Collagen Fiber Diameter in the Rabbit Cornea After Collagen Crosslinking by Riboflavin/UVA

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Objective: Collagen crosslinking of the cornea has been developed recently as a quasiconservative treatment of keratoconus. Biomechanical in vitro measurements have demonstrated a significant increase in biomechanical stiffness of the crosslinked cornea. The aim of the present study was to evaluate the effect of this new procedure on the collagen fiber diameter of the rabbit cornea.

Methods: The corneas of the right eyes of 10 New Zealand White albino rabbits were crosslinked by application of the photosensitizer riboflavin and exposure to UVA light (370 nm, 3 mW/cm^2) for 30 minutes. The left fellow control eyes were either left untreated (rabbits 1–4), deepithelialized (rabbits 5–7), or deepithelialized and treated with riboflavin/dextran solution (rabbits 8–10) to exclude an influence of epithelial debridement or hydration changes on the fiber diameter. On ultrathin sections of samples from the anterior and posterior cornea, the collagen fiber diameter was measured semiautomatically with the help of morphometric computer software.

Results: In the anterior stroma, the collagen fiber diameter in the treated corneas was significantly increased by 12.2% (3.96 nm), and in the posterior stroma by 4.6% (1.63 nm), compared with the control fellow eyes. In the crosslinked eyes, the collagen fiber diameter was also significantly increased by, on average, 9.3% (3.1 nm) in the anterior compared with the posterior stroma within the same eye.

Conclusions: Collagen crosslinking using riboflavin and UVA leads to a significant increase in corneal collagen diameter. This alteration is the morphologic correlate of the crosslinking process leading to an increase in biomechanical stability. The crosslinking effect is strongest in the anterior half of the stroma because of the rapid decrease in UVA irradiance across the corneal stroma as a result of riboflavin-enhanced UVA absorption.

Key Words: collagen fiber diameter, crosslinking, cornea, UVA, riboflavin

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We have recently developed a technique of collagen crosslinking in the cornea using UVA and the photosensitizer riboflavin to stiffen the cornea. In a prospective clinical pilot study including 22 patients with moderate or advanced progressive keratoconus and with a follow-up time of up to 4 years, the progression of keratoconus could be stopped in all treated eyes. Regression with a reduction of the maximal keratometry readings by 2 diopters was achieved in 70% of patients. Corneal and lens transparency as well as endothelial cell density remained unchanged.[1] Collagen crosslinking might therefore become a new way to stop the progression of keratectasia in keratoconus patients. By this means, the need for penetrating keratoplasty in keratoconus might be significantly reduced in the future. Given the simplicity and minimal costs of the treatment, it might also be well suited for developing countries. Another possible clinical use of collagen crosslinking lies in the field of refractive surgery, corneal ulcers, and stromal melting and thinning.

In extensive experimental studies including biomechanical stress-strain measurements, we could show a significant increase in corneal rigidity by about 70% in rabbit and porcine corneas after the crosslinking treatment[2–4] and an increased resistance to enzymatic digestion by collagenases.[5]

Crosslinking is a widespread phenomenon and can also be found in the aging and cataractous lens, where crosslinking of the lens crystallins leads to increased rigidity of the lens and to an increase of the molecular weight of the crystallin proteins from 20,000 to over 50,000.[6]

The aim of the present study was to elucidate the morphologic correlate of the new treatment by evaluating the influence of the crosslinking treatment on the corneal collagen fiber diameter.

MATERIALS AND METHODS

Animals

Ten female New Zealand White albino rabbits weighing 2–2.5 kg were used for the experiment. The right eyes of rabbits 1–10 were crosslinked with riboflavin and UVA. The left fellow eyes served as intraindividual controls, remaining completely untreated, in rabbits 1–4. In the left eyes of rabbits 5–7, epithelial debridement alone, and in rabbits 8–10 epithelial de-

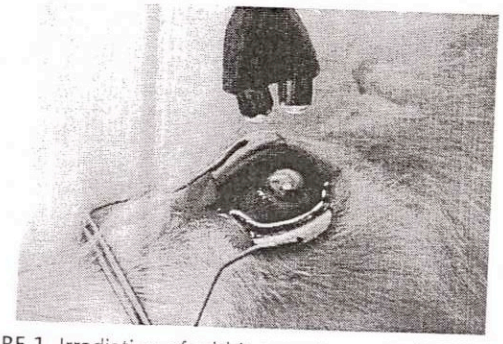

FIGURE 1. Irradiation of rabbit eye using a double UVA diode (370 nm, 3 mW/cm^2, 30 minutes) at a 1-cm distance.

bridement plus the application of dehydrating dextran/riboflavin solution was performed to exclude an influence on fiber diameter of epithelial abrasion or associated hydration changes. All animal procedures were approved by the ethics comittee and conformed to the ARVO Resolution on the Use of Animals in Ophthalmic and Vision Research.

Crosslinking Treatment

Ten rabbits were anesthetized with subcutaneous injection of a mixture of 1.5 mL ketamine hydrochloride 10% (35 mg/kg) and 0.5 mL xylazine hydrochloride (5 mg/kg). For premedication diazepam (10 mg) and atropine (0.5 mg) were used. After anesthesia, the central 5 mm of the cornea were deepithelialized mechanically using a blunt hockey knife. After the debridement, riboflavin photosensitizer solution containing 0.1% riboflavin-5-phosphate and 20% dextran T-500 was dropped onto the cornea 5 minutes before the irradiation and every 5 minutes during the irradiation. The UVA irradiation (370 nm) was applied using a double UVA diode (Roithner Lasertechnik, Vienna, Austria) with an irradiance of 3 mW/cm^2 for 30 minutes at a 1 cm distance from the cornea (Fig. 1). The animals were killed 4 hours after treatment in general anesthesia using an overdose of pentobarbital.

Transmission Electron Microscopy

After enucleation, the whole eye globes were fixed in 2.5% glutaraldehyde in 0.1 M PBS buffer at 4°C. After fixation, the globes were bisected, and 4 mm^2 samples of the central cornea were dissected at 50 μm depth for the anterior and at 350 μm corneal depth for the posterior stroma (Fig. 2) and embedded in epon. Ultrathin epon sections 50–70 nm thick were cut and contrasted with uranyl acetate and lead citrate and evaluated morphometrically using the electron microscope Morgagni 268D (Philips, Eindhoven, The Netherlands) at ×89,000 magnification. The EM pictures were transferred to a computer screen by an attached MegaView II camera. The diameters were marked manually with a computer mouse (Fig. 3) and calculated with the help of the semiautomatic software program Analysis (Soft Imaging System GmbH, Münster, Germany).

In each case, the diameters of 80 to 160 contiguous fiber section profiles were measured for the anterior and posterior stroma (Fig. 3). Only fiber profiles with clearly defined borders of high contrast were included; profiles with low contrast and indistinct borders were discarded. These were fewer than 1% of the total fiber count.

In some sections with a slightly ellipsoidal section profile (Fig. 4) as a result of oblique sectioning, the minimal transverse diameter of the collagen fibers was measured because in ellipsoidal section profiles the shortest diameter is equal to the diameter of the corresponding circular section profile.

Statistics

The outcome variables were compared statistically using one-way ANOVA followed by Sidak post hoc test and ex-

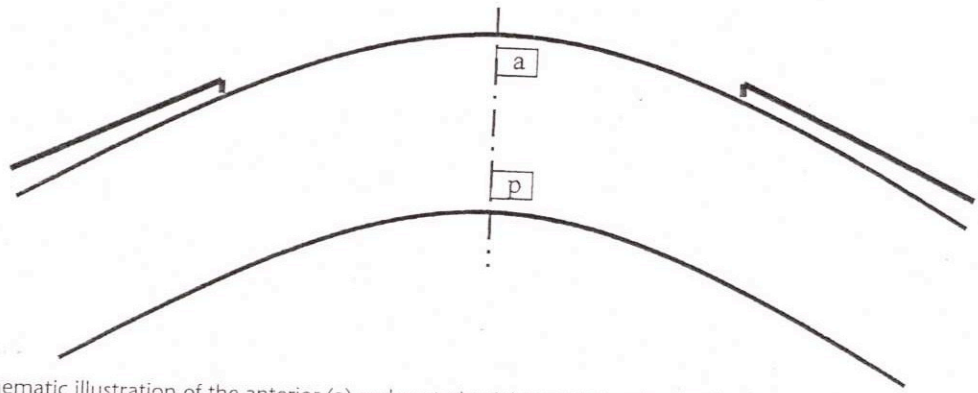

FIGURE 2. Schematic illustration of the anterior (a) and posterior (p) sample localization in the center of the cornea at 50 μm and 350 μm depth.

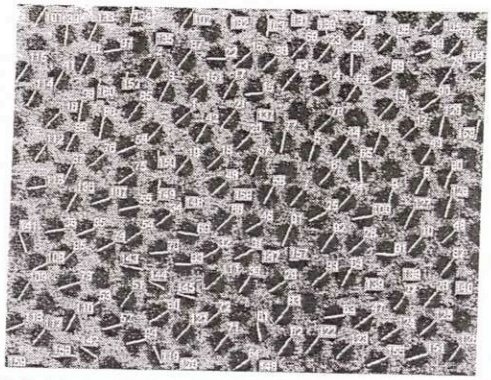

FIGURE 3. Measurement of collagen fiber diameter using morphometric computer software. The numbers indicated correspond to the number of measured profiles (n = 160), the drawn lines to the minimum diameter. TEM, ×89,000.

pressed as mean ± standard deviation. All statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS GmbH, Munich, Germany).

RESULTS

ANOVA testing showed statistical significance at the level of significance 0.001 between the treated and untreated corneas in their anterior and posterior localization. In brief, the following observations were made:

1. In the anterior stroma, the collagen fiber diameter of the crosslinked corneas was significantly increased by 3.96 ± 2.5 nm (12.2%) on the average (Sidak test, $P = 0.008$) compared with the untreated control fellow eyes (Table 1).
2. In the posterior stroma, the collagen fiber diameter of the crosslinked corneas was increased by 1.63 ± 1.45 nm (4.6%) on the average (Sidak test, $P = 0.023$) compared with the untreated control eyes (Table 2). Only in the posterior stroma of cases 5 and 6 was the collagen fiber diameter not statistically significantly increased.
3. In the crosslinked eyes, the collagen fiber diameter was also significantly increased by on the average 3.12 ± 2.1 nm (9.3%) in the anterior compared with the posterior stroma within the same cornea (Table 3). The control eyes only showed a tendency for an increased diameter in the anterior stroma with no statistically significant difference compared with the posterior collagen fiber diameter in the same eye.

DISCUSSION

This study has shown a statistically significant increase in corneal collagen fiber diameter, by an average of 12.2% (3.96 nm) in the anterior and by 4.6% (1.63 nm) in the posterior stroma, as a result of riboflavin/UVA-induced collagen crosslinking. In addition, the collagen fiber diameter was also significantly increased by an average of 9.3% (3.1 nm) in the anterior compared with the posterior stroma in the crosslinked corneas.

Similar to our results, a statistically significant increase in the collagen fiber diameter of the cornea by 4.5% with age-related crosslinking has been shown by others.[7,8] In photosensitized reactions as occurs with riboflavin and UVA treatment, an excited so-called triplet state of the sensitizer is induced by

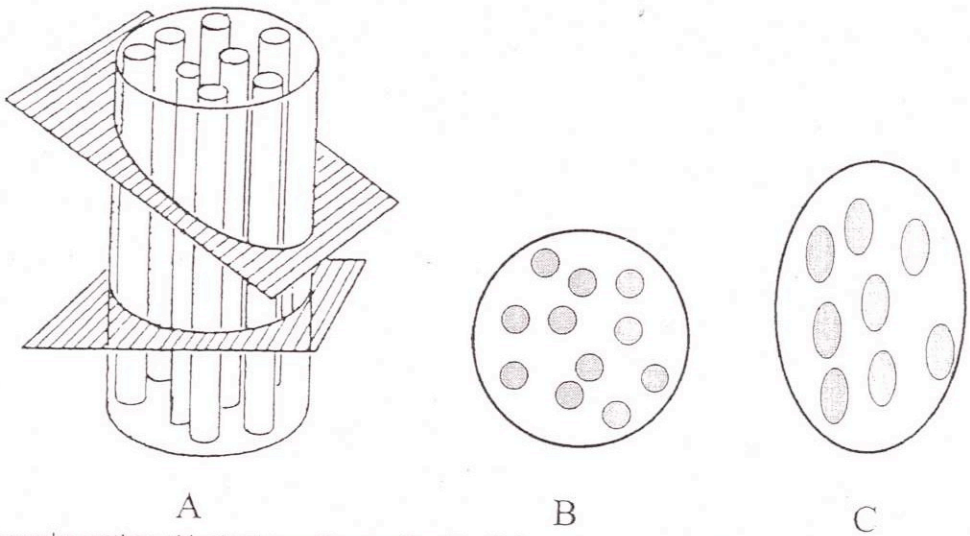

FIGURE 4. Rectangular section with circular section profiles (B). Oblique sections with ellipsoidal section profiles (C). The minimum profile diameter is identical in both section profiles (A).

TABLE 1. Difference in Collagen Fiber Diameter in Anterior Stroma Between Crosslinked and Control Fellow Eyes

Rabbit	Treated Eye (nm)	Control Eye (nm)	Rel. Difference (nm)	Significance (*P*)
1	36.78 ± 2.94	27.65 ± 2.19	9.13	0.0001
2	36.96 ± 2.81	33.40 ± 2.77	3.56	0.0001
3	37.11 ± 3.49	34.26 ± 3.20	2.85	0.005
4	36.76 ± 3.08	32.67 ± 2.60	4.09	0.0001
5	33.15 ± 2.43	31.64 ± 2.82	1.51	0.009
6	40.43 ± 2.52	33.15 ± 2.28	7.28	0.0001
7	33.23 ± 3.24	31.73 ± 3.24	1.50	0.036
8	37.89 ± 2.33	33.67 ± 2.56	4.22	0.0001
9	35.04 ± 2.67	31.78 ± 3.14	3.26	0.0003
10	36.21 ± 2.54	33.97 ± 2.43	2.24	0.005
Mean			3.96 ± 2.47	

the absorption of UVA light. So-called reactive oxygen species (ROS) or free radicals are generated that can cause, on the one hand, photooxidative damage of cells and, on the other hand, physical crosslinking of collagen, thereby increasing the fiber diameter and the mechanical stiffness of the collagen involved.[9] An increase in collagen fiber diameter caused by crosslinking induced by aging[8] or diabetes mellitus is a general phenomenon and has been measured in various other collagenous tissues.[10] It has been elegantly demonstrated in vitreous samples of diabetic patients using scanning electron microscopy.[11] The reason for the increased fiber diameter is that the induced crosslinks push the collagen molecules apart, resulting in an increased intermolecular spacing and diameter of the collagen fibers.[12] Interestingly, also in cataract formation crosslinking mediated by endogenous riboflavin has been found to lead to a massive increase in the molecular weight of crystallin proteins and increased lens hardness.[6,13]

The relative increase in the collagen fiber diameter compared with the untreated eyes was more pronounced in the anterior than in the posterior stroma. In addition, the relative difference in collagen fiber diameter between anterior and posterior stroma of the same eye was statistically significant in the treated eyes. This finding can be explained by the rapid loss of UVA irradiance across the cornea because of the increase in UVA absorption by the photosensitizer riboflavin. In an earlier experiment, we measured a 95% reduction of UVA irradiance at the endothelial level[2] after riboflavin/UVA treatment, which could explain the smaller degree of crosslinking in the posterior portion of the cornea. In the untreated eyes, only a nonsignificant tendency for a greater collagen fiber diameter in the anterior compared with the posterior stroma of the same eye was found, which has already been described by others.[14] The anterior localization of the crosslinking effect is a great advantage in the clinical application because the corneal endothe-

TABLE 2. Difference in Collagen Fiber Diameter in Posterior Stroma Between Crosslinked and Control Fellow Eyes

Rabbit	Treated Eye (nm)	Control Eye (nm)	Rel. Difference (nm)	Significance (*P*)
1	30.69 ± 2.88	29.49 ± 2.81	1.2	0.018
2	33.48 ± 2.94	32.07 ± 2.49	1.41	0.003
3	34.27 ± 3.42	32.13 ± 3.41	2.14	0.0001
4	34.40 ± 2.86	29.71 ± 2.63	4.69	0.0001
5	33.51 ± 2.62	33.86 ± 2.17	−0.35	0.922
6	34.72 ± 2.46	33.81 ± 2.37	0.91	0.092
7	31.90 ± 3.31	28.49 ± 3.12	3.41	0.0001
8	32.54 ± 2.45	31.67 ± 2.67	0.87	0.005
9	33.13 ± 2.89	32.34 ± 3.21	0.79	0.006
10	33.76 ± 2.93	32.54 ± 2.87	1.22	0.003
			Mean diff.: 1.63 ± 1.45	

TABLE 3. Anterior-posterior Difference of Collagen Fiber Diameter in the Crosslinked Eyes

Rabbit	Rel. Difference (nm)	Significance (*P*)
1	6.09	0.0001
2	3.48	0.0001
3	2.84	0.0001
4	2.36	0.0001
5	−0.36	0.943
6	5.71	0.0001
7	1.33	0.113
8	5.35	0.0001
9	1.91	0.008
10	2.45	0.0001
Mean	3.12 ± 2.07	

lium is therefore not affected by photooxidative damage and is spared.

The increase in collagen fiber diameter by 3.96 nm after riboflavin/UVA-induced corneal crosslinking should not lead to a loss of corneal transparency because the induced inhomogeneity is much lower than the critical threshold value for corneal opacification of 150 nm (one third of the wavelength of white light).[15–17] Accordingly, loss of transparency has never been observed so far in our 4-year clinical study of riboflavin/UVA treatment.[1]

The influence of fixation on corneal collagen fibril diameter has been examined systematically by others using x-ray diffraction measurements of unfixed cornea as the gold standard. They found an increase of the collagen fiber diameter through crosslinking induced by the fixative glutaraldehyde and a reduction in fiber diameter by the embedding resin. The two opposite effects cancel each other out.[18] In our series, all the specimens underwent the same fixation and processing so that the relative differences between the specimens cannot have been influenced by tissue processing anyway. In all but the untreated control eyes of cases 1–4, a slight postoperative corneal edema was observed. The collagen fiber diameter, however, is not affected by corneal hydration as demonstrated in the control eyes of cases 5–10 where epithelial debridement with and without dehydrating dextran solution was compared and not significantly different, which has also been shown by others similarly.[19,20]

In conclusion, riboflavin/UVA-induced collagen crosslinking causes an increase of the corneal collagen fiber diameter that is most pronounced in the anterior portion of the stroma. This feature is the main morphologic alteration underlying the increased biomechanical stiffness of the cornea after collagen crosslinking using riboflavin and UVA.

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