Article

Sustained Dorzolamide Release Prevents Axonal and Retinal Ganglion Cell Loss in a Rat Model of IOP–Glaucoma

Ian Pitha[1–3], Elizabeth C. Kimball[3], Ericka N. Oglesby[3], Mary Ellen Pease[3], Jie Fu[1,2], Julie Schaub[3], Yoo-Chun Kim[1,2], Qi Hu[1,2], Justin Hanes[1,2], and Harry A. Quigley[1–3]

[1] Department of Ophthalmology, The Johns Hopkins University School of Medicine, Baltimore, MD, USA
[2] Center for Nanomedicine, The Johns Hopkins University School of Medicine, Baltimore, MD, USA
[3] Glaucoma Center of Excellence, The Johns Hopkins University School of Medicine, Baltimore, MD, USA

Purpose: To determine if one injection of a sustained release formulation of dorzolamide in biodegradable microparticles (DPP) reduces retinal ganglion cell (RGC) loss in a rat model of glaucoma.

Methods: We injected either DPP or control microparticles intravitreally in rats. Two days later, unilateral ocular hypertension was induced by translimbal, diode laser treatment by a surgeon masked to treatment group. IOP and clinical exams were performed until sacrifice 6 weeks after laser treatment. RGC loss was measured by masked observers in both optic nerve cross-sections and RGC layer counts from retinal whole mounts.

Results: Cumulative IOP exposure was significantly reduced by DPP injection (49 ± 48 mm Hg × days in treated versus 227 ± 191 mm Hg × days in control microparticle eyes; $P = 0.012$, t-test). While control-injected eyes increased in axial length by 2.4 ± 1.7%, DPP eyes did not significantly enlarge (0.3 ± 2.2%, difference from control, $P = 0.03$, t-test). RGC loss was significantly less in DPP eyes compared with control microparticle injection alone (RGC axon count reduction: 21% vs. 52%; RGC body reduction: 25% vs. 50% [beta tubulin labeling]; $P = 0.02$, t-test).

Conclusions: A single injection of sustained release DPP protected against RGC loss and axial elongation in a rat model of IOP glaucoma.

Translational Relevance: Sustained release IOP-lowering medications have the potential to stop glaucoma progression.

Introduction

Vision loss from glaucoma is due to death of retinal ganglion cells (RGCs), whose axons project to the brain through the optic nerve head. It is well demonstrated in humans that lowering of intraocular pressure (IOP) prevents glaucomatous vision loss.[1,2] IOP reduction can be accomplished by daily eye drop application, laser procedures, or by incisional surgery. Presently, more patients receive eye drops to lower IOP than the other methods.[3] There are, however, limitations to effective treatment with eye drops.

The success of eye drops is hindered by poor patient adherence, preservative, and medication toxicity, and limited bioavailability. In the Travatan Dosing Aid Study, nearly half of the patients used their drops less than 75% of the time as judged by an electronic monitoring device.[4] This low adherence was present despite the facts that patients knew that they were being monitored and that they were provided with free medication. Preservatives used in drop formulation can cause significant ocular surface toxicity and irritation. Once a drop is applied to the eye, rapid tear turnover and clearance through the nasolacrimal duct limits the amount of drug that reaches the target tissue within the eye, while facilitating adverse systemic effects. Given these factors, it is not surprising that less than 3% of the drug applied reaches the target tissue.[5] Further, it is

demonstrated that chronic eye drop use causes scarring of the conjunctiva, possibly decreasing success of later surgery.[6–9] The limitations of topical delivery provide a strong rationale to create IOP-lowering medications that eliminate the need for eye drops and work for several months after a single administration. This approach would eliminate the need for daily drop adherence and reduce the need for preservatives, reducing ocular surface toxicity.

Over 50 years ago, Friedenwald[10] and Becker[11] showed that inhibition of carbonic anhydrase (CA) activity lowers IOP. There are five distinct CA families and their isozymes are widely expressed in tissues, including the gastrointestinal tract, kidney, liver, and skeletal muscle. CA II, IV, and XII are present in the ciliary processes of the eye and CA II is the dominant CA in aqueous humor production.[12] Oral CA inhibitors are effective at IOP lowering, but have limiting systemic side effects that prohibit extended use in most patients.[13] Dorzolamide was synthesized in the 1980s and a 2% topical formulation, Trusopt, was Food and Drug Administration approved in 1995. Trusopt reduces IOP 20%; however, its pH, 5.65, and the use of hydroxyethyl cellulose to increase the viscosity of the drop generate burning and irritation and reduces tolerability of the drop.[14] Trusopt was approved as monotherapy to be used 3 times daily, further reducing the likelihood of strict drop adherence.

We described a microparticle formulation for sustained delivery of dorzolamide (DPP) that lowered IOP for 35 days after a single subconjunctival injection in normotensive rabbits.[15] IOP following subconjunctival DPP injection was reduced to a similar extent as following Trusopt administration without the requirement for frequent administration. In this study, we use a model of rat glaucoma to determine whether treatment with DPP particles reduces glaucomatous axonal injury and RGC loss.

Materials and Methods

Microparticle Preparation

Preparation of poly(ethylene glycol)-co-poly(sebacic acid) (PEG_3-PSA) polymer, encapsulation of dorzolamide (Chempacific, Baltimore, MD) into PEG_3-PSA microparticles, and characterization of dorzolamide releasing PEG_3-PSA (DPP) microparticles was described previously.[15] Briefly, drug loading of these microparticles was 14.9% wt/wt and they were 9.7 ± 2.7 μm in diameter. They released dorzolamide over 12 days under in vitro, infinite sink conditions.[15]

Animals

Wistar rats (male, 12 weeks of age) were used in experimental protocols approved by the Animal Care and Use Review Board of Johns Hopkins University School of Medicine. Rats were handled in a manner consistent with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the Guide for the Care and Use of Laboratory Animal (Institute of Laboratory Animal Resources, the Public Health Service Policy on Humane Care and Use of Laboratory Animals).

Eye Drop Delivery and Intravitreal Injection of Microparticles

For the topical, eye drop experiment, a single, 2% dorzolamide hydrochloride eye drop (10 μL, HiTech Pharmacal Co., Amityville, NY) was administered to the cul-de-sac of one eye of normotensive, Wistar rats. Each group of rats ($n = 6$) underwent IOP measurement at 0.5, 1, 2, or 4 hours after drop administration. Sample size was calculated to detect a 20% reduction in IOP following drop administration (mean IOP 16.0 ± 1.7 mm Hg, power = 0.80 and α = 5%).

Intravitreal injection of DPP and control microparticles was performed on six normotensive, Wistar rats ($n = 6$ per group) and glaucomatous rats ($n = 10$ per group). Prior to intravitreal injection of microparticles, sedation was achieved using subcutaneous injection of a mixture of ketamine (25 mg/kg) and xylazine (2.5 mg/kg). An eye drop of 1% proparacaine was followed by 5% betadine eye drop to the operative eye. Either DPP microparticles or control, PEG_3-PSA microparticles containing no drug (2 μL in 0.25% hyaluronic acid [HA]; Lifecore, Chaska, MN) were injected through the pars plana in the superonasal quadrant of the eye using a 30-G needle attached to a Hamilton syringe (Hamilton, Reno, NV). Presence of the particles in the vitreous was confirmed by direct observation. Erythromycin ointment was applied to prevent infection and animals were inspected daily for 3 days following injection.

IOP Measurement

IOP was measured with the TonoLab tonometer calibrated for the rat eye (iCare, Vantaa, Finland) under sedation by isoflurane inhalation. The tonometer was used according to the manufacturer's instructions with the magnetic probe in a horizontal position. All measurements occurred between 10 AM and 2 PM and were performed by the same technician

who was masked to treatment group. Prior to measurements, sedation was induced for 3 to 4 minutes in an induction chamber. The rat was then transferred to a nose cone for IOP measurement. Three measurements, each consisting of the mean of six recordings, were taken of each eye. Topical anesthesia was not used for IOP measurement.

Experimental Glaucoma

Translimbal, diode laser treatment in rats induces a prolonged IOP increase and loss of RGC judged by both axonal and cell body loss.[16] Rats were given unilateral, intravitreal injections of either DPP or control microparticles (day 0). Translimbal diode laser treatment was applied 2 days after microparticle injection (day 2). Animals were sedated with a subcutaneous injection of ketamine-xylazine, and a drop of 0.5% proparacaine hydrochloride was used to anesthetize the eye. A 532-nm laser was used to induce ocular hypertension by scarring the trabecular meshwork as previously described. Each rat received a single treatment with 45 to 55 spots at 50-μm size, 0.6-W power, and 0.6-second duration by a surgeon who was masked to treatment group. Topical 5% erythromycin ointment was applied at the end of each procedure. IOP was measured on days 1, 4, 6, 9, 11, 16, 22, and 44 following microparticle injection.

Axial Length and Width Measurement

Measurement of axial length and width was described previously.[17] Briefly, after sacrifice, intracardiac perfusion with 4% paraformaldehyde, and enucleation, IOP was set at 15 mm Hg with a needle placed in the eye and connected to a fluid-filled reservoir. Measurements were performed using a digital caliper (Instant-Read-out Precision Digital Caliper; Electron Microscopy Sciences, Hatfield, PA). The length was measured from the center of the cornea to a position just temporal to the optic nerve, and width was measured at the largest dimension at the equator, midway between the cornea and optic nerve.

Optic Nerve Axon and RGC Counting

Rats were killed by exsanguination under deep ketamine-xylazine anesthesia. They were perfused transcardially with 4% paraformaldehyde in phosphate buffer, and the eyes with attached optic nerve were harvested.

RGC loss was determined by quantifying the number of RGC layer cells positive for β tubulin III

and 4′-6-diamidodino-2-phyenylindole (DAPI) in flat mounts of both glaucoma and fellow control eyes. Retinas were removed from perfusion-fixed eyes and incised for flat mounting. Retinas were incubated in 1:1000 β-tubulin III (Biolegend, San Diego, CA) followed by appropriate secondary antibody (Jackson Immunoresearch, West Grove, PA) with overnight incubation at 4°C. Finally, retinas were placed in a 1:1000 dilution of DAPI stain for 5 minutes (Invitrogen, Carlsbad, CA) then coverslipped with Fluorescent Mounting Medium (DakoCytomation, Carpinteria, CA). Samples were imaged using the Zeiss LSM 510 Meta Confocal Microscope (Zeiss MicroImaging, Thornwood, NY). Forty-eight ×40 images were taken per retina, 12 fields from each of four quadrants (superior, nasal, inferior, temporal), equaling a sampled area equivalent to 4% of the overall retina. Each image was taken for both β-tubulin III and DAPI at the level of the RGC layer avoiding the nerve fiber layer and astrocytes. Images were then analyzed for both cell labels with Metamorph Image Analysis software (Molecular Devices, Downingtown, PA) by trained technical staff masked to experimental group. Experimental retinas were compared with mean RGC layer counts from the data of pooled, fellow eyes.

Axonal loss was quantified using a published method.[16] Briefly, a cross section of the optic nerve was removed 1.5 mm posterior to the globe and postfixed in 1% osmium tetroxide in phosphate buffer. Nerves were processed into epoxy resin, sectioned at 1 μm, and stained with 1% toluidine blue. The area of the optic nerve cross section was measured by outlining its outer border at ×10 magnification on an image analysis system (Sensys digital camera and Metamorph software; Universal Imaging Corp., West Chester, PA). To measure the density and fiber diameter distributions, we captured images with a ×100 phase-contrast objective from 10 randomly spaced nerve regions. These were edited by a technician blinded to treatment group to eliminate nonneural objects, and the size of each axon internal to its myelin sheath (minimum diameter) and the density of axons per square millimeter were calculated for each image and for the entire nerve. The mean density was multiplied by nerve area to yield fiber number for each nerve. The total axon number in the glaucomatous eye was compared with pooled, control fellow eyes to yield a percentage loss value. The counting process was performed by observers masked to the protocol used in each nerve.
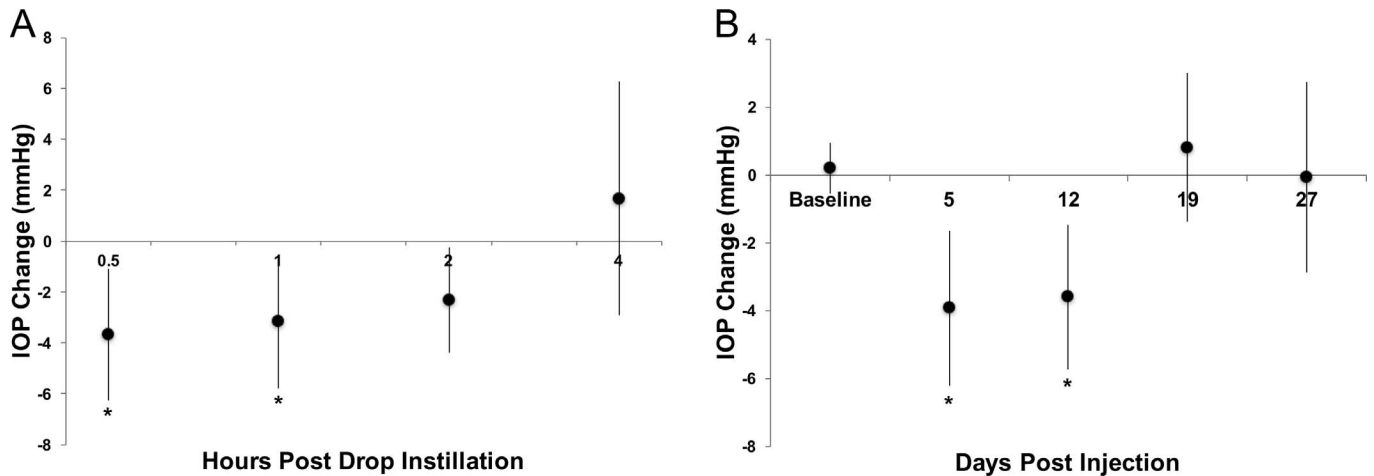
Figure 1. IOP lowering after topical eye drops and intravitreal DPP microparticle injection. IOP changed compared with untreated, fellow eyes was significantly reduced after administration of topical dorzolamide eye drops ([A] $n = 6$) and intravitreal DPP microparticle injection ([B] $n = 6$) (*$P \leq 0.05$). *Error bars*: ±SD.

Statistical Analysis

Outcomes are presented as either mean ± standard deviation (SD) or median. The difference between treated and untreated fellow eye IOP was compared using the mean over time, the peak IOP, and the cumulative IOP exposure (area under the IOP—time curve minus the area under the fellow control eye in mm Hg × days). One-way analysis of variance (ANOVA) was used for means. Dunnett's test ($\alpha = 0.05$) was performed to determine statistical significance for individual time points. Area under the curve (AUC) of IOP reduction compared with fellow, untreated eyes was calculated using the trapezoid rule. The cumulative IOP exposure was calculated using the trapezoid rule and statistical significance was calculated for both DPP and control groups using a paired t-test. Mann-Whitney U tests were performed for P values of independent samples when data were not normally distributed. P values ≤ 0.05 were considered statistically significant.

Results

Intravitreal Injection of DPP Microparticles Lowered IOP Longer Than Eye Drops in Normotensive Rats

Normotensive, Wistar rats had a significant but transient reduction of IOP compared with untreated eyes after delivery of dorzolamide eye drops (Fig. 1A). IOP was reduced by 3.7 ± 2.6 mm Hg 30 minutes after the drop compared with untreated fellow eyes (P

$= 0.01$, $n = 6$), but was not significantly lower after 2 hours. In contrast, intravitreal DPP microparticle injection reduced IOP to a similar extent for a much longer duration. IOP was reduced 3.9 ± 2.3 mm Hg (26%, $P = 0.01$) and 3.6 ± 2.1 mm Hg (20%, $P = 0.02$) at 5 and 12 days after injection, respectively, in DPP microparticle injected compared with control eyes ($n = 6$; Fig. 1B). Nineteen days after microparticle injection, the difference in IOP between DPP and control eyes was not significant. AUC of IOP reduction compared with fellow, untreated eyes following DPP microparticle injection was 34.1 ± 17.0 mm Hg × days, while the AUC after a single drop of 2% dorzolamide was 7.29 ± 3.13 mm Hg × hours. After DPP injection, animals did not show symptoms of eye pain, there was no hyperemia, signs of ocular inflammation, or retinal toxicity noted on clinical exam. Particles were observed in the vitreous 5 and 12 days after injection in all but one injected eyes. In the one eye in which particles were not found on clinical exam 5 days after injection, there was no IOP reduction. While we observed no leakage of DPP at the time of injection, we presume that they exited the eye shortly thereafter. IOPs in Figure 1 are shown as the difference between treated and control fellow eye. IOP measurements are shown in Supplementary Figure S1.

DPP Microparticles Reduce the Ocular Hypertensive Response to Laser Treatment

All eyes in the experimental glaucoma groups received equal laser energy (0.6-W power and 0.6 second duration). DPP microparticle and control
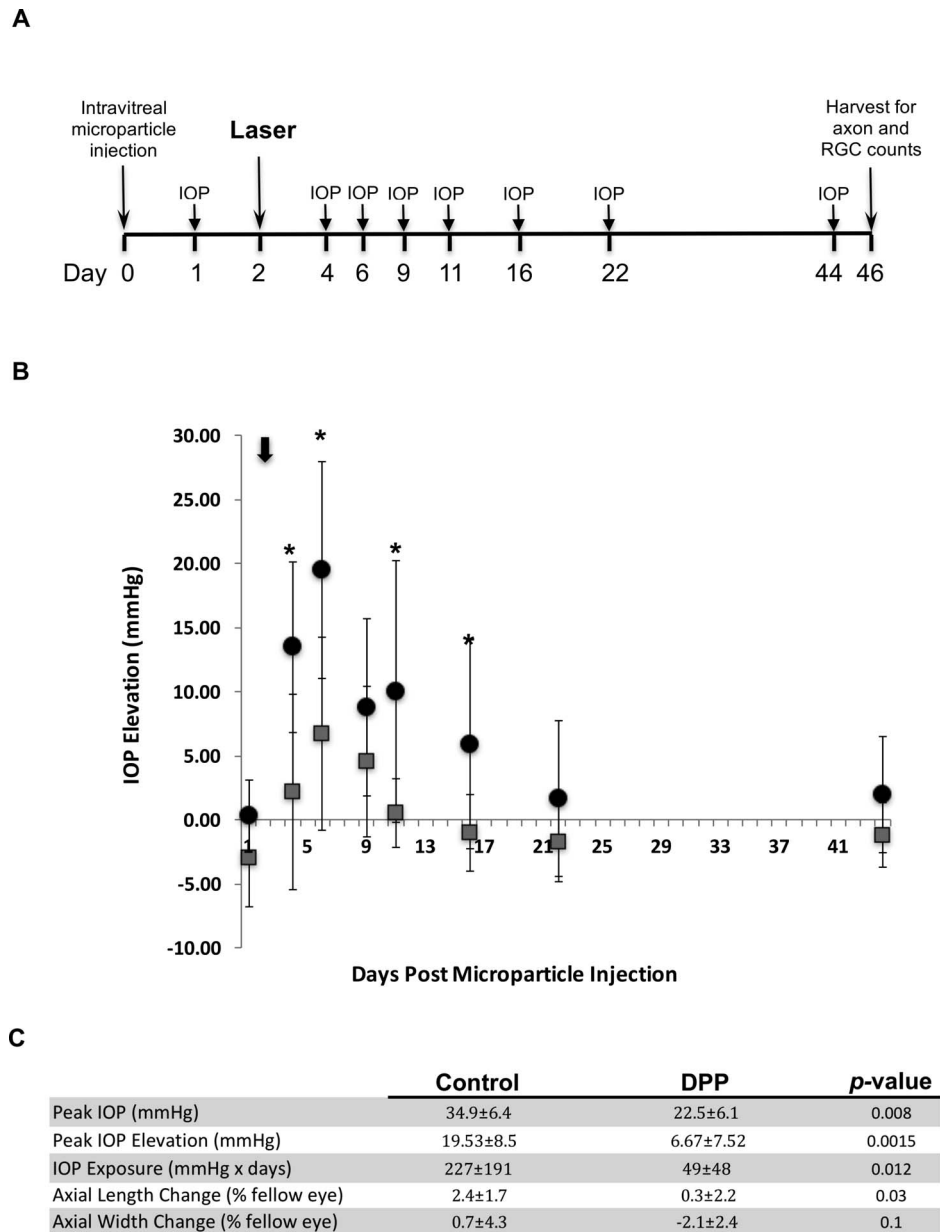
A

B

C

	Control	DPP	*p*-value
Peak IOP (mmHg)	34.9±6.4	22.5±6.1	0.008
Peak IOP Elevation (mmHg)	19.53±8.5	6.67±7.52	0.0015
IOP Exposure (mmHg x days)	227±191	49±48	0.012
Axial Length Change (% fellow eye)	2.4±1.7	0.3±2.2	0.03
Axial Width Change (% fellow eye)	0.7±4.3	-2.1±2.4	0.1

Figure 2. DPP microparticles lower IOP after translimbal laser. (A) Schematic of rat glaucoma experiment. (B) IOP was followed after DPP (*squares*, $n = 10$) or blank (*circles*, $n = 10$ per group) microparticle injection at day 0 and translimbal laser performed at day 2 (*arrow*) (*$P \leq$ 0.05). Y axis shows IOP elevation relative to fellow, untreated, nonglaucomatous eye. (C) Peak IOP, peak IOP elevation compared with fellow eye, cumulative IOP exposure, as well as axial length and width was compared between eyes injected with blank or DPP microparticles. *Error bars*: ±SD.

microparticle injected eyes received an average of 52.9 ± 3.4 and 53.7 ± 3.6 laser applications, respectively ($P = 0.61$). Intravitreal DPP microparticle injection significantly reduced IOP elevation after laser when compared with control microparticles at 4, 6, 11, and 16 days after particle injection (Fig. 2B). Cumulative IOP exposure was also significantly larger in control microparticle injected eyes (227 ± 191 mmHg·days)

as compared with DPP-treated eyes (49 ± 48 mm Hg × days, $P = 0.012$). IOPs in Figure 2 are shown as the difference between treated and control fellow eye. IOP values of the right and left eyes are shown in Supplementary Figure S2. The mean peak IOP (defined as the mean of the highest postlaser IOP for each animal) was significantly less in DPP microparticle–treated eyes (22.5 ± 6.1 mm Hg) than

Table 1. Axon Loss By Treatment Group

Treatment Group	n	Analysis	Control Eye	Glaucoma Eye	% Loss (Pooled Control)
Dorzolamide + glaucoma	9	Mean (SD)	117,782 (15,432)	86,170 (32,161)	26.4%*
		Median	124,502	93,397	20.7%*
Blank particles + glaucoma	10	Mean (SD)	111,073 (16,614)	45,633 (29,940)	61.3%**
		Median	111,763	56,274	52.2%**

n, number of animals providing data per group.
* $P = 0.01$, t-test for difference from zero percent loss.
** $P = 0.00003$, t-test for difference from zero percent loss.

in control microparticle injected eyes (34.9 ± 6.4 mm Hg, $P = 0.008$). The mean IOP elevation in the control injection group (compared with their fellow eyes) was highest at 4 days after laser (an increase of 19.5 ± 8.5 mm Hg). In DPP-injected eyes the mean IOP elevation was significantly less than control microparticle eyes at the same time point (6.7 ±7.5 mm Hg, $P = 0.0015$). The ranges of IOP measured in glaucomatous eyes were 10.0 to 19.0 mm Hg (control microparticles) and 10.0 to 17.0 mm Hg (DPP) prior to translimbal laser, and 15.3 to 42.0 mm Hg (control microparticles) and 10.0 to 34.0 mm Hg (DPP) after translimbal laser. The ranges of IOP measured in control eyes were 10.0 to 19.0 mm Hg (control microparticles) and 12.0 to 19.0 mm Hg (DPP) prior to translimbal laser, and 15.3 to 21.0 mm Hg (control microparticles) and 14.7 to 22.0 mm Hg (DPP).

DPP Microparticles Reduce Ocular Expansion Following Translimbal Laser

Experimental glaucoma in mice and rats is known to increase ocular width and length within the first week of IOP elevation. The bead-injection model of mouse glaucoma is associated with a 5% to 25% increase in axial length and width depending on the mouse strain tested.[17] Control injected, rat glaucoma eyes had an increase in axial length of 2.4 ± 1.7% ($P = 0.04$) compared with fellow eyes. This increase was not observed in DPP microparticle–treated eyes (difference from fellow length = 0.3 ± 2.2%, $P = 0.89$). The group difference in eye length increased between control and DPP microparticle–treated eyes was significant ($P = 0.03$, t-test). There were no significant changes in axial width measurements in either glaucoma group (Fig. 2C).

DPP Microparticles Prevent RGC Loss in the Glaucoma Model

The extent of RGC damage observed in rat laser-induced glaucoma increases with greater cumulative IOP exposure, higher peak IOP, and greater maximal IOP difference between the control and glaucoma eye.[16] Because DPP injection significantly decreased peak IOP and cumulative IOP exposure, we hypothesized that DPP microparticle–treated eyes would be protected from loss of both RGC bodies and axons. The median axon loss in the DPP–glaucoma group compared with pooled fellow eyes was 20.7%, significantly less than the 52.2% loss in the control microparticle group (Table 1, Fig. 3). The mean DPP group loss = 26.8% ($P = 0.01$, t-test compared with fellow eyes), while the mean control microparticle group lost more than twice as many axons compared with fellow eyes (61.3%, $P = 0.00003$). The axon loss in DPP–glaucoma group was significantly less than that in control microparticle–glaucoma group when compared with contralateral eye axonal counts ($P = 0.02$) and pooled controls ($P = 0.01$).

RGC body counts from retinal whole mounts labeled with β-tubulin and DAPI demonstrated similar comparative loss to the axon counts (Table 2, Fig. 4). The more specific label for RGCs is β-tubulin, which identifies only RGC and not amacrine cells that comprise approximately half the neurons there. The β-tubulin data showed 61% mean loss of RGC in the control-particle group, but only 19% mean loss in the DPP-particle group. The control group loss was significant, but the DPP group difference from fellow eye data was not ($P = 0.012$ and 0.4, respectively). DAPI staining labels all nuclei in the RGC layer, both RGC and amacrines. Only RGC die in glaucoma and glaucoma models, so the potential decrease in RGC layer cells would be only 50%, even if all RGCs died.[18] Thus, change in DAPI-labeled nuclei would be expected to be half of that identified by the more specific β-tubulin label. Consistent with this hypothesis, DAPI label data showed twice as many cells in the control, fellow eye RGC layer compared with β-tubulin labeling (Table
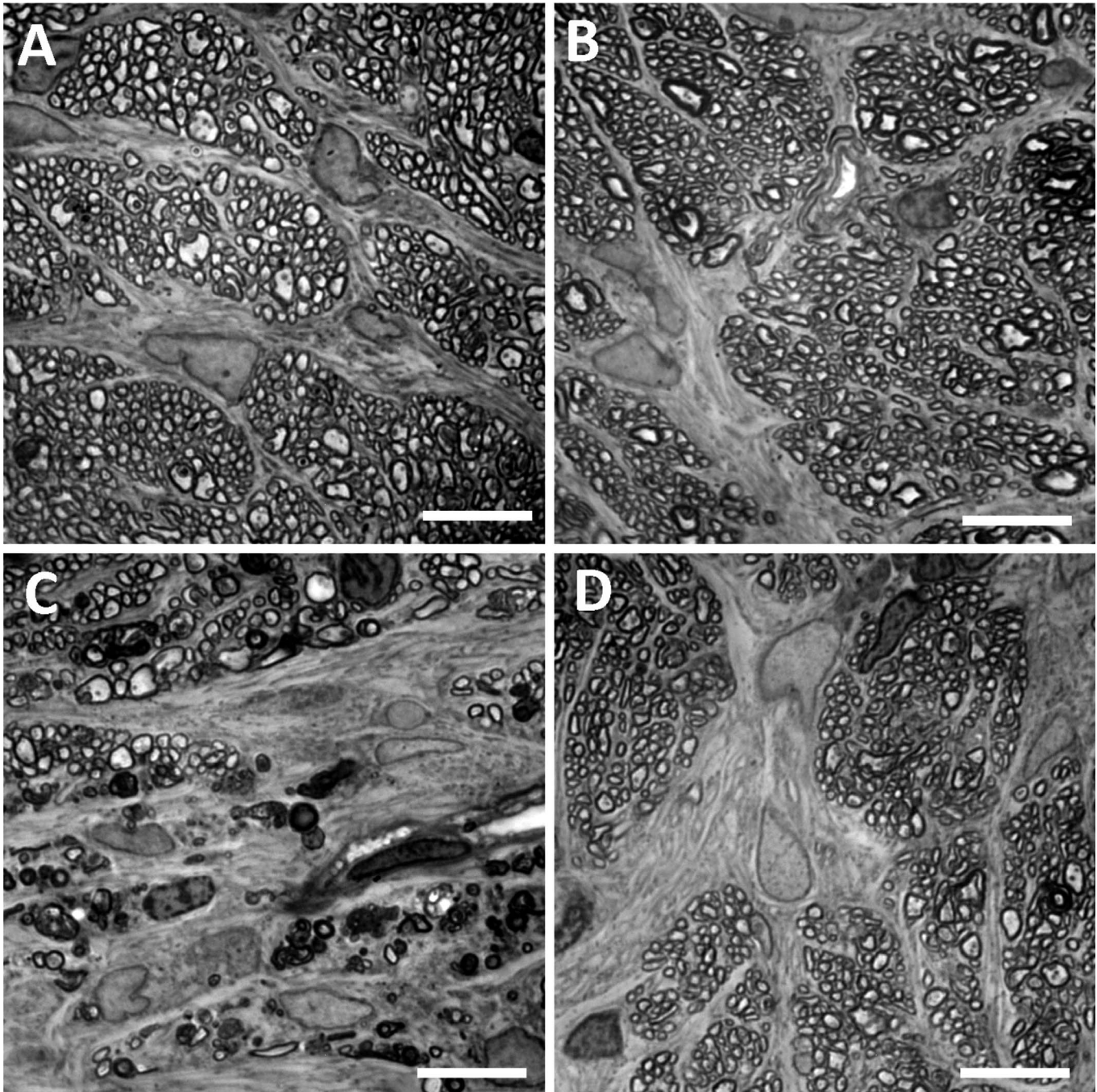
Figure 3. DPP microparticles prevent glaucomatous axon loss. Cross-sectional imaging of the optic nerves of rats treated with blank (A, C) or DPP (B, D) microparticles. Optic nerves were isolated 6 weeks after induction of ocular hypertension in glaucomatous (C, D) as compared with control (A, B) eyes. *Scale bar*: 10 μm.

2). Likewise, the mean loss by DAPI counts in control-particle glaucoma eyes was 38% compared with 6% loss in the DPP–particle glaucoma eyes. Again, the control particle loss was significant, while the DPP group loss was not ($P = 0.021$ and 0.5, respectively).

Discussion

We demonstrated four important results from a single injection of a sustained release, microparticle formulation of dorzolamide. First, it lowered IOP

Table 2. RGC Loss By Treatment Group

Treatment Group	n	Analysis	Control Eye	Glaucoma Eye	% Difference	P Value
DAPI Label						
Dorzolamide microparticles	5	Mean (SD)	1500 (185)	1403 (201)	−6	
		Median	1440	1510	5	0.53
Blank microparticles	5	Mean (SD)	1741 (350)	1084 (244)	−38	
		Median	1717	1005	−41	0.021
B-tubulin Label						
Dorzolamide microparticles	5	Mean (SD)	770 (192)	625 (399)	−19	
		Median	771	575	−25	0.4
Blank microparticles	5	Mean (SD)	839 (181)	324 (225)	−61	
		Median	818	405	−50	0.012

after intravitreal injection for a sustained period. Second, DPP reduced the degree of IOP elevation caused by translimbal, diode laser treatment. Third, it prevented the axial eye elongation associated with chronic IOP elevation in rats. Fourth, it reduced the degree of RGC loss in laser-induced rat glaucoma in quantitative measures of both cell bodies and axons.

In both rabbits and rats, one injection of DPP produced sustained IOP lowering compared with eye drop delivery.[15] Only a small amount of agent was needed for an equivalent maximal IOP lowering. In the transition from pulsed to sustained delivery of IOP-reducing medications, significant dose sparing was described previously. Intracameral bimatoprost, sustained-release implants reduced IOP for over 4 months in glaucoma patients while delivering less than one one-hundredth of the topical dosage per day.[19] In the present animal experiments, there was also significant dose sparing with intravitreal delivery of a carbonic anhydrase inhibitor. The total amount of dorzolamide delivered within DPP microparticles was 99 μg. Each drop of 2% dorzolamide, in contrast, contains 700 μg of drug (assuming 35 μL per drop). While DPP particles lowered IOP in normotensive rabbits for over 30 days after subconjunctival injection,[15] we observed a shorter duration (12 days) of significant IOP lowering after intravitreal injection in normotensive rats. Microparticles were not observed in the rat vitreous after 12 days following injection, which more closely reflects drug release kinetics seen in vitro rather then in vivo IOP reduction seen in rabbits. This reduced duration of IOP reduction could be due to the lower dosage of dorzolamide that was delivered intravitreally (99 μg of dorzolamide delivered intravitreally in rats versus 5 mg delivered subconjunctivally in rabbits). Alterna-

tively, there are likely to be interspecies differences in drug clearance that affect duration of IOP reduction. Lastly, the site of drug injection likely affects the extent of IOP lowering and duration of action. The relatively rapid particle clearance described in these studies suggests that for these microparticles, drug release pharmacokinetics occurs more rapidly in the vitreous space of rats than in the subconjunctival space of rabbits.

In humans, the role of IOP lowering in the prevention and treatment of glaucoma is well established. However, there are limited data on the effect on RGC damage by IOP-lowering treatments in animal models. Morrison et al.[20] showed a reduced mean IOP in 10 glaucomatous rats treated with either daily apraclonidine or betaxolol eye drops compared with rats given artificial tear drops. The degree of axon damage seemed reduced in semiquantitative grading of optic nerve cross sections. IOP reduction in glaucomatous DBA/2J mice was previously obtained by application of topical medications and ciliary body ablation. This reduction prevented RGC loss,[21] preserved vision,[22] and prevented axonal transport disruption[23] when compared with nonglaucomatous controls. We showed IOP reduction with DPP particles in normotensive rats that lasted between 12 and 19 days after injection. We demonstrated not only sustained lowering of IOP in normal rat eyes, but significantly lower IOP in glaucoma model eyes given one injection of DPP particles. This IOP reduction was sufficient to reduce significantly glaucomatous axonal loss and RGC death. Carbonic anhydrase II inhibition exerts IOP-independent effects such as increased choroidal and optic disc blood flow, however, there is no evidence of IOP-independent, neuroprotective activity of DPP in this model. In
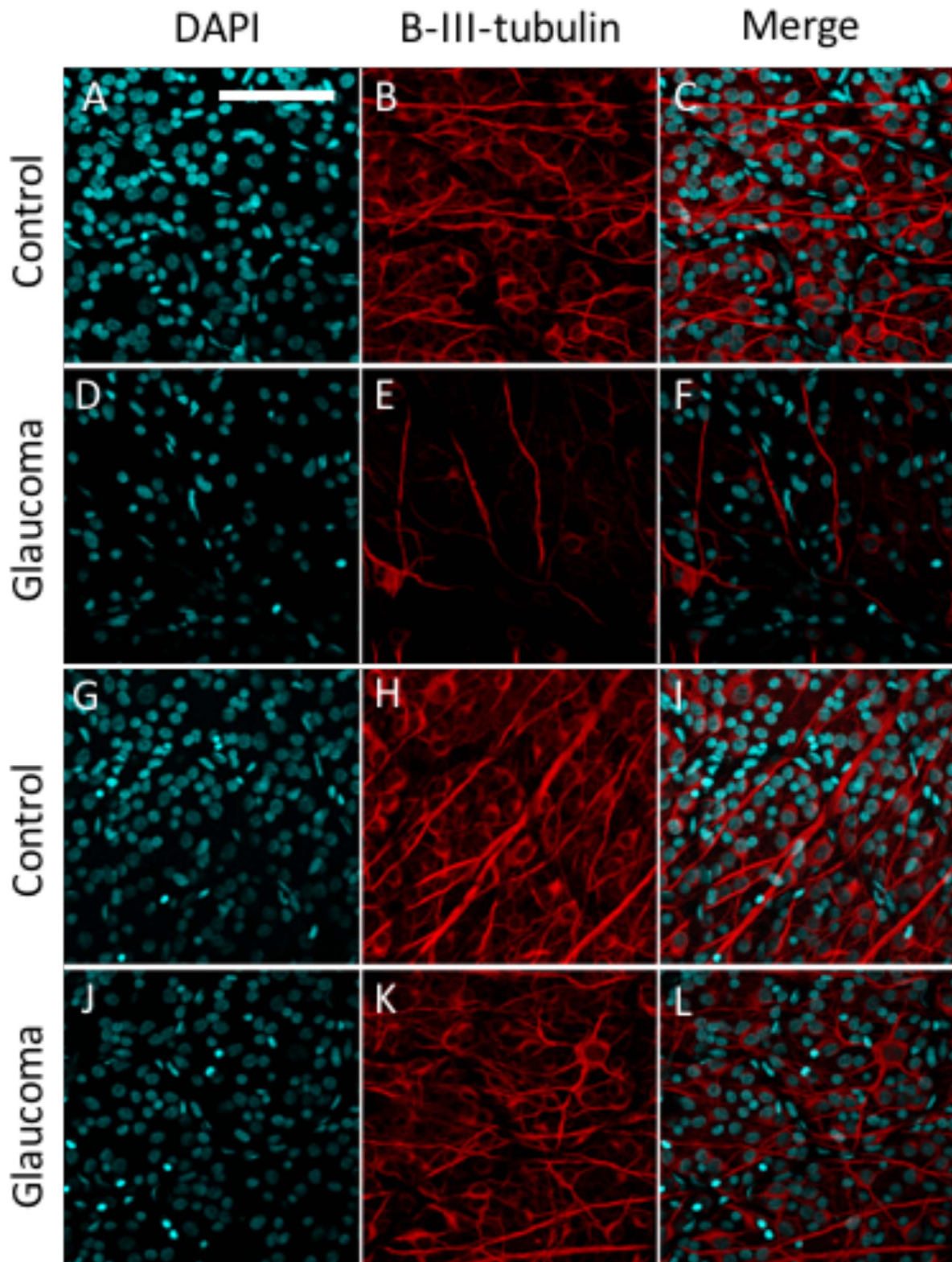
Figure 4. DPP microparticles prevent glaucomatous RGC loss. Immunolabelling for DAPI (*blue*) and β-tubulin (*red*) in the retina of fellow eyes from rats treated with blank (A–C) and DPP (G–I) microparticles and glaucomatous eyes treated with blank (E–F) or DPP (J– L) microparticles. Retinas were isolated 6 weeks after induction of ocular hypertension. *Scale bar*: 100 μm.

addition, DPP treatment prevented axial globe elongation corroborating the successful reduction in IOP-generated strain on the sclera. In mouse, bead-induced glaucoma axial length increases to a greater extent than width. The axial length increase in the rat model described here was modest in compared with mouse, bead-induced glaucoma (2.4% vs. 5%–25%). Given this modest increase, it is not surprising that an increase in axial width was not detected. Interestingly, axonal damage was observed in the DPP-treated group despite significant reduction in peak IOP and cumulative IOP exposure to almost baseline levels. It was previously reported that relatively small IOP elevations could cause axonal damage in this model of IOP glaucoma.[16] This phenomena could be due to RGC death occurring with relatively minor IOP elevations, the inability to precisely measure the maximal IOP elevation, or inability to quantify IOP elevation due to circadian variations in IOP elevation.

Intravitreal injection is one of several options for delivery of controlled release, IOP-lowering formulations. Intravitreal delivery was chosen in this study because subconjunctival delivery might interfere with laser transmission to the limbus. Intravitreal injection allowed delivery of the DPP particles in close proximity to the target tissue (the ciliary body). Intravitreal delivery, however, is associated with transient elevation of IOP[24] and increased risk of intraocular infection, bleeding, and retinal detachment.[25] We and others have shown IOP reduction following subconjuntival injection of controlled release formulations.[15,26–29] Additional formulations for sustained IOP reduction were developed for placement in the suprachoroidal space[30] as well as the ocular surface.[31] Each of these delivery sites likely has unique considerations regarding drug pharmacokinetics, pharmacodynamics, risk profile, and patient preference that warrant thorough investigation.

There are several limitations to this study. Topical dorzolamide was not evaluated in the translimbal laser model of ocular hypertension. It was not the aim of this study to compare the protective effect of DPP microparticles with that of topical dorzolamide. Given the brief IOP reduction observed following topical dorzolamide application, we did not anticipate significant RGC protection with topical dorzolamide, even if it were practical to give multiple eye drops per day. DPP treatment could have been started after induction of IOP glaucoma. Unfortunately, intravitreal injection of DPP into the vitreous space hypertensive rat eyes can lead to inconsistent delivery of microparticles by causing wound leakage. In addition, penetrating a hypertensive eye can lower IOP independent from DPP activity. Injection of particles prior to induction of ocular hypertension overcomes these limitations. Last, visual function was not evaluated following DPP or control microparticle treatment. Although the microparticles aggregated outside of the visual axis after intravitreal injection, translimbal laser can be associated with some corneal opacity, which prevents precise measurement of visual function. Future studies could include measurement of visual function with an alternate IOP–glaucoma model.

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