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Functional Characterization of Retina and Optic Nerve after Acute Ocular Ischemia in Rats

Sinisa D. Grozdanic,¹ Donald S. Sakaguchi,² Young H. Kwon,³ Randy H. Kardon,³ and Ioana M. Sonea¹

PURPOSE. To functionally characterize the status of the rat retina and optic nerve after acute elevation of intraocular pressure (IOP) and to determine the dynamics of the pathologic changes in the ischemic retina and optic nerve.

METHODS. Retinal ischemia was induced in rats by acutely increasing the IOP (110 mm Hg/60 minutes). Direct and indirect pupil light reflexes (PLRs) were recorded from the noninjured eye, and electroretinograms (flash and flicker ERG) were recorded from the injured and control eyes before and after surgery. Amplitudes and latencies were calculated for each recording session.

RESULTS. Preoperative PLR_{ratio}s (indirect/direct PLR) were 76.7 ± 2.6 (mean \pm SEM). Twenty-four hours after surgery the PLR_{ratio} was 15.2 ± 12.8 , 10 days after surgery, 11.6 ± 9.8 ; 20 days after surgery, 26.5 ± 8.0 ; and 28 days after surgery, 33.27 ± 9.3 . However, at day 35, the PLR had significantly recovered (41.1 ± 7.3) when compared with the 24-hour postoperative ratios ($P < 0.01$, repeated-measures ANOVA). Forty-two days after surgery, the PLR_{ratio} started to decrease once again in the injured eyes (28.7 ± 5.9). Electroretinographic amplitudes (full-field flash ERG) followed a similar pattern. Cone responses (flicker ERG) were measured 42 days after surgery and revealed defects in injured eyes (control eyes: $46.6 \pm 2.9 \mu\text{V}$, injured eyes: $3.4 \pm 1.7 \mu\text{V}$). Histologic analysis revealed ischemic damage to all retinal layers, with the primary defects localized to the central retina.

CONCLUSIONS. Acute ocular ischemia causes a significant decrease in retinal function, as measured by PLR and ERG, although over time the rat retina and optic nerve show partial regain of function. (*Invest Ophthalmol Vis Sci.* 2003;44:2597-2605) DOI:10.1167/iovs.02-0600

Ischemic insults to the retina and optic nerve are frequently observed in glaucoma, acute ocular hypertension, diabetic retinopathy, hypertension, and vascular occlusion, and giant

cell arteritis and can lead to serious perturbation of neuronal and glial retinal elements and can ultimately lead to blindness. Traditionally, retinal damage due to ischemia has been considered a potentially incurable condition in humans and animals because of the mammalian central nervous system has no regenerative capacity. Few reports have described the recovery of visual function in patients who have severe retinal ischemia after central retinal artery occlusion,^{1,2} whereas more reports have confirmed that severe ischemic events are followed by almost complete and irreversible loss of visual function.³⁻⁶ To understand the pathologic mechanisms in retinal and optic nerve hypoxia it is essential to develop strategies for the continuous and objective monitoring of visual function in easily accessible and reproducible animal models. One of the most frequently used models for the investigation of molecular mechanisms and potential therapeutic strategies for retina and optic nerve ischemia has been a rat model of acute elevation of intraocular pressure (IOP), characterized by ischemia-reperfusion injury. Numerous studies have identified molecular mediators with neurotoxic properties during ischemia-reperfusion injury of the rat retina and optic nerve, such as excitatory amino acids,⁷ free oxidative radicals,^{8,9} and cytokines.^{10,11} However, quantitative analysis of the damage has been achieved only by end-stage counting of the cells in the different retinal layers¹² or retrograde fluorescent labeling of retinal ganglion cells (RGCs).¹³ Relatively few studies have included a functional analysis of the retina after ischemia-reperfusion injury using electroretinography (ERG) for a prolonged period,¹⁴⁻¹⁶ and, to our knowledge, there is only one published study that actually documents function of the retina and optic nerve in rats after ischemia-reperfusion injury (Clarke RJ, Gamlin PDR, ARVO Abstract 4593, 1998). Although morphologic studies provide important information about the number of surviving cells, only electrophysiological studies offer precise information about the functional status of the retina and the dynamics of ischemic injury.^{17,18} The goal of this study was the functional characterization of the electrophysiological status of the retina and optic nerve after ischemia-reperfusion injury in rats over time (6 weeks), using ERG and computerized pupillometry. Also, we were interested in observing the dynamics of the functional deficit in vivo and in correlating electrophysiological changes with histologic and morphometric analysis of the retina from the injured eyes.

MATERIALS AND METHODS

Induction of Retinal Ischemia by Elevated IOP

All animal studies were conducted in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research, and procedures were approved by the Iowa State University Committee on Animal Care. A previously published procedure to generate an ischemia-reperfusion insult in rats was used, with slight modification.¹⁹ Briefly, adult Brown Norway rats ($n = 11$) were initially anesthetized with 4% halothane, 30% nitrous oxide (NO), and 70% oxygen (O₂). Anesthesia was maintained with 2.5% halothane, 30% NO, and

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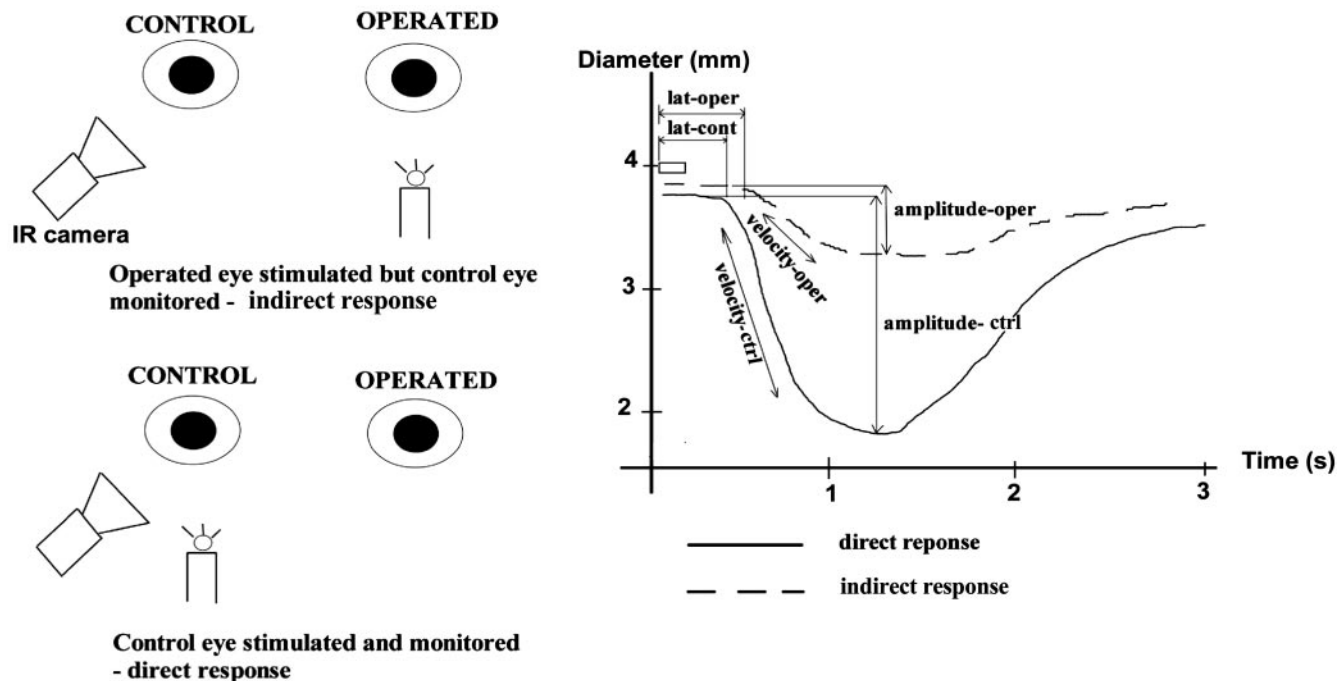


FIGURE 1. The PLR monitoring technique. A one-channel computerized pupillometer was used to record the movement of the pupil from the control (noninjured) eye while the stimulus light was alternated between the injured and noninjured eye. Software routines were used to analyze the recorded tracings of the pupil movements in response to light stimuli and to determine objectively the latency (lat-ctrl and lat-oper), velocity (velocity-ctrl and velocity-oper), and amplitudes of responses for control (ctrl) and injured (oper) eyes.

70% O₂, and body temperature was maintained with a heating pad. The pupils were dilated with topical 2.5% phenylephrine hydrochloride and 1% tropicamide. After topical instillation of 0.5% proparacaine hydrochloride, the anterior chamber was cannulated with a 25-gauge needle connected to a reservoir containing 0.9% NaCl. The IOP in experimental eyes was controlled by the height of the reservoir, to

maintain a pressure of 110 mm Hg for 60 minutes. Retinal ischemia was confirmed by the blanching of the iris and retinal circulation. At the end of the period of elevated IOP, the needle was removed, and reperfusion of the retinal vasculature was confirmed by ophthalmoscopic examination. To prevent potential infection, antibiotic ointment (neomycin+polymyxin B+bacitracin; Bausch & Lomb

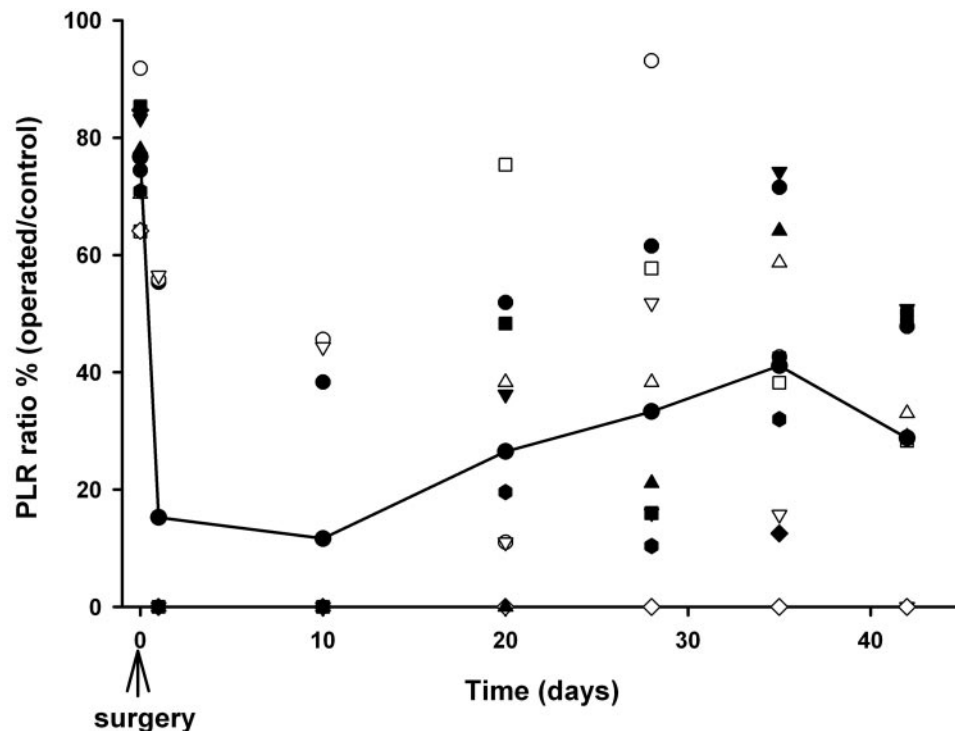


FIGURE 2. The dynamics of PLR recovery. The PLR was detectable in 3 of 11 injured animals 24 hours after surgery. However, at day 35 the PLR displayed significant recovery when compared with the 24-hour postoperative values ($P < 0.01$, repeated-measures ANOVA with Bonferroni posttest). Forty-two days after surgery the PLR started to decrease once again in the injured eyes and was not significantly different compared with the condition 24 hours after surgery ($P > 0.05$, repeated-measures ANOVA with Bonferroni posttest). Solid line connects mean values for each group, symbols represent individual animals.

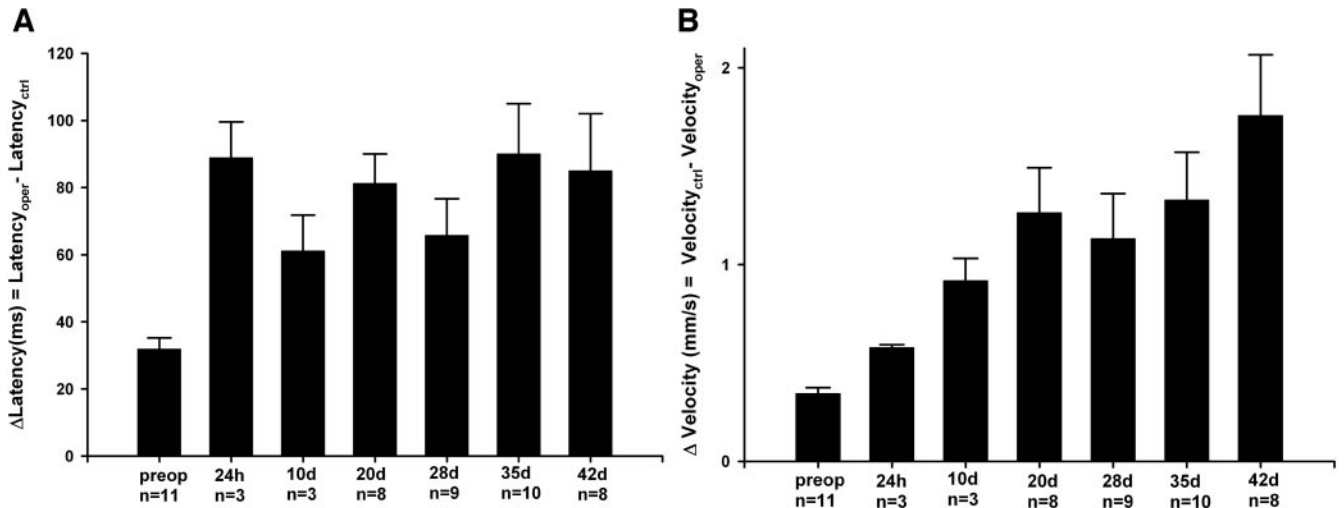


FIGURE 3. Combined data for injured eyes that had detectable pupil responses when injured eye was stimulated and control (noninjured) eye monitored. **(A)** Whereas mean levels of the ratio between indirect and direct pupillary response showed recovery of function with a peak at 35 days, latency defects (statistical comparison to preoperative values showed significant difference: $P < 0.05$, Kruskal-Wallis nonparametric test with Dunn's posttest) were detected at 20 and 35 days after surgery but not at 28 days after surgery ($P > 0.05$). **(B)** The velocity deficit followed a similar pattern.

Pharmaceuticals Inc; Tampa, FL) was applied topically after the procedure.

Computerized Pupillometry

The pupil light reflex (PLR) was evaluated with a custom-made computerized pupillometer (University of Iowa, Iowa City, IA) before surgery and on days 1, 10, 20, 28, 35, and 42 after surgery. Animals were anesthetized initially with 4% halothane, 30% NO and 70% O₂. A light plane of anesthesia was maintained with 1% halothane, 30% NO, and 70% O₂ to avoid suppression of the PLR response, as detected with the use of higher doses of anesthetic. The computerized pupillometer was attached to two infrared-sensitive, closed-circuit television video cameras for simultaneous visual monitoring of both pupils. However, a one-channel computerized pupillometer was used to record the movement of the pupil from the control (noninjured) eye, whereas the stimulus light was alternated between the control and injured eye. The stimuli were delivered through a mask equipped with four green (light-stimulus-delivering) and three infrared (iris-illuminating) diodes per eye. Optimal positioning of the light stimulus in relation to the eye was obtained by inserting calibrated photosensitive diodes into the orbits of a preserved rat head. The diameter of each diode corresponded approximately to the diameter of the dilated rat pupil (4 mm). Alternating light stimuli were delivered to each diode and the system adjusted until the light stimuli measured by both diodes were identical. This ensured that the alternated light stimulus used in animals was equal in luminance for right- and left-eye stimulation. Each camera was equipped with cutoff filters, to avoid detection of the corneal reflection of the stimulus light produced by the green diodes. Custom-made software routines (Winnana software; University of Iowa) were used to analyze the recorded tracings of the pupil movements in response to light stimuli and to determine objectively the timing and amplitude of the pupil reflex responses. Because the opposite, noninjured eye was used as a control at the same testing time, any defect that was caused by ischemia-reperfusion injury, was monitored longitudinally over time (Fig. 1).

Electroretinography

To quantitate postschemia recovery after acute elevation of IOP, ERG was performed 48 hours and 12, 22, 32, and 42 days after surgery. Animals were dark adapted for at least 6 hours, anesthe-

tized as described previously, and the pupils dilated with 1% tropicamide and 2.5% phenylephrine. The animals were placed in a specially designed dome with the interior completely covered with aluminum foil to obtain a Ganzfeld effect. Body temperature was maintained with a microwave-heated thermal pad (R. G. Barry Corp., Pickerington, OH). A light stimulus was delivered through the ceiling of the dome with a stimulator (PS-22; Grass-Telefactor, West Warwick, RI). To avoid any possibility of direct illumination of the eyes from the light source, the ceiling port was protected by a foil-wrapped baffle that prevented direct dispersion of the light to the animal eyes, which would result in the unequal illumination of the whole retinal field. The homogeneity of the light field at the level of the rat eyes was examined by using a cadmium-sulfide photosensitive diode with a 50-mm² surface area (the approximate surface area of the dilated rat pupil). The luminance of the Ganzfeld dome's illuminated surface was measured with a photometer (model J17LumaColor) equipped with a luminance head (model J1803; both from Tektronix, Wilsonville, OR) in five different quadrants of the dome positioned in the front, dorsally, ventrally, and laterally (right and left) from the rat head. Measured luminance in all quadrants was 1600 ± 200 cd/m². Two cotton-wick electrodes, containing Ag-AgCl cells immersed in saline, were used to obtain signals from both eyes simultaneously. The reference electrode was positioned in the ear, and the ground electrode was placed subcutaneously on the back. An evoked potential measuring system (Neuropack-MEB 7102; Nihon-Kohden America, Foothill Ranch, CA) was used to deliver a triggered output to the flash stimulator and collect signals from both eyes. A flash ERG routine was delivered at a 0.2-Hz frequency (20 averaged signals per recording session, sensitivity 100 $\mu\text{V}/\text{division}$, low-cut frequency 0.5 Hz, high-cut frequency 10 kHz, analysis time 500 ms). Isolated cone responses were recorded from previously light-adapted eyes by delivering stimuli at 20 Hz (100 averaged signals per recording session, sensitivity 50 $\mu\text{V}/\text{division}$, low-cut frequency 0.5 Hz, high-cut frequency 10 kHz, analysis time 500 ms). To avoid potential bias due to electrode differences, recordings were repeated with electrodes switched to the opposite eyes. The difference between right and left eyes before surgery was never more than 20% of recorded amplitudes.

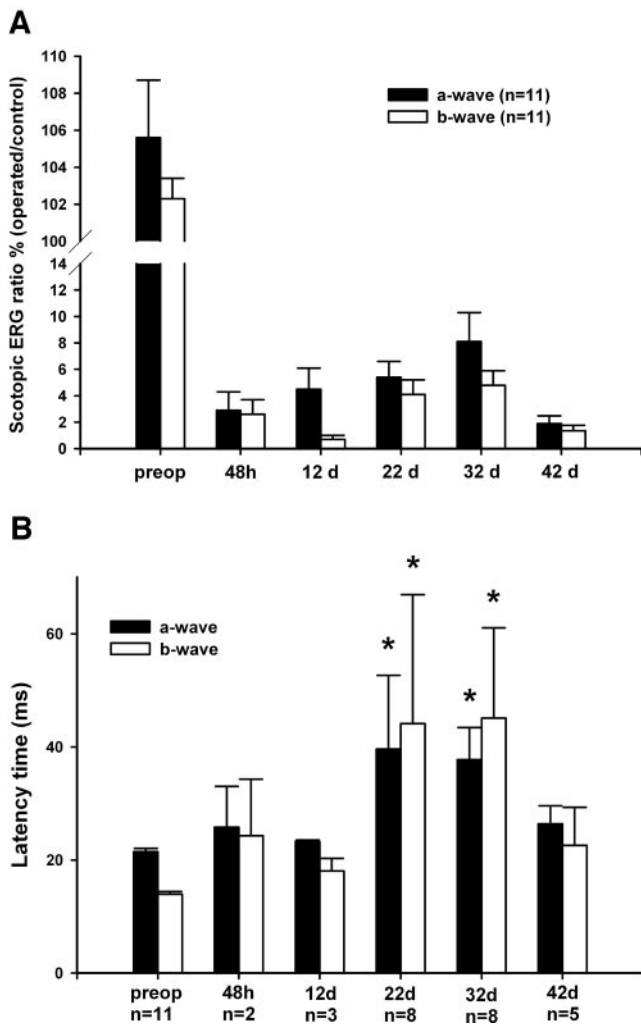


FIGURE 4. The dynamics of the full-field flash ERG amplitude recovery (A) followed dynamics of the PLR recovery (ratio for rats without detectable response was calculated as zero); however, latencies (B) at the peak recovery time points were significantly longer than in the noninjured control eyes (32 days: a-wave latency $P = 0.02$, b-wave latency $P = 0.03$; 42 days: a-wave latency $P = 0.007$, b-wave latency $P = 0.024$, paired t -test).

Histologic Examination

Forty-nine days after surgery, rats were deeply anesthetized with a high dose of phenobarbital (100 mg/kg) and perfused intracardially with ice-cold heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Optic nerves (dissected 1 mm posterior to the sclera) and eye globes were postfixed and embedded in paraffin, and 7- μ m-thick sections of the retina were collected onto poly-L-lysine-coated glass slides, and stained with hematoxylin and eosin, and transverse sections of the optic nerves were stained with 1% methylene blue. Tissue sections were examined with a photomicroscope (Microphot FXA; Nikon Corp., Melville, NY). Images were then captured (Megaplus camera, model 1.4; Eastman Kodak Corp., San Diego, CA, connected to a MegaGrabber Framegrabber in a Macintosh 8100/80 AV computer; Apple Computer, Cupertino, CA, using NIH Image 1.58 VDM software (available by ftp from zippy.nimh.nih.gov/ or from <http://rsb.info.nih.gov/nih-image>; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). The morphometric analysis of the retina was performed with slight modification of a previously described procedure.

12 Briefly, sections of the retina at the level of the optic nerve head were prepared, and each section was divided into 12 optic fields (6 fields of the central retina and 6 fields of the peripheral retina). Within each field we measured the thickness of the inner plexiform (IPL), inner nuclear (INL), and outer nuclear (ONL) layers by measuring four thickness per layer and calculating averaged measurements per section. Measurements of retinal layer thickness were performed with a calibrated scale of the objective of the light microscope.

Statistical Analysis

Statistical analysis was performed by using Student's t -test, paired t -test, repeated-measurement ANOVA, and Kruskal-Wallis nonparametric test on computer (GraphPad, San Diego, CA).

RESULTS

Assessment of Optic Nerve Function Using the PLR

The measurement of the PLR was used as an assay to investigate possible changes in retina and optic nerve function after acute elevation of the IOP. The reflex contraction of the pupil to a light stimulus provides an objective measure of the afferent conduction of the visual system. Damage to the retina or optic nerve reduces the amplitude of the pupil contraction to light. Because the motor output of the neuronal reflex of pupil contraction to light is distributed to both pupils, monitoring the pupil from just the noninjured eye is sufficient to assess any asymmetry of light input between the injured and noninjured eye. Because the fellow eye was used as a control at the same testing time, any defect that was caused by acute elevation of the IOP was monitored longitudinally over time. All pupil parameters were calculated by comparing data from the injured and noninjured (control) eyes in the same animal. Evaluation of the PLR revealed defects in all components (amplitude, latency, and maximum velocity) in all injured eyes (Figs. 2 and 3).

Preoperative PLR_{ratio} s (indirect PLR/direct PLR, Fig. 2) were 76.7 ± 2.6 (mean \pm SEM). Twenty-four hours after surgery, the PLR_{ratio} was 15.2 ± 12.8 ; 10 days after, 11.6 ± 9.8 ; 20 days after, 26.5 ± 8.0 ; and 28 days after, 33.27 ± 9.3 . However, at day 35, the PLR displayed significant recovery when compared with the 24-hour postoperative ratio (41.1 ± 7.3 , $P < 0.01$, repeated-measures ANOVA with Bonferroni posttest). Forty-two days after surgery, the PLR started to decrease once again in the injured eyes (28.7 ± 5.9) and was not significantly different than the condition 24 hours after surgery ($P > 0.05$, repeated-measures ANOVA with Bonferroni posttest). The PLR_{ratio} never recovered to the preoperative level ($P < 0.0001$, repeated-measures ANOVA; Fig. 2).

We analyzed latency and velocity of the PLR (latency is the time from onset of stimuli to the start of pupil constriction; velocity is the speed of constriction) as parameters of the retina and optic nerve function after ischemic insult and detected significant decrease of PLR velocity ($P < 0.0004$, Kruskal-Wallis nonparametric test) and increase in the latency ($P < 0.004$, Kruskal-Wallis nonparametric test) after stimulation of the injured eye compared with the stimulation of the noninjured (control) eye (Fig. 3).

The indirect latency was greatly prolonged after surgery. By subtracting latencies ($latency_{inj} - latency_{ctrl}$) we determined the following latencies: before surgery, 31.8 ± 3.4 ms (mean \pm SEM; $n = 11$); 24 hours, 88.9 ± 10.7 ms ($n = 3$); 10 days, 61.1 ± 10.7 ms ($n = 3$), 20 days, 81.3 ± 8.8 ms (significantly different from preoperative latency, $P < 0.05$, Kruskal-Wallis

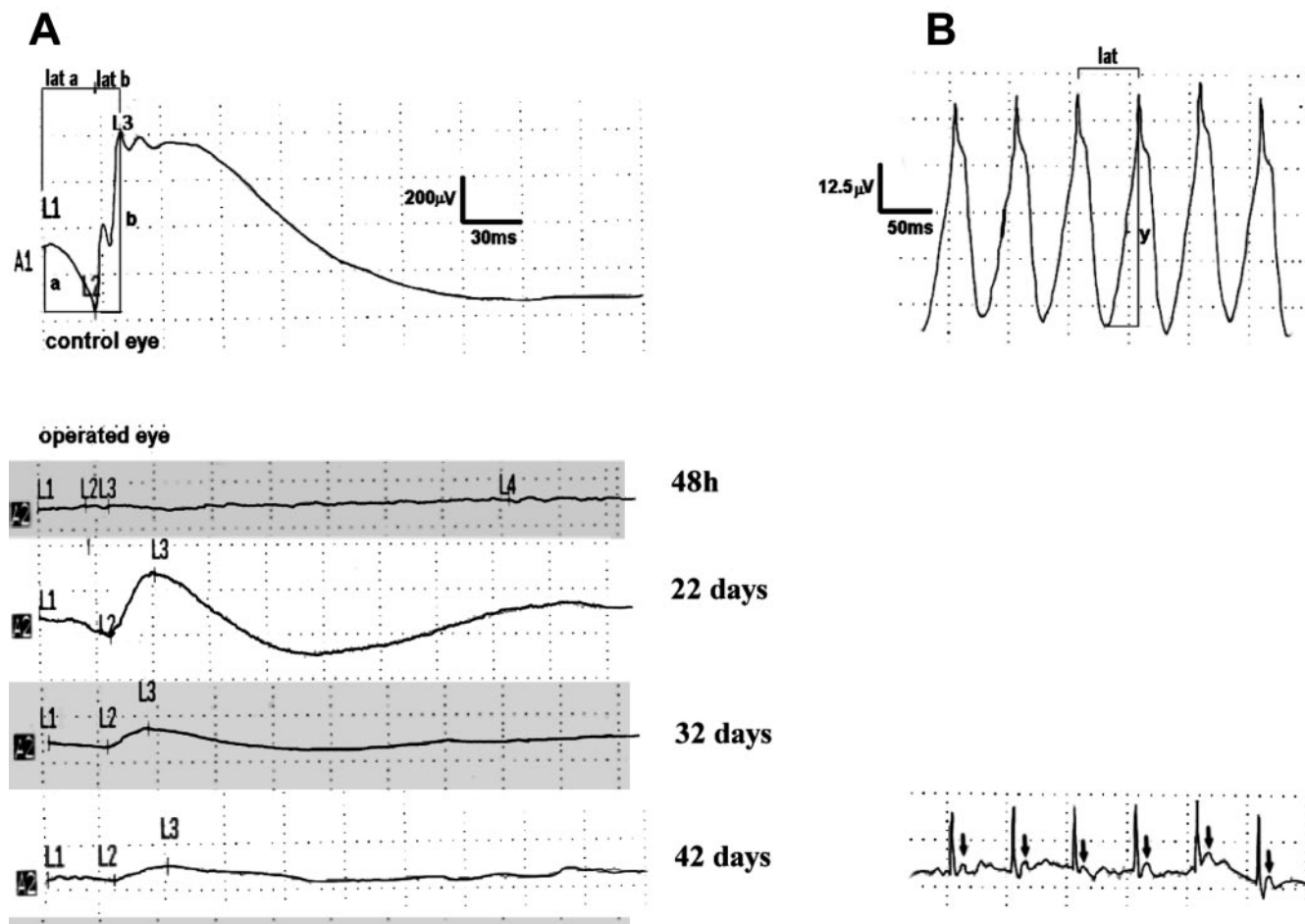


FIGURE 5. Electoretinographic characteristics from one animal that displayed detectable ERG responses 42 days after injury by acute elevation of IOP. (A) Acute elevation of IOP caused dramatic reduction in the amplitudes of a- and b-waves in injured eyes. L1, flash onset; L2, peak a-wave; L3, peak b-wave, lat a, latency of a-wave; a, amplitude of a-wave; lat b, latency of b-wave; b, amplitude of b-wave; A1, ERG recording channel for control eye; A2, ERG recording channel for injured eye. (B) The recording of the isolated cone response (flicker ERG) revealed barely detectable amplitudes in the injured eyes 42 days after surgery. y, amplitude of the response. Arrows indicate flicker ERG amplitudes in injured eye 42 days after surgery.

nonparametric test with the Dunn posttest, $n = 8$); 28 days, 65.7 ± 10.9 (difference was not statistically significant, $P > 0.05$, $n = 9$), 35 days, 90 ± 15 ($P < 0.05$, $n = 10$); and 42 days, 85 ± 17 ($P < 0.05$, $n = 8$) after surgery. Calculation of the velocity parameters (velocity_{ctrl} - velocity_{inj}) showed the following: before surgery, 0.34 ± 0.03 mm/s (mean \pm SEM) and at 24 hours, 0.58 ± 0.02 mm/s ($n = 3$); 10 days, 0.92 ± 0.11 mm/s ($n = 3$), 20 days, 1.26 ± 0.23 mm/s (significantly different from preoperative velocity: $P < 0.01$, Kruskal-Wallis nonparametric test with the Dunn posttest, $n = 8$); 28 days, 1.13 ± 0.22 mm/s ($P < 0.05$, $n = 9$); 35 days, 1.33 ± 0.24 mm/s ($P < 0.01$, $n = 10$); and 42 days, 1.76 ± 0.31 ($P < 0.001$, $n = 8$) after surgery.

Analysis of amplitude parameters revealed loss of function in 8 of 11 injured eyes (PLR was not detectable when the injured eye was stimulated) 24 hours and 10 days after surgery. However, 20 days after the ischemic event the PLR function was detected in 8 of 11 injured eyes, although latency and maximum velocity did not show signs of recovery. The peak in PLR recovery was detected 35 days after surgery, when 10 of 11 injured eyes had detectable activity. However, 7 days later, pupil parameters declined, leaving 8 of 11 injured eyes with detectable responses. One animal never recovered pupil or

ERG responses in the injured eye after acute elevation of the IOP. Because we were monitoring function of the healthy noninjured eye while stimulating the injured eye, we excluded the potential effect of ischemia on the iris sphincter muscle as a source of the deficit in analyzed pupil responses.

Electoretinography

ERG was used as an objective method to evaluate the functional status of the inner and outer retina, with the exception of the RGCs. To evaluate the effect of the acute elevation of the IOP on different populations of photoreceptors we used full-field flash and flicker ERG. Acute elevation of the IOP caused a dramatic reduction of ERG activity (Figs. 4, 5, 6).

The dynamics of the recovery (number of animals with detectable signals) revealed the peak of recovery of the ERG and pupil responses at 22 and 32 days after surgery (Fig. 4). Rats with a- and/or b-wave amplitudes smaller than 3μ V (noise level) were considered to have no detectable signal.

ERG data expressed as a ratio between control and injured eyes revealed higher a-wave amplitudes at all tested time points compared with b-wave amplitudes, but without statistical significance ($P > 0.05$, paired *t*-test; Fig. 4A): 48 hours: a-wave

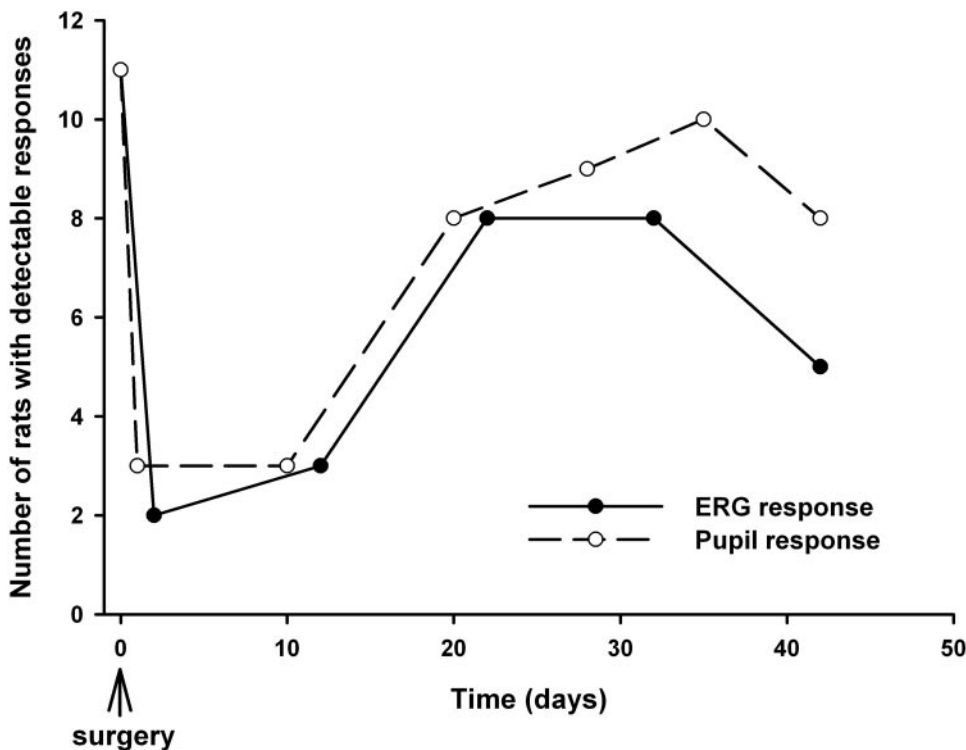


FIGURE 6. Recovery dynamics of ERG and PLR. Injured eyes showed the peak of recovery of PLR responses (10/11 injured eyes) at day 35 after surgery and of ERG responses at day 22s and 28 (8/11 injured eyes). Only rats with a- and/or b-wave amplitudes more than 3 μV (scotopic flash ERG noise level) and 1 μV (photopic flicker ERG noise level) were considered to have a detectable signal.

2.9 ± 1.4 (mean \pm SEM), b-wave 2.6 ± 1.1 ; 12 days: a-wave 4.5 ± 1.6 , b-wave 0.7 ± 0.3 ; 22 days: a-wave 5.4 ± 1.2 , b-wave 4.1 ± 1.1 ; 32 days: a-wave 8.1 ± 2.2 , b-wave 4.8 ± 1.1 ; and 42 days: a-wave 1.89 ± 0.6 , b-wave 1.35 ± 0.42 , after surgery.

Complete absence of activity was detected in 9 of 11 injured eyes after 48 hours of reperfusion period, whereas amplitudes of the a-wave ($45 \pm 24 \mu\text{V}$; mean \pm SEM) and b-wave ($95 \pm 10 \mu\text{V}$) in the remaining two injured eyes were dramatically reduced compared with control (noninjured) eyes (a-wave: $236.5 \pm 14 \mu\text{V}$; b-wave: $595.1 \pm 37.3 \mu\text{V}$). Ten days after acute elevation of the IOP, amplitudes further decreased (a-wave: $10 \pm 7 \mu\text{V}$; b-wave: $3.3 \pm 2.3 \mu\text{V}$), but an ERG was detectable in three injured eyes. Twenty-two and 32 days after surgery, ERG responses were detected in eight injured eyes (22 days: a-wave $39.6 \pm 13.1 \mu\text{V}$, b-wave $44.1 \pm 22.8 \mu\text{V}$; 32 days: a-wave $37.8 \pm 5.7 \mu\text{V}$, b-wave $45.1 \pm 16 \mu\text{V}$). Forty-two days after surgery ERG amplitudes were detectable in five injured eyes (a-wave $12.5 \pm 2 \mu\text{V}$, b-wave $31.3 \pm 1 \mu\text{V}$).

The latency (Fig 4B) was significantly increased compared with control noninjured eyes at 22 and 32 days after surgery (22 days: a-wave latency $39.6 \pm 13.1 \text{ ms}$, $P = 0.02$, paired t -test; b-wave latency $44.1 \pm 22.8 \text{ ms}$, $P = 0.04$; 32 days: a-wave latency $37.8 \pm 5.7 \text{ ms}$, $P = 0.007$; b-wave latency $45.1 \pm 16 \text{ ms}$, $P = 0.03$). The latency for a-wave ($12.5 \pm 2.0 \text{ ms}$) and b-wave ($31.3 \pm 1.0 \text{ ms}$) were not significantly decreased ($P > 0.05$) compared with control eyes at 42 days after surgery (the last recorded time point) but the number of injured eyes that had detectable ERG signals decreased to five.

Isolated cone response (flicker ERG) revealed existence of recorded activity in 3 of 11 injured eyes 42 days after surgery with average amplitude of $34.3 \pm 5.3 \mu\text{V}$ and latency of $52.7 \pm 0.7 \text{ ms}$ in control eyes and $3.9 \pm 1 \mu\text{V}$ and latency of $61.3 \pm 0.1 \text{ ms}$ in injured eyes. Statistical analysis revealed significant amplitude ($P = 0.0069$, paired t -test) and latency ($P = 0.0012$, paired t -test) defects in the injured eyes compared with control eyes.

Histologic Analysis

Our electrophysiological studies revealed a dramatic reduction of function in all retinal layers, and thus we performed a histologic analysis to determine whether functional defects were in agreement with the morphologic appearance of the injured eyes.

Light microscopy revealed vacuolization of the optic nerve axons (Figs. 7A, 7B), complete loss of inner retinal layers at the level of central retina (Figs. 7E, 7F), and significant thinning of inner retinal structures at the periphery (Figs. 7I, 7J). Dramatic reduction of the cellular organization of the central retina and reduced thickness compared with control noninjured eyes suggests that ischemia damaged both the inner and outer retinal structures (Figs. 7E, 7F).

For the analysis, we compared measurement of the thickness of the ONL, INL, and IPL between the control and injured eyes at central and peripheral retinal locations (Table 1). Retinal thickness, expressed as a ratio in percentages (injured/control), in the peripheral retina was: ONL, $97.3 \pm 8.5\%$ (mean \pm SEM); INL, $67.6 \pm 5.6\%$; and IPL, $36.4 \pm 3.6\%$; and in the central retina: ONL, $23.9 \pm 6.2\%$; INL, $5.5 \pm 1.8\%$; and IPL, $16.5 \pm 5.1\%$. The ONL, INL, and IPL absolute values were measured and showed significant reduction in thickness (Table 1). The photoreceptor layer was completely devoid of outer segments. Organization of the retinal layers was preserved at the retinal periphery, with no reduction in the thickness of the ONL. However, the thickness of the INL and IPL were dramatically reduced in the peripheral retina compared with control, noninjured eyes.

Functional monitoring and histologic analysis revealed dramatic effects of the ischemia-reperfusion injury on retinas and optic nerves of the injured eyes. Histologic analysis revealed the major impact of injury on all retinal layers in the central retina and the inner layers of the peripheral retina.

Whereas morphologic data (retinal thickness) showed relative preservation of the retinal structures (particularly the

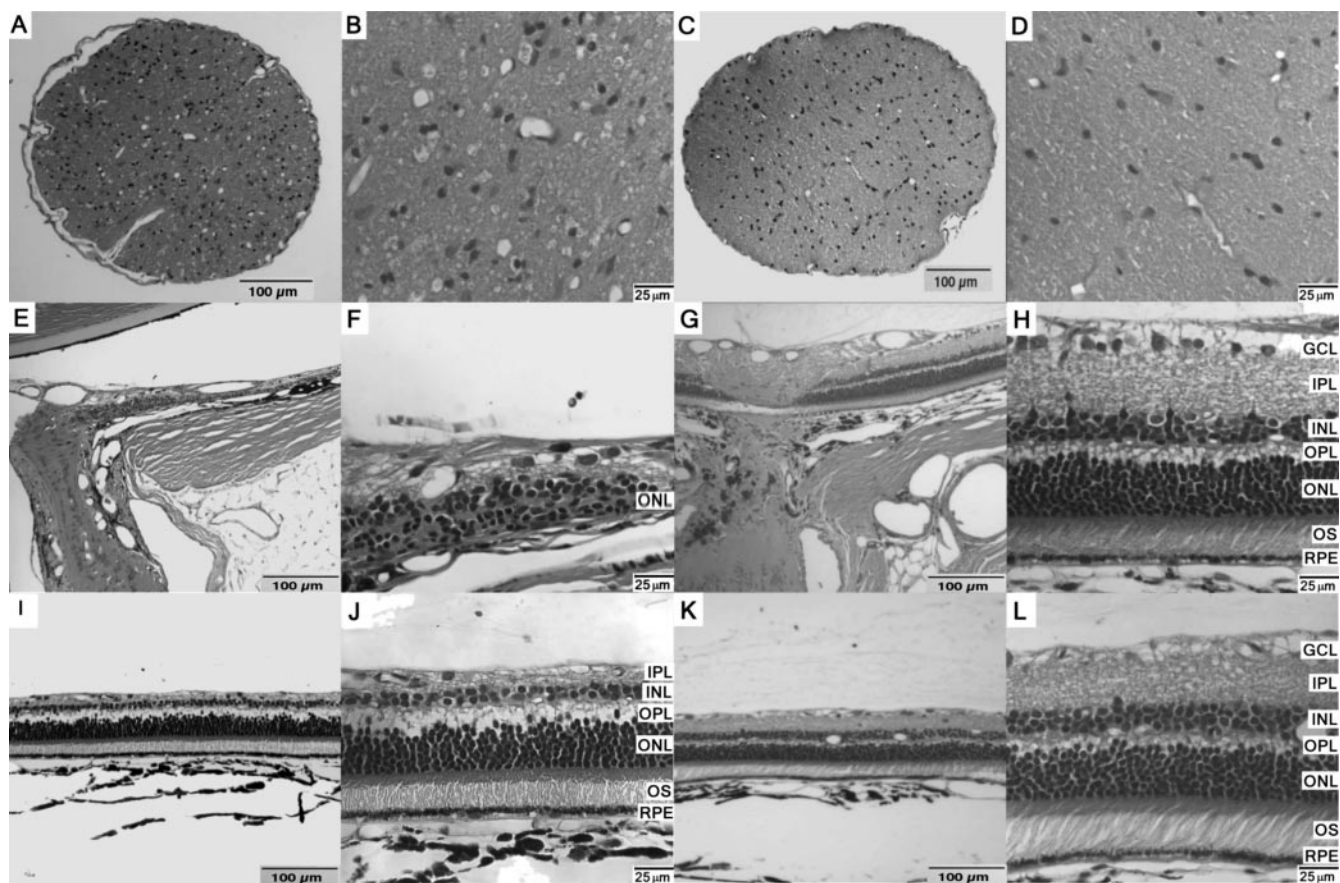


FIGURE 7. Histologic examination of the injured eyes revealed (A, B) optic nerve degeneration (C, D, noninjured eye), (E, F) complete loss of central retina architecture (G, H, noninjured eye), and (I, J) dramatic reduction of the IPL and INL of the peripheral retina (K, L, noninjured eye). GCL, ganglion cell layer; OPL, outer plexiform layer; OS, outer segments; RPE, retinal pigment epithelium. Magnification: (A, E, I) $\times 100$; (B, J) $\times 400$; (F) $\times 200$.

outer retina), functional analysis showed that even morphologically preserved retina may not be functional after acute ischemia. Calculated correlation coefficients did not show good correlation between morphologic and functional analysis (last recording time point, 42 days): a-wave ratio versus outer retinal thickness ratio ($r^2 = 0.37$), b-wave ratio versus inner retinal thickness ($r^2 = 0.23$), pupil ratio versus inner retinal thickness ratio ($r^2 = 0.007$), pupil ratio versus outer retinal thickness ratio ($r^2 = 0.04$), and pupil ratio versus combined retinal thickness ratio ($r^2 = 0.01$).

DISCUSSION

The experimental approach used in this study allowed us to monitor precisely the dynamics of ischemic retinal damage for

TABLE 1. Morphometric Analysis of the Retina

Retinal Layer	Central Retina		Peripheral Retina	
	Control	Ischemic	Control	Ischemic
ONL	50.2 \pm 5.2	12.8 \pm 4.6*	38.8 \pm 4.4	38.0 \pm 5.2 NS
INL	16.5 \pm 1.1	2.6 \pm 0.9*	12.1 \pm 1.0	7.9 \pm 0.6*
IPL	34.2 \pm 1.1	1.8 \pm 0.6*	25.3 \pm 1.5	9.0 \pm 1.0*

Significant reduction of the thickness of ONL, INL and IPL layers was observed in ischemic eyes with the exception of the ONL in the peripheral retina. Data represented as mean micrometers \pm SEM (* $P < 0.001$; NS, not significant ($P > 0.1$); paired *t*-test).

prolonged periods (up to 6 weeks) after acute elevation of the IOP, by using computerized pupillometry and ERG. Because ischemia itself can damage iris muscles, we recorded pupil responses from the untreated (noninjured) eye while stimulating the injured eye to obtain data regarding the direct effect of ischemia on the retina and optic nerve.

Use of Computerized Pupillometry to Identify Deficits of the Retina and Optic Nerve

Analysis of the PLR parameters (amplitude of pupil constriction, latency, and velocity) was an effective strategy for monitoring of the retina and optic nerve status after injury induced by acute ocular hypertension (ischemia-reperfusion). Investigators in a previous study reported that a relative afferent pupillary defect (RAPD) of 0.7 log units or more can be used as a very sensitive parameter, to differentiate ischemic from non-ischemic central retinal vein occlusion (CRVO) in 88% of patients.²⁰ The same group reported that ischemic CRVO is characterized by RAPD of at least 0.6 log units, whereas 91% of the patients who had ischemic CRVO had deficits larger than 1.2 log units.²¹ We used the PLR parameters more as an objective method to evaluate function of the optic nerve rather than the photoreceptors, because reports have shown that PLR activity does not correlate with the number of photoreceptors in rodents²² and recent evidence suggests that the PLR may be driven by light-sensitive, melanopsin-containing cells in the inner retina, even without presence of the photoreceptors (rods and cones).²³ Our results indicate dramatic suppression

of the PLR in the first 20 days of the reperfusion period. The PLR amplitude partially recovered in almost all (10/11) rats in the injured group 35 days after injury, but showed a decrease at 42 days after injury. The latency did not improve; however, maximum velocity recovered 28 days after injury but afterward continuously decreased until the end of the experiment. Recent data have implied that a specific population of melanopsin-containing RGCs that project into the suprachiasmatic nucleus (SCN) may respond with slow and delayed firing after light stimuli independently of the photoreceptor input.²⁴ Because melanopsin-containing RGCs are projecting to the pre-tectal olivary nucleus,²⁴ there is a possibility that detected recovery of the PLR with latency and velocity deficits may be due to primary recovery of this population of RGCs. In support of this hypothesis are data from three rats that did not have any detectable ERG responses (scotopic flash or photopic flicker ERG) 42 days after surgery, but had detectable pupil responses (Fig. 6). Unfortunately, we still do not know enough about the physiology of the melanopsin-containing RGCs, but findings in initial studies point toward significantly different physiological properties of these cells compared with the other populations of RGCs, which may potentially influence survival of these cells after acute ischemic insult.^{24,25} Results in a recent study involving retrograde labeling of RGCs after temporary retinal ischemia have confirmed that death of RGCs is an ongoing process that lasts up to 3 months after ischemic injury,²⁶ which may offer an explanation for the late decrease (42 days after surgery) in the quality of the pupil function.

Electroretinography as a Method for the Evaluation of Retinal Integrity

To obtain information about the status of the photoreceptors and inner nuclear layer neurons we used full-field flash and flicker ERG. Contrary to previous reports,^{14,15} which described only inner retina deficits but not significant alteration of photoreceptor function, we observed a dramatic reduction of a- and b-wave amplitudes 24 hours after pressure-induced ischemia-reperfusion insult. However, our data are consistent with previous electrophysiological^{27,28} and ultrastructural²⁹ studies in which significant decrease in outer retinal function (a-wave amplitude)^{27,28} and damage of the ONL with combined features of apoptosis and secondary necrosis were noticed after acute elevation of IOP.²⁹ ERG revealed minimal and statistically nonsignificant recovery of function at 22 and 32 days of the reperfusion period. Latencies for a- and b-waves were significantly increased, which may be attributable to changes in physiological activity of the retina after acute ischemia. The kinetics of the ERG response (number of rats with detectable flash ERG responses) revealed minimal recovery of the function 22 and 32 days after injury. However, 42 days after ischemic insult, the number of rats with detectable ERG responses started to decrease again. Because histologic analysis revealed complete destruction of the central retina, it is possible that electrical activity detected by ERG reflected the minimal activity of the peripheral outer retina, which appeared to be relatively spared after ischemic injury.

Histologic and Morphometric Analysis of the Retina and Optic Nerve

The morphometric analysis of the retinal layer thickness implicated location of the insult to ONL, INL, and IPL layers in the central retina. These types of changes revealed the existence of neurotoxic damage at both the inner and the outer retina (photoreceptors) in central retinal regions. However, the peripheral retina was more spared from the damage. We do not know the particular mechanism responsible for the better

survival of the peripheral retina; however, we can hypothesize that the central retina is more likely to sustain permanent damage, because it is thicker, has more cellular elements, and would have greater metabolic requirements. The peripheral retina which is thinner, may receive enough oxygen diffusion from the choroid to sustain it through ischemia and hence allow recovery of the affected cells.

CONCLUSION

The continuous monitoring of the retina and optic nerve function by ERG and pupillometry revealed dynamic functional events in ischemic rat retinas and optic nerves. Functional analysis seems to better represent the actual condition of the retina and optic nerve after acute ischemic insult. The exact characterization of the timing and identification of the mechanisms responsible for the transient recovery of function may open new avenues for the treatment of ischemic retinal and optic nerve diseases.

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