Normal Physiological and Pathophysiological Effects of Trypan Blue on the Retinas of Albino Rabbits

Gad Heilweil,[1] *Izabela Komarowska,*[2] *Esther Zemel,*[2] *Anat Loewenstein,*[1,3,4] *and Ido Perlman*[2,4]

PURPOSE. To determine whether intravitreal injection of trypan blue is toxic to the retina of the albino rabbit.

METHODS. Sixteen albino rabbits were studied for the effects of intravitreal trypan blue (eight with 0.06% solution and eight with 0.15% solution). Saline was injected into the fellow control eye of all rabbits. The electroretinogram and visual evoked potentials were recorded from each rabbit at different time intervals after injection. The rabbits were killed at the termination of the follow-up period, and their retinas were prepared for histologic examination under light microscopy.

RESULTS. In all rabbits, short-term follow-up showed significant reduction of ERG responses in the experimental eye, with the b-wave more affected than the a-wave. Partial to complete recovery was observed during follow-up. After 4 weeks, negligible ERG deficit was observed in the rabbits treated with 0.06% trypan blue, whereas significant ERG deficit was measured in rabbits tested by the 0.15% trypan blue. No differences in flash VEP responses between experimental and control rabbit eyes were found. Light microscopy showed no significant histologic effects in the retinas exposed to the 0.06% solution. Marked disorganization of all retinal layers was observed in areas close to the site of injection in the rabbits injected with the 0.15% solution.

CONCLUSIONS. Trypan blue exerts transient physiological effects on the distal retina of the rabbit, but in concentrations of 0.15% it can induce permanent toxic effects. Therefore, caution should be used when using this dye in vitreoretinal surgery. (*Invest Ophthalmol Vis Sci.* 2010;51:4187–4194) DOI:10.1167/iovs.09-4675

Internal limiting membrane (ILM) removal has become a common procedure in surgeries of macular hole and cystoid macular edema. Removal of the ILM is a difficult surgical procedure primarily because of difficulties in visualization of this thin and transparent structure.

In the past two decades, vitreoretinal surgeons have used indocyanine green (ICG), to stain the ILM, thus assisting in its visualization and its surgical removal. Clinical studies regarding the outcome of ICG-assisted ILM peeling vitrectomies have mostly shown good results. However, in the past few years, several clinical and animal model studies have shown toxic effects of this dye to the retina and retinal pigment epithelium (RPE).[1–3]

The potential retinal toxicity of ICG has prompted vitreoretinal surgeons to try trypan blue as an ILM staining dye.[4–6] Trypan blue has been used by cataract surgeons for many years to stain the anterior capsule before capsulorrhexis.[7,8] Follow-up of patients[8] and studies on human corneal cells[9] and on rabbit corneal endothelial cells[10] revealed no damage by exposure to trypan blue.

Published clinical studies regarding the use of trypan blue in vitreoretinal studies do not report signs of retinal toxicity. In several clinical studies, there was no statistical difference in the visual acuity of patients who had been exposed to trypan blue and those who had not.[4–6] However, histologic examination of three human donor eyes showed marked disorganization of the inner retina in eyes that were exposed to 0.15% and 0.25% trypan blue.[11]

Several studies examined the toxicity of trypan blue on RPE cultures. Two recent studies have shown marked toxic effect of ICG on RPE cultures, whereas no such effect was found for trypan blue, even when using high concentrations.[12,13] In contrast, other studies showed a dose-dependent toxic effect of trypan blue on RPE culture cells[14] and toxicity after long-term exposure of RPE cultures to trypan blue.[15] In an in vivo study on rabbits, ERG was normal for both the 0.06% or 0.2% trypan blue solutions. However, damage to the photoreceptors was found by histologic examination with the 0.2% concentration.[16] In contrast, subretinal administration of a solution containing trypan blue (0.15%) in pigmented Dutch Belted rabbits revealed significant histologic damage to the RPE and neuroretina[17,18] that was enhanced when hypo-osmotic trypan blue solution was used.[18]

The present study was performed to evaluate the toxic effects of different concentrations of trypan blue on the rabbit's retina. We used intravitreal rather than subretinal injection to mimic the clinical use of trypan blue during vitrectomy for the removal of epiretinal membrane without retinal hole. Electrophysiological tests (ERG and VEP) were used to assess retinal function to separate transient from permanent drug effects.

METHODS

Animals

Sixteen adult albino rabbits, each weighing 2.5 to 3.5 kg, were included in this study. The rabbits were housed under 12-hour/12-hour light/dark cycle and were allowed free access to water and food. Before intravitreal injection and electrophysiological recordings, rabbits were anesthetized by intramuscular injection (0.5 mL/kg body

weight) of a mixture containing ketamine hydrochloride (10 mg/mL), acepromazine maleate solution (10%), and xylazine solution (2%) at a volume ratio of 10:2:3. Topical anesthesia (benoxinate hydrochloride 0.4%) was administered to prevent any discomfort. The pupils were fully dilated with cyclopentolate hydrochloride (1%). All experimental procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and to institutional guidelines.

Drug Administration

Trypan blue solution (MembraneBlue [0.15%] and VisionBlue [0.06%]; Dutch Ophthalmic Research Center International B.U., Zuidland, The Netherlands) was used. In both preparations, trypan blue is dissolved in phosphate buffer to reach a pH of 7.3 to 7.6 and an osmolarity of 257 to 314 mOsm/kg (according to the manufacturer's specifications).

The animals were divided into two groups of eight rabbits each, based on the concentration of trypan blue injected intravitreally. In each rabbit, 0.1 mL trypan blue solution was injected into the right eye, and a similar volume (0.1 mL) of saline (BSS; Alcon, Fort Worth, TX) was injected into the left eye, which served as the control.

Intravitreal injection was performed, as described previously,[19] using a 28-gauge needle attached to a 1-mL tuberculin syringe. The needle was inserted into the eye approximately 1 mm posterior to the limbus and was advanced under visual control with an indirect ophthalmoscope (Neitz Instruments, Tokyo, Japan) toward the center of the vitreous above the optic disc. Injection was performed as close as possible to the retina, with the needle bevel pointing away from the retina to avoid possible physical damage by the injection procedure.

Electroretinography

Electroretinographic (ERG) recording was performed on each rabbit before intravitreal injection (time 0) and at 3 hours, 1 day, 3 days, 7 days, 14 days, and 28 days after injection. ERG responses were recorded from both eyes using corneal electrodes (Medical Workshop, Gröningen, The Netherlands). Reference and ground electrodes were inserted into the ears. ERG signals were amplified ($\times$20,000) and filtered (0.3–300 Hz) by differential amplifiers (Grass, West Warwick, RI). Light stimuli were obtained from a Ganzfeld light source (LKC Technologies, Gaithersburg, MD) with a maximum energy of 5.76 cd · s/m^2. ERG responses were recorded in the dark-adapted state after at least 3 hours in darkness. Six responses elicited by identical flashes delivered at 10-second intervals (0.1 Hz) were averaged by the data acquisition system and stored for off-line analysis.

ERG analysis was based on amplitude measurements. The a-wave was measured from baseline to trough, and the b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave. The a-wave and b-wave amplitudes were plotted as a function of log stimulus energy for each eye of each rabbit. The response-stimulus energy relationship was fitted to a Michaelis-Menten type hyperbolic function,[20]

$$V/V_{\max} = I/(I + \sigma) \qquad (1)$$

where V and $V_{\max}$ are, respectively, the amplitudes of the ERG b-wave (or a-wave) elicited by a flash of energy I and by a flash of supersaturating energy. The semisaturation constant σ is the stimulus energy eliciting half maximal b-wave (or a-wave) amplitude.

Functional damage to the experimental eye was assessed from the $V_{\max}$ ratio (experimental/control) and the difference in log σ (experimental-control), thus minimizing technical variability in ERG responses between different recording sessions.[1]

Visual Evoked Potentials

Flash visual evoked potentials (VEPs) were recorded using a stainless steel needle as the active electrode that was inserted under the skin above the area of the visual cortex midway between the two ears. The reference and ground electrodes were inserted in the ears. The signal was amplified ($\times$200,000) and filtered (1–100 Hz) by a differential amplifier (Grass). Fifty responses of identical light stimuli, delivered at a rate of 1.1 Hz, were digitized and averaged by the data acquisition system. With this procedure, monocular light stimuli yielded similar VEPs in nontreated animals.

VEP responses were assessed quantitatively from their temporal patterns and amplitudes. The most easily identifiable waves of the flash VEP in rabbits are an initial negative wave, appearing at an implicit time of 50 to 70 ms, followed by a prominent positive wave. The VEP amplitude was measured from the trough of the first negative wave to the peak of the following positive wave. Temporal properties of the VEP were assessed from the implicit time, defined as the time interval from stimulus onset to the trough of the first negative wave.

Histologic Analysis

At the termination of the electrophysiological follow-up period, animals were killed by intraperitoneal injection of an overdose of sodium pentobarbital (80 mg/kg body weight). Retinas from three rabbits of each experimental group were processed for morphologic analysis. Both eyes were enucleated and immersed in 4% paraformaldehyde (in phosphate buffer; 0.1 M; pH 7.4) for fixation. After 10 minutes, eyes were opened 2 mm posterior to the limbus to facilitate fixation. After 72 hours, the anterior segment of the eye was removed by a circumferential incision, the vitreous was removed, and the posterior eyecup was bisected at the level of the optic disc. Half of each eyecup was rinsed with water and dehydrated in ethanol (twice in 70%, twice in 96%; 1.5 hours each). Then the eyecups were embedded in resin (JB-4; Bio-Rad, Wadford, UK). Retinal sections were cut by a microtome (Reichert Jung, Heidelberg, Germany) at 3-μm thickness and mounted onto slides. For light microscopy, the sections were stained with Richardson's stain.

Statistical Analysis

Analysis of variance with repeated measures was performed on ERG data. For VEP analysis, Wilcoxon signed-rank tests for paired data were performed.

RESULTS

Clinical Observations

Rabbits in both groups showed no evidence of inflammation in any of the eyes during the follow-up period. In all eyes, the corneas and lenses remained clear. Immediately after intravitreal injection of trypan blue, the vitreous cavity turned blue, preventing retinal examination for up to 2 weeks in most eyes. Thereafter, the vitreous cavity appeared bluish, but it was sufficiently clear to visualize the retina. No visible differences were seen between the retinas of the experimental eyes and the control eyes.

Electrophysiological Studies

Dark-adapted ERG follow-up data of two rabbits, one injected with 0.06% trypan blue solution and the other with 0.15% trypan blue solution, are shown in Figures 1 and 2, respectively. Each rabbit was tested at different time intervals after trypan blue injection for retinal function by recording of ERG responses elicited in the dark-adapted state by stimuli of different energies. In Figures 1 and 2, the ERG responses to the brightest (Log $I = 0.76$ cd · s/m^2) white stimuli are shown in the upper part of the figure, and the corresponding response-stimulus energy relationship for the ERG b-wave are shown in the lower part. The response-stimulus energy relationships were fitted to the hyperbolic function (equation 1) to derive the maximal b-wave amplitude ($V_{\max}$) and the semisaturation constant (σ).

In both rabbits (Figs. 1, 2), ERG responses of the experimental eyes, recorded 3 hours after intravitreal injection, were
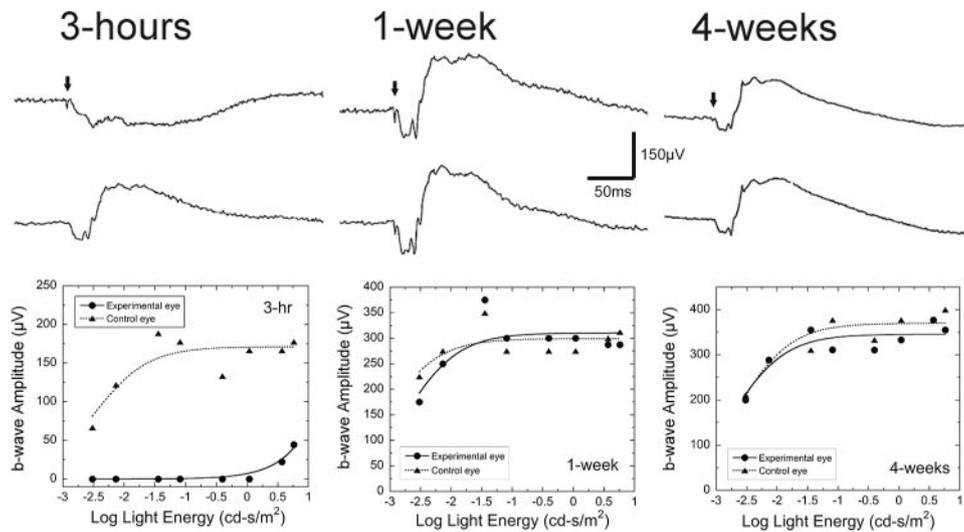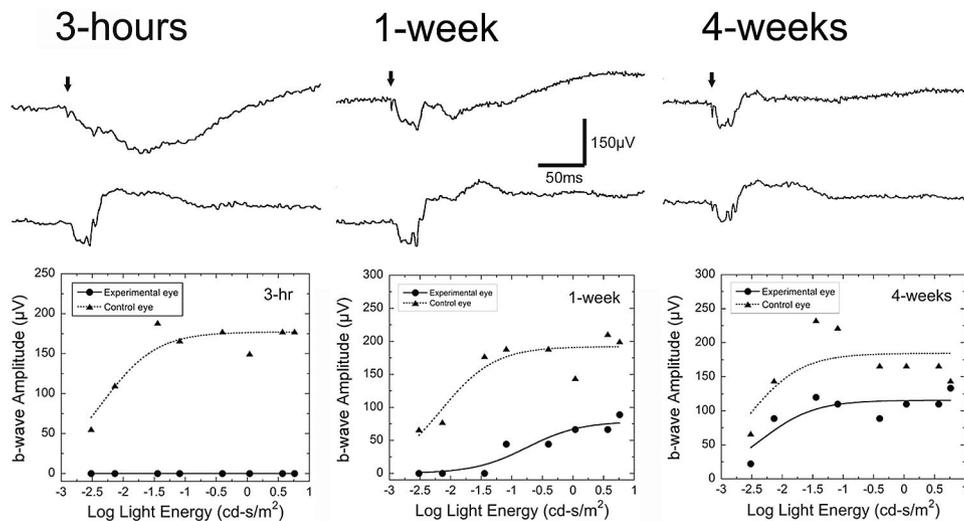
FIGURE 1. ERG follow-up of one albino rabbit injected with 0.06% trypan blue solution into the right eye and saline into the left eye. (**A**) Dark-adapted ERG responses that were elicited by light stimuli of bright (Log I = 0.76 cd · s/m^2) white light recorded 3 hours, 1 week, and 4 weeks after injection. Each pair of responses compares the ERG of the experimental eye (*upper trace*) with that of the control eye (*lower trace*). (**B**) The ERG responses were elicited by light stimuli of different energies. Response-stimulus energy data for the dark-adapted ERG b-waves were measured for the rabbit whose ERG responses are shown. ERG data of the experimental and control eyes are compared. The data were fitted to a Michaelis-Menten hyperbolic type relationship (equation 1; *continuous curves* for the experimental eye and *dotted curves* for the control eye). *Arrows*: timing of light stimuli.

characterized by a marked decrease in the b-wave amplitude with little change in the a-wave, resulting in an electronegative pattern. Within 1 week after injection, ERG responses of the experimental eye in the rabbit treated with 0.06% trypan blue solution completely recovered and were similar in amplitude and pattern to those of the control eye (Fig. 1, second column). No further changes in the ERG responses of this rabbit were seen with continued follow-up (Fig. 1, 3rd column). The ERG

responses of the experimental eye in the rabbit injected with 0.15% trypan blue solution also exhibited recovery during the follow-up period but remained smaller in amplitude and electronegative in pattern compared with those of the control eye throughout the follow-up period (Fig. 2).

To assess the effects of trypan blue on the functional integrity of the experimental eye, we also plotted the response-stimulus energy relationships for the ERG a-wave (not shown

FIGURE 2. ERG follow-up of one albino rabbit that was injected with 0.15% of trypan blue solution into the right eye and saline into the left eye. (**A**) Dark-adapted ERG responses elicited by light stimuli of bright (Log I = 0.76 cd · s/m^2) white light that were recorded 3 hours, 1 week, and 4 weeks after injection. Each pair of responses compares the ERG of the experimental eye (*upper trace*) with that of the control eye (*lower trace*). (**B**) The ERG responses were elicited by light stimuli of different energies. Response-stimulus energy data for the dark-adapted ERG b-waves were measured for the rabbit whose ERG responses are shown. ERG data of the experimental and control eyes are compared. The data were fitted to a Michaelis-Menten hyperbolic type relationship (equation 1; *continuous curves* for the experimental eye and *dotted curves* for the control eye). *Arrows*: timing of light stimuli.
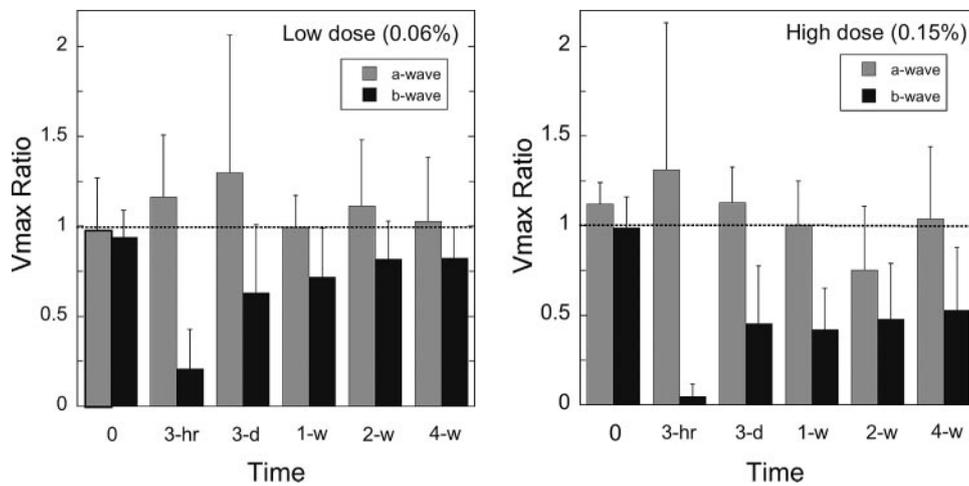
FIGURE 3. ERG follow-up for eight albino rabbits injected with a solution containing 0.06% trypan blue concentration (*left*) and eight rabbits injected with 0.15% trypan blue solution (*right*). Each rabbit at each ERG recording session was subjected to light stimuli of different energies to derive the response-stimulus energy relationship for the a-wave and the b-wave. Average (±SD) V_{max} ratios (experimental eye/control eye) for the a-wave and the b-wave are shown as a function of time after trypan blue injection. For clarity, only the upper part of the SD is shown.

here) and calculated the V_{max} ratio for the ERG a-wave (experimental eye/control eye).[21] Figure 3 shows average (± SD) time-dependent and dose-dependent effects of trypan blue on the a-wave V_{max} ratio and the b-wave V_{max} ratio of all the rabbits treated with the low-dose (0.06%) trypan blue solution (left part) and all those injected with high-dose (0.15%) concentration (right part). The maximum amplitude of the ERG a-wave did not change (V_{max} ratio approximately 1.0) throughout the entire follow-up period for both concentrations of trypan blue. In contrast, the maximum amplitude of the ERG b-wave was markedly reduced (V_{max} <1.0) shortly after injection (3 hours) in both groups of rabbits and showed close to complete recovery in the low-dose (0.06%) group (Fig. 3, left part) and only partial recovery in the high-dose (0.15%) group (Fig. 3, right part). In the rabbits treated with 0.15% trypan blue solution, the V_{max} ratios for the ERG b-wave were significantly ($P < 0.05$) lower than unity for the entire follow-up period.

ERG responses in both groups of rabbits, measured 3 hours after injection, were characterized by selective reduction in the b-wave compared with the a-wave (Figs. 1–3). A similar observation was seen in the rabbits treated with the high-dose (0.15%) trypan blue solution throughout the ERG follow-up period (Figs. 2, 3). Such an electronegative pattern of ERG response is indicative of a selective trypan blue effect on synaptic transmission from photoreceptors to ON-center bipolar cells or selective damage to the ON-center bipolar cells.[22] To assess the degree of this trypan blue effect and its time dependency, we plotted the b-wave as a function of the a-wave for the ERG responses of all rabbits (Fig. 4). Figure 4 was constructed from ERG responses, elicited by bright light stimuli, having measurable a-wave and b-wave, from all the rabbits belonging to the same experimental group that underwent ERG testing in the same time period after trypan blue injection.

Relationships between the b-wave amplitude and the a-wave amplitude are compared in Figure 4 between the control and experimental eyes (open and filled circles, respectively). Rabbits injected with low-concentration (0.06%) trypan blue solution showed a markedly decreased b-wave to a-wave relationship at the 3-hour recording session (Fig. 4A). During follow-up (Figs. 4B, 4C), the b-wave to a-wave relationship of the experimental eyes in these rabbits slowly recovered and approached the normal relationship within 4 weeks after injection (Fig. 4D). ERG responses of the rabbits injected with the high concentration (0.15%) of trypan blue solution also showed time-dependent recovery of the ERG pattern but were characterized by a subnormal b-wave to a-wave relationship throughout the entire follow-up period (Figs. 4E–H).

Visual Evoked Potentials

VEPs were recorded to test the possibility that trypan blue was toxic to the ganglion cells and/or nerve fiber layer. The typical pattern of a negative wave appearing 50 to 70 ms after the light stimulus, followed by a positive wave, was seen in all recordings, as demonstrated in Figure 5 for two rabbits, one injected with low-dose (0.06%) trypan blue solution and the other injected with high-dose (0.15%) trypan blue solution. Although the flash VEP varied in amplitude and temporal properties between rabbits studied in a given recording session and between recording sessions of the same rabbit, the differences between the two eyes of each rabbit in a given recording session were minor, as seen in Figure 5.

The implicit times of the first negative wave (Fig. 5, arrow) and the amplitude difference between the trough of the first negative wave and the peak of the following positive wave were measured from the VEP responses of all the rabbits, recorded 4 weeks after trypan blue injection. The average (± SD) amplitude ratio (experimental eye/control eye) was 0.88 ± 0.22 in the 0.06% group and 0.91 ± 0.33 in the 0.15% group. These values did not differ significantly from a ratio of 1. The average implicit time difference (in ms) was −4.94 ± 11.96 in the 0.06% group and 5.16 ms ± 5.28 in the 0.15% group. These values did not differ significantly from a difference of 0.

Histologic Findings

The retinas of rabbits treated with low (0.06%) trypan blue concentration did not exhibit histologic evidence for drug-induced damage, as shown for one rabbit in Figure 6. Retinal sections from an area close to the site of injection and from a remote region (upper and lower rows, respectively) from the experimental and control eyes (left and right columns, respectively) are compared. Retinal sections from the experimental and control eyes retained their normal layered structure and did not differ in width of the nuclear and plexiform layers. The retinal histology, illustrated in Figure 6, was obtained from a rabbit that exhibited negligible ERG deficit in the 4-week recording session, just before the eye were enucleated and prepared for histology. The V_{max} ratio of the dark-adapted a-wave and b-wave were within the normal range—0.79 and 0.94, respectively—in agreement with the normal retinal histology. We obtained histologic preparations from three of the eight rabbits tested for the effects of low (0.06%) trypan blue concentration, and all were similar to those shown in Figure 6. Quantitative analysis of retinal layer widths indicated no significant differences between experimental and control eyes (Table 1).
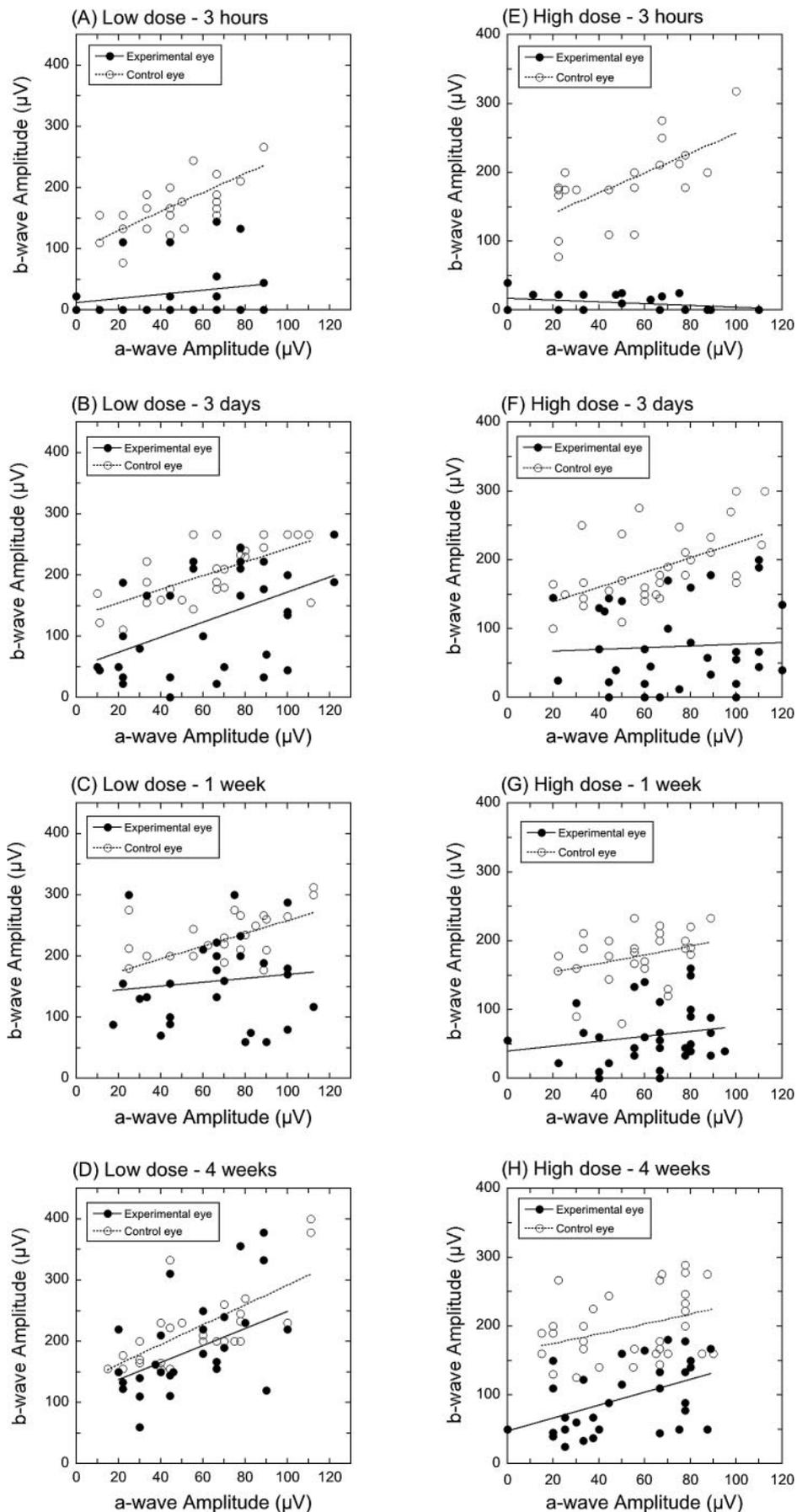
FIGURE 4. Comparing b-wave to a-wave relationships between the experimental eyes and control eyes for rabbits injected with the low (0.06%) trypan blue concentration (**A–D**) and for rabbits injected with the high (0.15%) trypan blue concentration (**E–H**). Each data point represents the b-wave to a-wave relationship of one ERG response of one rabbit, recorded from either the experimental eye or the control eye in a given recording session. The *straight lines* through the data points were obtained by linear regression (*continuous line* for experimental eyes and *dotted line* for control eyes).
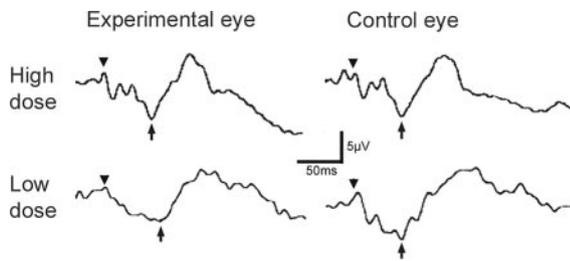
FIGURE 5. Flash VEP responses elicited by bright white light stimuli (Log I = 0.76 cd · s/m^2) in two rabbits, one injected with low-dose (0.06%) and the other with high-dose (0.15%) trypan blue solution. VEP responses were elicited by monocular stimulation of the experimental (*left*) and control (*right*) eyes at the termination of the follow-up period (4 weeks after injection). *Arrows*: first negative wave used to measure VEP implicit time; *arrowheads*: timing of light stimuli.

Retinal sections from one of the rabbits treated with the high (0.15%) trypan blue concentration are shown in Figure 7. The retina exposed to drug (left column) underwent considerable damage in regions close to the site of injection (upper row), as expressed by complete disorganization of retinal layers, whereas remote retinal regions were considerably less damaged and could have normal structures (lower row). Corresponding retinal regions from the control eye are also shown in Figure 7 (right column) for comparison. Retinal micrographs in Figure 7 were obtained from a rabbit showing severe ERG deficit. The V_{max} ratios of the dark-adapted a-wave and b-wave, measured 4 weeks after trypan blue injection (before eye enucleation), were significantly reduced— 0.44 and 0.21, respectively—in agreement with the structural damage shown on Figure 7. We conducted histologic analysis in three of the eight rabbits tested for the effects of high (0.15%) trypan blue concentration. In all three rabbits, the retina close to the injection site appeared disorganized, which prevented reliable quantitative analysis of identified retinal layers. At sites remote from the injection site, the retina appeared normal and did not differ significantly in width from corresponding areas in the control eye (Table 1).

DISCUSSION

Electrophysiological findings, described here, demonstrated dose-dependent and time-dependent functional and structural retinal effects, as expressed in the ERG responses and morpho-logic tests, of a single dose of trypan blue that was injected into the vitreous cavity of albino rabbits.

The strongest effect of trypan blue on the ERG responses was evident shortly (3 hours) after its injection in both groups of rabbits (Figs. 1–3). With longer periods of follow-up, the ERG responses of both groups of rabbits showed partial to complete recovery. The short-term transient effects of trypan blue on the ERG responses could reflect light absorption by the opaque blue mass of trypan blue that was seen in the vitreous (not shown here) immediately after injection. The degree of vitreal opacity reduced with time; after 4 weeks, the vitreous of the experimental eye was clear and similar to that of the control eye. Four lines of evidence argue against this possibility and suggest a functional effect of trypan blue.

First, if trypan blue acted only as an optical filter interposed in the light path between the cornea and the retina, its effect should have been expressed as a lateral and parallel shift of the response-stimulus energy relationship toward higher stimulus energies, but V_{max} should have been normal.[21] This was not the case (Figs. 1–3).

Second, light absorption lowers the light intensity reaching the photoreceptors and is expected to affect the ERG amplitude but not its pattern. Therefore, the b-wave to a-wave relationship was expected to remain normal.[22] However, we found that shortly (3 hours) after intravitreal injection, when vitreal opacity was most evident, the ERG responses of all rabbits in both experimental groups were of electronegative pattern; the a-wave was only slightly changed, and the b-wave was significantly reduced in amplitude. This pattern of ERG recovered completely toward the normal pattern during the 4 weeks of follow-up in the rabbits injected with the low trypan blue concentration but not in rabbits injected with the high dose (Fig. 4).

Third, the ERG responses of the rabbits tested with the low (0.06%) trypan blue concentration started to recover a few days after injection, even though the dye was not washed away.

Fourth, the ERG responses in the rabbits treated with the high (0.15%) trypan blue concentration remained subnormal even 4 weeks after injection, when the mass of trypan blue disappeared (Fig. 3).

Thus, we suggest that trypan blue in the vitreous probably reduces slightly the light energy reaching the retina, causing some reduction in the ERG responses, but the major effect of the drug arises from direct action on retinal structures.
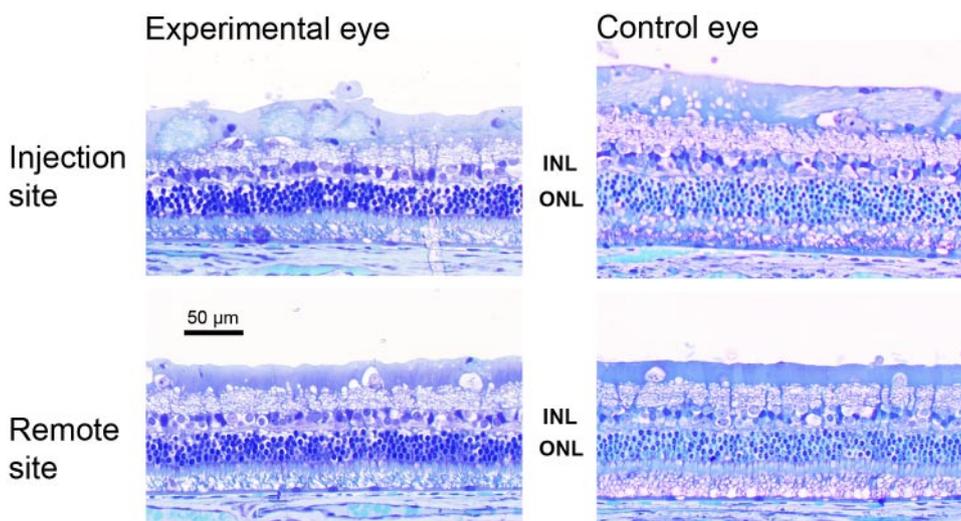
FIGURE 6. Morphologic effects of low-concentration (0.06%) trypan blue in one rabbit. Retinal sections from the experimental eye injected intravitreally with trypan blue (*left*) exhibit normal layered retinal structure in the site of injection (*upper row*) and in remote sites (*lower row*) compared with retinal sections from the control eye (*right*). INL, inner nuclear layer; ONL, outer nuclear layer. Calibration bar applies to all micrographs.

TABLE 1. Effects of Trypan Blue on Retinal Morphology

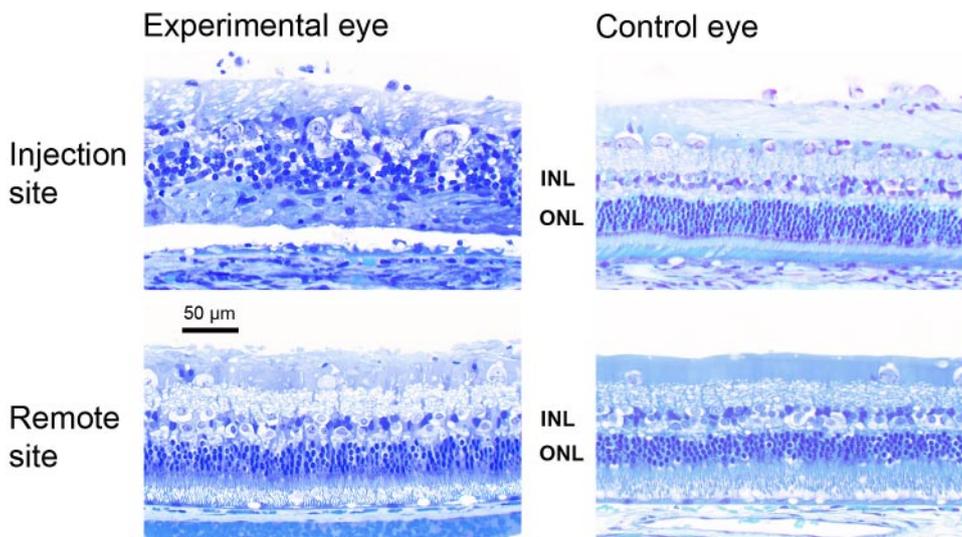| | 0.06% Trypan Blue | | | | 0.15% Trypan Blue | | | |
| | Injection Site | | Remote Site | | Injection Site | | Remote Site | |
Width	Exp	Control	Exp	Control	Exp	Control	Exp	Control
Ph	43.0 (18.8)	36.6 (9.2)	37.0 (6.1)	39.7 (9.2)	—	34.9 (10.3)	39.9 (11.4)	44.0 (16.5)
ONL	32.5 (7.9)	33.0 (3.3)	27.6 (6.1)	30.7 (5.8)	—	31.2 (7.6)	30.0 (2.9)	30.7 (8.3)
INL	16.3 (4.9)	18.5 (1.5)	16.1 (1.0)	18.3 (1.8)	—	18.1 (0.3)	16.9 (3.3)	19.6 (6.4)
Retina	152 (35)	154 (12)	127 (20)	138 (13)	—	145 (32)	130 (12)	158 (22)

Data in the table are given as average (SD) micrometers. Morphologic analysis was conducted in three rabbits of each trypan blue group. The analysis was based on width measurements of ONL and INL and total width of the photoreceptors and of the retina. Width measurements were determined in corresponding retinal regions relative to the optic disc. Ten measurements were determined in 10 slides of each retina and were averaged. Reliable measurements could not be obtained in retinal regions close to the site of injection in the rabbits treated with 0.15% trypan blue because the retina was completely disorganized (Fig. 7, upper row, left column). Exp, experimental; INL, inner nuclear layer; ONL, outer nuclear layer; Ph, photoreceptor.

The transient effect of trypan blue on retinal function that was seen even with the low concentration (0.06%) could be revealed only when noninvasive tests of retinal function, such as ERG measurements, were used, in contrast to studies using only histologic techniques to assess drug toxicity.[17,18] To analyze possible causes for the transient effect of trypan blue, we have to remember that the electroretinogram is an extracellular response to a light stimulus that reflects the summation of different components originating from different retinal structures, primarily in the distal retina.[23] The two most dominant components of the ERG are the negative P-III wave originating from light-induced electrical activity in the photoreceptors with involvement of Müller cells[23] and the positive P-II component reflecting primarily light-induced activity in ON-center bipolar cells with some involvement of Müller cells.[23–25] Because the a- and b-waves of the ERG depend on the summation of P-II and P-III, selective damage to retinal structures may be reflected in the b-wave to a-wave relationship.[22] Thus, a reduction in the stimulus energy reaching the photoreceptors or selective damage to the photoreceptors is expected to lead to a reduction in the ERG amplitude with normal b-wave to a-wave relationship.[22] In contrast, selective damage to postreceptoral mechanisms (e.g., synaptic transmission from photoreceptors, ON-center bipolar cell function) is expected to reduce the P-II component of the ERG, leading to a decrease in the b-wave with a concomitant augmentation of the a-wave, resulting in an ERG response of an electronegative pattern.[22]

The short-term effects of trypan blue on the rabbit retina were expressed in small amplitude ERG responses of electronegative pattern (Figs. 1, 2, 4), suggesting that the major effects of the drug were on the ON-center bipolar cells and/or on synaptic transmission from the photoreceptors to the second-order retinal neurons.

The exact action of trypan blue is unknown, but one possible explanation is interference with synaptic transmission from photoreceptors to second-order neurons. A variety of dyes, including Evans blue and trypan blue, were found to act as efficient competitive inhibitors of L-glutamate uptake into synaptic vesicles in CNS neurons.[26–29] L-glutamate is the neurotransmitter of the photoreceptors in the vertebrate retina; therefore, blocking its transport into presynaptic vesicles is expected to interfere with synaptic transmission. This was the case in a mouse knockout for glutamate vesicular transporter 1.[30] Therefore, we suggest that inhibition of glutamate vesicular transporters by trypan blue interferes with synaptic transmission from the photoreceptors to second-order retinal neurons, leading to selective reduction in the light-induced responses of ON-center bipolar cells and thus to a selective reduction in the b-wave leading to an electronegative ERG. This can account for the short-term effects of trypan blue of both concentrations on the b-wave to a-wave relationships (Figs. 4A, 4E). Furthermore, long-lasting inhibition of L-glutamate uptake into presynaptic vesicles in retinal neurons may lead to accumulations of L-glutamate in the extracellular space

FIGURE 7. Morphologic effects of high-concentration (0.15%) trypan blue in one rabbit. The retinal section from the experimental eye injected intravitreally with trypan blue (*left*), from an area close to the injection site (*upper*), exhibits abnormal retinal structure compared with a corresponding region in the control eye (*right*). The retina appeared disorganized in this region and lost its layered structure. In contrast, the retinal section from a site remote from the injection site (*lower*) appeared unharmed in the experimental eye (*left*) compared with the corresponding area in the control eye (*right*). INL, inner nuclear layer; ONL, outer nuclear layer. Calibration bar applies to all micrographs.

to levels that are toxic to retinal neurons. Retinal toxicity because of the failure of glutamate removal by its transporters has been demonstrated[31] and can explain the permanent toxic action of the high-concentration trypan blue. The subnormal b-wave to a-wave relationship, measured 4 weeks after the injection of high concentration trypan blue solution (Fig. 4H), probably reflects selective damage to bipolar cells and partial sparing of the photoreceptors.

In our study we have demonstrated short-term effects of trypan blue that were dose independent (Figs. 3, 4), probably reflecting normal physiological effects of trypan blue on synaptic transmission between photoreceptors and second-order retinal neurons. This effect was transient, as evident by the recovery of the b-wave to a-wave relationship during 4 weeks of follow-up; however, the high dose (0.15%) of trypan blue also induced permanent ERG deficit (Figs. 3, 4). Such a dose-dependent finding is similar to previous reports.[14,16] The normal VEP responses indicated that the ganglion cells were not involved and that the damage to the distal retina was relatively mild to be expressed in the retinal output (flash VEP).

Are the findings presented here relevant to practicing ophthalmologists? One limitation of this study is the time period in which the retina was exposed to trypan blue. When trypan blue is used in vitreoretinal surgery to stain epiretinal membranes, the retina is exposed to the drug for a short period (10–20 seconds) and is then washed away. In contrast, in our study, trypan blue was injected into the vitreous and was left until its removal by the vascular system. It should be noted that the retinal vascular system in the rabbit is not as developed as in humans, and drug removal may be slower. Despite this limitation, the possibility that trypan blue will remain in the retina for longer periods of time after vitreoretinal surgery should be considered, and extra care is needed washing away trypan blue after the surgery.

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