Stress-strain measurements of human and porcine corneas after riboflavin–ultraviolet-A-induced cross-linking

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Purpose: To evaluate the biomechanical effect of combined riboflavin–ultraviolet A (UVA) treatment on porcine and human corneas.

Setting: Department of Ophthalmology, Technical University of Dresden, Dresden, Germany.

Methods: Corneal strips from 5 human enucleated eyes and 20 porcine cadaver corneas were treated with the photosensitizer riboflavin and irradiated with 2 double UVA diodes (370 nm, irradiance = 3 mW/cm^2) for 30 minutes. After cross-linking, static stress-strain measurements of the treated and untreated corneas were performed using a microcomputer-controlled biomaterial tester with a prestress of 5×10^3 Pa.

Results: There was a significant increase in corneal rigidity after cross-linking, indicated by a rise in stress in treated porcine corneas (by 71.9%) and human corneas (by 328.9%) and in Young's modulus by the factor 1.8 in porcine corneas and 4.5 in human corneas. The mean central corneal thickness was 850 μm ± 70 (SD) in porcine corneas and 550 ± 40 μm in human corneas.

Conclusions: Riboflavin−UVA-induced collagen cross-linking led to an increase in mechanical rigidity in porcine corneas and an even greater increase in human corneas. As collagen cross-linking is maximal in the anterior 300 μm of the cornea, the greater stiffening effect in human corneas can be explained by the relatively larger portion of the cornea being cross-linked in the overall thinner human cornea.

J Cataract Refract Surg 2003; 29:1780–1785 © 2003 ASCRS and ESCRS

The biomechanical properties of the cornea are primarily determined by the collagen fibers comprising 15 000 km and the degree of interfibrillar linkage.[1] Therefore, collagen cross-linking induced by combined riboflavin–ultraviolet A (UVA) treatment has been used successfully to mechanically stabilize the cornea in keratoconus (thereby stopping the progression of keratectasia[2]) and in corneal melting processes.[3] No adverse effects have been observed clinically.[2]

However, biomechanical experimental measurements of the effect of combined riboflavin–UVA treatment on human corneas have not been done.[4,5] This study measured the biomechanical effect of riboflavin–UVA-induced collagen cross-linking in human corneas and compared it to the effect in cross-linked porcine corneas having comparable biomechanical properties.[6,7]

Accepted for publication March 17, 2003.

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None of the authors has a financial interest in any product mentioned.

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0886-3350/03/$–see front matter
doi:10.1016/S0886-3350(03)00407-3

Materials and Methods

Specimen Preparation

Five freshly enucleated human eyes with intact and clear corneas that had been removed because of endophthalmitis (1 patient), choroidal melanoma (3 patients), and a nonhealing retinal detachment (1 patient) were used within 1 to 2 hours of enucleation. Before treatment, the corneal epithelium was completely scraped using a blunt hockey knife. The 12 o'clock position was marked with a nylon thread for orientation of the superior–inferior cut. The corneoscleral ring was then removed. With a self-constructed triple-blade scalpel, the cornea was cut into 2 equal strips of 4.0 mm width, 550 μm central corneal thickness, and 14.0 mm length including 1.0 mm sclera on both ends. Human cadaver eyes had been measured but were not included in the study as the variances in the stress-strain measurements were too great because of different postmortem times and degrees of autolysis.

Twenty fresh porcine cadaver eyes with intact epithelia and clear corneas were retrieved from the local slaughterhouse within 2 to 5 hours post-mortem. The eyes were deepithelialized mechanically, and the corneoscleral ring was removed using a scissors. With a self-constructed double-blade scalpel, 1 corneal strip of 5.0 mm width, 850 μm central corneal thickness, and 14.0 mm length including 1.0 mm sclera on both ends was cut in a superior–inferior fashion from the 12 o'clock position of the cornea, which was easily identified by its oval shape. Because of the natural thickness of the porcine cornea, only 1 corneal strip with clearly cut perpendicular edges could be prepared properly from each eye. There were shear artifacts in the deeper layers otherwise. Ten corneas were treated with riboflavin–UVA irradiation, and 10 were used as untreated controls.

Pachymetry

Using ultrasound pachymetry (Pachette, Technomed), the central corneal thickness was determined in the human and porcine eyes.

Treatment

Starting 5 minutes before the treatment, 0.1% riboflavin (vitamin B_2) photosensitizer solution (10 mg riboflavin-5-phosphate in 10 mL 20% dextran-T-500) was dropped on the treated strips and 20% dextran solution on the control strips at 5-minute intervals. Ultraviolet A irradiation (370 nm) was applied using 2 double UVA diodes (Roithner Lasertechnik) with an irradiance of 3 mW/cm^2 at a distance of 1.0 cm from the cornea for 30 minutes (Figure 1). This is equal to a dose of 5.4 J/cm^2. The parameters of exposure were chosen according to the treatment procedure used in keratoconus patients.[2] Three 1.3 V accumulators were used as a power generator. Before treatment, the desired irradiance of 3 mW/cm^2 was controlled with a calibrated UVA meter (LaserMate-Q, Laser 2000) at a distance of 1.0 cm and, if necessary, regulated with a potentiometer.

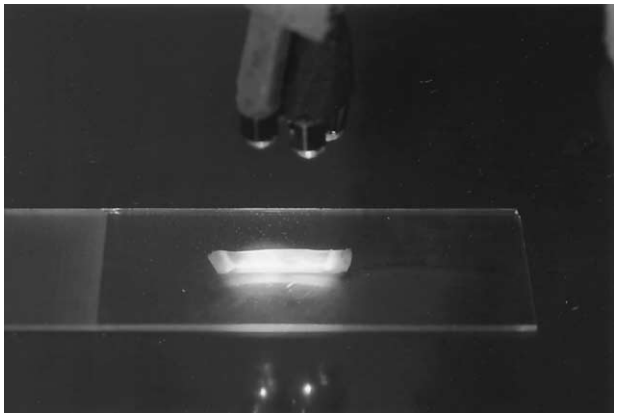

Figure 1. (Wollensak) Irradiation of riboflavin-treated human corneal strip using 2 double-UVA diodes (irradiance 3 mW/cm^2 , exposure time 30 min) at 1.0 cm distance.

Static Stress-Strain Measurements

The human and porcine corneal strips were clamped horizontally at a distance of 8.0 mm between the jaws of a commercially available microcomputer-controlled biomaterial tester (Minimat, Rheometric Scientific GmbH (Figure 2). To include the physiological stress range, a prestress of 5×10^3 Pa (1 Pa = 1 N/m^2) was used, which required a force of 10 mN in human corneas and 20 mN in porcine corneas because of the different thicknesses. The strain was then increased linearly with a velocity of 1.5 mm min^{-1}, and the stress was measured up to 2×10^5 Pa.[4,5] The stress-strain values were fitted by an exponential function $\sigma = A \exp (B \times \varepsilon)$ using the SPSS-calculation program (SPSS GmbH Software, Munich). Young's modulus (E) was calculated for 4%, 6%, and 8% strain as the gradient of the stress-strain graph ($E = d\sigma/d\varepsilon = A \times B \exp (B \times \varepsilon)$).

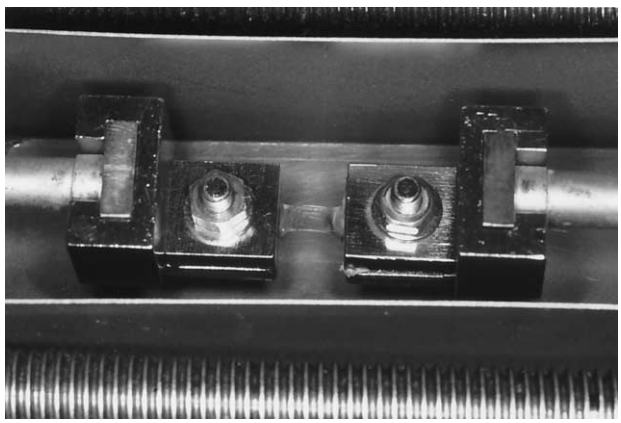

Figure 2. (Wollensak) Human corneal strip between the clamps of the stress-strain biomaterial tester (Minimat).

Statistical Evaluation

The stress data necessary for a strain of 4%, 6%, and 8% in treated and untreated corneas (separately for human and porcine eyes) and in human and porcine corneas were compared using the Student *t* test.

Results

Stress-Strain Curves

The stress-strain curves showed the typical exponential increase of a bioviscoelastic solid (Figure 3).

In porcine corneas, the stress using 6% strain was $98.5 \pm 29.77 \times 10^3$ Pa in the treated corneas and $57.3 \pm 17.3 \times 10^3$ Pa in the untreated corneas, corresponding to a 71.9% increase (Table 1). The difference was statistically significant ($P = .014$).

In human corneas, the stress using 6% strain was $227.3 \pm 95.7 \times 10^3$ Pa in the treated corneas and $53.0 \pm 11.5 \times 10^3$ Pa in the untreated corneas, corresponding to a 328.9% increase (Table 1). The difference was statistically significant ($P = .012$).

In untreated corneas in porcine and human eyes, the difference in the stress-strain values was not statistically significant ($P = .87$). In treated corneas in porcine and human eyes, the difference was statistically significant ($P = .01$). The increased biomechanical stiffness was also reflected in the different bending behaviors (Figure 4).

Young's Modulus

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 $E = d\sigma/d\varepsilon = A \times B \exp(B \times \varepsilon)$.

In porcine corneas at 6% strain, Young's modulus was 1.5×10^6 Pa in the untreated eyes and 2.7×10^6 Pa in the treated eyes (increase factor 1.8). In human cor-

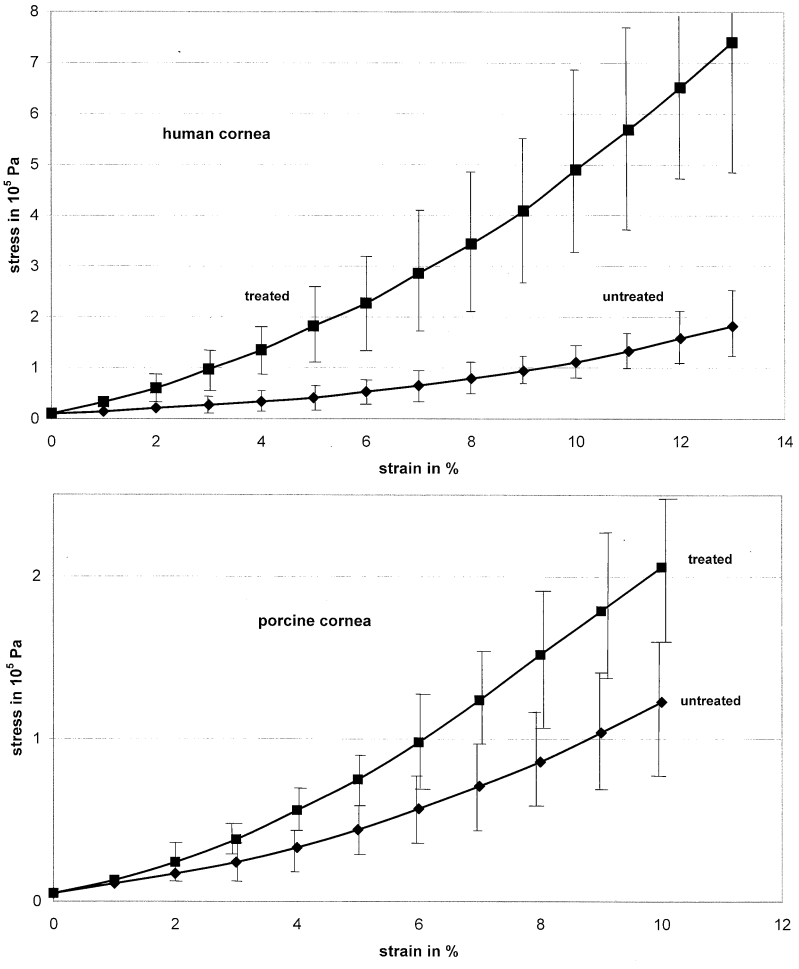

Figure 3. (Wollensak) *Top:* Stress-strain measurements of human corneas (n = 5) treated with riboflavin–UVA (irradiance = 3 mW/cm^2). *Bottom:* Stress-strain measurements of porcine corneas (n = 20) treated with riboflavin–UVA (irradiance = 3 mW/cm^2).

Table 1. Stress values for 4%, 6%, and 8% strain and calculated Young's modulus in brackets.

Type of Cornea	Stress at 4% (10^3 Pa)	Stress at 6% (10^3 Pa)	Stress at 8% (10^3 Pa)
Porcine			
Untreated	33.7 ± 9.3 ($E = 0.8 \times 10^6$ Pa)	57.3 ± 17.3 ($E = 1.5 \times 10^6$ Pa)	86.5 ± 29.9 ($E = 2.6 \times 10^6$ Pa)
Treated	55.8 ± 17.6 ($E = 1.4 \times 10^6$ Pa)	98.5 ± 29.7 ($E = 2.7 \times 10^6$ Pa)	151.8 ± 44.7 ($E = 5.3 \times 10^6$ Pa)
Human			
Untreated	34.3 ± 5.5 ($E = 0.8 \times 10^6$ Pa)	53.0 ± 11.5 ($E = 1.3 \times 10^6$ Pa)	79.3 ± 21.2 ($E = 2.2 \times 10^6$ Pa)
Treated	135.7 ± 61.4 ($E = 3.0 \times 10^6$ Pa)	227.3 ± 95.7 ($E = 5.9 \times 10^6$ Pa)	344.7 ± 141.9 ($E = 11.8 \times 10^6$ Pa)

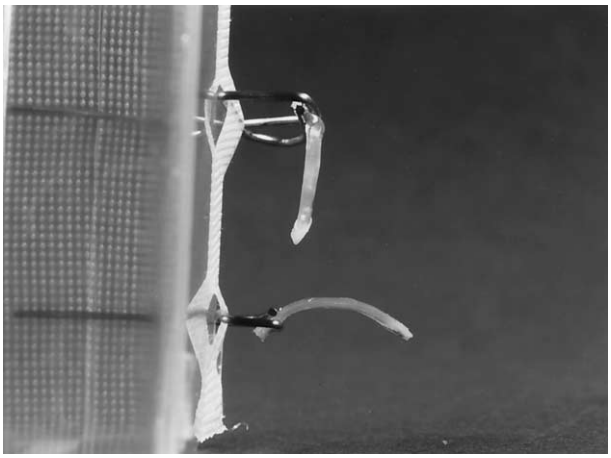

Figure 4. (Wollensak) Treated (below) and untreated (above) porcine corneal strips with preservation of the corneal curvature in the treated cornea due to the increase in bending stiffness by collagen cross-linking.

neas at 6% strain, Young's modulus was 1.3×10^6 Pa in the untreated eyes and 5.9×10^6 Pa in the treated eyes (increase factor 4.5).

Pachymetry

The mean central corneal thickness was 850 ± 70 μm in porcine eyes and 550 ± 40 μm in human eyes.

Discussion

We found a significant increase in biomechanical rigidity by a factor of 4.5 in human corneas, as indicated by Young's modulus, following riboflavin–UVA-induced collagen cross-linking. The increase in biomechanical stiffness in porcine eyes was also significant, but only by a factor of 1.8.

The increase in biomechanical stiffness in the human corneas was surprisingly high. From previous measurements of porcine eyes, using slightly different treatment conditions (ie, treatment time [45 minutes], UVA irradiation source [mercury lamp with 365 nm UV filter], UVA irradiance [2 mW/cm^2]), we expected an increase in the range of a factor of 2, as found in porcine eyes in the present study.[4]

From other experiments on the diameter of corneal collagen fibers,[8] resistance to enzymatic digestion,[9] and keratocyte loss after riboflavin–UVA treatment,[9,10] we know that the cross-linking and cytotoxic effects are significantly higher in the anterior portion of the cornea. This is caused by the significant increase in UVA absorption by riboflavin, leading to a rapid reduction of UVA irradiance and collagen cross-linking across the cornea.[11]

Given the uneven distribution of collagen cross-linking, with the maximum cross-linking effect in the anterior 300 μm of the corneal stroma, the treated corneas can be regarded as 2-layer structures. The anterior portion amounts to 35% cross-linking volume in the porcine cornea (300 μm/850 μm) and to 54% cross-linking volume in the human cornea (300 μm/550 μm), resulting in higher rigidity of the total cornea in human eyes, as reflected in the stress-strain measurements (Figures 3 and 5). In normal eyes, the more rigid anterior stroma also accounts for maintenance of the corneal curvature.[12,13]

The anterior localization of the main cross-linking effect has the advantage of allowing us to achieve a relatively high increase in corneal rigidity in human eyes because of the relatively small thickness of the human cornea and to spare the endothelium and the lens from cytotoxic damage provided the corneal stroma is normally thick.

To avoid additional cross-linking inhomogeneities in the horizontal dimensions, the irradiation was performed over the entire length of the corneal strips using 2 double

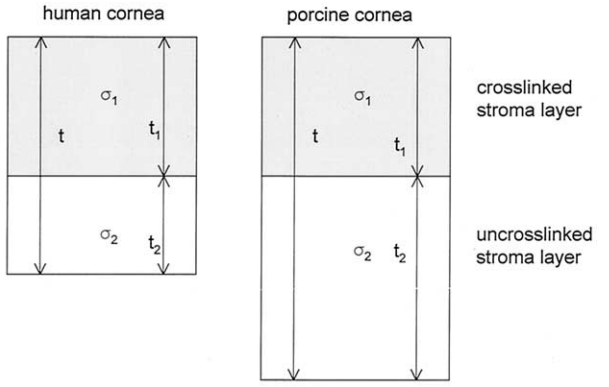

Figure 5. (Wollensak) Two-layer model of cross-linked cornea with the anterior cross-linked and posterior non-cross-linked corneal stroma. In porcine corneas, the relative portion of the cross-linked cornea (t_1/t) is less than in human corneas due to the greater corneal thickness in pig eyes (850 μm versus 550 μm), resulting in less rigidity for the total porcine cornea (as indicated by a lower σ_{total}) (σ = total stress of the cornea, σ_1 = partial stress of the cross-linked cornea layer, σ_2 = partial stress of the noncross-linked cornea layer, t = total thickness of the cornea, t_1 = partial thickness of the cross-linked cornea layer, t_2 = partial thickness of the noncross-linked cornea layer; $\sigma = \sigma_1 \times t_1/t + \sigma_2 \times t_2/t$).

UVA diodes, whereas in clinical applications, we irradiate only the central cornea using 1 double UVA diode.[2]

The rise in biomechanical rigidity after collagen cross-linking in human and porcine corneas is probably caused by an increase in the collagen fiber diameter due to intrafibril cross-links.[8]

Other cross-linking methods that have been tested successfully in vitro have a biomechanical effect on the cornea comparable to riboflavin–UVA treatment but cannot be used clinically because of the development of corneal haze and scarring, as after glutaraldehyde treatment (W.J. Dupps, ARVO abstract 147, 2002),[14] or application problems and prolonged treatment time, as with glyceraldehyde (F.J. Tessier, ARVO abstract 3234, 2002).[14]

In keratoconus, a 50% decrease in the stress necessary for a defined strain has been found.[15] Accordingly, we have used the cross-linking treatment mainly in progressive keratoconus and less frequently in corneal melting processes. Further applications lie in the field of refractive surgery and are being explored in conditions such as after iatrogenic laser in situ keratomileusis (LASIK) induced keratectasia[16,17] or for preventive treatment before LASIK for high myopia to avoid or reduce possible postoperative myopic regression or keratectasia. Collagen cross-linking might also help to avoid so-called central islands in broad-beam laser surgery (W.J. Dupps, ARVO abstract 147, 2002), often caused by differential corneal hydration,[18] which is markedly reduced after cross-linking (E. Spoerl, ARVO abstract 2339, 1997).

In conclusion, the present study showed a stronger increase in the biomechanical rigidity of the human cornea after riboflavin–UVA treatment than in the porcine cornea because of the relatively larger portion of cross-linking in the thinner human cornea. Practical applications of the new method are for progressive keratoconus, corneal-melting processes, and LASIK in high myopia.

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