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Assessment of Visual and Retinal Function Following In Vivo Genipin-Induced Scleral Crosslinking

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Purpose: Genipin has been proposed as a possible neuroprotective therapy in myopia and glaucoma. Here, we aim to determine the effects of prolonged genipin-induced scleral stiffening on visual function.

Methods: Eyes from Brown Norway rats were treated in vivo with either a single 15 mM genipin retrobulbar injection or sham retrobulbar injection and were compared to naïve eyes. Intraocular pressure, optomotor response, and electroretinograms were repeatedly measured over 4 weeks following retrobulbar injections to determine visual and retinal function. At 4 weeks, we quantified retinal ganglion cell axon counts. Finally, molecular changes in gene and protein expression were analyzed via real-time polymerase chain reaction (RT-PCR) and proteomics.

Results: Retrobulbar injection of genipin did not affect intraocular pressure (IOP) or retinal function, nor have a sustained impact on visual function. Although genipin-treated eyes had a small decrease in retinal ganglion cell axon counts compared to contralateral sham-treated eyes ($-8{,}558 \pm 18{,}646$; mean $\pm$ SD), this was not statistically significant ($P = 0.206$, $n = 9$). Last, we did not observe any changes in gene or protein expression due to genipin treatment.

Conclusions: Posterior scleral stiffening with a single retrobulbar injection of 15 mM genipin causes no sustained deficits in visual or retinal function or at the molecular level in the retina and sclera. Retinal ganglion cell axon morphology appeared normal.

Translational Significance: These results support future in vivo studies to determine the efficacy of genipin-induced posterior scleral stiffening to help treat ocular diseases, like myopia and glaucoma.

Introduction

Scleral collagen crosslinking has been proposed as a therapeutic treatment for myopia and glaucoma. In myopia, refractive error is largely caused by scleral remodeling, which leads to axial elongation,[1,2] and crosslinking the posterior sclera has been hypothesized as a treatment to slow or reverse this process.[3,4] In glaucoma, intraocular pressure (IOP) causes excessive biomechanical strains on the optic nerve head, which is the main and early site of retinal ganglion cell (RGC) damage. It is hypothesized that reducing such excessive strains by crosslinking the posterior sclera surrounding the optic nerve may protect against RGC loss in glaucoma.[5,6]

Ocular collagen crosslinking has recently been clinically approved for the treatment of keratoconus, where the cornea becomes weak and misshapen.[7] This treatment uses a collagen crosslinker, riboflavin, that is photoactivated by ultraviolet-A (UVA) light to strengthen the cornea. Unfortunately, scleral

crosslinking using riboflavin and UVA light is known to be toxic to the retina.[8,9] Thus, alternative collagen crosslinking approaches have been evaluated to stiffen the posterior sclera for myopia and glaucoma treatments.

Genipin, a naturally occurring, non-photoactivated, collagen crosslinking agent extracted from the Gardenia fruit, has been previously investigated as a collagen crosslinker for ocular use.[3,4,10–13] Genipin acts as an anti-inflammatory and anti-angiogenic agent[14] and can induce stable crosslinks in biological tissue.[15] Genipin-induced crosslinking increases tissue stiffness by similar magnitudes as do glutaraldehyde[16] and riboflavin,[17,18] yet is significantly less cytotoxic than are glutaraldehyde[19,20] and riboflavin.[17,18]

Of several studies that have evaluated genipin-induced scleral crosslinking, only two have evaluated both the efficacy and safety of genipin-induced scleral crosslinking in vivo.[4,12] These studies examined the biomechanical properties of genipin-stiffened scleral strips, gross ocular anatomy via slit-lamp examinations after treatment, and histological and immunohistological sections of ocular tissues in rabbits[4] and guinea pigs.[12] These studies found that genipin successfully stiffened the sclera and did not cause any gross changes in ocular structures. However, to date, no study has evaluated whether genipin-induced scleral collagen crosslinking affects visual and retinal function in vivo.

We have previously shown the efficacy of genipin (15 mM) to induce sustained scleral stiffening for up to 4 weeks after retrobulbar injection in Brown Norway rat eyes.[21] However, it is important to ensure that long-term scleral stiffening is safe and has negligible adverse effects on visual function. Here, we aim to evaluate the potential use of genipin-induced collagen crosslinking in the sclera on visual and retinal function over 4 weeks.

Methods

Animals

This study used 37 retired breeder (7–13 months old) Brown Norway rats (Charles River Laboratories, Inc., Wilmington, MA). Most rats used in this study ($n = 34$ rats) were male, whereas a small group of female rats ($n = 3$) were used in a preliminary proteomics study. All rats were housed on a 12-hour light (fluorescent 25–200 lux)/12-hour dark cycle and were provided with food and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee at the Georgia Institute of Technology, Duke University, and the Atlanta VA Healthcare System. All procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The number of rats used for each outcome parameter are listed in the Table.

Experimental Groups and Crosslinking Procedure

Rats were randomly assigned to one of three groups for this study, as displayed in Figure 1. The first group of rats (naïve/naïve, $n = 7$ animals) were from a previous study in our laboratory in which both eyes were left completely naïve. Optic nerves were removed from these eyes and used for RGC axon count comparisons.[22] Rats in the second group (Hank's Balanced Salt Solution [HBSS]/naïve, $n = 10$) received a single (unilateral) retrobulbar injection of HBSS (150 µl) unilaterally, whereas the contralateral eye was left as a naïve control. Rats in the third group (genipin/HBSS, $n = 20$) received a single retrobulbar injection of genipin (Wako Pure Chemical Industries, Ltd., Richmond, VA) mixed in HBSS (15 mM and 150 µl) unilaterally, whereas the contralateral eye received a single retrobulbar injection of HBSS (150 µl). For all retrobulbar injections, rats in the remaining two groups were anesthetized with a cocktail of ketamine (60 mg/kg) and xylazine (7.5 mg/kg) and a drop of topical tetracaine (0.5%; Alcon, Geneva, Switzerland) was applied as a local anesthetic to both eyes. All retrobulbar injections were performed using a sterile 31G insulin syringe needle (BD 300 µl Insulin Syringe Ultra-Fine needle; Becton, Dickinson and Company, Franklin Lakes, NJ) inserted into the inferior quadrant. A genipin concentration of 15 mM was previously used in our in vivo stiffening study,[21] and successfully stiffened the posterior rat sclera for 4 weeks. Rats received a topical antibiotic (Certi-sporyn, Kansas City, MO) to avoid infection and antisedan (1 mg/kg) to reverse anesthesia.[23] All rats were euthanized (via CO_2 overdose) either 1 day, 1 week, or 4 weeks postinjection, depending on the experiment.

Tonometry and Eye Examinations

IOP measurements were taken between 9:00 AM and 11:00 AM using a Tonolab rebound tonometer (Icare Finland Oy, Vantaa, Finland) in HBSS/naïve ($n = 5$) and genipin/HBSS ($n = 6$) rats. The tonometer was previously calibrated on a cannulated eye, in which we externally set IOP ranging from 5 to 50 mm Hg using an external reservoir (data not shown). Awake rats were gently restrained by hand while eight tonometer readings were recorded on each eye. We removed the lowest and highest IOP values and averaged the remain-

Table. Number of Rats in Each Group for Each Outcome Measure

Outcome Measure	Time Points Measured	Naïve/Naïve ($n = 7$)	HBSS/Naïve			Genipin/HBSS ($n = 20$)				
IOP (Tonometry)	Days 1, 3, 7, 10, 14, 21, and 28		5*			6*				
Visual function (OMR - spatial frequency)	Days 0, 1, 7, 14, 21, and 28		5*			6*				
Visual function (OMR - contrast sensitivity)	Days 0, 14, and 28		5*			6*				
Retinal function (ERG)	Days 0, 7, 14, and 28	7	5*			6*				
RGC Axon morphology (Axon counts)	Day 28			2		4*	5			
Scleral and retinal mRNA expression (RT-PCR)	Day 7				3			3		
	Day 28								3	
Scleral expression of ECM proteins (Proteomics)	Day 28									3
Total rats of cohort		7	5*	2	3	6*	5	3	3	3

Columns indicate cohorts of rats within each group. Asterisks indicate rats that were used in multiple outcome measures.

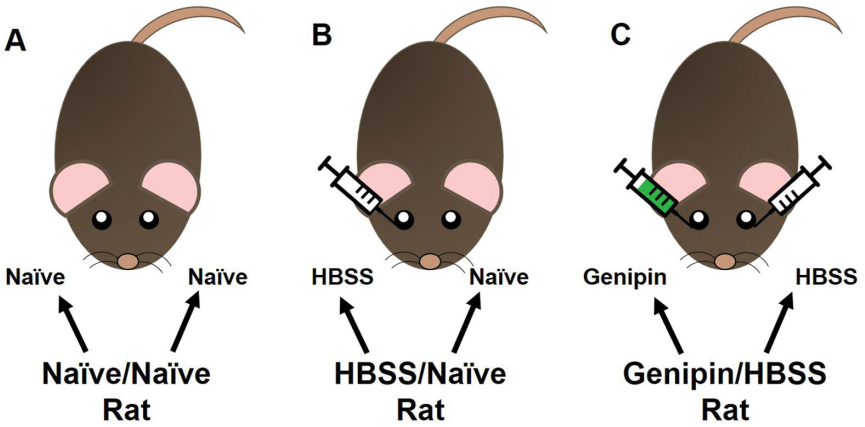

Figure 1. **Schematic of three groups of rats used in this study:** Naïve/naïve rats (**A**) were completely naïve control rats. HBSS/naïve rats (**B**) received a single (unilateral) retrobulbar injection of HBSS, and genipin/HBSS rats (**C**) received a unilateral retrobulbar injection of genipin and a contralateral retrobulbar injection of HBSS.

ing six measurements to represent IOP from that eye. IOP measurements were recorded at 0 (baseline, just before injection), 3, 7, 10, 14, 21, and 28 days relative to the time of retrobulbar injection. At each of these time points, rat eyes were also grossly observed for any abnormalities arising from the retrobular injections.

Optomotor Response

The optomotor response (OMR) was used to assess visual function (OptoMotry; Cerebral-Mechanics, Lethbridge, AB, Canada[24]) at 0 (baseline), 1, 7, 14, and 28 days after retrobulbar injection. We evaluated the OMR in HBSS/naïve ($n = 5$) and genipin/HBSS ($n = 6$) rats. In brief, awake rats were placed on a platform in the center of a chamber consisting of four flat screen computer monitors. Each monitor displayed vertical black and white gratings, which produced a virtual drum rotating at a speed of 12 degrees/second (deg/s). A video camera above the platform was used by a trained observer to visualize the rat's reflexive head movements during the experiment. Gratings rotated in a clockwise or counter clockwise direction to separately stimulate the responses of the left and

right eyes, respectively.[24] To determine a rat's spatial frequency threshold, the vertical bands were displayed at 100% contrast starting at 0.042 cycles/degree and the spatial frequency of the bands was adjusted until the rat no longer demonstrated an OMR, as determined by a lack of reflexive head movement. Contrast sensitivity was measured at baseline, week 2, and week 4. To determine contrast sensitivity, spatial frequency was set to 0.064 cycles/degree, whereas the contrast was adjusted from 100% following a staircase paradigm until the animal no longer displayed a reflexive response. Contrast sensitivity is reported as the reciprocal of the Michelson contrast from the screen's luminance, as previously described.[25]

Electroretinogram

To assess inner and outer retinal function we performed electroretinograms (ERGs). ERG measurements on HBSS/naïve ($n = 5$) and genipin/HBSS ($n = 6$) rats were taken at baseline, and 1, 2, and 4 weeks postinjection. For each time point, rats were dark-adapted for 30 minutes and anesthetized using a cocktail of ketamine (60 mg/kg) and xylazine (7.5 mg/kg), after which drops of tetracaine (0.5%) and tropicamide (1%) were applied topically to anesthetize corneas and dilate pupils, respectively. Reference needle electrodes were carefully inserted subcutaneously in each cheek and a ground electrode was placed in the tail. Custom gold-loop corneal electrodes were placed on the cornea of each eye under a layer of carboxymethylcellulose (Celluvisc; Allergan, Dublin, Ireland) to ensure electrical conductivity and prevent the eye from drying. Electrical responses to various full-field flash stimuli in a Ganzfield dome were recorded and differentially amplified (1–1500 Hz) using a signal-averaging ERG system (UTAS BigShot; LKC Technologies, Gaithersburg, MD) that was calibrated according to manufacturer's specifications. Under dark-adapted conditions, a five-step series of increasing flash intensities (−3.0 to 2.1 log cd s/m^2) was used to selectively isolate rod and mixed rod/cone dominated photoreceptor responses. Interstimulus intervals increased from 2 to 70 seconds at each stimulus level to provide full recovery of the retina before the next flash. Three to 10 flashes were averaged to generate a waveform for each scotopic flash. Rats were then light-adapted for 10 minutes (30 cd/m^2) before being presented with a three-step series of increasing light stimuli (0.4 to 1.4 log cd s/m^2) followed by a flickering light stimulus (1.9 log cd s/m^2 at 6 Hz) to isolate cone photoreceptor responses. Each photopic waveform was averaged from 25 flashes. Oscillatory potentials (OPs) were filtered with a 65 to 275 Hz bandpass fifth order Butterworth filter and then measured on the leading edge of the b-wave starting with the first trough. Amplitudes and implicit times were measured as follows: baseline to trough (a-waves and photopic negative responses [PhNR]) and trough to peak (OPs, b-waves, and flicker response).

RGC Axon Counting

RGC axon numbers were quantified in genipin/HBSS ($n = 9$) and naïve/naïve ($n = 7$) rats at 4 weeks postinjection. Immediately after euthanasia, optic nerves were dissected from the enucleated eye and fixed in isotonic phosphate-buffered saline (PBS; Sorensen's buffer) containing 2.5% glutaraldehyde and 2% paraformaldehyde (PFA; EMS, Hatfield, PA). The tissue was then post-fixed in 1% osmium tetroxide, dehydrated through an ethanol series, embedded in Araldite 502/Embed 812 resin (EMS), and cured in a 60°C oven for 24 hours. Semi-thin cross sections (0.5 µm thick) were then cut approximately 1.0 mm posterior to the sclera, using a Leica EM UC7 ultramicrotome (Leica Microsystems, Buffalo Grove, IL) with a diamond or glass knife. Cross-sections were then dried and stained with 1% toluidine blue on a 70°C hotplate for 15 seconds, and imaged with a Leica DM6 microscope (Leica Microsystems) at an objective power of × 100. Tiled images were collected and merged together to produce a montage of the entire optic nerve cross-section. From this image, normal-appearing axons were then automatically counted using the AxoNet fully convolutional neural-network software, as described previously.[26]

Real-Time Polymerase Chain Reaction

A preliminary study was performed to assess genipin treatment's effect on abundance of extracellular matrix (ECM) proteases in the sclera and inflammatory markers in the retina. Real-time polymerase chain reaction (RT-PCR) was performed on HBSS/naïve ($n = 6$) and genipin/HBSS ($n = 6$) rats at 1 week and 4 weeks ($n = 3$/group) postinjection. Whole globes were enucleated immediately after euthanasia and stored in RNA later (Thermo Fisher Scientific, Waltham, MA) until processing. Sclerae were cleaned of fat, tissue, and muscle, cut along the limbus, and opened to create four quadrants. Retinas were separated and put in a biomasher tube (Kimble, Tokyo, Japan) with trizol (Thermo Fisher Scientific). The retinal pigment epithelium (RPE) was gently peeled and then scraped off from the sclera with a blade and the sclera was washed in PBS, cut into small pieces and then put into a

biomasher tube with trizol. Retina and sclera tissues were then homogenized, mixed with chloroform (200 µl per mL of trizol) and centrifuged at 4°C for 15 minutes at 12,000 g. The upper phase containing RNA was collected, cleaned, and concentrated using RNeasy MiniElute Cleanup Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions.

Once RNA was acquired, strand cDNA was synthesized from total RNA (700 ng) by reverse transcription using oligodT and Superscript III reverse transcriptase (Thermo Fisher Scientific) according to manufacturer's instructions. Quantitative-polymerase chain reaction (q-PCR) reactions were performed in 20 µl mixture containing 1 µl of the cDNA preparation, 1X iQ SYBR Green Supermix (Biorad, Hercules, CA) and 10 µM primers, using the following PCR parameters: 95°C for 5 minutes followed by 40 cycles of 95°C for 15 seconds, 55°C for 15 seconds, and 72°C for 15 seconds. β-Actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal standards of mRNA expression. The absence of nonspecific products was confirmed by the analysis of the melt curves. The primers used for q-PCR amplification are shown in Supplementary Table S1. During this processing, one globe from a 4 week old HBSS/naïve rat was damaged and could not be used for further analysis.

Proteomics

Sample Preparation

Proteomics analysis was performed on sclerae from genipin/HBSS ($n = 3$) rats at 4 weeks postinjection to determine the effect of genipin treatment on the abundance of various proteins involved in various functions, including protein binding, cell motility, and ECM structural support. Eyes were enucleated immediately after euthanasia and kept in cold PBS until processing. The sclera was first cleaned of conjunctiva, retina, muscle, and fat, and the RPE was gently peeled and scraped off from the sclera with a blade. The sclera was then washed in PBS, cut into smaller pieces, transferred to an Eppendorf tube, and frozen in liquid nitrogen. Frozen scleral pieces were ground using a CryoGrinder kit (OPS Diagnostics, Lebanon, NJ) and then suspended in 300 µl of lysis buffer (10 mM HEPES, 42 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM DTT; all from Sigma-Aldrich, St. Louis, MO), 1 × protease inhibitor from Thermo Fisher Scientific) and homogenized in a biomasher tube. Samples were sonicated on ice 3 times for 10 seconds and sodium dodecyl sulfate (SDS) was added for a final concentration of 2% (w/v). Samples were incubated at room temperature for 10 minutes to lyse the cells and extract the proteins, then spun for 45 minutes at 14,000 × g, at room temperature. SDS-soluble proteins were kept on ice, whereas SDS-insoluble proteins were processed further by adding 10 volumes of urea buffer (8M urea; 4% SDS, and 60 mM Tris-HCl; Sigma-Aldrich; 12.5 EDTA in deionized water) to samples, incubating 30 minutes at room temperature, and then centrifuging at 16,000 g for 5 minutes. Supernatant was precipitated using methanol/chloroform, resuspended on 2% SDS, and combined with SDS-soluble protein. Samples were reduced with dithiothreitol (DTT; 10 mM) at 60°C for 20 minutes, alkylated with iodoacetamide (IAA, 25 mM; Sigma-Aldrich) for 30 minutes at room temperature in the dark. The IAA was then quenched with DTT. Samples were precipitated using methanol/chloroform, and resuspended in digestion buffer (8M urea and 0.1M Tris pH 8.0) containing trypsin-Lys-C mix (Promega) and incubated overnight at 37°C. The following morning, 50 mM ammonium bicarbonate (Sigma-Aldrich) was added containing 1:40 trypsin/Lys-C and samples were incubated for 3 more hours with 10% trifluoroacetic acid (Sigma-Aldrich). Peptides were cleaned up using C18 tips (Nest Group, Southborough, MA), following manufacturer's instructions and dried by Speed Vac.

Mass Spectrometry Analysis and Protein Quantification

Tryptic peptides eluted from the beads were dried under vacuum and dissolved in 2% acetonitrile and 0.25% formic acid. Peptides (typically 0.5–1 µg) were analyzed using a NanoAcquity UPLC system coupled to a Synapt G2 HDMS mass spectrometer (Waters Inc., Milford, MA) using a liquid chromatography tandem mass spectrometry (LC-MS/MS) experiment in a data-independent acquisition mode complemented with ion mobility separation (high definition MSE [HDMSE]). Samples were analyzed in duplicate on a 1.7 mm 75 mm x 150 mm C18 130 A BEH column (Waters Inc.) using a 90 minute, 5% to 30% gradient of acetonitrile in 0.1% formic acid at a flow rate of 0.3 mL/min at 35°C. Eluting peptides were sprayed into the ion source of the Synapt G2 using the 10 µm PicoTip emitter (Waters Inc.) at a voltage of 2.5 kV.

Duplicate data-independent analyses (HDMSE) for each sample were conducted with similar liquid chromatography (LC) settings for simultaneous peptide identification and quantification. For robust peak detection and alignment of individual peptides across all HDMSE runs, we performed automatic alignment of ion chromatography peaks representing the same mass/retention time features using Progenesis QI software. To perform peptide assignment to the features, PLGS version 2.5.1 (Waters Inc.) was used

to generate searchable files that were submitted to the IdentityE search engine incorporated into Progenesis QI for Proteomics. For peptide identification, we searched against the UniProt rat protein database (July 2016 release) using Cys carbamidomethyl as constant modification and Met oxidation as variable modification. Protein abundances in control and treated samples were calculated from the sum of all unique peptide ion intensities for each protein normalized to the same total ion current intensity of all peptides in all experimental samples. Conflicting peptides for different proteins and their isoforms were excluded from the calculations. All identified proteins were ranked based on their abundance ratios between control (HBSS) and treated (genipin) samples.

Statistical Analysis

All data were analyzed using one of the following approaches, depending on the number of groups and independent variables: 2-way repeated measures (RMs) ANOVA with Tukey post hoc (2-way ANOVA with Sidak post hoc, 1-way ANOVA with Tukey post hoc, multiple *t*-tests with Holm-Sidak correction, or one sample *t*-test (GraphPad Software version 8, San Diego, CA). For 2-way RM ANOVA, the reported F statistic is the interaction effect unless otherwise stated. All statistical tests used for each assay are reported in figure legends and results.

For RT-PCR results, fold change was computed as the increase in expression of the experimental eye normalized to the control eye. In HBSS/naïve rats, the experimental eye was the HBSS eye and for genipin/HBSS rats the experimental eye was the genipin-treated eye. Outliers were detected and removed based on Graphpad's ROUT method with a threshold of 0.1% to remove definitive outliers.[27] Proteomic results were analyzed using Progenesis QI for Proteomics software (Waters Inc.), which was used to compute q-values and p-values for each protein. Significant changes in protein expression were defined as any $q < 0.05$.[28] All results are presented as mean ± SD.

Results

Throughout the experiments, animals were carefully observed to determine whether complications occurred from the retrobulbar injection itself (HBSS eyes) or from genipin retrobulbar injections (genipin eyes). A bleb was visible in the nasal/inferior region of the conjunctiva immediately after retrobulbar injection in all eyes, regardless of injection fluid (Fig. 2A). All blebs resolved within 1 to 3 days postinjection. In approximately 5% of genipin-treated eyes, we saw chemosis, which lasted approximately 1 week after injection (Fig. 2C; compared to a normal-appearing genipin-treated eye 1 week after injection (Fig. 2B). Additionally, we observed a small amount of conjunctival bleeding upon removal of the needle after the retrobulbar injection, likely due to damaging small conjunctival blood vessels when inserting the needle. After blotting with gauze, bleeding quickly subsided and, in a few cases, caused a subconjunctival hemorrhage that was visible 1 week postinjection (Fig. 2D).

Genipin treatment of the posterior sclera did not affect IOP for up to 4 weeks following injection (Fig. 3; RM ANOVA, interaction of time and treatment: F(21,

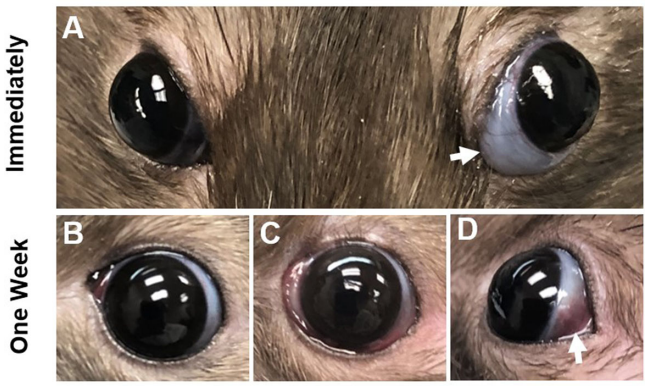

Figure 2. Ocular examination of eyes immediately and one week after retrobulbar injections show mild transient complications. In all eyes receiving a retrobulbar injection (HBSS or genipin), a bleb (**A**) appeared in the nasal quadrant immediately after injection. One such bleb is indicated by an *arrow* (OS) and can be compared to the naïve OD eye (prior to retrobulbar injection). Typically, the bleb would resolve 1 week after injection (**B**). In a few cases, eyes had mild conjunctival chemosis (**C**) or subconjunctival hemorrhage (**D**, *arrow*). All images taken 1 week after injection are oriented such that the nasal portion of the eye is on the left.

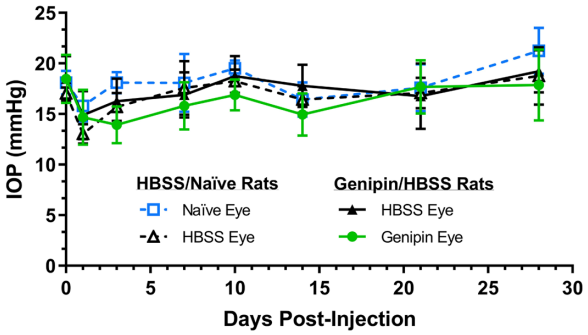

Figure 3. Genipin-induced scleral stiffening did not affect IOP. No significant differences in IOP were found in any group at any timepoint up to 4 weeks postinjection. RM ANOVA, $F(21, 133) = 0.976$; $P = 0.497$. All data shown as mean ± SD, all $n \geq 5$.

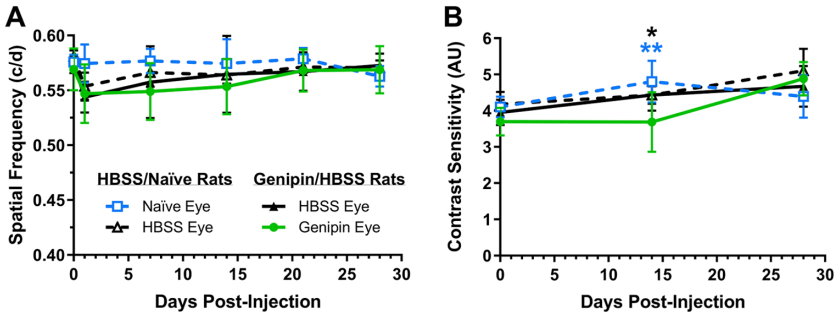

Figure 4. **Genipin treatment did not have a sustained effect on spatial frequency or contrast sensitivity**. Spatial frequency (**A**) and contrast sensitivity (**B**) for HBSS/naïve and genipin/HBSS rats. Spatial frequency was not significantly decreased in any of the groups over the course of the experiment (RM ANOVA, $F(15, 95) = 1.33$; $P = 0.201$). Contrast sensitivity was transiently decreased at day 14 in genipin eyes compared with naïve eyes ($P = 0.002$, denoted by *double asterisks*) and in HBSS eyes (of genipin/HBSS rats) versus genipin eyes ($P = 0.043$, denoted by *asterisk*). All data shown as mean ± SD and analyzed by RM ANOVA, Tukey post hoc used when appropriate; all $n \geq 5$.

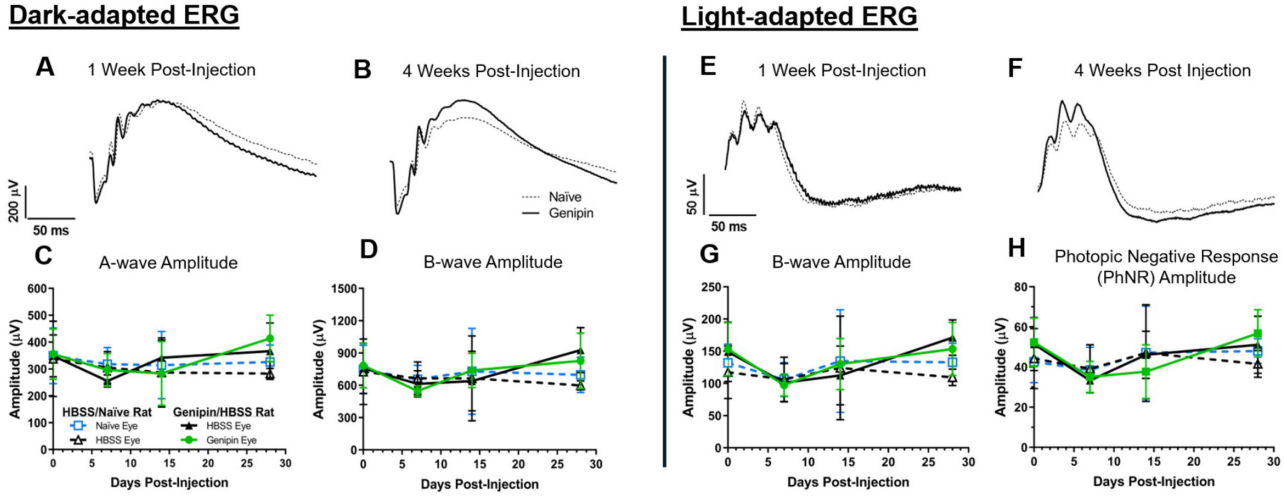

Figure 5. **Retinal function was not altered by HBSS or genipin injections up to 4 weeks postinjection.** Electroretinogram naïve responses for dark-adapted (**A–D**) and light-adapted (**E–H**) testing conditions. Plotted are representative waveforms at 1-week (**A, E**) and 4 weeks (**B, F**) postinjection for naïve (*black dotted*) and genipin (*black solid*) eyes. Mean amplitude and implicit time of all genipin (or naïve) eyes were computed at each time point and flash intensity to select waveforms that most closely matched the means to ensure proper representative waveforms. A-wave and B-wave amplitudes from the brightest dark-adapted flash (2.1 log cd s/m^2) are plotted versus time in **C** and **D**, respectively. Additionally, B-wave and PhNR amplitudes from the brightest single photopic flash (1.4 log cd s/m^2) are plotted versus time in **G** and **H**. All ERG data was analyzed with a 2-way RM ANOVA. No significant interactions of time and treatment were found for any flash intensity (all $P > 0.05$). All data shown as mean ± SD, all $n \geq 5$.

133) = 0.976; P = 0.497), although there was a mild transient decrease in IOP in all injected eyes. OMR assessment of visual function did not reveal any changes in spatial frequency (Fig. 4A; RM ANOVA, interaction of time and treatment: $F(15, 95) = 1.33$; $P = 0.201$), but did show mild transient changes in contrast sensitivity (Fig. 4B, RM ANOVA, interaction of time and treatment: $F(3, 38) = 3.099$; $P = 0.014$). Specifically, at 2 weeks postinjection, the contrast sensitivity of genipin treated eyes was lower than in naïve eyes (Tukey post hoc, $P = 0.002$) and in HBSS eyes from genipin/HBSS rats (Tukey post hoc, $P = 0.043$). This deficit recovered by 4 weeks postinjection (all $P >$ 0.05).

Retinal function assessment via full-field ERGs showed no differences between treatment groups in implicit time or amplitude over time at any flash intensity for both dark adapted and light adapted ERGs (Fig. 5; RM ANOVA, all $P > 0.05$). Representative waveforms from genipin and naïve eyes at 1- and 4-weeks postinjection from the brightest flash in dark adapted (2.1 log cd s/m^2; Figs. 5A, 5B) and light adapted (1.4 log cd s/m^2; Figs. 5E, 5F) protocols are plotted for qualitative analysis.

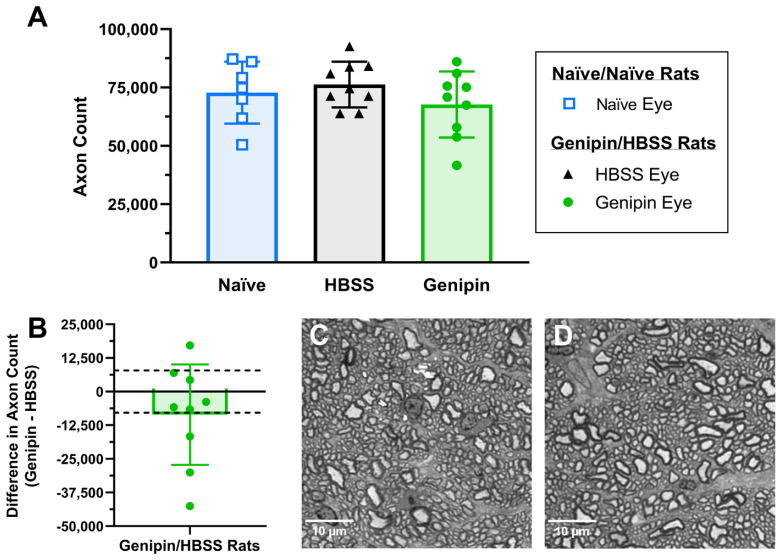

Figure 6. **Genipin treatment results in a minor, non-statistically significant, loss of RGC axons.** (**A**) Whole nerve counts from naïve ($n = 7$, randomly selected as OD or OS eye), HBSS ($n = 9$), and genipin ($n = 9$) eyes. Nerve counts were not different in any cohort (1-way ANOVA, F(2, 22) = 1.067, $P = 0.361$). (**B**) Contralateral optic nerve axon count differences for genipin/HBSS rats at 4 weeks postinjection. Differences are computed as whole nerve axon count in genipin eye minus whole nerve axon count in contralateral HBSS eyes. (One sample *t*-test, t = 1.377, df = 8, $P = 0.206$, *black dashed lines* represent SD of axon count differences from 5 naïve rats). Data shown as mean ± SD. (**C**, **D**) show representative subregions from the central region of optic nerves from a genipin/HBSS rat, with **C** being the HBSS eye and **D** being the genipin eye. Axons appear to be normal with homogenous interiors surrounded by uniform myelin sheaths.

RGC axon counts from naïve, HBSS, and genipin eyes were not significantly different from one another (Fig. 6A; 1-way ANOVA, F(2, 22) = 1.067, $P = 0.361$). However, the mean axon counts of genipin treated eyes (67,714 ± 14,194, mean ± SD) showed a trend toward being lower than that of HBSS (76,272 ± 9,792) and naïve (72,805 ± 13,252) eyes. We further analyzed the axon counts in a paired manner by computing the difference in axon count between genipin and HBSS eyes (Fig. 6B), with a negative value indicating the genipin-treated eye had fewer axons than the contralateral HBSS injected eye. The difference in axon count was not significantly different than zero (one sample *t*-test, t = 1.377, df = 8, $P = 0.206$), although the mean value was negative (−8,558 ± 18,646 axons). These differences translate to a 9.4% ± 23.8% axonal loss in genipin-injected eyes compared to fellow HBSS-injected eyes and were also not statistically significant (one sample *t*-test, t = 1.183, df = 8, $P = 0.271$, data not shown). Qualitative evaluation of a genipin/HBSS rat shows healthy axon morphology with uniform myelin sheath surrounding homogenous axonal interior in both HBSS (Fig. 6C) and genipin (Fig. 6D) eyes.

RT-PCR was used to evaluate message abundance for ECM proteases in the sclera and inflammatory markers in the retina (see Supplementary Table S1) at 1 and 4 weeks after injection. No significant changes in message for ECM proteases in the sclera or pro-inflammatory cytokines in the retina were found at 1 week or 4 weeks after injection (Supplementary Figure S1, multiple *t*-test, all $P > 0.05$). We also used proteomic analysis to determine the amount of various scleral proteins involved in protein binding, cell motility, and ECM structural support at 4 weeks postinjection (Supplementary Table S2). Protein levels were not significantly different in genipin-injected eyes compared to their contralateral HBSS-injected eyes (all $q > 0.05$).

Discussion

Previous work from our laboratory demonstrated successful stiffening of the posterior rat sclera for 4 weeks after a single retrobulbar injection of genipin[21] as a possible therapy to mitigate axial elongation in myopia or optic neuropathy in glaucoma. In the present study, we have used the same genipin injection procedure to evaluate potential adverse effects of genipin-induced scleral crosslinking at the molecular and functional levels in the eye. We conclude that genipin-induced scleral crosslinking had no sustained effect on visual and retinal function over a 4-week period, although there was a trend toward a slight (nonstatistically significant) loss of retinal ganglion cell

axons. These results are the first to assess functional outcomes of genipin stiffening, required for eventual clinical translation of a potential therapy for myopia or glaucoma.

Although no change in steady-state IOP was found in this study, it is important to note that fluctuations in IOP are a risk factor for glaucomatous damage.[29] Clayson et al. (2017) found that scleral stiffening with glutaraldehyde increased the magnitude of IOP spikes in ex vivo porcine eyes, although to a much lesser extent than corneal crosslinking.[30] Future research with genipin-induced scleral crosslinking should evaluate the potential effects on the magnitude of IOP fluctuations.

A slight decrease in visual acuity (spatial frequency) was measured at 1 day postinjection in all eyes receiving a retrobulbar injection with any fluid (HBSS or genipin). This initial deficit was most likely due to the retrobulbar injection itself, which seemed to cause a nonsignificant recoverable deficit. In addition, at 2 weeks postinjection, genipin-treated eyes had significantly lower contrast sensitivity compared to naïve and HBSS (of genipin/HBSS rats) eyes; however, this deficit recovered by week 4. Because contrast sensitivity was only evaluated at baseline, week 2, and week 4, no conclusions can be made about whether genipin affected contrast sensitivity within the first 2 weeks after injection. However, there appears to be no sustained deficit in spatial frequency or contrast sensitivity due to a single retrobulbar injection of genipin.

A robust ERG protocol was implemented in this study to selectively stimulate retinal layers and cell types. Amplitude and implicit time of each wave were analyzed to evaluate retinal function and kinetics. In all parameters evaluated, no significant effects of treatment over time were found. Therefore, genipin-induced scleral stiffening does not appear to affect retinal function in any retinal layer as detectable by ERGs.

Even though no sustained visual or retinal functional deficits were found, it was important to assess RGC axon counts at 4 weeks after injection. Genipin/HBSS rats did not have a statistically significant loss in RGC axons in the genipin-treated eyes, but these eyes showed, on average, a loss of approximately 8500 axons, or 9.4% of total axons (using paired analysis). The mean difference between axon counts in genipin and contralateral HBSS eyes was within one standard deviation of axon count differences from contralateral eyes of naïve rats. This amount of axonal loss appears not to have been functionally significant, but is nonetheless a potential concern. Clinical studies have estimated that patients with glaucoma with the earliest detectable vision loss may already have, on average, approximately 30% RGC loss.[31,32] If one extrapolates from human clinical data to the rat, one could state that the amount of RGC axon loss that we observed is roughly three times smaller than the threshold for visual deficits; however, any RGC axonal loss is worrisome, especially if genipin is to be considered as a treatment for glaucomatous optic neuropathy. Therefore, if genipin treatment is used to treat glaucoma or myopia in the future, RGC axonal loss due to minor genipin toxicity should be carefully evaluated. Additionally, a lower dose of genipin (volume or concentration, or a combination of the two) could be considered to potentially mitigate the slight loss of RGC axons that we observed, although we note that the dose and concentration that we used was selected because it was the lowest dose that achieved a desired level of scleral stiffening.[21]

Several studies have found that genipin is cytotoxic at low concentrations (0.02–0.50 mM) when cultured with various cell types.[33–36] These concentrations of genipin are much lower than the 15 mM concentration used in this study. It is important to note that 15 mM was the concentration that was injected into the periocular space, and therefore the exact concentration of genipin that scleral fibroblasts or retinal cells were exposed to is unknown. In our pilot study evaluating molecular changes in gene expression and protein levels, we also did not find any significant changes in scleral gene expression related to ECM turnover, or in retinal gene expression for inflammatory cytokines. Because these molecular analyses were based upon a small number of animals, these data should be considered as a pilot study to identify large effects of genipin treatment on transcript and protein levels. We did not identify any such effects, but further studies are warranted.

Retrobulbar injections are used clinically to anesthetize the globe and extraocular muscles for cataract and retinal surgery.[37] Some of the major complications of retrobulbar injections include: inadvertent globe perforation, retrobulbar hemorrhage, central retinal artery occlusion, and even death.[38,39] None of these serious complications occurred in any of the injected rats in this study. Other minor complications of retrobulbar injections include chemosis and subconjunctival hemorrhage.[38,40] Here, we did observe a few eyes with chemosis that persisted for approximately 1 week after injection, but which spontaneously resolved by 2 weeks after injection. Because no signs of chemosis were seen in HBSS injections, we infer that the genipin treatment itself could have caused minor transient conjunctival inflammation. On the other hand, subconjunctival hemorrhage

was seen in both HBSS- and genipin-injected eyes, and thus we conclude that subconjunctival hemorrhage was a complication from the injection rather than from the genipin per se.

Conclusion

Previous research has shown that retrobulbar injection of 15 mM genipin produces sustained posterior scleral stiffening. Here, we find that the same injection protocol does not cause prolonged decrease in visual or retinal function. However, we did observe some possible signs of toxicity when evaluating RGC axons and gross anatomy of eyes after injection. This work lays the groundwork for future in vivo studies to evaluate genipin-induced scleral crosslinking for treatment of myopia and glaucoma.

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