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An Experimental Animal Model of Photodynamic Optic Nerve Head Injury (PONHI)

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Abstract

Purpose—Anterior ischemic optic neuropathy (AION) is the most common cause of non-glaucomatous optic nerve head (ONH) injury among older adults. AION results from a sudden ischemic insult to the proximal portion of the optic nerve, typically leading to visual impairment. Here, we present an experimental model of photodynamically induced ONH injury that can be used to study neuroprotective modalities.

Methods—Intraperitoneal injection of mesoporphyrin IX was followed by photodynamic treatment of the ONH in one eye of Brown-Norway rats; the fellow eye received the reverse sequence as a sham control. Fluorescein angiography (FA), spectral domain optical coherence tomography (SD-OCT), and visual evoked potential (VEP) recordings were performed at different time points following laser treatment. Immunohistochemistry was used to monitor apoptotic cell death (TUNEL) and macrophage infiltration (CD68). Cytokine levels were evaluated using enzyme-linked immunosorbent assay (ELISA).

Results—FA showed early hyperfluorescence and late leakage of the ONH, while SD-OCT revealed optic nerve edema. No leakage or other abnormalities were detected in control eyes. VEPs were significantly reduced in amplitude and showed prolonged responses compared to sham eyes. The number of apoptotic retinal ganglion cells was elevated one day after laser treatment (13.77 ± 4.49 , $p < 0.01$) and peaked on day 7 (57.22 ± 11.34 , $p < 0.01$). ONH macrophage infiltration also peaked on day 7 (101.8 ± 9.8 , $p < 0.05$). ELISAs performed showed upregulation

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Declaration of interest

The authors report no conflicts of interest

of macrophage chemoattractant protein-1 and macrophage inflammatory protein-2 on days 3 and 1, respectively.

Conclusions—Photodynamic treatment of the ONH after administration of mesoporphyrin IX leads to macroscopic, histologic, and physiologic evidence of ONH injury. Given the long half-life of mesoporphyrin IX and the ease of intraperitoneal injections, this new model of photodynamically induced ONH injury may be a useful tool for studying optic nerve injury and possible neuroprotective treatments.

Keywords

Animal model; mesoporphyrin IX; neuroprotection; optic nerve head injury; photodynamic treatment

Introduction

Ischemic optic neuropathy is the most common form of non-glaucomatous optic neuropathy in older adults. The variant with the highest incidence is non-arteritic anterior ischemic optic neuropathy (NAION), which, in the United States alone, affects 6000 people per year.¹ Patients with NAION typically develop an altitudinal visual field defect, with 11.7% of cases having their central vision affected, thus presenting with decreased visual acuity of 20/200 or worse.² In addition, there is a 14.7–30% risk that the fellow eye will develop NAION within 5 years after the original insult.³

Although NAION was first described over 50 years ago,⁴ the pathophysiology that ultimately leads to loss of retinal ganglion cell axons is not clear. Though the disorder is believed to be secondary to “ischemia”, the exact site of vascular disruption and the mechanism of injury remain unknown. There is debate as to whether the process is primarily the result of arterial or venous occlusion,⁵ with some researchers even calling the vascular basis into question.⁶ There is good evidence, however, that patients who have a small cup-disc ratio are at higher risk for developing NAION, perhaps because this small ratio causes a “disc at risk” for an “ischemic event”, with subsequent development of a compartment syndrome secondary to edema of the optic nerve head (ONH).^{7–10} Evidence also exists that nocturnal hypotension and sleep apnea are predisposing or perhaps precipitating risk factors of NAION in some patients.^{11–13} In terms of treatment, there is no effective way to prevent vision loss once an attack of NAION occurs, or is there any known strategy for reducing the risk of developing NAION in the fellow eye.

Whatever the mechanism of injury, it is important to develop a neuroprotective strategy to help reduce the degree of injury to the retinal ganglion cells that pass through the ONH, thus reducing the degree of visual loss suffered by patients. Even in the absence of a clear understanding of the mechanism of NAION, it would be valuable to establish an animal model of acute ONH injury that could be used to assess the potential benefit of various treatment options.

The first step in this direction was made by Bernstein et al.,¹⁴ who developed a photodynamic model of NAION in rodents by photoactivating rose Bengal with a 532 nm

laser.^{14,15} This model was further refined and used to test neuroprotective agents,¹⁶ but due to a number of factors, the severity of AION induced in rodents appears to be variable at times, despite using consistent laser settings and techniques. These factors include the normal variation in vasculature of each animal, idiosyncratic responses to laser treatment, and varying angles of incidence of laser exposure.¹⁷ The photosensitizer used, as well as its route of administration, may also contribute to any possible variability observed between animals. The Bernstein model utilizes rose Bengal as the photosensitizing agent. Although extensively used in animal models, rose Bengal has a very short half-life of a just few minutes.¹⁸ Once administered, its photoactivation must be performed almost immediately. Any delay may lead to vessel occlusion of varying degree, given the rapid decrease of rose Bengal in the systemic circulation. Furthermore, rose Bengal is injected into animals via their tail vein and intravenous injections in rodents can be technically challenging. Consistent and timely delivery of the appropriate amount of photosensitizing agent is key to having reproducible findings in this type of animal photothrombotic model.

In this study, we describe an alternative approach to induce photodynamic optic nerve head injury (PONHI) in a rat model.

Methods

Animals

All animal experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the protocols were approved by the Animal Care Committee of the Massachusetts Eye and Ear Infirmary. Adult male Brown Norway rats (Charles River, Wilmington, MA, USA) weighing 250–300 g were fed standard laboratory chow and allowed free access to water in an air-conditioned room with a 12 h light/12 h dark cycle.

Optic nerve head injury induction

Rats were anesthetized with 400 µl/kg of a mixture of 5 parts ketamine (100 mg/ml, Phoenix Pharmaceutical, Inc., St Joseph, MO, USA), 1 part xylazine (100 mg/ml, Lloyd Laboratories, Inc., Shenandoah, IA, USA), and 4 parts phosphate buffered saline (PBS). Anesthetized animals were kept on a heating pad throughout the experiment and until full recovery. Both pupils were dilated with drops of phenylephrine 5% and tropicamide 0.5% (Mass Eye and Ear Infirmary Pharmacy). Sham control experiments were performed first. The ONH of the left eye of each animal was irradiated with a 532 nm laser (Oculight GLx Laser System, IRIDEX Corporation, Mountain View, CA, USA) attached to a slit lamp; a coverslip was used to appanate the cornea, in order to adjust the optics and clearly visualize the fundus. The ONH received 50mW of laser energy across a 500-µm diameter spot size for 14 consecutive seconds. 10mg/kg of the photosensitizing agent mesoporphyrin IX dihydrochloride (Sigma-Aldrich, St. Louis, MO, USA), diluted in dimethyl sulfoxide (Sigma-Aldrich), were subsequently administered by intraperitoneal (IP) injection. Thirty minutes after the photosensitizer administration, laser was applied on the ONH of the right eye of each animal, in order to induce ONH injury. The laser apparatus and parameters used were exactly the same as for the sham eyes.

The laser settings applied were chosen after a series of preliminary experiments in animals that had received the photosensitizer prior to irradiation. A laser spot size of 500µm, previously used by Bernstein et al.,^{14,15} was sufficient to treat the rat optic nerve of the same diameter,¹⁷ without causing direct damage to the peripapillary retina. Laser treatments of different duration (10–25 seconds) were tested; prolonged laser treatment (> 20 seconds) led to undesired sequelae, including retinal vessel occlusion, severe optic disc pallor, retinal pallor, and pre-retinal hemorrhages, as determined by fundus visualization and fluorescein angiography. The combination of a 50mW laser power output for duration of 14 seconds, using a 500 micron spot, was finally deemed optimal in maximizing ONH injury, while minimizing the aforementioned complications.

Fluorescein angiography and optical coherence tomography

Fluorescein angiography (FA) was performed using a TRC 50 VT camera and IMAGENet 1.53 system (Topcon, Paramus, NJ, USA). Following the IP injection of 0.1 mL of 2% fluorescein sodium (Akorn, Decatur, IL, USA), a series of photographs of the retinal vasculature focused on the ONH were taken using a 20-diopter lens. Using a spectral domain optical coherence tomography system (SD-OCT, Bioptigen Inc., Durham, NC, USA), retinal scans centered on the optic disc were performed before and immediately after laser treatment, in both control and injured eyes. Scans were also performed on days 1, 3 and 7 after laser application.

Visual evoked potentials (VEPs)

Rodent VEP recordings were performed in a similar manner to what has been previously described.¹⁹ Briefly, rats were dark-adapted overnight, had their pupils dilated, and were then anesthetized. Responses were elicited with full-field white flashes of light (1.7×10^4 cd/m²) presented at 1 Hz to both eyes. VEPs were monitored with a subdermal electrode in the scalp over the visual cortex as the positive electrode and in the snout as the reference electrode, with the system grounded. This arrangement reduced electrical noise from the line (60 Hz) and the heartbeat. All responses were differentially amplified at a gain of 1000 (–3 db at 2 and 300 Hz), digitized at 16-bit resolution with an adjustable peak-to-peak input amplitude, and displayed on a personal computer using custom software. Consecutive waveforms were averaged ($n = 4$ to 25) after automatically rejecting waveforms containing movement artifacts by an adjustable voltage window. Amplitudes and latencies were measured from baseline and flash onset, respectively, to the trough of the first principal negative deflection (N_1).

For the purpose of VEP recording alone, bilateral treatment was performed in a small subgroup of animals. Specifically, three animals were sham-treated (i.e. they had not received mesoporphyrin IX prior to laser application, and thus had no neural injury); three other animals received laser treatment after having received mesoporphyrin IX, and thus neural injury (but not complete blindness) was induced bilaterally. Based on our previous experience, recording VEPs after unilateral eye treatment can confound the interpretation of the results, since neural activity from an untreated eye can contribute to the VEP waveform of the treated eye. Given that both treatments were part of the same experiment and same study, and were performed to facilitate interpretation, this approach falls within the

acceptable guidelines for the Guide to the Care and Use of Laboratory Animals and the ARVO resolution for the Use of Animals for Ophthalmic and Visual Research. The animals that received bilateral photodynamic treatment did not demonstrate any obvious change in visual behavior or feeding after treatment, and there were still easily recordable VEP waveforms. These observations indicate that complete blindness was not induced in any animal.

Immunohistochemistry

Animals were euthanized by IP injection of pentobarbital at day 1, 4, 7, or 14 after laser application. Eyes were enucleated, fixed in 4% paraformaldehyde (PFA) in PBS for 4 hours and cryoprotected in 30% sucrose overnight. Subsequently, eyes were embedded in Tissue-Tek O.C.T compound (Sakura, Torrance, CA) and 8- μ m cross sections were performed. To identify macrophages, sections were incubated with mouse anti-CD68 monoclonal antibody (MAB1435, Millipore, Billerica, MA, USA) at a 1:100 dilution, at 4°C overnight. Alexa Fluor 488-conjugated goat anti-mouse IgG (Life Technologies, Carlsbad, CA, USA) was used as a secondary antibody, for 2 hours. Finally, sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) nuclear stain (AnaSpec Inc, Fremont, CA, USA).

To prepare retinal flat mounts, animals were anesthetized with ketamine/xylazine and then perfused intracardially with 4% PFA–PBS at various time points after laser application (day 0, 1, 4, 7, 14, or 21). Both eyes were enucleated, the anterior chamber and lens were removed, and the remaining eyecups were fixated in 4%PFA–PBS for 1 hour. Retinas were dissected out of the eyecups, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed to assess cell death. The kit used was the ApopTag Fluorescein Apoptosis Detection Kit by Millipore (Billerica, MA). Briefly, retinas were permeabilized in a solution of ethanol:acetic acid (2:1) for 15 minutes, equilibrated for 5 minutes in the corresponding buffer, and then placed in TdT working solution at 37° C for 2 hours. Retinas were subsequently washed for 20 minutes, at room temperature, using the stop buffer. Retinas were then incubated overnight with an antibody for the neuronal nuclear antigen NeuN (MAB377, Millipore), diluted in 10% goat serum in PBS, at a concentration of 1:200. Finally, fluorochrome-conjugated secondary antibodies were added, i.e. anti-digoxigenin (ApopTag Fluorescein Apoptosis Detection Kit, Millipore) and Alexa Fluor 555 goat anti-mouse IgG (Life Technologies), for the TUNEL and NeuN staining respectively. Retinas were mounted on slides using Permafluor aqueous mounting medium (Thermo Scientific, Fremont, CA, USA), and examined with a Leica fluorescence microscope.

In situ apoptosis analysis

Similar to what was previously described,¹⁵ the retinal ganglion cell apoptosis in our photodynamically treated eyes had marked variability across regions, and thus, when analyzing random areas of the retina, the results obtained showed high variability. To minimize this variation, we followed the methodology described previously,¹⁵ and analyzed three areas from adjacent retinal quadrants, specifically one peripapillary (0–0.8 mm from the ONH), one mid-peripheral (0.8–1.6 mm from the ONH) and one peripheral (1.6–2.4 mm from the ONH) region. For the quantification of TUNEL-positive cells, Image J software (<http://imagej.nih.gov/ij/>) was utilized to analyze images (32 $\times$ magnification) using the

methodology previously described.²⁰ To confirm that the quantified TUNEL-positive cells were also retinal ganglion cells (RGCs), the NeuN retinal staining performed concurrently was used to exclude any non-RGCs that might be undergoing apoptosis; only double positive cells were taken into consideration.

Enzyme-linked immunosorbent assay (ELISA)

Control and treated eyes were enucleated 24 and 72 hours after laser application. The retinas and optic nerves were isolated, lysed in 50 µl of protein lysis buffer, sonicated for 10 seconds, centrifuged for 10 minutes at 10,000 rpm, and the resulting supernatants were used for ELISA. To estimate the concentration of chemotactic cytokines Macrophage Chemoattractant Protein 1 (MCP-1) and Monocyte Inflammatory Protein 2 (MIP-2/CXCL2), the corresponding ELISA kits were used (R&D Systems Inc., Minneapolis, MN, USA) following the company's instructions. The results were normalized for the concentration of total protein per sample, as measured using the DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA), as well as the concentration of the cytokine under study in the sham-treated eyes.

Statistical analysis

Group data are expressed as means $\pm$ 1 standard deviation. Where fellow eyes had received different treatments, interocular difference scores were analyzed by the nonparametric Wilcoxon matched pairs test (The Shapiro–Wilk test for normality rejected the null hypothesis and the distributions were either bimodal or trimodal). However, for the VEP data involving two groups of 3 rats each, analyses were performed with the non-parametric Mann–Whitney U-test. A *p*-value $\leq$ 0.05 was considered statistically significant.

Results

Fluorescein angiography findings after photodynamically induced ONH injury

FA performed within one hour after photodynamic treatment showed early ONH hyperfluorescence that increased in intensity and size with time, consistent with fluorescein leakage from the ONH vasculature (i.e. “optic disc leakage”). This finding was present consistently in the photodynamically treated eyes, but was not observed in the sham-treated eyes (Figure 1A, B, C). Occlusion of major retinal vessels was a rare complication of the laser treatment applied; these animals were excluded from any further analysis (Figure 1D).

SD-OCT imaging of photodynamically induced ONH injury

SD-OCT performed 24 hours after treatment didn't reveal any abnormal changes in the sham-treated eyes (Figure 2A). In the photodynamically treated eyes, OCT imaging at day 1 showed hyporeflectivity of the inner retinal layers around the disc and loss of clear distinction among the layers of the retina (Figure 2B). There was mild swelling of the tissue consistent with optic nerve edema, which became more pronounced 3 days after the treatment (Figure 2C). However, the edema resolved by day 7 (Figure 2D). Shallow subretinal fluid was detected in a small number of treated eyes.

Visual evoked potentials (VEPs)

Cortical responses to flashes of white light were abnormal in photodynamically treated rats compared with sham-treated rats (Figure 3). Specifically, VEPs recorded from photodynamically treated eyes one day after treatment showed only subtle, non-significant changes. However, at 10 days after treatment, VEPs were significantly reduced in amplitude (mean $\pm$ standard deviation, $14.0 \pm 1 \mu\text{V}$) compared with those recorded from sham-treated eyes ($29.7 \pm 12.3 \mu\text{V}$) by the Mann–Whitney U Test ($p = 0.05$). Similarly, VEP latency was significantly delayed in the photodynamically treated eyes ($83.3 \pm 23.7 \text{ msec}$) compared with sham-treated eyes ($50.0 \pm 2 \text{ msec}$) by the same test ($p = 0.05$).

The temporal and spatial pattern of ganglion cell death

To examine the pattern of cell death, TUNEL staining was performed on retinal whole mounts. The decision to assess whole mounts over cryosections was based on the finding that the pattern of RGC loss induced by our ONH ischemia model is patchy. As seen in Figure 4, photodynamically treated eyes showed a significant number of TUNEL-positive retinal cells. Most, but not all, TUNEL-positive cells were also positive for the neuronal cell marker NeuN, suggesting that the majority of apoptotic cells were indeed neuronal cells (Figure 4-F). When assessing apoptotic cell death in our model, only cells staining positive for both NeuN and TUNEL were counted. As seen in Figure 4-G, the number of TUNEL-positive cells in photodynamically treated eyes was significantly increased on day 1 (13.77 ± 4.49 , $p < 0.01$) and peaked on day 7 (57.22 ± 11.34 , $p < 0.01$), after which it started declining. TUNEL-positive cells were still present 21 days after PONHI (16.11 ± 4.76 , $p < 0.01$).

PONHI is associated with increased CD68-positive cell infiltration

To investigate the degree of macrophage infiltration, the number of CD68-positive cells on the ONH was quantified. We focused our assessment on the optic nerve, including the posterior remnant of the hyaloid artery. As seen in Figure 5, photodynamically treated eyes showed an increase of CD68-positive cells in the optic nerve from the first day (43.4 ± 4.5 , $p < 0.05$), reaching a peak on day 7 (101.8 ± 9.8 , $p < 0.05$) (Figure 5C). Macrophage infiltration was less prominent the following days, but a significant number of CD68-positive cells remained 14 days after laser-induced ONH injury (67.3 ± 6.5 , $p < 0.05$).

PONHI induced changes in cytokine levels

Immune cell recruitment usually correlates with upregulation of chemoattractant agents. The levels of key cytokines were assessed at different time points after the induction of ONH injury. Specifically, as seen in Figure 6, the concentration of MCP-1 in photodynamically treated eyes was increased 2.8 fold at 24 hours (2.81 ± 0.99 , $p < 0.01$) and 5.51 fold at 72 hours (5.51 ± 1.31 , $p < 0.01$). The levels of MIP-2 were significantly increased at 24 hours (2.72 ± 0.43 , $p < 0.05$), but not at 72 hours (1.31 ± 0.18 , $p = 0.08$).

Discussion

The goal of our study was to develop a new and technically easier experimental model of photodynamic treatment that can be used to evaluate ischemic injury of the rodent optic

nerve. The Bernstein group first developed a photodynamically induced model of NAION using an iodine derivative of fluorescein, rose Bengal, and a 532 nm wavelength laser.^{14,15} This model was further refined and utilized by several groups.^{15,17,21,22} However, in our experience, tail injections can be technically challenging, leading to variable blood levels of the photosensitizer across users and experiments. Moreover, blood disappearance curves of rose Bengal, after its administration in doses between 0.01 and 10 mg/kg in the rat, show that its initial biological half-life is only 2 minutes.¹⁸ Even a 1-minute delay post-injection of the laser treatment can result in reduced serum concentration of the photosensitizer, thus leading to attenuation of the vasoocclusive effects of photodynamic treatment performed on the ONH. These factors are likely to contribute to the variability of results frequently observed when working on such experimental models. Drawing upon our previous experience with porphyrin-induced photodynamic treatment in other experimental models,^{23,24} we sought an alternative approach to induce ONH injury using a dye with a significantly longer half-life and an easier route of administration.

Mesoporphyrin IX is a member of the porphyrin family and is produced during the preparation of metal mesoporphyrin compounds which have been proposed for the treatment of human diseases, such as porphyrias.²⁵ Previous studies have shown that it has an approximate half-life of 3.6 hours after being intraperitoneally injected in rodents,^{25,26} which increases the probability that a near maximal level of photosensitizer is present in the animal's blood stream at the time of laser treatment. Intraperitoneal injection not only facilitates the experimental process because of its ease of administration, but it also ensures that all animals originally receive the same dose of photosensitizing agent. From a biochemical standpoint, mesoporphyrin IX has been shown to be a photosensitizer with high uptake by cell membranes and partitioning to liposomes, factors that are considered essential when choosing a dye for successful photodynamic therapy.²⁷

Given the aforementioned advantages of mesoporphyrin IX use in photothrombotic models, we were pleased to discover that our model was indeed successful in inducing damage to the rodent optic nerve. FA performed soon after photodynamic treatment of the ONH consistently showed hyperfluorescence of the optic nerve, increasing in intensity and size with time; there were no similar findings in the sham-treated eyes. The optic disc leakage observed is indicative of injury sustained by the small vessels feeding the ONH.

OCT imaging of the photodynamically treated eyes didn't show significant changes compared with the sham-treated group immediately after laser application. However, optic nerve edema was detected on day 1, peaking on day 3 and resolving by day 7 after injury induction. Another finding observed on OCT images on day 1 was the accumulation of fluid in the subretinal space in a few photodynamically treated eyes. Subretinal fluid has been documented in human NAION cases,²⁸ as well as experimental NAION.²⁹ In our animals, the presence and amount of subretinal fluid, though variable, could have contributed to the death of retinal cells outside the ganglion cell layer, thus producing a TUNEL-positive but NeuN-negative staining pattern. Despite the fact that the laser spot size in our experimental model (500µm) is not larger than the rat optic head diameter (500µm),¹⁷ we cannot exclude the possibility that the observed subretinal fluid is the result of photodynamic treatment of the peripapillary area either inadvertently or indirectly.

Apoptotic cell death was evaluated by TUNEL staining. TUNEL-positive cells were detected in the ganglion cell layer one day after photodynamic treatment. This finding cannot be interpreted as degeneration secondary to axon injury since that would take longer to manifest.^{30,31} Photodynamic therapy is known to cause apoptotic cell death through other mechanisms, including depletion of oxygen and nutrients, activation of the immune response, cytochrome C release and Bcl-2 damage.³² The RGC loss in our model reached a peak 7 days after ONH injury, and subsequently declined. TUNEL-positive cells were still detectable on day 21. This delayed temporal pattern is similar to the one previously described by Bernstein et al. regarding their rodent anterior ischemic optic neuropathy (rAION) model,¹⁵ even though the cell death in our model had a time course with one peak instead of two as reported by Bernstein et al. In our analysis, RGCs were identified using the marker NeuN, a neuron-specific protein which may be downregulated by TUNEL (+) cells; in that case, some TUNEL (+) RGCs may appear as NeuN (-), thus leading to an underestimation of the total number of neuronal cells that are undergoing apoptosis. We don't believe, however, that this parameter affects the shape of the resulting cell death curve. Furthermore, in our study the apoptotic cells were sparsely distributed in the ganglion cell layer, and similar regional RGC loss has been described in the rAION animal model,¹⁵ other rodent models of optic nerve injury,³³ as well as a human study of NAION.³⁴

The inflammatory response triggered by the RGC death in our PONHI model was studied using the CD68 marker, which recognizes tissue macrophages and peripheral blood granulocytes. We quantified the macrophage accumulation during the first two weeks after PONHI. Infiltration of CD68 positive cells increased as early as day 1, peaked on day 7, and started to trend down thereafter. Focusing on the pattern of macrophage accumulation in experimental eyes, we noticed most CD68 macrophages in the anterior optic nerve and peripapillary choroid, with little expansion toward the intrascleral, and especially the retroscleral portion of the optic nerve. It is also interesting to note that the apoptotic cell death in PONHI followed a temporal pattern similar to the one of macrophage infiltration; however, no cause-effect relationship can be attributed to this observation. The importance of inflammation in the course of ischemic optic neuropathies was highlighted in the rAION model.²¹

Upon confirming the presence of inflammatory cells, we then evaluated the levels of key inflammatory cytokines in the retina and optic nerve complex, specifically MCP-1 and MIP-2. MCP-1 was significantly increased on both days 1 and 3 after PONHI. The upregulation of MCP-1 has been previously noted in optic nerve disorders, such as neuromyelitis optica,³⁵ but it has never been reported in an experimental model of photodynamic optic nerve injury. The decision to evaluate MIP-2 was based on the functional homology between human IL-8 and rodent MIP-2,^{36,37} and the finding of elevated plasma levels of IL-8 in patients during the acute phase of NAION.³⁸ We found MIP-2 levels to be significantly increased on day 1, but not on day 3 post-PONHI. This result is similar to the findings of an ischemic optic neuropathy mouse model, in which MIP-2 levels were elevated in both the plasma and optic nerve, although at slightly different time points.³⁹

The anatomical and histological changes seen after PONHI were associated with functional deficits demonstrated by changes in the VEP. The PONHI animals, as a group, showed subnormal VEP amplitudes and latencies at 10 days post-treatment, but not at 1 day post-treatment, when compared with sham-treated rats. Since recordings were not done between these two time points, our results do not exclude the possibility that VEP reductions would have been found before the 10-day time point. The finding that the VEP amplitudes were subnormal at 10 days is remarkable, since it suggests that degeneration at the cellular level continued to proceed even after the optic nerve edema resolved. Previous studies showing abnormal VEPs in rat models of rAION with loss of RGCs have reported reduced amplitudes^{14,22} and prolonged latencies,²² consistent with the present findings. In each of these models, loss of RGCs is associated with a reduction in light-evoked signal transmission to the visual cortex, which is consistent with loss of vision.

The new model of PONHI that we have described results in a successful injury that can be demonstrated by FA, OCT, and VEP, and confirmed with immunohistochemical staining for inflammation and RGC death. This model is unique because it uses a photosensitizer with a long half-life, that can be administered intraperitoneally rather than intravenously, factors that we think facilitate the process of ONH lesion induction. Our aim is neither to replace existing models of ischemic optic neuropathy, nor to directly compare our model to others. The main goal of our study is to provide an additional, technically easier model, which can be utilized to test the potential neuroprotective effects of novel agents. Even though experimental models of ischemic optic neuropathy show distinct differences from corresponding human diseases, their role is essential in the process of discovering new strategies that can potentially help to limit RGC loss and improve visual outcomes in humans.

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Figure 1.

Fundus images of fluorescein angiography. Mid phase fluorescein angiography of sham treated eyes without any areas of abnormal hyperfluorescence or hypofluorescence. The white arrow shows the embryologic remnant of hyaloid artery normally seen in rats (A). Fluorescein angiography showed early-mid hyperfluorescence of the disc after PONHI (B), while the mid-late phase showed disc hyperfluorescence secondary to leakage (arrowhead) (C). Occlusion of a major retinal vessel (white arrow); a rare complication of our PONHI model (D).

Figure 2.

Evaluation of PONHI using SD-OCT. The retinal layers are clearly demarcated around the embryonic remnant of the hyaloid artery (white arrowhead) in the sham treated eyes (A). 24 hours after PONHI, the margin between the retinal layers is less clear in the peripapillary area, while subretinal fluid (white arrow) can also be detected (33.3% of animals) (B). By day 3, the mild tissue edema noted is accompanied by further loss of clear distinction between the retinal layers (C). Note that by day 7 the edema and subretinal fluid have resolved (D).

Figure 3.

Visual Evoked Potentials (VEPs) in PONHI. Representative VEPs recorded from injured and sham-treated eyes. The figure illustrates a reduction in amplitude (baseline to trough of N₁) and a delay in latency (trace onset to the trough of N₁) 10 days following ONH injury. ($n = 3$).

Figure 4.

Immunofluorescent staining of retinal whole mounts assessing cell death in the ganglion cell layer. Anti-NeuN antibody was used to label ganglion cells (A, D) and TUNEL assay to detect apoptotic cells (B, E). For quantification of cell apoptosis the double positive cells (C, F) from six areas per retina, from a total of six retinas were used. In our PONHI model, cell death was significant from day 1 (13.77 ± 4.49 , $p < 0.01$) and peaked on day 7 (57.22 ± 11.34 , $p < 0.01$), after which it started declining. TUNEL-positive cells were still present on day 21 (16.11 ± 4.76 , $p < 0.01$) (G). Data expressed as mean $\pm$ 1 standard deviation. ($n = 6$), ** $p < 0.01$ Field of $200 \times 200 \mu\text{m}$.

Figure 5.

Macrophage infiltration in the PONHI model. Double immunofluorescence staining with CD68-positive and DAPI was performed on frozen sections at the level of the optic nerve in the sham (A), as well as the PONHI group (B). Quantification of cells stained with CD68 showed increase of CD68-positive cells from the first day (43.4 ± 4.5 , $p < 0.05$), with further increase on day 4 (62.5 ± 9.2 , $p < 0.05$). The number of CD68-positive cells was maximum on day 7 (101.8 ± 9.8 , $p < 0.05$), after which it started trending back down; a significant number of positive cells was counted on day 14 (67.3 ± 6.5 , $p < 0.05$). (C) Data expressed as mean $\pm$ 1 standard deviation. ($n = 6$). * $p < 0.05$. Scale bar = 100 μ m.

Figure 6.

ELISA analysis of cytokine upregulation after PONHI. While MCP-1 activity was elevated on both day 1 ($n = 6$, $p < 0.01$) and day 3 ($n = 5$, $p < 0.01$) after PONHI (A), in the case of MIP-2 the rise was significant on day 1 ($n = 6$, $p < 0.05$), but not on day 3 ($n = 5$, $p = 0.08$) (B). Data expressed as mean $\pm$ 1 Standard Error. * $p < 0.05$, ** $p < 0.01$.