Collagen crosslinking of human and porcine sclera

Gregor Wollensak, MD, Eberhard Spoerl, PhD

Purpose: To develop methods of collagen crosslinking the sclera to increase its biomechanical strength for the treatment of progressive myopia.

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

Methods: Sagitally oriented scleral strips of 4.0 mm × 8.0 mm were prepared from 5 human postmortem eyes and 50 porcine cadaver eyes and treated with various crosslinking methods including physical crosslinking by combined riboflavin–ultraviolet A (UVA) or rose bengal/white-light irradiation and chemical crosslinking by incubation with glucose, ribose, glyceraldehyde, and glutaraldehyde solutions. Parallel scleral strips from the same eye were used as untreated controls. After crosslinking, stress-strain measurements of the treated and control scleras were performed using a microcomputer-controlled biomaterial tester.

Results: A statistically significant increase in scleral rigidity was found after crosslinking with riboflavin–UVA, with a rise in stress in treated porcine (157%) and human (29%) sclera, and after treatment with glyceraldehyde, with a rise in stress in treated porcine (487%) and human (34%) sclera, and with glutaraldehyde, with a rise in stress in treated porcine (817%) and human sclera (122%) at 8% strain. The other crosslinking methods proved ineffective. The untreated human sclera had a 4-fold higher stiffness than porcine sclera.

Conclusions: Collagen crosslinking induced by riboflavin–UVA, glyceraldehyde, and glutaraldehyde led to a significant increase in biomechanical strength in human and porcine sclera. Using these methods, collagen crosslinking might become a treatment possibility for progressive myopia. Future animal and clinical studies must determine the best application methods and the long-term effects of increased crosslinking on scleral rigidity and prevent potential toxicity or serious side effects.

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Myopia is a common ocular disorder that was described by the ancient Greeks. It has been corrected by concave lenses since the 16th century. The prevalence of myopia is around 30% of the general population in the United States and Europe and as high as 60% in Asian countries.[1] The prevalence of pathologic myopia with a refractive error greater than −8.0 diopters (D) is around 0.3% of the general population in the United States and 1% in Japan.[2]

Myopic progression (around −0.5 D in a 2-year interval) in up to 50% of myopes is an unsolved problem.[3] Therapeutic attempts to arrest myopic progression comprise nonsurgical treatments such as administration of atropine, tropicamide, pirenzipine, timolol, apomorphine[4] and the use of bifocal and multifocal glasses or rigid contact lenses to assist in near work and accommodation. Surgical means of scleral strengthening such as

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From the Department of Ophthalmology, Technical University of Dresden, Dresden, Germany.

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Reprint requests to Priv.-Doz. Dr. med Gregor Wollensak, University Eye Clinic Dresden Fetscherstrasse 74, D-01307 Dresden, Germany. E-mail: gwollens@hotmail.com.

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injection of a polymeric composition forming a gel under Tenon's capsule and inducing scar tissue[5] or so-called scleral reinforcement operations in which donor sclera, fascia lata, or synthetic bands[6,7] are placed around the back of the globe and sutured to the sclera to provide scleral support and prevent axial elongation have shown limited success.

The etiology of myopia is controversial, and a multifactorial cause is assumed. Besides classic risk factors such as genetic predisposition, high level of education, near work, scleral weakness with deranged metabolism of the sclera,[8,9] and abnormal scleral collagen[10] have been proposed. High myopia, blue sclera, and keratoconus have been described in Ehlers-Danlos syndrome in which a lack of intermolecular crosslinks in collagen is known.[11] Conversely, in diabetic patients, where glucose-induced collagen crosslinking is known, axial myopia is rare.[12] Moreover, recent animal experiments in tree shrews show the influence of collagen crosslinking on the development of experimental myopia.[13]

Collagen crosslinking, which was recently introduced for the treatment of progressive keratoconus,[14,15] may be a way to strengthen the sclera and prevent myopic progression. In contrast to other treatments, this would correct a cause rather than an effect. In the present in vitro study, we evaluated the efficiency of various methods of collagen crosslinking in human and porcine sclera to increase the biomechanical strength of the sclera. Future animal studies, however, must exclude potential toxic side effects before collagen crosslinking can be applied clinically.

Materials and Methods

Specimen Preparation

Five human eye-bank eyes from nonmyopic subjects with a donor age of 45 to 63 years were obtained within 8 hours post-mortem and were used after the corneal button was removed for transplantation. In addition, 50 porcine cadaver eyes were retrieved within 5 hours post-mortem from the local abattoir. After the episcleral tissue was removed, the anterior segment of the globe was cut off. The opened globe was mounted inversely on a globular-shaped plastic block.

In the porcine eyes, 2 parallel 8.0 mm × 4.0 mm scleral strips were dissected sagitally at the 12 o'clock position (Figure 1) starting 10.0 mm from the limbus using a self-constructed triple-blade scalpel. In the human eyes, 2 parallel 8.0 mm × 4.0 mm scleral strips were dissected in each quadrant beginning 10.0 mm from the limbus, yielding 8 strips per eye. The tissues adherent to the scleral strips internally were carefully peeled off.

Figure 1. (Wollensak) Location of the longitudinally excised porcine scleral strips.

Pachymetry

The scleral strips were excised from the equatorial region 10 to 18 mm from the limbus because the scleral thickness there is relatively uniform in human and porcine sclera.[15–17]

The thickness of the strips was determined using ultrasound pachymetry (Pachette, Technomed).

Crosslinking Treatment

In the human scleras, 4 scleral strips were used per treatment group and compared to the adjacent parallel control strips from the same eye. In the porcine scleras, 10 strips were used per treatment group and compared to the respective parallel control strips from the same eye. All scleral strips including the controls were soaked in 20% dextran solution to avoid hydration-induced swelling. The crosslinking times were chosen according to our previous experience in crosslinking the cornea.[15]

The following crosslinking treatments were tested:

Physical Crosslinking. (1) Riboflavin−UVA: Ten minutes before the treatment, 0.1% riboflavin photosensitizer solution (10 mg riboflavin-5-phosphate in 10 mL 20% dextran-T-500) was dropped on the treated strips. 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 cm from the sclera for 30 minutes. (2) Rose bengal and white light: Following application of rose bengal for 10 minutes, the strips were irradiated with a white-light fiber optic for 1 hour.

Chemical Crosslinking. (1) Incubation in modified Eagle medium (MEM) plus glyceraldehyde (0.2 mol) for 5 days.

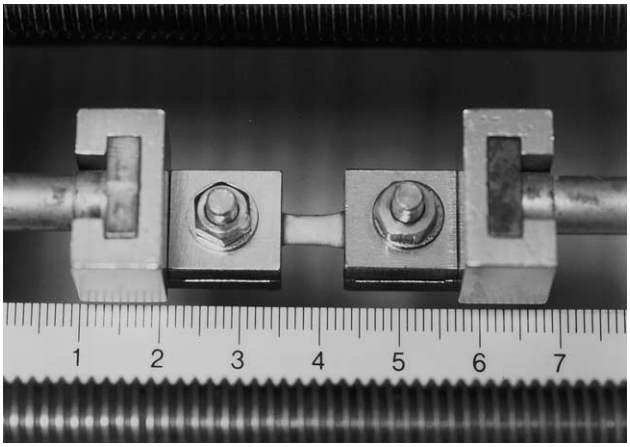

Figure 2. (Wollensak) Material tester with scleral strips beween the clamps.

(2) Incubation in glutaraldehyde (0.1%) for 1 hour. (3) Incubation in MEM plus ribose (0.5 mol) for 1 week. (4) Incubation in MEM plus glucose (0.5 mol) for 1 week.

Stress-Strain Measurements

The human and porcine scleral strips were clamped horizontally with 6.0 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 10×10^3 Pa was used, which required a force of 20 mN in both human and porcine sclera. The strain was increased linearly with a velocity of 1.5 mm min^{-1}, and the stress was measured up to 3.5×10^6 Pa. The stress-strain values were fitted by an exponential function $\sigma = A \exp (B \times \varepsilon)$ using the SPSS calculation program (SPSS GmbH Software). Young's modulus (E) was calculated for 8% strain as the gradient of the stress-strain graph ($E = d\sigma/d\varepsilon = A \times B \exp (B \times \varepsilon)$).

Statistical Evaluation

The stress data necessary for a strain of 8% in the untreated control group and the various treatment groups were compared using an analysis of variance.

Results

Stress-Strain Measurements

The stress at 8% strain was $236.8 \pm 179.5 \times 10^3$ Pa in untreated porcine scleras and $1037.3 \pm 477.6 \times 10^3$ Pa in untreated human scleras, demonstrating a 4-fold higher stiffness in natural, untreated human sclera (Tables 1 and 2; Figures 3 and 4). The difference was statistically significant ($P = .02$, Student t test).

In both porcine and human sclera, a significant increase in corneal rigidity was found after crosslinking by glutaraldehyde, glyceraldehyde, and riboflavin–UVA in decreasing order. The achieved absolute stress values for crosslinked porcine and human sclera were similar, but the relative increase in scleral rigidity was markedly higher in porcine sclera.

Pachymetry

The scleral thickness of the human sclera at the equator was 0.4 ± 0.15 mm and of the porcine sclera at the equator, 0.8 ± 0.17 mm.

Discussion

Efficacious collagen crosslinking with a significant rise in biomechanical strength up to 120% in human sclera and 817% in porcine sclera was achieved after crosslinking by glutaraldehyde, glyceraldehyde, or combined riboflavin−UVA treatment.

Collagen crosslinking is a widespread phenomenon, as seen in aging and diabetes mellitus, and causes mechanical strengthening of collagenous tissue by the induction of intrafibrillar and interfibrillar chemical bonds.[18] However, there are differences in the strength of crosslinking among the various crosslinking methods, as shown in the present study. Glutaraldehyde is known to be 1 of the strongest crosslinking agents with 1 of the highest number of crosslinks, and therefore its strong efficiency is not surprising.[19] Glyceraldehyde leads to glycation-induced crosslinking, as shown in lens proteins,[20] and is used successfully in the crosslinking of gelatin microspheres as pharmaceutical drug-delivery systems.[21] Riboflavin−UVA induces physical crosslinking by UVA and the photosensitizer riboflavin and has been used successfully as a relatively strong crosslinking method for the treatment of progressive keratoconus.[14] Crosslinking by rose bengal [22] was probably not effective because of poor scleral penetration, which has also been described in corneal stroma.[23] Glucose and ribose lead to glycation-induced crosslinking but are relatively ineffective crosslinkers.[15,24,25]

Effective crosslinkers such as glutaraldehyde and glyceraldehyde led to a relatively higher increase in mechanical stiffness in porcine sclera because of the lower baseline values of untreated porcine sclera, but the maximum stress values after glutaraldehyde and glyceraldehyde crosslinking were remarkably similar in porcine and human sclera. The reason for this phenomenon

Table 1. Stress and Young's modulus at *8%* strain in porcine sclera.

Porcine Sclera	Stress (10^3 Pa)	Young's Modulus (10^6 Pa)	Increase in % (*P* Value)
Untreated control	237 ± 180	5.95	—
Rose bengal/light	228 ± 77.0	5.80	NS
Glucose 1 week	204 ± 147	4.87	NS
Ribose 1 week	123 ± 21	3.69	NS
Riboflavin–UVA	608 ± 319	14.63	157 (.03)
Glyceraldehyde	1388 ± 433	30.88	487 (.02)
Glutaraldehyde	2166 ± 927	52.76	817 (.04)

NS = not significant

seems to be a sort of saturation process for these 2 types of crosslinking that leads to almost identical maximum mechanical strength in human and porcine sclera after strong crosslinking, despite different baseline values. However, the lower mechanical strength of untreated porcine sclera (factor 3) is at least partially compensated by greater thickness (factor 2).

The scleral strength and the degree of collagen crosslinking in the sclera seem to play a pivotal role in the development of myopia[10] because in myopia one finds axial elongation with a weakened, often staphylomatous, sclera of reduced thickness with a reduction in fibril diameter.[26,27] High myopia of up to −18.0 D with thin blue sclera and keratoconus is described in Ehlers-Danlos syndrome in which a lack of reducible intermolecular crosslinks is present in collagen and a deficiency of lysyl oxidase, an enzyme involved in the formation of collagen crosslinks, has been found.[11] In diabetic patients, however, where glycation-induced collagen crosslinking is increased, a reduced axial length was found[12] and axial myopia is rare.[28] Similarly, in animal models, deprivation myopia was increased to −23.0 D[6] compared to −11.0 D in a control group by giving monocularly deprived tree shrews systemic injections of the lathyrogenic agent beta–aminopropionitrile, which prevents collagen crosslinking by inhibiting lysyl oxidase.[13] In addition, in the myopic tree shrews, the scleral creep rate was increased.[29]

Therefore, scleral collagen might be a good target for the crosslinking treatment in progressive myopia and should be tried clinically and experimentally[10] with the methods we have developed in the present study, ie, glutaraldehyde, glyceraldehydes, and riboflavin–UVA, which proved to be reasonably effective. The somewhat moderate increase in mechanical strength after crosslinking in vitro for human sclera (30% to 120%) should be higher in myopes because they have a significantly lower level of mechanical stiffness with a significantly decreased scleral tensile strength and increased scleral extensibility,[8–10,30] as in the untreated porcine scleras, so the degree of crosslinking can be markedly increased by the treatment.

As for the best location of the crosslinking treatment, we know from other studies that in myopia, the

Table 2. Stress and Young's modulus at *8%* strain in human sclera.

Human Sclera	Stress (10^3 Pa)	Young's Modulus (10^6 Pa)	Increase in % (*P* Value)
Untreated control	1037 ± 478	22.82	—
Rose bengal/light	1063 ± 387	23.1	NS
Glucose 1 week	1000 ± 421	22.38	NS
Ribose 1 week	1010 ± 373	22.48	NS
Riboflavin–UVA	1338 ± 453	29.91	29 (.04)
Glyceraldehyde	1388 ± 822	33.26	34 (.04)
Glutaraldehyde	2300 ± 448	54.69	122 (.03)

NS = not significant

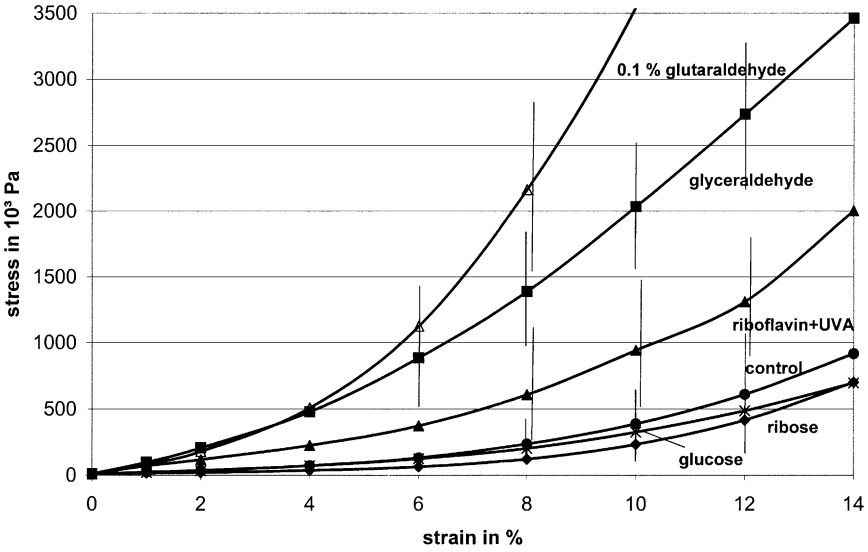

Figure 3. (Wollensak) Stress-strain curves in porcine scleral strips.

pathological changes in the sclera such as thinning, reduction in fibril diameter,[26] and increased scleral extensibility are highest at the posterior pole.[9,27,29,30] Therefore, the posterior sclera should be the main target of the crosslinking treatment. Special caution, however, must be applied because of the closeness of the macula.

Different modes for in vivo application must be developed for the 3 most effective crosslinking treatments. The sclera has a large and relatively easily accessible surface area and is permeable to molecules as large as 150 000 d, as demonstrated in rabbit sclera.[31] Therefore, glutaraldehyde (MW 100 d), glyceraldehyde (MW 90.08 d), and riboflavin-5-phosphate (MW 456 d) should easily penetrate the sclera. They could be administered, for example, by peribulbar injection or by direct intrascleral injection after surgical access to the bare sclera had been opened. For repeated or long-term treatments, an osmotic pump,[31] a soaked scleral buckle, or less traumatically, repeated parabulbar sub-Tenon's injections as in Avetisov's scleral strengthening injections[5] or steroid injections in uveitis could be performed.

To develop practical methods of application and to demonstrate the effect of the crosslinking treatment against the development or progression of myopia, animal studies with form deprivation or optical defocus models in the tree shrew or monkey[13] must be performed. Riboflavin–UVA irradiation could be applied only after direct surgical access to the sclera, but the extent of the treatment area should be easily controllable because of the visible UVA-light-induced fluorescence.

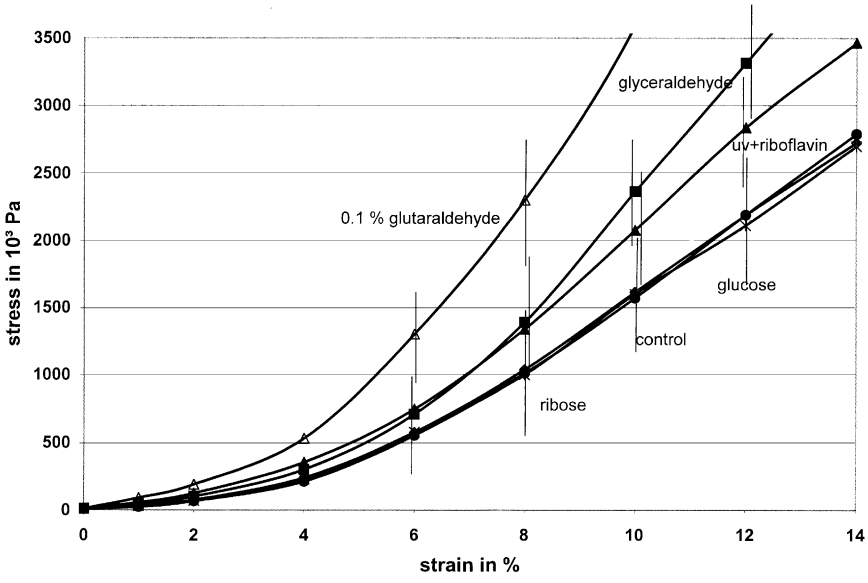

Figure 4. (Wollensak) Stress-strain curves in human scleral strips.

Glyceraldehyde and glutaraldehyde would have the advantage of a more extended application zone with a larger spatial distribution but reduced spatial control.

Before clinical application, all the crosslinking treatments must be tested for possible toxic effects on the retina, lens, and other ocular structures. Glutaraldehyde is known to have a high cytotoxicity and if used must be tried cautiously as with other cytotoxic agents, for example, 5-fluorouracil or mitomycin in glaucoma filter operations, to avoid damage to the choroid and retina. Glyceraldehyde is usually considered to be nontoxic and is a natural product of metabolism.[21,32] Still, specific cytotoxicity to ocular structures must be excluded in animal experiments. Riboflavin–UVA treatment should mainly damage the fibrocytes of the superficial layers of the treated sclera because of the high absorption rate of UVA in sclera.

In summary, we have shown that human and porcine scleral collagen can be crosslinked effectively using glyceraldehyde, glutaraldehyde, and riboflavin–UVA irradiation, leading to a statistically significant increase in the biomechanical rigidity of the sclera. Future in vivo animal and clinical studies must determine the best method of application, exclude side effects, and prove the in vivo effectiveness of the crosslinking treatment in stopping the progression of myopia.

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