**Sustained Neuroprotection From a Single Intravitreal Injection of PGJ\textsubscript{2} in a Nonhuman Primate Model of Nonarteritic Anterior Ischemic Optic Neuropathy**

Neil R. Miller,\textsuperscript{1,2} Mary A. Johnson,\textsuperscript{2} Theresa Nolan,\textsuperscript{3} Yan Guo,\textsuperscript{2} Alexander M. Bernstein,\textsuperscript{2} and Steven L. Bernstein\textsuperscript{2}

\textsuperscript{1}Wilmer Eye Institute, the Johns Hopkins Medical Institutions, Baltimore, Maryland, United States  
\textsuperscript{2}Department of Ophthalmology and Visual Science, University of Maryland Medical Center, Baltimore, Maryland, United States  
\textsuperscript{3}Department of Veterinary Resources, University of Maryland Medical Center, Baltimore, Maryland, United States

Correspondence: Neil R. Miller, Woods 458, Wilmer Eye Institute, Johns Hopkins Hospital, 600 North Wolfe Street, Baltimore, MD 21287, USA; nrmiller@jhmi.edu.

Submitted: January 29, 2014  
Accepted: September 18, 2014  
Citation: Miller NR, Johnson MA, Nolan T, Guo Y, Bernstein AM, Bemstein SL. Sustained neuroprotection from a single intravitreal injection of PGJ\textsubscript{2} in a nonhuman primate model of nonarteritic anterior ischemic optic neuropathy. Invest Ophthalmol Vis Sci. 2014;55:7047-7056. DOI: 10.1167/iovs.14-14065

**PURPOSE.** Prostaglandin J2 (PGJ\textsubscript{2}) is neuroprotective in a murine model of nonarteritic anterior ischemic optic neuropathy (NAION). After assessing for potential toxicity, we evaluated the efficacy of a single intravitreal (IVT) injection of PGJ\textsubscript{2} in a nonhuman primate model of NAION (pNAION).

**METHODS.** We assessed PGJ\textsubscript{2} toxicity by administering it as a single high-dose intravenous (IV) injection, consecutive daily high-dose IV injections, or a single IVT injection in one eye of five adult rhesus monkeys. To assess efficacy, we induced pNAION in one eye of five adult male rhesus monkeys using a laser-activated rose bengal induction method. We then injected the eye with either PGJ\textsubscript{2} or phosphate-buffered saline (PBS) intravitreally immediately or 5 hours post induction. We performed a clinical assessment, optical coherence tomography, electrophysiological testing, fundus photography, and fluorescein angiography in all animals prior to induction and at 1 day, 1 week, 2 weeks, and 4 weeks after induction. Following analysis of the first eye, we induced pNAION in the contralateral eye and then injected either PGJ\textsubscript{2} or PBS. We euthanized all animals 5 weeks after final assessment of the fellow eye and performed both immunohistochemical and light and electron microscopic analyses of the retina and optic nerves.

**RESULTS.** Toxicity: PGJ\textsubscript{2} caused no permanent systemic toxicity regardless of the amount injected or route of delivery, and there was no evidence of any ocular toxicity with the dose of PGJ\textsubscript{2} used in efficacy studies. Transient reduction in the amplitudes of the visual evoked potentials and the N95 component of the pattern electroretinogram (PERG) occurred after both IV and IVT administration of high doses of PGJ\textsubscript{2}; however, the amplitudes returned to normal in all animals within 1 week. Efficacy: In all eyes, a single IVT dose of PGJ\textsubscript{2} administered immediately or shortly after induction of pNAION resulted in a significant reduction of clinical, electrophysiological, and histopathological damage compared with vehicle-injected eyes ($P = 0.03$ for both VEP and PERG; $P = 0.05$ for axon counts).

**CONCLUSIONS.** In nonhuman primates, PGJ\textsubscript{2} administered either intravenously or intravitreally produces no permanent toxicity at even four times the dose given for neuroprotection. Additionally, a single IVT dose of PGJ\textsubscript{2} is neuroprotective when administered up to 5 hours after induction of pNAION.

Keywords: anterior ischemic optic neuropathy, white matter, ischemia, 15d-prostaglandin J2, PGJ\textsubscript{2}, intravitreal injection, neuroprotection

Nonarteritic anterior ischemic optic neuropathy (NAION) is caused by inadequate blood supply to the optic nerve head (ONH; i.e., the optic disc) and is the leading cause of sudden optic nerve-related vision loss in individuals over 50 years old.\textsuperscript{1} In the United States, it affects 2.3 to 10.2 per 100,000 people over 50 years of age,\textsuperscript{2,3} resulting in up to over 10,000 new cases per year. Very little is understood concerning the pathophysiology of the disease, and there currently is no medical or surgical treatment that has been proven to be consistently beneficial.\textsuperscript{3}

Reproducible rodent and nonhuman primate models of NAION (rNAION, pNAION) have been developed by intravenous (IV) injection of rose bengal (RB) followed by intraocular laser photoactivation of the dye at the ONH that produces an optic neuropathy that, despite having a different etiology, clinically, electrophysiologically, and histopathologically resembles human NAION.\textsuperscript{5-8} These models provide an invaluable resource because they can be used to analyze critically the mechanisms resulting in postischemic ON damage and because they can be used as tools in the evaluation of potential neuroprotective and neuroreparative therapies for patients who experience NAION.

15-deoxy 12, 14 delta prostaglandin J\textsubscript{2} (PGJ\textsubscript{2}) is a naturally occurring prostaglandin that has been found to be neuroprotective in a number of murine models of stroke, both in vitro and in vivo.\textsuperscript{9,10} PGJ\textsubscript{2} influences at least two major pathways, both of which are key modulators of inflammation. First, PGJ\textsubscript{2} is an...
Intrinsically, the nuclear transcription factor kappabeta is likely involved in the pathogenesis of optic neuropathy. The upregulation of this factor has been linked to cell death in retinal ganglion cells (RGCs). This upregulation is associated with the activation of the nuclear factor peroxisomal proliferator activated receptor-gamma (PPARγ). In the brain, PPARγ expression occurs in microglia and astrocytes, two cell types that play an important role in inflammation. The systemic administration of 15d-PGJ₂ results in a PPARγ-dependent decrease in neuronal apoptosis and necrosis in a murine model of brain stroke. Thus, it is not surprising that PPARγ agonists are associated with neuroprotection and reduced degenerative neuroinflammation.

We previously reported that PGJ₂, whether systemically administered or directly injected into the eyes of adult rats immediately following induction of our rNAION model, results in electrophysiological and histopathological evidence of preservation of optic nerve function as well as preservation of both retinal ganglion cells (RGCs) and RGC function compared with control animals injected with phosphate-buffered saline (PBS) alone 30 days post injection. Visual evoked potentials (VEPs) obtained 7 days after a single intravital (IVT) injection of PGJ₂ in rNAION-induced eyes had amplitudes similar to baseline measurements. Thirty days post induction, electron microscopic analysis of optic nerves from PGJ₂-treated eyes demonstrated significant preservation of axons and minimal demyelination compared with eyes injected with PBS. RGC counts revealed significant RGC preservation in PGJ₂-treated eyes compared with PBS-injected. Similar results also were reported after systemic injection of PGJ₂.

Despite the encouraging results described above in our rNAION model, a major problem with the assessment of potential treatments of ON ischemia as well as ischemia in other parts of the central nervous system (CNS) is that, to date, treatments successful in murine models have rarely been successful in human clinical trials. One reason is that rodent and primate physiologic responses can be considerably different. Thus, it is our opinion that the only way truly to determine if a drug is neuroprotective in humans is to test it in humans or, initially, in a species that responds in a similar manner. For this reason, we elected to test the efficacy of PGJ₂ in our pNAION model.

**Methods**

**Animals**

All animal protocols were approved by the University of Maryland Institutional Animal Care and Utilization Committee (IACUC) and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For induction of pNAION, male rhesus monkeys (Macaca mulatta, age 4–6 years, 6–14 kg) were anesthetized with a mixture of ketamine (10 mg/kg) and xylazine (2 mg/kg). For subsequent assessments, the animals were anesthetized initially with intramuscular (IM) ketamine, followed by an IM injection of atropine to reduce oral secretions. After initial anesthetization, the animals (n = 10) were intubated, and assessments performed while the animals were supported with a continuous infusion of either isoflurane (α = 2) or propofol (α = 8). Propofol was a far better agent than isoflurane for obtaining in vivo electrophysiological measures because isoflurane suppresses cortical electrical responsiveness. Intermittent IV or IM injections of ketamine were used throughout the assessment to reduce spontaneous eye movements.

**PGJ₂ Toxicology**

Five animals (T1–T5) underwent toxicological studies of PGJ₂ before efficacy experiments were begun. Prior to administration of PGJ₂, both eyes of each animal were assessed clinically as well as electrophysiologically with VEPs, pattern electroretinograms (PERGs), ganzfeld ERGs, color photography, red-free photography, and fluorescein angiography (FA). Additionally, blood obtained by venipuncture was used to assess each animal’s systemic biochemical (i.e., electrolyte), hepatic, and hematologic status. The dosages of PGJ₂, routes and length of times of administration, and results are illustrated in Figure 1. Briefly, two animals (T1, T2) were injected with a single IV dose of PGJ₂ (T1 = 40 μg/kg; T2 = 125 μg/kg). The animals then were assessed for changes in behavior and examined clinically, electrophysiologically, and with blood drawn for electrolytes, liver enzymes, and hematology at days 1 and 3 and then weekly for up to 4 weeks for potential PGJ₂-induced toxicity. Two months after completion of these studies, one of these animals (T1) and a third animal (T3) were injected with five consecutive daily IV doses of PGJ₂ (T1 = 40 μg/kg daily; T3 = 100 μg/kg daily) and assessed in the same manner.

Several months after completion of the assessments of the animals given the five consecutive daily IV (systemic) injections, four animals (T1, T3, T4, and T5) were injected with a single IVT dose of PGJ₂ (T1 = 200 μg; T3 = 150 μg; T4 = 50 μg; T5 = 50 μg). The volume injected was the same for all doses, and all injections were performed under aseptic conditions using a 31-gauge needle attached to an insulin syringe (BD) and inserted through the pars plana. A tran-scleral paracentesis was performed within 1 minute after injection to reduce intraocular pressure, and antibiotic ointment was applied immediately after the injection. All four animals were again assessed clinically, electrophysiologically, and hematologically for up to 4 weeks following the IVT injection.

**pNAION Induction**

We induced pNAION in five animals (E1–E5) for our efficacy studies. Although we induced pNAION in both eyes of all five animals, these inductions were sequential, not simultaneous, with the second eye induced 5 weeks after the first eye. The procedure for induction of pNAION has been described previously. Briefly, after anesthetization with ketamine and xylazine, the pupil of one of the monkey’s eyes was dilated with a combination of 1% tropicamide and 2.5% phenylephrine. The
Neuroprotection From PGJ2 Injection

Control, and the other for PGJ2 treatment. All animals had lesions that enabled us to use one eye of each animal as a vehicle generated lesion was consistent between eyes of a given animal. 2.5 mg/kg of lean body weight, followed 30 seconds later by dye pNAION was induced by injecting RB intravenously in a dose of 2.5 mg/kg of lean body weight, followed 30 seconds later by dye activation at the optic disk using the 1.06-mm spot size, at 200 mW, for times ranging from 8 to 9 seconds. With equivalent induction times, laser power and dye administration, the generated lesion was consistent between eyes of a given animal. Thus, we were able to generate submaximal lesions that enabled us to use one eye of each animal as a vehicle control, and the other for PGJ2 treatment. All animals had preserved visual function after each induction, as assessed by overall behavior, pupillary responses to light stimulation, and electrophysiologic assessment. Thus, despite being visually impaired from pNAION induction in both eyes, the animals were not blinded during the course of this study. This approach considerably reduced the total number of animals needed for the study and enabled us to compare directly responses between eyes of the same animal.

Neuroprotection Experiments, IVT Injection

Eyes were injected with PGJ2 or vehicle either 15 minutes (E1-E5) or 5 hours (E4, E5) after pNAION induction. Prior to injection, eyes were prepped three times with 5% povidone idone and anesthetized with viscous ophthalmic tetracaine (Tetravisc). Topical ciprofloxacin drops were instilled, and the induced eye received an IVT injection consisting of either 50 μl of sterile filtered PBS 20% ethanol (vehicle) or 50 μg of sterile filtered PGJ2 dissolved in 50 μl of PBS 20% ethanol (PGJ2). The 50 μg dose of PGJ2 was chosen for our efficacy experiments based on the dosage that we previously used in our rNAION model and the difference in size between adult rat and adult rhesus monkey eyes. Five weeks following pNAION induction and injection of the first eye, pNAION was induced in the contralateral eye, and PGJ2 or vehicle administered, using the same technique. Neither the individual who performed the induction nor the individual who performed the IVT injections was masked as to which substance was being injected; however, the individual who performed the injections did not participate in the subsequent assessments of the animals. In addition, the individuals who performed the assessments could not be masked as to the substance injected because IVT PGJ2 causes slight clouding of the ocular media for several days following the injection.

Optical Coherence Tomography

Optical coherence tomography (OCT) was used to assess the thickness of the peripapillary retinal nerve fiber layer (RNFL), volume of the peripapillary retina, and volume of the macula in four of the five animals (E2-E5): the OCT instrument was obtained after the first animal, E1, was treated). We used a Heidelberg Spectralis spectral-domain HRA + OCT (SD-OCT) instrument (Heidelberg Engineering, Heidelberg, Germany) with 5.4b-US software and equipped with an automated real time eye-tracking system (ART). Before imaging, each animal’s pupils were dilated with topical 2.5% phenylephrine and 1.0% tropicamide. For assessment of the RNFL, the circular scan mode was employed, which uses a circle measuring 3.5 mm in diameter. One hundred images were averaged. A minimum of three scans was obtained for each eye at the same location, manually segmented, and the average value determined. The posterior border of Bruch’s membrane was used as the outer boundary for all retinal thickness measurements. For total peripapillary and retinal thickness, we obtained ON and macular volume scans with 30 images averaged per frame, and image spacing between scans of 120 μm. At least two sets of measurements were obtained at each site. The set with the best quality was used for manual segmentation. All manual segmentation was performed by the same investigator (NRM).

Electrophysiology

Pattern VEPs and PERGs were performed prior to induction and at 1 day, 1, 2, and 4 weeks post induction of pNAION. Ganzfeld ERGs were performed at baseline, at 1 day, and at 4 weeks post induction. All recordings were performed with both of the animal’s pupils dilated with topical 2.5% phenylephrine and 1.0% tropicamide. Simultaneous PERG and VEP recordings were performed with the monkey in the prone position and a Burian-Allen bipolar contact lens electrode placed on the eye. Potentials were recorded from each eye separately. To record simultaneous VEP and PERG waveforms, a Topcon fundus camera (TOPCON Corporation, Tokyo, Japan) was modified by inserting a 2-cm OLED screen from a head-mounted display into the split-viewer pathway. The input to the screen was an alternating (1.9 Hz) black-and-white checkerboard pattern having a luminance of 109 cd/m2 at a nominal contrast of 100%, generated by a LKC UTAS visual diagnostic system (LKC Technologies, Inc., Gaithersburg, MD, USA). The location of the screen was adjusted so that it was conjugate to the plane of the animal’s pupil. In this way, when the monkey’s retina was in focus, the image of the checkerboard was in focus on the monkey’s retina. The macula was positioned using an infrared light source and an infrared-sensitive charge-coupled device camera to avoid bleaching visual pigment. The field size stimulated was 45°. A 32 × 32 matrix was used, producing a check size of 50°. At intervals during the testing, the position of the animal’s eye was reassessed using the infrared camera, followed by a resting period of 5 to 7 minutes to reduce the effects of light exposure.

For VEP recordings, the active electrode (Oz) was placed above the inion in the midline over the shaved skull, the reference electrode was placed in the midline frontal position (Fz), and the ground electrode was placed on an arm using Grass gold surface electrodes and EC2 electrode paste (Grass Instruments, Warwick, RI, USA). Recordings were repeated at least 10 times per eye; 8 to 10 consistent recordings were averaged offline to produce a waveform, the parameters of which were used in comparative analyses. For analysis of PERGs, the amplitudes of P50 (N35 trough to P50 peak) and N95 (P50 peak to N95 trough) waves were measured.

Ganzfeld ERGs were performed following 30 minutes of dark adaptation. A Burian-Allen bipolar electrode was placed in each eye and a ground electrode placed on an arm. Responses were elicited following the International Society for Clinical Electrophysiology of Vision (ISCEV) protocol. High and low band-pass filters were set at 0.5 Hz and 500 Hz, respectively. Oscillatory potentials were extracted using software developed by Severns et al. available in existing LKC software.
Table. Animals Used in the Efficacy Studies and the Clinical Testing Performed on Them

<table>
<thead>
<tr>
<th>Animal</th>
<th>Injection Time Post pNAION for Both PGJ₂ and Vehicle</th>
<th>Eye Injected First/Second Substance Injected</th>
<th>OCT</th>
<th>VEP/PERG</th>
<th>Ganzfeld ERG</th>
<th>Color Photos and FA</th>
<th>H &amp; I</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>&lt;15 min</td>
<td>OD/PGJ₂</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>E2</td>
<td>&lt;15 min</td>
<td>OD/PGJ₂</td>
<td>†</td>
<td>†</td>
<td>†</td>
<td>†</td>
<td>X</td>
</tr>
<tr>
<td>E3</td>
<td>&lt;15 min</td>
<td>OD/PGJ₂</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>E4</td>
<td>5 h</td>
<td>OD/Vehicle</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>E5</td>
<td>5 h</td>
<td>OD/Vehicle</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

H & I, histopathological and immunohistochemical assessment; OD, right eye; VEP/PERG, visual evoked potentials/pattern-evoked electroretinography.

† Animal developed corneal opacity in one eye following IVT injection, preventing testing in that eye until 4 weeks post induction.

Optic Nerve Vascular Imaging

Clinical optic nerve vascular imaging was performed using FA. Fluorescein angiography was performed at baseline, 1 day, and at 1, 2, and 4 weeks post pNAION induction by intravenously injecting 0.30 mL of 25% fluorescein dye (AK-Fluor; AMP; Akorn, Decatur, IL, USA). Retinal and ON photographs were obtained using a Topcon fundus camera with a standard excitation filter transmitting blue-green light at 465 to 490 nm, the peak excitation range of fluorescein, and a barrier filter transmitting a narrow band of yellow at fluorescein’s peak emission range of 520 to 530 nm.

Tissue Collection and Preparation

Animals were euthanized approximately 70 days following induction and injection of the second eye under deep surgical plane anesthesia by intracardiac saline perfusion followed subsequently with 4% paraformaldehyde in PBS (PF-PBS). Following dissection and postfixation in PF-PBS, ON tissues were prepared for standard histology either by paraffin embedding (7-μm thick sections) or by cryopreservation in 30% sucrose and frozen section embedding (10-μm thick sections) in optical cutting temperature compound. All nerves were embedded on end. After removal of the anterior segment, globes were incised to form a Maltese cross pattern, with the macula in the center of one of the arms. Each arm was bisected longitudinally, with half the arm saved for paraffin embedding and the other half for frozen section. Paraffin-embedded retinal sections (7-μm thick) were dehydrated and evaluated by staining with hematoxylin and eosin (H&E).

For transmission electron microscopy (TEM), ON tissue was postfixed in glutaraldehyde-paraformaldehyde buffer. All ONs were placed on end and divided into six pie-shaped sections, impregnated with uranyl acetate, and shadowed with osmium. Each section was then embedded on end in Araldite-Epon.

Immunohistochemistry

Seven-micrometer-thick paraffin sections of the ON were dewaxed and rehydrated. Following boiling citrate buffer (pH 6.0)-antigen retrieval, the sections were blocked with 2% donkey serum and incubated with mouse monoclonal antibody to neurofilaments (SMI312; Sternberger Monoclonals, Lutherville, MD, USA) for intact axons or rabbit anti-human glial fibrillary acidic protein (GFAP; ab72626; Abcam, Cambridge, MA, USA) for glial scarring at 1:1000 dilution overnight at 4°C. Slides then were washed and incubated with the appropriate fluorescein-labelled secondary donkey antibody (Jackson Immunoresearch, West Point, PA, USA) for 4 hours at room temperature, followed by extensive washing. Slides were mounted with aqueous mounting medium and examined at the appropriate wavelengths using an Olympus four-channel confocal microscope.

Electron Microscopic Analysis and Axon Quantification

Two hundred nanometer-thick cross-sections were prepared from each embedded ON section, floated onto copper mesh grids, and examined by TEM using a Tecnai FEI T12 electron microscope. One individual (AB) blinded to the substance injected in the eye from which the ON was obtained generated all axon counts. Counts were performed stereologically using 60 randomly selected axon fields after dissection of each monkey ON into six equivalent regions. Ten random sites from each region were imaged at 21000× to yield 60 151-μm² fields from each ON (total 7825-μm²). A minimum of 600 axons was counted in each nerve. We calculated the coefficient of error (COE) for each nerve. The COE provides information on the accuracy of the population count used to supply stereologically obtained estimates and is a standard statistical value that is used extensively in stereological studies (www.stereology.info/ coefficient-of-error; provided in the public domain by MBF Bioscience, Williston, VT, USA). COEs were in the best-practice boundary range (0.05-0.18). This was particularly crucial given that, in normal adult macaques, the age-independent mean of ON axons is approximately 1,100,000, with a range of 785,532 to 1,280,474 and a mean difference of 0.6% (SD = 1.7%) between the two eyes.

Figure 2. Representative color photographs of the optic discs of the vehicle-injected and PGJ₂-treated eyes after pNAION (E4). In this animal, induction of pNAION was followed 5 hours later by a single IVT injection of either 50 μL of vehicle or 50 μL of PGJ₂ (total dose = 50 μg). Note that there is less swelling of the left optic disc than the right optic disc at both 1 day and 1 week post injection and that at 4 weeks post injection, there is more optic disc and PRNFL atrophy in the vehicle-injected eye than in the PGJ₂-treated eye. Note also the peripapillary retinal hemorrhages present in the vehicle-injected eye but not in the PGJ₂-treated eye. Optic disc photographs of three of the other animals are available as Supplementary Figures S1 through S3.
FIGURE 3. Fluorescein angiograms of the optic discs of the PGJ2-treated and vehicle-injected eyes in monkey E1 1 day and 1 week after induction of pNAION followed within 15 minutes by a single IVT injection of either 50 µL of PGJ2 (total dose = 50 µg) or 50 µL of vehicle. Note that there is less fluorescein leakage in the PGJ2-treated eye than in the vehicle-injected eye at both time periods.

FIGURE 4. Results of OCT. Left, OCT of the vehicle-injected and PGJ2-treated eyes of animal E4 at baseline and at 1 day, 1 week, and 4 weeks post pNAION induction. Note that there is considerably more intraretinal and subretinal swelling at both 1 day and 1 week post pNAION in the vehicle-injected eye than in the PGJ2-treated eye (asterisks) and that there is better preservation of the retinal architecture in the PGJ2-treated eye than in the vehicle-injected eye at 4 weeks post pNAION induction. Right, PRNFL thickness for each animal (n = 4) and for the group as a whole at baseline (hatched bars) and 1 month post pNAION induction followed by IVT injection of either PGJ2 (black bars) or vehicle (white bars). (Vehicle versus baseline: P = 0.06; PGJ2 versus baseline: P = 0.63; PGJ2 versus vehicle: P = 0.03, Wilcoxon Rank Sum Test.) Error bars are ±1 SD except for composite, which is ±1 SE.

FIGURE 5. VEP (left) and PERG N95 (right) amplitudes measured at baseline (hatched bars), and 1 month post pNAION and injection with either PGJ2 (black bars) or vehicle (white bars). Error bars are ±1.96 SE. VEP amplitudes were significantly higher in three of five eyes (E1, E4, and E5) injected with PGJ2 compared with vehicle. PERG N95 amplitudes were significantly higher in three of five eyes (E1, E3, and E5) injected with PGJ2 compared with vehicle. Neither VEP nor PERG N95 amplitudes were significantly different from baseline in the PGJ2-treated eye of any animal or for the group as a whole (for both VEP and PERG, vehicle versus baseline: P = 0.03; PGJ2 versus baseline: P = 0.44; PGJ2 versus vehicle, P = 0.03, Wilcoxon Rank Sum Test).
RESULTS

PGJ$_2$ Toxicity

PGJ$_2$, whether injected intravenously as a single dose, intravenously in five daily doses, or intravitreally as a single dose was not associated with systemic toxicity as assessed by changes in daily animal behavior and serologic parameters in any of the five animals assessed. Specifically, there were no changes in animal behavior, liver enzymes showed no consistent or significant alterations after treatment, and there were no significant alterations in serum electrolytes or complete blood count and differential. No changes in the appearance of the ONs or retinas were detected in the eyes of any of the animals. In addition, no significant changes were detected in the ganzfeld ERG in any of the five animals after either IV or IVT administration of PGJ$_2$; however, both VEP and PERG amplitude reductions were detected at 1 day in animal T2 after a single IV injection of 125 µg/kg of PGJ$_2$, in animal T3 after five consecutive daily IV injections of 100 µg/kg of PGJ$_2$, in animal T1 after a single IVT injection of 200 µg of PGJ$_2$, and in animal T3 after a single IVT injection of 150 µg of PGJ$_2$, suggesting that at these doses, both intravenously and intravitreally administered PGJ$_2$ had a toxic effect on ON function. Nevertheless, all amplitudes recovered to baseline values within 7 days of the last injection, and neither of the animals that received an IVT injection of 50 µL of PGJ$_2$, the dose ultimately chosen for efficacy studies, showed any clinical or electrophysiological evidence of ocular toxicity.

PGJ$_2$ Efficacy

The Table describes the conditions and testing on the five animals used for efficacy experiments.

Clinical examination revealed that in all five animals assessed for efficacy of PGJ$_2$ versus vehicle (E1–E5), optic disc and peripapillary swelling were substantially reduced in the PGJ$_2$-treated eye, compared with the vehicle-injected eye (Fig. 2, Supplementary Figs. S1–S5).

Fluorescein angiography revealed a variable amount of leakage of dye from the optic disc in all pNAION eyes. The leakage was maximal at day 1 and decreased over time such that within 4 weeks, there was variable late staining of all optic discs but no leakage (Fig. 3, Supplementary Figs. S4–S6).

All four animals assessed by SD-OCT (E2–E5) showed a variable amount of both peripapillary intraretinal edema and peripapillary subretinal fluid that gradually resolved over approximately 2 weeks (Fig. 4).

In all four of the animals assessed by SD-OCT two of which (E2, E3) had been injected immediately after induction and two of which (E4, E5) had been injected 5 hours after induction,
were euthanized at least 50 days after pNAION was induced in the second eye. Significant histological differences between all vehicle-injected and PGJ$_2$-treated eyes were present in all animals (Fig. 8). H&E staining revealed increased regional cellular infiltration in the ON cross-sections of vehicle-injected eyes compared with PGJ$_2$-treated eyes (Figs. 8A, 8D: compare 8A [vehicle-injected] with 8D [PGJ$_2$-treated]). Gross structural differences in the individual septated regions also were noted, with increased cellularity present within individual compartments (compare 8A [PGJ$_2$-treated] with 8D [vehicle-injected]). This suggested that PGJ$_2$ treatment after pNAION-associated ischemia not only reduced overall damage but also altered the type of damage associated with the lesion, with increased focal damage in the ONs of vehicle-injected eyes, compared with the ONs of PGJ$_2$-treated eyes.

We confirmed the impressions made from observing the H&E ON staining patterns with analysis of ON-axon loss and scarring using immunohistochemical staining for intact axons (SMI312; Figs. 8B, 8E) and glial scarring (GFAP; Figs. 8C, 8F). In all five animals, ONs from vehicle-injected eyes had severe axon loss with dense regions of dropout (Fig. 8B) similar to the severity and pattern of loss identified in the only histologically assessed clinical case of NAION. In contrast, PGJ$_2$-treated eyes had ONs with reduced axon loss, and the axon loss that was present was more diffuse (Fig. 8E). The difference in severity and pattern of axon loss between ONs from PGJ$_2$-treated and vehicle-injected eyes was mirrored by the comparative differences in the glial scarring patterns seen between ONs from vehicle-injected and PGJ$_2$-treated eyes (Figs. 8C, 8F). ONs from vehicle-injected eyes had larger areas of glial scarring and stronger GFAP signal (Fig. 8C) than did ONs from PGJ$_2$-treated eyes (Figs. 8A, 8D). In every animal, ONs from PGJ$_2$-treated eyes revealed [8D [vehicle-injected]] with 8D [PGJ$_2$-treated]). This suggested that PGJ$_2$ treatment after pNAION-associated ischemia not only reduced overall damage but also altered the type of damage associated with the lesion, with increased focal damage in the ONs of vehicle-injected eyes, compared with the ONs of PGJ$_2$-treated eyes.

Histopathological Findings

All five animals assessed for efficacy of PGJ$_2$ were euthanized at least 50 days after pNAION was induced in the second eye. Significant histological differences between all vehicle-injected and PGJ$_2$-treated eyes were present in all animals (Fig. 8). H&E staining revealed increased regional cellular infiltration in the ON cross-sections of vehicle-injected eyes compared with PGJ$_2$-treated eyes (Figs. 8A, 8D: compare 8A [vehicle-injected] with 8D [PGJ$_2$-treated]). Gross structural differences in the individual septated regions also were noted, with increased cellularity present within individual compartments (compare 8A [PGJ$_2$-treated] with 8D [vehicle-injected]). This suggested that PGJ$_2$ treatment after pNAION-associated ischemia not only reduced overall damage but also altered the type of damage associated with the lesion, with increased focal damage in the ONs of vehicle-injected eyes, compared with the ONs of PGJ$_2$-treated eyes.

There were no abnormalities in the ganzfeld ERG in any of the eyes injected with vehicle or PGJ$_2$ (Fig. 7).

**Histopathological Findings**

All five animals assessed for efficacy of PGJ$_2$ were euthanized at least 50 days after pNAION was induced in the second eye. Significant histological differences between all vehicle-injected and PGJ$_2$-treated eyes were present in all animals (Fig. 8). H&E staining revealed increased regional cellular infiltration in the ON cross-sections of vehicle-injected eyes compared with PGJ$_2$-treated eyes (Figs. 8A, 8D: compare 8A [vehicle-injected] with 8D [PGJ$_2$-treated]). Gross structural differences in the individual septated regions also were noted, with increased cellularity present within individual compartments (compare 8A [PGJ$_2$-treated] with 8D [vehicle-injected]). This suggested that PGJ$_2$ treatment after pNAION-associated ischemia not only reduced overall damage but also altered the type of damage associated with the lesion, with increased focal damage in the ONs of vehicle-injected eyes, compared with the ONs of PGJ$_2$-treated eyes.

**Histopathological Findings**

All five animals assessed for efficacy of PGJ$_2$ were euthanized at least 50 days after pNAION was induced in the second eye. Significant histological differences between all vehicle-injected and PGJ$_2$-treated eyes were present in all animals (Fig. 8). H&E staining revealed increased regional cellular infiltration in the ON cross-sections of vehicle-injected eyes compared with PGJ$_2$-treated eyes (Figs. 8A, 8D: compare 8A [vehicle-injected] with 8D [PGJ$_2$-treated]). Gross structural differences in the individual septated regions also were noted, with increased cellularity present within individual compartments (compare 8A [PGJ$_2$-treated] with 8D [vehicle-injected]). This suggested that PGJ$_2$ treatment after pNAION-associated ischemia not only reduced overall damage but also altered the type of damage associated with the lesion, with increased focal damage in the ONs of vehicle-injected eyes, compared with the ONs of PGJ$_2$-treated eyes.

**Histopathological Findings**

All five animals assessed for efficacy of PGJ$_2$ were euthanized at least 50 days after pNAION was induced in the second eye. Significant histological differences between all vehicle-injected and PGJ$_2$-treated eyes were present in all animals (Fig. 8). H&E staining revealed increased regional cellular infiltration in the ON cross-sections of vehicle-injected eyes compared with PGJ$_2$-treated eyes (Figs. 8A, 8D: compare 8A [vehicle-injected] with 8D [PGJ$_2$-treated]). Gross structural differences in the individual septated regions also were noted, with increased cellularity present within individual compartments (compare 8A [PGJ$_2$-treated] with 8D [vehicle-injected]). This suggested that PGJ$_2$ treatment after pNAION-associated ischemia not only reduced overall damage but also altered the type of damage associated with the lesion, with increased focal damage in the ONs of vehicle-injected eyes, compared with the ONs of PGJ$_2$-treated eyes.

**Histopathological Findings**

All five animals assessed for efficacy of PGJ$_2$ were euthanized at least 50 days after pNAION was induced in the second eye. Significant histological differences between all vehicle-injected and PGJ$_2$-treated eyes were present in all animals (Fig. 8). H&E staining revealed increased regional cellular infiltration in the ON cross-sections of vehicle-injected eyes compared with PGJ$_2$-treated eyes (Figs. 8A, 8D: compare 8A [vehicle-injected] with 8D [PGJ$_2$-treated]). Gross structural differences in the individual septated regions also were noted, with increased cellularity present within individual compartments (compare 8A [PGJ$_2$-treated] with 8D [vehicle-injected]). This suggested that PGJ$_2$ treatment after pNAION-associated ischemia not only reduced overall damage but also altered the type of damage associated with the lesion, with increased focal damage in the ONs of vehicle-injected eyes, compared with the ONs of PGJ$_2$-treated eyes.
agreement among the different tests used to evaluate structure and function validated the use of any one of them to evaluate damage from NAION. We found that for most animals, PGJ\textsubscript{2} treatment, whether given immediately post pNAION or 5 hours after, substantially reduced the amount of pNAION-associated damage compared with contralateral vehicle-injected control eyes. The majority of PGJ\textsubscript{2}-treated eyes showed reduced ON and PRNFL edema by fundus photography and OCT imaging, and reduced ON capillary leakage at 1 day and 7 days post NAION induction. None of the pattern VEP and PERG N95 amplitudes in PGJ\textsubscript{2}-treated eyes was statistically different than baseline values, suggesting that PGJ\textsubscript{2} is strongly neuroprotective in eyes with pNAION compared with vehicle-injected controls. The relative functional preservation in PGJ\textsubscript{2}-treated ONs documented by electrophysiological findings was in close agreement with axon stereology. Thus, by all measures, a single IVT injection of PGJ\textsubscript{2} was neuroprotective following sudden anterior ON ischemia when given either immediately after or within 5 hours post induction. PGJ\textsubscript{2} is therefore the first candidate drug confirmed to be effective in the NAION models of both rodents and nonhuman primates, supporting its potential use in humans.

OCT studies have revealed that in many cases, human NAION is characterized not only by optic disc edema but also by the development of peripapillary retinal edema associated with intraretinal and subretinal fluid.\textsuperscript{27,28} We observed similar phenomena in our pNAION model, with PGJ\textsubscript{2}-treated eyes showing both less intraretinal edema and subretinal fluid and more rapid resolution of the edema and fluid than seen in vehicle-injected eyes.

PGJ\textsubscript{2}'s neuroprotective effect is apparent when the IVT injection is given either immediately or even several hours following induction of pNAION. This suggests that a single injection of PGJ\textsubscript{2} may be able to reduce significant visual loss in humans with NAION. Nevertheless, more work needs to be done to determine PGJ\textsubscript{2}’s treatment time window before there is irreversible damage.

The mechanism by which PGJ\textsubscript{2} limits ON damage and improves ON function is not entirely clear, but given our previous findings of significant inflammation in the area of ischemia in both our murine\textsuperscript{29} and our nonhuman primate\textsuperscript{26} models of NAION as well as in the only human case of NAION that has been evaluated,\textsuperscript{26} we believe that PGJ\textsubscript{2} reduces early inflammation and, thus, limits the posts ischemia compartment

---

**Figure 9.** Electron microscopic analysis of ONs of post pNAION vehicle-injected and PGJ\textsubscript{2}-treated eyes (n = 5). (A, B) Representative TEMs of animal E5. (A) Vehicle-injected ON. (B) PGJ\textsubscript{2}-treated ON. There is relative reduced axon packing density in the ON of the vehicle-injected eye compared with the PGJ\textsubscript{2}-treated eye. Although degenerating axons are seen in both ONs (arrowheads), there is increased interaxonal debris in the vehicle-injected nerve (compare [A] and [B]), and the myelin sheaths are thinner in the nerve from the vehicle-injected eye than in the ON from the PGJ\textsubscript{2}-treated eye. Demyelinated axons are seen in both nerves (arrowheads). (C) ON axon quantification in vehicle-injected and PGJ\textsubscript{2}-treated eyes. Sixty axon fields were randomly selected in each ON, and axons counted in each 135.5-\textmu m\textsuperscript{2} field, using a stereological frame (see Methods). Total counted axons are indicated in the first (vehicle-injected) and third (PGJ\textsubscript{2}-treated) columns. Mean axons/field are indicated in the second (vehicle-injected) and fourth (PGJ\textsubscript{2}-treated) columns. Significance between axon counts was determined using a two-tailed t-test for individual animals and Wilcoxon Rank Sum test for the group as a whole. PGJ\textsubscript{2}-treated eyes had more preserved axons than vehicle-injected eyes in all animals. The difference was significant in four animals (E1: P < 0.001; E2: P = 0.01; E4: P = 0.02; E5: P = 0.02) as well as for the group as a whole (P = 0.05). (D) Bar graph showing relative axon preservation in the five individual test animals as well as for the group as a whole (for vehicle-injected eyes, SD = 2.04 and SE = 1.01; for PGJ\textsubscript{2}-treated eyes, SD = 3.84 and SE = 1.34). Scale bar: 500 nm. Error bars are ±2 SE.
Neuroprotection From PGJ₂ Injection

Intravitreal administration of PGJ₂ enables delivery of high local concentrations of the drug with reduced risk of systemic side effects. The 50-μg intraocular dose used in our efficacy studies was derived from the dose used in our previous study of IVT PGJ₂ in rat eyes with rNAION. Specifically, the volume of the adult rat vitreous cavity is 60 μL, whereas the volume of the adult rhesus monkey vitreous cavity is 3500 μL (i.e., ~50× that of the rat). We had injected 1 μg IVT in our rats. Accordingly, we injected 50 μg in our primates. There were no changes in the VEP, PERG, or ganzfeld ERG after a single administration of this dose. There were transient reductions in the amplitudes of both the VEP and the N95 component of the PERG after single IVT injections of 150 μg and 200 μg were given in two animals, respectively. In both cases, the amplitudes returned to baseline values within 1 week post injection. These doses are 3 and 4 times higher, respectively, than the dose (50 μg) we used in our efficacy studies. Although these results do not totally eliminate the possibilities of long-term systemic or ocular toxicity from IVT PGJ₂, they do suggest that PGJ₂’s therapeutic index is likely to be sufficient for early treatment of NAION, particularly given that PGJ₂ would be used as a single dose for a serious, vision-disabling disease, rather than as a chronic treatment. More work is needed to elucidate further any potential deleterious effects, whether systemic or local, of intravitreally administered PGJ₂.

This is the first study to use an experimental primate model of NAION to evaluate the potential therapeutic efficacy of a new agent for the treatment of human NAION. Because of the precious nature of primate resources, we wanted to maximize the information obtained from each individual animal and minimize the number of primates used for both the toxicity and the efficacy arms of the study. We therefore assessed different doses of PGJ₂ as well as different routes of administration of drug in five animals, allowing sufficient time between studies for the animals to recover from administration. The use of multiple functional tests (assessment of pupillary responses to light, VEP, PERG, ganglion cell loss, immunohistochemical analyses of the eyes of all animals, compared with initial baseline analyses, further enabled us to identify and compare ON ischemia-induced changes in retinal and ON structure and function in each eye with a high degree of sensitivity. Our ability to predictably and reproducibly generate a NAION-type defect is one of the great strengths of the current pNAION model. It should be emphasized again that although we induced visual impairment in all pNAION-induced eyes, none of the animals was blinded; all continued to see and respond appropriately. Judicious use of the pNAION model for analysis of potential clinical NAION treatments enabled us to use each animal as its own control. This approach can minimize variability and reduce the number of animals needed to analyze treatment efficacy, for future preclinical analyses.

Acknowledgments

Supported by NEI Grant R01 EY019529 and a grant from the Hirschlhorn Foundation.

Disclosure: N.R. Miller, None; M.A. Johnson, None; T. Nolan, None; Y. Guo, None; A.M. Bernstein, None; S.L. Bernstein, P

References

Neuroprotection From PGI2 Injection


