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Quantification of Efficacy of Collagen Cross-linking Agents to Induce Stiffening of Rat Sclera

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1 **ABSTRACT**

2 The concept of scleral stiffening therapies has emerged as a novel theoretical approach for
3 treating the ocular disorders glaucoma and myopia. Deformation of specific regions of the
4 posterior eye is innately involved in the pathophysiology of these diseases, and thus targeted
5 scleral stiffening could resist these changes and slow or prevent progression of these diseases.
6 Here, we present the first systematic screen and direct comparison of the stiffening effect of
7 small molecule collagen cross-linking agents in the posterior globe, namely using
8 glyceraldehyde, genipin, and methylglyoxal (also called pyruvaldehyde). To establish a dose-
9 response relationship, we used inflation testing to simulate the effects of increasing intraocular
10 pressure (IOP) in freshly harvested rat eyes stiffened with multiple concentrations of each agent.
11 We used Digital Image Correlation to compute the mechanical strain in the tissue as a metric of
12 stiffness, using a novel treatment paradigm for screening relative stiffening by incubating half of
13 each eye in cross-linker and using the opposite half as an internal control. We identified the
14 doses necessary to increase stiffness by approximately 100%, namely 30 mM for glyceraldehyde,
15 1 mM for genipin, and 7 mM for methylglyoxal, and we also identified the range of stiffening
16 possible to achieve with such agents. Such findings will inform development of *in vivo* studies of
17 scleral stiffening to treat glaucoma and myopia.

18 **KEYWORDS**

19 Biomechanics; Rat; Sclera; Collagen; Crosslinking; Digital Image Correlation
20
21

22 **INTRODUCTION**

23 Vision loss has been ranked in patient surveys as the worst possible type of health outcome,
24 equivalent to a diagnosis of cancer, HIV/AIDS, and losing a limb [1]. It is therefore unfortunate
25 that there is no known cure for either glaucoma, the second leading cause of blindness [2], or
26 myopia, the most common vision disorder [3] with incidence rates approaching 90% in some
27 countries [4]. Although both diseases can be treated, these treatments are not successful in all
28 patients and are not a true cure. In glaucoma, for example, 25-45% of patients continue to lose
29 vision even with treatment [5-7]. At present, all therapies for glaucoma are based upon the notion
30 of reducing intraocular pressure (IOP); when these approaches fail, there is no alternative treatment
31 paradigm. Thus, there is significant clinical need for novel treatments for vision loss from
32 glaucoma and myopia.

33
34 Some evidence suggests that stiffening the sclera may be a beneficial treatment for these diseases
35 (reviewed extensively in [8]). In glaucoma, the elastic modulus of the peripapillary sclera (the
36 region immediately surrounding the optic nerve) has been shown in computer models and physical
37 tests to strongly influence deformation of the lamina cribrosa, the region where axonal damage
38 first starts [8-13]. In myopia, the stiffness of the sclera may play a role as well, although conflicting
39 data exists, warranting further study [14, 15]. Finally, corneal stiffening is currently used as a
40 clinical treatment for keratoconus [16], suggesting that the eye can tolerate local modulation of the
41 stiffness of its collagenous tissues.

42
43 Pursuant from this evidence, *in vivo* testing of scleral stiffening therapies for disorders of the
44 posterior eye is indicated. This requires dose-response relationships for suitable such agents to be
45 well understood. Collagen crosslinking agents have been used in the orthopedic and ophthalmic
46 literature to modulate stiffness, and based upon this evidence, three agents have emerged with

1 potential for posterior eye scleral stiffening: glyceraldehyde [17-24], genipin [25-32], and
2 methylglyoxal [20, 31, 33] (also called pyruvaldehyde). Glutaraldehyde is known to increase
3 scleral stiffness [12, 19] but is toxic *in vivo* [34], and riboflavin, used in treatment of keratoconus,
4 requires ultraviolet light to induce crosslinking [16], which adds complications for posterior eye
5 delivery in a clinical setting.
6

7 Although these agents have been identified and studied in an ocular context, no studies to date
8 have directly compared the dose-stiffening relationship of all these agents for sclera. A few studies
9 have examined multiple agents [20, 31] or more than two concentrations of a single agent [25, 29,
10 33] side by side, but the paucity of agents interrogated with identical testing methodologies limits
11 the ability to widely compare the dose-stiffening relationship of scleral collagen crosslinking
12 agents. Given the prevalence and acceptance of rodent models in pre-clinical studies of treatments
13 for vision disorders, there is also significant need for a well-characterized dose of scleral stiffening
14 agents to be used in animal trials. Here, we hypothesize that incubation in collagen cross-linking
15 agents will locally reduce the strain in the sclera resulting from elevated IOP in a dose-dependent
16 manner. In this study, our specific objective is to determine the dose-response of each agent's
17 effect on scleral stiffness with a goal of approximately doubling scleral stiffness (roughly the
18 magnitude observed in prior trials [19]) for future use *in vivo*.
19

20 **METHODS**

21 **Animals:**

22
23 Eyes were freshly harvested from a total of 67 euthanized male, retired breeder (approximately 9–
24 12 months old) Brown Norway rats (Charles River Laboratories, Inc., Wilmington, MA) that were
25 otherwise experimentally naïve. All procedures were approved by the Institutional Animal Care
26 and Use Committee at the Georgia Institute of Technology, and all experiments were performed
27 in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision
28 Research. Female retired breeders were not used in this initial study, as estrogen is known to
29 modulate collagen density and turnover with mechanical consequences [35], and female rats that
30 have had numerous litters (such as retired breeders) may have atypical estrogen levels. Further
31 work will consider animals of both genders.
32

33 Based upon the results of preliminary studies performed during methods development, we used an
34 *a priori* power analysis to estimate that we needed 3 rats/concentration/agent (nested 2-factor
35 ANOVA [agent and concentration]; $\alpha=0.05$; ratio of treatment effect to error effect size = 1.2; 95%
36 power). To be conservative, we harvested 5 eyes per group and used all that were not excluded
37 due to methodological problems (e.g. puncture while cleaning or air bubble when inflating) except
38 for two groups where we harvested 8 eyes (62.5 mM and 125 mM glyceraldehyde). Eyes were
39 randomized
40

41 **Tissue Preparation:**

42 *Stiffening Agents:*

43 Stiffening agents and administered concentrations were chosen based on published studies [17-
44 33]. We used three agents: Genipin (078-03021, Wako Pure Chemical Industries, Ltd., Richmond,
45 VA), Glyceraldehyde (G5001-5G, Sigma-Aldrich Corp., St. Louis, MO), and Methylglyoxal
46 (W296902-100G, Sigma-Aldrich Corp., St. Louis, MO). Several concentrations (Table 1) of each

1 agent were used to establish a dose-response curve of concentration and relative stiffness. All
2 dilutions were made in phosphate-buffered saline (PBS) except for glyceraldehyde, which was
3 made at stock concentration (500 mM) in deionized water to obtain an osmolality similar to
4 extracellular fluid, then diluted further with PBS.

5 6 *Partial Incubation Technique:*

7 Intact eyes were incubated in stiffening agents overnight, such that half the sclera was immersed
8 in the stiffening solution (treated) and the other half (control) was moistened by PBS. Freshly
9 harvested rat eyes were cleaned under a dissecting microscope by carefully removing excess fat,
10 connective tissue, and musculature from the posterior sclera. A 3 ml polypropylene transfer pipette
11 (225, Samco Thermo Scientific, Waltham, MA) was then trimmed to yield a cone approximately
12 the diameter of the eye (approximately 6.5 mm). The eye was then gently placed into the cut pipette
13 with the anterior-posterior axis (identified by the position of the optic nerve) parallel to the cut
14 (Fig. 1A) with the ophthalmic blood vessels aligned with the cut and serving as natural landmarks
15 to aid in identifying the scleral region exposed to stiffening agent. Two small (approximately 1
16 mm diameter) droplets of glue (Loctite Super Glue Ultragel Control, Henkel Corporation,
17 Westlake, OH) were applied to the cornea with a toothpick, attaching the cornea to the pipette, and
18 a third droplet was carefully applied to the face of the distal optic nerve so that no glue touched
19 the sclera. Drops of PBS were applied to keep the eye moist during handling. Stiffening agent
20 (Table 1) was injected slowly into the pipette tip with a hypodermic needle until all air was
21 evacuated.

22
23 Once the pipette was filled with the agent, a small rectangle (4 x 8 mm) of paraffin film was tightly
24 wrapped around the opening at the bottom of the pipette to prevent any stiffening agent from
25 leaking out. A Kimwipe was cut into a 5 x 5 cm cross shape, wetted with PBS, draped over the top
26 of the eye, and then wetted with PBS to maintain moisture in the region not immersed in stiffening
27 solution. The entire assembly (pipette, eye, and Kimwipe) was then placed into a PBS-filled 1.5
28 ml microtube with the dangling strips of the Kimwipe allowing PBS to wick up to keep the control
29 portion of the eye moist. To further maintain physiological conditions overnight, the microtube
30 was placed in a floating rack in a 37°C water bath (Precision Shallow Chamber Water Bath 280,
31 Thermo Scientific, Waltham, MA) and misted from above (Monsoon RS400, EXO TERRA,
32 Mansfield, MA) every 3 minutes with PBS (Fig. 1C). Eyes were carefully removed from the tube
33 the next day (approximately 16 hours incubation time) and mounted for inflation testing.

34 35 **Inflation Testing:**

36 Stiffening agents were evaluated by comparing mechanical strain measurements (stiffened vs.
37 control regions) during whole globe inflation tests. We modulated the intraocular pressure of each
38 eye while submerged in a PBS bath at physiological temperature. Calibrated stereo cameras
39 (including compensation for refraction through PBS) imaged a speckle pattern on the surface of
40 the eye throughout the inflation test, and 3D digital image correlation (DIC) was used to quantify
41 surface strain (Q-400 DIC, Dantec Dynamics, Holtsville, NY).

42 43 *Testing Chamber Construction:*

44 The eye was submerged in a temperature controlled, PBS-filled plastic chamber (Kritter Keeper,
45 Lee's Aquarium & Pets, San Marcos, CA) during experimentation. To model physiological
46 conditions *ex vivo*, the temperature of the PBS in the chamber was maintained at 37°C ± 2°C

1 throughout the experiment by pumping saline through a thermoelectric heater assembly (LA-045-
2 24-02-00-00, Laird Technologies, London, UK; temperature controller TC-XX-PR-59; measured
3 by thermistor TC-NTC-1 immersed next to the eye) using a peristaltic pump (BT300L, Golander
4 LLC, Duluth, GA; pump head DT15-44; tubing #25 [ID 4.8 mm, OD 8 mm]) at 60 ml/min. This
5 low flow rate was selected so as not to produce any turbulence and resultant optical distortion in
6 the PBS around the eye.

7
8 To avoid evaporation of PBS during experimentation, a 1/8" thick borosilicate glass sheet was
9 placed over the chamber and warmed to 70°C to prevent condensation (3682K25, McMaster Carr,
10 Douglasville, GA; PID controller 36815K71). The mounted eye was then illuminated from above
11 with dual gooseneck lighting (Mi-LED-US-DG, Dolan-Jenner Industries, Boxborough, MA).

12
13 An adjustable-height pressure reservoir [36] was connected to the base of the chamber through
14 silicone tubing connected to a bulkhead fitting. This presented a female luer connection on the
15 inside surface of the chamber where we could attach mounted eyes and modulate their IOP using
16 hydrostatic pressure.

17 *Mounting Procedure:*

18 Prior to experimentation, custom-made mounting blocks (Fig. 2) were manufactured from acrylic
19 sheets (8560K369, McMaster Carr, Douglasville, GA). A 1/4" diameter ball end mill created a
20 hemispherical cradle for rat eyes, and a thin channel was drilled through the block with a 1/16"
21 drill bit. This hole was widened opposite the indentation for the eye using a 3/16" drill bit that
22 could accept a luer fitting adaptor.

23
24
25 Following overnight (16 hours) incubation, the orientation of the eye relative to the solution was
26 recorded. The cornea was blotted dry with a Kimwipe, and a small, continuous bead of gel
27 superglue was applied along the inner rim of the mounting block hemisphere. The eye was then
28 placed onto the hemisphere, cornea-side down, with the optic nerve centered upwards, and excess
29 glue was scraped away. The mounting block was marked with a waterproof marker to record the
30 region of the eye that was incubated in stiffening solution.

31
32 In order for DIC to evaluate displacements, a speckle pattern must be applied to the tissue. For this
33 study, the speckle pattern was applied to the posterior sclera with graphite powder (#970 PG,
34 General Pencil Company, Inc., Redwood City, CA). Graphite was poured onto a fine mesh sieve
35 (Tensile bolting cloth #60, Amazon) and an airbrush was used to blow the powder through the
36 sieve onto the external surface of the eye and allowed to dry briefly. This method was repeated
37 until the graphite powder formed a speckle pattern that did not detach from the surface of the eye
38 when submerged in PBS. Eyes were immersed in ice-cold PBS until testing began.

39 *Experimental Procedure:*

40 Prior to testing each day, the PBS chamber was filled and heated to temperature, and the intrinsic
41 stereo calibration parameters of the cameras were determined using a standardized checkerboard
42 calibration target. To inflate the eye, the cornea was punctured by inserting a 1 mm biopsy punch
43 through the 3/16" hole in the mounting block and twisting gently until slight collapse of the eye
44 was observed. Care was taken to ensure the eye not detach from the mounting block, nor the biopsy
45

1 punch deeply penetrate the eye. A threaded male luer fitting (EW-45505-84, Cole-Parmer, Vernon
2 Hills, IL) was then glued into the 3/16" hole.

3
4 The pressure reservoir was set to the height corresponding to the baseline IOP of 3 mmHg
5 (approximately the minimum necessary to prevent the eye globe from buckling under its own
6 weight). PBS was injected through polyethylene tubing into the lumen of the mounting block to
7 purge all air bubbles. The eye was then submerged in the PBS chamber 25 mm below the surface,
8 imparting an external pressure of approximately 2 mmHg to the eye, and attached to the luer fitting
9 at the base of the chamber connected to the pressure reservoir. Extrinsic camera calibration
10 parameters were then determined after the eye was mounted to account for refraction through the
11 borosilicate glass sheet and PBS[37].

12
13 Effective IOP was calculated by subtracting the external pressure on the eyes (2 mmHg from the
14 tissue bath) from the internal hydrostatic pressure from the reservoir. Images were captured every
15 30 seconds at an exposure time of 20 milliseconds for 30 minutes (see DIC system characterization
16 results) at each of 3 pressures: 3 (low/hypotensive IOP), 13 (normal/normotensive IOP), and 28
17 mmHg (high/hypertensive IOP). The pressure reservoir was raised after each set of 60 images to
18 the next height via stepper motor at a speed of 5 mm/second. Eyes were not preconditioned prior
19 to inflation testing.

20 21 *Strain calculation:*

22 Dantec's Istra 4D software (v4.4.1) was used to compute displacement and resulting principal
23 strains from the image dataset using DIC. Correlation settings were: 99 pixel facets, 45-pixel grid
24 spacing, maximum permissible start points accuracy 0.2 pixels, residuum of 30 gray values, and
25 3D residuum of 1.1 pixels. All strain calculations were performed from smoothed displacement
26 data using a 2D bicubic spline function to the data set. The grid reduction factor (minimizes the
27 difference between the data point and the spline function) was set to 3 for displacement and 2 for
28 contours, and the smoothness factor (straightens the filtered data) was set to 0 for both items.

29
30 Strain was computed relative to the reference state (3 mmHg after 30 minutes). Exported strain
31 data for each image was then segmented (Fig. 4) in custom MATLAB software (R2016a,
32 MathWorks, Natick, MA) by manually tracing the experimental and control regions of the
33 posterior sclera (excluding the optic nerve) based upon the markings made on the mounting block
34 prior to testing. Relative stiffness as a percent change between E_{exp} (elastic modulus in the
35 experimental region) and E_{con} (elastic modulus in the control region, see Appendix 1 for derivation)
36 was defined as:

$$37 \text{ Relative Stiffness} = \frac{E_{exp} - E_{con}}{E_{con}} = \frac{\epsilon_{con} - \epsilon_{exp}}{\epsilon_{exp}} \times 100\% \quad (1)$$

38
39
40 where ϵ_{con} represents strain in the control region and ϵ_{exp} represents strain in the stiffened region.
41 The calculation was performed following outlier removal, as described in *Data analysis*.

42 43 *Data analysis:*

44 DIC data is noisy, particularly when dealing with small strains, as tiny errors in displacements
45 become amplified in strain computations. Although smoothing displacements helps minimize this
46 type of error, we required outlier detection to remove spurious data points. Having verified that

1 the data was normally distributed within both the experimental and control regions of each eye at
2 each time point (Anderson-Darling normality test, $p>0.05$), the median absolute deviation (MAD)
3 was calculated according to 1.4826 times the median of the absolute values of the difference
4 between each data point and the median [38]. Any values that were more than 2 MADs away from
5 the median were considered to be outliers and removed from the data set.

6
7 We then computed the mean and standard deviation of the 1st principal Lagrange strain, as this
8 metric is sensitive to deformation in the direction of local stretching were then calculated for a
9 given control or experimental region at each time point. The primary deformation mode of a
10 spherical eye is expected to be a hoop deformation, which would result in in-plane extension of
11 the sclera; thus, the principal Lagrange strains should capture this effect. Following outlier
12 removal, we used a weighted linear fit of this strain metric (weighted by $1/\sigma^2$) using Matlab's
13 `lmfit` function using strains from the final 10 minutes at normotensive and hypertensive IOPs
14 each. If the slope of this fit was above 0.5 millistrain (mStrain) per minute, we assumed the eye
15 was creeping and had not reached its steady state, and thus the eye was discarded from further
16 analysis (2 of 73 total inflation tests were excluded under this criterion). We then recorded the
17 intercept of fits that were not excluded as well as the 95% confidence interval of the intercept of
18 this fit as an indication of the uncertainty of the test.

19
20 Finally, we used Equation 1 to compute the relative stiffness at normotensive and hypertensive
21 IOPs for both the control and experimental halves of the eye. Using a nested 2-factor ANOVA
22 (relative stiffening as a function of pressure nested within concentration; R 3.3.1), we compared
23 the relative stiffness of each ocular region as a function of treatment and inflation pressure.

24 **RESULTS**

25 *DIC system characterization:*

26 We characterized two aspects of our inflation testing system. First, to estimate the baseline
27 correlation noise of the system, we speckled a glass sphere approximately the same radius as a rat
28 eye (3.25mm radius, 8996K25, McMaster-Carr), immersed it in our PBS bath, and imaged it for 8
29 hours. Noise was < 2 mStrain, indicating this level as the minimum resolvable strain magnitude.

30
31 To study the viscoelastic relaxation of pressurized rat eyes, we also imaged an untreated pair of
32 rat eyes at pressure levels corresponding to baseline/hypotensive, normotensive, and hypertensive
33 IOPs (3, 13, and 28 mmHg) for 2 hours per pressure level. We fit a standard Kelvin-Voigt model
34 of viscoelastic relaxation

$$35 \quad \epsilon(t) = A \left(1 - e^{-\frac{1}{\tau}t} \right) + C \quad (2)$$

36
37 to this strain ϵ as a function of time t in Matlab with fitting constants A , C , and τ , and we found
38 that the time constant τ was approximately 1 minute. Out of abundance of caution, specifically to
39 avoid confounding our stiffness findings with biomechanical creep of the scleral shell, we thus
40 maintained our treated eyes for 30 minutes at these same three pressure levels and only analyzed
41 data from the final 10 minutes of each pressure step.

42 *Partial immersion of eyes in collagen crosslinking agents:*

43 Eyes were partially immersed in various stiffening agents overnight such that approximately half
44 the eye was exposed to the collagen cross-linking agent and the other half to PBS as a control.
45 Genipin, which is also used as a blue dye, acted as a visual reporter of its presence, confirming

1 that the agent stayed constrained to the incubation region and did not diffuse or wick into the
2 control region (Figure 1B). We also visually confirmed that agents did not adversely affect the
3 structure of the eye. In preliminary experiments (not shown), we incubated eyes overnight in 500
4 mM glyceraldehyde, as has been done previously [19-21]. However, the eyes were visibly
5 dehydrated the following day. We calculated that the osmolarity of 500 mM glyceraldehyde is
6 approximately 800 mOsm, whereas the osmolarity of aqueous humor and PBS is about 300 mOsm
7 [39]. Thus, we diluted the glyceraldehyde and only used lower concentrations in these
8 experiments.

9 *Average strain magnitudes:*

10 In almost all eyes, the mean 1st and 2nd principal strains (representing stretch in the direction of
11 greatest local deformation and the stretch orthogonal to this direction, both tangent to the surface
12 of the eye) in the control half the eye were on the order of 40-150 mStrain at 13 and 28 mmHg,
13 respectively, relative to the reference configuration at 5 mmHg. These strain values are well above
14 the noise floor of our system. In the stiffened half of the eye, strains were lower, generally 10-50
15 mStrain, again above the noise floor. Strains stabilized within minutes of a change in pressure in
16 all but 2 cases, and the difference in strain between baseline/hypotensive and normotensive
17 pressures was always considerably larger than between the normotensive and hypertensive
18 pressures (Fig 4). 2nd principal strains in the posterior sclera were approximately half the
19 magnitude of 1st principal strains, consistent with current understanding that there is a direction of
20 preferential collagen fiber alignment but that the posterior sclera is quasi-transversely isotropic
21 tangent to the scleral surface [40]. The distribution of strains within each region at any given
22 timepoint followed a normal distribution (Anderson-Darling test; $p > 0.05$).

24 *Relative stiffening:*

25 We observed a significant ($p = 1.03 \times 10^{-9}$) stiffening effect (relative stiffening as a function of
26 pressure nested within concentration; Table 2) pooled over all agents. All three agents
27 demonstrated a dose-dependent stiffening effect where increasing the concentration of the solution
28 increased the relative stiffness of the treated region. However, at very high concentrations (for
29 genipin, above 7.5 mM; for glyceraldehyde, above 62.5 mM), increasing concentration did not
30 increase stiffness. For genipin (Fig. 5A), we observed stiffness increases between 14.7% and
31 1320%. For glyceraldehyde (Fig. 5B), stiffness increased between 21.8% and 273%, and for
32 methylglyoxal (Fig. 5C), stiffness increased between 11.9% and 310% at the concentrations
33 included in these studies. In order to achieve a target increase in stiffness of approximately 100%
34 [13], the appropriate dose for a rat eye overnight is therefore approximately 1 mM for genipin, 30
35 mM for glyceraldehyde, and 7 mM for methylglyoxal.

37 *Data availability*

38 The datasets supporting this article have been uploaded as part of the supplementary material.
39

40 **DISCUSSION**

41 This study offers the first quantification of the efficacy of scleral stiffening agents in the rat eye, a
42 common and important animal model of experimental glaucoma. It also offers the first
43 demonstration of the efficacy of genipin and methylglyoxal in the rodent eye, an important
44 milestone for use in mice, whose eyes have similar collagen composition to rats and are widely
45 used in glaucoma and myopia research. We found that each agent is capable of stiffening the sclera
46

1 by several hundred percent but that there exists an upper bound to this stiffening effect. This
2 quantification sets a range on the magnitude one might be able to achieve using collagen
3 crosslinking approaches to scleral stiffening. Researchers investigating the physiological
4 consequences of scleral stiffening using these agents should not expect to increase stiffness by
5 more than several hundred percent.
6

7 While we cannot determine the mechanism causing genipin to achieve its highest stiffness around
8 7 mM and glycerinaldehyde around 30 mM from the data at hand, we hypothesize that the collagen
9 crosslinking sites have become fully saturated at these higher concentrations. Thus, the presence
10 of additional crosslinker may have no further effect. Although we did observe a drop in relative
11 stiffness at the highest concentrations of genipin and glycerinaldehyde in this study, this resulted
12 from a decrease in strain in the control portion of the eye without change in the treated portion.
13 Thus, this phenomenon should not be interpreted as a drop in efficacy at the highest concentrations.
14 Instead, it is likely that the agents at these very high concentrations diffused through the eye into
15 the internal tissues or even into the control portions, potentially crosslinking them and reducing
16 strain, thereby decreasing the relative stiffness of the eye.

17 Our novel approach to treating approximately half an eye with stiffening agent overnight while
18 using the other half as a control provides a powerful tool for studying the efficacy of small
19 molecule collagen crosslinking agents. Although these agents may diffuse outside the desired
20 region of the eye at very high concentrations, we do not believe this is a problem at the more
21 moderate concentrations examined in this study. Genipin yields a visible blue dye at sites where it
22 is present (Figure 1), and this color change has previously been shown to correlate with scleral
23 stiffness [28], suggesting its relevance as a visual reporter of cross-linking. Glycerinaldehyde and
24 methylglyoxal have approximately half the molecular weight of genipin, and thus they may diffuse
25 slightly faster but are not expected to considerably enter the control half of the eye. Strain maps
26 for these eyes similar to Figure 3 show a relatively sharp line of demarcation between the two
27 halves. To avoid confounding our analysis with any diffusion effects, however minor, we also
28 avoided including regions closest to the line of demarcation when computing average strain. As
29 seen in Figure 3, the perimeters of the regions of interest do not overlap perfectly where they come
30 closest to intersecting (there are no points included that are underneath the visible perimeters) in
31 order to exclude strain measurements in the transition zone. Additionally, it is important to
32 remember that the relatively higher strains in the stiffened region close to its boundary with the
33 control region result from cross-linker not fully diffusing into this region, so we err on the side of
34 under-diffusion, not cross-linker bleed-over. We also used outlier removal to eliminate any data
35 points that deviated considerably from the median, such as those resulting from edge effects, and
36 by computing the mean relative stiffness from several hundred data points per region after outlier
37 removal, the effect of any small bleed-over should be small.
38

39 Our method of using half of each eye as experimental and control groups to compute relative
40 stiffening is especially powerful when we consider the inter-eye variability in strains in naïve
41 regions of eyes (and confirmed in fully untreated eyes, data not shown). Even in two eyes from a
42 single rat, strains in PBS-treated regions can vary by a factor of approximately three (see dispersion
43 of data points along X-axis in Figure 5). Thus, by using the two halves of each eye as an internal
44 comparison, we can minimize the effects of inter-eye variability. While it is certainly true that
45 strains are somewhat heterogeneous even within regions of a single eye (as in Figure 3) our

1 technique for computing relative strain allows us to only introduce intra-eye variability without
2 adding the effect of inter-eye variability to each relative strain calculation.

3
4 An additional benefit of using half the eye as an internal control and making a relative comparison
5 is that the need for preconditioning is greatly reduced. Prior work such as that of Wong et al. [31]
6 used up to 10 cycles of preconditioning before the eye converged to a stable relation between
7 inflation and strain. With our testing methodology, we are comparing the relative stiffness of two
8 halves of a single eye such that preconditioning effects, or lack thereof, should be approximately
9 uniform between the two halves. Thus, the strain magnitudes quantified in this study may not
10 exactly equal ocular strains in the rat eye at various magnitudes of IOP, but the relative stiffening
11 effect should still be relevant to future *in vivo* studies in the rat.

12
13 This study focused exclusively on eyes *ex vivo*, although we took care to freshly harvest eyes and
14 maintain them at physiological temperatures during testing. We treated eyes overnight in order to
15 simulate the stiffening effect that might result if such agents were delivered to the posterior eye
16 within the Tenon's capsule. However, because this is a relatively un-explored frontier of
17 ophthalmology, it is unclear what the body's clearance of such agents would be *in vivo*. Recently,
18 Kimball et al. investigated the efficacy of glutaraldehyde scleral stiffening *in vivo* in a mouse
19 model of glaucoma and found that its use was detrimental to visual function [19]. In an attempt to
20 recreate the conditions of their study, we first attempted to study eyes incubated in 500 mM
21 glyceraldehyde, identical to the Kimball et al. paper. However, eyes became significantly
22 dehydrated and collapsed with this treatment, presumably from a significant osmolarity mismatch.
23 Such an effect would clearly be problematic *in vivo* and could explain the negative findings of the
24 Kimball study, but active transport of fluids in a living mouse also might be able to compensate
25 for any osmolar mismatch. Further investigation is certainly warranted.

26
27 Eyes were freshly harvested from rats daily and randomized to a treatment agent and concentration.
28 However, in an effort to ensure that our stiffening solutions were freshly-mixed from a stock
29 solution, all eyes studied in a single day (usually 2-3 pairs) were incubated in a single agent,
30 although often at different concentrations of that agent. Thus, one limitation of the present work is
31 that some treatments were from both eyes of a single rat. Although Brown Norway rats are an
32 inbred strain and thus should have low genetic variability, as previously mentioned, in initial
33 testing prior to this study using naïve eyes (not shown), variability in average strain between two
34 eyes from a single rat were high enough that, for this study, we assumed that each eye was an
35 independent sample regardless of which rat it came from.

36
37 Although the eyes in this study were all studied within 24 hours of harvest, another unknown factor
38 for scleral stiffening therapies is the temporal efficacy of such agents. Previously, Wollensak et al.
39 showed that glyceraldehyde increases scleral stiffness for at least 8 months in rabbits [22], a
40 promising finding. However, further work is necessary to characterize the temporal profile of the
41 stiffening agents in this study, both in terms of how long the eye must be incubated in order to
42 derive a stiffening effect as well as in terms of how long the eye maintains its increased stiffness
43 before collagen turnover and remodeling negates the effects of treatment.

44
45 Eyes were incubated in agents for approximately 16 hours each, with an unavoidable variability
46 of several hours as result of practical aspects of the eye mounting procedure, tissue cleaning, etc.

1 Per Fick's law, the rate of diffusion into the tissue should drop as the concentration of cross-linker
2 equalizes between the solution and the tissue. Additionally, we can approximately estimate an
3 upper bound on the effects of different incubation times using the fact that the diffusion distance
4 is proportional to the square root of elapsed time, so a deviation of two hours less than our
5 approximated 16 hours would lead to a variation in the extent of cross-linking of roughly 8-9%
6 ($\sqrt{14/16}$), considerably smaller than the stiffening effects of 100% or more observed in this study.

7 Further study is certainly warranted to better characterize the dynamics of cross-linking treatments
8 to ocular tissues, but in the present study, we attempted to characterize the role of collagen cross-
9 linking at various starting concentrations with the understood limitation that some modest
10 variability in stiffening may result from variations in tissue preparation.

11
12 Having characterized these three collagen crosslinking agents *ex vivo*, our next step will be to
13 deliver them to rats *in vivo* to answer the questions raised by this and other studies. Future work
14 will need to characterize how well-tolerated these agents are by delicate neural tissues of the retina
15 and optic nerve head, as well as by the scleral fibroblasts providing collagen turnover in the eye.
16 If the stiffening agents have any sort of toxic effect to these components of the eye, it may be
17 necessary to take care to use highly targeted delivery of such agents using novel drug delivery
18 techniques. Such approaches might involve delivering agents with an activatable reservoir of
19 cross-linker or by flushing away agents from undesired locations, but such techniques will need to
20 be evaluated after determining whether scleral stiffening offers any benefit for glaucoma or myopia
21 as well as which agents are the safest for *in vivo* use. It will also be important to quantify whether
22 the same magnitude of relative stiffening for the concentrations of the agents measured here exists
23 *in vivo* and how long the stiffening is maintained. Most importantly, future studies should build
24 upon this foundation in order to evaluate the efficacy of various scleral stiffening approaches for
25 ocular diseases such as glaucoma and myopia in order to improve our clinical ability to preserve
26 vision.

27 28 **CONCLUSION**

29 Here, we have reported the first direct comparison of the dose-response relationship of three
30 stiffening agents in sclera. All three collagen cross-linking agents examined in this study, genipin,
31 glyceraldehyde, and methylglyoxal, exhibited dose-dependent stiffening behavior, with maximum
32 relative stiffening of several hundred percent at higher concentrations. Thus, all 3 agents can be
33 titrated to achieve a desired magnitude of stiffening. Future studies will examine the efficacy of
34 these agents *in vivo* to ensure the stiffening effect is maintained in longitudinal studies and, more
35 importantly, to assess whether scleral stiffening agents protect against vision loss in diseases like
36 glaucoma and myopia.

37 38 **ACKNOWLEDGEMENTS**

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42 Jonathan Suever (Geisinger Health Systems) for providing Matlab segmentation code.

43 44 **COMPETING INTERESTS**

45 The authors have no competing interests.

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AUTHOR CONTRIBUTIONS

IC, BH, ATR, and SS collected data, performed data analysis, drafted the manuscript, and designed the study. JS performed data analysis, drafted the manuscript, and performed statistical analysis. CRE designed the study, coordinated the study, and drafted the manuscript. All authors gave final approval for publication.

TABLES

Stiffening Agent	Concentration (mM)	Concentration (% w/v in PBS)
Genipin	0.25, 0.50, 1.0, 7.5, 15, 30	0.06, 0.11, 0.23, 1.7, 3.4, 6.8
Glyceraldehyde	10.0, 30.0, 62.5, 125	0.90, 2.7, 5.7, 11
Methylglyoxal	3.5, 7.0, 14	0.25, 0.50, 1.0

Table 1: Concentrations of all stiffening agents tested

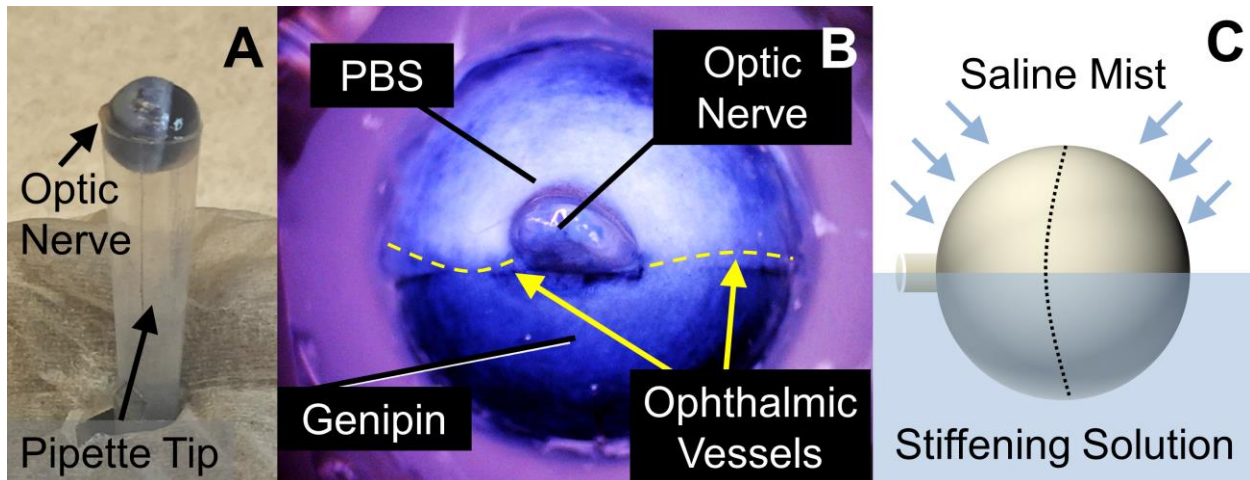
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Agent	Concentration (mM)	13 mmHg	28 mmHg	Number of Eyes (n)
Genipin	0.25	15 ± 14	19 ± 17	6
	0.50	64 ± 23	54 ± 23	4
	1.0	108 ± 28	86 ± 16	4
	7.5	1,321 ± 703	577 ± 220	5
	15	503 ± 252	253 ± 130	4
	30	576 ± 164	348 ± 137	5
Glyceraldehyde	10.0	22 ± 26	20 ± 20	4
	30.0	73 ± 64	55 ± 40	6
	62.5	273 ± 143	165 ± 75	8
	125	192 ± 214	131 ± 120	7
Methylglyoxal	3.5	12 ± 20	11 ± 19	4
	7.0	108 ± 52	81 ± 38	5
	14	310 ± 222	160 ± 119	5

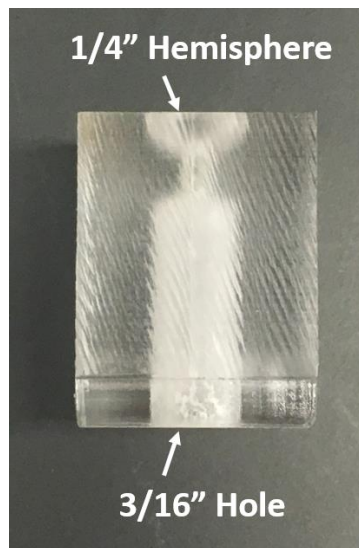
Table 2: Relative stiffening expressed as a percentage. Values are mean ± standard deviation.

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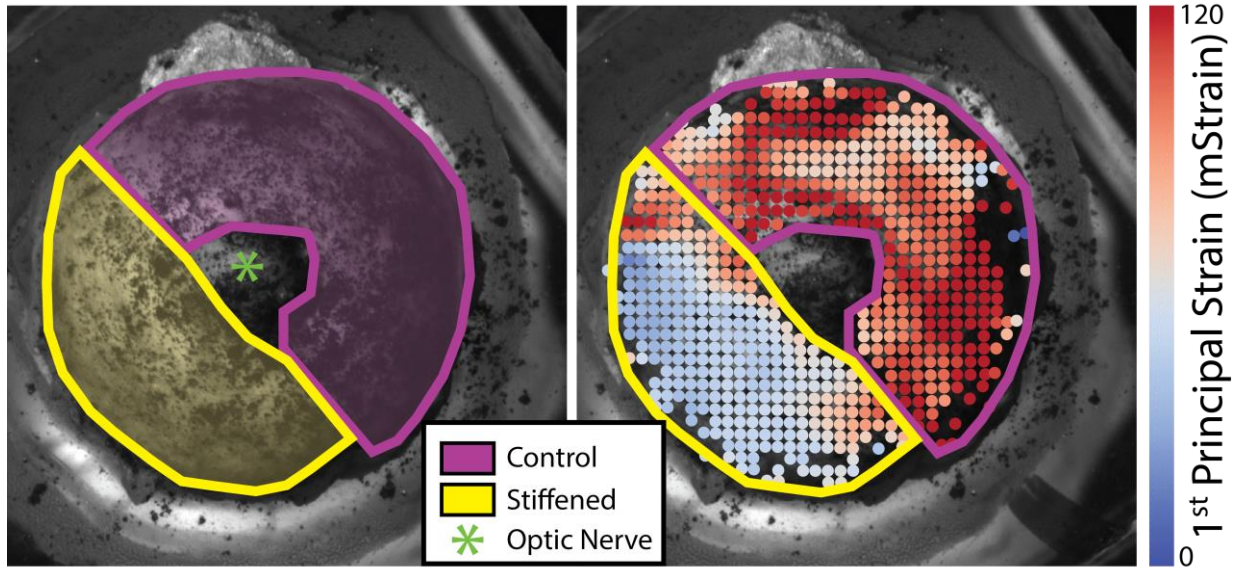
FIGURE CAPTIONS



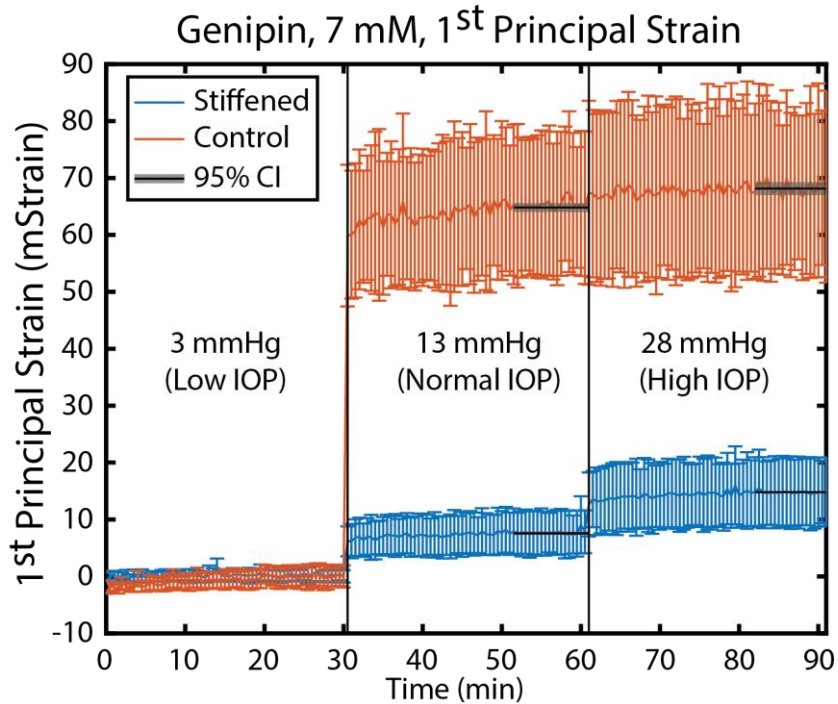
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Figure 1: Eyes were partially immersed in cross-linking agents, exposing approximately half the eye to a stiffening agent overnight by mounting it in a trimmed pipette tip (A). Genipin, which is also used as a blue dye, provides a visual indicator of its location (B). This is closely localized to the treated region and demonstrates little evidence of wicking. Regions appearing blueish near the top of panel (B) are actually thin regions of translucent sclera where choroid is visible, not regions exposed to genipin. Eyes were then incubated overnight while misting the tissue-draped control half with PBS to keep it moist (C). Dashed line indicates limbus.



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Figure 2: Side view of acrylic mounting block. Eyes are placed in the hemisphere at top, and a threaded luer fitting mates with the hole in the bottom.



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Figure 3: Digital image correlation was used to spatially resolve the surface strains in individual eyes. At left, the speckle pattern on the posterior sclera is overlaid with manually-traced masks (made prior to calculating strain) denoting the locations treated with cross-linking agent or PBS as a control, taking care not to include the optic nerve. At right, we have overlaid these same masks on the computed surface strains at an inflation pressure of 13 mmHg (normotensive). Regions of comparatively low and high strain match closely with the treatment and control zones.



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Figure 4: Representative plot of average 1st principal strain as a function of time from a single eye during our inflation experiment. Eyes were maintained for 30 minutes at each of 3 pressures representing different ranges of IOP. Strains were considerably higher in the control region of eye than in the treated region, indicating that the treated region is stiffer. Black overlays represent the 95% confidence interval about the mean during the final 10 minutes of each pressure step, when the eye reached steady state. Error bars: standard deviation over the interrogated region. Raw data to generate these figures is included in Supplemental Data.

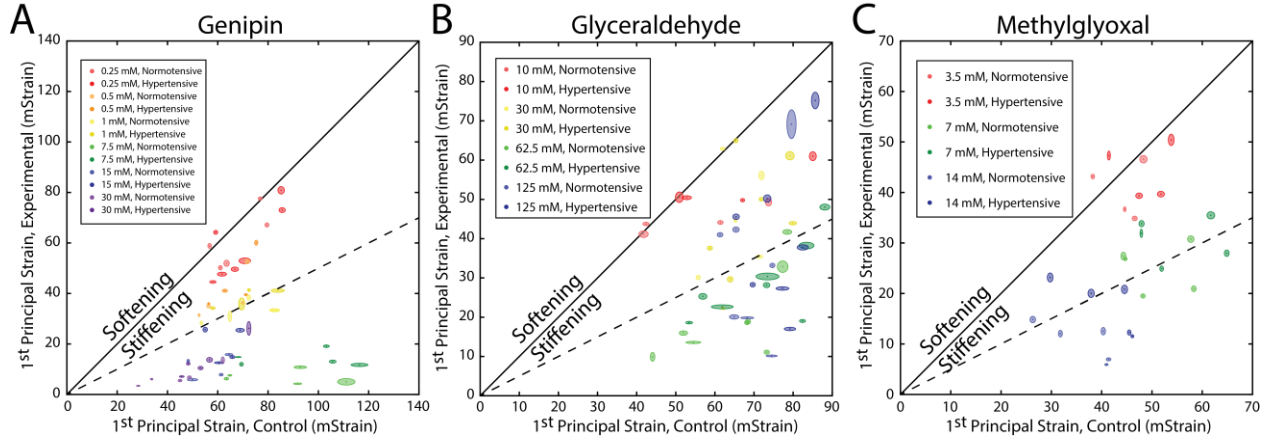


Figure 5: Average 1st principal strains for control (horizontal axis) and stiffened (vertical axis) regions of eyes treated with (A) genipin, (B) glyceraldehyde, or (C) methylglyoxal. Each dot represents the mean steady-state strain for one eye, and the surrounding oval represents the 95% confidence interval of that point from linear fitting. Points falling below the unity line (black line) indicate that the treated eye has been stiffened relative to the control, and mutatis mutandis. The dotted line represents 100% stiffening. Eyes in red lie very close to the unity line, suggesting that this low dose has minimal stiffening effect. Higher concentrations lie farther from the unity line until reaching a maximum effective dose around 7 mM for genipin, 62.5 mM for glyceraldehyde, and 14 mM for methylglyoxal. Higher concentrations do not further stiffen the experimental half the eye but do reduce strain in the control portion of the eye, possibly as result of diffusion into the internal tissues of the eye and crosslinking them.

APPENDIX 1:

Relative Stiffness in Terms of Strain Derivation:

Relative stiffness is defined as the stiffness of the experimental material relative to the control:

$$\text{Relative Stiffness} = \frac{E_{exp} - E_{con}}{E_{con}} \quad (3)$$

where E_{exp} is the experimental effective Modulus and E_{con} is the control effective modulus.

By modeling the eye as a thin-walled pressure vessel and assuming a constant radius and thickness throughout the eye, we can compute the applied stress at each pressure step:

$$\sigma = \frac{PR}{2t} \quad (4)$$

where σ is the hoop stress in a sphere, P is the internal pressure, R is the radius, and t is the thickness of the sphere (see Supplemental Figure).

Since the internal pressure applied is the same for the entire eye the stresses in the experimental and control portions of the eye are the same:

$$\sigma = \sigma_{con} = \sigma_{exp} \quad (5)$$

where σ_{con} and σ_{exp} are the hoop stresses in the control and experimental portions of the eye, respectively.

We approximate the tissue behavior as incrementally linear elastic within this loading regime to write:

$$\frac{\sigma_{con}}{\varepsilon_{con}} = E_{con}, \quad \frac{\sigma_{exp}}{\varepsilon_{exp}} = E_{exp} \quad (6)$$

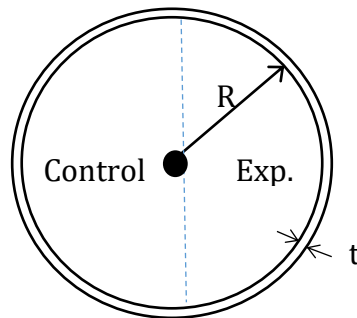
where ε_{con} and ε_{exp} are the control and experimental first principal strains, respectively.

Using equation (6) we can write:

$$\frac{E_{exp} - E_{con}}{E_{con}} = \frac{\frac{\sigma_{exp}}{\varepsilon_{exp}} - \frac{\sigma_{con}}{\varepsilon_{con}}}{\frac{\sigma_{con}}{\varepsilon_{con}}} = \frac{\sigma \left(\frac{1}{\varepsilon_{exp}} - \frac{1}{\varepsilon_{con}} \right)}{\sigma \left(\frac{1}{\varepsilon_{con}} \right)} \quad (7)$$

Simplifying by multiplying by $\varepsilon_{exp}\varepsilon_{con}$, we obtain the relative stiffness equation in terms of strains at a given pressure step:

$$\text{Relative Stiffness} = \frac{E_{exp} - E_{con}}{E_{con}} = \frac{\varepsilon_{con} - \varepsilon_{exp}}{\varepsilon_{exp}} \quad (8)$$



Supplemental Figure: Schematic diagram of regions used in calculation of relative stiffening

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