Intravitreal Colchicine Causes Decreased RNFL Birefringence without Altering RNFL Thickness

Brad Fortune, Lin Wang, Grant Cull, and George A. Cioffi

PURPOSE. To test the hypothesis that longitudinal differences between retinal nerve fiber layer (RNFL) birefringence, measured by scanning laser polarimetry (SLP), and RNFL thickness, measured by optical coherence tomography (OCT), are informative about the state of axonal degeneration.

METHODS. Colchicine was injected into the vitreous cavity of Chlorocebus sabaeus; estimated vitreal concentration: 1 mM, n = 3; 2 mM, n = 1; 10 mM, n = 2; an equivalent volume (approximately 0.1 mL) of sterile saline was injected into fellow control eyes. RNFL birefringence was measured by SLP before injection and every 10 minutes after injection for 2 hours. RNFL thickness was measured by OCT before injection and 2 hours later. After isolating each retina, biopsy specimens were obtained from the inferotemporal arcade region, approximately 2 mm from the center of the optic disc, using a 2-mm trephine and were processed for transmission electron microscopy (TEM). Retinas were then flat-mounted and stained with an antibody against polymerized β-III-tubulin.

RESULTS. RNFL birefringence measured by SLP decreased over time in all six colchicine-injected eyes, appearing to reach a plateau of −20% ± 7% (P < 0.0001) approximately 100 minutes after injection. There were no significant differences between quadrants (P = 0.44) and no apparent dose effect (P = 0.87). The change in vehicle-injected control eyes was −3% ± 3% (P = 0.06; NS). The change in RNFL thickness measured by OCT was +1% ± 4% (P = 0.81; NS) in colchicine-injected eyes and +6% ± 6% (P = 0.13; NS) in control eyes. There was no evidence of macular edema by fundus biomicroscopy, stereo fundus photography, or OCT. TEM revealed disorganization of microtubules, swelling of mitochondria, and blurred axonal membranes in the colchicine-injected eyes. Flat-mounted retinas stained with an antibody against polymerized β-III-tubulin showed only a mild reduction of peripapillary stain intensity in the colchicine-injected eyes compared with controls.

CONCLUSIONS. Intravitreal injection of colchicine caused microtubule disruption within the axons of the RNFL in nonhuman primates. This was manifest as a reduction of RNFL birefringence, without alteration of RNFL thickness, suggesting that such discrepancies can be informative about the status of axonal degeneration. (Invest Ophthalmol Vis Sci. 2008;49:255–261) DOI:10.1167/iovs.07-0872
designed as a proof of this principle: its purpose was to test the hypothesis that longitudinal differences between RNFL birefringence, measured by SLP, and RNFL thickness, measured by OCT, can exist and be informative about the state of axonal degeneration.

METHODS

Animals

Six adult female vervet monkeys (Chlorocebus sabaeus), weighing 4 to 6 kg each, were included in this study. All experimental methods and animal care procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the local Institutional Animal Care and Use Committee.

Anesthesia

The experimental protocol began with the induction of general anesthesia with intramuscular injection of 15 mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA), along with a single subcutaneous injection of atropine sulfate (0.05 mg/kg). Animals were then intubated so that anesthesia could be maintained using 2% to 3% isoflurane (Baxter, Deerfield, IL) mixed with oxygen. Heart/pulse rate and arterial oxyhemoglobin saturation were monitored continuously (Propaq Encore model 200EL; Protocol Systems, Inc., Beaverton, OR) and were maintained above 80 min⁻¹ and 95%, respectively. Body temperature was maintained with a warm-water heating pad set at 37°C. Pupils were fully dilated (≥7 mm) with 1.0% tropicamide and 2.5% phenylephrine (Alcon Laboratories Inc., Fort Worth, TX). Topical anesthetic (0.5% proparacaine; Alcon Laboratories Inc.) and lubricating drops (Refresh; Allergan, Irvine, CA) were applied before insertion of a plano-powered, rigid gas-permeable contact lens in each eye.

Baseline Imaging

Before intravitreal injections, baseline images were obtained in each eye. Simultaneous stereoscopic photographs were taken of the optic disc and macula (3-Dx; Nidek Co., Ltd., Aichi, Japan), first without any filter, then with the camera’s red-free filter in place. SLP measurements were obtained using a GDxVCC instrument (Carl Zeiss Meditec, Inc., Jena, Germany). Anterior segment birefringence measurements were obtained before the initial baseline scan and were used to “compensate” all subsequent scans. Three SLP scans were averaged for the baseline and for each subsequent time point. The medium locus of RNFL values (the peripapillary profile or TSNIT curve) was exported for analysis. Each of the 64 values in the TSNIT series is an average from an 8-pixel-wide band centered on the optic disc. The outer limit of the medium band is 43 pixels from the center of the optic disc, so the center of the band has a radius of 39 pixels. This corresponds to a scan angle of 6.1°, which translates to a total diameter of 3.5 mm (assuming 45 μm per pixel for an emmetropic human eye with average axial length). OCT measurements of RNFL thickness were obtained using an OCT instrument (Stratus, v3.0.1; Carl Zeiss Meditec, Inc.). The RNFL algorithm was applied with a 3.4-mm diameter scan centered on the optic disc. The 3.4-mm designation is more accurately stated as 3.46 mm for an emmetropic human eye with an axial length of 24.46 mm, which closely matches the center of the medium band (3.5-mm diameter) of the SLP. Empiric alignment procedures during pilot studies confirmed that the center of the medium band was a good match to the 3.4-mm diameter scan locus from the OCT in these relatively small vervet monkey eyes. Magnification differences resulting from the shorter axial length of the vervet eye would apply equally to both the SLP and the OCT instrument. The 3.46-mm diameter scan would have an actual transverse distance on the monkey retina of 2.9 mm (calculation based on measured axial length of 20 mm). Peripapillary retinal thickness (RT, using a 3.4-mm diameter scan) and macular thickness scans were also obtained in each eye during baseline and final time points.

Intravitreal Injections

Intravitreal injections were made through the temporal pars plana using a 30-gauge needle. Colchicine (Sigma-Aldrich, St. Louis, MO) was diluted into sterile balanced salt solution (Alcon Laboratories Inc.) to achieve an injected dose of 0.83 mg (0.083 mL), which provides an estimated vitreal concentration of 1 mM assuming a 2-mL vitreous volume (the latter based on measurements of vervet eye dimensions). This 1-mM dose was injected in three of the six experimental eyes; higher doses were injected in the remaining three experimental eyes (2 mM, n = 1; 10 mM, n = 2). The fellow (control) eye of each animal received an intravitreal injection of an equal volume (0.083–0.1 mL) of vehicle only (balanced salt solution).

Repeat Imaging

SLP scans were repeated in approximately 10-minute intervals; three scans were averaged at each of these time points. The series of postinjection time points consisted of three sequential time points in the experimental eye, followed by one time point in the control eye,
which was then repeated twice more (see Results). After this series of SLP time points was completed, the postinjection OCT scans and fundus photographs were obtained. The duration of the entire experiment was approximately 2 hours. Note that one animal remained supine for 1 hour after receiving a 10-mM injection, reflecting an attempt to minimize and equalize colchicine concentration across the posterior pole. SLP imaging resumed at the 60-minute time point.

**Histopathology**

On completion of in vivo imaging, animals were immediately killed by intravenous injection (Euthanol; Diamond Animal Health, Inc., Des Moines, IA). After a bolus of IV heparin, perfusion fixation ensued through bilateral pre-cannulated carotid arteries using approximately 1 L 4% buffered paraformaldehyde. Eyes were enucleated, retinas were dissected, and the retinal pigment epithelium and vitreous were removed. With the use of a 2-mm trephine, a biopsy sample was obtained from the inferotemporal arcade region of each retina and prepared for transmission electron microscopy (TEM). A wedge-shaped incision was used to orient the sample so that ultrathin sections could be made perpendicular to the direction of RNFL bundles. Sections thus began tangentially along the biopsy edge closest to the optic disc.

After biopsy, each pair of retinas was stained simultaneously with mouse monoclonal antibodies against βIII-tubulin (TUJ-1 [Abcam Inc., Cambridge, MA] or SMI-62 [Covance Research Products Inc., Berkeley, MOines, IA). After a bolus of IV heparin, perfusion fixation ensued through bilateral pre-cannulated carotid arteries using approximately 1 L 4% buffered paraformaldehyde. Eyes were enucleated, retinas were dissected, and the retinal pigment epithelium and vitreous were removed. With the use of a 2-mm trephine, a biopsy sample was obtained from the inferotemporal arcade region of each retina and prepared for transmission electron microscopy (TEM). A wedge-shaped incision was used to orient the sample so that ultrathin sections could be made perpendicular to the direction of RNFL bundles. Sections thus began tangentially along the biopsy edge closest to the optic disc.

After biopsy, each pair of retinas was stained simultaneously with mouse monoclonal antibodies against βIII-tubulin (TUJ-1 [Abcam Inc., Cambridge, MA] or SMI-62 [Covance Research Products Inc., Berkeley, CA]) according to the following protocol. Retinas were treated with 0.2% triton X-100 in 0.01 M PBS for 1 hour at room temperature, then with methanol and 0.3% H2O2 for 1 additional hour. Retinas were placed in blocking serum solution (2% horse serum and 2% BSA) for 12 hours, washed once with PBS, and incubated at 4°C with the primary antibody (1:500) for 5 days. They were then washed in 0.01 M PBS for 1 hour at least three times each. Finally, retinas were incubated at 4°C for 3 days with the FITC-conjugated horse anti-mouse secondary antibody (1:100) and were flat mounted for microscopy.

**Statistical Analysis**

Repeated-measures analysis of variance was used to test for the effects of treatment, time, and quadrant.

**RESULTS**

Figure 1A shows the change in RNFL birefringence over time obtained by SLP for each eye in the study. Each data point represents the average of three SLP scans acquired at the corresponding time point (the latter was taken as average time after baseline for the three scans). The data are presented as the TSNIT average RNFL thickness as output by the SLP instrument. In each case, intravitreal injection occurred at $t = 0$. SLP measurements of RNFL birefringence declined over time in colchicine-injected eyes (open symbols, dashed curves; $P < 0.0001$) but not in vehicle-injected control eyes (filled symbols, solid curves; $P = 0.06$). Four of the experimental eyes followed a similar time course, whereby the effect of colchicine appeared to plateau toward the end of the 2-hour measurement sequence (m1, 1 mM; m6, 2 mM; m7, 10 mM; m5, 10 mM). However, the effect in the other two eyes (m3, 1 mM, green squares; and m4, 1 mM, orange triangles) seemed to be either slightly delayed or more variable; both had received the 1-mM dose. However, there was no statistically significant effect of colchicine concentration ($P = 0.87$).

Figure 1B shows the group average SLP measurements of RNFL birefringence as a percentage change from baseline versus time for colchicine-injected eyes (open circles, dashed curves) and vehicle-injected control eyes (filled circles, solid curves). At the final measurement point, the SLP measurement of RNFL birefringence declined by an average ($\pm$SD) of 20% ± 7% in the colchicine-injected eyes ($P < 0.0001$) and by 3% ± 3% ($P = 0.06$, NS) in the control eyes. There were significant effects of time ($P < 0.0001$) and a time-treatment interaction ($P < 0.0001$) for the SLP measures of RNFL birefringence. Analysis by quadrant indicated that the effect of colchicine over time was uniform around the optic nerve head (quadrant, $P = 0.44$; time, $P = 0.01$; quadrant-time interaction, $P = 1.0$).

Figure 2 compares the group average (n = 6) TSNIT curves obtained at baseline (dashed curves) with those obtained at final time point (solid curves) for both the SLP measure of...
birefringence (Figs. 2A, 2C) and the OCT measure of RNFL thickness (Figs. 2B, 2D). The data for colchicine-injected eyes are shown in the top row (Figs. 2A, 2B), and the data for control eyes are shown in the bottom row (Figs. 2C, 2D). The TSNIT average loss of 20% ± 7%, shown in Figure 1, can be seen here again in Figure 2A for the SLP measure of RNFL birefringence. Also shown again is the lack of any significant change measured by SLP in control eyes (Fig. 2C). Figures 2B and 2D show that there was no significant change in the OCT measure of RNFL thickness for either group; it increased by 1% ± 4% (P = 0.81, NS) in colchicine-injected eyes and increased by 6% ± 6% (P = 0.13, NS) in control eyes.

Figure 3 shows that there was also no significant change measured by OCT for peripapillary full retinal thickness in either the colchicine-injected eyes (Fig. 3A) or the control eyes (Fig. 3B). Figure 4 shows that there was no significant effect of colchicine injection on macular thickness measured by OCT. There were no clinical signs of macular edema or any other macular abnormality evident by OCT macular imaging, stereoscopic photography, or fundus biomicroscopy. There was no clinically apparent decline in the relative brightness (i.e., contrast) of RNFL striations after colchicine injection or vehicle injection in any of the eyes. A representative example is provided in Figure 5. Similarly, there was no apparent decline in the internal reflectance of the RNFL after colchicine injection in any of the eyes; a representative example is shown in Figure 6.

Figure 7 shows a pair of flat-mounted retinas stained with an antibody against polymerized β-III-tubulin (SMI-62). There was only a minor difference in pixel density (brightness) between this pair of retinas, with the colchicine-injected eye showing a 3% lower density. Although one other pair did show a 20% lower density in the colchicine-injected eye compared with its fellow control eye, no significant difference was observed across the group of five pairs stained with the SMI-62 antibody (3% ± 16% difference; P = 0.8, paired-Wilcoxon signed rank test). Similarly, in the sixth pair of eyes, the alternative antibody against β-III-tubulin (TUJ-1) did not show any visible difference between the colchicine-injected and control eye (though this pair was not quantified). However, the TUJ-1 antibody is purported to recognize any β-III-tubulin subunits, polymerized or not.

The site of the biopsy obtained for TEM can also be seen in each flat-mounted retina shown in Figure 7 (arrows). Figure 8 shows a representative example of the results obtained by TEM. In general, TEM revealed disorganization of microtubules, swelling of mitochondria, and blurred axonal membrane borders in the colchicine-injected eyes. In all five pairs studied by TEM, the colchicine-injected eye showed a more homogeneous internal structure compared with the regular array of microtubules seen in cross-sections through the RNFL of control eye samples.

**DISCUSSION**

Intravitreal injection of colchicine caused microtubule disruption within the axons of the RNFL in nonhuman primate eyes, manifest as a reduction of RNFL birefringence when measured by SLP, without any accompanying change in RNFL thickness as measured by OCT, indicating that RNFL birefringence can change independently of axonal caliber. This, in turn, suggested that discrepancies between these two imaging modalities can be informative about the status of axonal degeneration and can perhaps identify a state during degeneration in which the birefringent elements have become abnormal before the ultimate demise of the axon.

Evidence indicates that NFs become abnormal at an early stage of experimental glaucoma (specifically, an altered phosphorylation state) and that the largest axons expressing the most NFs are the most vulnerable in the same monkey model.
It has also been shown that mRNA for NF decreases immediately after axotomy in the adult rat optic nerve, whereas β-tubulin mRNA initially increases. Perhaps even more relevant are the findings that a decrease in axonal caliber after optic nerve crush is delayed by approximately 1 month relative to the decrease in NF expression.

It seems that more is known about changes in NF than MT after RGC injury, but the work of Knighton and colleagues suggests that MT are the primary source of birefringence in the RNFL. Although it is possible that other thin cylindrical parallel structures, such as NFs or the axonal membranes themselves, could also contribute to form birefringence of the RNFL, Knighton et al. found that RNFL birefringence declines to essentially zero after colchicine treatment of rat retinal explant preparations. The latter results are consistent with their earlier theoretical work and a model in which MT are the primary contributor to RNFL birefringence, with other mechanisms contributing less than 15%.

The results obtained in the current experiment showed, on average, a 20% decline in the SLP-derived estimate of RNFL thickness around the optic nerve. The GDxVCC instrument measures retardance, then calculates its estimate of RNFL thickness using a linear conversion factor of 0.67 nm/μm (as stated in the instrument manual). Because the conversion is linear, the decline in RNFL “thickness” measured by SLP represents a 20% decline in RNFL birefringence within approximately 100 minutes of intravitreal colchicine injection. This effect is smaller than that obtained by Huang and Knighton, which might be attributed to one or several differences in the experimental conditions. Their experiment was carried out on rat retinal explants with the use of a custom polarimeter device and a colchicine dose of 10 mM; our experiment was carried out in vivo using available clinical instrumentation and in doses ranging from 1 to 10 mM (it is difficult to achieve an estimated vitreous concentration of 10 mM because colchicine does not easily remain in solution at the concentration required before it is diluted into the vitreous volume). Although it is possible that the dose was not strong enough to observe the same magnitude of the effect obtained by Huang and Knighton (Huang X-R, personal communication, 2006), we did not see a substantive difference between the effects of 1 mM and 10 mM colchicine. Moreover, using intravitreal colchicine doses 10 to 100 times lower than what was used here, Davidson et al. found near total loss of neurotubules within the intraretinal axons of the monkey after only 1 hour.

In a separate study, Huang et al. also observed an approximately 50% decline in the reflectance of the rat RNFL after colchicine treatment. We did not see evidence of reflectance
changes in the monkey RNFL, by clinical indirect ophthalmoscopy, inspection of fundus photography, or the strength of the internal reflectance signal on OCT. If proportional effects of colchicine on RNFL birefringence and reflectance followed that observed in the Huang et al.\textsuperscript{26} study, then reflectance should have declined by only approximately 10% in this study, which is perhaps below the detection threshold. It is also possible that some of the differences between our results and those of previous studies\textsuperscript{15–18} suggest that MT account for less than 85% to 100% of RNFL birefringence and less than 50% of RNFL reflectance in the primate eye.

There was a trend toward a greater effect of colchicine at the superior and inferior poles of the optic disc, where the RNFL is thickest (measured by OCT) and birefringence is greatest (measured by SLP), though our study was underpowered to characterize this relatively subtle differential spatial effect with statistical confidence. Variation in the degree of birefringence per unit thickness\textsuperscript{18,27–30} suggests that even in normal eyes not all bundles contain axons with the same density of birefringent elements. Consistent with those earlier findings, the ratio of birefringence to RNFL thickness\textsuperscript{18,27–30} suggests that even in normal eyes not all bundles contain axons with the same density of birefringent elements. Consistent with those earlier findings, the ratio of birefringence to RNFL thickness was not uniform around the optic disc in our study, varying from approximately one third nasally and 30° temporal to the poles to a peak of two thirds or more at the superior and inferior poles. Even within a given bundle, not all axons contained an equivalent density of MT or NF. That was evident in all the TEM samples obtained in our study, and it is clearly demonstrated in Figures 9–71 through 9–75 in Hogan et al.\textsuperscript{31} This means that detecting birefringence changes relative to RNFL thickness will be complicated by normal variation not considered within the clinical instrument’s software, but it may also indicate that certain axons may be more or less susceptible to particular insults, depending on their internal structural composition. This could be an important avenue for future study in glaucoma or in other optic neuropathies or RGC diseases.

The observation that flat-mounted retinas stained with antibodies against β-III-tubulin did not consistently reveal differences between colchicine-injected and control eyes while the SLP measurement selectively showed reduced RNFL birefringence and the TEM results showed disorganized MT in colchicine-injected eyes suggests that the presence of MT subunits, even if more or less polymerized, is insufficient to produce normal RNFL birefringence. It seems that the three-dimensional MT architecture must also remain intact to produce normal RNFL birefringence. Thus, abnormal RNFL birefringence (in the presence of normal RNFL thickness) may signal states of RGC stress that include not only potentially abnormal production, modification, transport, and maintenance of tertiary structure but perhaps also a failure of proper three-dimensional spacing and alignment.

In summary, the results of this study demonstrate that SLP-derived measures of RNFL birefringence can change inde-
pendently of RNFL thickness, as derived by OCT. Based on current hypotheses about the major source(s) of birefringence in the RNFL, this suggests that longitudinal comparisons between birefringence and RNFL thickness (e.g., by SLP and OCT or by polarization-sensitive OCT) may be capable of signaling an intermittent state of axonal degeneration whereby internal structural elements such as MT or NF have become abnormal before changes in axonal caliber. It remains to be determined whether the results from this acute experimental manipulation will be applicable to the slower course of most disease states, though a similar differential time course has been observed after optic nerve crush. However, in diseases with a substantially slower course such as glaucoma, gliotic changes within the RNFL are likely to complicate measurement of this relationship. It is nonetheless interesting that Mohammadi et al. have recently shown SLP-derived measures of RNFL birefringence were independent predictors of future vision loss in glaucoma suspects who began the study with normal SAP visual fields, regardless of their age, IOP, or optic disc appearance.

Acknowledgments
The authors thank Robert Kayton (CROET Histology/Electron Microscopy Facility, Oregon Health and Science University) for conducting transmission electron microscopy.

References