Effect of Scleral Crosslinking Using Multiple Doses of Genipin on Experimental Progressive Myopia in Tree Shrews

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Introduction

Pathologic or degenerative myopia represents a subgroup of myopia with high and progressing levels of myopia and it is one of the leading causes of blindness worldwide.¹⁻⁵ Although the underlying mechanism is unclear, pathologic myopia is thought to be due to uncontrolled, progressive scleral remodeling leading to excessive axial elongation and posterior staphylomas.⁶⁻⁹ Currently, there are no universally accepted methods to prevent, slow, or control myopia progression. All of the currently available nonsurgical treatment options have either a small transient effect on slowing myopia progression or have significant side effects.¹⁰,¹¹ Posterior scleral reinforcement is a surgical procedure that showed the potential to control the progression of pathologic myopia, but it is considered controversial owing to its invasive nature and risk of complication.¹²⁻¹⁹ Scleral crosslinking (SXL) was first proposed by Wollensak et al.²⁰ as a promising strategy for myopia control. Since then, SXL has been proposed as a potential treatment option, in particular, for very severe cases of progressive myopia, where less invasive...
Scleral Crosslinking in Experimental Myopia

As in other soft tissues, collagen crosslinks accumulate naturally in the sclera with aging. The accumulation of natural collagen crosslinks has been proposed to underlie the age-dependent decrease in susceptibility to myopia. Natural and exogenous collagen crosslinks are known to alter the biomechanical properties of the sclera, that is, to increase stiffness and ultimate strength and decrease cyclic softening, but their role in impacting scleral remodeling and myopia is mostly unknown. McBrien and Norton have shown that preventing natural collagen crosslinking doubles the axial elongation rate during lens-induced myopia but not during normal visual experience in juvenile tree shrews, suggesting that collagen crosslinks modulate scleral remodeling during myopia progression. Wang and Corpuz demonstrated that weekly SXL (three times) using genipin (GEN) can prevent form deprivation (FD) myopia in guinea pigs for 21 days. Recently, controversial and contradicting results were reported in two animal studies using two different SXL modalities. Chu et al. performed sub-Tenon’s injections using two different SXL modalities. Chu et al. performed sub-Tenon’s injections using glyceraldehyde, whereas Liu et al. used ultraviolet light-activated riboflavin for SXL in guinea pigs. In both studies, SXL increased scleral stiffness, but only Liu et al. observed a significant treatment effect of SXL on experimental myopia. Collagen crosslinking using ultraviolet light-activated riboflavin has been successfully performed in human patients with keratoconus since 1998. A single treatment session of corneal crosslinking has been shown to achieve long-term stabilization of keratoconus progression (10 years postoperatively) with a good safety profile. However, using an ultraviolet light-activated crosslink agent for SXL remains challenging as current techniques require the excision of ocular muscles to expose the sclera to the ultraviolet light. Transpupillary light-activated crosslinking methods are being developed currently using light at a nonhazardous spectrum such as near infrared. Of the known low-cytotoxic collagen crosslinking agents that do not require light activation, GEN is one of the best characterized and most potent agents. It is a naturally occurring organic compound derived by enzymatic hydrolysis of Geniposide extract from the Gardenia jasminoides plant. In addition to being commonly used in Chinese traditional medicine and as a colorant in the food industry, GEN is a naturally occurring biodegradable compound with lower cytotoxicity as compared with the commonly used synthetic crosslinkers. Although alternative crosslinking agents have been shown to be less cytotoxic than GEN at equal concentrations, GEN is more potent and requires a lower concentration to effectively stiffen the sclera and slow myopia progression compared with other agents. For instance, Chu et al. reported that SXL using glyceraldehyde at a concentration of 0.5 M has no treatment effect on preventing experimental myopia development in guinea pigs. In contrast, Wang and Corpuz used a much lower concentration of GEN (0.022 M) but observed a significant treatment effect on experimental myopia. Liu and Wang reported a significant increase in scleral stiffness after four sub-Tenon’s injections of GEN within 4 weeks in rabbit eyes. In a recent study, Hannon et al. reported a sustained stiffening effect in rat sclera for 4 weeks after a single retrobulbar injection of GEN at a concentration of 0.015 M and proposed its potential use as a therapeutic approach for glaucoma. In addition to scleral stiffening, in situ experiments suggest that GEN may have a direct effect on collagen degradation and synthesis by restoring messenger RNA levels of miR-29, MMP2, and alpha1 chain type I collagen in experimental myopia. In light of these findings and the properties of GEN, SXL using GEN can be regarded as a potential promising therapeutic approach to combat myopia and glaucoma prevalence. In terms of safety, GEN has been proven to be safe for many applications, including tissue-engineered implants, Chinese traditional medicine, and in the food industry. Within the context of using GEN for SXL, no adverse effects have been identified based histologic investigations. For instance, Wang and Corpuz reported a significant thickening of scleral collagen fibrils after sub-Tenon injections of GEN in guinea pigs but no histologic damage was observed in the retina or choroid. Liu and Wang found no signs of cytotoxicity in the scleral, choroidal, and retinal cells after four sub-Tenon’s injections of GEN in rabbits. More recently, Hannon et al. reported that retrobulbar injections of GEN in rat eyes did not compromise retinal function or lead to any abnormality in retinal ganglion cell axon morphology. Furthermore, human retinal pigment epithelial cells were found to be cytocompatible with a GEN crosslinked chitosan in culture, supporting the safe use of GEN for the treatment of posterior segment pathologies.

A series of studies have confirmed that GEN can effectively stiffen the sclera, but only one study has investigated its effect on slowing myopia progression. Although the results from Wang and Corpuz suggest that sub-Tenon’s injections of GEN can inhibit experimental myopia in guinea pigs, these results are based on one GEN dose, in vivo measurements at baseline versus...
the study end point, and did not include a group with sham injections. To date, no study has investigated the dose-dependent effect of SXL using GEN on myopia control or used repeated longitudinal in vivo measurements to confirm the potential treatment effect of GEN on myopia progression. The aims of the present study are to (i) clarify if SXL using sub-Tenon’s injections of GEN can slow axial elongation and progressive experimental myopia and (ii) determine if this potential treatment effect is dose dependent. We investigate these aims using longitudinal biometric and refractive measurements in the translational tree shrew model of myopia.

Materials and Methods

Experimental Groups and Procedure

This study was carried out using northern tree shrews (*Tupaia glis belangeri*) housed in individual cages on a 14-hour light/10-hour dark cycle. Experimental subjects were randomly assigned to one of five different experimental groups. Experimental groups were balanced to include both males and females and multiple pups from the same parents were not used in the same group. To induce progressive myopia, animals of all groups were exposed to 11 days of FD starting at 24 days of visual experience (DVE) by wearing a lightweight aluminum goggle frame with a translucent diffuser covering one eye while the other eye has unobstructed vision through an open goggle frame. FD treatment was randomly assigned to one eye. The other eye remained untreated and served as control. Four groups received sub-Tenon’s injections in the FD-treated eye of either buffer (sham) or GEN at different concentrations and frequencies. The groups were named based on the visual treatment, GEN concentration, and injection frequency as follows:

- FD: FD treatment with no GEN injections (*n* = 8)
- FD + 5 × sham: FD treatment and 5 sham injections using buffer (*n* = 6)
- FD + 3 × 10 mM GEN: FD treatment and 3 GEN injections at 10 mM (*n* = 6)
- FD + 3 × 20 mM GEN: FD treatment and 3 GEN injections at 20 mM (*n* = 6)
- FD + 5 × 20 mM GEN: FD treatment and 5 GEN injections at 20 mM (*n* = 6)

The GEN dose was varied by either increasing the concentration or increasing the number of injections as indicated above. Treatment effects were compared against our reference group (FD). All procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were also approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Genipin

GEN (Wako Chemicals USA, Corp, Richmond, VA; Fisher Scientific, Waltham, MA) injections used in this study were prepared from a stock solution of 50 mg/mL that was made by dissolving GEN powder in dimethyl sulfoxide (DMSO; Corning DMSO, Fisher Scientific) to ensure maximum solubility. In addition, DMSO provides optimal storage conditions because GEN can crosslink itself and lose its potency if stored in aqueous buffers for more than 24 hours according to the manufacturer. The stock solution was vortexed and filtered through a 0.22-μm filter and then split into aliquots that were stored at –25°C until used. Aliquots were thawed and further diluted to the required concentration using a balanced salt solution (BSS; BSS ophthalmic irrigating solution; Alcon, Geneva, Switzerland) before each injection. All aliquots were freshly prepared on the day of the first injection for each experimental subject. Sham injections were prepared using the same DMSO to BSS ratio that was used to prepare GEN injections.

Crosslinking Procedure and Experimental Timeline

Isoflurane anesthesia was induced using the animal’s nesting box (3% in 100% oxygen at 1 L/min). The skin around the eye was prepared for surgery by using gauze pads to apply povidone iodine (5%) three times followed by rinsing with phosphate buffer saline. The cornea was anesthetized using topical proparacaine hydrochloride ophthalmic solution (0.5%; Sandoz; Holzkirchen, Germany). A traction suture (Ethicon Perma-Hand 7/0, 18 Silk Black, Braided) was placed through the inferior conjunctiva at the limbus and the eye was supraducted. The inferotemporal conjunctiva was incised using curved Vannas scissors (World Precision Instruments, Sarasota County, FL) and dissection through tenon’s was carried down to the bare sclera. Using tying forceps, the tenon’s capsule was dissected bluntly from the sclera and the muscle cone identified. The tying forceps were used to open the muscle cone and visualize the intracanal fat. A 25G blunt curved needle was used to deliver a volume of 0.4 mL of GEN solution to the sub-Tenon’s space directly behind the posterior sclera (Fig. 1A). The
conjunctiva was closed around the needle and held in position with tying forceps in an effort to minimize egress of GEN out the surgical incision. The needle was kept in place as the solution was slowly injected over a period of 15 minutes to ensure the exposure of the sclera to GEN for this time period. The cornea was flushed with BSS then treated with an antibiotic ophthalmic ointment (neomycin and polymyxin B sulfates and Bacitracin Zinc) after each injection. The first injection was performed at 18 DVE in all injected animals. Injections were repeated every other day according to the group injection frequency as illustrated in Figure 1B. All injections, except the last injection of the five injection groups, were started before FD treatment to evaluate whether SXL could inhibit myopia development completely. Our experimental strategy does not mimic a real clinical case scenario where the treatment starts after diagnosing the problem. Instead, our intention was to evaluate whether SXL using GEN could completely inhibit myopia development during the highest susceptibility period. Animals were followed daily and measurements of the refractive state and axial dimensions were taken daily from 18 to 35 DVE. Sub-Tenon’s injections were performed by an experienced surgeon who was blinded with respect to the specific GEN concentration and substance (sham vs. GEN) that was administered. The animals were examined daily for potential weight loss, signs of discomfort, pain, retinal damage, corneal damage, inflammation, and scar tissue formation at the site of injection. However, none of these complications were detected in any of our treated experimental subjects.

Refractive and Biometry Measurements

Noncycloplegic refractive and biometry measurements were performed daily in fully awake animals at approximately 10:00 AM using the Nidek ARK-700A infrared auto-refractor (Marco Ophthalmic, Jacksonville, FL) and Lenstar LS-900 optical biometer (Haag-Streit USA, Mason, OH) following previously established protocols. All refractive measurements were corrected for the small eye artifact, which has been estimated to be 4 diopters (D) in tree shrews. As in previous studies, noncycloplegic refractive measurements were made because cycloplegic drugs may interfere with the emmetropization process and myopia development. Also, noncycloplegic refractive measurements have been shown to provide a valid estimate of a wide range of refractions in tree shrews with minimal differences to cycloplegic measurements. Central corneal thickness (CT), anterior chamber depth (ACD), lens thickness (LT), vitreous chamber depth (VCD) and axial length (AL) were calculated from the raw Lenstar data using species-specific refractive indices.

Corneal Power

In addition to axial dimensions, we have extracted the corneal curvature from the Lenstar raw data to calculate corneal power. We performed three biometry measurements per eye and imaging session. During each biometry measurement, the Lenstar acquires four images of the anterior corneal surface showing two concentric rings of reflective light spots (32 spots in total). Unfortunately, the internal analysis of the Lenstar does not provide corneal curvature results when used with tree shrew eyes. To overcome this limitation, we have developed our own algorithm to extract corneal curvature from the Lenstar raw data. The algorithm consists in extracting the raw images with the reflective spots and the distance parameter from the Lenstar database. We assume that the distance parameter correlates with the distance between the Lenstar camera and the anterior corneal surface. The reflective light spots were segmented automatically.
Figure 2. (A) Lenstar image of a ball bearing showing the automatically segmented reflective light spots and best fit ellipses for inner and outer ring rings. (B) Surface plots showing the linear correlations between the known radius of curvature of the imaged ball bearings, the radius of the best fit ellipses, and the distance parameter.

and grouped into inner ring, outer ring, and outliers using our developed algorithm. A visual check was performed for each automated segmentation of the reflective light spots. Best fit ellipses were determined for the reflective spots of the inner and outer ring independently (Fig. 2A). The algorithm was tuned by imaging 14 ball bearings with known radii of curvature ranging between 6.91 mm and 15.86 mm. A strong linear correlation was identified between the mean radii of best fit ellipses ($R_{inner, ellipse}$, $R_{outer, ellipse}$), the distance parameter, and the radii of curvature of the ball bearings (Fig. 2B). The consideration of the distance parameter was critical in establishing a good fit of the ball bearing data. The following established correlations were then used to calculate the radius of curvature for the inner $r_{inner}$ and outer ring reflections $r_{outer}$

$$r_{inner} [mm] = -0.6817 + 0.1120 \cdot R_{inner, ellipse}[px] + 137.2 \cdot \text{distance},$$

$$r_{outer} [mm] = -0.6888 + 0.0792 \cdot R_{outer, ellipse}[px] + 139.8 \cdot \text{distance}. \quad (1)$$

Corneal power $K$ was calculated as $K[D] = (1.3375 - 1)/r[m]$. Finally, corneal power was averaged over the inner and outer ring reflection data and over repeated measures.

**Statistical and Data Analysis**

All animals were raised and housed under identical conditions. If animals are not subjected to any treatment, refractive and biometric parameters are known to be very similar between the left and right eyes. Therefore, we used the differences between the treated and control eyes to evaluate the effect of sub-Tenon’s injections on the refractive and biometric parameters. A mixed design analysis of variance known as split-plot analysis of variance was used to test for differences in the measured parameters (axial components and refraction) between groups in terms of time-group interaction (rate of change over time) and main effects between-subjects (overall means). Each experimental group was compared with the reference group (FD group). The statistical analysis was performed using SPSS Statistics V24. $P$ values and partial eta squared ($\eta^2$; effect size) were reported. We used the Greenhouse–Geisser correction for time–group interaction because most of our datasets did not satisfy the sphericity assumption owing to the high number of within-subject independent variables (18 time points). Cohen’s $d$ (effect size) was also reported to estimate the overlap of overall means of each experimental group and the FD group. The significance level was set to 0.05.

**Results**

**Refraction**

The sham injections showed no significant effect on development of refractive error compared with the FD group (Fig. 3A; Table 1). A significant treatment effect (interaction and main effect) on slowing FD-induced myopia was observed in the two highest GEN dose group interaction (rate of change over time) and main effects between-subjects (overall means). Each experimental group was compared with the reference group (FD group). The statistical analysis was performed using SPSS Statistics V24. $P$ values and partial eta squared ($\eta^2$; effect size) were reported. We used the Greenhouse–Geisser correction for time–group interaction because most of our datasets did not satisfy the sphericity assumption owing to the high number of within-subject independent variables (18 time points). Cohen’s $d$ (effect size) was also reported to estimate the overlap of overall means of each experimental group and the FD group. The significance level was set to 0.05.

**Table 1.** Summary of the Statistical Analysis of the Difference in Refractive Error for Each Experimental Group Compared With the FD Group

| Experimental Groups     | Interaction $P$ Value | $\eta^2$ | Main Effect $P$ Value | $d$ |
|-------------------------|-----------------------|----------|-----------------------|-----|
| FD + 5 × sham           | 0.313                 | 0.086    | 0.125                 | 0.17|
| FD + 3 × 10 mM GEN      | 0.053                 | 0.171    | 0.419                 | 0.12|
| FD + 3 × 20 mM GEN      | **0.043**             | 0.189    | **0.043**             | 0.40|
| FD + 5 × 20 mM GEN      | **0.020**             | 0.213    | **0.040**             | 0.92|

$P$ value, $\eta^2$, and $d$ represent the significance level, partial eta squared (effect size), and Cohen’s $d$ (effect size), respectively. Statistically significant values are highlighted in bold.
Figure 3. Daily development of the difference in refractive error (treated minus control eye, mean and individual subject responses) for each injected group: (A) FD + 5 × sham, (B) FD + 3 × 10 mM GEN, (C) FD + 3 × 20 mM GEN, and (D) FD + 5 × 20 mM GEN. The reference group (FD) is shown for comparison in each plot. The ↑ represent days of sub-Tenon's injections and the gray-shaded area represents FD treatment period. (E) Box plots representing the difference in refractive error (treated minus control eye) from 18 to 35 DVE. The * and # indicate significant differences in terms of time-group interaction and main effect with respect to the FD group, respectively.

Figure 4. Daily development of the CT difference (treated minus control eye, mean and individual subject responses) for each injected group: (A) FD + 5 × sham, (B) FD + 3 × 10 mM GEN, (C) FD + 3 × 20 mM GEN, and (D) FD + 5 × 20 mM GEN. The reference group (FD) is shown for comparison in each plot. The ↓ represent days of sub-Tenon's injections and the gray-shaded area represents FD treatment period. (E) Box plots representing the distribution of CT difference (treated minus control eye) from 18 to 35 DVE. The * and # indicate significant differences in terms of time-group interaction and main effect with respect to the FD group, respectively. CT in normal eyes at 35 DVE is approximately 0.24 mm.

Table 2. Summary of the Statistical Analysis of the Difference in CT for Each Experimental Group Compared With the FD Group

| Experimental Groups               | Interaction P Value | Interaction $\eta^2$ | Main Effect P Value | Main Effect d |
|-----------------------------------|---------------------|----------------------|---------------------|--------------|
| FD + 5 × sham                     | 0.396               | 0.080                | 0.0210              | 1.12         |
| FD + 3 × 10 mM GEN                | 0.427               | 0.078                | 0.0001              | 4.74         |
| FD + 3 × 20 mM GEN                | 0.001               | 0.341                | 0.0001              | 2.73         |
| FD + 5 × 20 mM GEN                | 0.002               | 0.425                | 0.0010              | 2.59         |

$P$ value, $\eta^2$ and $d$ represent the significance level, partial eta squared (effect size) and Cohen’s $d$ (effect size), respectively. Statistically significant values are highlighted in bold.

Sham treatment showed a slight but significant effect on CT (Figs. 4A,E; Table 2). All groups with GEN injections showed a significant main effect for the CT difference when compared with the FD group (Figs. 4B–E; Table 2). The overall CT difference increased with an increased dose of GEN suggesting a dose-dependent corneal swelling of the treated eye (Figs. 4B–E). In the two highest GEN dose groups, the CT difference increased during the injection phase

CT and Power

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$P$ value, $\eta^2$ and $d$ represent the significance level, partial eta squared (effect size) and Cohen’s $d$ (effect size), respectively. Statistically significant values are highlighted in bold.
Figure 5. Daily development of the difference in corneal power (treated minus control eye, mean and individual subject responses) for each injected group: (A) FD + 5 × sham, (B) FD + 3 × 10 mM GEN, (C) FD + 3 × 20 mM GEN, and (D) FD + 5 × 20 mM GEN. The † represent days of sub-Tenon’s injections and the gray-shaded area represents FD treatment period. (E) Box plots representing the distribution of the difference in corneal power (treated minus control eye) from 18 to 35 DVE. The # indicate significant differences in terms of main effect with respect to the FD group. Corneal power in normal eyes at 35 DVE is approximately 102 D.

Table 3. Summary of the Statistical Analysis of the Difference in Corneal Power for Each Experimental Group compared with the FD Group

| Experimental Groups | Interaction P Value | Interaction η² | Main Effect P Value | Main Effect d |
|---------------------|---------------------|----------------|---------------------|---------------|
| FD + 5 × sham       | 0.623               | 0.058          |                     |               |
| FD + 3 × 10 mM GEN  | 0.350               | 0.087          | 0.845               | 0.27          |
| FD + 3 × 20 mM GEN  | 0.510               | 0.069          | 0.470               | 0.60          |
| FD + 5 × 20 mM GEN  | 0.463               | 0.074          | 0.014               | 1.43          |

P value, η² and d represent the significance level, partial eta squared (effect size) and Cohen’s d (effect size), respectively. Statistically significant values are highlighted in bold.

and decreased after the last GEN injection suggesting that the GEN induced corneal swelling was transient and reversible. This time-dependent change in CT was also reflected in a significant difference in terms of time-group interaction in the two highest GEN dose groups (Figs. 4C–E; Table 2).

The corneal power was significantly altered in terms of main effects (overall means) in the two groups with five repeated injections (Figs. 5A,D,E; Table 3). Although the groups receiving five injections (FD + 5 × sham group and FD + 5 × 20 mM GEN) had a significantly altered corneal power in terms of main effects (overall means), no significant differences were found in corneal power in terms of time–group interaction with a low effect size ($\eta^2 < 0.1$) for all groups. This suggests that the corneal power development was not significantly impacted by the treatment despite the changes seen in CT. Interestingly, subject 1721 of the lowest GEN dose group showed a large change in corneal power (Fig. 5B), which may have contributed to the strong treatment effect seen in this subject in Figure 3B.

Table 3. Summary of the Statistical Analysis of the Difference in Corneal Power for Each Experimental Group compared with the FD Group

Anterior Chamber Depth

Sham injections showed no significant effect on the ACD difference (Fig. 6A; Table 4). All groups with GEN injections showed a significant difference in terms of time–group interaction and main effect (Figs. 6B–E; Table 4). Overall, the ACD difference compared with the FD group increased with increased GEN dose suggesting a dose-dependent shortening of the ACD in the GEN treated eyes (Figs. 6B–E). In contrast with the CT, the ACD differences remained and did not recover after the injections ended (Figs. 6B–D) suggesting that the GEN injections had a prolonged effect on the ACD.

Lens Thickness

Neither the sham nor the lowest GEN dose groups showed a significant difference in LT difference when compared with the FD group (Figs. 7A,B; Table 5). A significant increase in the overall LT difference (main effect) was only observed in the highest GEN dose group (Fig. 7D; Table 5). This effect seemed to be transient because the LT difference recovered by the end of the experiment (Fig. 7D). A significant time–group interaction effect was seen in the second highest GEN dose group (Fig. 7C), but not in the highest dose group.

Vitreous Chamber Depth

As expected, FD alone caused a progressive increase in the VCD difference between the treated and control eye after the diffuser was put on at 24 DVE (Fig. 8). The sham injections had a significant effect on the VCD (time–group interaction and main effect, Table 6), which can be clearly seen in daily development of the VCD difference in Figure 8A. In contrast to the sham
Figure 6. Daily development of the ACD difference (treated minus control eye, mean and individual subject responses) for each injected group: (A) FD + 5 × sham, (B) FD + 3 × 10 mM GEN, (C) FD + 3 × 20 mM GEN, and (D) FD + 5 × 20 mM GEN. The reference group (FD) is shown for comparison in each plot. The ↑ represent days of sub-Tenon’s injections and the gray-shaded area represents FD treatment period. (E) Box plots representing the distribution of ACD difference (treated minus control eye) from 18 to 35 DVE. The * and # indicate significant differences in terms of time-group interaction and main effect with respect to the FD group, respectively. ACD in normal eyes at 35 DVE is approximately 0.8 mm.

Figure 7. Daily development of the LT difference (treated minus control eye, mean and individual subject responses) for each injected group: (A) FD + 5 × sham, (B) FD + 3 × 10 mM GEN, (C) FD + 3 × 20 mM GEN, and (D) FD + 5 × 20 mM GEN. The reference group (FD) is shown for comparison in each plot. The ↑ represent days of sub-Tenon’s injections and the gray-shaded area represents FD treatment period. (E) Box plots representing the distribution of LT difference (treated minus control eye) from 18 to 35 DVE. The * and # indicate significant differences in terms of time-group interaction and main effect with respect to the FD group, respectively. LT in normal eyes at 35 DVE is approximately 3.35 mm.

Table 4. Summary of the Statistical Analysis of the Difference in ACD for Each Experimental Group Compared with the FD Group

| Experimental Groups | Interaction P Value | $\eta^2$ | Main Effect P Value | $d$ |
|---------------------|---------------------|----------|---------------------|-----|
| FD + 5 × sham       | 0.7420              | 0.045    | 0.6320              | 0.37 |
| FD + 3 × 10 mM GEN  | **0.0001**          | 0.470    | **0.0030**          | 2.14 |
| FD + 3 × 20 mM GEN  | **0.0001**          | 0.613    | **0.0001**          | 2.05 |
| FD + 5 × 20 mM GEN  | **0.0001**          | 0.553    | **0.0001**          | 2.45 |

$P$ value, $\eta^2$ and $d$ represent the significance level, partial eta squared (effect size) and Cohen’s $d$ (effect size), respectively. Statistically significant values are highlighted in bold.

Table 5. Summary of the Statistical Analysis of the Difference in LT for Each Experimental Group Compared With the FD Group

| Experimental Groups | Interaction P Value | $\eta^2$ | Main Effect P Value | $d$ |
|---------------------|---------------------|----------|---------------------|-----|
| FD + 5 × sham       | 0.658               | 0.055    | 0.357               | 0.64 |
| FD + 3 × 10 mM GEN  | 0.470               | 0.073    | 0.971               | 0.03 |
| FD + 3 × 20 mM GEN  | **0.033**           | 0.161    | 0.284               | 0.92 |
| FD + 5 × 20 mM GEN  | 0.163               | 0.116    | **0.0001**          | 2.30 |

$P$ value, $\eta^2$ and $d$ represent the significance level, partial eta squared (effect size) and Cohen’s $d$ (effect size), respectively. Statistically significant values are highlighted in bold.

Group with five injections, the lowest GEN dose group with three injections at 10 mM of GEN showed no significant effects (Fig. 8B; Table 6). The FD-induced increase in VCD difference was significantly reduced in the two highest GEN dose groups (Figs. 8C–E, Table 6). The daily development of the VCD difference suggests that the treatment effect was transient in the FD+3 × 20 mM GEN group (Fig. 8C). In the highest GEN dose group, however, the VCD difference was reduced after the first injection and remained negative throughout the experiment indicating that the VCD of the treated eye was shorter compared with the
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Figure 8. Daily development of the VCD difference (treated minus control eye, mean and individual subject responses) for each injected group: (A) FD + 5 × sham, (B) FD + 3 × 10 mM GEN, (C) FD + 3 × 20 mM GEN and (D) FD + 5 × 20 mM GEN. The reference group (FD) is shown for comparison in each plot. The ↑ represent days of sub-Tenon’s injections and the gray-shaded area represents FD treatment period. (E) Box plots representing the distribution of VCD difference (treated minus control eye) from 18 to 35 DVE. The * and # indicate significant differences in terms of time-group interaction and main effect with respect to the FD group, respectively. VCD in normal eyes at 35 DVE is approximately 2.85 mm.

Table 6. Summary of the Statistical Analysis of the Difference in VCD for Each Experimental Group Compared with the FD Group

| Experimental Groups | Interaction P Value | η² | Main Effect P Value | d |
|---------------------|-------------------|----|-------------------|---|
| FD + 5 × sham       | 0.0050            | 0.238 | 0.0001 | 0.58 |
| FD + 3 × 10 mM GEN  | 0.5280            | 0.056 | 0.5210 | 0.20 |
| FD + 3 × 20 mM GEN  | 0.0540            | 0.168 | 0.0020 | 0.75 |
| FD + 5 × 20 mM GEN  | 0.0001            | 0.473 | 0.0001 | 2.28 |

P value, η² and d represent the significance level, partial eta squared (effect size) and Cohen’s d (effect size), respectively. Statistically significant values are highlighted in bold.

control eye despite 11 days of FD (Fig. 8D). The GEN-injected groups showed a dose-dependent treatment effect on slowing FD-induced VCD elongation. This was reflected by the dose-dependent increase in effect size (partial eta squared η² and Cohen’s d; Table 6) and by the dose-dependent decrease in the VCD difference compared with the FD group (Figs. 8B–E). Subject 1721 of the lowest GEN dose group stands out showing a significant decrease in VCD elongation as opposed to the group response (Fig. 8B).

Axial Length

The AL was computed as the sum of CT, ACD, LT, and VCD. Consequently, the effect of SXL on the development of AL is the result of the combined effect that SXL had on each individual compartment. Similar to the development of VCD and as intended, FD alone causes progressive axial elongation after the diffuser lens was put on (24 DVE). Sham injections had a significant effect (interaction and main effect)

Figure 9. Daily development of the AL difference (treated minus control eye, mean and individual subject responses) for each injected group: (A) FD + 5 × sham, (B) FD + 3 × 10 mM GEN, (C) FD + 3 × 20 mM GEN and (D) FD + 5 × 20 mM GEN. The reference group (FD) is shown for comparison in each plot. The ↑ represent days of sub-Tenon’s injections and the gray-shaded area represents FD treatment period. (E) Box plots representing the distribution of AL difference (treated minus control eye) from 18 to 35 DVE. The * and # indicate significant differences in terms of time-group interaction and main effect with respect to the FD group, respectively. AL in normal eyes at 35 DVE is approximately 7 to 7.5 mm.
on the AL difference compared with the FD group (Fig. 9A; Table 7). This sham effect was mainly driven by changes in the VCD (Fig. 8A) because all other compartments remained unaffected by the sham injections. The lowest GEN dose group showed a similar trend as the sham group, but the effect was not significant (Fig. 9B; Table 7). The treatment effect of SXL on slowing FD-induced AL elongation increased with a higher dose of GEN, as can be seen in the daily trends (Figs. 9B–D), and the dose-dependent increase in the estimated effect size given by partial eta squared and Cohen’s $d$ (Table 7). Again, subject 1721 of the lowest GEN group showed a strong treatment effect on slowing AL elongation in contrast with the overall group response (Fig. 9B).

### Discussion

We have presented a preclinical study that evaluates the potential treatment effect of SXL using GEN at different doses on slowing the excessive expansion of the posterior scleral shell seen in progressive myopia. To this end, GEN sub-Tenon’s injections have been used to induce artificial collagen crosslinks in the sclera of tree shrew eyes undergoing FD-induced myopia. Our results showed that SXL using GEN can effectively slow axial elongation and FD myopia in tree shrews. To our best knowledge, this is the first study that investigated the treatment effect of SXL at different doses using different GEN concentrations (10 and 20 mM) and numbers of injections (three and five injections). Our results suggest that the treatment effect of SXL is dose dependent, with both increasing the concentration and frequency of GEN injections increased the treatment effect with respect to refraction, AL elongation, and VCD elongation. Previous single-dose studies showed controversial results, where SXL was found to be effective using GEN and ultraviolet light-activated riboflavin, and ineffective using glyceraldehyde in slowing myopia progression. Our dose-dependent study clarified that SXL can slow myopia progression if used at a high enough dose. Similar to our lowest GEN dose, the crosslinking dose used by Chu et al. was likely insufficient in slowing myopia progression suggesting that a sufficient amount of crosslinks have to be formed before a treatment effect can be observed.

Interestingly, our sham treatment caused a significant effect on the VCD that resulted in slight, but not significant changes in the refractive state as compared with the FD group. We were not able to discern the exact cause of this sham effect because the sham treatment was a combination of the surgical procedure and the injected buffer. However, a similar sham effect was observed by Garcia et al., where sub-Tenon’s injections of a hydrogel had a significant treatment effect on FD myopia in guinea pigs. Because we used a different buffer than Garcia et al., for our sham injections, the sub-Tenon’s injections per se are likely the cause of the sham effect and not a chemical interaction of the buffer with the sclera. Surprisingly, although the sham-injected group showed a significant effect on slowing the VCD elongation, the lowest GEN dose group (Fig. 8B) showed no such effect. In contrast with the lowest GEN dose group, the sham group had two additional sub-Tenon’s injections (three vs. five injections) to match our highest GEN dose group. Furthermore, the additional two injections occurred during the FD period, which may have enhanced the sham effect. The sham injections had a significant effect on the AL, which was a direct consequence of the effect on the VCD. However, the sham injections had no significant effect on the CT, ACD, LT, or, surprisingly, on refraction. The sham group showed a small but significant main effect on the corneal power difference. The source of this effect is unclear, but it may explain why the sham injections significantly impacted the AL and VCD but not the refractive development of the injected eyes.

Although our primary treatment goal was to slow VCD elongation in FD eyes, other compartments were also impacted by our SXL procedure. For instance, a dose-dependent increase in CT and a decrease in the ACD difference between the GEN-treated and control eyes has been observed. Our daily measurements provided valuable insights into the time-dependent effect of SXL. Although the corneal thickening mostly recovered by the end of the experiment, the significant decrease in the ACD remained stable until the end of the experiment. The overall mean (main effect) of corneal power was significantly increased in the
highest dose group. The observed corneal changes were likely caused by the direct exposure of the cornea to the GEN solution that leaked out of the conjunctival incision during the sub-Tenon’s injections or immediately postoperatively.

Note that a profound and prolonged treatment effect on slowing axial elongation was seen in the highest dose group, where the AL difference remained negative until the end of the experiment (Fig. 9D). The same group showed a lesser treatment effect with respect to the refractive state, where a significant amount of myopia still developed at the end of the experiment (Fig. 3D). This observation supports the idea that an additional effect altered the refractive power of the ocular system in the GEN-treated eyes and counteracted the refractive treatment effect. It is likely that this effect was caused by either a change in corneal curvature owing to its exposure to leaked GEN or a change in the lens curvature that was caused by the observed changes in the LT, or perhaps a combination of both. The exposure of the cornea to the crosslinking agent should be prevented by optimizing the injection technique in future studies.

Similar to the CT, the ACD was significantly impacted by SXL; the GEN-treated eyes had a significantly shallower ACD than their follow control eyes. In contrast with the cornea, the SXL-induced decrease in the ACD was not restored at the end of the experiment. The ACD difference increased steadily during the GEN injection period until it reached a stable state that remained consistent until the end of the experiment (Figs. 6B–D). This prolonged reduction of ACD cannot be explained by the also observed corneal thickening, because the CT was mostly recovered at the end of the experiment whereas the ACD was not (compare Figs. 4B–D with Figs. 6B–D). Reviewing the data of the treated eyes alone reveals that the ACD was actually decreased over the entire period of the experiment, suggesting that the GEN injections actually shortened the ACD of the crosslinked eyes. This decrease in the ACD might have decreased the anterior chamber volume. Together with the overall stiffening of the ocular coats, SXL might have impacted the intraocular pressure in our experimental subjects. Unfortunately, we did not measure the intraocular pressure and this potential effect needs to be evaluated in future studies.

Surprisingly, the LT difference was significantly increased in the two groups injected with the greatest amount of GEN (Figs. 7C, D). Because the lens was not directly exposed to our GEN treatment, it is unclear if the observed thickening was caused directly by GEN diffusing into the eye or indirectly. A SXL-induced stiffening of the ocular coats might have changed the interaction between the ciliary body, suspensory ligaments, and ocular coats, and indirectly affected the shape of the lens. However, the LT changes might have altered lens curvature and therefore the lens power that might have contributed to myopic shift in the highest group dose toward the end of the experiment, despite the decrease seen in the VCD and AL.

The accumulation of collagen crosslinks has been proposed as a mechanism that slows and/or inhibits the scleral remodeling that would eventually result in slowing myopia progression with age.26 Our experimental subjects were treated with GEN before starting FD-induced progressive myopia to evaluate if SXL could completely inhibit the development of myopia. Our treatment showed a significant effect in the highest dose group regarding refraction (Fig. 3D) and VCD elongation (Fig. 8D). However, this treatment effect seems to fade away; the progression of the highest dose group was similar to that of the FD group, suggesting that such a treatment effect is unlikely to be sustained beyond the end point of our experiment. These findings indicate that SXL using GEN can significantly slow down myopia progression, but it may not inhibit it completely.

In summary, we have presented the first longitudinal study that investigates the dose dependent effect of SXL on progressive myopia using sub-Tenon’s injections of GEN in tree shrew eyes. We have shown that SXL using GEN can slow axial elongation, VCD elongation, and FD myopia. Furthermore, we have provided evidence that these treatment effects can be increased by increasing the GEN concentration or injection frequency. Our findings support the notion that excessive scleral expansion in myopia can be slowed by inducing artificial collagen crosslinks in the sclera. Some results were unexpected (corneal thickening, decrease in the ACD, sham effect) and need further evaluation. Future optimizations of this potential treatment modality should include an improved methodology to deliver GEN so as to avoid the exposure of the cornea to the crosslinking agent and to decrease the number of injections. A sustained delivery of GEN may be needed for a prolonged treatment effect. GEN is a promising crosslinking agent for SXL because it can slow the progression of myopia and does not require light activation.

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