavin (Sigma) prepared in PBS.

119   Group 3 (PBS vs. Riboflavin + Dextran): As for Group 2, except that the riboflavin was

120   prepared in 20% dextran (Sigma).   Group 4 (Dextran vs. Riboflavin + Dextran + UVA): 10

121   corneas; one half treated following the conventional Dresden protocol[3], the anterior surface

122   of the corneas were treated with 5ml of 0.1% riboflavin in 20% dextran every 3-minutes

123   intervals for 30 minutes, followed by UVA (370nm) illumination at 3 mW/cm^2 (Opto XLink,

124   Mehra Eyetech Pvt Ltd, Delhi, India) for a further 30 minutes. Topical dosing of riboflavin

125   with dextran drops was continued every 3 minutes manner during the UVA irradiation (for

126   full cross-linking details following the standard convention, see table 1)[20]. Cross-linked

127   corneas were compared to fellow segments treated with 20% dextran only.

128

129   Following treatment, the corneal tissues were dissected for mechanical testing and for

130   ultrastructural analysis (Fig 1).

131

132   **Tensile Testing**

133   Corneal stiffness was examined using a uniaxial tensile tester, Instron 3366 machine (Instron

134   Engineering Corp., MA, USA), equipped with a 10N load cell. Two strips (3mm width x

135   6mm length) cut in the superior-inferior direction at the central cornea were inserted

136   vertically into custom designed clamps and the protocol set to apply a maximum loading

137   stress of 0.125 MPa, slightly above the stress expected under an intraocular pressure of 80

138   mmHg[21]. A constant extension of 1 mm/min was applied and the corresponding stress

139   (applied force divided by cross-sectional area) and strain (extension over original length)

140   measured continuously. Five conditioning cycles[21], with four-minute recovery periods

141   between each two cycles, were performed. The tangent modulus (Et), the gradient of a

142   tangent of the stress-strain behavior pattern, was calculated at different stress levels to derive

143   the overall stiffness of the tissue[22,23].

144

145   **Transmission electron microscopy**

146   Transmission electron microscopy (TEM) was performed as described previously[6]. Briefly,

147   the specimens were isolated from the central regions of cornea (Fig 1) and fixed overnight

148   with 2.5% glutaraldehyde (TAAB Laboratories Equipment Ltd, Reading, UK) in 0.1% tannic

149   acid. Thereafter, specimens were dissected into 1mm x 2mm blocks and incubated with 4%

150   osmium (TAAB), followed by serial dehydration through an acetone gradient (30%, 50%,

151   70%, 90%, 100%)(Sigma). Specimens were then infiltrated and embedded in medium resin

152   (TAAB) and ultrathin 70 nm-thickness sections cut using a diamond knife microtome and

153   collected onto 200 mesh copper grids (Ted Pella, Inc., CA, USA). Sections were examined

154   using a Tecnai G2 spirit BioTWIN Transmission electron microscope (FEI Company, OR,

155   USA) operated at 120kV and 60k-fold magnification with a CCD camera. Tissues were

156   sampled at five depth intervals from top of the anterior stroma 0-50μm, 80-150μm,

157   200-250μm, 300-350μm, 400-450μm.

158

159   Collagen fibrils in longitudinal, frontal and oblique profiles were observed in TEM images;

160   only those in frontal profiles were used for quantitative analysis with analyses performed

161   using Fiji software (National Institutes of Health, Bethesda, MA, USA). Ultrastructural

162   parameters evaluated were; i) mean diameter of collagen fibrils ii) interfibrillar spacing and

163   iii) number of fibrils per unit area. For i) circular spots in frontal profiles were isolated,

164   exported and diameters measured. ii) The calculation of interfibrillar spacing was generated

165   from equation (1) while assuming that the collagen fibrils were evenly distributed.

166
$$\text{R} + \text{D} = \sqrt{A/N} \tag{1}$$

167   Here R was defined as interfibrillar spacing (nm), D as mean diameter of collagen fibrils

168   (nm), A as the area of selected zone of measurement (nm^2), N as the number of fibrils

169   (circular spots) within the selected zone. iii) fibrils per unit area was determined by randomly

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

171   counted.

172   The distribution curve of collagen fibrils was assessed with a bespoke code using MATLAB

173   2016b (The MathWorks Inc., Natick, MA, USA). Original images were converted to binary

174   using adaptive thresholding and a custom designed collagen fibril detection system, using the

175   Circular Hough Transform (CHT) based algorithm [24,25], was established to analyse the radius

176   of circular objects in frontal profiles of TEM images. Distribution curves were generated by

177   plotting the frequency in 2nm increments against fibril radius.

178

179   **Fourier Transform Infrared (FTIR) Absorption Spectroscopy**

180   Fresh porcine corneas were treated with either PBS, 0.1% riboflavin in 20% dextran or the

181   full Dresden protocol (6 per group), lyophilized for 3 days, then FTIR spectra measured using

182   a Nicolet 6700 FTIR spectrometer (Thermo Scientific, MA, USA) with a attenuated total

183   reflection (ATR) module. 64 accumulative scans were taken with a resolution of 4 cm^{-1}

184   between 800 cm^{-1} to 4000 cm^{-1}. Data was collected using OMNIC software (Thermo

185   Scientific, MA, USA). Analyses of FTIR spectra of each condition were obtained with a

186 combination of four spectral intervals [ν(C=O) absorption of amide I (1,680–1,630 cm^{-1}),

187 δ(NH$_2$) absorptions of amide II (1,570–1,515 cm^{-1}), ν(C–N) absorptions of amide III (1,350–

188 1,200 cm^{-1}), and ν(C–O) absorptions of carbohydrate moieties (1,150–1,000 cm^{-1})]. Area

189 under the curve at each interval was analyzed using KnowItAll® ID Expert™ (Bio-Rad, CA,

190 USA).

191

192 **Statistical Analysis**

193 Results for mechanical, ultrastructural and FTIR spectra analyses are presented as means ±

194 standard deviation (SD) and statistical significance calculated using one-way analysis of

195 variance (ANOVA) with Turkey's HSD post-hoc tests. The Mann-Whitney U test (two-tailed)

196 was used for analyzing the statistical difference of the distribution curves, and significance

197 differences accepted where $p < 0.05$.

198

199   **Results**

200   **UVA/riboflavin treatment and dextran mediated dehydration both contribute to**

201   **mechanical property changes in corneal cross-linking.**

202   In order to isolate the effect of the individual components of UVA/riboflavin crosslinking

203   procedure, uniaxial tensile experiments and transmission electron microscopy were

204   conducted on a set of porcine split into paired comparison groups. To account for

205   inter-animal variability, each cornea was cut in two and one half treated with the treatment

206   and compared against the control (Fig 1), tensile measurements and ultrastructural analyses

207   were performed on the same cornea). Comparison groups were: PBS vs. PBS, riboflavin in

208   PBS vs. PBS, riboflavin in 20% dextran vs. PBS and riboflavin in 20% dextran+UVA vs.

209   20% dextran (Fig 1).

210

211   The tangent modulus (Et) versus stress (σ) for each corneal strip was determined and the

212   overall stiffening effect indicated by the ratio of the tangent modulus ($Et_{experimental}$ /$Et_{control)}$.

213   Comparisons concentrated on tangent modulus ratios at a stress of 0.03MPa, which is

214   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 ($Et_{experimental\ PBS}$

217   vs. $Et_{control\ PBS}$: 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

279   80-150μm, intrafibril spacing increases, and collagen fibril density decreases each displayed

280   correlation with tangent modulus increases across the test population (diameter; r^2 =0.23, Fig.

281   5A, spacing r^2= 0.39, Fig. 5B, density r^2=0.52, Fig. 5C). When percentage change on an

282   individual eye basis was plotted, the none of the individual ultrastructural parameters were

283   independently indicative of the overall tissue stiffness (Fig. 5D). However, when considered

284   in combination, the ultrastructural measurements performed better, with a positive correlation

285   of 0.177 (collagen fibril diameter + fibril density - interfibrillar spacing, Fig. 5E).

286

287   **Fourier Transform Infrared Spectroscopy Reveal the Formation of New Amide Bonds**

288   **in Cross-linked Corneas**

289   To assess the detailed chemical reactions and the conversion of chemical bonds within the

290   cornea tissue after UVA/riboflavin cross-linking, Fourier Transform Infrared Spectroscopy

291   measurements were performed on corneas treated with either PBS, riboflavin+dextran or

292   riboflavin+dextran+UVA (Fig 6A and B). The relevant characteristic bands were; amide I

293   C=O stretching vibration (1,680–1,630 cm^{-1}), amide II NH$_2$ bending vibration (1570-1515

294   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

304 Discussion

305 In this study, we have characterized how corneal mechanical properties relate to

306 ultrastructural changes following Dresden protocol treatment, and identified the contribution

307 of the different components of the protocol to observed effects. Specifically, our data

308 demonstrate that the increases in corneal stiffness measured following Dresden protocol

309 treatment arise from combination of dextran-mediated dehydration along with

310 UVA/riboflavin induced new bond formation and depth-dependent increases in collagen fibril

311 diameter.

312 Tissue stiffness is considered as a combination of the internal geometry of the tissue and the

313 properties of the material itself. Therefore, although our data indicate that almost half of the

314 measured stiffening effect of the Dresden protocol comes from the dextran component of the

315 protocol, these effects can be explained by dehydration effects. However, we did observe an

316 increase in C-O stretching force in the riboflavin in dextran treated corneas compared with

317 controls indicating that dextran does itself induce collagen changes. A potential explanation

318 for these new bonds is that the dextran induced dehydration increases the swelling pressure of

319 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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References

364 **1.** Andreassen TT, Simonsen AH, Oxlund H. Biomechanical properties of keratoconus and normal corneas. *Exp. Eye Res.* Oct 1980;31(4):435-441.

366 **2.** Wollensak G. Crosslinking treatment of progressive keratoconus: new hope. *Curr. Opin. Ophthalmol.* Aug 2006;17(4):356-360.

368 **3.** Wollensak G, Spoerl E, Seiler T. Riboflavin/ultraviolet-a-induced collagen crosslinking for the treatment of keratoconus. *Am. J. Ophthalmol.* May 2003;135(5):620-627.

371 **4.** Brummer G, Littlechild S, McCall S, Zhang Y, Conrad GW. The role of nonenzymatic glycation and carbonyls in collagen cross-linking for the treatment of keratoconus. *Invest. Ophthalmol. Vis. Sci.* Aug 11 2011;52(9):6363-6369.

374 **5.** Hayes S, Kamma-Lorger CS, Boote C, et al. The effect of riboflavin/UVA collagen cross-linking therapy on the structure and hydrodynamic behaviour of the ungulate and rabbit corneal stroma. *PloS one.* 2013;8(1):e52860.

377 **6.** Akhtar S, Almubrad T, Paladini I, Mencucci R. Keratoconus corneal architecture after riboflavin/ultraviolet A cross-linking: ultrastructural studies. *Mol. Vis.* 2013;19:1526-1537.

380 **7.** Spoerl E, Wollensak G, Dittert DD, Seiler T. Thermomechanical behavior of collagen-cross-linked porcine cornea. *Ophthalmologica.* Mar-Apr 2004;218(2):136-140.

383 **8.** Spoerl E, Wollensak G, Seiler T. Increased resistance of crosslinked cornea against enzymatic digestion. *Curr. Eye Res.* Jul 2004;29(1):35-40.

385 **9.** Wollensak G, Wilsch M, Spoerl E, Seiler T. Collagen fiber diameter in the rabbit cornea after collagen crosslinking by riboflavin/UVA. *Cornea.* Jul 2004;23(5):503-507.

388 **10.** Wang X, Huang Y, Jastaneiah S, et al. Protective Effects of Soluble Collagen during Ultraviolet-A Crosslinking on Enzyme-Mediated Corneal Ectatic Models. *PloS one.* 2015;10(9):e0136999.

391 **11.** Wollensak G, Aurich H, Pham DT, Wirbelauer C. Hydration behavior of porcine cornea crosslinked with riboflavin and ultraviolet A. *J. Cataract Refract. Surg.* Mar 2007;33(3):516-521.

394 **12.** Boote C, Dennis S, Newton RH, Puri H, Meek KM. Collagen fibrils appear more

395   closely packed in the prepupillary cornea: optical and biomechanical

396   implications. *Invest. Ophthalmol. Vis. Sci.* Jul 2003;44(7):2941-2948.

397 **13.** Depalle B, Qin Z, Shefelbine SJ, Buehler MJ. Influence of cross-link structure,

398   density and mechanical properties in the mesoscale deformation mechanisms of

399   collagen fibrils. *Journal of the mechanical behavior of biomedical materials.* Dec

400   2015;52:1-13.

401 **14.** Whitford C, Studer H, Boote C, Meek KM, Elsheikh A. Biomechanical model of the

402   human cornea: considering shear stiffness and regional variation of collagen

403   anisotropy and density. *Journal of the mechanical behavior of biomedical*

404   *materials.* Feb 2015;42:76-87.

405 **15.** Raiskup F, Spoerl E. Corneal crosslinking with riboflavin and ultraviolet A. I.

406   Principles. *The ocular surface.* Apr 2013;11(2):65-74.

407 **16.** Schumacher S, Mrochen M, Wernli J, Bueeler M, Seiler T. Optimization model for

408   UV-riboflavin corneal cross-linking. *Invest. Ophthalmol. Vis. Sci.* Feb 16

409   2012;53(2):762-769.

410 **17.** Kohlhaas M, Spoerl E, Schilde T, Unger G, Wittig C, Pillunat LE. Biomechanical

411   evidence of the distribution of cross-links in corneastreated with riboflavin and

412   ultraviolet A light. *J. Cataract Refract. Surg.* 2006;32(2):279-283.

413 **18.** Seifert J, Hammer CM, Rheinlaender J, et al. Distribution of Young's modulus in

414   porcine corneas after riboflavin/UVA-induced collagen cross-linking as

415   measured by atomic force microscopy. *PloS one.* 2014;9(1):e88186.

416 **19.** Choi S, Lee S-C, Lee H-J, et al. Structural response of human corneal and scleral

417   tissues to collagen cross-linking treatment with riboflavin and ultraviolet A light.

418   *Lasers in medical science.* 2013;28(5):1289-1296.

419 **20.** Randleman JB, Santhiago MR, Kymionis GD, Hafezi F. Corneal Cross-Linking

420   (CXL): Standardizing Terminology and Protocol Nomenclature. *J. Refract. Surg.*

421   Nov 1 2017;33(11):727-729.

422 **21.** Elsheikh A, Wang D, Brown M, Rama P, Campanelli M, Pye D. Assessment of

423   corneal biomechanical properties and their variation with age. *Curr. Eye Res.*

424   2007;32(1):11-19.

425 **22.** Boyce B, Jones R, Nguyen T, Grazier J. Stress-controlled viscoelastic tensile

426   response of bovine cornea. *J. Biomech.* 2007;40(11):2367-2376.

427 **23.** Wollensak G, Spoerl E, Seiler T. Stress-strain measurements of human and

428   porcine corneas after riboflavin–ultraviolet-A-induced cross-linking. *J. Cataract*
429   *Refract. Surg.* 2003;29(9):1780-1785.

430 **24.** Atherton TJ, Kerbyson DJ. Size invariant circle detection. *Image and Vision*
431   *computing.* 1999;17(11):795-803.

432 **25.** Yuen H, Princen J, Illingworth J, Kittler J. Comparative study of Hough transform
433   methods for circle finding. *Image and vision computing.* 1990;8(1):71-77.

434 **26.** Hatami-Marbini H, Etebu E, Rahimi A. Swelling pressure and hydration behavior
435   of porcine corneal stroma. *Curr. Eye Res.* 2013;38(11):1124-1132.

436 **27.** Hodson SA. Corneal stromal swelling. *Prog. Retin. Eye Res.* 1997;16(1):99-116.

437 **28.** Spoerl E, Huhle M, Seiler T. Induction of cross-links in corneal tissue. *Exp. Eye Res.*
438   1998;66(1):97-103.

439 **29.** McCall AS, Kraft S, Edelhauser HF, et al. Mechanisms of corneal tissue
440   cross-linking in response to treatment with topical riboflavin and
441   long-wavelength ultraviolet radiation (UVA). *Invest. Ophthalmol. Vis. Sci.*
442   2010;51(1):129-138.

443 **30.** Hahn RA, Birk DE. beta-D xyloside alters dermatan sulfate proteoglycan
444   synthesis and the organization of the developing avian corneal stroma.
445   *Development.* 1992;115(2):383-393.

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448

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 ±

465 standard deviation are indicated by the bold line and error bars. The red dash line represented

466 the value of 1; i.e. no difference between paired samples. Gray shaded region in A-E plots

467 represents 0.03MPa. (F) average thickness measurement of each comparison group after

468 treatment. (G) the stiffening ratio at 0.03MPa with and without correction for tissue thickness

469 changes. Values in F and G denote mean ± standard deviation (SD). * *p* value of < 0.05, **

470 p<0.01.

471

472 **Fig 3. Transmission electron microscopy (TEM) images of porcine corneas imaged at**

473 **depth of 80-150μm (A)** Representation TEM images of PBS, riboflavin in dextran, and

474 dextran only and riboflavin/dextran/UVA cross-linked corneas. Bar 500nm.An area of 300 x

475 300 dpi (yellow dash-square) is shown at higher magnification in (B) with example of the

476 measurement of the area and density of collagen fibrils.

477

478 **Fig 4. Measurement of ultrastructural parameters and collagen fibril distribution at**

479 **different depth intervals.** (A) Mean collagen fibril diameters of PBS and riboflavin in

480 dextran at each depth interval. (B) Collagen fibril diameter distributions plotted as either

481   uncorrected values from of riboflavin in dextran group (top panel, black filled squares), or

482   corrected for dehydration (top panel, gray squares) or its corresponding PBS control (middle

483   panel, blue filled squares). Traces are shown overlaid in bottom panels. Grey filled

484   background added to aid visualization. (C) Collagen fibril diameter distribution curve of

485   cross-linking group (green boxes) and its dextran control (black boxes) at each depth interval.

486   (D) Relative collagen fibril diameter, (E) relative interfibrillar spacing, (F) relative collagen

487   density of cross-linking group relative to its dextran group at each depth interval. Values are

488   plotted as mean±SD from n=6 (A and B) or n=10 (C, D, E and F). Asterisks denote

489   significant differences from control groups with * $p < 0.05$, ** $p<0.01$.

490

491   **Fig 5. Correlation between ultrastructural parameters and tangent modulus.** Tangent

492   modulus at 0.03MPa versus (A) collagen fibril diameter, (B) interfibrillar spacing and (C)

493   collagen fibril density. Black boxes; dextran treated eyes, green boxes;

494   Riboflavin+UVA+Dextran treated eyes, (D) percentage change in tangent modulus versus

495   percentage change in each ultrastructural parameter. Each box represents the measurements

496   from one eye for either fibril diameter (red), interfirbil distance (orange) or fibril density

497   (yellow). (E) Percentage change in tangent modulus plotted against the cumulative effects of

498   percentage change in fibril diameter+interfibril spacing-fibril density. Each box represents

499   one eye. Green line; linear line of best fit. Red dotted line; 100% correlation.

500

501   **Fig 6. Absorption FTIR spectra of porcine corneas following cross-linking treatments.**

502   (A) Representative FTIR spectra of corneas treated with PBS (bottom, gray),

503   riboflavin+dextran (middle, black) or riboflavin+dextran+UVA (top, green). (B) Regions

504   where the characteristic bands are located shown at higher magnification and regions of

505   interest indicated: Amide I (1,680–1,630 cm^{-1}), Amide II (1570-1515 cm^{-1}), Amide III

506   (1,350–1,200 cm^{-1}), and CO absorption band (1,150–1,000 cm^{-1}). (C) Relative intensity ratio

507   of each characteristic band and (D) conversion rate of amide II to amide III.

508

509   **Fig 7. Proposed model of UVA/riboflavin cross-linking with the presence of dextran.** The

510   black solid circle, blue solid line, green solid line and red solid line represent the collagen

511   molecules, proteoglycans, original cross-links formed between collagen molecules and

512   additional cross-links induced by UVA/riboflavin cross-linking treatment, respectively.

513

S1 Fig.Transmission electron microscopy (TEM) images of porcine corneas imaged at

different depth intervals following PBS, dextran and/or UVA/riboflavin treatment.

Representative TEM images of PBS (A), Riboflavin+PBS (B), Riboflavin+Dextran (C), or

Riboflavin+Dextran+UVA (D) and their corresponding controls at five depth intervals of

$0\text{-}50\mu m$, $80\text{-}150\mu m$, $200\text{-}250\mu m$, $300\text{-}350\mu m$, $400\text{-}450\mu m$.

520

S2 Fig. Collagen radius distribution of each treatment group at each depth intervals. The

distribution of control segments (blue); (A) PBS, (D) PBS, (G) PBS, and (K) Dextran, and

the experimental segments (orange) (B) PBS, (E) Riboflaivin+PBS, (H) Ribflavin+Dextran,

and (L) Riboflaivn+Dextran+UVA. Dashed lines represent trend curves. The overlapped

curves (C, F, I, J, M) were used for detecting the shifting of distribution in radius between

control and experimental segments. The data were obtained at five depth intervals of $0\text{-}50\mu m$,

$80\text{-}150\mu m$, $200\text{-}250\mu m$, $300\text{-}350\mu m$, $400\text{-}450\mu m$. p value of < 0.05 is indicated by an

asterisk compared with its internal control.

529

530   S1 Table. Summary of the average thickness and stiffening ratio at 0.03MPa of each group

531   before and after thickness correction. Values denote mean ± standard deviation (SD). *p* value

532   of < 0.05 is indicated by an asterisk compared with its control. Red asterisks indicate increase

533   compared with control, green represents decrease.

534

535   S2 Table. Mean diameter, interfibrillar spacing, and density of collagen fibrils of corneal

536   segments at different depth intervals following PBS, dextran and/or UVA/riboflavin

537   treatment. Values denote mean ± standard deviation (SD). *p* value of < 0.05 is indicated by an

538   asterisk compared with its control. Red asterisks indicate increase compared with control,

539   green represents decrease.

540

541   S3 Table. Summary of area ratio corresponding to indicatged characteristic bands in FTIR

542   spectra. Values denote mean ± standard deviation (SD). *p* value of < 0.05 is indicated by an

543   asterisk compared with its control. Red asterisks indicate increase compared with control,

544   green represents decrease.

545

546   S4 Table. Parameters (decreased percentage, hydration loss percentage, remaining hydration

547   percentage and swelling factor) used in correction of the collagen fibril radius distribution

548   curves.

549