



PRECLINICAL INVESTIGATION OF THE RETINAL BIOCOMPATIBILITY OF SIX NOVEL VITAL DYES FOR CHROMOVITRECTOMY

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Purpose: To investigate the retinal biocompatibility of six novel vital dyes for chromovitrektomy.

Methods: An amount of 0.05 mL of 0.5% and 0.05% light green (LG), fast green (FG), Evans blue (EB), brilliant blue (BriB), bromophenol blue (BroB), or indigo carmine (IC) was injected intravitreally in the right eye, whereas in the left eye balanced salt solution was applied for control in rabbits' eyes. Clinical examination, fluorescein angiography, histology with light microscopy, and transmission electron microscopy were performed after 1 and 7 days. Retinal cell layers were evaluated for morphologic alterations and number of cells. The electroretinographic changes were assessed at baseline, 24 hours and 7 days.

Results: Fluorescein angiography disclosed hypofluorescent spots only in the 0.5% EB group. Light microscopy and transmission electron microscopy disclosed slight focal morphologic changes in eyes exposed to 0.05% IC, FG, BriB, similar to the control at 1 and 7 days. In the lower dose groups, EB, LG, and BroB caused substantial retinal alterations by light microscopy. At the higher dose, BroB and EB produced diffuse cellular edema and vacuolization within the ganglion cells, bipolar cells, and photoreceptors. FG and IC at 0.5% caused slight retinal alterations similar to balanced salt solution injection. LG at 0.5% caused diffuse vacuolization of bipolar cells after 1 and 7 days. Injection of 0.5% EB caused a significant decrease in neuroretinal cell counts in comparison to control eyes in the 7-day examination ($P < 0.05$). Electroretinography revealed intermittent prolonged latency and decreased amplitude in eyes injected with 0.5% EB, LG, BriB, and BroB, while at the lower dose, only LG and EB induced few functional changes.

Conclusion: The progressive order of retinal biocompatibility, from safest to most toxic, was IC, FG, BriB, BroB, LG, EB.

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Chromovitrectomy refers to the staining of preretinal membranes and tissues to facilitate their intraoperative visualization and removal.¹ Initially, indocyanine green (ICG) dye was shown to stain avidly the internal limiting membrane (ILM).² However, nu-

merous reports have raised concerns over retinal toxicity after intravitreal ICG injection.^{3–5} Subsequently, trypan blue (TB), triamcinolone acetonide and patent blue arose as newer alternative vital dyes.^{6–8} Nonetheless, the blue dyes may stain epireti-



nal membranes (ERM) rather than ILM elements, whereas triamcinolone acetonide deposits well in the acellular vitreous.^{9–13} Moreover, the exact risk of patent blue and TB toxicity to retinal tissue is under investigation.^{14–16} Recently, research groups released their preliminary data on the good biocompatibility of brilliant blue (BriB) and bromophenol blue (BroB) for chromovitrectomy.^{17–21} However, the safest vital dye to the retina with high affinity for the acellular ILM and ERM is yet to be determined.

For this study, a total of six vital dyes were selected from the di-azo, aminoarylmethane, and thiazine chemical groups: light green (LG), fast green (FG), Evans blue (EB), indigo carmine (IC), BriB, and BroB.²² The aim of this paper was to investigate in vivo the retinal biocompatibility of these six dyes injected intravitreally into rabbits' eyes with clinical examination, fluorescein angiography, histology and cell counting with light microscopy (LM), transmission electron microscopy (TEM), and electroretinography (ERG).

Methods

Dye Solutions, pH, Osmolarity, and Light Absorbing Properties

A total of 5 and 50 mg of LG, FG, EB, IC, BriB (Merck, Darmstadt, Germany) and BroB (Sigma-Aldrich, Munich, Germany), in the powder form, were weighed with an analytical balance (Mettler-Toledo Inc., Columbus) and suspended in 10 mL of solvent, glucose 5%, water or balanced salt solution (BSS), to obtain a concentration of 0.5% and 0.05%. The mixtures were shaken for 5 minutes and sonicated (Unique Ind., Idaiatuba, Brazil) to obtain a complete solution. The pH was measured with a pH meter (Quimis Inc., Diadema, Brazil), whereas osmolarity was determined with an osmometer (Advanced Instruments Inc., Norwood). Vital dyes with pH and osmolarity closest to 7.0 and 290 mOsm were selected for the experiments. Spectral absorption was measured

with a spectrometer between 200 and 1,000 nm immediately after preparation (Spectronic Genesys 5, Milton Roy).

Animals and Surgical Technique for Intravitreal Injection of Vital Dyes in Rabbits' Eyes

A total of 90 male rabbits, 1.8 to 2.5 kg, were used in accordance with the university guidelines for research in animals. After the preparation of the drug solutions, 0.05 mL of 0.05% or 0.5% EB, IC, FG, LG, BriB, or BroB was injected into the vitreous cavity of the right eye, and 0.05 mL of BSS (290 mOsm) into the left eye was used as control.²³ After the placement of a lid speculum, a drop of topical povidone-5% iodine was instilled followed by BSS wash-out. The vitreous cavity was entered through the superotemporal sclera 2 mm posterior to the limbus using a 27-gauge needle connected to a 1-mL syringe containing dye or control solution. Eyes were immediately examined by indirect ophthalmoscopy, one drop of antibiotic-steroid solution was then instilled.

Clinical Evaluation with Fundoscopy and Fluorescein Angiography

Fluorescein angiography and indirect ophthalmoscopy were performed at 24 hours and 7 days after the surgical procedure. Indirect ophthalmoscopy (Keeler Instruments, Broomall) was performed for detection of corneal clarity, appearance of the lens and retina, conjunctival reaction, and vitreous hazing. For fluorescein angiography, a slow (10–20 seconds) intravenous injection of 0.3 mL of 10% sodium fluorescein (Ophthalmos, São Paulo, Brazil) into the marginal ear vein was performed, and the photographs were taken with the Heidelberg Retinal Angiography 2 (Heidelberg Engineering, Heidelberg, Germany) within 30 seconds, 1 minute, and 5 minutes after injection.²⁴

In Vivo Morphologic Retinal Toxicity and Cell Counting with LM

The rabbits were euthanized with an intravenous injection of 120 mg/kg sodium pentobarbital 24 hours and 1 week after surgery, and their eyes were enucleated. All eyes were sectioned in half and fixed at 4°C in a mixture of 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 moles/L phosphate buffer at pH 7.4. The specimens were stained en bloc with lead citrate, washed three times in 0.1 moles/L phosphate buffer, and dehydrated with ethyl alcohol. The specimens were embedded and stained with 1% toluidine blue

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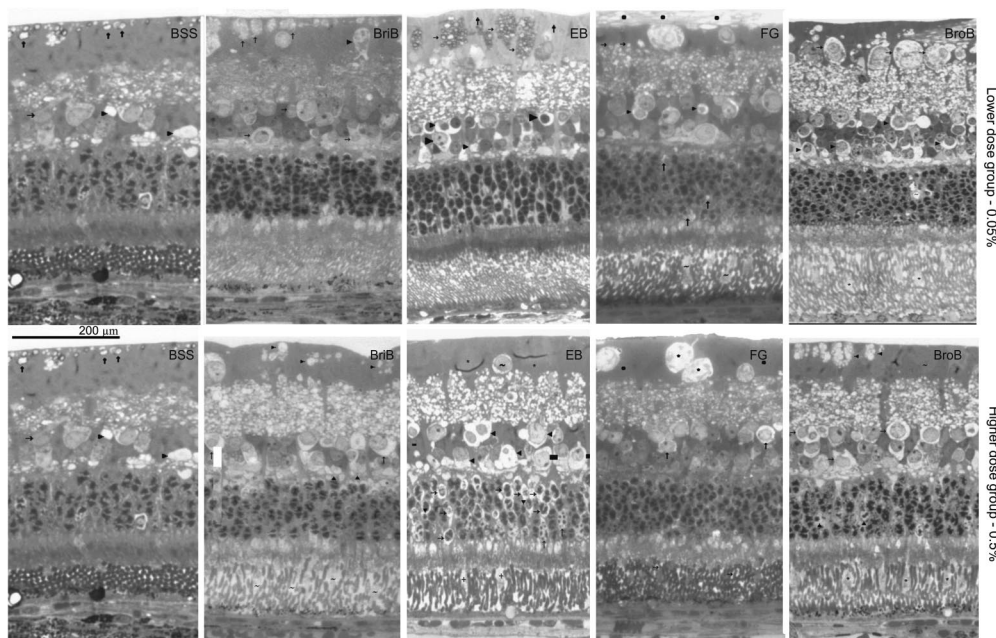


Fig. 1. Retinal findings by histology with LM 24 hours after intravitreal BSS, BriB, EB, FG, and BroB dyes. Row at the top shows the group injected with the lower dose of 0.05%, whereas at the bottom the higher dose of 0.5% is shown. BSS control group (top and bottom): Nerve fiber layer (NFL): focal areas of vacuolization (vertical arrows). GC layer: normal ultrastructural morphology. INL: focal cytoplasmic vacuolization (horizontal arrowheads) and focal swelling (horizontal arrow). Outer nuclear layer (ONL): normal ultrastructural findings. BriB 0.05% (top): NFL: diffuse vacuolization (vertical arrows). GC: one focal region of slight swelling and vacuolization (horizontal arrowhead). INL: small foci of vacuolization with cytoplasmic vacuolization (horizontal arrows). ONL and photoreceptor outer segment (POS)/PIS: normal. BriB 0.5% (bottom): Superficial retina-NFL and GC: focal areas of slight vacuolization, arrowheads. INL: areas of cytoplasmic vacuolization with cellular swelling (vertical arrows) and some foci of necrosis (vertical arrowheads). ONL: totally normal findings. POS & photoreceptor inner segments (PIS): few areas of disorientation and cellular swelling (—). EB 0.05% (top): NFL: swelling (vertical bold arrows). GC: diffuse areas of small cytoplasmic vacuolization (horizontal arrows). INL: diffuse cellular elements with edema (horizontal larger arrowheads), vacuolization (horizontal smaller arrowheads). ONL: normal as the POS and PIS. EB 0.5% (bottom): GC: cellular edema (—) and focal areas of vacuolization (*). INL: diffuse areas of vacuolization (horizontal arrowheads) and focal cellular edema (—). ONL: diffuse cytoplasmic vacuolization, cellular swelling (vertical arrows), and focal regions of chromatin condensation (horizontal arrowheads). POS/PIS: focal regions of disorientation at deeper level (+). FG 0.05% (top): Some artifactual changes on the retinal surface may be observed due to the adherent vitreous represented as (●). GC: focal vacuolization (horizontal arrows). INL: focal areas of vacuolization only (horizontal arrowheads). ONL: focal areas of cytoplasmic vacuolization (vertical arrows). POS/PIS structures: some artifacts (—). FG 0.5% (bottom): NFL and vitreous: normal findings. GC: focal areas of swelling (●) and vacuolization (*). INL: focal vacuolization (vertical arrows). ONL: normal retinal architecture, also slight disorientation of the POS/PIS (horizontal arrows). BroB 0.05% (top): The retinal cytoarchitecture is well organized. GC: remarkable diffuse vacuolization and swelling (horizontal arrows). INL: diffuse areas of cellular vacuolization (horizontal arrowheads) with some additional focal cell edema. ONL: focal areas of cytoplasmic vacuolization (—). POS/PIS: some artifacts or cellular swelling due to the histologic preparation (*). BroB 0.5% (bottom): NFL and GC: multiple areas of vacuolization (horizontal arrowheads) and cellular swelling (—). BC: focal areas of swelling and vacuolization (horizontal arrows). ONL: normal retinal architecture, with slight swelling of the photoreceptors outer and inner segments (*). LM, toluidine blue staining. Original magnification $\times 400$. Scale bar: 200 μm .

and examined with an Optiphot-2TM (Nikon, Tokyo, Japan) for LM. Samples were collected from two different areas in all the dye-injected eyes in three serial sections: 500 μm inferior to the optic nerve and 4 mm from the optic nerve at the temporal-inferior quadrant. A horizontal diameter of the retinal surface of 1,100 μm was used for detailed analysis of retinal toxicity. For histologic evaluation of the degree of cellular injury, cellular abnormalities such as vacuolization, edema, or necrosis were described as “no” changes, “focal” abnormalities with less than 12 cells/structures damaged, or “diffuse” changes with more than 12 cells/structures damaged. Eyes were analyzed by two masked examiners in regard to dye, concentration and exposure to avoid bias.

The inner (INL) and outer nuclear layers were counted for number of cells with the software program NIH ImageJ (NIH, Bethesda) within the 1,100 μm horizontal surface. The eyes of the dye group were compared with the BSS-control group; statistical analysis was performed using analysis of variance (ANOVA) followed by the Newman-Keuls test with a specific software program (GraphPad Software Inc., San Diego).

In Vivo Morphologic Retinal Toxicity by TEM

The eyes were enucleated for TEM evaluation at 24 hours and 7 days after the surgical procedure and the retinas were fixed with 2% osmium tetroxide and embedded in Epon (Electron Microscopy Sciences). Ultra-

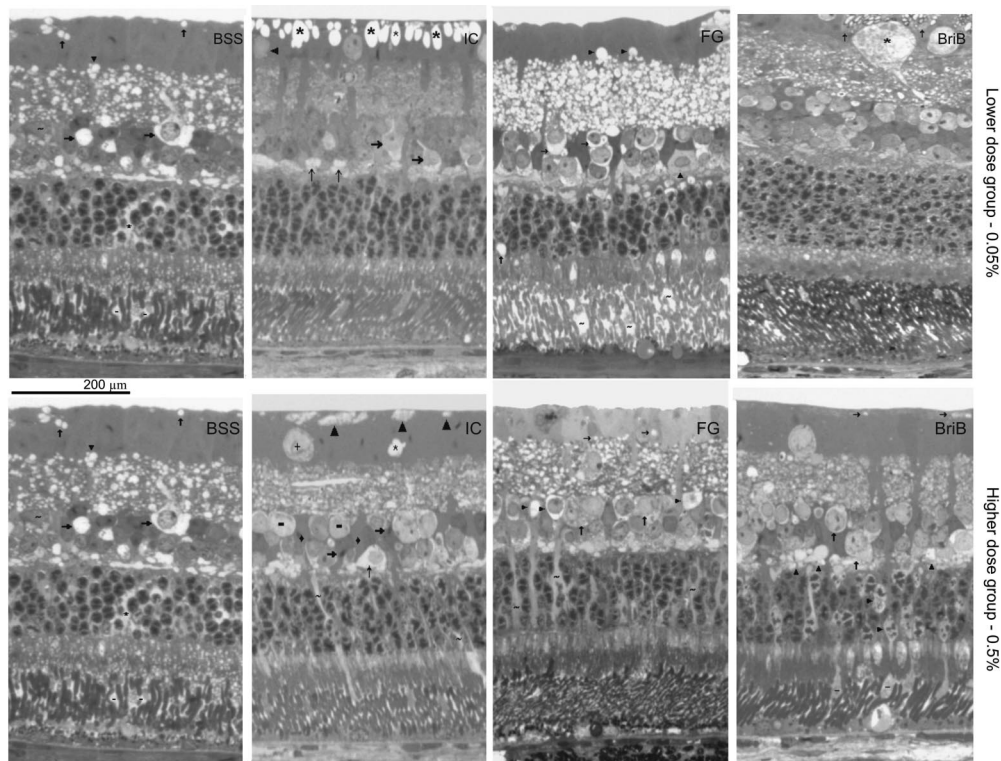


Fig. 2. Retinal histology 7 days after intravitreal BSS, IC, FG, BriB dyes. Row at the top shows group injected with the lower dose of 0.05%, whereas at the bottom the higher dose of 0.5% is shown. BSS control group (top and bottom): NFL: focal areas of vacuolization (vertical arrows). GCL: normal findings except for small focal areas of vacuolization (arrowhead). INL: focal cytoplasmic vacuolization (horizontal arrows) and focal cellular swelling (~). ONL: some focal areas of vacuolization like artifactual findings (*). Photoreceptor outer segment (POS) and PIS: slight focal cellular swelling and disorientation (-). IC 0.05% (top): NFL: diffuse necrosis (*). GC: one focal region of swelling (arrowhead). INL: small foci of vacuolization (vertical arrow) and areas of cytoplasmic vacuolization with cellular edema (bold horizontal arrow). ONL and POS/PIS: no abnormalities. IC 0.5% (bottom): NFL: diffuse areas of vacuolization, shown in arrowheads. GC: one single focal area of vacuolization of the layer detected (*). GC: sparse swelling (+). INL: some artifactual interstitial changes (■), focal swelling (◆), focal cytoplasmic vacuolization with cellular edema and contraction of the nuclear ultrastructure suggestive of pyknosis (vertical arrow), and focal region of chromatin condensation (bold horizontal arrow). ONL: some artifactual findings secondary to fixation (~). FG 0.05% (top): GC: some focal regions of vacuolization in the basal part of the cellular layer (horizontal arrowheads). INL: few focal areas of cytoplasmic vacuolization with cellular edema (horizontal arrows). INL: few cells with cellular edema (vertical arrowhead). Photoreceptors: normal ultrastructural organization except for minor rare foci of vacuolization (vertical arrow). POS/PIS: some artifacts (~). FG 0.5% (bottom): Superficial retina of the NFL: normal structure. GC: few scattered areas of vacuolization (horizontal arrows). INL: focal cellular elements with vacuolization (horizontal arrowheads). ONL, POS, and PIS of photoreceptors: normal findings. BriB 0.05% (top): NFL: normal findings. GC: focal areas of vacuolization, vertical arrows; in addition, a large normally present multinucleated glial cell is present in the superficial retina (*). INL: totally normal retinal structure, no areas of edema or vacuolization. ONL of photoreceptors and the POS/PIS: normal ultrastructural findings. BriB 0.5% (bottom): Superficial retina of the NFL: vacuolization (horizontal arrows). INL: few areas of cytoplasmic vacuolization with cellular edema (vertical arrows), with slight necrosis in some foci (vertical arrowheads). ONL: some localized regions of slight cellular edema with cytoplasmic vacuolization (horizontal arrowheads). POS and PIS of photoreceptors: slight foci of disorientation and cellular swelling (-).

thin sections were stained with uranyl acetate and lead citrate, and observed with a JEOL 1,200 EXII TEM at 80 kV (Jeol Inc., Tokyo, Japan). Samples collected from two sections obtained in similar retinal areas as for LM, were submitted to serial sectioning. Cellular injury on TEM was characterized by rupture, twisting, swelling or structure disorganization in any retinal cellular element, or when signs of pyknosis were found.

In Vivo Functional Retinal Toxicity by ERG

Electroretinography recordings were taken at baseline, 24 hours and 7 days after intravitreal injection of 0.05% or

0.5% EB, IC, FG, LG, BriB, or BroB. The rabbits were kept in a dark room for 3 minutes, and anesthetized by intramuscular injection of a solution containing 1 mL of ketamine (50 mg/mL) and 0.4 mL of xylazine (10 mg/mL). The pupils were dilated and the cornea was anesthetized with 1% proparacaine drops. The rabbits were placed on a heating pad during the experiment, unipolar contact lenses with ERG jet electrodes (Universe SA, La Chaux-de-Fons, Switzerland) were put on both corneas with 2% methylcellulose (Ophthalmos, São Paulo, Brazil). A reference electrode filled with electrolytic gel was placed at the temporal cantus of the eyelid, whereas the ground electrode also filled with



Table 1. Retinal Findings by Histology With Light Microscopy After Intravitreal Injection of Six Dyes in Rabbits

BSS-Control NA	Evans Blue			Light Green			Fast Green			Indigo Carmine			Brilliant Blue			Bromophenol Blue		
	0.5 mg/mL			5 mg/mL			0.5 mg/mL			0.5 mg/mL			0.5 mg/mL			0.5 mg/mL		
	24 Hours	7 Days	24 Hours	7 Days	24 Hours	7 Days	24 Hours	7 Days	24 Hours	7 Days	24 Hours	7 Days	24 Hours	7 Days	24 Hours	24 Hours	7 Days	7 Days
Vitreous	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Inflammatory cells	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Opacities/hazing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Swelling	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Nerve fiber layer																		
Vacuolization	f+	0	f+	0	f+	0	0	0	0	0	d+	0	f+	0	0	0	f+	f+
Swelling	0	0	0	0	f+	f+	f+	f+	f+	f+	f+	f+	0	f+	0	d+	f+	d+
Vacuolization	0	f+	d+	0	0	0	0	0	0	0	f+	f+	0	f+	0	d+	d+	d+
Ganglion cells																		
Chromatin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
condensation																		
Dead cells	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Swelling	f+	f+	f+	f+	f+	f+	f+	f+	f+	f+	f+	f+	0	f+	0	0	f+	d+
Vacuolization	f+	f+	f+	f+	f+	f+	f+	f+	f+	f+	f+	f+	0	f+	0	d+	f+	d+
Inner nuclear layer																		
Chromatin	0	0	f+	0	f+	0	0	0	0	0	0	0	0	0	0	0	0	0
condensation																		
Dead cells	0	0	f+	0	f+	0	0	0	0	0	0	0	0	0	0	0	0	f+
Swelling	0	f+	f+	0	0	0	0	0	0	0	0	0	0	0	0	0	f+	d+
Outer nuclear layer/photoreceptors																		
Vacuolization	0	0	d+	0	f+	0	0	0	0	0	0	0	0	0	0	f+	f+	d+
Chromatin	0	0	f+	0	f+	0	0	0	0	0	0	0	0	0	0	0	0	f+
condensation																		
Dead cells	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Swelling	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
POS/PIIS																		
Disorientation	0	f+	0	f+	0	f+	f+	f+	f+	0	0	0	0	0	0	0	f+	f+
Tubulization and enovelling	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

NA, not applicable; d, diffuse changes, more than 12 per histologic field of examination; f, focal abnormalities, less than 12 per histologic field of examination.



gel, was placed on the earlobe. They were then presented in a Ganzfeld stimulator of the Veris system (Electro-Diagnostic Imaging Inc., San Mateo). After 30 minutes of dark adaptation, the procedure was performed according to four types of responses: scotopic rod response, scotopic maximal response, oscillatory potential, and photopic 30-Hz flicker. Peak-to-peak amplitude and b-wave implicit time were measured for all responses, except OPs. The responses at 1 day and 1 week after injection were compared with baseline levels. A decrease in the postinjection response of >50% was considered remarkable.

Results

Dye Solutions, Osmolarity Measurements, and Light Absorbing Properties

Dye preparations that were well mixed and sonicated produced complete solutions for injection. The

following dyes were selected. At the higher concentration of 0.5%, BroB was chosen in the BSS solvent, whereas FG, IC, EB, and BriB were selected as solutions in glucose 5%. LG 0.5% was further diluted to 0.45% to decrease the osmolarity. At 0.05%, LG, BroB, and BriB solutions were in BSS, while FG, EB, and IC were in glucose 5%. The osmolarity of selected dyes for experiments ranged from 267 mOsm to 315 mOsm, whereas pH values were within 5 to 8.93. Most vital dyes showed substantial absorption between 450 and 650 nm, whereas no agent demonstrated spectral absorption above 700 nm.

Clinical Evaluation with Fundoscopy and Fluorescein Angiography

After intravitreal injection, the green or blue dye appeared as a floating green or blue mass in the

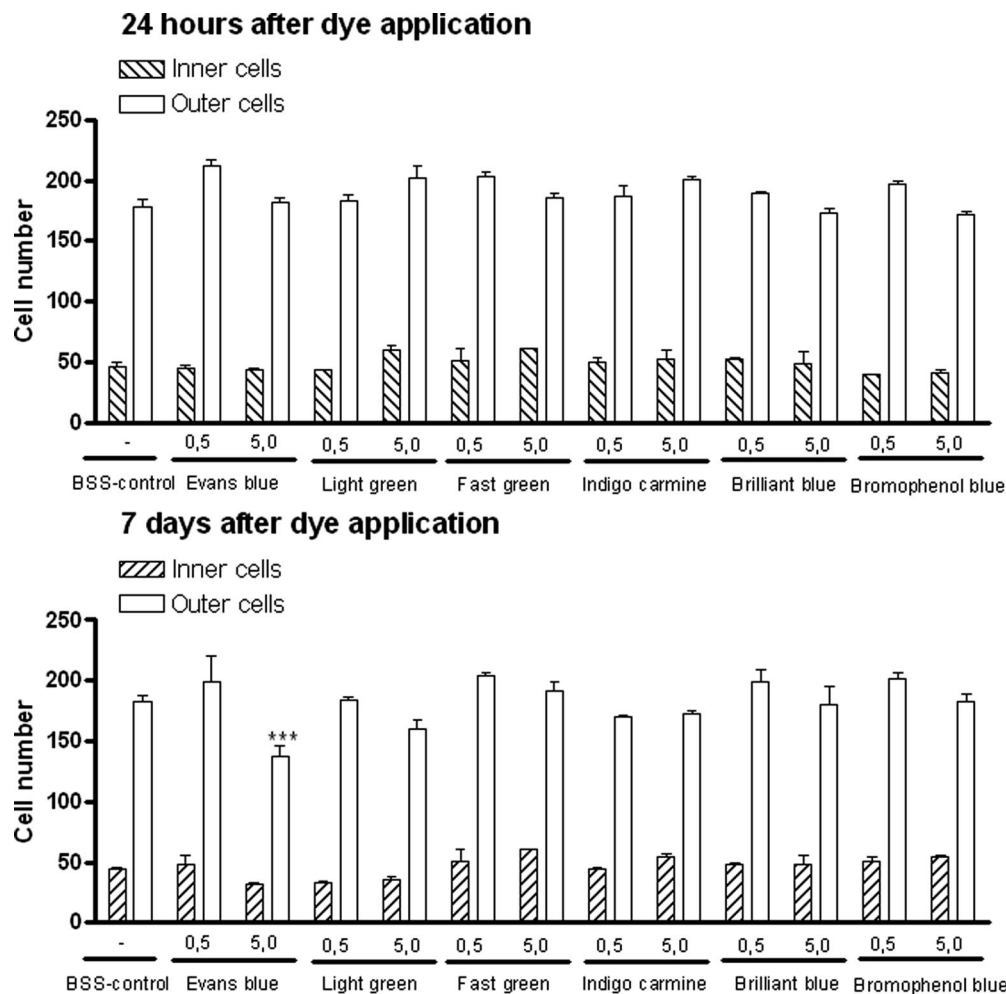


Fig. 3. Cell counting analysis of the INL and ONL with the ImageJ program (NIH, Bethesda) within the 1,100 μ m horizontal surface. The groups of eyes injected with dyes were compared with control group. Top, Cell counting disclosed no cellular loss in any groups injected with 0.05% vital dyes. Bottom, Eyes injected with 0.5% EB revealed statistically significant reduction in the number of outer retinal layer cells ($P < 0.001$). The remaining eyes injected with the higher dose of vital dye demonstrated no reduction in INL or ONL.



vitreous. At clinical examination by indirect ophthalmoscopy, 24 hours and 7 days after dye injection, all eyes were negative for cataract, hemorrhage, retinal detachment, and intraocular opacities. Fluorescein angiography disclosed normal findings without hyper- or hypo-fluorescence in all animals, except for few focal hypofluorescent areas at 7 days in one animal injected with 0.5% EB.

Morphologic Retinal Toxicity With LM and Cell Counting

The histology of both eyes treated with dyes and control eyes showed no major anatomic signs of toxicity. The gross histopathologic appearance of the retina, choroids, sclera, and optic nerve was within normal limits without any signs of severe retinal necrosis or cystic degeneration.

In regard to the 24-hour examination, the control group showed focal areas of vacuolization and edema within retinal layers. At 0.05%, FG, IC, and BriB caused just focal cellular changes in various retinal layers at 1 day, similar to control. Exceptionally, 0.05% BriB produced severe vacuolization of the nerve fiber layer at 24 hours. BroB and EB at the lower dose of 0.05% induced diffuse changes within the ganglion cells (GC), whereas BroB also produced INL damage at 24 hours.

In regard to the 24-hour results of the injection of higher dose dyes, retinas submitted to 0.5% EB injection showed diffuse cellular alterations at the GC layers, INL and photoreceptors, whereas LG at 0.5% caused only diffuse vacuolization of bipolar cells (BC). FG, IC and BriB caused overall just various focal alterations in retinal cells after 1 day. However, BriB at 0.5% also caused remarkable changes within the photoreceptors 24 hours after injection (Figures 1 and 2). Table 1 describes in detail the cellular changes after 24 hours within the retinal tissue with LM.

Concerning the retinal damage 7 days after intravitreal dye injection, the control group examination also showed only sparse regions in the retina of vacuolization and edema. The lower dose of the dyes FG, IC, and BriB caused focal cellular changes within the retinal tissue at 7-days, not differing from control. BroB and EB at the lower dose of 0.05% caused some diffuse changes within the GC and INL but mostly focal changes after 1 week. LG at 0.05% yielded remarkable cellular changes within the GC layer and INL after 7 days.

Histology examination showed more severe alterations to cellular elements 7-days after intravitreal injection of dyes with the higher dose, especially with LG, BroB, and EB. LG at 0.5% induced diffuse vac-

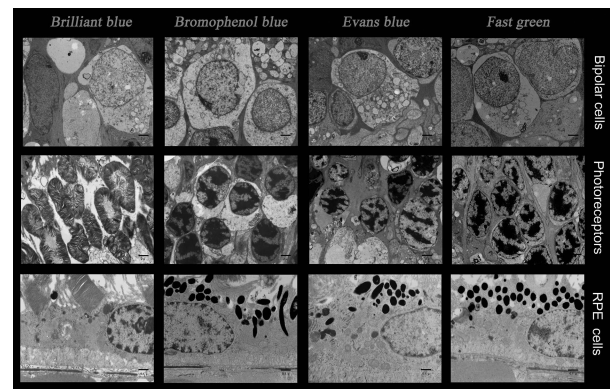


Fig. 4. TEM evaluation at 24 hours after injection of high dose 0.5% BriB, BroB, EB, and FG enabled detailed evaluation of cellular injury. BCs (row above): focal areas of vacuolization at the BC were found in every subgroup in this study, similar to BSS group. Photoreceptors (row in the middle): BriB caused cellular swelling of photoreceptors and some intracellular disruption. BroB and EB induced vacuolization of photoreceptors with areas of cell loss. Eyes injected with FG showed normal photoreceptor layer on TEM. RPE (Bottom row): RPE cells were overall within normal limits in every subgroup.

uolization of BC after 7 days. The remarkable changes in the photoreceptors observed after 1 day with BriB at 0.5% decreased in severity after 1 week follow-up. BroB induced more diffuse edema and vacuolization of the GC, BC and photoreceptors after 1 week. Interestingly, IC-mediated diffuse vacuolization of the nerve fiber layer was found 7 days after dye application with both the lower and higher dose (Figures 1 and 2). The retinal cell damage after 7 days with LM is also summarized in Table 1.

Cell counting with the ImageJ program revealed no cellular loss in any group of eyes injected with 0.05%

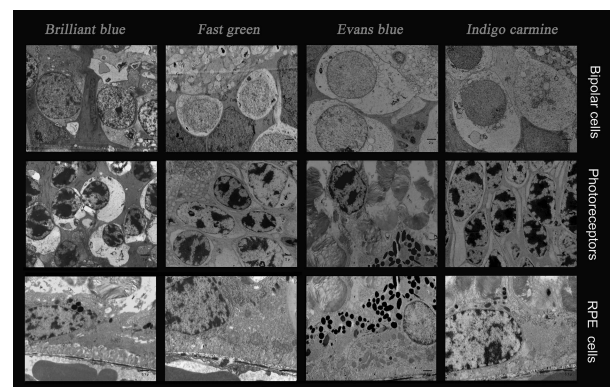


Fig. 5. TEM evaluation at 7 days after injection of high dose 0.5% BriB, FG, EB, IC enabled detailed analysis of cellular injury. BCs (row above), Normal cellular elements of BCs were encountered in these eyes. Photoreceptors (row in the middle), BriB caused few areas of vacuolization and pyknosis of photoreceptors, whereas EB induced additionally some areas of cell death. Both FG and IC groups showed normal appearance of the photoreceptors on TEM. RPE (bottom row), At Day 7 in the higher dose group, RPE cells showed normal findings.



vital dyes. Eyes injected with 0.5% EB showed a statistically significant reduction in the number of outer retinal layer cells ($P < 0.001$). The remaining eyes with high dose injection demonstrated no reduction in cellular elements (Figure 3).

Retinal Toxicity by TEM

Ultrastructural morphology was normal in the retinal layers of the ultrathin sections of the control groups. The nerve fiber layer, retinal pigment epithe-

lium (RPE) and choriocapillaris appeared normal on TEM at 1 and 7 days in every group analyzed. In all eyes, TEM showed that the intracellular structure of RPE was well preserved with good intercellular connections with photoreceptor outer segments. Overall, TEM disclosed only sparse intracellular alterations in eyes exposed to low dose dye injection, similar to control eyes. Cellular injury on TEM as characterized by rupture or twisting of cytoplasmic organelles of GC and BC was observed within the eyes exposed to

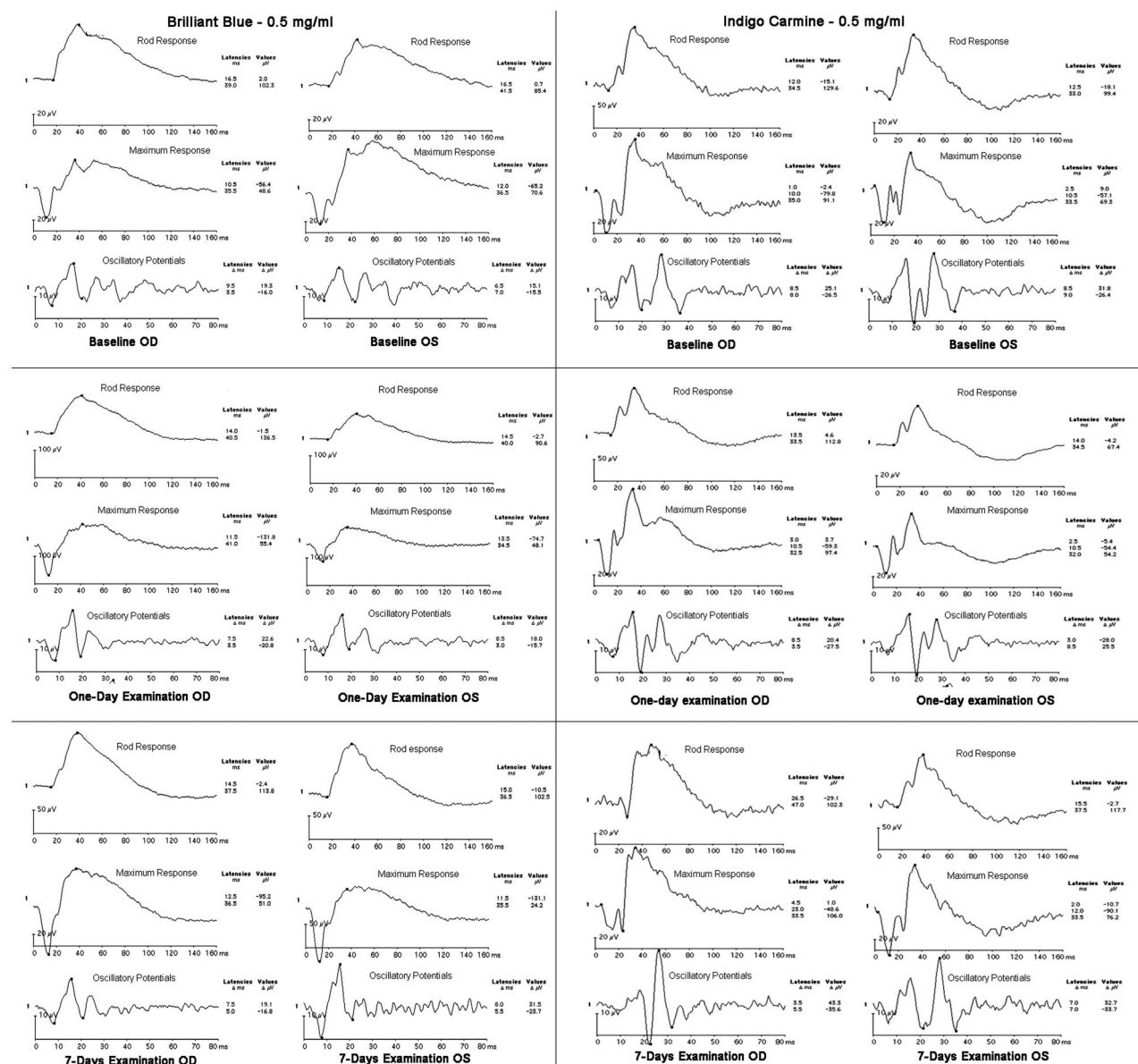


Fig. 6. ERG examination at baseline (top panel), 1 day (middle panel), and 7 days (bottom panel) after intravitreal injection with dyes at the low dose of 0.05%. BriB (right eye) and BSS (left eye) (left images), Rod response and maximum response amplitudes were within normal limits indicating the safety of these two dyes. In this rabbit injected with BriB, at 7 days there was reduction in the ratio of amplitude of OPs of 19.1 in comparison to 31.5 for matched controls. IC (right eye) and BSS (left eye) (right images), Rod response, maximum response, and OPs amplitudes were within normal limits in eyes injected with IC when compared with control in the left eyes.



intravitreal EB, BroB, and LG at the low dose after 7 days.

In the groups of eyes exposed to the higher dose, for 24 hours, diffuse cellular twisting and swelling was observed in the photoreceptor layers of eyes exposed to BriB, whereas BroB and EB induced areas of photoreceptor cytoplasmic vacuolization and sparse pyknosis (Figure 4). After 7 days, TEM revealed vacuolization and cellular edema of photoreceptors in eyes injected with 0.5% BriB, whereas EB caused cellular disorganization, rupture and edema (Figure 5). The examination of BC-treated eyes revealed only

focal vacuolization in every group at 1 and 7 days after injection with 0.5% dyes (Figures 4 and 5).

Functional Retinal Evaluation With ERG

At a dose of 0.05%, FG, BroB, BriB, and IC caused no major changes in ERG-examination during the follow-up period, except for mean reduction in the dye-normal ratio for IC and BriB in the b-wave amplitude of the 30-Hz flicker examination after 7 days and decrease in b-wave amplitude of OPs 7 days after BriB injection (Figures 6 and 7). LG at 0.05% caused

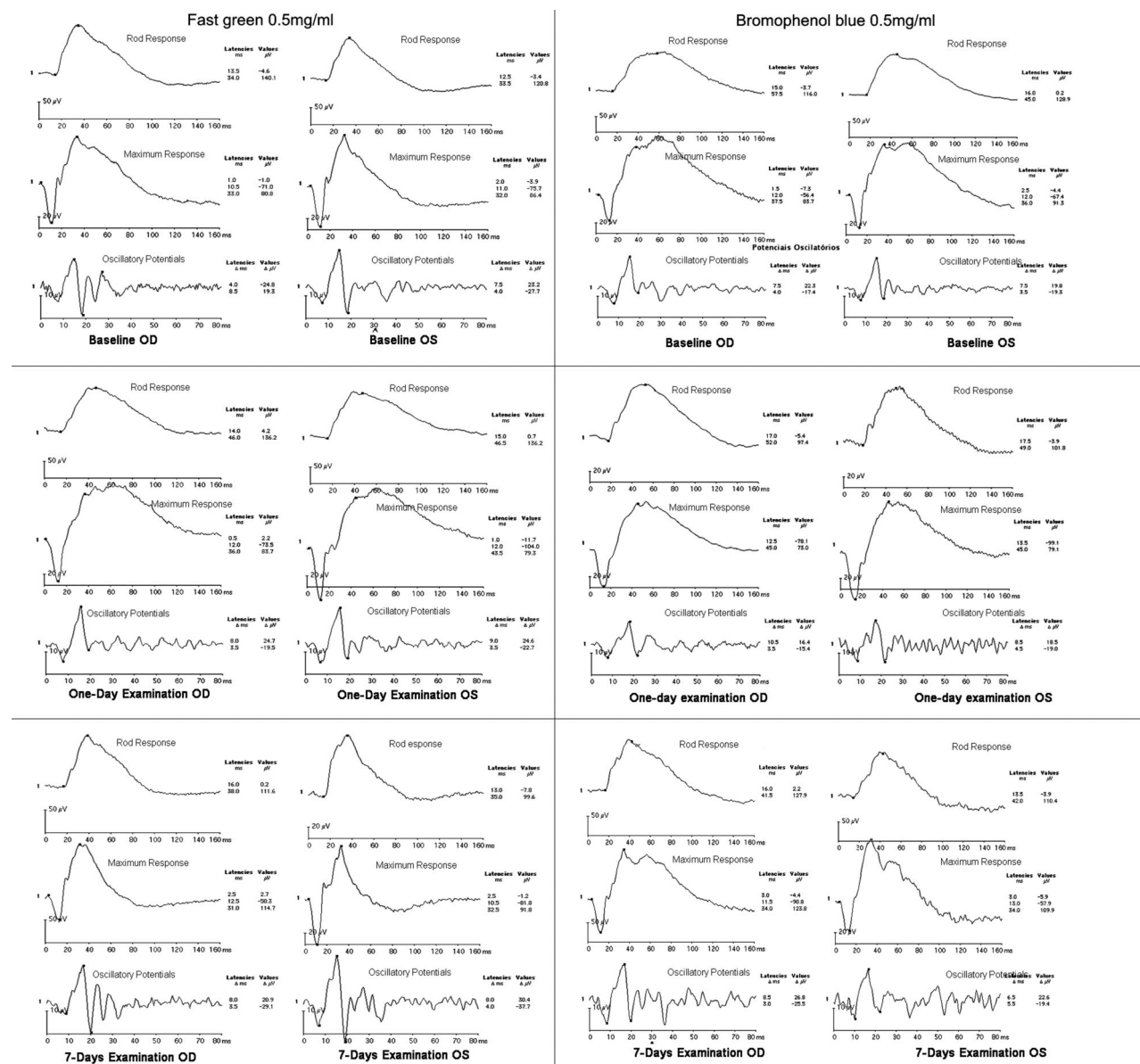


Fig. 7. ERG examination at baseline (top panel), 1 day (middle panel), and 7 days (bottom panel) after intravitreal injection of dyes at the low dose of 0.05%. FG (right eye) and BSS (left eye) (left images), Rod response, maximum response, and OPs amplitudes were within normal limits. BroB (right eye) and BSS (left eye) (right images), Rod response, maximum response, and OPs amplitudes revealed normal findings indicating the safety of the two dyes.



Table 2. Descriptive Analysis of Mean Results for Each Eye in Rabbits Treated with 0.05% Injection of Novel Vital Dyes in the Right Eye and Saline Solution in the Left Eye

Vital Dye Parameter Evaluated	Bromophenol Blue						Evans Blue						Light Green					
	Baseline		1 Day		1 Week		Baseline		1 Day		1 Week		Baseline		1 Day		1 Week	
	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS
Rods response																		
b-Wave amplitude	119.7	129.1	102.8	105.7	125.7	114.3	179.3	114.8	39.3	173.1	61.0	124.3	194.6	171.1	134.8	107.2	77.4	77.4
b-Wave amplitude ratio	0.93		0.97		0.99		1.56		0.23		0.49		1.14		1.26		1	
b-Wave latency	57.5	45.0	52.0	49.0	41.5	42.0	28.5	31.0	29.0	32.0	35.5	33.0	38.0	42.5	40.0	34.0	43.0	40.0
b-Wave latency ratio	1.28		1.06		0.99		0.92		0.91		1.07		0.89		1.18		1.07	
Maximal response																		
b-Wave amplitude	140.1	158.7	151.1	178.2	214.6	167.8	253.4	141.8	42.2	215.6	107.2	190.2	253.9	214.6	180.9	152.8	104.7	262.0
b-Wave amplitude ratio	0.88		0.85		1.28		1.78		0.19		0.56		1.19		1.18		0.39	
b-Wave latency	37.5	36.0	45.0	45.0	34	34	33.0	33.5	26.0	32.0	33.0	33.5	38.0	36.5	33.0	31.0	39.0	40.0
b-Wave latency ratio	1.04		1		1		0.98		0.81		0.98		1.04		1.06		0.97	
a-Wave amplitude	49.1	63	NA	NA	95.2	63.8	141.1	73.2	122.1	95.2	88.8	102.3	NA	NA	98.9	80.8	NA	NA
Oscillatory potentials																		
b-Wave amplitude	22.3	19.8	16.4	18.5	26.8	22.6	46.3	21.3	19.0	40.7	17.8	30.9	41.2	26.6	25.4	29.8	10.6	41.6
b-Wave amplitude ratio	1.13		0.88		1.18		2.17		0.47		0.58		1.55		0.85		0.25	
Flicker 30 Hz																		
b-Wave amplitude	39.1	27.6	18.5	15.6	24.5	24.5	91.5	40.7	30.0	83.3	19.7	60.4	54.1	53.2	38.8	45.8	18.2	65.0
b-Wave amplitude ratio	1.42		1.18		1		2.25		0.36		0.32		1.02		0.85		0.28	
b-Wave latency	24.5	24.0	40.0	39.5	49.1	35.6	28.5	35.5	24.0	23.5	25.5	23.0	23.5	24.0	24.0	24.0	25.0	24.5
b-Wave latency ratio	1.02		1.01		1.38		0.80		1.02		1.11		0.98		1		1.02	

NA, not available.

a mean reduction in dye-control ratio of b-wave amplitude of OPs and 30-Hz flicker at 1 and 7 days after dye injection. EB induced more severe ERG alterations, and reduced ratios of amplitude in rod response, maximum response, OPs and 30-Hz flicker (Table 2).

Injection of the vital dyes at the higher dose produced overall more severe mean functional changes detected by ERG. BroB caused reduction in total b-wave amplitude and to the dye-control ratio in rod response at 7 days after injection. EB at 0.5% resulted in a reduction in b-wave amplitude, OPs and 30-Hz flicker at 7 days examination, whereas 0.5% LG caused alterations in b-wave amplitude of rod response, maximum response, OPs and 30-Hz flicker at 1 day or 7 day of follow-up testing. Eyes injected with 0.5% FG showed mean reduction in b-wave amplitude of OPs, which recovered after 7 days. FG also caused prolonged implicit time in rod response after 7 days. BriB caused a >50% mean reduction in b-wave amplitude in rod response, OPs and 30-Hz flicker testing. The ERG responses of the rabbits injected with 0.5% IC did not change appreciably during the 1-week follow-up period (Table 3).

Discussion

Chromovitrectomy facilitates intraoperative surgical procedures such as ILM- or ERM-peeling.¹⁻³ The two dyes available for chromovitrectomy, ICG and TB, may lead to complications in macular surgery.^{24,25} Recent preliminary data revealed BroB and BriB as

good alternative dyes for chromovitrectomy.¹⁷⁻²¹ In this rabbit model study, the retinal biocompatibility of six vital dyes, EB, IC, BriB, BroB, LG, and FG, belonging to the group of arylmethane, thiazine, and azo dyes was determined to evaluate these dyes as alternatives in chromovitrectomy.

Azo dyes constitute a group of dyes containing an azo chemical group linked to benzene, naphthalene, or aromatic heterocyclic rings. EB ($C_{34}H_{24}N_4Na_4O_{14}S_4$; 980 Da) is an azo dye with a large planar aromatic system which has an absorption peak of 611 nm in water.²⁶ In ophthalmology, EB has been used as a dye for endothelial function evaluation, and animal studies have demonstrated that the dye is not toxic to corneal endothelial cells.²⁷ The toxicity and staining affinity of EB for retinal structures has been evaluated in cell culture, where EB induced slight retinal damage at 0.02%, and it showed favorable staining affinity for ILM.¹⁸ In our investigation, EB caused severe functional and morphologic retinal toxicity at the higher dose of 0.5%. Based on this finding, EB at least at high doses should not be considered for human application in chromovitrectomy.

Thiazine dyes are small and cationic molecules containing a chromophore called thiazinium. IC ($C_{16}H_8N_2Na_2O_8S_2$; 466 Da) is part of the group of carbonyl dyes. It has an absorption peak at 608 nm in water and is known to stain collagen tissues well.^{22,28} To our knowledge, the present study is the first time IC has been evaluated for vitreoretinal surgery, where it showed overall good safety about the retina. Such



Table 2. (Continued)

Fast Green						Indigo Carmine						Brilliant Blue					
Baseline		1 Day		1 Week		Baseline		1 Day		1 Week		Baseline		1 Day		1 Week	
OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS
111.4	107.4	132.0	135.5	111.8	107.4	144.7	117.5	108.2	71.6	131.4	120.4	104.3	86.1	138	93.3	116.2	113.0
1.04		0.97		1.04		1.23		1.51		1.09		1.21		1.47		1.03	
38.0	35.0	46.0	46.5	38.0	35.5	34.5	33.0	33.5	34.5	47.0	37.5	39	41.5	40	40	37.5	36.5
1.08		0.99		1.07		1.04		0.97		1.25		0.94		1		1.03	
165.0	172.8	157.2	183.3	165.0	173.6	170.9	126.4	156.7	108.6	154.6	166.3	105	135.2	187.2	122.8	146.2	155.3
0.95		0.86		0.95		1.35		1.44		0.93		0.77		1.52		0.94	
31.0	32.5	36.0	43.5	31.0	32.5	35.0	33.5	32.5	32.0	33.5	33.5	35.5	36.5	41	34.5	36.5	35.5
0.95		0.83		0.95		1.04		1.01		1		0.97		1.19		1.03	
53.0	83.0	75.7	92.3	52.9	80.6	77.4	66.1	63	59.8	49.6	79.4	NA	NA	NA	NA	NA	NA
24.8	23.2	24.7	24.6	20.9	30.4	25.1	35.8	20.4	28.0	43.3	32.7	19.3	15.1	22.6	18.0	19.1	31.5
1.07		1.00		0.69		0.70		0.73		1.32		1.28		1.25		0.61	
24.9	31.3	29.7	44.7	30.5	31.5	41.4	36.4	28.1	24.4	13.6	36.5	42.8	35.7	40.3	34.2	37.6	50.0
0.79		0.67		0.97		1.14		1.15		0.37		1.20		1.18		0.75	
24.5	23.5	25.0	25.5	24.5	23.5	23.5	23.5	24.0	24.5	26.5	24.5	25.0	25.0	24.5	24.0	24.5	24.5
1.04		0.98		1.04		1		0.98		1.08		1		1.02		1	

findings may indicate that IC and possibly other thiazine dyes may be useful in chromovitrectomy, but staining affinity issues should be evaluated.

Amino di- and tri-arylmethane dyes are heterogeneous groups of dyes characterized by the presence of an amino moiety and by a strong dyeing property because of their physicochemical properties such as ionic charges and the presence of bases in the chemical structure.²² In this group of vital dyes there are four particular dyes tested in our investigation: BroB, BriB, LG, and FG. LG ($C_{37}H_{34}N_2Na_2O_9S_3$; 792 Da) is a very hydrophilic diaminotriarylmethane dye with two benzylated amino groups, which has an absorption peak of 630 nm in water. FG ($C_{37}H_{34}N_2Na_2O_{10}S_3$; 808 Da) is a large hydrophilic anionic diaminotriarylmethane dye with an absorption peak around 620 nm in aqueous ethanol.²⁹ FG was applied in vitreoretinal surgery by Sorsby in 1939, as he injected the green agent endovenously for the recognition of breaks in the treatment of retinal detachments.³⁰ More recently, Jackson et al described poor staining of the retina and no toxicity for FG in a laboratory in vitro study.¹⁸ In regard to the application of LG in chromovitrectomy, Haritoglou et al found in their in vitro toxicity studies no toxic effect of LG on ARPE-19 and primary RPE cell proliferation, but cell viability was affected at a concentration of 0.2%.¹⁹ In our current investigation, very good in vivo retinal biocompatibility was found for FG, whereas LG induced some remarkable histologic and functional retinal abnormalities, especially at the higher dose of 0.5%.

BroB ($C_{19}H_{10}Br_4O_5S$; 670 Da) is a sulfonated weakly hydrophilic hydroxytriarylmethane dye, which has been used as an acid–base indicator or as a marker

to monitor gel electrophoresis procedures.³¹ Recent experimental investigations proposed BroB as an alternative dye for chromovitrectomy. Preclinical experiments demonstrated that among six biologic dyes, BroB stained the ERM and ILM better, and caused no damage in vitro to either ARPE-19- or RPE-cells at concentrations of 0.2% and 0.02%.¹⁹ Further in vivo studies in rodent and porcine eyes demonstrated that BroB at concentrations of 0.5% and 0.02% did not result in any significant retinal toxicity assessed by histology and GC counting at various concentrations, while providing enhanced ILM staining and identification.¹⁷ In contrast to those studies, we found that BroB promoted significant retina toxicity manifested by cellular damage such as cytoplasmic vacuolization and pyknosis of the GC, INL and photoreceptors as well as ERG dysfunction, more severe in the higher dose of 0.5%. Such different results may be explained by different animal models applied or biochemical factors such as solvents or source of dyes.³² We also found that 0.5% BroB induced more frequent retinal damage than 0.5% BriB, especially after 7 days.

BriB ($C_{47}H_{48}N_3S_2O_7Na$; 854 Da) is a synthetic triarylmethane dye which has an absorption peak of 630 nm in water. This stain has been used as a protein marker in cardiovascular and neurologic diseases.¹ Animal and human data on the safety profile of BriB for chromovitrectomy and anterior lens capsule staining were released in 2006. In the rat and primate eyes, no significant retinal change was observed with LM and TEM after low-dose BriB injection; there was also no reduction in the amplitude of the ERG waves. However, TEM revealed that the high-dose groups



Table 3. Descriptive Analysis of Mean Results for Each Eye in Rabbits Treated with 0.5% Injection of Novel Vital Dyes in the Right Eye and Saline Solution in the Left Eye

Vital Dye Parameter Evaluated	Bromophenol Blue						Evans Blue						Light Green					
	Baseline		1 Day		1 Week		Baseline		1 Day		1 Week		Baseline		1 Day		1 Week	
	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS
Rods response																		
b-Wave amplitude	126.7	125	96.0	110.4	96.4	144.1	167.7	147.2	85.4	97.1	60.3	89.8	120.6	123.0	20.8	101.3	28.4	78.7
b-Wave amplitude ratio	1.01		0.87		0.18		1.14		0.88		0.67		0.98		0.21		0.36	
b-Wave latency	37.0	36.5	44.5	39.0	38.0	36.0	35.0	36.0	32.5	33.0	43.5	41.0	30.5	32.5	22.0	34.0	49.5	33
b-Wave latency ratio	1.01		1.14		1.05		0.97		0.98		1.06		0.94		0.65		1.5	
Maximal response																		
b-Wave amplitude	174.3	193.8	134.7	174.0	154.3	208.3	226.6	194.8	141.6	176.0	56.9	115.7	144.1	154.3	30.3	126.2	72.3	138.2
b-Wave amplitude ratio	0.90		0.78		0.74		1.16		0.80		0.49		0.93		0.24		0.52	
b-Wave latency	33.0	31.5	30.5	31.5	30.0	33.3	36.0	37.5	32.0	32.5	34.0	33.5	30.0	27.5	30.9	26.0	32.0	36.0
b-Wave latency ratio	1.05		0.97		0.90		0.96		0.98		1.01		1.09		1.19		0.89	
a-Wave amplitude	83.0	94.4	66.4	76.4	51.3	84.5	NA	NA	62.8	69.8	29.5	59.6	96.5	96.9	50.5	84.2	35.4	60.1
Oscillatory potentials																		
b-Wave amplitude	23.4	28.2	16.3	20.2	18.1	24.2	41.1	32.1	22.0	30.0	14.9	17.9	30.6	26.4	17.7	26.9	17.0	16.6
b-Wave amplitude ratio	0.83		0.81		0.75		1.28		0.73		0.83		1.16		0.66		1.02	
Flicker 30 Hz																		
b-Wave amplitude	37.6	31.5	23.8	31.1	20.7	61.0	68.9	49.1	23.0	23.5	15.9	47.4	53.4	31.3	NA	28.4	17.1	36.4
b-Wave amplitude ratio	1.19		0.76		0.33		1.40		0.98		0.33		1.70				0.47	
b-Wave latency	24.5	24.5	24.0	24.0	24.5	23.5	24.0	23.5	26.8	43.6	26.0	24.0	24.0	24.5	NA	25.5	29.0	25.0
b-Wave latency ratio	1		1		1.2		1.02		0.61		1.08		0.98				0.97	

NA, not available.

(0.1% and 1%) showed vacuolization in the GC and Müller cell processes in the nerve fiber layer.^{20,21} Ueno et al compared the toxicity of BriB with that of ICG and TB in a rat model of subretinal injection. Their results showed that BriB induced no detectable toxic effects after 2 months, in contrast to the retinal and RPE degeneration induced by TB and ICG.³³ In humans, BriB caused suitable ILM staining in an iso-osmolar solution of 0.025% for ERM and macular hole treatment, with 85% of patients showing improved vision and no clinical signs of toxicity.²⁰ In 2007, BriB was approved for intraoperative use for chromovitrectomy in Europe at a concentration of 0.025%. Overall, we observed herein good retinal biocompatibility of 0.05% BriB in our experiments in rabbits' eyes, which confirm previous experimental investigations with the arylmethane dye. Further experiments should elucidate the acute toxic effects of BriB to the photoreceptors found in the current investigation.

The investigation of dyes and drugs applied on the retina may be performed in cell culture experiments or animals. Cell culture models with retinal pigment epithelial or neuroretinal cells, e.g., R28 cells, may allow systematic evaluation of drug toxicity in a controlled fashion. In a cell culture system, a chemical agent may be found to be safe at certain concentrations, while in vivo evaluation may show specific signs of damage. These observations may result from the complexity of the intraocular milieu, making it difficult to replicate in a cell or tissue culture system. Compared with previous experiments in RPE and neuronal cell cul-

ture, for instance by Mennel et al, our study found a similar safety for BriB at two doses.³⁴ Usually research in animals is considered better evidence of pharmacological activity than cell culture alone, since it may better simulate the actual human retinal environment. However, there are interspecies variations which may produce results that may not be translated to human experience in chromovitrectomy. Previous studies in rabbits, rats, and primates revealed an overall safe profile for BroB and BriB dyes.^{21,32,33} In our research, we found a novel finding of some photoreceptor changes after high dose BriB, as well as significant toxicity of high dose BroB. Some explanations for those suspicious signs of retinal toxicity of BriB and BroB include interspecies variation, different chemical composition of the dyes among studies, various regions of the retina examined, or pH/osmolarity differences in solutions injected. In the future, we aim to pursue studies in primates with intravitreal injection of iso-osmolar neutral BriB and BroB for elucidation of retinal safety concerns.

One advantage of our study concerns the comparison of potential and novel dyes in an animal model. BriB has been recently approved in Europe for intraocular injection in humans, whereas BroB may soon enter the market for application in chromovitrectomy. We recently have found that FG, LG, and EB stain intraocular membranes very well, and therefore, they may be pursued for surgical use in the future. The disadvantage of our current investigation is that only two concentrations were tested, in contrast to the large



Table 3. (Continued)

Fast Green						Indigo Carmine						Brilliant Blue					
Baseline		1 Day		1 Week		Baseline		1 Day		1 Week		Baseline		1 Day		1 Week	
OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS
92.8	118.4	91.0	76.0	82.3	98.6	111.1	117.9	109.4	170.4	85.9	128.4	166.5	95.7	64.5	61.0	54.5	69.3
0.78		1.20		0.83		0.94		0.64		0.67		1.74		1.06		0.79	
33.0	37.0	34.0	36.5	55.5	15.0	33.5	32.5	30.5	32.0	33.5	33.5	38.5	37.5	48.5	46.5	48.5	45
0.89		0.93		3.7		1.03		0.95		1		1.02		1.04		1.08	
153.5	147.7	142.5	97.4	84.2	75.2	116.2	134.0	155.0	203.6	123.1	163.3	177.0	143.8	124.5	102.6	73.2	122.4
1.04		1.46		1.12		0.87		0.76		0.75		1.23		1.21		0.60	
30.0	31.5	34.5	32.5	38.5	37.5	29.5	29.0	34.0	33.5	30.0	32.5	33.5	30.5	33.5	36.0	31	34
0.95		1.06		1.03		1.02		1.01		0.92		1.10		0.93		0.91	
NA	NA	NA	NA	NA	NA	59.1	60.5	108.7	93.5	63.5	68.8	119.2	62.5	111.6	26.6	44.9	56.4
33.0	15.9	18.5	18.5	18.6	10.5	16.9	22.1	35.5	32.7	22.7	17.8	32.6	19.4	23.4	11.9	18.7	35.5
2.07		1		1.77		0.76		1.08		1.27		1.68		1.97		0.52	
67.6	47.6	64.7	47.8	8.4	11.2	28.7	37.6	73.3	83.3	56.0	37.3	61.2	41.3	31.1	37.6	14.9	38
1.42		1.35		0.75		0.76		0.88		1.5		1.48		0.82		0.39	
24.5	25.0	24.5	26.0	34.0	26.5	24.0	23.5	24.0	24.0	23.0	23.5	24.0	24.0	25.5	24.5	26.0	24.0
0.98		0.94		1.28		1.02		1		0.98		1		1.04		1.08	

number of concentrations that can be examined in cell culture research.

In conclusion, our study showed that new dyes exerted dose-dependent toxicity to the retinal tissue in rabbits. The progressive order of retinal biocompatibility encountered in our investigation from higher to lower was IC (safest), FG, BriB, BroB, LG, EB (toxic). The next step is to test the staining affinity of safe vital dyes for preretinal membranes and tissues. Further on, surgical experience will help to improve usage and interpretation of these new alternative dyes in chromovitrectomy.

Key words: chromovitrectomy, indocyanine green, fast green, vital dye, trypan blue, retina, brilliant blue, bromophenol blue, toxicity.

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