Effect of Intraocular Pressure on Optic Disc Topography, Electroretinography, and Axonal Loss in a Chronic Pressure-Induced Rat Model of Optic Nerve Damage

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PURPOSE. To characterize the effect of intraocular pressure (IOP) on optic disc topography, retinal function, and axonal survival in a model of IOP-induced optic nerve damage in rat.

METHODS. Hypertonic (1.75 M) saline was injected into an episcleral vein of one eye of 49 Brown Norway rats, with the fellow untreated eye serving as the control. During the 1 to 3 months of follow-up, IOP was measured twice weekly in conscious animals with a handheld tonometer, and changes in disc topography and retinal function were monitored with scanning laser tomography and electroretinography (ERG), respectively. Peak IOP elevation in the experimental eye compared with the fellow control eye (peak ΔIOP), integral of IOP elevation over time (ΔIOP integral), and days of IOP elevation were calculated. Axon counts were obtained from electron micrographs of the sectioned optic nerves.

RESULTS. Progressive cupping was found in 9 (56.3%) of 16 eyes with peak ΔIOP of more than 15 mm Hg and in none of 21 eyes with peak ΔIOP less than 15 mm Hg. A strong correlation between ΔIOP integral and progressive cupping was also found, but not with days of IOP elevation. ERG abnormalities (limited to the b-wave) were found in 11 (64.7%) of 17 eyes found, but not with days of IOP elevation. ERG abnormalities were best correlated to peak ΔIOP, integral of IOP elevation over time (ΔIOP integral), and days of IOP elevation were calculated. Axon counts were obtained from electron micrographs of the sectioned optic nerves.

CONCLUSIONS. Structural and functional changes in this model are best correlated to peak ΔIOP and not to duration of IOP elevation, suggesting the existence of an IOP-related damage threshold. (Invest Ophthalmol Vis Sci. 2002;43:2969–2976)

Elevated intraocular pressure (IOP) is the most important known risk factor for the development of open-angle glaucoma.1 Prevalence data show that persons with IOP between 25 and 29 mm Hg are approximately 13 times more likely to have glaucoma than those with IOP less than 16 mm Hg,2 whereas incidence data show that the risk of developing glaucoma in persons initially with IOP of 24 mm Hg or more is approximately 11 times higher than the risk in those with IOP less than 16 mm Hg.3 More recent evidence also suggests that IOP is a significant factor, even in patients with glaucoma who have statistically normal IOP.4,5 Clinicopathologic studies show significant retinal ganglion cell (RGC) loss, a hallmark of glaucoma, in patients with statistically abnormal IOP but normal visual fields.6

The noxiousness of IOP across the spectrum of glaucoma means that knowledge of its effects on the optic nerve and retina is critical for understanding and treating the disease. To this end, models of pressure-induced optic neuropathy have been developed in experimental animals. Laser-induced damage to the trabecular meshwork to elevate IOP in monkeys7–10 has been used by several laboratories. Results of this model have allowed characterization of the nature and extent of RGC loss,11–13 ultrastructural changes in the optic nerve head,14–17 alterations in RGC axoplasmic transport and receptor regulation,18–20 and measurement of electrophysiological21–23 and visual field24 changes.

Although primate models remain the most relevant to human glaucoma, several investigators have used the relatively inexpensive rat models of pressure-induced optic neuropathy. A variety of techniques have been employed to elevate IOP, including injection of hypertonic saline into an episcleral vein to sclerose the trabecular meshwork,25 cautery,26 or laser treatment27 of the episcleral veins, and laser treatment of the trabecular meshwork after intracameral injection of India ink.28 These models have been similarly used to study the effects of IOP elevation on the rat optic nerve and retina in addition to investigating potential avenues for protec- tion.29–31

Optic disc cupping, a hallmark of glaucoma, has been demonstrated in optic nerve sections of monkeys with IOP elevation.32 Although noninvasive imaging of the optic disc is feasible in monkeys, it is more difficult in rats, because of the poorer optics and relatively small pupil size, even when dilated. Cupping, measured noninvasively, has been demonstrated in rats,30,33 but the relationship between the degree of IOP elevation and cupping is not known. The objective of this study was to determine the relationship between a variety of IOP parameters and cupping in a rat model of IOP-induced optic neuropathy, by using a modification of an in vivo imaging technique scanning laser tomography.34 We also report on the relationship between these IOP parameters and functional loss, as measured by electroretinography (ERG).

MATERIALS AND METHODS

Animals

Adult male Brown Norway rats (250–300 g) were housed in a constant low-light (80 lux) environment and given food and water ad libitum. All
procedures complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and ethics approval was obtained from the Dalhousie University Committee on Laboratory Animals. Animals were anesthetized for surgery with a ketamine-xylazine cocktail and killed with an overdose of pentobarbital sodium (340 mg/ml). Both drugs were administered intraperitoneally.

**Method of IOP Elevation**

The animals were allowed to acclimate for 1 week, during which they were handled daily. The first of two episcleral vein injections was then performed, according to the method described by Morrison et al. Briefly, a lateral canthotomy was performed, and a 5.5-mm polypropylene ring with a 1-mm gap placed around the equator. The ring was oriented so that flow in all vessels was blocked with the exception of one episcleral vein. The unobstructed episcleral vein was then exposed by cutting the overlying conjunctiva. The microneedle (a glass micropipette coupled to a tapered polyethylene tube connected to a 1-ml tuberculin syringe with a broken 23-gauge needle) was inserted along the long axis of the vein. To standardize the injection force, we used a syringe pump (SP 2001; World Precision Instruments, Sarasota, FL) and experimentally determined, in a separate set of animals, the flow rate of 50 μl of a hypertonic saline solution (1.75 M NaCl) that just (but consistently) produced blanching of the limbal artery: 250 μl/min. One week later, a second episcleral vein was injected in the same eye. Injections were given in one eye only of each animal, with the fellow eye serving as the untreated control.

**Measurement of IOP**

A handheld tonometer (Tonopen-XL; Innova Corp., North York, ON, Canada) was used to measure IOP in conscious animals. The rats were removed from their cages and handled for a few minutes before measurement. After a drop of topical anesthetic (proparacaine hydrochloride; Alcon Inc., Missisauga, ON, Canada) was instilled, animals were gently cradled in a manner allowing full exposure of the measured eye, but at the same time allowing sufficient restraint. At no time was any external pressure applied to the eye or adnexa during handling. Approximately 10 readings were obtained from each eye, of which the mean was taken as the IOP for the day. A set of baseline IOP measurements was taken before the injection of the episcleral vein. Follow-up measurements were taken twice a week at approximately the same time of day. Before each session, the tonometer was calibrated according to the manufacturer’s instructions. We have previously performed calibration experiments in cannulated rat eyes and have shown close agreement between the actual IOP and tonometer-measured IOP from 15 to 55 mm Hg. The reliability of this method of tonometry in rats has been demonstrated by others.

Three IOP parameters were calculated: peak ΔIOP, defined as the maximum IOP in the experimental eye compared with the fellow untreated eye for the duration of the follow-up; ΔIOP integral (in mm Hg days), defined as the integral of the IOP difference between the experimental and control eyes during the follow-up; and days of IOP elevation, defined as the number of days between the second hypertonic saline injection and the death of the animal.

**Scanning Laser Tomography**

A commercial scanning laser tomograph (Heidelberg Retina Tomograph [HRT]; Heidelberg Engineering GmbH, Dossenheim, Germany) was modified for use in the rat eye. Briefly, the scanning angles of the horizontal and vertical mirror were reduced and the laser output increased. The tomograph was mounted on an operating microscope stand (OPMi 6; Carl Zeiss, Thornwood, NY) and maneuvered with a custom-built remote arm that allowed four degrees of movement (horizontal, vertical, height, and rotation).

The rat was placed in a stereotactic frame (Kopf Instruments, Tujunga, CA) and the pupils fully dilated with 1% cyclopentolate (Diopentolate; Diotpic Laboratories, Markham, ON, Canada). A custom-built glass plano-concave lens was placed on the cornea to neutralize the corneal power and minimize corneal aberration. A ×20 infinity-corrected microscope lens objective (Olympus, Melville, NY) was screwed onto a plastic lens holder fixed onto the objective tube of the tomograph. After centering the optic disc in the image frame, a minimum of three images was obtained. After processing, a mean reflectivity and topography image was obtained for each session. Baseline images were taken for each eye, and follow-up images were taken at approximately 1- to 2-week intervals.

A circular contour line was drawn well outside the optic disc margin in the baseline image. This contour line was imported to subsequent mean images and was checked for proper placement. In images in which the alignment was not accurate, the same size contour line was placed manually. The ratio of cup volume in the final image to that in the baseline image was computed as the index of change in cupping.

**Electroretinography**

Pups were fully dilated (as described earlier) and a silver-impregnated nylon fiber (Retina Technologies, Scranton, PA) was laid down on the cornea as the active electrode. A custom-made plano hard contact lens was then placed on the cornea to prevent drying, with 0.5% methylcellulose as a conductive medium. Platinum subdermal electrodes (Grass Instruments, Quincy, MA) were inserted at the base of the nose (reference) and at the right hind leg (ground). Electrodes were connected to a differential amplifier and the signal amplified 10,000-fold with an opened bandwidth of 3 to 1,000 Hz (P511; Grass Instruments). Three hundred points were digitized, at a sampling rate of 1,000 Hz (A/D InstruNet converter; GW Instruments, Millersville, SC). Averages of five sweeps were recorded with an interstimulus interval of 5 seconds.

A scotopic bright flash response with a well delineated a- and b-wave was obtained with a flash stimulus (10.1 cd/m²; PS3 photostimulator; Grass Instruments) in a 15-cm Ganzfeld bowl. The a-wave amplitude was measured from the baseline to the most negative trough. The b-wave was measured from the a-wave trough to the maximum positive peak that follows that trough. We expressed changes in the a- and b-wave amplitudes in the experimental eye as a ratio of the corresponding values in the fellow control eye. However, to control for electrode impedance, we defined an ERG amplitude ratio in the last ERG examination before sacrifice as Δ(b-wave/a-wave)_{exp}/(b-wave/a-wave)_{con} where exp is the experimental eye and con is the control eye. Hence, a relative decrease in b-wave amplitude in the experimental eye would result in a lower ratio. To determine the distribution of the ERG ratio in normal animals, we tested a separate group of 14 animals under identical conditions.

**Tissue Preparation**

After the animals were killed, eyes were enucleated, taking care to preserve the maximum length of optic nerve. The nerve was marked for orientation and then cut approximately 1 mm behind the globe. The nerve stump was fixed immediately in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) overnight. The stump was rinsed with the buffer, placed in 1% OsO₄, for 2 hours and then in 0.25% uranyl acetate for an additional 2 hours. The nerves were dehydrated with acetone and embedded in Epon Araldite (Mirvac; Halifax, NS, Canada). They were thin sectioned (100–130 nm), poststained with 2% uranyl acetate, and viewed on an electron microscope (Phillips EM300; Phillips, Eindhoven, The Netherlands). After the center of the optic disc was located, three equidistant micrographs (83 × 58 μm) on a prefixed rectangular grid were taken in each quadrant. Axon counts were made by a single observer in micrographs printed on 12.5 × 12.5-cm paper (12 per nerve) with a print magnification of ×4800. The proportion of surviving axons in the experimental eye was expressed as a ratio of the mean number of axons in the experimental versus control eyes. Criteria for intact axons included an intact myelin sheath, visible neurofilament and absence of obvious swelling or shrinkage.
RESULTS

Forty-nine animals were used in the study. The mean ± SD peak ΔIOP was 13.22 ± 7.85 mm Hg, whereas the respective results for the ΔIOP integral and days of IOP elevation were 292.43 ± 263.87 mm Hg days and 60.10 ± 17.86 days, respectively.

Serial optic disc images were obtained in 37 animals with scanning laser tomography. The mean ± SD time difference between the baseline and final image in these animals was 56.69 ± 18.47 days. The relationship between change in cup volume (expressed as a ratio of cup volume in the final-to-baseline images) and the three IOP parameters is shown in Figure 1. The 5th and 95th percentiles of the distribution of the change in cup volume of the fellow untreated eyes is superimposed (Fig. 1) to identify those eyes in which the change in cup volume was outside normal limits. There was a strong association between peak ΔIOP and progressive cupping. None of the 21 eyes that had peak ΔIOP of less than 15 mm Hg exhibited cupping that was statistically significant, whereas 16 eyes that had peak ΔIOP more than 15 mm, 9 (56.3%) showed cupping (Fig. 1, P < 0.001, Fisher’s Exact Test). However, in the latter subgroup, there was no correlation between peak ΔIOP and cup volume ratio (Spearman r = 0.350, P = 0.184). There was also a strong association between ΔIOP integral and progressive cupping. Twenty-five (89.3%) of 28 eyes with ΔIOP integral below 400 mm Hg days had a cup volume ratio within normal limits, whereas 6 (66.7%) of 9 eyes above this cutoff showed progressive cupping (P < 0.001). There was no association between days of IOP elevation and progressive cupping (P > 0.403).

Representative serial optic disc images in animals with and without progressive cupping are shown in Figure 2. The onset of the cupping was usually abrupt, and progression occurred rapidly thereafter. Histopathologically, these eyes showed deep excavation with marked loss of the nerve fiber and RGC layers (Fig. 3). Scleral canal expansion was also evident in both the tomographic images (Fig. 2) and the histologic sections (Fig. 3). In some animals, there were also thinning of the inner plexiform layer; however, the outer retina appeared normal.

There was a weak, but statistically significant, correlation between peak ΔIOP and the ERG ratio (n = 40; r = −0.409, P = 0.009; Fig. 4). The correlations between the other IOP parameters and ERG ratio were not significant (P > 0.342; Fig. 4). Two of the 23 (8.7%) eyes with peak ΔIOP less than 15 mm Hg had an ERG ratio lower than the 5th percentile in normal animals, whereas 11 (64.7%) of the 17 animals with a peak ΔIOP of more than 15 mm Hg had a statistically reduced ERG ratio. Although relatively more eyes with peak ΔIOP higher than 15 mm Hg had abnormal ERG ratios compared with those below this cutoff value (P < 0.001), the other IOP parameters could not effectively segregate those eyes with an abnormal ERG ratio (P > 0.127). There was no relationship between any of the IOP parameters and the a-wave ratio (P > 0.249); however, the b-wave ratio was negatively correlated with peak ΔIOP (r = −0.490; P = 0.001). Representative recordings in animals with and without progressive changes in the ERG are shown in Figure 5.

Electron micrographs were available in 26 animals. There was a strong negative correlation between peak ΔIOP and the proportion of surviving axons (r = −0.751, P < 0.001; Fig. 6) and between ΔIOP integral and the proportion of surviving axons (r = −0.621, P = 0.001; Fig. 6). In addition to reduced axon counts, signs of damage included axonal degeneration, swelling or shrinkage, loss of neurofilament and myelin debris (Fig. 7). Most animals who had both serial optic disc images and axon counts showed a change in cup volume that was statistically within normal limits. A change in cupping was evident only when more than 55% of axons were lost (Fig. 8). Although many animals showed ERG ratios within normal limits with moderate axonal loss, all animals who had more than 70% axonal loss had markedly abnormal ERG ratios (Fig. 9). Similarly, the b-wave ratio was affected only when more than 70% of axons were lost; however, there was no relationship between the a-wave ratio and the proportion of surviving axons (r = 0.301, P = 0.174).

DISCUSSION

Animal models on the effects of chronic elevation of IOP on the optic nerve head are providing important information on the structural changes in both the optic nerve and retina. This information will ultimately lead to a better understanding of the pathogenesis and treatment of the glaucomas. The objective of this study was to characterize the relationship between...
FIGURE 2. Color-coded serial optic disc images showing cupping (top) in an eye with peak ΔIOP of 21.6 mm Hg, ΔIOP integral of 495.7 mm Hg days, and 58 days of IOP elevation. This eye had an ERG ratio of 0.41 and 84% axonal loss. Serial disc images showing absence of cupping (bottom) in an eye with peak ΔIOP of 7.9 mm Hg, ΔIOP integral of 357.9 mm Hg days, and 76 days of IOP elevation. This eye had an ERG ratio of 1.27 and 4% axonal loss.

FIGURE 3. Optic disc image showing deep cupping in an experimental eye (top left) immediately prior to death. This eye had a peak ΔIOP of 20.4 mm Hg, ΔIOP integral of 525.0 mm Hg days, 58 days of IOP elevation, and an ERG ratio of 0.64. Section of the retina and optic nerve in the same eye stained with cresyl violet (top right) showing deep excavation to the disc edge, loss of the nerve fiber and RGC layer, reduced thickness of the inner plexiform layer, and markedly altered pattern of staining in the optic nerve. Disc image (bottom left) and section of the retina and optic nerve (bottom right) of the fellow control eye. Scale bars, 100 μm.
the level of IOP and the ensuing structural and functional damage.

Our data show that optic disc cupping, as measured with noninvasive scanning laser tomography, and changes in the ERG were dependent on the peak elevation of IOP in the experimental eye compared with that in the fellow untreated eye (peak ΔIOP). These changes were also dependent on the product of the IOP elevation (compared with the fellow eye) and days of IOP elevation (ΔIOP integral). Optic nerve axonal loss additionally depended on these IOP parameters. We were not able to show a relationship between the days of IOP elevation and either of the structural or functional parameters, suggesting that there may an IOP threshold below which measurable damage does not occur.

The IOP in normal eyes of conscious Brown Norway rats is approximately 20 mm Hg. Our results show that if peak ΔIOP

![Figure 4](image)

**Figure 4.** Relationship between peak ΔIOP (left), ΔIOP integral (center), and days of IOP elevation (right) with ERG ratio. Shaded area: central 90% confidence interval of the ERG ratio in a set of control animals in which both eyes were untreated.

![Figure 5](image)

**Figure 5.** Serial ERG recordings in an experimental eye (A, left) with peak ΔIOP of 26.1 mm Hg, ΔIOP integral of 700.8 mm Hg days, and 61 days of IOP elevation, showing progressive loss of the b-wave with an intact a-wave. This eye had 79% axonal loss. Serial ERG recordings from the fellow control eye (A, right). Experimental eye in another animal (B, left) with peak ΔIOP of 17.1 mm Hg, ΔIOP integral of 460.2 mm Hg days, and 76 days of IOP elevation, showing no alteration in the ERG. This eye had 35% axonal loss. Serial ERG recordings from the fellow control eye (B, right). Arrows: flash onset.
exceeds 15 mm Hg (∼35 mm Hg, or a factor of 1.75 above normal), extensive axonal loss (mean, 69.2%), optic disc cupping, and electrophysiological loss usually occur. If peak ΔIOP exceeds 20 mm Hg, that is a factor of 2 above normal, profound structural (with a mean of 76.7% axonal loss), and electrophysiological losses occur. It should be noted that the equivalent thresholds for mean IOP elevation would be substantially less. The relationship between peak ΔIOP and axonal loss is in agreement with Morrison et al.\textsuperscript{29} In another study, the same group reported 100% nerve degeneration when mean ΔIOP was higher than 20 mm Hg.\textsuperscript{25}

Compared with the present study and that by Morrison et al.,\textsuperscript{29} RGC losses for equivalent elevations in IOP are more modest in a rat model in which argon laser irradiation of the trabecular meshwork was used\textsuperscript{31} and in a model in which argon laser photocoagulation of the episcleral and limbal veins was used.\textsuperscript{27} Our results diverge, however, from those obtained in studies in which the thermal cautery model, as described by Sharma et al.,\textsuperscript{26} was used. Their group reported approximately 35% RGC loss in animals in which IOP was increased by an average factor of 2 for 42 days.\textsuperscript{37} At 42 days, using a similar model, Sawada and Neufeld\textsuperscript{33} reported approximately 11% and 6% RGC loss in the peripheral and central retina, respectively, in experimental eyes with an IOP 1.7 times higher than in fellow control eyes. At 182 days (6 months), the respective RGC losses were 39% and 12%, whereas the IOP was 1.5 times higher than in fellow control eyes. Finally, using the cautery model with application of 5-fluorouracil, Mittag et al.\textsuperscript{38} showed only qualitative patchy ganglion cell losses in eyes with IOPs elevated by a factor of 2 to 3 for 3 to 4 months. It is likely that methods of elevating IOP, strain of rats, methods of measuring IOP, state of consciousness of the animal during measure-
ments, and methods of quantifying axonal or ganglion cell loss have contributed to the differences in results observed in the literature. Furthermore, unlike the present study, these studies did not report the relationship between IOP and damage on an individual basis, but rather averages of IOP and neural damage parameters across groups.

To the best of our knowledge this is the first study to show progressive cupping in the same animal in a rat model of IOP-induced optic neuropathy. Scanning laser tomography allows monitoring of structural changes and measurements at different time points. Our results show that progression of optic disc cupping can occur relatively quickly, suggesting that these changes may develop after an IOP threshold has been reached. That cupping was not noted with scanning laser tomography until there was considerable axonal loss indicates either that the technique is not sensitive enough or that in this model cupping is primarily a mechanical phenomenon related to the level of IOP. Caution should be exercised in extrapolation of this finding to other species, because in the rat the large area occupied by the blood vessels on the optic disc and their fanned distribution on the optic disc and peripapillary retina may provide considerable structural support to the disc surface. In other species, cupping may occur with more modest axonal loss and may therefore be detectable earlier by imaging techniques. The normal rat disc also has little or no physiological disc cupping and therefore development of even a small degree of cupping would result in large changes in the cup volume change ratio as observed in this study.

Inspection of the optic disc images and histologic sections (Figs. 2, 3, respectively) shows evidence of expansion of the scleral canal. This phenomenon has been described previously in monkeys with experimentally IOP elevation59 and may be due to increased scleral wall stress at the posterior pole.48 Estimating the expansion of the scleral canal and its dependence on IOP can only be performed reliably with histologic analysis since outlining the scleral canal in topographic analysis is not accurate, because of the nature of the rat optic disc. For this reason we placed the contour line well outside the actual optic disc margin to avoid potential inaccuracies due to serial image misalignment or scleral canal expansion. Therefore, although what was defined as cup area and volume may not be accurate, this approach is better suited to detect changes in topography within the contour line and is less influenced by any size changes in the scleral canal.

The a-wave of the Ganzfeld ERG is classically thought to represent photoreceptor activity, whereas the b-wave reflects bipolar and Müller cell function.41 These components of the ERG have been shown to be affected in some patients with glaucoma42,43 which suggests either that, at least in some cases, glaucoma affects retinal cells besides RGCs or that there is a component of the ERG that reflects ganglion cell activity. Previous experimental work in rats has shown time-dependent changes in ERG parameters58,44; however, the relationship between IOP and the ERG findings was not reported. We were not able to show systematic alterations in the a-wave with IOP elevation, which suggests that, at least functionally, the photoreceptor layer is unaffected in this model. Both the b-wave and ERG ratio, as defined in this study, were affected only at high IOP, suggesting loss of inner retinal function in these animals. Histologic evidence corroborates these electrophysiological findings (Fig. 3). The ERG ratio was generally unaffected with early or moderate axonal losses but was outside normal limits with extensive axonal loss when presumably damage had affected the inner retina. This was due to alterations in the b- and not the a-wave. However, because IOP is a covariate, it follows that the electrophysiologic changes may occur as a result of IOP elevation itself and not solely as a result of neuronal loss.

In summary, our study shows that in an IOP-induced model of optic neuropathy, both cupping as measured by scanning laser tomography and ERG changes were strongly related to the peak IOP and IOP integral in the experimental eye, compared with the fellow untreated eye. These structural and function changes were independent of the days of IOP elevation, suggesting that they may be dependent on an IOP elevation threshold. Our data also show that these in vivo structural and functional parameters can be unaltered in spite of moderate levels of axonal loss.

References


