Minocycline Delays Death of Retinal Ganglion Cells in Experimental Glaucoma and After Optic Nerve Transection

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Objective: To evaluate the effect of minocycline hydrochloride on the survival of retinal ganglion cells (RGCs) in glaucomatous rat eyes and rat eyes after optic nerve transection (ONT).

Methods: The effect of intraperitoneal injections of minocycline at dosages of 15 mg/kg per day, 22 mg/kg per day, and 45 mg/kg per day was evaluated and compared with saline in ONT (n=174) and experimental glaucoma (n=51).

Results: The mean±SEM survival rate of RGCs 1 week after ONT was significantly higher with minocycline at dosages of 15 mg/kg per day (36%±3%; n=9; P=.04), 22 mg/kg per day (44%±2%; n=15; P=.001), and 45 mg/kg per day (39%±3%; n=10; P=.008) compared with saline (29%±2%; n=28). Minocycline at a dosage of 22 mg/kg per day was also significantly neuroprotective compared with saline 2 weeks after ONT (mean±SEM survival rate, 5%±1% vs 3%±0.4%, respectively; n=20 [10 rats in each group]; P=.03). In experimental glaucoma, the mean±SEM percentage of RGCs after 4 weeks was 84%±4% in the minocycline group (n=15) compared with 65%±4% in the saline group (n=18) (P=.003). Apoptosis of RGCs was significantly delayed by minocycline 4 days and 1 week after ONT.

Conclusion: Minocycline significantly enhances the survival of RGCs after ONT and in experimental glaucoma by delaying the apoptosis pathway.

Clinical Relevance: The safety record of minocycline and its ability to penetrate the blood-brain barrier suggest that this drug is a promising neuroprotective drug for optic nerve injuries.

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Glaucoma is the second leading cause of blindness worldwide. Both glaucoma and optic nerve transection (ONT) lead to retinal ganglion cell (RGC) death by apoptosis. Considerable research efforts are being devoted to the search for compounds with the ability to delay and prevent apoptosis of neurons and RGCs. With respect to glaucoma research, there is interest in neuroprotective agents that could supplement the proven but incomplete therapeutic effect of lowering intraocular pressure (IOP).

It has recently been shown that RGC injury in ONT and experimental glaucoma involve activation of the caspase enzyme family, especially caspase 3 and caspase 8, as well as activation of the mitogen-activated protein kinase pathway, including p38 mitogen-activated protein kinase and c-Jun, after ONT and in experimental glaucoma. Another factor implicated in the pathophysiology of RGC diseases, including glaucoma, is nitric oxide. Minocycline hydrochloride, a second-generation tetracycline, was recently found to be neuroprotective in many animal models of neuronal injury. This drug effectively crosses the blood-brain barrier and is commonly used in humans because of its beneficial antimicrobial and anti-inflammatory actions. Interestingly, it also has remarkable neuroprotective qualities in animal models of cerebral ischemia, traumatic brain injury, Huntington disease, and Parkinson disease. Its neuroprotective effect is associated with marked inhibition of inducible nitric oxide synthase, caspase 1 and caspase 3 expression, and p38 mitogen-activated protein kinase. Minocycline also inhibits cytochrome c release and delays progression of amyotrophic lateral sclerosis in mice, and it depresses oxygen free radical release and matrix metalloproteinase activity. Recently, mino-
cycline was also found to be neuroprotective in models of photoreceptor death.\textsuperscript{30-32}

While minocycline has been shown to be protective in cerebral disease models, there are no data on its effect on RGC survival. Because it may inhibit members of the caspase family, the mitogen-activated protein kinase pathway, and nitric oxide synthase, factors that are important in RGC apoptosis, we hypothesized that it may delay the death of RGCs in experimental glaucoma and after ONT. The purpose of this study was to investigate whether minocycline has a neuroprotective effect on the survival of RGCs in 2 models of optic nerve injury: experimental glaucoma and ONT.

### METHODS

#### ANIMALS

Wistar rats (n = 289) weighing 330 to 400 g were treated with procedures approved and monitored by the Animal Care Committee, Tel Aviv University School of Medicine, Tel Aviv, Israel, and following the procedures outlined in the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were housed with a 14-hour light and 10-hour dark cycle, standard chow, and water ad libitum.

#### EXPERIMENTAL GLAUCOMA

Experimental glaucoma was induced 3 days following the initiation of treatment. Elevated IOP was induced in 1 eye in each of 51 animals by treating the outflow channels of the rat eye through the peripheral cornea with a diode laser (Coherent Radiation, Clement-Ferrand, France).\textsuperscript{33} Treatment was repeated at 1 week if the difference in IOP between the rat’s 2 eyes was less than 6 mm Hg. The IOP was measured under anesthesia, and we recorded the average of 10 readings with the TonoPen XL tonometer (Mentor, Norwell, Mass). The IOP measurements were taken immediately before, 1 day after, and 3 days after each treatment, then weekly. Rats were sacrificed at 2 and 4 weeks after the first laser treatment.

#### OPTIC NERVE TRANSECTION

Optic nerve transection was performed 3 days following the initiation of treatment. The surgical procedure was performed unilaterally under anesthesia with intraperitoneal (IP) ketamine hydrochloride (50 mg/kg), IP xylazine hydrochloride (3 mg/kg), and topical 1% proparacaine hydrochloride eye drops. The optic nerve was transected by a diamond knife 3 to 4 mm behind the globe, taking care not to interfere with the blood supply. The retinas were examined ophthalmoscopically to assure blood vessel patency.

#### IP MINOCYCLINE AND SALINE TREATMENT GROUPS

The rats were randomly divided into a minocycline (Sigma-Aldrich Corp, St Louis, Mo) treatment group and a saline treatment group (controls). Treatment (minocycline or saline) was given by IP injections initiated 3 days before injury and continuing daily until sacrifice. The neuroprotective effects of different dosages of minocycline were tested 1 week after ONT; the 3 dosages were 15 mg/kg per day (9 rats), 22 mg/kg per day (15 rats), and 45 mg/kg per day (10 rats). These groups were compared with a daily saline injection group (28 rats), which was also sacrificed at 1 week. The minocycline dosage of 22 mg/kg per day was compared with saline at 2 additional time points after ONT: at 4 days (7 rats in each group) and 2 weeks (10 rats in each group). The minocycline dosage of 22 mg/kg per day was also investigated 2 and 4 weeks after the induction of glaucoma.

Nineteen additional rats composed a group of pure controls (ie, no treatment and without injury). This group was used to calculate the percentage of RGC loss in the treatment groups (discussed later).

#### ORAL TREATMENT

The bioavailability of oral minocycline is 90% of IP delivery,\textsuperscript{34} with an oral dosage of 25 mg/kg per day achieving the same plasma level as an IP dosage of 22 mg/kg per day. Each rat habitually drank a mean amount of 30 mL of water per day in our animal care unit. A minocycline dosage of 25 mg/kg per day was prepared by dissolving the minocycline in their drinking water. Control animals were given plain water. The bottles were changed daily, and the rats were carefully observed to monitor their drinking. Because the minocycline was dissolved in the drinking water of rats that were kept 3 per cage, it was difficult to accurately assess how much water each rat drank.

#### RGC BODY LABELING WITH RHODAMINE DEXTRAN

The RGCs were labeled by backfilling with rhodamine dextran (Molecular Probes, Eugene, Ore) applied to the orbital optic nerve 1 day before sacrifice. The rats were anesthetized as mentioned earlier, and ONT was performed unilaterally in the experimental eye 1 to 2 mm behind the globe, ie, closer to the globe than the initial ONT. In eyes that had initial ONT as the insult and fibrosis that had developed at this area, the fibrous tissue was gently removed by fine forceps and the optic nerve was exposed. Twenty-four hours later, the eyes were enucleated and the retinas were prepared as whole mounts. The rats were sacrificed by IP pentobarbital sodium (60 mg/mL). The surgeon (H.L.-V.) was masked to the drug assignment group in all of the procedures.

#### RGC COUNTING PROCEDURE

Retinal whole mounts were viewed with an Olympus BX51 fluorescence microscope (Olympus Optical Co, Tokyo, Japan) and an appropriate filter to identify rhodamine dextran–labeled cells. Labeled RGCs were counted with a 40X superwide field objective along 4 radii in 4 directions (ie, superior, temporal, inferior, and nasal areas) centered on the position of the optic nerve head. Three fields were counted along each radius, yielding a total of 12 fields per retina. The counting process was performed by an experienced observer who was masked to the procedure that had been performed and to the treatment that was given.

#### HOECHST STAINING FOR APOPTOSIS

To investigate the effect of the minocycline dosage of 22 mg/kg per day on RGC apoptosis, the rats were treated with daily IP injections of either a minocycline dosage of 22 mg/kg per day or saline beginning 3 days before they underwent ONT. The animals were sacrificed at different time points after ONT, and both eyes were cryopreserved. Ten-micrometer retinal cryosections were labeled with cell-permeable form of bisbenzimide (Hoechst 33342; Molecular Probes), which stains the condensed chromatin of apoptotic cells.
cells, at a concentration of 4 µg/mL for 30 minutes at 37°C. The cells were visualized with a UV fluorescent microscope, and the number of apoptotic cells with condensed and fragmented chromatin was counted in each section, 4 sections per eye. In addition, the total number of nuclei in the RGC layer was counted and the proportion exhibiting apoptosis was calculated per eye. The counting process was done by a masked observer. We refer here to the RGC nuclei as those identified by their presence in the RGC layer and by their size and morphology. It is possible that some of the cells identified in the RGC layer were amacrine cells.

DATA ANALYSIS

The area of each field in our microscope is 0.34 mm², yielding a total counted area of 4.1 mm², which is an 8% sample of the average 50.1-mm² Wistar rat retina. The total number of surviving RGCs per retina was calculated by multiplying the mean density of RGCs by the total retinal area. The number of RGCs in each rat retina was compared with pooled control values to yield the survival rate. Data are presented as means±standard errors of the mean. The neuroprotective effect of minocycline was evaluated for each time point by comparing the number of surviving RGCs in the minocycline-treated group with the number of surviving RGCs in the saline-treated group using paired and unpaired t-tests with a significance level set at P<.05.

RESULTS

Of the original 293 Wistar rats, 16 died within 24 hours after being anesthetized (9 in the saline group and 7 in the minocycline group), leaving a total of 273 animals.

RGC DENSITY

The mean±SEM for RGC density as measured in 38 normal eyes of 19 rats was 1507±65 cells/mm² (75 500±3256 cells/retina). Thus, the number of RGCs identified by rhodamine dextran is within the range of the reported normal number of RGCs in Wistar rats.35,36
DOSE-DEPENDENT EFFECT OF IP INJECTIONS OF MINOCYCLINE

One week after ONT, each dose of minocycline, ie, 15 mg/kg per day, 22 mg/kg per day, and 45 mg/kg per day, was significantly neuroprotective compared with saline injections ($P = .04$, .001, and .008, respectively) (Table 1). The greatest protective effect by minocycline was achieved at 22 mg/kg per day. The mean±SEM RGC survival rate with IP minocycline administration of 22 mg/kg per day was 44±2% compared with 29±2% in the saline-treated group at 1 week after ONT (Figure 1). The highest minocycline dosage (45 mg/kg per day) was significantly more neuroprotective than the dosage of 15 mg/kg per day but less neuroprotective than the dosage of 22 mg/kg per day.

NEUROPROTECTIVE EFFECT OF IP MINOCYCLINE OVER TIME

Because the best neuroprotective effect was achieved with a minocycline dosage of 22 mg/kg per day, we studied this dose in greater detail at 2 more time points: 4 days and 2 weeks after ONT. At 4 days following ONT, the mean number of RGCs per retina in the group that received a minocycline dosage of 22 mg/kg per day was higher than in the saline-treated group, but not significantly ($P = .40$) (Table 1 and Figure 1). At 2 weeks, the mean number of RGCs per retina in the group that received a minocycline dosage of 22 mg/kg per day was significantly higher than in the saline-treated group ($P = .03$) (Table 1 and Figure 1).

EFFECT OF ORAL MINOCYCLINE

With the oral minocycline dosage of 25 mg/kg per day (theoretically similar to the IP dosage of 22 mg/kg per day), there was a trend toward greater RGC survival, but the difference was not statistically significant ($P = .27$) (Figure 2). The mean±SEM number of RGCs per retina at 1 week after ONT was 29 754±2230 RGCs (n = 16) in the minocycline group (n = 16) compared with 25 718±2892 RGCs in the water-drinking group (n = 17) ($P = .30$).

NEUROPROTECTIVE EFFECT OF IP MINOCYCLINE IN EXPERIMENTAL GLAUCOMA

All of the experimental eyes had significantly elevated IOP compared with the control eyes (Table 2). This was evident both in mean IOP and peak IOP. The mean and peak IOPs were similar in the minocycline and saline groups, without any effect of minocycline on IOP. Two weeks following the induction of glaucoma, the mean number of RGCs per retina in the group that received a minocycline dosage of 22 mg/kg per day was higher than in the saline-treated group, but not significantly (mean±SEM number of RGCs per retina, 64 151±3594 RGCs [n = 11] vs 60 258±6589 RGCs [n = 7], respectively; $P = .58$) (Figure 3 and Figure 4). At 4 weeks, the mean number of RGCs per retina in the group that received a minocycline dosage of 22 mg/kg per day was significantly higher than in the saline-treated group (mean±SEM number of RGCs per retina, 63 527±3395 RGCs [n = 15] vs 49 032±2928 RGCs [n = 18], respectively; $P = .003$). Between 2 and 4 weeks after the laser treatment, there was a substantial RGC loss in the saline-treated group, but almost none in the minocycline group. The short-term effect of minocycline was neuroprotective, but this was not significant. In our experimental model for glaucoma, the mean RGC loss at 2 weeks was variable, and any neuroprotective effect may be masked by the variability between animals. At 4 weeks, when RGC loss was substantial, minocycline was significantly neuroprotective.

Table 2. Intraocular Pressure History in Rats With Experimental Glaucoma

<table>
<thead>
<tr>
<th>Time Point</th>
<th>IOP, Mean ± SD, mm Hg</th>
<th>Peak IOP, Mean ± SD, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glaucoma</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>Glaucoma</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>IOP, Mean ± SD, mm Hg</td>
<td>Peak IOP, Mean ± SD, mm Hg</td>
</tr>
<tr>
<td>2 wk (n = 18)</td>
<td>29.9 ± 2.5</td>
<td>21.4 ± 1.9</td>
</tr>
<tr>
<td>4 wk (n = 33)</td>
<td>30.1 ± 2.2</td>
<td>22.3 ± 1.2</td>
</tr>
</tbody>
</table>

Abbreviation: IOP, intraocular pressure.
The effect of minocycline on apoptosis of RGCs was evaluated at 3 time points by comparing the mean percentages of apoptosis between the minocycline and saline groups using Hoechst staining (Table 3 and Figure 5).

Four days after ONT, there were only half as many apoptotic cells in the RGC layer of minocycline-treated eyes compared with control eyes. At this time point, the mean total number of cells (apoptotic and normal) identified in the RGC layer was equivalent in the minocycline and control groups since the major phase of cell death had likely not started (as seen in Table 1, only one third of RGCs or fewer are not labeled 4 days after ONT). Therefore, the percentage of all of the nuclei in the RGC layer that were Hoechst positive (dividing the number that were positive by the total nuclear count) was also only half in the drug-treated eyes.

One week after ONT, the mean number of apoptotic RGCs was slightly lower than in controls, but not significantly (P = .37). The mean number of total nuclei in the RGC layer was significantly higher in the minocycline group, confirming the protective effect seen when RGCs were specifically identified by backfilling (Table 1). When the number of apoptotic cells was divided by the total number of nuclei to give the percentage of cells exhibiting apoptosis, the 1-week group also showed a significant reduction in apoptosis (Table 3).

Two weeks after ONT, the number and percentage of nuclei in the RGC layer with condensed chromatin were dramatically increased over either 4-day or 1-week time points, indicating that the wave of RGC death was maximal at this time. The difference between minocycline-treated and control groups was not significant (Table 3). Interestingly, the number of visible nuclei was only modestly reduced compared with the 1-week time point, at about 50% of normal values. However, the number of RGCs that could be successfully backfilled from the nerve at 2 weeks was reduced by 90% from controls (Table 1).

At 2 weeks after ONT, there may be many RGCs that have not yet begun to undergo overt nuclear condensation but nonetheless have dysfunctional axons and do not backfill with rhodamine dextran.

### ADVERSE EFFECTS

Rats treated with minocycline seemed to behave more agitatedly right before injections (note that the minocycline solution has a lower pH than the saline solution, and this feature could make the former more painful). Otherwise, all of the rats behaved similarly and gained a similar amount of weight. No ocular adverse effects were detected.

### COMMENT

In this study, we found that minocycline, a commonly used drug in humans, is neuroprotective in 2 models of optic nerve injury: glaucoma and ONT. Optic nerve transection represents the most severe form of optic nerve injury, leading to an almost total loss of RGCs at 2 weeks largely due to apoptosis. Consequently, any potential neuroprotective drug must be very powerful to de-
lay the apoptotic cascade in this type of injury, and even a delay in cell death may indicate a possible benefit. We found that minocycline can significantly enhance the survival of RGCs in this severe injury model. The neuroprotective effect of minocycline was statistically significant at 1 and 2 weeks after ONT. Additionally, minocycline was significantly neuroprotective in an experimental model of glaucoma. Compared with ONT, glaucoma is a progressive disease of the optic nerve with a more gradual apoptotic loss of RGCs.\(^2\) Two weeks after the induction of glaucoma, minocycline enhanced the survival of RGCs, but not significantly. However, 2 weeks later, there was a progressive loss of RGCs in the control group but a significantly delayed RGC loss in the minocycline-treated group.

The smallest protective effect was achieved with the lowest minocycline dosage (15 mg/kg per day) whereas greater protection was achieved with the dosages of 22 mg/kg per day and 45 mg/kg per day. The dose-response relationship of the 3 doses was not classic, as the highest dose had a slightly lower benefit than the middle dose. The 45-mg/kg solution was, however, highly concentrated, and the solubility of the minocycline content may not be optimal. The dose range selected in our study was based on past descriptions in various central nervous system disease models. A minocycline dosage of 5 mg/kg per day delayed disease progression and inhibited caspase 1 and caspase 3 mRNA up-regulation in a mouse model of Huntington disease.\(^2\) A minocycline dosage of 10 mg/kg per day delayed the disease onset and extended the survival in another model of amyotrophic lateral sclerosis in mice.\(^2\) In mice subjected to traumatic brain injury, however, only higher minocycline dosages (45 mg/kg per day) were effective in reducing tissue injury, neurological deficits, and activation of caspase 1.\(^2\) The same high dosage reduced cortical infarction volume and inhibited activation of microglia in a rat model of transient middle cerebral artery occlusion.\(^2\) Even higher dosages of minocycline (90-180 mg/kg per day) were used to inhibit global brain ischemia in rats, and they did so without adverse effects.\(^2\)

We could not show that oral treatment with minocycline was significantly protective, although survival of the RGCs in the minocycline group was numerically higher than in controls. The oral treatment was given by dissolving minocycline in drinking water to try to conduct an experiment that can easily be repeated in primates. Perhaps the effect would be more demonstrable with consistent dosing by gavage.

We chose to begin minocycline treatment 3 days before ONT to achieve a sufficient drug level before injury. Interestingly, in models of central nervous system disease and injury, minocycline was neuroprotective when administered before or even shortly after the onset of injury, but the effect was more marked when given before the injury.\(^1,2\) Minocycline is not the only tetracycline with known neuroprotective effects. Doxycycline, another semisynthetic second-generation tetracycline, is also known for its neuroprotective effect.\(^30,31\) Like minocycline, it is also well tolerated by humans and penetrates the blood-brain barrier. We chose to investigate minocycline because its neuroprotective effect was better than that of doxycycline in most studies.

The specific mechanism by which minocycline induces its neuroprotective effect on RGCs is unknown. Now that a benefit has been shown in models of optic nerve injury, the pathways that may be operative should be investigated in detail. In this study, we demonstrated that minocycline significantly delays apoptosis of RGCs after the severe injury of ONT. More work should be done to explore the specific mechanisms involved in this neuroprotective effect.

To our knowledge, our study is the first to show that minocycline can delay apoptosis and RGC death in glaucoma and after ONT. This study supports findings in previous articles about the neuroprotective effect of minocycline in other ocular injury and disease models, including photoreceptor death\(^30,31\) and retinas of diabetic mice. To evaluate the neuroprotective effect of minocycline in patients with glaucoma, we must first investigate its effect on experimental glaucoma in primates, including evaluation of the best dosage and route of administration. Nevertheless, we believe that the safety record of minocycline and its ability to penetrate the blood-brain barrier make it an attractive candidate for further investigation.
brain barrier suggest that this drug is a promising neuroprotective drug for treating diseases leading to RGC death.

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