**Patterns of Intraocular Pressure Elevation after Aqueous Humor Outflow Obstruction in Rats**

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**PURPOSE.** To determine the diurnal intraocular pressure (IOP) response of Brown Norway rat eyes after sclerosis of the aqueous humor outflow pathways and its relationship to optic nerve damage.

**METHODS.** Hypertonic saline was injected into a single episcleral vein in 17 animals and awake IOP measured in both the light and dark phases of the circadian cycle for 34 days. Mean IOP for light and dark phases during the experimental period were compared with the respective pressures of the uninjected fellow eyes. Optic nerve cross sections from each nerve were graded for injury by five independent masked observers.

**RESULTS.** For fellow eyes, mean light- and dark-phase IOP was 21 ± 1 and 31 ± 1 mm Hg, respectively. For four experimental eyes, mean IOPs for both phases were not altered. Six eyes demonstrated significant mean IOP elevations only during the dark phase. Of these, five showed persistent, large circadian oscillations, and four had partial optic nerve lesions. The remaining seven eyes experienced significant IOP elevations during both phases, and all had extensive optic nerve damage.

**CONCLUSIONS.** Episcleral vein injection of hypertonic saline is more likely to increase IOP during the dark phase than the light. This is consistent with aqueous outflow obstruction superimposed on a circadian rhythm of aqueous humor production. Because these periodic IOP elevations produced optic nerve lesions, both light- and dark-phase IOP determinations are necessary for accurate correlation of IOP history to optic nerve damage in animals housed in a light-dark environment.

*Invest Ophthalmol Vis Sci. 2000;41:1380–1385*

Although long considered a major risk factor for glaucomatous optic neuropathy, the cellular mechanisms by which elevated intraocular pressure (IOP) damages the optic nerve remain unknown. Acquiring this knowledge relies heavily on the use of animal models of chronically elevated IOP.

Although nonhuman primates are anatomically most appropriate for such studies,1,2, their use is limited by cost and decreased availability. This has led to increased interest in developing a well-characterized model of aqueous humor outflow obstruction in rodents with identifiable, predictable optic nerve damage, along with reliable methods for documenting IOP.3–9 This model should be economical enough to allow experimentation with sufficient numbers of animals to provide a complete picture of both the ultrastructural consequences and the cell biology of pressure-induced optic nerve damage. Understanding these mechanisms will provide important insights for developing new methods of counteracting the effects of pressure on the optic nerve.

Such a model will also provide a system for assessing the efficacy of treatments designed to protect the optic nerve, either during elevated IOP or after IOP is controlled by medications or surgery.9 However, successful evaluation of such neuroprotective strategies requires a thorough understanding of the relationship between pressure and optic nerve damage, beginning with careful documentation of the IOP to which the optic nerve is subjected.

Our demonstration that IOP can be monitored in awake animals represents a major advance in this regard,10 because it avoids general anesthetics that may in themselves alter the pressure–damage relationship by artifactually lowering IOP.11,12 We found that Brown Norway rats normally experience a large circadian fluctuation in IOP, ranging from approximately 20 mm Hg during the light phase to 30 mm Hg in the dark.10

We have devised a method of producing chronically elevated IOP in Brown Norway rats by injecting hypertonic saline into episcleral veins.3 Because this treatment is likely to increase resistance to aqueous outflow, we might expect these animals to experience a larger than usual fluctuation in IOP. Such a fluctuation could be missed if IOP were monitored only during the day or light portion of the cycle.

In these experiments, we determined the effect of episcleral vein injection of hypertonic saline on rat IOP during the light and dark phases of the circadian cycle. The results provide important insight into the pressure–damage relationship in this model and strongly support our supposition that ob-
struction of aqueous humor outflow is the primary mechanism of pressure elevation.

**METHODS**

All experiments complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Seventeen male Brown Norway rats (Rattus norvegicus), weighing 300 to 400 g, were housed in a standard animal room with food and water provided ad libitum and the temperature maintained at 21°C. The room was lighted by fluorescent lights (530 lux) that were turned on and off automatically every 12 hours, with the lights on from 6:00 AM to 6:00 PM (light phase) and off from 6:00 PM to 6:00 AM (dark phase). Animals were weighed weekly to monitor their general health.

A TonoPen XL (Mentor, Norwell, MA) tonometer was used to measure awake intraocular pressure, with one drop of 0.5% proparacaine hydrochloride applied to each eye, as described previously. Ten IOP readings were obtained from each experimental and fellow (control) eye daily by using firm contact with the cornea and omitting readings obtained as the instrument was removed from the eye (off readings). The mean of these readings for each eye was recorded as the IOP for that date and used to calculate the mean IOP for each eye during the dark and light phases over the course of the experiment. Dark-phase measurements were made using a long-wavelength 16-W bulb (Bright Laboratory Jr.; CPM Inc., Dallas, TX), which emits light in the far-red range of the visible spectrum and does not affect circadian rhythms.

The animals were acclimated to daily handling and awake IOP measurement before episcleral injection. Animals were confirmed to have normal IOP, 21 ± 1 mm Hg and 31 ± 1 mm Hg (mean ± SD) for the light and dark phases, respectively. One eye of each animal was then injected with 50 μl 1.75 M hypertonic saline solution through an episcleral vein, using a force sufficient to just blanch the limbal artery. IOPs were determined daily from the first day after injection throughout the experimental period, alternating between light-phase (9–11 AM) and dark-phase (7–9 PM) measurements. These time windows were chosen because our previous experience demonstrated that IOP in these animals reaches its peak or trough within 1 hour of the light change and varies little during each phase.

After 34 days of IOP monitoring, animals were anesthetized with halothane and perfused transcardially with 5% glutaraldehyde, as previously described. Optic nerve segments 1 mm from the back of the globe were dissected, washed, post-fixed, dehydrated, and embedded. Sections (0.70 μm) were cut on a microtome (Ultra cut E; Reichert, Vienna, Austria) and stained with 1% toluidine blue.

**Optic Nerve Injury Evaluation**

Optic nerve cross sections from injected eyes and fellow eyes were masked and assessed by light microscopy for damage by five independent observers. For each nerve, a cross section from approximately 2 mm behind the globe was chosen at random, and each observer based the assessment of injury on an evaluation of the entire cross section. A graded scale of nerve injury ranging from 1 (normal) to 5 (total degeneration) was used, based on prior observations of a stereotyped pattern of injury in this model.

As illustrated and described in Figure 1, the extent of injury varies in both the intensity of axonal degeneration, shown in the photomicrographs, and the extent of the nerve cross section affected. In general, early mild lesions (grade 2) begin in one, focal region of the nerve, with moderate concentration of degenerating axons. Greater injury (grade 3) is characterized by an increasing density of degenerating axons, with spread to other portions of the optic nerve. As the entire nerve becomes affected, the intensity of damage progresses, until degenerated and normal-appearing axons are equal in number (grade 4). Finally, nearly all axons are degenerating across the entire optic nerve (grade 5). From this analysis, each eye was then assigned a grade of injury determined by calculating the mean of the five independent grade scores.

**Statistical Analyses**

For each experimental eye, the light- and dark-phase IOP (mean ± SD) from injection to death was compared with the corresponding IOP of the fellow eye by Student’s t-test. Eyes were also categorized into three groups by extent of optic nerve injury and the mean light- and dark-phase IOPs for each group compared with the mean light- and dark-phase IOPs of the fellow eyes, by using the same test.

Interrater agreement for the analysis of optic nerve cross-section injury was evaluated by calculating the kappa statistic according to Fleiss. The kappa statistic has been divided into categories describing varying degrees of agreement: less than 0, poor agreement; 0.0 to 0.20, slight agreement; 0.21 to 0.40, moderate agreement; 0.41 to 0.60, moderate agreement; 0.61 to 0.80, substantial agreement; and 0.81 to 1.0, almost perfect agreement. All analyses were performed using a statistical software package (Excel 97; Microsoft, Redmond, WA) except the kappa analysis, which was performed with KAPPA (Shareware, Allison Park, PA) by David Zubrow.

**RESULTS**

Mean IOPs for light and dark phases of the circadian cycle for each experimental eye from the time of injection to death are shown in Table 1, along with the SD and grade of optic nerve injury. When mean light- and dark-phase IOPs for each experimental eye were compared with the respective mean IOPs of their control eyes, the animals segregated into three groups.

Four animals (group 1) demonstrated at most a 1 mm Hg increase in IOP over that of the uninjected, fellow eyes for the corresponding phase. All these eyes had an injury grade of 1.0, indicating no evidence of optic nerve damage.

Six eyes (group 2) showed significantly (P < 0.05) increased IOP only during the dark phase. The mean dark-phase IOPs were at least 3 mm Hg higher than the corresponding IOP of the control group. The difference between the mean dark and light IOP was equal to or greater than 13 mm Hg, with a mean of 16 ± 3 mm Hg. Large circadian IOP fluctuations persisted throughout the experimental period, without sustained elevation during the light phase. Figure 2 illustrates the IOP history and the histologic appearance of the optic nerve cross section of one experimental eye that was typical of this group. Of these eyes, four had unequivocal, partial optic nerve lesions.
All seven eyes in the third group had mean IOPs significantly elevated ($P < 0.001$) relative to the fellow eye in both light and dark phases. Their corresponding optic nerves all had extensive lesions with injury grades of 4.9 or higher. The IOP history record and experimental optic nerve section illustrated in Figure 3 demonstrates a typical example from this group.

When all 17 experimental eyes were analyzed based on the extent of their optic nerve injury, they also segregated into three groups. Six showed no injury, four had partial optic nerve injury (focal lesions), and seven had degeneration of nearly all optic nerve axons (global lesions). Table 2 compares the light- and dark-phase mean IOP of each group with the mean IOP of the control eyes.

For experimental nerves with no lesions ($n = 6$), the mean light-phase IOP for the experimental eyes was $21 \pm 1$ mm Hg, identical with that of the control group. The dark-phase mean was $33 \pm 3$ mm Hg. Although this was slightly higher than that of the control eyes, the difference was not statistically significant ($P = 0.08$).

Four nerves showed a partial optic nerve lesion, ranging in grade from 1.7 to 2.6. IOP in all these eyes showed persistent wide fluctuations between light- and dark-phase IOPs. The light-phase IOP for this group overall was $22 \pm 1$ mm Hg and that for the dark phase was $39 \pm 3$ mm Hg. Only the dark-phase value was significantly greater than that in the corresponding fellow eye value ($P < 0.001$).

In the final group, all seven eyes with optic nerve injury involving nearly all axons had sustained mean IOP elevations during both the light and dark phases. Overall mean pressures for the light and dark phases were $37 \pm 4$ and $42 \pm 2$ mm Hg,
TABLE 1. Analysis by IOP Level

<table>
<thead>
<tr>
<th></th>
<th>Light Phase (mm Hg)</th>
<th>Dark Phase (mm Hg)</th>
<th>Nerve Injury Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fellow eyes*</td>
<td>21 ± 1</td>
<td>31 ± 1</td>
<td>1.0</td>
</tr>
<tr>
<td>Experimental</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>20 ± 1</td>
<td>32 ± 2</td>
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</tr>
<tr>
<td></td>
<td>22 ± 1</td>
<td>31 ± 3</td>
<td>1.0</td>
</tr>
<tr>
<td>Group 2</td>
<td>21 ± 1</td>
<td>34 ± 2</td>
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<td></td>
<td>21 ± 3</td>
<td>41 ± 7</td>
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<td></td>
<td>22 ± 3</td>
<td>40 ± 8</td>
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<td></td>
<td>22 ± 3</td>
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<td></td>
<td>22 ± 2</td>
<td>37 ± 6</td>
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<td>31 ± 10</td>
<td>44 ± 5</td>
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<td></td>
<td>34 ± 10</td>
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<td>44 ± 7</td>
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</table>

Data are light- and dark-phase mean IOP (±SD) and injury grade for fellow eyes and individual experimental eyes.

* Represents the mean of 17 fellow (uninjected) eyes.

respectively. Both were significantly higher than in their respective control eyes ($P < 0.001$).

Agreement among observers regarding optic nerve injury grades using this evaluation system was almost perfect (kappa $0.90 \pm 0.07$ SE, Z [Test Statistic] 12.8).

**DISCUSSION**

Accurate, noninvasive, repeatable measurements of IOP provide the cornerstone for acquiring a thorough understanding of the relationship between IOP and optic nerve damage. This relationship must be understood if we are to use animal models of pressure-induced optic nerve damage to study mechanisms of injury and to test the efficacy of potential neuroprotective strategies.

We initially showed that readings with the tonometer in rat eyes correlate linearly with transducer measurements of actual IOP. Subsequently, we found that these measurements could be repeated over months without ocular side effects. We finally determined that IOP measured in awake animals using only topical anesthesia could record subtle, physiologic variations in IOP in normal rat eyes. This strongly suggested that measurement of IOP without general anesthesia would provide the most accurate assessment of the IOP responsible for optic nerve damage in eyes with experimentally elevated IOP.

In the present study, we have made frequent observations of IOP in awake animals during both the light and dark phases of a strictly controlled circadian cycle. This points to important refinements of our techniques to measure IOP in rats, particularly when applied to our model of pressure-induced optic nerve damage. The results confirm our initial observations on the circadian fluctuation of IOP in normal rat eyes and offer several similarities in IOP behavior between our model and human glaucoma. The results also provide important insights into the mechanism of pressure elevation in this model, the relationship between this pressure elevation and optic nerve damage, and methods of measuring IOP in rats housed in a light-dark environment.

Measurements in the uninjected fellow eyes confirmed our prior observation that IOP in the normal rat eye varies in a distinct circadian fashion, between 21 mm Hg during the light phase and 31 mm Hg in the dark. Rhythmic oscillations of IOP, centered around the 24-hour biological clock, represent a common cause of IOP fluctuation both in humans and in laboratory animals. In humans, some studies report that peak IOP occurs during the day, whereas others have found that peak IOP in normal humans occurs during the dark, or sleep, portion of the cycle. In contrast, animal studies in both rabbits and rats have consistently demonstrated IOP elevation during the dark phase. Fluorophotometric studies in rabbits indicate that this increase results from increased rates of aqueous humor production during the dark. Fluctuations of aqueous humor formation and IOP are both inhibited by removing sympathetic input to the eye.

In the initial description of our model, we demonstrated that episcleral vein injection of hypertonic saline produced variable degrees of trabecular meshwork sclerosis and angle closure. From this, we hypothesized that the mechanism of IOP elevation

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932905/)
and more than 20 mm Hg for some glaucoma patients, whereas a study reported a mean fluctuation of approximately 11 mm Hg, for the experimental eye, with injury grade 5. Magnification, of aqueous humor formation significantly lowers IOP in this model.9

Because increased IOP during the dark in normal eyes probably depends, at least in part, on an increase in the rate of aqueous humor formation, we would expect aqueous outflow obstruction to accentuate the dark-phase IOP increase. This is exactly what we observed in the present study. Elevations in IOP were much more common during the dark phase of the circadian cycle. Overall, six animals (Table 1, group 2) demonstrated persistent, wide fluctuations in IOP, due in every case to a greater than normal mean IOP increase during the dark. Thus, this study provides a further physiologic correlate to our prior observations of anatomic angle closure in this model and suggests strongly that injection of hypertonic saline accomplishes our original intent: to sclerose aqueous outflow pathways and increase the resistance to outflow.

The increased circadian pressure variation shown in several of these eyes has strong parallels with observations in human glaucoma. Although IOP fluctuation is generally less than 5 mm Hg in normal individuals, glaucoma patients can have an exaggerated phasic variation of IOP at all stages of the disease.26–29 One study reported a mean fluctuation of approximately 11 mm Hg, and more than 20 mm Hg for some glaucoma patients, whereas another showed a mean variation of 18 mm Hg and more than 30 mm Hg in two individuals. As in our model, the increased IOP fluctuations in glaucoma patients most likely result from obstruction of aqueous humor outflow superimposed on the normal circadian rhythm of IOP. In humans with elevated IOP, aqueous humor outflow obstruction can be confirmed by tonography, as well as by the effectiveness of glaucoma drops, which reduce aqueous humor formation.

With this in mind, the complex array of pressure histories seen in our study probably resulted from differing degree of angle closure among these eyes. We suspect that those eyes with only mild elevation of mean IOP, or none at all, had the least amount of outflow obstruction. Eyes with more striking mean IOP elevation only during the dark phase had greater obstruction, enough to accentuate elevation during the periods of highest aqueous production. Extensive angle closure apparently reduced aqueous outflow enough to produce elevated IOP during both the light and the dark phases, at both rates of aqueous humor formation.

Although sustained IOP elevations during both the light and dark phases clearly result in devastating optic nerve injury (Table 1, group 3), persistent dark-phase elevations in IOP with intervening normal light-phase values can also produce axonal degeneration. Of the six animals in group 2, four demonstrated partial, but unequivocal evidence of optic nerve damage. The absence of histologic evidence of nerve damage in the other two probably reflects individual variation in optic nerve sensitivity to IOP and the relatively short duration of this experiment. More prolonged dark-phase elevations would probably have resulted in identifiable injury in these animals as well.

In humans, exaggerated IOP fluctuations are also thought to be associated with optic nerve damage and may have important implications for the prognosis of glaucoma.50–53 Undetected increases in pressure may precede the development of disc cupping and field loss. Other investigators have noted that patients with progressive field defects tend to have a greater magnitude of IOP circadian variation than do patients with stable visual fields.52

In this study, large fluctuations in IOP usually appeared as repetitive, periodic, sharp increases in IOP during the dark that nearly doubled the normal circadian oscillation, as illustrated in Figure 2. Because four of these eyes also demonstrated partial optic nerve injury without experiencing sustained IOP elevation during both phases, this shows that repeated, periodic, sharp increases, or spikes, in IOP are capable of producing optic nerve damage.

Similarly, undetected intermittent IOP elevations in humans have been identified as a potential mechanism for optic nerve damage in eyes with otherwise normal IOP.53–55 Both situations

![Figure 3](image_url)

**Figure 3.** (A) IOP history for experimental and control eyes in an animal with marked IOP elevation in both the light and dark phases of the circadian cycle. (B) High-power view of optic nerve cross section for the experimental eye, with injury grade 5. Magnification, ×360.
suggest that the mechanisms by which elevated IOP damages the optic nerve may be cumulative, with a limited capacity to recover after restoration of normal IOP. How or why this occurs is currently unknown, but our model may provide the means to study this problem and lead to important insights into understanding this puzzling facet of glaucomatous optic neuropathy.

Our findings validate the reliability of using the tonometer (TonoPen) to measure IOP in awake rats. They also confirm that the timing of pressure measurement is critical to the accurate documentation of IOP history. Because IOP is typically higher in the dark phase, measurements at the same time of day should yield more reproducible values for monitoring IOP over time or for comparisons among groups of animals or treatments. However, several of our animals demonstrated large dark-phase IOP elevations, without a significant mean elevation in the light. This demonstrates that light-phase measurements alone did not accurately reflect the true IOP exposure of the eye. In these eyes, pressure measurements obtained during the light phase only would have yielded normal pressure readings. The optic nerve damage observed in several of these eyes would have gone unexplained or resulted in mistakenly attributing this damage to abnormal sensitivity to normal IOP or to the injection procedure itself.

Conversely, these data suggest that animals with IOP elevated during the light phase are likely to have extensive, near total optic nerve damage. Thus, analyzing only optic nerves from eyes that manifest elevated IOP, determined by light-phase measurements alone, could give the erroneous impression that IOP-induced damage in rats is an all-or-none phenomenon. Rats housed in a light-dark cycle after aqueous humor outflow obstruction should have IOP monitored during both the light and dark phases of the circadian cycle.

References