Damage threshold in adult rabbit eyes after scleral cross-linking by riboflavin/blue light application

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Abstract  
Several scleral cross-linking (SXL) methods were suggested to increase the biomechanical stiffness of scleral tissue and therefore, to inhibit axial eye elongation in progressive myopia. In addition to scleral cross-linking and biomechanical effects caused by riboflavin and light irradiation such a treatment might induce tissue damage, dependent on the light intensity used. Therefore, we characterized the damage threshold and mechanical stiffening effect in rabbit eyes after application of riboflavin combined with various blue light intensities. Adult pigmented and albino rabbits were treated with riboflavin (0.5 %) and varying blue light (450 ± 50 nm) dosages from 18 to 780 J/cm² (15 to 650 mW/cm² for 20 min). Scleral, choroidal and retinal tissue alterations were detected by means of light microscopy, electron microscopy and immunohistochemistry. Biomechanical changes were measured by shear rheology. Blue light dosages of 480 J/cm² (400 mW/cm²) and beyond induced pathological changes in ocular tissues; the damage threshold was defined by the light intensities which induced cellular degeneration and/or massive collagen structure changes. At such high dosages, we observed alterations of the collagen structure in scleral tissue, as well as pigment aggregation, internal hemorrhages, and collapsed blood vessels. Additionally, photoreceptor degenerations associated with microglia activation and macroglia cell reactivity in the retina were detected. These pathological alterations were locally restricted to the treated areas. Pigmentation of rabbit eyes did not change the damage threshold after a treatment with riboflavin and blue light but seems to influence the vulnerability for blue light irradiations. Increased biomechanical stiffness of scleral tissue could be achieved with blue light intensities below the characterized damage threshold. We conclude that riboflavin and blue light application increased the biomechanical stiffness of scleral tissue at blue light energy levels below the damage threshold. Therefore, applied blue light intensities below the characterized damage threshold might define a therapeutic blue light tolerance range.

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1. Introduction  
Corneal cross-linking was introduced as a possible treatment technique for keratoconus by Spoerl, Seiler and colleagues, about 15 years ago (Spoerl et al., 1998; Spoerl and Seiler, 1999). Meanwhile, corneal cross-linking (CXL) by application of riboflavin and UVA light became an established and increasingly common treatment to...
stabilize the cornea and preventing keratoconus progression (Raiskup-Wolf et al., 2008; Wollensak et al., 2003). Aside from this application on the cornea, cross-linking was suggested and investigated as a method to increase scleral rigidity (Wollensak et al., 2005; Wollensak and Spoerl, 2004). Treating the sclera with riboflavin and UV-light is aimed at strengthening the biomechanically weak sclera (Wang et al., 2012) in cases of progressive myopia and therefore, to reduce the axial eye elongation (McBrien and Gentle, 2003). This would reduce the severity of pathological outcomes such as retinal degeneration, tears, and detachments which can cause blindness (Tano, 2002). Other suggested treatments of progressive high myopia (e.g. a variety of surgical scleroplasty procedures, injection of polymer composition on the scleral surface or peri-scleral reinforcement by grafting donor tissue or synthetic materials) were not effective, and thus were not introduced into accepted clinical practice (Nooram, 2002). Therefore, various scleral cross-linking (SXL) methods might possess the potential to be translated into therapeutic use. However, SXL with riboflavin and UVA light irradiation with an intensity of 4.2 mW/cm² for 30 min (7.6 J/cm² with 0.1 % riboflavin solution) was shown to cause serious side effects in retinal tissue (Wollensak et al., 2005). A reduction of the UV light intensity down to 3 mW/cm² for 30 min (5.4 J/cm² with 0.1 % riboflavin solution) during the SXL treatment avoided such degenerative side effects and maintained strengthening effects for the scleral tissue (Wollensak and Iomdina, 2009). Recently, it was demonstrated that riboflavin and UVA light irradiation (57 mW/cm² for 200 s = 11.4 J/cm² with 0.1 % riboflavin solution) prevent occlusion-induced axial elongation in young rabbit eyes (Dotan et al., 2014). Iseli et al. first introduced scleral cross-linking with blue light (26 mW/cm² for 20 min = 31.2 J/cm² with 0.5 % riboflavin solution) instead of UVA light, and proved both its efficacy in respect to stiffening of the sclera and its safety in pigmented adult rabbits (Iseli et al., 2008). However, all these studies investigated only one UV or blue light intensity per study. In general, the grade of tissue damage seems to be dependent on applied light energy doses and on used riboflavin solution. Therefore, we investigated the dose-dependent impact of the blue light irradiation on the ocular tissue during the SXL treatment using 0.5 % riboflavin solution.

High myopia is commonly accompanied with choriotretal atrophy, including attenuation of the retinal pigment epithelium and disappearance of choroidal melanocytes (Curtin and Karlin, 1970; Fujiwara et al., 2009). Furthermore, it is reported that the sclera, particularly at its posterior pole, was significantly thinner in highly myopic eyes than in emmetropic eyes (McBrien and Gentle, 2003; Rada et al., 2006). We investigated rabbit eyes, which possess a thinner sclera than humans in order to increase the safety of SXL treatment in cases of application to humans. As pigmentation of ocular tissue in human is often reduced in highly myopic eyes we investigated both, pigmented and non-pigmented eyes after SXL. The aim of the presented study was to determine a damage threshold for ocular tissue after an in vivo SXL treatment with increasing blue light intensities. Such a threshold is an absolutely essential precondition for a possible translational of the SXL treatment into clinical use.

2. Materials and methods

2.1. Animals

Animals were bred, handled, and finally euthanized in accordance with the applicable. European laws (European Communities Council Directive 86/609/EEC) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. In a first series of preliminary experiments 34 adult rabbits were used to establish a suitable surgical intervention, a successful and stable anesthesia for the entire SXL treatment, a procedure to apply riboflavin, to place an adjusted blue light intensity and axial eye length measurement using ultrasonic A-scan. After optimization of all procedures and methods we started with the investigation of the damage threshold for the ocular tissue. For damage threshold experiments (second series of experiments) 25 pigmented (Chinchilla bastard) and 42 albino (New Zealand White) rabbits (3–4 kg body weight) were obtained from the Medizinisch-Experimentelles Zentrum of the University of Leipzig and kept on a 12-h light–dark cycle with ad libidum access to water and food. Adult rabbits of both genders (older than 3-month) were randomly selected into groups to perform scleral cross-linking with/without riboflavin and different blue light intensities (15, 40, 80, 150, 200, 400 and 650 mW/cm²). Each light intensity was applied in at least 3 different albino and/or pigmented rabbits (see Fig. 1 for illustration of the procedure). Our strategy was to start with low blue light intensities in albino rabbits and then increase the intensity until we achieved ocular tissue damage. Then we evaluated the damage threshold in pigmented rabbits around the already determined blue light intensity from the albino animals.

2.2. Surgery and SXL treatment

To perform the riboflavin/blue light scleral cross-linking the animals were anesthetized by an intramuscular injection of ketamine hydrochloride (50 mg/kg body weight; Ketamin 5 %, Ratilpharm, Ulm, Germany) and xylazine hydrochloride (10 mg/kg body weight; Rompun, Bayer Vital GmbH, Leverkusen, Germany). For maintenance of the anesthesia ketamine hydrochloride (25 mg/kg body weight) and xylazine hydrochloride (5 mg/kg body weight) were injected intramuscularly if necessary. Only the right eye underwent treatments whereas the contra-lateral untreated left eye served as intra-individual control. The control eye was treated with Floxal® eye ointment (Dr. Gerhard Mann GmbH, Berlin, Germany) to avoid corneal damage/drying while the entire surgery/anesthesia procedure. Conjunctivae was used for local anesthesia of the conjunctiva. Atraumatic forceps and a 23G needler were used for temporal canthotomy and conjunctivectomy and a 5/0 Prolene suture (Ethicon, Somerville, New Jersey, USA) was used for closure of the corneal incision. The superior rectus muscle and the temporal rectus muscle were dissected bluntly in the superior temporal quadrant. The conjunctiva was incised at the limbus to open the Tenon's space. Then Tenon's space was bluntly dissected in the superior temporal quadrant. The superior rectus muscle and the temporal rectus muscle were dissected and fixed by means of 5/0 Prolene sutures (Ethicon, Nesoddtangen, Norway) to avoid better exposition of the sclera and easier manipulation of the eye position during scleral treatment. Riboflavin-5'-phosphate (vitamin B2, 0.5 % in PBS without any dextran admixture, Streuli Pharma, Uznach, Switzerland) was dropped every five minutes on the exposed sclera to assure a plain penetration of riboflavin into the scleral stroma (Fig. 1D). After 20 min of riboflavin soaking time one light spot (1.3 cm diameter corresponding with an area of 1.1 cm²) with a defined blue light intensity was applied on the temporal sclera (Fig. 1E and F) for 20 min irradiation time. A commercial dental light source (blue-light, 250 mW/cm² for 200 s, IvoKer Vivadent GmbH, Ellwangen-Jagst, Germany) was used to achieve blue light intensities (15, 40, 80, 150, 200, 400 and 650 mW/cm²). The real emitted wavelengths of our blue light source was checked and measured by a spectrometer (Horiba Jobin Yvon iHR320 Imaging Spectrometer; Fig. 1A). The adjustment of the light intensities (15 mW/cm² up to 400 mW/cm²) was realized by custom-built polypropylene spacing tubes (Fig. 1B) and checked by different power-meters.

The Bluephase meter (Ivoclar Vivadent GmbH, Ellwangen-Jagst,
Germany) and the LEDRadiometer (Kerr Corp Demetron Kerr Corp., Orange, CA, USA) were used to guarantee the promised light intensity of the light source according to the manufacture information. The SDI (SDI N. America Bensenville, IL, USA) and the FieldMax II þ thermopile sensor PowerMax PS19 (Coherent Inc., Santa Clara, CA, USA) were used for measuring the adjusted light intensities of the blue light source with the spacing tubes because of the possibility to measure a wide light intensity range. The variations (approx. 10 %) of light intensity measurements were comparable in these three power-meters: FieldMax II þ PowerMax PS19, Bluephase meter and LEDRadiometer. The SDI displayed a variance of 37 % to the manufacture information and was not able to measure light intensities below 50 mW/cm². Therefore, the FieldMax II þ PowerMax PS19 was used to evaluate the different light intensities of the blue light source with the spacing tubes because of the possibility to measure a wide light intensity range. The variations (approx. 10 %) of light intensity measurements were comparable in these three power-meters: FieldMax II þ PowerMax PS19, Bluephase meter and LEDRadiometer. The SDI displayed a variance of 37 % to the manufacture information and was not able to measure light intensities below 50 mW/cm². Therefore, the FieldMax II þ PowerMax PS19 was used to evaluate the different light intensities of the blue light source with the spacing tubes because of the possibility to measure a wide light intensity range. A light intensity of 650 mW/cm² was realized by the light source without an additional spacing tube. Unfortunately, a variation of 20 % light intensity occurred inside the illuminated area caused by an inhomogeneous illumination.

Riboﬂavin drops were alternately applied every 5 min during the entire irradiation period to avoid excessive photo-bleaching of the fluorophore and drying of the eye. The blue light application was performed in a pulse-like fashion with an interruption break of several seconds because the light device (bluephase 16i) switched off every 30 s for safety reasons. An irradiation of the cornea had to be avoided because of the destructive properties of blue light for cornea, lens and retinal tissue. After irradiation, the sutures around the muscles were removed and the connective tissue was ﬁxed on the sclera using absorbable surgical sutures. Finally the canthotomy was readapted with absorbable surgical sutures. Both eyes were treated with Floxal® eye ointment (Dr. Gerhard Mann GmbH, Berlin, Germany) into the conjunctival fornix and the cornea avoiding infection and drying. The animals were monitored till awakening and kept in the Medizinisch-Experimentelles Zentrum of the University of Leipzig for 3 weeks. The same surgery and treatment was performed in the comparison group (only blue light irradiation) by only omitting the riboﬂavin solution (PBS was used to moisten the eye).

2.3. Specimen preparation

After 3 weeks survival time, the animals were anesthetized by an intramuscular injection of ketamine hydrochloride (50 mg/kg body weight) and xylazine hydrochloride (10 mg/kg body weight). Then the eye length was measured using an A-scan ultrasound biometry system (AL-010, Shin-Nippon, Japan). After measurement the animals were sacriﬁced by an intracardial injection of T61 (3 ml, embutramide mebezonium iodide, Hoechst, Unterschleissheim, Germany). Treated and control eyes were excised for investigation. The treated area of the right eye and corresponding part of the left eye were broadly isolated (as eye patches) and bisected. The samples were always taken 2–3 mm behind the limbus, from temporal superior parts of the sclera; likewise in untreated and treated eyes. One half of each patch (that means the treated area was localized in both patches) was ﬁxed in 4 % paraformaldehyde (Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS; Sigma, Deisenhofen, Germany), pH 7.4 at 4 °C for 3 h for immunohistochemical analysis; the other half of each patch was ﬁxed in phosphate-buffered 4 % paraformaldehyde with 1 % glutaraldehyde (Sigma, Steinheim, Germany) solution, pH 7.4 over-night at 4 °C for light and electron microscopic studies.

Fig. 1. A) Absorption spectrum of riboﬂavin (red line) and emission spectrum (blue area) of the used blue light applicator “bluephase 16i”. The absorption maximum of riboﬂavin is 447 nm in the blue light range. The blue light applicator emits light with wavelengths from 400 to 500 nm with a maximum at 450 nm. B) Picture of the applied custom-built polypropylene spacing tubes with assigned light intensity in mW/cm². C) Blue light applicator “bluephase 16i”. D) Riboﬂavin application by a curved hollow needle into the eye-socket during the cross-linking surgery. The eye is ﬁxed and manipulated by sutures to protrude the eye. E) Blue light irradiation of the temporal area of the eye without a polypropylene tube to achieve the highest light intensity of 650 mW/cm². F) Drawing of an irradiated eye area (blue). The anatomical position is shown as an “orientation cross”, a— anterior, i— inferior, n— nasal, p— posterior, s—superior, t— temporal.
2.4. Light and electron microscopy

For light and electron microscopic examinations the fixed tissue (4 % paraformaldehyde and 1 % glutaraldehyde) was rinsed with PBS, post-fixed in 1 % osmium tetroxide for two hours, dehydrated in acetone, and post-contrasted overnight in 70 % acetone saturated with 1 % uranyl acetate (Serva, Heidelberg, Germany). After further dehydration in absolute acetone, the samples were gradually embedded in a non-hardening epoxy resin (Durcupan TM ACM Fluka; Sigma, Steinheim, Germany) and acetone mixture (1:3; 1:1; 3:1; each 60 min). They were incubated overnight in pure Durcupan and successively replaced by hardening Durcupan. The embedded tissue was sectioned by a microtome (Reichert Ultracut S, Leica, Wetzlar, Germany) with a diamond knife (Diatome, Leica microsystems, Wetzlar, Germany). Semithin sections (0.5 μm) were stained with toluidine blue, and visualized using a conventional light microscope. Serial ultrathin sections (approx. 60 nm) were studied by means of scanning electron microscope (Zeiss, Sigma-0231, Oberkochen, Germany) operated at 27 kV using a STEM de-stained with toluidine blue, and visualized using a conventional vibratome slicer (Microm HM 650 V Vibration microtome, Thermo Electron Corporation, Waltham, MA, USA). Some retinae in eyes treated with riboflavin and high blue light intensities (400 or 650 mW/cm² = 480 or 780 J/cm²) showed internal hemorrhages right beneath the treated sclera (Fig. 2B). An obvious degeneration of inner eye tissue and pigment aggregations were observed in pigmented rabbits treated with the highest blue light intensities (Fig. 2C). Visible macroscopic destructions were limited to the treated part of the eye while the untreated areas appeared intact.

2.5. Immunohistochemistry

For immunohistochemistry the retina was carefully isolated from the fixed (4 % paraformaldehyde) temporal half of the bisected eye patch. After several washing steps in PBS the retinal tissue was embedded in low-temperature melting agarose gel medium (3 %, Sigma–Aldrich, Taufkirchen, Germany) for sectioning with a vibratome slicer (Microm HM 650 V Vibratome microtome, Thermo Scientific, Pittsburgh, PA, USA). The 30 μm-sections were pre-incubated in PBS with normal donkey serum (3 mg/ml) plus 1 % DMSO and 0.3 % Triton X-100 (both Carl ROTH GmbH & Co. KG, Karlsruhe, Germany) for 1 h at room temperature to block nonspecific binding of antibodies. Primary immunostaining was performed at 4 °C overnight with mouse anti-glial fibrillary acidic protein (anti-GFAP; GA5 clone, 7 μg/ml, Sigma–Aldrich, Taufkirchen, Germany) antibody to label reactive Müller cells and a rabbit anti- ionized calcium binding adaptor molecule 1 antibody (anti-Iba1; 1 μg/ml, Wako Bioproducts, Neuss, Germany) to label retinal microglia cells. After washing, the sections were incubated 2 h at room temperature with Cy2-conjugated donkey anti-mouse IgG (7.5 μg/ml) and Cy3-conjugated donkey anti-rabbit IgG (7 μg/ml; both Jackson Immunoresearch Laboratories; Inc., West Grove, PA). All probes were diluted in PBS plus 1 % DMSO and 0.3 % Triton X-100. To visualize cell nuclei the slices were incubated with PBS with normal donkey serum (3 mg/ml) plus 1 % DMSO and 0.3 % Triton X-100 (both Carl ROTH GmbH & Co. KG, Karlsruhe, Germany) for 1 h at room temperature to block nonspecific binding of antibodies. Primary immunostaining was performed at 4 °C overnight with mouse anti-glial fibrillary acidic protein (anti-GFAP; GA5 clone, 7 μg/ml, Sigma–Aldrich, Taufkirchen, Germany) antibody to label reactive Müller cells and a rabbit anti-ionized calcium binding adaptor molecule 1 antibody (anti-Iba1; 1 μg/ml, Wako Bioproducts, Neuss, Germany) to label retinal microglia cells. After washing, the sections were incubated 2 h at room temperature with Cy2-conjugated donkey anti-mouse IgG (7.5 μg/ml) and Cy3-conjugated donkey anti-rabbit IgG (7 μg/ml; both Jackson Immunoresearch Laboratories; Inc., West Grove, PA). All probes were diluted in PBS plus 1 % DMSO and 0.3 % Triton X-100. To visualize cell nuclei the slices were incubated with Hoechst 33258 (10 μg/ml, Molecular Probes, Eugene, Oregon, USA) in PBS for 1 h at room temperature and then mounted with ImmuMount (Thermo Scientific, Pittsburgh, PA, USA). The probes were analyzed by means of a confocal laser scanning microscope LSM 510 Meta (Carl Zeiss, Oberkochen, Germany).

2.6. Shear rheology

The isolated scleral patch was aligned between the two parallel plates of a dynamic shear rheometer (Ares; TA Instruments, New Castle, DE, USA). We used the internal gap finding routine of the rheometer to compensate for different thicknesses of the scleral tissue patches and an axial/normal force equivalent to 0.05 N was exerted. The applied force was sufficient to avoid slippery of the scleral tissue during the measurement. Each sample was subjected to three consecutive test routines (dynamic frequency sweep, dynamic strain sweep, creep test) at 37 °C, controlled by the rheometer device. By means of the shear rheometry we were able to measure the stress-strain behaviour of the sclera and thus calculating their visco-elastic properties. In different test routines the rheometer measures the complex shear modulus (G’) and the phase angle (δ) of the scleral patches and automatically calculate from these information the elastic G’(f) and viscous shear modulus G”(f) (Verdier, 2003). Furthermore, we calculated a relative elastic modulus ΔG’(f) of each pair of patches (treated vs. untreated) from one individual animal for better comparison of SXL effects. Therefore, the dynamic frequency sweep data G’ treated(f) of the treated patch were normalized (divided) by the untreated patch data G’ untreated(f) at a frequency of 1 Hz and a strain of 1.5 %. We applied the same procedure to calculate the relative viscous modulus ΔG”(f). Detail information about the rheological measurements and the analysis procedure were described by Schulte et al. (2014).

3. Results

3.1. Surgery and gross anatomical outcome

The surgery and the cross-linking treatments were performed in all animals without any complications occurring during or after our SXL treatments. No apparent macroscopic complications such as inflammatory reactions of the cornea or the connective tissue, problematic scar formation or remnants of surgical sutures were observed. However, treated scleral areas of adult albino rabbit eyes showed an apparent yellow staining at high blue light intensities (>200 mW/cm² = 240 J/cm²), Fig. 2A). Moreover, treated scleral areas of adult pigmented rabbit eyes appeared as darker spots at such high light intensities (200 mW/cm²) suggesting changes of optical properties of the scleral tissue.

Some retinae in eyes treated with riboflavin and high blue light intensities (400 or 650 mW/cm² = 480 or 780 J/cm²) showed internal hemorrhages right beneath the treated sclera (Fig. 2B). An obvious degeneration of inner eye tissue and pigment aggregations were observed in pigmented rabbits treated with the highest blue light intensities (Fig. 2C). Visible macroscopic destructions were limited to the treated part of the eye while the untreated areas appeared intact.

3.2. Histology

All animals were SXL treated at the same temporal/superior location, applying various blue light intensities. Tissue samples including the treated – and a part of the surrounding – area were histologically analyzed by light microscopy, electron microscopy and immunohistochemistry, and compared with the corresponding ocular tissues of the untreated eye (Figs. 3–6). The results of the light microscopic and immunohistochemical examination of all rabbit eyes are summarized in Tables 1 and 2. Pathological changes in the scleral, choroidal and retinal tissue components were separately assessed by light microscopy. Cellular alterations and possible neurodegenerations in the retina were examined by means of changes in the immunoreactivity and/or morphological changes of microglial cells, Müller cells and/or retinal layers, respectively. However, on one hand, we observed cellular degenerations, loss of cells and massive alterations of the collagen structure as obvious signs of a tissue damage threshold at high blue light intensities (dark gray highlighted in Tables 1 and 2). On the other hand, we observed cellular activation of fibroblasts in the scleral tissue and/or activation of microglial and Müller glial cells in retinal tissue without any obvious signs of cellular (neuro-) degeneration (light gray highlighted in Tables 1 and 2). Cellular degenerations, detected cell loss as well as ambiguous structural tissue alterations (indicated with * in Tables 1 and 2) were defined as signs of damage. Thus the “damage threshold” indicates the light intensity used in scleral cross-linking with riboflavin and blue light in rabbits, which causes pathological structural alterations in the
retinal, choroidal and scleral tissue. Furthermore, any pathological

tissue alteration (see definition of damage threshold) in only one

individual animal of the several examined animals included in each
group (same SXL treatment) was defined as damage threshold for
the SXL treatment.

3.3. Light microscopy

The treatment with riboflavin and blue light intensities up to
200 mW/cm² (240 J/cm²) did not result in structural changes of
sclera, choroid and/or retina; rather, all tissues appeared normal
and healthy compared with the control eyes (Fig. 3A). Several
structural and degenerative changes co-occurred in all three tissues
after treatments with blue light intensities of 400 and 650 mW/cm²
(480 and 780 J/cm²; Figs. 3 and 4). In most severe cases, retinal
layers could no longer be distinguished, neuronal cell somata were
lost and the anatomical borders between retina, retinal pigment
epithelium and choroid were destroyed. Accumulations of pig-
mented cells occurred in retina, choroid and lamina fusca scleræ
(the innermost part of the sclera facing the choroid) (Figs. 3B,C
and 4). The collagen bundles of the scleral stroma appeared disorga-
nized and morphologically altered; moreover, enlarged fibroblasts
and pigmented cells were observed (Fig. 4B). Surprisingly, these
changes (with exception of the melanin granules) were present in
albino as well pigmented rabbit eyes after treatment with ribo-
flavin and high blue light intensities (400 and 650 mW/cm²) and no
difference in the damage threshold could be revealed after com-
plete SXL treatment. Thus, the damage threshold for both rabbit

Fig. 2. Complications observed in rabbit eyes after treatment with riboflavin and a blue light intensity of 650 mW/cm². A) Yellow pigmentation (arrowheads) of the sclera of an
adult albino rabbit three weeks after treatment. B) Hemorrhage and degenerative retina (arrowheads) of an adult albino rabbit eye. C) Localized retina degeneration of an adult
pigmented rabbit after treatment (arrowheads). In the centre of this area yellow pigment aggregations are visible. The optic nerve head and the medullary rays are marked by an
asterisk.

Fig. 3. Comparison of Toluidine blue-stained semithin sections of an untreated healthy (A) and a damaged (B and C) eye of a pigmented adult rabbit treated with riboflavin and
high-intensity blue light. A) The retina (ret), choroid (cho) and sclera (scl) of the untreated control eye show their typical appearance. A blood vessel lumen in the choroid is marked
by an arrowhead. B) Appearance of the degenerated retina of an eye treated with riboflavin and a blue light intensity of 400 mW/cm². The retinal cells were completely degenerated
and all retinal layers were lost. The choroidal blood vessels were collapsed, the tissue appeared melted, and pigment aggregations (arrow) occurred in hypertrophic cells of the
lamina fusca sclerae (LF). C) Detail of the transition zone between choroid and LF of an eye treated with riboflavin and a blue light intensity of 400 mW/cm². Hypertrophic cells with
pigment aggregations occur in the LF (arrow). The asterisk indicates a choroidal blood vessel within a massive collagen matrix. Scale bar, 50 µm (A and B); 20 µm (C).
Fig. 4. Toluidine blue-stained semithin sections of a pigmented adult rabbit eye treated with riboflavin and a blue light intensity of 650 mW/cm². A) Section of the retina and choroid; the integrity of the retinal layers and its anatomical borders are lost. Pigment granules were observed within cells in the retinal remnant (arrow). Melanocytes with aggregated melanin granules (asterisk) were found in the choroid. B) Section of the lamina fusca sclerae (LF) and scleral stroma (scl); melanocytes with aggregated melanin granules (asterisk) and thickened processes of fibroblasts (arrowhead) were found in the lamina fusca sclerae at the border to the choroid. The scleral fibril bundles appear irregular and disorganized, and the inter-bundle space appears enlarged. Retina (ret), Bruch’s membrane (BM), choroid (cho), lamina fusca sclerae (LF), sclera (scl); scale bar, 20 μm.

Fig. 5. Electron micrographs of the sclera of untreated (A and D) and treated (riboflavin and a blue light intensity of 650 mW/cm²; B, C and E) adult albino rabbit eyes. A) Collagen fibril bundles appeared regularly arranged in an untreated control eye. Fibroblasts (f) were located between the bundles having a spindle-like shape with thin cytoplasmic processes, and were orientated in parallel to the surface of the eye. B) In the treated sclera enlarged fibroblasts (f) bear thick cell processes which were arbitrarily oriented to the scleral surface. The collagen fibril bundles appeared disorganized. C) In the treated eye the defined collagen fibril bundle borders disappeared and the collagen fibril bundles appeared disorganized. Enlarged fibroblast processes (f) and erythrocytes (e) were distributed between collagen bundles around blood vessels (asterisk) indicating scleral hemorrhage (arrow). Macrophage-like cells (m) were prominent next to the hemorrhage. D) Magnified image of a collagen fibril bundle of the untreated sclera shown in Fig. 5A. The collagen fibrils in one bundle are orientated in the same direction, with similar fibril cross-sectional diameter. A thin fibroblast cell process is located between two collagen bundles (f). E) Magnified image of a collagen fibril bundle from the treated sclera shown in Fig. 5C. Small-diameter collagen fibrils (arrowhead) appeared in combination with large-diameter collagen fibrils in proximity to a macrophage-like cell (m) and a fibroblastic process (f). The collagen fibrils were irregular distributed within this collagen fibril bundle and the inter-fibril space appeared enlarged. Scale bars, 10 μm (A–C); 1 μm (D and E).
in the comparison group (only blue light irradiation without riboflavin application) the damage threshold was similar to that of the complete SXL treatment, even though pathological changes were only observed in retinal but not in choroidal and scleral tissue in one of the examined animals. There was regional dependent loss of retinal cells. In the pigmented animals morphological alterations and/or tissue damage could be observed even at lower blue light intensities than in albinotic animals (Table 1). But the morphological tissue alterations were comparable to those after the complete SXL treatment (e.g. loss of collagen fibril bundle organization and pigment aggregation). However, these tissue alterations were detected in only one animal of each of the two groups (150 and 200 mW/cm² = 180 and 240 J/cm²) whereas the other animals in the group were without pathological findings. Accordingly, we defined the lowermost light intensity which induced any kind of tissue damage as a damage threshold (see above).

3.4. Electron microscopy

Electron microscopic examinations were conducted to evaluate changes of sub-cellular structures, especially of the collagen fibril/fibril bundle organization in scleral tissue after SXL treatment. In the untreated healthy sclera and in treated sclera up to 200 mW/cm² (240 J/cm²) blue light and riboflavin, collagen fibril bundles appeared irregularly and the lamellar-like character changed (Fig. 5A). The collagen fibrils inside each bundle displayed the same orientation with almost similar fibril diameters (Fig. 5D). The fibroblasts show spindle-like somata with long small processes oriented parallel to the scleral surface (Fig. 5A). In contrast, eyes
treated with riboflavin and high blue light intensities (400 and 650 mW/cm² = 480 and 780 J/cm²) displayed an altered collagen structure, cellular reactivity, and indications of inflammatory processes in the sclera. Collagen fibril bundles appeared disorganized and the superordinated lamellar character seems to be lost (Fig. 5B and C). The collagen fibrils of one bundle appear heterogeneously with various particularly small diameters (Fig. 5E). Scleral fibroblasts of treated scleral tissue show thickened cell processes. Noteworthy, the cells were then arbitrarily oriented in respect to the orientation of the fibril bundles. Additionally, blood derived cells like erythrocytes were identified in the scleral tissue, indicating the occurrence of hemorrhage. Near these hemorrhages macrophages occurred (Fig. 5C).

### 3.5. Immunohistochemistry

The immunohistochemical examinations confirmed the light microscopic observations regarding the determination of a damage threshold after SXL treatment (Table 2). In particular, we examined the retinal tissue to evaluate neurodegeneration and cellular responses of glial cells, which often precede neurodegenerative effects. In untreated control eyes, retinæ with resting microglial cells were ramified and localized in the inner retinal layers and Müller glial cells were immunonegative for GFAP (Fig. 6A). In eyes treated with a blue light intensity beyond the damage threshold of 400 mW/cm² (480 J/cm²), microglial cells appear activated, display an amoeboid cell shape, and are localized in all retinal layers towards areas of photoreceptor degeneration. The retinal structures were severely destroyed and Müller glial cells became GFAP-immunopositive as evidence of a reactive gliosis (Fig. 6D). Cellular activation of both microglia and Müller cells was observed coincidentally in the examined retinæ often accompanied with massive neurodegeneration in the animals SXL treated beyond the damage threshold (with some exceptions, see below).

In the comparison group (only blue light irradiation without riboflavin application) retinal tissue alterations occurred at similar damage thresholds as observed after SXL treatment with riboflavin and blue light irradiation. In some sham-treated pigmented animals microglial activation and GFAP-immunoreactivity in Müller glial cells could be detected without obvious signs of neurodegeneration at the damage threshold of 400 mW/cm² blue light irradiation (Table 2, light grey highlighted).

### 3.6. Rheological measurements

Rheological measurements were performed to examine whether the biomechanical stiffness of the sclera is increased after an in vivo SXL treatment (as an in vivo proof of principle). Preliminary examinations with isolated scleral tissue revealed that various applied light intensities (from 12 mW/cm² up to 200 mW/cm² for 20 min = 14.4 up to 240 J/cm²) increased the biomechanical stiffness of scleral tissue (Schuldt et al., 2014). Therefore, we conducted complete (riboflavin and blue light) and comparison (only light) SXL treatments with two blue light intensities: 40 mW/cm²
48 J/cm²) as a mean effective dose in isolated scleral tissue and 200 mW/cm² (240 J/cm²) near the determined damage threshold. And indeed, an in vivo SXL treatment with riboflavin and 40 mW/cm² blue light increased the elastic ($\Delta G^0$) as well as the viscous ($\Delta G^00$) proportion of scleral tissue by a factor of 2 immediately after the surgery (Table 3). Additionally, riboflavin and an applied blue light intensity of 200 mW/cm² without riboflavin application result in a slight decrease of the biomechanical stiffness of scleral tissue as compared to control tissue (Table 3).

4. Discussion

In the present study, we have successfully established a reliable ophthalmologic surgery procedure and a scleral cross-linking treatment with riboflavin application and various blue light intensities. Furthermore, we determined blue light irradiance damage thresholds for the ocular tissues in pigmented and albino rabbits in vivo. Our results show that blue light intensities up to 200 mW/cm² (240 J/cm²) applied in combination with riboflavin did not result in any harm to the retina, choroid, and sclera of adult pigmented and albino rabbits. A biomechanical strengthening of scleral tissue was demonstrated by this in vivo SXL treatment as it was earlier shown in other in vivo SXL treatments with riboflavin and UVA light application (Wollensak and Iomdina, 2009; Wollensak et al., 2005). Additionally, our findings confirm the results of another study in which 26 mW/cm² (31.2 J/cm²) blue light and riboflavin had been applied to rabbit sclera and the scleral tissue was strengthened without any damage to the retina (Iseli et al., 2008). An exactly determined damage threshold for this cross-linking approach is an important safety issue for a possible treatment.

Table 2
Summarized results of the immunohistochemical examinations determining the damage threshold in adult pigmented and albino rabbit eyes after SXL treatment with riboflavin and various blue light intensities or after blue light irradiation alone (only light).

<table>
<thead>
<tr>
<th>blue light intensity [mW/cm²]</th>
<th>15</th>
<th>40</th>
<th>80</th>
<th>150</th>
<th>200</th>
<th>400</th>
<th>650</th>
</tr>
</thead>
<tbody>
<tr>
<td>adult/ pigmented</td>
<td>-/-*</td>
<td>+/+/+</td>
<td>+/+/+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>adult/ pigmented (only light)</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>+/+/-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>adult/ albino</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>+/+/+</td>
<td>+/+/+</td>
<td></td>
</tr>
<tr>
<td>adult/ albino (only light)</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>-/-</td>
<td>+/+/+</td>
<td>+/+/+</td>
<td></td>
</tr>
</tbody>
</table>

*Damage and cellular changes of the retinal tissue were determined, and represented in following order: microglia cells/Müller cells/neurodegeneration for the different blue light intensities from 15 to 650 mW/cm² (18 up to 780 J/cm²).

A (+) symbol indicates the detection of any kind of morphological alteration, cellular reactivity, changed immunoreactivity or tissue damage after treatment even if only one animal of the examined group (means equal SXL treatment) was affected and all other animals in the group were without abnormalities. Any kind of detected cell degeneration, cell loss and/or massive tissue alteration were defined as damage threshold and highlighted with dark grey. Any kind of cell activations, cell reactivity and/or increased immunoreactivity without obvious cellular degeneration and/or tissue damage were defined as SXL induced alterations and highlighted in light grey.

Table 3
Changes of the elastic and viscous properties of scleral tissue after SXL treatment.

<table>
<thead>
<tr>
<th>Ribo + 40 mW/cm²</th>
<th>Ribo + 200 mW/cm²</th>
<th>Only 40 mW/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta G^0$</td>
<td>$\Delta G^00$</td>
<td>$\Delta G^0$</td>
</tr>
<tr>
<td>Immediately after SXL treatment n = 3 each</td>
<td>3.35</td>
<td>2.05</td>
</tr>
</tbody>
</table>

$^a$ $\Delta G$ represents the relative elastic modulus and $\Delta G^0$ represents the relative viscous modulus as calculated (normalized) by dividing $G'$ and $G''$ from the treated by the untreated scleral patch, respectively (modulus >1 means the tissue was stiffer after SXL treatment; <1 means it was softer than the untreated control eye).
translation into the clinical practice.

From preliminary investigations we know that the biomechanical stiffening effect, if measured in isolated rabbit scleral tissue, is dose-dependent on the applied blue light intensity up to 150 mW/cm² (180 J/cm²) and decreased again at 200 mW/cm² (240 J/cm²) (Schuldt et al., 2014). Therefore, it is important to know the most appropriate blue light energy value as a compromise to strengthen the sclera and to avoid ocular tissue damage. In comparable in vivo studies in adult rabbits, SXL with riboflavin and 4.2 mW/cm² UVA light irradiation for 30 min (7.56 J/cm²) caused serious undesirable side effects in retinal tissue (Wollenksa et al., 2005). A reduction of the UV light intensity down to 3 mW/cm² for 30 min (5.4 J/cm²) of SXL treatment avoided these degenerative side effects and maintained strengthening effects for the scleral tissue (Wollenksa and Iomdina, 2009). In another study, a total UVA light dose of 57 J/cm² and riboflavin were used for the SXL treatment in very young rabbits and no retinal damage was detected (Dotan et al., 2014). The surprising differences in the damage thresholds of approximately 5.4 J/cm² or 57 J/cm² UVA vs. 240 J/cm² blue light (200 mW/cm² for 20 min in the presented results) might be explained by different absorption characteristics of the scleral tissue/proteins/molecules for different wavelengths, causing different tissue damaging effects (Wu et al., 2006). Furthermore, blue light (around 450 nm), having a longer wavelength than UVA light (around 365 nm), possesses less photon energy. Therefore, blue light might exert less tissue damage. Another reason might be the fact that light with shorter wavelengths leads to a stronger production of oxygen radicals than light with longer wavelengths (Rozanowska et al., 1995, 1998).

In general, on one hand the vulnerability for retinal/ocular tissue damage correlates with the wavelength of the light, the absorption/scattering/reflection properties of the tissue components and on the other with the total applied light energy amount (J/cm²): i.e. the lower the wavelength of the light and/or the higher the intensity and/or the longer the exposure time the more the probability of tissue damage (Ham et al., 1976; Rapp et al., 1990; van Norren and Schellekens, 1990). The application regime might also influence tissue damaging effects besides an equal total light energy amount (e.g. high light intensities combined with short time application vs. long time exposure with low light intensities). Additionally, the vulnerability of light induced photoreceptor damage correlates mostly with the absorption spectrum of the photo-pigment. However, experiments demonstrated that in general the susceptibility for damage sharply increased towards shorter wavelengths with an irradiant dose from 4 J/cm² at 379 nm to 2000 J/cm² at 559 nm (van Norren and Schellekens, 1990). Similarly, irradiation of rat eyes through the cornea by UV light (380 nm) at a light dose of 1.2 up to 1.6 J/cm² (achieved by an irradiation time of 4–7 min) induces a complete loss of rod photoreceptors. In contrast, blue light (470 nm) application at a light dose of 250 up to 500 J/cm² (achieved by an irradiation time of 50–60 min) only leads to retinal pigment epithelium (RPE) damage (Gorgels and van Norren, 1998).

Despite the wavelength dependent differences of tissue specific vulnerabilities, these results support the assumption that UV light might be more harmful to photoreceptors and/or ocular tissue and that the blue light tolerance range of ocular tissue is higher. Our experiments support these observations because the blue light energy levels are lower at a certain damage threshold compared to the SXL experiments in rabbit with UVA light (Wollenksa and Iomdina, 2005; Wollenksa et al., 2005). This fact might open a wider irradiation/light energy range of the applied blue light for an optimal therapeutic effect compared to an UV light application. The applied blue light energy might be technically better controlled and regulated and a dose-dependent stiffening effect on sclera (Schuldt et al., 2014) might be optimal adjusted.

Another observation was made when only blue light was applied, without riboflavin. In some of the pigmented rabbits of the comparison group (only blue light application), we detected tissue alterations in choroid and sclera and retinal degeneration at light intensity levels of 150 and 200 mW/cm² (180 and 240 J/cm²). This finding can be explained if riboflavin exerts some kind of protection for the deeper (more distant from the irradiation source) tissues (choroid, RPE and retina). Such an effect is to be expected because riboflavin shows maximum absorption of blue light wavelength, even if the light absorption of riboflavin induces the production of various chemical radicals (Choe et al., 2005). Interestingly, we observed these pure blue light–induced alterations only in pigmented rabbits and not in albinos. This suggests that blue light absorption by the melanin pigment might induce degenerative processes in the affected tissues (Hunter et al., 2012; Wu et al., 2006). However, Putting et al. stated, in a comparative study of albin and pigmented rabbits that “melanin does not have a damaging nor a protective role in phototoxicity since (1) the presence of melanin is not essential for blue-light-induced photochemical damage to the blood-retinal barrier at the retinal pigment epithelium, and (2) protection from this sort of damage is not greater in melanin containing epithelia than in non-melanin containing epithelia” (Putting et al., 1994). Additionally, Gorgels and Norren demonstrated that melanin was not involved in the phototoxic effect inducing photoreceptor death after irradiation but described specific damaging effect of blue light (vs. UV light) in RPE cells (Gorgels and van Norren, 1998). In summary, we determined the same damage threshold for a SXL treatment with riboflavin and blue light in pigmented and albino rabbits suggesting a protecting property for riboflavin for the inner ocular tissues (choroid, RPE and retina). However, with regard to a therapeutic application it should be avoided to irradiate unprotected (i.e. without riboflavin application) ocular tissue during the SXL treatment.

Furthermore, the present study revealed an inter-individual variability of the responses to the SXL procedure, as sometimes only one or few animal(s) in a given group showed some specific type of tissue alterations. Moreover, it remains to be shown whether (some of) the tissue alterations represent an irreparable damage or whether they may be reversible (one exception are neurodegenerative processes within the retina which certainly are irreparable at the present state of the art).

High light intensity treatment (650 mW/cm²) leads to an increase of small collagen fibrils. Whether these fibrils are the result of collagen new synthesis, collagen degradation or both remained unclear at this time. But we assume that there is a functional relationship between altered collagen fibril structure/organization and the biomechanical properties of the sclera. (Parry, 1988; Schuldt et al., 2014).

Thus, in the present animal study, we tried to mimic human myopic conditions as well as possible. For this purpose, we used both pigmented and non-pigmented albino eyes. High-grade myopia is commonly accompanied by choriotinal atrophy including attenuation of the retinal pigment epithelium and disappearance of melanin pigmentation (Curtin and Karlin, 1970; Fujiwara et al., 2009). Furthermore, the sclera at the posterior pole was reported to be significantly thinner in highly myopic eyes than in emmetropic eyes (McBrien and Gentile, 2003; Rada et al., 2006). The sclera of adult (pigmented and non-pigmented) rabbits is thinner (anterior 0.25 mm, posterior 0.33 mm) (Trier et al., 1999) than the human sclera, (equatorial 0.39 mm, posterior pole 0.9 to 1.0 mm), and therefore, the rabbit sclera might be similar to a thinned myopic human sclera (Olsen et al., 1996). Using adult albino rabbits addresses both aspects of the myopic human eye, thinning of the sclera and lack of melanin pigmentation. Notwithstanding all these similarities, a clinical damage threshold for humans remains to be determined in future.
Generally, several scleral cross-linking methods (chemical and physical) were suggested/proved to increase scleral stiffness, each with its advantages and disadvantages, and possible undesired side effects. The most effective and safe of these approach(es) have the capacity to be translated into the clinical practice (Elsheikh and Phillips, 2013), thus each of them has to be examined in-depth as closely as possible to clinical application on human patients.

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