Tracking Longitudinal Retinal Changes in Experimental Ocular Hypertension Using the cSLO and Spectral Domain-OCT

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PURPOSE. Involvement of the outer retina is controversial in glaucoma. The aim of this study was to test, first, whether the outer retina is affected in experimental ocular hypertension (OHT) and, second, whether whole retinal thickness can be used as a surrogate marker of glaucomatous change.

METHODS. OHT was surgically induced in 20 Dark Agouti rats. Animals were imaged using a modified Spectralis OCT (Heidelberg Engineering, Heidelberg, Germany) at baseline and at 3 and 8 weeks after OHT induction. Measurements were recorded for whole and individual retinal layer thickness in four regions—temporal, superior, nasal, and inferior—around the optic nerve head.

RESULTS. Whole retinal thickness in normal eyes was $172.19 \pm 5.17 \mu\text{m}$, with no significant regional differences. OHT caused a significant reduction in whole retinal thickness and the outer nuclear layer (ONL) at 3 and 8 weeks ($P < 0.05$), along with the expected thinning of the retinal nerve fiber layer (RNFL). Whole retinal thickness correlated well with RNFL ($P = 0.035$) and ONL ($P \leq 0.001$) changes. Sensitivity of RNFL and ONL to IOP exposure appeared greater at 3 than at 8 weeks. In addition, regional profiles were significantly altered in the ONL and RNFL after OHT induction.

CONCLUSIONS. Adaptation of the Spectralis OCT enables tracking of structural damage in experimental rat OHT. Here the authors show evidence of glaucomatous damage in the outer retinal layers of this model with significant regional changes and highlight whole retinal thickness in the rat as a useful surrogate marker of inner and outer retinal changes. The authors believe that the OCT data can provide useful information with regard to clinical management. (*Invest Ophthalmol Vis Sci.* 2010;51:6504–6513) DOI:10.1167/iovs.10.5551

Glaucoma, which is characterized by the progressive degeneration and loss of retinal ganglion cells (RGCs) and their axons, is a leading cause of irreversible blindness world-

wide.^{1,2} Although RGC death is a key pathologic event in glaucoma, damage may not be limited to the inner retina. Understanding early identified structural patterns related to RGC death may have potential in the early diagnosis, intervention, and management of glaucoma.

Advances in optical imaging technology have made it increasingly possible to detect structural changes in the retina. One emerging technology is optical coherence tomography (OCT), a noninvasive, low-coherence, interferometry-based imaging technique that can be used to acquire cross-sectional tomographic images of the retina and the optic nerve head (ONH).³ This state-of-the-art technology has become an increasingly accepted method for assessing retinal nerve fiber layer (RNFL) thickness and other morphologic changes in the retina in humans because of its accuracy and reproducibility. This technology has been greatly enhanced by the recently developed spectral domain (SD) or Fourier domain (FD) OCT.^{4–6} SD-OCT has been available since 2004, and clinical data has shown that the SD-based OCT system is a promising technology to image changes in the retina because of its higher resolution and rapid data acquisition.^{4–6} Recent studies^{7,8} on glaucoma patients have confirmed that SD-OCT enables the detection of localized structural damage in the RNFL as opposed to existing OCT devices. Moreover, the SD-OCT has been recently applied to small animals in both normal^{9–12} and disease models^{13–18} and has been shown to enable the identification of retinal layer thinning, including the outer retina, in various retinal degeneration models.^{13–18}

It has been a topic of debate that the outer retinal layers are affected in glaucoma. Histologically, swelling and loss of photoreceptors have been observed in human and primate glaucoma.^{19,20} Decreased cell density in the outer nuclear layer, along with a similar change in the inner retina, has also recently been shown in human glaucomatous eyes.²¹ However, this is not supported by other studies that suggest minimal cellular loss in the outer retina in both human glaucoma and primate glaucoma.^{22–25} Recent OCT imaging studies have also failed to demonstrate outer retinal layer damage in glaucoma patients.^{26–28} In addition, electroretinography (ERG) studies have revealed conflicting results, though some have reported both outer and inner retinal involvement in glaucoma, with evidence of reduction and delay in the dark-adapted ERG a- and b-waves, reduced focal ERG amplitudes, reduced flicker ERG even harmonics (the nonlinear components in the pattern ERG), or aberrant responses in the multifocal (mf) ERG in humans^{29,30,31,32} and primates.³³ This is also evident in experimental rodent models of OHT^{34,35} (Georgiou AL, et al. *IOVS* 2008;49:ARVO E-Abstract 1557). However, other electrophysiological studies have demonstrated selective functional loss in RGCs only, rather than any abnormalities in the outer retina in human^{36–39} and primate glaucoma,^{23,25} and in rat models of OHT.⁴⁰

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Supported by a Wellcome Trust ViP Award and by the University of Rome Campus Bio-Medico, Rome, Italy.

Submitted for publication March 18, 2010; revised June 11, 2010; accepted July 4, 2010.

Disclosure: L. Guo, None; E.M. Normando, None; S. Nizari, None; D. Lara, None; M.F. Cordeiro, None

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In this study, we tested the hypothesis that the outer retinal layer is affected during the course of ocular hypertension (OHT) in the rat using a modified Spectralis OCT (Heidelberg Engineering, Heidelberg, Germany). Because not all OCT devices have high enough resolution to distinguish between separate layers within the retina, we also assessed whether whole retinal thickness may be used as a surrogate marker of glaucomatous changes in the rat.

We modified a commercial SD-OCT (Spectralis OCT, Heidelberg Engineering GmbH) to enable imaging of the rat eye. We demonstrated that this customized apparatus is capable of tracking structural changes longitudinally in the retina in experimental OHT. We show evidence of significant thinning in the outer retinal layers with significant regional changes in the rat model. We also show whole retinal thickness as a useful, reproducible, and easily measured parameter in the rat and advocate its use in OCT techniques when separate layer identification is not possible.

METHODS

Animals

All animal experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with procedures approved by the United Kingdom Home Office. Adult male Dark Agouti (DA) rats weighing 150–200 g each were maintained in a 12-hour light/12-hour dark cycle with a room illuminance of 140 to 260 lux during the bright portion of the cycle. Animals were provided standard food and water ad libitum. In vivo experiments were conducted under general anesthesia with a cocktail of 37.5% ketamine (Ketaset; Fort Dodge Animal Health, Fort Dodge, IA), 25% medetomidine (Dormitor; Pfizer, New York, NY), and 37.5% sterile water administered at 2 mL/kg intraperitoneally.⁴¹

Glaucoma Model of Rat

The OHT model was surgically induced in 20 DA rats using a well-established method.^{41–45} IOP was elevated in the left eye of each animal by the injection of 50 μ L hypertonic saline solution (1.80 M) into the episcleral vein⁴² using a syringe pump (50 μ L/min; UMP2; World Precision Instruments, Sarasota, FL). A propylene ring, with a 1-mm gap cut out of its circumference, was placed around the equator to prevent injected saline outflow from other aqueous veins.⁴² Contralateral, unoperated eyes served as controls. Under inhalational anesthesia of 0.4% isoflurane in oxygen, the IOP of both eyes in each rat was measured at regular intervals with a tonometer (TonoLab; Tiolat Oy, Heisinki, Finland) before surgery and weekly after it. For each animal, cumulative IOP exposure, defined as the integral of IOP elevation over time (mm Hg days), was calculated from the area under the curve, as previously described.^{43,46} The animals were killed 8 weeks after IOP elevation.

Optical Modification of the Spectralis OCT to the Rat Eye

The Spectralis OCT can acquire simultaneous confocal scanning laser ophthalmoscope (cSLO) and SD-OCT images of the living human retina. This important feature allows consistent registration of the OCT depth scans with the aerial image of the retina. Because the device was originally designed to work in the human eye, some imaging features had to be adjusted to acquire high-quality images of the rodent retina. The key differences of the optics between human eyes and rat eyes include typical refraction power, eyeball length, equivalent focal length, and maximum dilated pupil size. These differences have four important effects on imaging of the rat retina. First, the retinal image at best focus in the rat requires a diopter compensation of approximately +20 D because of the typical strong negative spherical aberration.⁴⁷ Second, the optical path (OP) of the sample arm in the OCT inter-

ferometer is significantly shorter in the rat eye than in the human eye. Third, the dispersion of the sample arm in the OCT instrument is significantly different between rat eyes and human eyes. Fourth, the amount of light returning to the instrument from the rat eye is smaller because the pupil is smaller.

Custom adaptation of the instrument regarding these points was made. With an achromatic +10 D doublet lens placed in front of the instrument, we were able to compensate a large portion of the refraction power of the rat eye (Fig. 1). The lens also helped balance the OP of the sample and reference arms. We then matched these OPs exactly by adjusting the reference arm length with the use of software available in the Spectralis OCT without internal alterations to the hardware. The positive lens also helped reduce the illumination pupil diameter from 3 mm to approximately 2.3 mm, according to approximate ray tracing. With the smaller pupil size, most of the light from the instrument was delivered to the rat eye, and the expected negative effect was decreased.

Our customized Spectralis OCT is still capable of acquiring simultaneous cSLO and SD-OCT images, both at best focus from the rat retina (Fig. 1). This important feature has made our experiments robust and repeatable. We do not require additional contact lenses, and our imaging method is still a noncontact technique.

In Vivo Spectralis OCT Imaging of Retina in the OHT Rat

To assess IOP-induced changes in the thickness of the whole retina and the individual retinal layers, we performed volume scans using the modified Spectralis OCT in OHT rats at baseline and a 3 and 8 weeks after IOP elevation. A typical raster scan was performed for each eye. The Spectralis OCT features a broadband superluminescent diode at $\lambda = 870$ nm as a low coherent light source. Each 2D B-scan recorded at a 30° field-of-view consisted of 1536 A-scans acquired at a speed of 40,000 per second. The OCT optical depth resolution was approximately 7 μ m, and the digital depth resolution was 3.5 μ m.

The OCT 3D volume images for each eye consisted of 19 OCT cross-sections (Figs. 2A, 2B), and the gaps between the cross-sections were nominally 240 μ m for the human eye. To convert the parameter from human to the rat, we assumed 4.5 mm as the equivalent focal length of the rat eye in air.⁴⁷ We calculated the gap width (Y) between B scans to be 63.2 μ m in the rat eye, as shown in Figures 2B and 2C. To apply consistent measurements across all eyes, a grid was used with predefined regions around the ONH to identify specific areas of interest, namely B scans within the temporal (T), superior (S), nasal (N), and inferior (I) regions (Fig. 2C). The area of each B scan was 364 μ m (lateral) by 350 μ m (depth), equivalent to 100 $\times$ 100 pixels. These specific areas were identified on the grid, which was centered on the ONH, and the preset locations are illustrated in Figures 2A to 2C. The OCT cross-section containing the ONH was located at the center of the grid. Superior and inferior measurements were made on the 5th and 15th sections, respectively. Temporal and nasal measurements were made on the middle cross-section (10th), at a distance of 252.8 μ m from the center of the ONH, temporally and nasally (Figs. 2B, 2C).

The OCT cross-section images, obtained from averaging 25 B-scans (Figs. 2D, 2E) from each preset location, were then processed into a single document using software (MATLAB, version 7; The MathWorks, Inc., Natick, MA). The images were then analyzed and calibrated to scale (1 lateral pixel = 3.64 μ m, 1 depth pixel = 3.5 μ m), and the measurements were made within an area of 100 $\times$ 100 pixels (364 μ m $\times$ 350 μ m). The thickness of the whole retina and its individual layers was manually segmented using an editing tool (Trace Region tool in MetaMorph Offline software, version 6.1; Universal Imaging Corporation, Downingtown, PA; Fig. 2E). The average height was obtained for each retinal layer, with 95% confidence interval (CI). Whole retinal thickness was outlined from the RNFL to the outer segment (OS) of photoreceptors, with individual retinal layer thickness measurements of RNFL, inner plexiform layer (IPL), INL, outer plexiform layer (OPL), ONL, and the inner segment (IS) and OS of photoreceptors, performed as illustrated in Figures 2D and 2E.

FIGURE 1. A customized adaptation of the Spectralis OCT to image rat retina. The normal Spectralis HRA (Heidelberg Engineering) objective with the +10 D lens adapter mounted to the lens (A), and the Spectralis HRA with the 100-mm focal length lens mounted in front of the HRA ophthalmic lens (B). A screen image shows that the modified Spectralis OCT can acquire simultaneous high-quality SLO and OCT images of the living rat retina (C).

Statistical Analysis

Mean values were calculated with 95% confidence intervals (mean $\pm$ 95% CI) for the thickness of whole retina and individual retinal layers and IOP profiles. The Student's *t*-test was applied to assess the reduction in retinal layer thickness caused by cumulative IOP exposure. OCT retinal layer thickness and regional analyses were performed using the repeated-measures procedure with ANOVA and the generalized linear model (GLM; SPSS 11.5 software; SPSS, Chicago, IL). This allowed comparison between the different layers and regions over the entire study period (matched for each animal eye) to assess whether there were statistical differences over time. To examine differences between each parameter at each time point (baseline, 3 weeks, 8 weeks), further comparative analysis was performed using ANOVA and Bonferroni's modification. Finally, because all retinal thickness data were parametric, Pearson's correlation coefficient was used to assess the strength of the correlation of whole retinal thickness to individual layers.

RESULTS

IOP Elevation in Surgical Eyes

OHT was induced in the left eye of each animal by injection of hypertonic saline solution into the episcleral vein. The IOP profiles for both eyes of the animals are shown in Figure 3A. Mean baseline IOP before surgery was 10.29 ± 0.59 mm Hg. Surgery induced an increase of IOP in all eyes, with a mean peak IOP elevation of 23.43 ± 3.50 mm Hg (Fig. 3A) and an integral IOP elevation of 429.79 ± 37.11 mm Hg days at 3 weeks and 918.92 ± 65.76 mm Hg days at 8 weeks after OHT induction (Fig. 3B).

Baseline Characteristics in the Normal Rat Retina

Analysis of the normal rat (parameters) revealed that the mean thickness of whole retina was 172.19 ± 5.17 μ m, with the four regions showing no significant difference (Fig. 4). Among the individual layers, IPL was the thickest and OPL was the thinnest, with mean thicknesses of 48.22 ± 2.51 and 21.56 ± 1.03 μ m, respectively. In contrast to the plexiform layers, the ONL (45.10 ± 1.73 μ m) was thicker than the INL (26.23 ± 1.31 μ m). The thickness of the RNFL and the IS/OS were similar, with an average of 36.65 ± 1.22 and 33.33 ± 2.31 μ m, respectively.

Analysis of regional thickness showed a trend, though not significant, for the profiles of individual layers according to region. For the RNFL, the thickest region was in the temporal sector, followed by the superior, nasal, and inferior sectors, or TSNI. The INL and ONL had a regional trend of NIST and INST, respectively, whereas the IPL and OPL showed a trend profile of SINT and NSIT, respectively. Finally, the IS/OS had a regional trend of TNIS (Fig. 4).

OHT-Induced Changes in the Thickness of Whole Retina and Individual Retinal Layers

We next assessed the effects of increased IOP on the thickness of whole retina and individual retinal layers. Using GLM with repeated measurements, we found a significant difference between the thicknesses of all OCT retinal layers over time ($P \leq 0.001$). Pairwise comparison between the whole retina and individual layers showed significant differences between the whole retina and OPL ($P \leq 0.001$), IPL ($P = 0.002$), IS/OS ($P = 0.002$), and INL ($P = 0.004$) over time but no significant differences between the whole retina and ONL ($P = 0.085$).

FIGURE 2. Measurements of thickness of the whole retina and individual layers from Spectralis OCT cross-sections. Representative 3D OCT volume automatic reconstruction (A), reflectance retinal image (B), and grid scale with precalibrated regions (C) illustrating the four locations (T, S, N, and I) used in measurements of rat left eyes. The area of each preset location is 100×100 pixels or $364 \mu\text{m} \times 350 \mu\text{m}$. OCT cross-section images obtained from averaging 25 B-scans (D, E) from a single preset location processed and analyzed to enable measurements of the whole retinal and individual layer thickness, after manual segmentation of each layer, outlined in red (E). RNFL/RGCL, retinal nerve fiber layer/retinal ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer.

and RNFL ($P = 0.058$). We then looked at the whole retina and individual layers at each specific time point using ANOVA and Bonferroni's modification. Statistical analysis revealed that OHT caused a significant reduction in whole retinal thickness at both 3 ($158.03 \pm 4.95 \mu\text{m}$; $P = 0.017$) and 8 ($156.80 \pm 7.34 \mu\text{m}$; $P = 0.011$) weeks compared with baseline ($172.19 \pm 5.17 \mu\text{m}$; Fig. 5A). Examination of the individual layers of the retina showed that RNFL thickness was significantly reduced at 3 weeks ($31.47 \pm 2.08 \mu\text{m}$; $P = 0.031$) but not at 8 weeks ($33.94 \pm 4.29 \mu\text{m}$; $P = 0.383$), compared with baseline ($36.65 \pm 1.21 \mu\text{m}$; Fig. 5B). Although there were no noteworthy changes in other inner retinal layers (IPL and INL), ONL thickness showed significant reduction at both 3 ($40.87 \pm 2.01 \mu\text{m}$; $P = 0.035$) and 8 ($37.74 \pm 2.54 \mu\text{m}$; $P \leq 0.001$) weeks after IOP elevation (Fig. 5B).

Given that GLM analysis suggested that there was a correlation among whole retina, ONL, and RNFL thickness over time, we next investigated this specifically. We found a strong correlation between whole retinal thinning and reduced thickness in the ONL ($r = 0.748$; $P \leq 0.001$) and the RNFL ($r = 0.500$; $P = 0.035$). This confirmed that whole retina, RNFL, and ONL followed a similar trend over time.

To further analyze the effects of IOP exposure on the thinning of the retinal layers, we normalized the reduction in retinal layer thickness to cumulative IOP exposure. Mean changes in retinal layer thickness from baseline were calculated and then divided by the integral IOP exposure at 3 and 8 weeks after OHT induction (Fig. 5C). This analysis suggested that IOP exposure caused more damage in the RNFL and ONL retinal layers at the earlier (3 weeks) than later (8 weeks) time points. In the RNFL, reduction in its thickness was $0.012 \pm 0.002 \mu\text{m}$ per mm Hg day at 3 weeks compared with $0.004 \pm$

$0.001 \mu\text{m}$ per mm Hg day at 8 weeks after IOP elevation ($P = 0.049$). Reduction in ONL thickness was $0.010 \pm 0.001 \mu\text{m}$ per mm Hg day at 3 weeks compared with $0.008 \pm 0.001 \mu\text{m}$ per mm Hg day at 8 weeks ($P = 0.051$). This suggests that RNFL and ONL were more susceptible and sensitive to IOP exposure in the early time points.

OHT-Induced Changes in Regional Thickness of Whole Retina and Individual Retinal Layers

We subsequently examined the effects of raised IOP on regional thickness (T, S, N, and I) in the whole retina and within each individual layer, as shown in Figure 6. Using GLM with repeated measurements, comparison between individual regions showed no significant difference between regions over time, including when performed by pairwise comparison within each individual layer. We then looked at the whole retina and the individual layers at each specific time point using ANOVA and Bonferroni's modification to compare differences between regions. We found that all four regions of the whole retina showed reduced thickness at 3 weeks after OHT induction, but significant thinning was seen only in the S ($158.94 \pm 3.55 \mu\text{m}$; $P = 0.01$) and I ($157.12 \pm 6.95 \mu\text{m}$; $P = 0.017$) regions compared with baseline levels ($174.27 \pm 6.24 \mu\text{m}$ and $170.63 \pm 4.49 \mu\text{m}$, respectively; Fig. 7A). Interestingly, though the S region ($162.84 \pm 7.73 \mu\text{m}$) and the I region ($157.86 \pm 5.99 \mu\text{m}$) underwent no significant changes since 3 weeks, thinning at the other two regions of T ($151.5 \pm 12.15 \mu\text{m}$; $P = 0.043$) and N ($155.30 \pm 10.74 \mu\text{m}$; $P = 0.034$) became significant at 8 weeks (Fig. 7A).

Analysis of the RNFL showed that the S region underwent a significant reduction in thickness at 3 weeks (30.38 ± 3.62

FIGURE 3. IOP profiles in OHT and control rat eyes. (A) Mean baseline IOP before surgery was 10.29 ± 0.59 mm Hg. Surgery induced an increase of IOP, with a mean peak IOP of 23.43 ± 3.50 mm Hg at 2 hours (0.08 day) after surgery. The IOP gradually decreased to near baseline level at 56 days (8 weeks). (B) Cumulative IOP exposure was calculated as an integral IOP at 3 (429.79 ± 37.11 mm Hg days) and 8 (918.92 ± 65.76 mm Hg days) weeks after OHT induction.

μm ; $P = 0.022$), compared with baseline ($38.18 \pm 2.25 \mu\text{m}$), whereas the other three regions underwent modest (not significant) thinning (Fig. 7B). However, the uneven damage in the four regions of the RNFL affected the physiological regional trend of TSNI, causing an altered regional profile of NTIS at 3 weeks.

In addition to the expected changes in the inner retina, the outer retina was notably altered by IOP elevation. Although the ONL showed significant thinning in the I region only at 3 weeks ($P = 0.02$), considerable widespread reduction was observed in all four regions—T ($35.75 \pm 3.40 \mu\text{m}$; $P = 0.014$), S ($36.81 \pm 1.78 \mu\text{m}$; $P = 0.003$), N ($34.90 \pm 4.27 \mu\text{m}$; $P = 0.008$), and I ($35.45 \pm 2.30 \mu\text{m}$; $P \leq 0.001$)—at 8 weeks compared with baseline levels (Fig. 7F). Analysis of the regional trend profiles revealed that although glaucomatous damage was more severe at 8 weeks than at 3 weeks, a similar pattern of regional profiles was observed, where the N and I regions appeared to be more altered than the T and S regions, shifting the physiological regional trend of INST to STIN (Fig. 7F).

No evidence of significant glaucomatous changes was found in any other retinal layer, though a slightly altered regional profile was seen in the IPL (Fig. 7C), INL (Fig. 7D), and photoreceptor segments (Fig. 7G). Interestingly, no change in the regional trend was found in the OPL (Fig. 7E).

DISCUSSION

In vivo imaging of changes in the retinal structure has been increasingly recognized as a valuable tool in the investigation of retinal neurodegeneration in animal models.^{13–17} Using a customized adaptation of the high-resolution Spectralis OCT to image a rat model of OHT, we have demonstrated that the outer retina is affected, along with the expected thinning of the RNFL. We have also shown that glaucomatous damage in the rat may be readily correlated with whole retinal thinning. Finally, we have established that increased IOP induces a pathologic change in regional patterns in the rat retina.

In the past decade, a variety of new imaging technologies have been developed to detect retinal structural changes in glaucoma, particularly RNFL loss and optic disc atrophy. Scanning laser polarimetry, first reported in 1990, has been used to assess RNFL thickness based on the linear relationship between the birefringence and thickness of the RNFL. The currently used device, the GDx (Carl Zeiss Meditec, Vista, CA), has been shown to be more reproducible and accurate in the identification of glaucoma.^{48,49} However, the GDx is less commonly used than other imaging technologies because of the limitations of indirect measurement of RNFL thickness.⁵⁰ A cSLO (Heidelberg Retinal Tomograph [HRT]; Heidelberg Engineering) has been commercially available for topographic mapping of the optic disc and surrounding retina. The new generation of this cSLO (HRT-3; Heidelberg Engineering) provides an advanced automatic evaluation of RNFL parameters, including RNFL thickness. However, its value is limited because cSLO measurement is based on an arbitrarily designated reference plane rather than on true RNFL thickness. Recent clinical studies have shown that it is less sensitive than OCT in the assessment of RNFL damage in early glaucoma.^{51,52}

OCT technology has been shown to be a dominant strategy for clinical imaging of the RNFL. The development of the SD-OCT in 2004 has greatly enhanced the imaging speed and sensitivity of the OCT technology^{53,54} compared with time domain OCT (TD-OCT), first introduced in 1991.³ With high image capture rates of 24,000 to 55,000 A-scans/s, SD-OCT devices provide a much better axial resolution of 3 to 6 μm compared with 10 to 15 μm for TD-OCT.⁵ Recent clinical observations have demonstrated that SD-OCT may have applications in the early detection of glaucoma.^{7,51,55} In this study, we have shown that the Spectralis OCT, with an optical adaptation, is also a valuable tool to track longitudinal retinal structural changes in experimental glaucoma.

FIGURE 4. Regional thickness of the whole retina and individual layers in the normal rat. Thickness is calculated by the mean value of 40 rat eyes. RNFL, retinal nerve fiber layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer.

FIGURE 5. OHT-induced changes in the overall thickness of the whole retina and individual retinal layers. Increased IOP caused significant reduction in whole retinal thickness at 3 and 8 weeks (A; $P < 0.05$). Analysis of individual layers revealed that the RNFL was significantly thinner at 3 weeks than at baseline (B; $P < 0.05$) and that the ONL was significantly thinner at both 3 ($P < 0.05$) and 8 ($P < 0.01$) weeks after IOP elevation (B). IOP exposure caused more damage in the retinal layers at the earlier (3 weeks) than at the later (8 weeks) time points, with a statistically significant greater reduction in RNFL thickness per mm Hg day at 3 weeks than at 8 weeks (C; $P = 0.049$). * $P < 0.05$; ** $P < 0.01$.

The RGC is conventionally believed to be the only neuron affected by glaucoma. Nonetheless, increasing evidence shows that other retinal neurons, including those in the outer retina,

are also injured in optic neuropathy in humans,^{19–21,29,30,31,56} primates,^{19,33} and rodents.^{34,35,57} However, glaucomatous changes in the outer retinal layers are not well established, and there are many conflicting findings in human glaucoma,^{22–24,26–28,36–39} primate glaucoma,^{23,25} and rat models of OHT.⁴⁰

In this study, we have confirmed *in vivo* that OHT causes a significant reduction in the ONL thickness at both 3 and 8 weeks after IOP elevation in the rat. We have further shown that cumulative IOP exposure induces more damage at the earlier stage of the OHT course. Moreover, we have found that the physiological regional pattern of the ONL is affected by increased IOP as early as 3 weeks.

OHT-induced changes in ONL thickness reflect photoreceptor injury, which is consistent with previous findings of photoreceptor injury and loss histologically,^{19–21} molecular biologically⁵⁸ and functionally^{29,30,31,33–35,58} in human glaucoma^{19–21,29,30,31} primate glaucoma^{19,58,33,34} and experimental rodent OHT models.^{34,55} Moreover, a recent study using fundus reflectometry has reported that the integrity of the foveal cone outer segments is impaired in human glaucoma.⁵⁹ We have previously shown that a significant reduction of a-wave amplitudes in the dark-adapted (scotopic) ERG occurs at 16 weeks after IOP elevation (Georgiou AL, et al. *IOVS* 2008;49:ARVO E-Abstract 1557). In the present study, we found that ONL thinning can be detected as early as 3 weeks, suggesting that structural injury in photoreceptors may precede functional damage in glaucoma. A similar phenomenon has been seen in the visual field test, in which visual functional defects cannot be detected until considerable percentages of RGCs (25%–35%) have been lost.⁶⁰ The mechanisms behind glaucomatous injury of photoreceptors remain unclear, but they could be attributed to reduced blood flow through the choriocapillaris, caused by choroidal shrinkage and ischemia.^{61–64}

Whole retinal thinning has been documented in patients with early and moderate stages of glaucoma using the retinal thickness analyzer, with evidence of reduced thickness in the perifoveal area that correlated well with visual field deficits and

FIGURE 6. *In vivo* Spectralis OCT cross-sectional serial images showing OHT-induced changes at the four regions of the retina at baseline (BL) and at 3 and 8 weeks after IOP elevation, in the left eye of the same rat.

FIGURE 7. OHT-induced changes in regional thickness in the whole retina and in individual layers. Regional thickness of the whole retina (A), RNFL (B), IPL (C), INL (D), OPL (E), ONL (F), and IS/OS of photoreceptors (G) at baseline (BL) and at 3 and 8 weeks after IOP elevation. The whole retina showed significant thinning in the regions of S and I at 3 weeks ($P < 0.05$) and of T, N, and I at 8 weeks ($P < 0.05$; A). For individual layers, the RNFL was significantly thinner in the S region at 3 weeks ($P < 0.05$; B). The ONL showed significant thinning in region I at 3 weeks ($P < 0.05$) and in all regions (T, S, N, and I) at 8 weeks ($P < 0.05$ to $P < 0.01$; F). There were no significant changes in other retinal layers (C, D, E, G). * $P < 0.05$; ** $P < 0.01$.

ONH cupping.^{65–69} This is supported by recent OCT measurements in which macular thickness is decreased in the early stage of glaucoma and reduced macular volume is correlated with changes in the RNFL and visual function.^{70–74} Whole retinal thinning has also been histologically demonstrated in transgenic DBA mice.³⁵

Using a rat model of OHT, our OCT measurements revealed that thinning of the whole rat retina appeared as early as 3 weeks after IOP elevation and up to 8 weeks later in vivo, with altered regional patterns. These findings in the rat are comparable to those in human glaucoma in terms of reduced whole retinal thickness in the early stage of the disease.^{65–68} Moreover, we show that OHT-induced whole retinal thinning is significantly correlated with reduced thickness in the RNFL and ONL, suggesting that the OCT measurement of whole

retinal thickness may be used as a surrogate parameter in glaucoma management when separate layer identification was not possible.

Optical modification of OCT allows the illustration of individual layers of the retina in rodents.^{14–18} In this study, we manually segmented individual retinal layers in the four regions (T, S, N, I) and found no significant regional variation in any layers in the normal rats. This differed from the findings in human and monkey, in which peripapillary RNFL thickness is characterized by a “double-hump” pattern with peaks in the superior and inferior quadrants and troughs in the temporal and nasal quadrants.^{75–77} This may be attributed to the difference in spatial distribution of the RNFL and RGCs in the fundus in the rodent eye. In humans, a large proportion of foveal fibers are derived from the temporal aspect of the ONH, and these

fibers appear to be displaced to more superior and inferior regions, which may account for the double-hump pattern.⁷⁸ In contrast, the rat has no macular region anatomically, and the spatial distribution of the RGC nerve fibers appears to occur in a radial symmetric pattern surrounding the ONH.⁷⁹ RGC counts in rats show no significant difference among the four regions (T, S, N, I),⁷⁹ though this is controversial.^{80,81}

Although we found no significant regional differences in the RNFL in normal rats, a regional trend reduction in thickness has been observed in an order of the temporal, superior, nasal and inferior regions ($T \geq S \geq N \geq I$). We subsequently found that this regional profile was affected as early as 3 weeks after OHT induction, with evidence of a shift of the regional pattern from TSNI to NTIS, after significant thinning in the superior region (Fig. 7B). This finding in the rat shares similarity with human glaucoma, in which the thicker regions are more vulnerable to damage than the thinner regions. In humans, the neuroretinal rim of the ONH, an intrapapillary extension of the RNFL, shows a characteristic configuration in normal eyes. Neuroretinal rim width is thickest in the inferior region, followed by the superior and nasal regions, and is thinnest in the temporal region. This pattern of rim width is widely known as the ISNT rule (inferior $\geq$ superior $\geq$ nasal $\geq$ temporal). Glaucoma preferentially damages superior and inferior optic nerve fibers before temporal and nasal fibers, leading to loss of the physiological rim shape.^{82,83} Thus, violation of the rule has been shown to have predictive value in the diagnosis of glaucoma.^{83,84}

In the present study, overall RNFL thickness in the OHT rat is thinnest at 3 weeks after IOP elevation. Interestingly, this is consistent with our previous findings that peak RGC apoptosis occurs at 3 weeks after OHT induction using the same animal model.^{44,85,86} The development of RGC death in glaucoma is thought to be caused by primary degeneration of the RGC or secondary degeneration from RGC axonal injury.^{87,88} Our results are unable to separate either mechanism because both RGC apoptosis and axonal degeneration reach their peaks as early as 3 weeks after OHT induction. In summary, we suggest that the Spectralis OCT may be a useful adjunct in the assessment of experimental OHT in the rat.

Acknowledgments

The authors thank the staff of Heidelberg Engineering for technical advice, William Cheung for assistance with MATLAB software for OCT imaging analysis, and Mukhtar Bizrah for proofreading the manuscript.

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