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ABSTRACT

Background: This study aims to assess the efficacy of the scleral collagen cross-linking method using glyceraldehyde solution for prevention of lens-induced axial elongation in New Zealand rabbits and investigate the biochemical and microstructural changes that occur.

Methods: The right eyes of New Zealand rabbits aged seven weeks were randomly divided into three groups: the cross-linking group (n = 6), non-crosslinking group (n = 5), and untreated control group (n = 5). Eyes in cross-linking and non-crosslinking groups were treated with a ~8.0 Diopter spherical lens over the course of two weeks. The cross-linking effects were achieved by a sub-Tenon’s injection of 0.15 ml 0.5 M glyceraldehyde to eyes in the CL group. Ocular parameters were measured on the 1st, 7th, and 14th days. Biomechanical testing, light and electronic microscopy were used.

Results: Following the cross-linking treatment, eyes in the cross-linking group had a shorter axial length compared to those in the non-crosslinking group (p = 0.006). Collagen fibrils larger than 240 nm were observed in the scleral stroma of cross-linking group, which were absent in the scleral stroma of the non-crosslinking and untreated control group. The mean ultimate stress and Young’s modulus was significantly greater in the cross-linking group compared to those in the non-crosslinking and untreated control group (p < 0.05). No histological damage observed in the retina or choroid.

Conclusions: This study demonstrates that lens-induced axial elongation in rabbits can be effectively blocked by cross-linking using glyceraldehyde, with anatomical and mechanical modification and no deleterious effects.

Introduction

Structural changes are noted with continuing myopia progression, and the resultant AXL elongation is often accompanied by scleral tissue loss, scleral thinning, and the long-term weakening of the scleral collagen fibril matrix. Therefore, early detection and surgical treatment are essential in the prevention of progressive myopia. In clinical practice, application of posterior reinforcement surgery is controversial, with the degradation of the implanted sclera and a loss of the reinforcement effect of the sclera over time. Accordingly, scleral collagen cross-linking (CXL) that aimed at mechanically reinforcing the sclera may yield a universal and effective therapy for preventing myopia progression by addressing the underlying causative factor.

Previous attempts at CXL for prevention of myopia have used riboflavin/UVA irradiation and riboflavin/blue light irradiation with equivocal effectiveness, but complicated by difficulty in exposing the posterior sclera to certain light sources and concerns about detrimental ultrastructural changes in the retinal layers. One potential treatment modality to circumvent these complications is the use of a sub-Tenon’s injection. This method could serve as a simple and minimally invasive approach to reach the posterior sclera, which has been demonstrated by ultrasound. It has been proven that scleral cross-linking using sub-Tenon injections of genipin can block the form-deprivation myopia of guinea pigs.

In this study, we used glyceraldehyde as the CXL agent because its efficacy in stiffening the sclera has been shown to be long-lasting in the rabbit, while Chu et al. found the scleral strips examined after injection of the glyceraldehyde treatment exhibiting significantly larger stress and Young’s elastic modulus compared to their controls. Glyceraldehyde was among the least toxic of all the available agents. The primary aim of this study is to investigate whether glyceraldehyde is an effective agent for CXL in preventing lens-induced axial elongation. A second aim of this study is to characterize the effects of CXL induced by glyceraldehyde in a large animal model is a crucial step before initiating human clinical trials. To the best of our knowledge, there are no

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other studies investigating the efficacy of CXL using glyceraldehyde for the prevention of myopia and the biomechanical as well as microstructure changes in the sclera in the rabbit after lens application.

**Materials and methods**

**Animals**

Sixteen seven-week-old New Zealand rabbits weighing between 1010 and 1255 grams were obtained from the Animal Care Center of the Eye and ENT hospital of Fudan University (Eye & ENT Hospital; Shanghai, China). The presence of ocular lesions or deficiencies of the refractive media and ocular fundi were excluded by a priori clinical examination. The rabbits were raised under natural diurnal lighting, housed in large individual cages and maternally reared, with water and food available ad libitum. All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the regulations of Eye & ENT Hospital for animal experimentation. The study protocol was approved by the Animal Ethics Committee of Fudan Eye & ENT Hospital. All efforts were made to minimize any suffering experienced by the animals.

**Lens induced anisometropia and scleral cross-linking using glyceraldehyde**

The right eyes of all rabbits were randomly divided into three groups by randomized number generated by a calculator: the cross-linking group (CL, \( n = 6 \)), non-crosslinking group (NCL, \( n = 5 \)), and the untreated control group (UC, \( n = 5 \)). The data of the right eyes in the non-crosslinking group were from the study of the changes in mechanical stress and ultrastructure of the right eyes and the contralateral left eyes of the rabbit after negative lens application.\(^{12}\) Eyes in the CL and NCL group were treated with a ~8.00 Diopter spherical lens monocularly over the course of two weeks (Figure 1A). Rabbits in the UC group did not receive any treatment at all. The concave lens (4 cm diameter, ~8.00 Diopter, polymethyl methacrylate) was placed in front of each right eye at a vertex distance of approximately 7 mm. The cross-linking effects were achieved by a sub-Tenon’s injection of 0.15 ml 0.5 M glyceraldehyde dissolved in physiologic saline solution (DL-glyceraldehyde from Sigma-Aldrich; isotonic sodium chloride solution 0.9% from Braun Melsungen AG) to the right eyes of rabbits in the CL group at days 0, 2, 4, 6, 8, 10, and 12 (Figure 1B). This method of sub-Tenon’s injection was previously described by Wollensak, Gregor and Iomdina, Elena.\(^8,9\) Throughout the experiment, the externally applied lens systems were inspected and adjusted if necessary at four-hour intervals throughout the day to ensure the sutures in the lens system were firmly in place and the lenses were clean and free of debris. Animals without lens in front of eye appropriately during inspection were excluded from the analysis.

**Measurements of the ocular components**

Ocular components were measured using A-scan ultrasonography (Opticon, Italian, software version: Opticon 2000SPA, instrument accuracy of ± 0.036 mm) by an investigator who was unaware the arrangement of groups. Topical anaesthetic (oxybuprocaine) was used to anaesthetize the cornea to allow applanation of the A-scan ultrasonography probe. Alignment of the probe on the cornea was adjusted after monitoring the ultrasonic graph for signal strength and quality. Proper alignment was defined as strong ultrasonic wave reflection peaks of lens surfaces and retina. Five consecutive readings of the ocular components, including the anterior chamber depth, lens thickness, AXL, and vitreous chamber length, were recorded and averaged after proper alignment was obtained.

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Figure 1. Lens-induced anisometropia and scleral cross-linking in the rabbit. (A) Spherical lenses were attached to arcs made of Velcro and sutured by strings to the soft frame also made from soft Velcro, which circled the ear and neck of the rabbit; (B) Subconjunctival glyceraldehyde injection, which led to elevation of the sclera and conjunctiva immediately and resolved within two days.
Specimen preparation

Lethal doses of ketamine and xylazine were administered to the animals at the end of the experiment. The eyes were enucleated immediately after death. We used the method for specimen preparation in our previous studies.1,2,13

Morphological observations

Histopathological examinations were performed on all the eyes using light microscopy and transmission electron microscopy, as described in our previous study.1,3 We utilized the method previously described by Funata and Tokoro of scleral lamination for analysis of the fibril diameter.14 Electron micrographs at 42,000X of the outer layers (10 µm inward from the boundary between the episclera and sclera), the inner layers (10 µm outward from the boundary between the suprachoroid and sclera), and the middle layers (midway between the outer and inner layers) were generated, by a researcher blind to groups arrangements. Eighteen micrographs were taken from the three layers of all samples, in order to ensure that each micrograph consisted of a different array of bundles. The diameter and number of the collagenous fibrils in the scleral sections were evaluated using an image-processing software (ImageJ ™ 1.51e, Wayne Rasband, National institutes of health, USA). The smaller diameter was measured when fibrils appeared to be oval in shape. These fibrils were all included in the calculations of the distribution of the fibril diameter.

Biomechanical measurements

Strips were dissected sagittally as described above. One strip per eye from three eyes in the CL group, one strip per eye from four eyes in the NCL group, and one strip per eye from four eyes in the UC group were analyzed. Before testing, each strip was measured with an eyepiece micrometer to a resolution of ± 0.01 mm. Biomechanical measurements were performed on the scleral specimens, using the Instron ™ 5565 universal testing machine. We used the parameters as detailed in our previous study.1,2,13 Ultimate stress (MPa) and ultimate strain (%) were calculated, and Young’s modulus (MPa) was determined as the slope of the stress–strain graph at 10% strain.

Statistical analysis

The data was analyzed using SPSS™ 24.0 (SPSS Inc., Chicago, IL). Data were tested for normality by Kolmogorov–Smirnov test and Shapiro–Wilk test and reported as the mean ± the standard deviation (SD) when normally distributed. Values are otherwise reported as the median with the range. Non-parametric Kruskal–Wallis, Mann–Whitney tests and One-way ANOVA were used to compare differences between the three groups. Statistical significance was set at the p = 0.05 level.

Results

Measurements of the refractive system

To assess the efficacy of the scleral CXL method using glyceraldehyde, the ophthalmological characteristics were measured and summarized in Tables 1 and 2. The baseline of the AXL in CL group, NCL group and UC group were not statistically significant (p = 0.622). The final measurements demonstrated that the eyes in the CL group had an AXL of 15.16 mm and a vitreous chamber depth of 6.67 mm, which was significantly shorter than those in the NCL group (p = 0.006). The change in AXL from baseline to the end of the experiment was statistically significantly different between the CL and NCL groups (0.39 vs. 0.84 mm, p = 0.006), with less AXL elongation noted in the CL group. A significantly different degree of change in VCL was also observed between the CL and NCL groups (0.15 mm, p = 0.006).

Table 1. Comparison of ocular dimension of the right eye between three groups.

<table>
<thead>
<tr>
<th></th>
<th>CL group</th>
<th>NCL group</th>
<th>UC group</th>
<th>p (K-S test)</th>
<th>p (K-S test)</th>
<th>p (K-S test)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Before</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>2.24</td>
<td>2.11, 2.35</td>
<td>2.25</td>
<td>2.12, 2.28</td>
<td>0.137</td>
<td></td>
</tr>
<tr>
<td>LENS</td>
<td>6.04</td>
<td>5.83, 6.18</td>
<td>5.98</td>
<td>5.91, 6.47</td>
<td>0.179</td>
<td></td>
</tr>
<tr>
<td>AXL</td>
<td>14.74</td>
<td>14.63, 14.80</td>
<td>14.66</td>
<td>14.60, 14.85</td>
<td>0.130</td>
<td></td>
</tr>
<tr>
<td>VCL</td>
<td>6.45</td>
<td>6.43, 6.59</td>
<td>6.45</td>
<td>6.07, 6.66</td>
<td>0.200</td>
<td></td>
</tr>
<tr>
<td><strong>One week</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>2.30</td>
<td>2.05, 2.41</td>
<td>2.15</td>
<td>2.03, 2.27</td>
<td>0.200</td>
<td></td>
</tr>
<tr>
<td>LENS</td>
<td>6.20</td>
<td>6.13, 6.34</td>
<td>6.45</td>
<td>6.19, 6.63</td>
<td>0.200</td>
<td></td>
</tr>
<tr>
<td>AXL</td>
<td>15.03</td>
<td>14.89, 15.30</td>
<td>15.02</td>
<td>14.84, 15.33</td>
<td>0.200</td>
<td></td>
</tr>
<tr>
<td>VCL</td>
<td>6.62</td>
<td>6.47, 6.69</td>
<td>6.53</td>
<td>6.12, 6.85</td>
<td>0.200</td>
<td></td>
</tr>
<tr>
<td><strong>Two weeks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>2.82</td>
<td>2.21, 2.31</td>
<td>2.29</td>
<td>2.25, 2.36</td>
<td>0.200</td>
<td></td>
</tr>
<tr>
<td>LENS</td>
<td>6.23</td>
<td>6.12, 6.33</td>
<td>6.35</td>
<td>6.19, 6.47</td>
<td>0.200</td>
<td></td>
</tr>
<tr>
<td>AXL</td>
<td>15.16</td>
<td>15.01, 15.19</td>
<td>15.53</td>
<td>15.44, 15.80</td>
<td>0.075</td>
<td></td>
</tr>
<tr>
<td>VCL</td>
<td>6.67</td>
<td>6.57, 6.70</td>
<td>6.82</td>
<td>6.73, 7.15</td>
<td>0.200</td>
<td></td>
</tr>
</tbody>
</table>

Kolmogorov–Smirnov Test (K–S test) was used to test normality.
Kruskall–Wallis Test (K–W test) and Mann–Whitney Test (M–W test) were used.

Data are expressed as the median (min, max).

*Indicate a significant difference between the three groups (p < 0.05), different letters (a, b, and c) indicate a significant difference between groups.
P (AXL group CL group vs. NCL group) = 0.006, P (VCL CL group vs. NCL group) = 0.006
P (AXL group NCL group vs. UC group) = 0.009, P (VCL NCL group vs. UC group) = 0.009
groups (0.15 vs. 0.53 mm, p = 0.006), with a smaller change again noted in the CL group.

**Morphological observations**

To investigate the microstructural changes that occur, light and electronic microscopies were used. Light microscopy suggested that the retina and choroid appear overall unaffected, although mild reversible side effects do occur from the glyceraldehyde treatment in the peripheral structures adjacent to the injection (Figure 2). The main findings of the scleral ultrastructure of the posterior regions of eyes obtained from electronic microscopy can be seen in Table 3, Figures 3 and 4. Collagen fibril diameters among the fibril bundles varied considerably, ranging from 18.993–272.303 nm. As shown in Table 3, the fibril diameters of the outer and middle layers did not differ significantly in the right eyes in the CL group (p = 0.209), while the fibril diameters exhibit a significant difference between the inner and middle layers in the CL group (p = 0.000). However, eyes in the UC group exhibited a gradient across the three layers of scleral stroma, with a significantly different fibril diameter between the three layers (p < 0.001). Fibril diameter in the inner layer of the scleral stroma was significantly larger in the right eyes of the CL group compared to the NCL group (74.177 vs. 63.533 nm, p < 0.001), but was similar to that in the UC group (74.177 vs. 76.747, p = 0.052). In the middle layer and outer layers, the fibril diameter of the CL and NCL group did not differ significantly. Collagen fibrils larger than 240 nm were observed in all the three layers of the scleral stroma in eyes in the CL group, but were absent in eyes in the NCL and UC groups (Figure 3). Dilated endoplasmic reticula and vacuoles within fibroblasts were observed in eyes in the CL and NCL groups (Figure 4B–F). Macrophage-like cells were observed within the inner layer of the scleral stroma in eyes from all three groups, and were also observed in the middle layer of the scleral stroma in eyes of the CL and NCL groups (Figure 4A, E, and G).

**Biomechanical measurements**

To investigate the biochemical changes that occur, biomechanical measurements were used. Table 4 shows statistically significant differences in the ultimate stress and Young’s modulus observed from the scleral strips of eyes from the CL, NCL, and UC groups. The thickness of the scleral strip did not differ significantly between the three groups. The ultimate stress of the scleral strips from the CL group were greater than those from the NCL (28.69 vs. 13.53 Mpa, p = 0.001) and UC groups (28.69 vs. 20.48 Mpa, p = 0.032). The Young’s modulus of the scleral strips from eyes in the CL group was greater than that in the NCL group.
Translational biomechanical strategies, such as sub-Tenon, have been applied in clinical practice, with the potential for more applications in the future. The present study aimed to develop and provide a new evidence of this scleral cross-linking method using glyceraldehyde for preventing lens-induced axial elongation in a New Zealand rabbit model, and to investigate the biochemical and microstructural changes that occur.

Lens-induced AXL compensation for imposed defocusing has been demonstrated in a number of species, including the rabbits. When raised with a ∼4D lens worn in front of one eye for 10 days, the guinea pig eyes were found to have significantly longer AXLs compared to their contralateral, non-lens-induced eyes. In the present study, we used a ∼8D lens applied monocularly with a soft frame to the right eyes of New Zealand rabbits to induce defocused effects on their eyes. Experiments were conducted using a New Zealand rabbit model because they have proven useful in revealing several important findings in previous studies involving CXL and myopia.

CXL is a process that occurs naturally in the cornea with aging via either a non-enzymatic or enzymatic pattern. The enzyme-regulated process involves the enzyme lysyl oxidase and results in oxidation of hydroxylysine and the amino acid lysine to their respective aldehydes, which then condense with other aldehydes to form intra and inter-molecular cross-links. The non-enzymatic glycation process, however, can reduce tissue stiffness and resistance to enzymatic degradation. The resultant covalent collagen cross-links promote enhanced advanced glycation end products, which are more stable. Protein molecules and can be further transformed to Maillard reactions, glyceraldehyde can be added to the ends of aldehyde of the open chain form of a simple sugar, which is permeable to molecules of 150,000 Da. As a natural CXL agent, glyceraldehyde represents the aldehyde of the open chain form of a simple sugar, which has been demonstrated to be effective in causing stiffening of biological tissue. With a molecular weight of 90 Da, glyceraldehyde can infiltrate the sclera efficiently and easily, which is permeable to molecules of 150,000 Da. The glycation-induced chemical CXL reactions resulting from glyceraldehyde are called the Maillard reactions. Through the Maillard reactions, glyceraldehyde can be added to the ends of protein molecules and can be further transformed to advanced glycation end products, which are more stable. The resultant covalent collagen cross-links promote enhanced tissue stiffness and resistance to enzymatic degradation.

Compared with earlier attempts at CXL using photochemical irradiation, a sub-Tenon’s injection may prove to be a much simpler and safer way to access the posterior sclera. In our study, AXL elongation was reduced by the administration of glyceraldehyde in the rabbits. Primate and Mammalian eyes have a monolayer fibrous sclera, and the additional fibrous capsule is likely to have a greater (1.74 vs. 0.47 Mpa, p = 0.001) and the UC group (1.74 vs. 0.99 Mpa, p = 0.011).

### Discussion

Previous ophthalmic research has helped to support our understanding that ocular biomechanics contribute, in part, to the mechanisms involved in almost all major ophthalmic disorders such as ametropia, presbyopia, and macular degeneration. Translational biomechanical strategies, such as the use of riboflavin/UVA corneal CXL for the treatment of keratoconus, have been applied in clinical practice, with the potential for more applications in the future. The present study aimed to develop and provide a new evidence of this scleral cross-linking method using glyceraldehyde for preventing lens-induced axial elongation in a New Zealand rabbit model, and to investigate the biochemical and microstructural changes that occur.

**Table 3.** Fibril diameter at different layers at the edge of the ONHs of the right eyes in the three groups.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Group</th>
<th>Median (min, max)</th>
<th>n</th>
<th>p (K-S test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inner</td>
<td>CL</td>
<td>74,176 (217.865)</td>
<td>238</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>NCL</td>
<td>92,441 (216.514)</td>
<td>318</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>UC</td>
<td>97,544 (237.502)</td>
<td>215</td>
<td>0.000*</td>
</tr>
<tr>
<td>Middle</td>
<td>CL</td>
<td>63,533 (247.492)</td>
<td>300</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>NCL</td>
<td>86,996 (226.568)</td>
<td>300</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>UC</td>
<td>92,647 (243.214)</td>
<td>217</td>
<td>0.000*</td>
</tr>
<tr>
<td>Outer</td>
<td>CL</td>
<td>61,162 (216.514)</td>
<td>300</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>NCL</td>
<td>83,987 (216.514)</td>
<td>300</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>UC</td>
<td>90,954 (216.514)</td>
<td>217</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

*indicates a significant difference (p < 0.05).

Within groups (a, b, c): p < 0.05.

Between groups (e, f, g): p < 0.05.
Figure 3. Collagen fibril diameter taken from representative electron micrographs of the three groups. The results showed sections taken from the outer, middle, and inner layers of scleral stroma and the corresponding distribution of the diameter of the collagen fibrils (shown as percentage). Collagen fibrils larger than 240 nm were observed in the inner, middle, and outer layers of the scleral stroma in eyes in the CL group, but were absent in eyes of the NCL and UC groups. The scale can be found in the figure. CL: cross-linking group, NCL: non-crosslinking group, UC: untreated control group.
influence on the biomechanical properties of the sclera and could possibly slow elongation of the eye. The eyes in the CL group in our study displaying an average AXL of 15.16 mm and a vitreous chamber depth of 6.67 mm, which was shorter than that in the NCL group (p = 0.006), but similar to that of the UC group; whereas Chu et al. found the development of form-deprived myopia in guinea pigs, which was induced by diffusers, was not retarded by the injection of glyceraldehyde to the sclera, despite the scleral strips examined after injection of the glyceraldehyde treatment exhibiting significantly larger stress and Young’s elastic modulus compared to their controls. This divergence of results might be explained by the different animals and paradigm of myopia model used:

Table 4. Overview of biomechanical parameters of the right eye of the three groups.

<table>
<thead>
<tr>
<th></th>
<th>CL group</th>
<th>NCL group</th>
<th>UC group</th>
<th>p (One-way ANOVA)</th>
<th>p values of Post Hoc Tests (LSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>S-W test</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Thickness (µm)</td>
<td>0.17</td>
<td>0.07</td>
<td>0.69</td>
<td>0.26</td>
<td>0.01</td>
</tr>
<tr>
<td>Ultimate strain (%)</td>
<td>21.69</td>
<td>5.80</td>
<td>0.76</td>
<td>33.40</td>
<td>9.82</td>
</tr>
<tr>
<td>Ultimate stress (MPa)</td>
<td>28.69</td>
<td>7.52</td>
<td>0.67</td>
<td>13.53</td>
<td>1.60</td>
</tr>
<tr>
<td>Young modulus (MPa)</td>
<td>1.74</td>
<td>0.53</td>
<td>0.27</td>
<td>0.47</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Shapiro-Wilk Test (S-W test) was used to test normality.
One-way ANOVA was used.

*p < 0.05 was considered significant for the difference between groups.

*indicates a significant difference.

CL: cross-linking group, NCL: non-cross-linking group, UC: untreated control group.
rabbits have relatively large eyes, which display similar microscopic retinal and scleral characteristics to those of humans of which changes in the scleral biomechanical properties may occur earlier in the development of myopia (within 24 hours). Structural changes were noted with scleral proteoglycan synthesis greater than normal 8 hours after a short period of negative lens-wear, whereas it took 27 hours for a significant difference in form-deprived eyes. The results of previous studies evaluating the efficiency of scleral reinforcement via a number of treatment regimens have also been controversial, and the divergence was attributed to the bilayer structure of the chick sclera.

Further structural differences were noted between the groups in our study, highlighting some of the changes that appear to occur with myopic change and AXL elongation. The diameter, distribution and orientation of collagen fibrils significantly influence the biomechanical properties of the sclera. Collagen fibrils of a large diameter generate a high tensile strength. In our study, the diameter of the inner layer of the scleral stroma at the posterior pole was significantly larger in the eyes of the CL group compared to those in the NCL group (74.177 vs. 63.533 nm, p < 0.001), but was similar to those in the UC group (74.177 vs. 76.747, p = 0.052). The fibril diameter for the outer layer is greatest for the UC group. Collagen fibrils larger than 240 nm were observed in the inner, middle and outer layers of the scleral stroma in eyes in the CL group, but were absent in eyes in the NCL and UC groups (Figure 3). It might be these large fibrils across the three layers contributed to the higher strength of the sclera in the CL group. A reduction in collagen fibril diameter in the three scleral layers of induced eyes compared with the contralateral eyes has been reported to occur at the posterior pole after two-week period of defocus in lens-induced New Zealand rabbits. Similar change was also observed in form deprived rabbits.

Myopia appears not to be affected by different collagen subtypes uniformly. A reduction in the collagen subtype ratio (V/I) has been linked to myopia, with speculation that it may be important in determining alterations in fibril diameter in myopia. As cross-links by glyceraldehyde promote resistance to enzymatic degradation, this might explain the large diameter fibrils in the CL group. In the direction of the inner-outer sclera, the collagen fibril diameter increased, which is defined as a normal gradient. Different from the right eyes in the UC group, the right eyes in the CL and NCL groups did not display a normal fibril diameter gradient from the inner to outer layers. The change of normal gradient in induced eyes were consistent with previous studies. It was reported that the nasal region between the optic nerve head and 10°nasal possessed significantly fewer cells in deprived eyes than either normal or control eyes. The inner half of sclera had significantly more cells than the outer half in the examined region in deprived eyes, normal and control eyes, while no significant differences were present for the myofibroblasts. In our study, macrophage-like cells were observed within the inner layer of the scleral stroma in eyes from all three groups, and were also observed in the middle layer of the scleral stroma in eyes of the CL and NCL groups. Dilated endoplasmic reticula and vacuoles within fibroblasts were observed in eyes in the CL and NCL groups, but were not observed in the eyes from the UC group (Figure 4B–F). These changes provide clues to the macro-molecular changes induced by therapeutic CXL technology and may provide a method to evaluate the potency of scleral CXL therapies. The adult sclera of human contains blood vessels but lacks true lymphatic vessels. Under physiological circumstances, lymphatic vessels transport fluid, proteins, and immune cells, whereas under pathological ones they can grow through the sclera into the intraocular space after trauma and during ocular melanomas invasion. Scleral distribution of immune cells under physiological conditions has not been investigated thoroughly. LYVE1+ CD68+ macrophages were located adjacent to the longitudinal axis of blood vessels, whereas the number of detected LYVE1+ CD68+ macrophages was comparable in all locations in the episclera; within the stroma, their number increased from anterior toward the posterior part of the eye. In our study, macrophage-like cells were not only observed in the inner layer of the scleral stroma in eyes across all three groups, but were also observed in the middle layer of the scleral stroma in eyes in the CL and NCL groups. C-reactive proteins and complement components found in human pathological myopia confirmed the similarity between myopia and autoimmune diseases.

The ultimate stress of the scleral strips from the CL group were greater than those from the NCL (28.69 vs. 13.53 Mpa, p = 0.001) and the UC groups (28.69 vs. 20.48 Mpa, p = 0.032). The Young’s modulus of the scleral strips from eyes in the CL group was greater than that in the NCL group (1.74 vs. 0.47 Mpa, p = 0.001) and the UC group (1.74 vs. 0.99 Mpa, p = 0.011). The greater ultimate stress and Young’s modulus in sclera from eyes in the CL group may be a result of the large diameter fibrils observed across all three layers, and this change in mechanical parameters was consistent with that of previous studies.

In our study, we used sub-Tenon’s injection of 0.15ml 0.5 M glyceraldehyde 7 times during 14 days. Light microscopy suggested the retina and choroid unaffected (Figure 4); which is consistent with results reported in previous studies investigating glyceraldehyde treatment. Nevertheless, this strategy is not devoid of complications and challenges. Making the sclera more rigid may be result in other long term side effects that were not discoverable in our short term study. As glyceraldehyde leads to the formation of advanced glycation end products, this could be a risk for the development of glaucoma where advanced glycation end products have been implicated in the development of. In the eyes of mice with bead-induced glaucoma, it was reported that a 0.5 M glyceraldehyde subconjunctival injection given over 1 week resulted in greater retinal ganglion cell axon loss from elevated intraocular pressure than that resulting from either buffer-injection or...
in control eyes. We did not measure the intraocular pressure in this study, which is a limitation of this study. In the future, we plan to perform a follow-up study investigating progressive high and long-term myopia, and will include measurements of intraocular pressure looking for any possible cytotoxic effects.

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Declaration of interest

The authors declare no conflict of interest.

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