The Effect of Acutely Elevated Intraocular Pressure on the Functional and Blood Flow Responses of the Rat Retina to Flicker Stimulation

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PURPOSE. To evaluate the effect of acutely elevated intraocular pressure (IOP) on the functional and blood flow responses of the rat retina to flicker stimulation.

METHODS. Brown Norway (n = 15) rats were dark-adapted before ketamine/xylazine anesthesia. IOP was raised acutely in one eye to ~45 mm Hg with a vascular loop. In 11 rats, white light flicker stimulus (10 Hz, 2 seconds duration, 0.80 log scotopic cd·s/m²) was applied before and during IOP elevation, and 10 minutes after loop removal. Changes in the total retinal blood flow (TRBF) and retinal function induced by the visual stimulus were measured simultaneously with a combined optical coherence tomography (OCT) + electroretinography (ERG) system. Systemic blood pressure was measured in the remaining four rats frequently from 10 to 90 minutes post anesthesia injection.

RESULTS. The systemic blood pressure remained at 99 ± 4 mm Hg throughout the measurements (n = 4). Under normal IOP the TRBF was 5.6 ± 1.9 μL/min, and the average retinal blood vessel size (BVS) in the vicinity of the optic nerve head (ONH) was 44.1 ± 4.5 μm. During IOP elevation, the TRBF was significantly lower (3.8 ± 1.2 μL/min, P < 0.01) and the BVS was significantly smaller (35.1 ± 2.6 μm, P < 0.01). Both TRBF and BVS returned to baseline within ~10 minutes from removal of the vascular loop. The flicker-induced TRBF change measured under normal IOP (6.0 ± 3.3%) was reduced significantly to 0.1 ± 0.3% (P < 0.01) during IOP elevation, and recovered to 5.9 ± 1.7% within 10 minutes after loop removal. During IOP elevation, the magnitude of the ERG second harmonic component (SHC) decreased to 55% of its baseline value (P < 0.01) and remained significantly smaller than baseline (P < 0.01).

CONCLUSIONS. Acute IOP elevation to 45 mm Hg caused suppression of the retinal functional and TRBF response to flicker stimulation.

Keywords: doppler optical coherence tomography, retina imaging, neurovascular coupling, intraocular pressure

Elevated intraocular pressure (IOP) is one of the most well-studied and documented pathogenic risk factors for open-angle glaucoma (OAG),1–5 and as such, numerous animal models have been developed to study the acute and chronic IOP elevation effect on optic nerve head (ONH) structure,4–8 retinal blood perfusion,9–11 retinal and ONH autoregulation,11 and ganglion cell function.12–15

Currently retinal nerve fiber layer thinning and ONH deformation are recognized as major IOP-induced retinal alterations in the retinal structure; however, these morphologic retinal changes develop over a fairly long time to the extent that they are measurable by ophthalmic imaging modalities. More recent studies have shown that hypertensive glaucoma patients exhibit reduced flicker-induced blood flow changes and vasodilation compared to healthy subjects.16–20 Specifically, Riva et al.19 used laser Doppler flowmetry and reported that the retinal blood flow (RBF) response at the optic disc rim to flicker stimulation is significantly reduced in early OAG and ocular hypertension patients. Similarly, Gugleta and colleagues17,20 conducted a study using a retinal vessel analyzer and reported attenuated flicker-induced vasodilation in early glaucoma and ocular hypertension patients. Garhöfer et al.19 used retinal vessel analyzer to show that vasodilation in retinal veins in response to flicker stimulation is greatly diminished in early glaucoma patients. Results from those studies suggest that visually evoked retinal blood vessel vasodilation and changes in the RBF could be affected by elevated IOP. However, as glaucoma is a multifactorial disease, direct correlation between the altered neurovascular coupling and elevated IOP lacks direct evidence.

So far, various imaging modalities have been used to investigate the neurovascular coupling in the retina, including the blue field entoptic method,21 laser Doppler velocimetry,22 fluorescence microspheres,23 and functional MRR(BOLD).24 Over the past few years, Doppler optical coherence tomography (OCT) and optical coherence tomography angiography (OCTA) have gained significant clinical importance for clinical diagnostics and treatment management of various retinal
diseases including glaucoma. Different OCT system designs and/or image acquisition protocols have been utilized in methods such as the en face integration method, the double concentric circle method, and the multidirectional beam, developed for accurate retinal total blood flow measurements in human subjects and animal studies.

Doppler OCT was also used to measure changes in the RBF in response to flicker stimulation in both humans and animals. Recently, our research group has developed a combined OCT-ERG system that allows for examination of the neurovascular coupling in the rat retina. A study conducted with this system in healthy rats under normal IOP showed that flicker-induced changes in the RBF are time-correlated with amplitude and latency changes of the ERG traces.

Here we present results from a study that used the OCT-ERG system to examine the effect of acutely elevated IOP on the functional and blood flow responses of the rat retina to flicker stimulation. The TRBF and retinal blood vessel size (BVS) before, during, and after acute IOP elevation are also reported.

**Materials and Methods**

**Animal Preparation**

All experiments described here were approved by the University of Waterloo Animal Research Ethics Committee and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male Brown Norway rats (n = 15, Harlan Laboratories, Inc., Indianapolis, IN, USA), weighing ~300 g, were dark adapted for at least 12 hours in a 12/12 light cycle room prior to the experiment. The rats were transferred from the animal room to the research lab in light-isolated cages, and all animal procedures except for the OCT-ERG data acquisition were conducted under a dim, red headlight (631 nm, <10.9 lux). The rats were anesthetized with 200 μL/100 g ketamine/xylazine cocktail (0.75 mL ketamine + 0.5 mL xylazine + 0.75 mL sterile water) through intraperitoneal injection (IP), followed by a 5 mL saline IP injection to keep the body hydrated. One drop of topical anesthetic (0.5% proparacaine hydrochloride, Alcaine; Alcon, Mississauga, ON, Canada) was applied to each eye, followed by one drop of tropicamide (Alcon) for pupillary dilation. To keep the cornea properly hydrated and therefore optically transparent, artificial tears were applied to both eyes every ~5 minutes. The rats were separated into two groups: OCT-ERG recordings were collected from 11 rats before, during, and after IOP elevation. Systemic blood pressure was measured from the remaining four rats with a CODA surgical monitor (Kent Scientific, Torrington, CT, USA) before, during, and after IOP elevation for the same duration as for a regular OCT-ERG recording session.

For the OCT-ERG imaging procedure, the animals were placed on a custom stereotaxic holder that includes a bite bar, a nose bar, and ear bars, designed to minimize head motion caused by breathing. The base of the animal holder was lined with an ear bar, designed to minimize head motion caused by breathing. The base of the animal holder was lined with an ear bar, designed to minimize head motion caused by breathing. The base of the animal holder was lined with an ear bar, designed to minimize head motion caused by breathing.

**IOP Elevation Protocol**

An adjustable vascular loop (Sentinal Loops; Sherwood-Davis & Geck, St. Louis, MO, USA), placed anterior to the equator of the eye, was used to elevate the IOP in one eye to ~45 mm Hg for a duration of 30 minutes, following a procedure developed by Joos et al. The IOP was measured with a corneal rebound tonometer (Icare; Tonolab, Tuik, Finland) before placement of the vascular loop, approximately 10 minutes after placement of the loop, immediately before the OCT-ERG imaging procedure, and approximately 10 minutes after removal of the loop.

**OCT-ERG System**

A research grade, ultrahigh-resolution spectral-domain OCT system, combined with a commercial ERG system (Diagnosys LLC, Lowell, MA, USA), was used for this study. The OCT-ERG system was developed by our research group for various animal retinal studies and was recently used to evaluate the effect of single-flash and flicker stimuli on the total axial RBF. In this study, a different scanning protocol was used in order to evaluate the total retinal blood flow (TRBF) before, during, and after IOP elevation. Briefly, the OCT system operates at the 1060-nm spectral region and provides ~3-μm axial and ~5-μm lateral resolution in the rat retina, at 92 kHz image acquisition rate. For this study, the camera data acquisition rate was reduced to 47 kHz in order to improve the signal-to-noise ratio of the Doppler OCT signal and ensure maximum measurable flow velocity of 8.6 mm/s, sufficient for quantitative blood flow measurements in the rat retina. The OCT imaging probe was designed to deliver a collimated imaging beam of 1.5-mm diameter and optical power of 1.7 mW to the rat cornea, and was integrated with a custom visual stimulator connected to the commercial ERG system. The visual stimulator was designed to focus the stimulus light at the pupil plane, thus providing a wide-angle, uniform Maxwellian illumination of the retina. The intensity and duration of the visual stimuli were controlled from the ERG console, and the OCT and ERG data acquisition were synchronized.

**Data Acquisition**

Doppler OCT and ERG data were acquired immediately after the IOP measurements for the three time points of the study: before IOP elevation (pre-loop), during IOP elevation (loop-on), and after loop removal (post-loop). A Doppler OCT scanning protocol based on two concentric circular scans centered at the ONH, similar to the protocol proposed by Shahidi et al., was used in our study to determine the Doppler angle necessary for the calculation of the TRBF. Figure 1A shows an en face maximum-intensity projection morphologic image of the rat ONH and vicinity, with colored dashed lines marking the Doppler OCT concentric circular scan pattern (O₁ = 0.65 mm, O₂ = 0.85 mm). The size of each circular OCT scan was 4000 × 512 (A-scans × pixels), and five repeated scans were acquired consecutively from the same location in the retina. A live en face OCT preview image of the rat retina was used to center the two concentric scans at the ONH to make adjustments in the alignment between consecutive recordings to correct for misalignment induced by eye motion. Data with this protocol were acquired prior to the simultaneous Doppler OCT and ERG recordings for each loop phase to determine the Doppler angle between each retinal blood vessel and the OCT imaging beam. A continuously repeated single-circle Doppler OCT scan pattern (O = 0.75 mm) was used for the simultaneous Doppler OCT and ERG recordings in order to achieve high temporal resolution of 85 ms, sufficient to track the blood flow pulsatile oscillations and the stimulus-induced TRBF changes. Each synchronous Doppler OCT and ERG recording lasted 6 seconds (1 second long pre-stimulus, 2 seconds long flicker stimulus, and 3 seconds long post-stimulus period). White light flicker stimulus (10 Hz, 2 seconds duration, 0.80 log scotopic cd/m²) with 20% duty
cycle and 100% modulation depth was used. Five OCT+ERG recordings were acquired for every loop phase, with a 3-minute rest interval between consecutive recordings. For the ERG recordings, the positive loop electrode (Ø = 4 mm) was placed onto the cornea, leaving a clear aperture for the OCT imaging beam. Artificial tears (Refresh Tears; Alcon) were applied to the imaged eye to ensure optimal impedance between the ERG loop electrode and the cornea. The negative electrode needle was placed subcutaneously behind the ear, and the ground electrode needle was placed in the skin between the ears. The ERG system has a 1.5-kHz sampling frequency and a built-in band pass filter (0.3–300 Hz).

DATA ANALYSIS

OCT Doppler Data

Retinal blood vessels in the vicinity of the ONH were manually selected from the circle Doppler OCT scans, and the axial blood flow was calculated by integrating the blood velocity over the selected blood vessel area. Arterial and venal blood flow were calculated separately due to the different polarities of the respective phase changes, and the axial flow rate was calculated based on the phase difference between adjacent A-scans. In order to compute the TRBF, knowledge of the Doppler angle between the OCT imaging beam and the orientation of each retinal blood vessel is required. The Doppler angle \( \theta \) was determined from the shift of the retinal blood vessel locations between the two concentric circular Doppler OCT scans:

\[
\text{Doppler Angle} = \arccos \left( \frac{\Delta z}{\sqrt{\Delta x^2 + \Delta y^2 + \Delta z^2}} \right)
\]

Here, \( \Delta y \) and \( \Delta z \) are the lateral and axial displacements of the blood vessel between the two circular scans, respectively, and \( \Delta x \) is the distance between the two circular scans. Figure 1A shows an en face maximum-intensity projection image of the rat retina with colored dashed lines marking the Doppler OCT concentric circular scans centered at the ONH. Figure 1B shows an overlay of the two OCT circular tomograms, color coded in violet and green. Figures 1C and 1D show a magnified view of the location in Figure 1B marked with the yellow dashed line and demonstrate the shift in location of the major retinal blood vessels between the two concentric circular scans, used for the calculation of the Doppler angle. TRBF was calculated by integrating over the total blood flow data for all retinal arteries and veins. A moving window (Savitzky-Golay, window size = 0.2 second, polynomial order = 3) smoothing algorithm was used to filter out cardiac oscillations in the temporal TRBF recordings arising from pulsatile blood flow. The BVS for all retinal vessels was also determined manually by two researchers by measuring the shadow underneath each vessel in the Doppler OCT images. The five repeated concentric circular OCT scans acquired from the same locations in the retina were aligned and averaged in order to reduce speckle noise and thus improve the precision of the BVS analysis.

ERG Data

ERG traces were averaged over the five recordings for each loop phase. In accordance with the standard for processing ERG recordings, the first negative and positive peaks in the ERG traces were used for calculating the ERG metrics. A Fourier transform was applied to the ERG recordings to extract the first harmonic component (FHC = 10 Hz) and the second harmonic component (SHC = 20 Hz), and explore their dependence on the elevated IOP.

Statistics

One-way ANOVA was used to detect any significant differences in all measured parameters such as the IOP, the TRBF, the BVS, the flicker-induced changes in the TRBF, and the ERG components. Bonferroni-corrected multiple comparison post hoc tests were applied to determine any significance between the pre-, during-, and post-loop conditions. Differences were considered significant when \( P < 0.05 \). All data in the text are presented as mean ± standard deviation (SD), while data in all figures are presented as mean ± standard error (SE).

RESULTS

Intraocular Pressure

The pre-loop IOP was 9.6 ± 1.1 mm Hg and when elevated, it stabilized at 43.1 ± 6.8 mm Hg after 10 minutes of loop wear (\( P < 0.01 \)). After 10 minutes of post-loop recovery, the IOP decreased and stabilized at 7.1 ± 1.1 mm Hg (\( P < 0.01 \)).
was no significant difference between the pre-loop and post-loop IOP values ($P = 0.54$).

TRBF and BVS

Acute elevation of the IOP caused temporary shape changes of the ONH (Figs. 2A–C), as well as temporary constriction of the retinal blood vessels (Figs. 2D–F), observed in the volumetric and cross-sectional OCT images, respectively. The red arrows in Figures 2D through 2F show the change in blood vessel diameters with IOP elevation. Figure 3 shows statistical results for the TRBF and the BVS measured during the pre-loop, loop-on, and post-loop phases in complete darkness (no visual stimulation). The TRBF decreased significantly from $5.6 \pm 1.9$ to $3.8 \pm 1.2 \mu L/min$ during loop wear ($P = 0.02$) and recovered to $5.5 \pm 1.2 \mu L/min$ after 10 minutes from loop removal ($P = 0.02$ relative to loop-on). No significant difference between the pre-loop and the post-loop data was observed ($P = 1.00$). On average (over all retinal arteries and veins and all animals), the elevated IOP had a significant effect on the retinal BVS. The average retinal blood vessel diameter was $44.1 \pm 4.5 \mu m$ at normal IOP (pre-loop), reduced significantly to $35.1 \pm 2.6 \mu m$ (20.0 \pm 4.7%, $P < 0.01$) during the IOP elevation (loop-on), and recovered to $42.4 \pm 3.3 \mu m$ within 10 minutes after loop removal ($P < 0.01$). There was no significant difference in the average blood vessel diameter between the pre-loop and post-loop measurements ($P = 0.27$).

Electroretinography

Figure 5A shows a typical ERG trace acquired in our study, with its single-flash and flicker components marked with the red and green line boxes. Acute IOP elevation caused a temporary increase in the a-wave and b-wave amplitudes and latencies of the ERG single-flash component for the duration of the loop-induced TRBF response recovered to $5.9 \pm 1.7\%$ ($P < 0.01$). No significant difference was detected in the visually evoked TRBF responses between baseline and recovery ($P = 1.00$).
wear, and recovery of the ERG signal to normal after 10 minutes of the vascular loop removal (Fig. 5B). The magnitude of the ERG flicker component decreased during loop wear and returned to normal after loop removal (Fig. 5C). Synchronously, the flicker-induced TRBF changes were suppressed during loop wear and recovered to normal within 10 minutes of loop removal (Fig. 5D).

Increase of the single-flash a-wave and b-wave magnitudes was observed during the IOP elevation (131.0 ± 21.3 and 166.5 ± 44.7 μV, respectively), compared to the pre-loop value (a-wave 98.5 ± 22.9 μV, P = 0.02; b-wave 63.8 ± 29.1 μV, P < 0.01) and at post loop value (a-wave 110.8 ± 21.2 μV, P = 0.06; b-wave 103.0 ± 17.5 μV, P < 0.01) (Figs. 6A, 6B, respectively). The FHC of the flicker ERG was not affected significantly by the loop wear (P = 0.18, Fig. 6C). However, the SHC decreased significantly from 26.7 ± 4.8 mV (pre-loop) to 12.4 ± 4.4 mV during loop wear (P < 0.01), corresponding to a 55% reduction in amplitude, and recovered to 18.4 ± 2.8 mV after loop removal (P < 0.01). The post-loop SHC value was significantly lower than the pre-loop measurement (P < 0.01).
**DISCUSSION**

Results from this study show that elevation of the IOP to 45 mm Hg is associated with reduced flicker-induced TRBF response. Previously, clinical studies conducted on high ocular tension glaucomatous patients revealed reduced flicker-induced vasodilation and RBF response. Specifically, by using 15-Hz green light flicker stimulation, Riva et al. found that the magnitude of the blood flow response to flicker stimulation at the neuroretinal rim of the optic disc decreased significantly in ocular hypertension and early glaucoma patients. Similarly, Gugleta et al. observed reduced flicker-induced vasodilation of retinal blood vessels in OAG patients and ocular hypertension patients for different ages and development stage of the glaucoma. Specifically, flicker-induced vasodilation in retinal veins was 0.8 ± 2.5% in early glaucoma patients, compared to 2.1 ± 2.1% in healthy subjects. Both these clinical studies and our animal study show similar trends (reduced flicker-induced TRBF and vasodilation); however, results from the studies cannot be compared directly for the following reasons. The subjects in the clinical studies had chronically elevated IOP, while in our animal study, the IOP level was acutely elevated; the IOP level in the clinical subjects was much lower (<30 mm Hg), while in our study, the IOP level was raised to 45 mm Hg to simulate intermittent, short-duration spiking of the IOP, which has been observed in some glaucomatous subjects. In a different study, Garhofer et al. used the ocular suction cup method to elevate the IOP up to 43 mm Hg for 60 seconds. ONH blood flow was measured by laser Doppler flowmetry and retinal vessel diameter was measured by retinal vessel analyzer, and as a result the response of retinal vessel diameter and ONH blood flow to the luminance flicker did not change significantly. It is likely that 60 seconds of IOP elevation to 43 mm Hg may not be sufficient to induce measurable changes in the human ONH blood flow, and different responses between ONH blood flow and RBF in response to acute IOP elevation also need to be considered.

The amplitude of the flicker-induced RBF change can be affected by the intensity, duration, and frequency of the flicker stimulation. In previous studies, researchers used flicker stimuli with durations ranging from 2 to 60 seconds, and the flicker-induced hyperemic RBF changes in rats varied from 4.7% to 28.1%. Specially, Kornfield et al. used the same flicker stimulus duration as in our study (2 seconds), as well as the same animal species (rats). Results from this study are very close to the results from our study (flicker-induced RBF change relative to baseline: ~4.7% and ~6%, respectively). The difference in the results between the two studies can be attributed to differences in the type of anesthesia, the flicker frequency (5 vs. 10 Hz), and the measurement methods (functional magnetic resonance imaging versus Doppler OCT).

The effect of elevated IOP on the retinal blood perfusion and physiological response to visual stimulation is complex. Results from multiple studies suggest that the observed decrease in the TRBF and the vasoconstriction with elevated IOP could be associated with insufficient metabolic supply to the retina, caused by activated astrocyte, endothelial dysfunction, and excess of nitric oxide (NO), though the exact physiological mechanism of these changes is unknown. The effect of elevated IOP on the functional response of the retina to visual stimulation has been studied extensively, and more groups report decrease of the ERG a- and b-wave amplitudes with elevated IOP. Our research group reported recently an initial pronounced increase of the ERG a- and b-wave amplitudes in rats during IOP elevation to a moderate level of 35 mm Hg by use of a vascular loop. Although such increase may be counterintuitive, it was observed consistently both in the current study...
and in other studies conducted on rats of different strains. Further increase of the IOP with a vascular loop to ischemic levels > 60 mm Hg showed progressive decrease in the ERG a- and b-wave amplitudes (data not shown). Such supranormal scotopic a-wave and b-wave amplitudes with normal implicit times have been reported for specific conditions, including loss of retinal dopaminergic amacrine cells, blockage of retinal dopamine receptors, gestational low-level lead exposure in rats and humans, and loss of a mitochondrial ATP transporter in Ant1−/− mice. Furthermore, Vielma et al. showed that low-level intravitreal injections of NO into rat eyes is associated with increases in the amplitudes of ERG a- and b-waves, oscillatory potentials, and positive scotopic threshold responses. Several groups have reported that chronic elevation of IOP to less than 35 mm Hg in the retina and the optic nerve.

Reduced ocular perfusion pressure (OPP), is another potential factor in reduced neurovascular coupling. OPP is usually calculated as the difference between mean arterial pressure and IOP. Reduced OPP could result in secondary insult to the retinal ganglion cells by breaking down autoregulation and altering neurovascular coupling. Riva et al. raised the IOP in cats to 57 mm Hg, which corresponds to OPP of ~40 mm Hg, and reported reduction of the neurovascular coupling at the ONH. When the IOP was raised further to ~77 mm Hg, corresponding to 20 mm Hg, no flicker-induced changes in the RBF were observed. In our study, we observed diminished flicker-induced TRBF response when the OPP was ~55 mm Hg, which is higher than what has been reported previously. The discrepancy can be attributed to differences between the imaging technologies, the species, and the disparity of ONH BF and RBF in response to reduced OPP.

The fundamental harmonic component (FHC) and the SHC of flicker ERG could provide significant insight into the physiological mechanisms of reduced neurovascular coupling. It has been reported that in humans and monkeys, the FHC and SHC of the flicker ERG are affected mostly by the outer retina and inner retina, respectively. Furthermore, a strong correlation between flicker-induced RBF change and SHC was reported in both monkeys (Bolay S, et al. IOVS 2003;44:ARVO E-Abstract 351), and humans, though in those cases the blood flow measurements and ERG recordings were acquired separately instead of synchronously. In this study, we observed synchronously a significant decrease in both the magnitude of the SHC of the flicker ERG and the flicker-induced TRBF change and during acute IOP elevation to a nons ischemic level of 45 mm Hg. Furthermore, the FHC of the flicker ERG remained unchanged during acute IOP elevation. As FHC is mostly affected by the outer retina, it is likely that the metabolic supply to the outer retina was not affected significantly by the IOP elevation. Most likely this is due to the fact that oxygen and nutrition to the outer retina are provided by the choroidal flow, which has been shown to resist changes in response to acute IOP elevation.

Using OCT+ERG to measure flicker-induced RBF and retinal function responses can be adapted for human clinical studies with some modifications. First, a faster camera is required to minimize artifacts associated with involuntary eye motion and remove the potential phase unwrapping associated with faster blood flow rate in human subjects compared to rats. Second, the OCT+ERG imaging probe needs to be redesigned to be adapted to the size and optics of the human eye.

In conclusion, results from our study showed that acute IOP elevation to the level of 45 mm Hg causes a significant decrease of both the flicker-induced TRBF change and the magnitude of the SHC of the ERG recordings. However, the ERG a-wave and b-wave amplitudes showed an increase. Future studies in which the IOP is raised progressively from normal to ischemic levels and the respective changes in the retinal morphology, TRBF, BVS, flicker-induced TRBF response, and ERG metrics are recorded simultaneously with the OCT+ERG system may provide valuable information regarding the early stages of IOP elevation–induced retinal damage. Such studies may prove more definitively that flicker-induced changes in the TRBF and the ERG metrics may be more sensitive markers to elevated IOP-induced damage compared to markers based purely on morphologic analysis such thickness changes in the nerve fiber layer or ganglion cell layer, or shape changes of the ONH.

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References


