Glaucoma Drops Control Intraocular Pressure and Protect Optic Nerves in a Rat Model of Glaucoma

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Purpose. To determine whether chronic topical glaucoma therapy can control intraocular pressure (IOP) and protect nerve fibers in a rat model of pressure-induced optic nerve damage.

Methods. Sixteen adult Brown Norway rats were administered unilateral episcleral vein injections of hypertonic saline to produce scarring of the aqueous humor outflow pathways. Twice daily applications of either artificial tears (n = 6), 0.5% betaxolol (n = 5), or 0.5% apraclonidine (n = 5) were delivered to both eyes, and awake pressures were monitored with a TonoPen XL tonometer for 17 days before the rats were killed.

Results. For animals administered artificial tears, the mean IOP of the experimental eyes was 39 ± 2 mm Hg compared with 29 ± 1 mm Hg for the control eyes. This difference was statistically significant (P < 0.001). Mean IOPs in the experimental eyes of animals administered betaxolol and apraclonidine were 29 ± 7 and 29 ± 4 mm Hg, respectively, whereas the mean IOP in the control eyes was 28 ± 1 mm Hg for both groups. There was no statistically significant difference among these values. The mean IOP for the experimental eyes in the betaxolol and apraclonidine groups was lower than that in animals administered artificial tears (P = 0.003). Quantitative histologic analysis of optic nerve damage in experimental eyes showed that four of the six animals administered artificial tears had damage involving 100% of the neural area. This degree of damage appeared in only 3 of 10 animals administered glaucoma therapy. Optic nerve protection was closely correlated with IOP history because damage was limited to less than 10% of the cross-sectional area in all animals in which the maximal IOP was less than or equal to 39 mm Hg, more than 2 SD below the mean value for eyes administered artificial tears.


Elevated intraocular pressure (IOP) is a major risk factor for glaucomatous optic nerve damage. Although other factors are postulated to play a role in glaucoma, IOP remains the best documented.1-2 Thus, nearly all our current glaucoma therapy is directed toward lowering IOP.

However, as a result of the protracted course of glaucoma and the slow progression of optic nerve damage, the protective effects of pressure-lowering therapy are difficult to demonstrate in humans. Several multicenter human trials are now in progress, but the results may not be available for several years. More rapid demonstration of the efficacy of current glaucoma therapy, as well as new drugs that may directly protect the optic nerve, must rely on suitable animal models of pressure-induced optic nerve damage.

Animal models of chronic IOP elevation primarily have used gradual scarring of the trabecular meshwork in monkeys using the argon laser.3-5 Although these models have provided important information on many aspects of chronic pressure-induced optic nerve damage, the expense of these animals and their dwindling supply make them unsuitable for studies that require establishing a relationship between IOP and nerve damage. The large number of animals required, and the need for frequent IOP measurements, preferably in the unanesthetized state, also preclude the use of monkeys for such studies.

We have developed an inexpensive model of chronic IOP elevation in Brown Norway rats.6,7 Rat aqueous humor exits the eye through an identifiable trabecular meshwork,6 into Schlemm’s canal, and then into a vascular plexus that circles the limbus. This plexus connects with the general circulation through multiple radial veins that run posteriorly within the episclera. We have shown that a retrograde injection of 1.75 M hypertonic saline through one of these episcleral veins toward the limbus produces trabecular meshwork scarring.7

The resultant elevation of IOP produces nerve fiber damage in a characteristic distribution, its extent dependent on the duration and degree of the pressure elevation. The docile nature of these animals and their relatively prominent globes allow us to measure IOP while the animals are awake8 without using general anesthetics, which can affect actual IOP. It is

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possible to document IOP frequently and to avoid overlooking pressure data crucial to understanding the relationship between IOP and optic nerve damage.

We have shown that standard glaucoma drops applied topically can lower IOP in this model.9 With these tools, we want to determine whether pharmacologic agents could control elevated IOP chronically in this model, and whether this control prevents optic nerve damage.

METHODS

All experiments complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Sixteen male Brown Norway retired breeder rats (Rattus norvegicus), each weighing 300 to 400 g, were initially housed in a standard animal room lit by fluorescent lights (330 lux), which were turned on and off automatically every 12 hours. After frequent daily handling to allow reliable IOP measurement while they were awake, all the animals were placed in constant light (40 to 90 lux) for a minimum of 3 days to stabilize the normal circadian variation of IOP.10 Room temperature was maintained at 21°C. Animals were provided food and water ad libitum, and they were weighed weekly to monitor their general health and tolerance to the medications.

After 3 days, one eye of each animal was injected with 1.75 M hypertonic saline using an episcleral vein, as previously described.7 A polypropylene ring was fitted around the equator of the globe to temporarily occlude aqueous veins draining the limbal venous plexus and Schlemm's canal and to confine the injected sclerosing agents to the limbus. A gap in the ring oriented over one radial aqueous-containing vein allowed the insertion of a specially designed microneedle into the vessel lumen. Fifty microliters of 1.75 M hypertonic saline solution was injected, with a force sufficient just to breach the limbal artery, thus avoiding excessive pressure. After 1 week, the same eye was reinjected in the same fashion, also using 1.75 M saline.

Animals were divided into three groups. One group (six animals) was given 10-μl applications of Tears Naturale (Alcon Laboratories, Fort Worth, TX), one group (five animals) was given 0.5% betaxolol (Alcon Laboratories), and the last group (five animals) was given 0.5% apraclonidine (Alcon Laboratories). All medications were begun 1 day after the second injection and were delivered twice daily to each eye. The examiner measuring IOP was unaware of which drops were administered to each animal.

IOP was measured between 6 AM and 6 PM using the TonoPen (Mentor O & O, Norwalk, MA) XL tonometer on awake animals and topical proparacaine anesthesia, as previously described.8 With gentle manual restraint, 10 readings from firm contact of the tonometer tip with the cornea were recorded for each eye. Readings resulting from the tip leaving contact with the eye and instrument-generated averages were ignored, because they have proved unreliable when compared with actual IOP. The average of these readings was recorded as the IOP for that day and was used to calculate the mean IOP in each eye for the course of the experiment.

Animal IOP was measured every 2 days during the week between the first and second episcleral vein injections. To detect the initial rise in IOP, pressures were measured daily for 4 days after the second injection and then every 2 days for the remainder of the study. In seven animals, three each administered betaxolol and apraclonidine and one administered artificial tears, daily measurements were maintained throughout the study.

The mean IOP (± SD) was calculated for experimental and control eyes from the time of the first injection to the time each animal was killed. The mean experimental and control IOPs for each group were then determined and compared using Student's t-test and one-way analysis of variance. To assess the duration of IOP elevation in each animal, all pressure readings in which the IOP of experimental eye was greater than that of the control by ≥3 mm Hg were noted, and the mean pressure over this time period was calculated.

Seventeen days after the second injection, animals were anesthetized with halothane and were perfused transcardially with 100 ml 4% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) after the intracardiac injection of heparin (1 ml/kg) containing 10 mg/ml sodium nitroprusside. Eyes with attached optic nerves were removed and were immersed in 5% glutaraldehyde for 24 hours at 4°C. Optic nerve segments 1 mm from the back of the globe were dissected, washed with fresh buffer, postfixed in 1% OsO4 in 25 mM potassium ferricyanide, dehydrated with ascending ethanol and aceton, and embedded in Spur's low-viscosity resin. Sections (1 μm) were cut on a Reichert Ultracut E microtome (Reichert AG, Vienna, Austria) and were stained with 1% toluidine blue. Optic nerve cross-sections from all injected eyes were assessed for damage by photographing sections on a Zeiss Photomicroscope III (Zeiss, Jena, Germany), by printing them to a final magnification of ×620, and by assembling them into a final montage.7 The identical sections were scanned twice at 100× by two masked observers, who marked the location of swollen axons and of axonal and myelin debris on the montages. Zones of axonal degeneration, defined as areas containing three or more affected axons separated by no more than 10 μm, were outlined on the montage, and these areas were quantitated on a Kontron (Munchen, Germany) image analysis system. By adding together these areas plus the areas of isolated degenerating or swollen axons, a semiquantitative measure of optic nerve injury was determined for each nerve cross-section. The total lesion area then was expressed as a percentage of the total optic nerve area.

RESULTS

Mean pretreatment IOP was 29 ± 2 mm Hg for all un.injected control eyes and 28 ± 2 for all experimental eyes. These values

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Preinjection</th>
<th>Postinjection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artificial Tears</td>
<td>29 ± 3</td>
<td>29 ± 2</td>
</tr>
<tr>
<td>Betaxolol</td>
<td>29 ± 3</td>
<td>29 ± 2</td>
</tr>
<tr>
<td>Apraclonidine</td>
<td>28 ± 3</td>
<td>29 ± 4</td>
</tr>
</tbody>
</table>

*The postinjection mean is for the 17 days after the second episcleral injection.

Values are mm Hg ± SD.
TABLE 2. Mean IOP After the Second Injection, Duration (and Mean) of Experimental Eye IOP Compared† with Fellow Eye, and Mean Maximum IOP Correlated with the Percentage of Area Damage of the Optic Nerve

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Mean IOP*</th>
<th>Mean Elevated IOP</th>
<th>Days</th>
<th>Maximum IOP</th>
<th>Damage</th>
</tr>
</thead>
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<tr>
<td></td>
<td>(mm Hg ± SD)</td>
<td>(mm Hg ± SD)</td>
<td></td>
<td>(mm Hg)</td>
<td>(%)</td>
</tr>
<tr>
<td>Artificial tears</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>128</td>
<td>35 ± 8</td>
<td>41 ± 6</td>
<td>11</td>
<td>46</td>
<td>100</td>
</tr>
<tr>
<td>129</td>
<td>40 ± 8</td>
<td>45 ± 7</td>
<td>13</td>
<td>47</td>
<td>100</td>
</tr>
<tr>
<td>135</td>
<td>41 ± 7</td>
<td>43 ± 5</td>
<td>15</td>
<td>48</td>
<td>100</td>
</tr>
<tr>
<td>149</td>
<td>40 ± 11</td>
<td>47 ± 2</td>
<td>11</td>
<td>51</td>
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<tr>
<td>153</td>
<td>39 ± 6</td>
<td>41 ± 5</td>
<td>16</td>
<td>48</td>
<td>100</td>
</tr>
<tr>
<td>175</td>
<td>38 ± 4</td>
<td>39 ± 3</td>
<td>13</td>
<td>42</td>
<td>6.1</td>
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<td>Apraclonidine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>137</td>
<td>22 ± 4</td>
<td></td>
<td>11</td>
<td>39</td>
<td>1.3</td>
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<tr>
<td>150</td>
<td>32 ± 9</td>
<td>40 ± 6</td>
<td>0</td>
<td>31</td>
<td>0.4</td>
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<tr>
<td>171</td>
<td>28 ± 4</td>
<td>38 ± 0</td>
<td>9</td>
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<tr>
<td>174</td>
<td>38 ± 6</td>
<td>40 ± 5</td>
<td>0</td>
<td>26</td>
<td>0.35</td>
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<tr>
<td>176</td>
<td>23 ± 4</td>
<td></td>
<td>2</td>
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<td>7.07</td>
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<td>Betaxolol</td>
<td></td>
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<td></td>
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<tr>
<td>151</td>
<td>31 ± 7</td>
<td>35 ± 3</td>
<td>0</td>
<td>28</td>
<td>0.14</td>
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<tr>
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<td>12</td>
<td>45</td>
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<tr>
<td>168</td>
<td>34 ± 6</td>
<td>40 ± 4</td>
<td>1</td>
<td>38</td>
<td>1.7</td>
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<tr>
<td>177</td>
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<td></td>
<td>12</td>
<td>46</td>
<td>100</td>
</tr>
<tr>
<td>178</td>
<td>29 ± 4</td>
<td>37 ± 0</td>
<td>0</td>
<td>29</td>
<td>0.53</td>
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</tbody>
</table>

* IOP, intraocular pressure.
† IOP was elevated 3 mm Hg or more.
‡ These IOP measurements were taken during the 17-day period after the second injection.
Optic Nerve Protection with Topical Glaucoma Therapy

FIGURE 2. Representative high-power views of optic nerves from three experimental optic nerves. (A) R151 (betaxolol) shows a normal-appearing nerve cross-section, compared with extensive degeneration of (B) R129 (artificial tears). (C) R171, administered apraclonidine, measured 100% damage, but it shows many more normal-appearing fibers than B. Magnification, ×620.

control remains the mainstay of current glaucoma therapy. However, little is known about the mechanism by which pressure damages optic nerve fibers, mostly as a result of the lack of a readily available, cost-effective animal model of pressure-induced optic nerve damage.

A successful glaucoma model must be anatomically relevant to the primate and amenable to the noninvasive measurement of IOP, and it must produce nerve damage with an abnormality similar to that seen in human glaucoma and provide opportunities to test the clinical efficacy of current and future glaucoma therapies. Our model meets these requirements, as illustrated by the following observations.

The connective tissue lamina cribrosa of the rat has a composition nearly identical to that of the primate. In addition, unmyelinated nerve fibers in the rat and primate optic...
nerve head are associated intimately with horizontally oriented astrocytes of the lamina cribrosa, and they become myelinated only at its posterior margin.13-15

We and others16-18 have shown that the TonoPen tonometer can be successfully used to measure IOP in the rat eye. TonoPen readings correlate linearly with actual IOP, as determined with a transducer in cannulated eyes.16,17 and repeated measurements can be performed over months without apparent ocular side effects or alteration of IOP.18 When used on awake rats, this instrument can even record subtle, physiological variations of IOP, including its circadian fluctuation during a 24-hour period.18

The IOP elevation in our model produces a progressive cupping of the optic nerve head19 and the appearance of extracellular materials at the level of the lamina cribrosa,20 similar to that described in glaucomatous eyes in humans and in primate models.21,22 The degeneration of nerve fibers occurs in a reproducible pattern,7 suggesting that this model eventually may provide important clues to the forces that underlie regional susceptibility in human glaucoma.23

The present experiments further support the usefulness of this model by showing that topical glaucoma therapy applied on a repeated basis can chronically control IOP. With the protocol used here, each experimental eye treated with artificial tears had a mean IOP during the entire period of drug treatment that was at least 6 mm Hg above that of the fellow eye. In contrast, only one eye administered betaxolol, and no apraclonidine eyes, had a similar IOP elevation. Considering only the time period in which the experimental eye IOP was elevated 3 mm Hg or more above the control eye, all six animals in the artificial tears group demonstrated mean IOPs of at least 39 mm Hg for a minimum of 11 days, whereas a similar combination of pressure and duration of elevation was seen in only two animals administered betaxolol and one administered apraclonidine.

In our initial study of awake IOP in Brown Norway rats, we found that the mean IOP in daylight was approximately 19 mm Hg, and in darkness it was 31 mm Hg.8 In the present study, the mean pretreatment IOPs for all eyes and the IOP for control eyes throughout the experiment were 28 and 29 mm Hg, respectively. IOP measurements after 6 PM showed similar results. The reason for the difference is that animals were housed in an environment of constant, low-intensity light, which produces a mild constant elevation of IOP. A similar effect has been reported in rabbits.10 We chose this protocol because the resultant mild IOP elevation improved the chance that episcleral injections would consistently produce a further pressure rise, as shown here by our 100% success rate in elevating pressure in the animals administered artificial tears.

It is unlikely that the constant levels of light used in these experiments influenced the effect of the glaucoma drugs on IOP because similar drug effects were found in animals housed in a regular light/dark environment.6 It is also unlikely that constant light alone affected our nerve damage findings because none of the control eyes showed damage greater than that previously reported in normal animals7 and because all eyes, regardless of drug treatment, were exposed to the same levels of light. Retinal phototoxicity is also an unlikely influence because eyes of pigmented rats exposed to similar light levels for as long as a year do not show histologic evidence of retinal damage.24 The total duration of light exposure in our animals was no greater than 2 months.

In addition to demonstrating that glaucoma therapy can control eye pressure, our results show that this control effectively protects optic nerve fibers. In the artificial tears group, all but two of the nerves showed degeneration involving 100% of the optic nerve cross-section. In contrast, only one nerve in the apraclonidine group and two in the betaxolol group showed this degree of damage.

Table 2 reveals that the eyes of the three glaucoma treatment nerves with 100% nerve damage attained maximal IOPs of 45, 45, and 46 mm Hg. In the remaining seven eyes, with less than 10% damage, the maximum IOP was less than 39 mm Hg, which is more than 2 SD below the maximal IOP in the artificial tears group. Glaucoma therapy protected optic nerves in those eyes in which it controlled IOP.

This study illustrates several important points for using animal models to evaluate optic nerve protection. Animals administered artificial tears gained weight steadily over the course of this study, indicating that the animal injections, regular IOP measurements, and frequent examinations had little effect on their well-being. By contrast, both groups administered glaucoma therapy lost weight, suggesting a systemic effect from the drops.

Some of this effect may be a result of the relatively high doses of medication used. Our purpose was to control IOP in this model and to determine whether this control would protect optic nerve fibers. To accomplish this, each eye received two hypertonic saline injections that elevated IOP in all the animals administered artificial tears. Because this treatment also produces a relatively high level of pressure, we chose dosages to maximize the chance that drug treatment could effectively control IOP. Preliminary experience indicated that this was sufficient to lower awake IOP in rat eyes after episcleral vein injections of hypertonic saline,9 but it did not affect IOP in the fellow, uninjected eye. This is supported by our present findings that mean IOP in control eyes was unaffected by either medication and was similar to the control IOP in the animals administered artificial tears.

Because of the relative dosages used here, our findings cannot be applied directly to humans. Determining in our model drug effects similar to what may be expected in humans would require a dose-response study, beginning with the dosages used here. Although such a study is beyond our original purpose, it would be a logical extension of the present findings, which show evidence that these drugs protect the optic nerve in our model.

It is unlikely that systemic drug effects produced the optic nerve protection seen here. Only three of the animals administered glaucoma medications developed a sustained rise in IOP comparable to that seen in the artificial tears group. Because each of these animals developed a 100% lesion, it seems that the drugs alone do not protect nerve fibers in the face of elevated IOP. A fourth animal, R137, had less than 10% damage and a pressure rise for 11 days. However, mean pressure over this time period was 35 mm Hg, lower than that seen for any of the animals that sustained 100% damage, regardless of treatment history. With the exception of the weight change and occasional agitation, no sluggishness or behavioral changes were noted that were suggestive of significant systemic hypotension. Even if this had occurred, it would have been expected to promote damage to nerve fibers, not to protect them.
As in glaucoma in humans, nerve damage in this model depends on the duration and the degree of the IOP elevation. In the present study, the extent of damage seemed to correlate with peak IOP readings, and it was possible to identify a threshold IOP below which nerves were relatively protected. In addition, the comparison of nerve damage with the duration of IOP elevation and mean pressure during this time period indicates that 100% damage results from mean pressure elevations of approximately 10 mm Hg or more above that of the fellow eye for at least 9 days. Although pressure increases for animals R168 and R176 were in this range, these lasted no more than 2 days and neither animal showed extensive nerve damage. Conversely, animal R137 had an 11-day pressure rise, but only to a mean of 35 mm Hg.

As a result of the short duration of this study, the time of exposure to the pressure elevation varied among animals by a few days. Within such short periods, pressure fluctuation and other factors have a greater influence on the degree of nerve damage, increasing the variability. More prolonged studies using milder pressures have more successfully demonstrated this effect.

How we define the extent of IOP elevation and the nerve damage will influence these correlations. As with all situations of increased outflow resistance, we found greater IOP fluctuation in eyes with elevated IOP. Although it is conceivable that an unusually high IOP at the beginning of the study, followed by a minimal elevation for the remainder of the observation period, could produce damage in excess of that expected by the mean IOP, our data failed to reveal this effect in any of the animals.

In Table 2, we quantitated nerve damage by measuring the percentage of the area of the nerve cross-section involved with axon damage. Using this definition, damage tends to group either at 100% or at less than 10%. Figure 2 shows that, within some nerves with 100% injury from eyes administered glaucoma therapy, there still existed many viable-looking axons compared with nerves from eyes administered artificial tears. This type of analysis is designed to detect early nerve damage, and the strong correlation between IOP control and nerve damage suggests that it is adequate to demonstrate the protective effects of glaucoma drops for the relatively short duration of this experiment. Different measures of optic nerve damage may be required in future studies using more prolonged treatment periods.

Finally, two animals administered artificial tears did not have extensive nerve damage despite IOP elevations comparable to the other animals in this group. Although a longer observation period may have allowed more extensive damage to occur, the relative nerve sparing in these eyes reflect a variation in susceptibility among individual nerves. A better understanding of why nerves vary in susceptibility in our model eventually may provide insight into similar situations, such as ocular hypertension, that exist in humans.

References