Administration of Novel Dyes for Intraocular Surgery: An In Vivo Toxicity Animal Study

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Purpose. To investigate the effect of intravitreal injections of new vital dyes on the retina, the retinal pigment epithelium (RPE) and the choroid in an in vivo rat model.

Methods. Rats were injected intravitreally with four dyes: light-green SF yellowish (LGSF), copper(II)phthalocyanine-tetrasulfonic acid (E68), bromphenol blue (BPB), and Chicago blue (CB) dissolved in physiologic saline solution (PSS) at concentrations of 0.5% and 0.02%. PSS served as the control. Additional animals were treated with single injections of 0.5%, 0.02%, 0.002%, and 0.0002% ICG or 0.002% E68 into one eye. Adverse effects on anterior and posterior segments were evaluated by slit lamp biomicroscopy and ophthalmoscopy. Retinal toxicity was assessed by histology and retinal ganglion cell (RGC) quantification 7 days after dye administration.

Results. Eyes treated with 0.5% E68, 0.5% ICG, or 0.5% CB showed discrete staining of both cornea and lens not seen at lower concentrations or with other dyes. Histology revealed dose-dependent reactions after E68 administration. ICG 0.5% induced significant thinning of inner retinal layers compared with PSS. ICG 0.02% caused focal degenerative changes of the outer retina in three of seven eyes, whereas 0.002% and 0.0002% ICG did not. CB led to heterogeneous morphologic alterations. BPB- or LGSF-treated eyes showed normal retinal morphology. ICG at all tested concentrations induced significant RGC loss, as did E68 at 0.5% but not at lower concentrations.

Conclusions. BPB or LGSF produced no significantly detectable toxic effects on the retina in vivo. The safety of these new dyes must be established in other models and/or in preclinical studies before the clinical use of any of these dyes. (*Invest Ophthalmol Vis Sci.* 2006;47:3573–3578) DOI:10.1167/iovs.06-0211

The thorough removal of epiretinal tissue and the internal limiting membrane (ILM) of the retina is an important element in the successful treatment of traction maculopathies such as macular holes or macular pucker.[1,2] The ILM represents the basement membranes of the underlying Müller cells and is therefore intimately associated with their plasma membrane. As a consequence, there has been a long and still ongoing discussion among surgeons about whether the ILM can be removed from the retinal surface without disrupting adjacent cellular components. The relevance of this becomes even clearer if one considers the fact that the ILM is less than 1 μm thick and barely visible. The safe removal of this delicate structure represents a great challenge to the vitreoretinal surgeon. As a result, ILM peeling long remained outside the standard surgical repertoire.

The introduction of vital dyes such as indocyanine green (ICG) to stain the ILM selectively during the surgical procedure[3,4] made the technique of ILM removal more popular, because ICG allowed for much better visualization. However, shortly after its introduction, some concerns were voiced about the safety of ICG for intraocular application, and some investigators reported adverse effects on functional outcome.[5–9] Because others did not observe any ICG-related adverse effects,[10–13] the role of ICG in macular surgery is still the subject of ongoing discussion in the ophthalmic community. Despite several clinical and experimental investigations, the underlying pathomechanism of potential ICG-related toxicity is still not completely understood, and reliable safety margins have not been established that would allow an evidence-based and safe intraocular application.

This motivated us to search for new vital dyes with good staining characteristics and better biocompatibility.[14] In view of the known photochemical properties of ICG, which seem to play an important role in ICG-related toxicity[15,16] we chose several dyes with high photochemical stability. These dyes were then first evaluated in an ex vivo approach to gain initial information on their staining characteristics and biocompatibility.[14] In the present study, these dyes were investigated more thoroughly for their biocompatibility profile in a well-established animal in vivo experimental setting using slit lamp biomicroscopy, ophthalmoscopy, histologic evaluation of the retina, and retinal ganglion cell (RGC) quantification.

Materials and Methods

Dye Preparation

Four dyes were chosen for the present study: light-green SF yellowish (LG SF), the tetrasodium salt of copper(II)phthalocyanine-tetrasulfonic acid (E68), bromophenol blue (BPB), and Chicago blue (CB). ICG (Pulsion, Munich, Germany) was used as a reference. All dyes were dissolved and diluted with physiologic saline solution (PSS; BSS plus; Alcon Laboratories Inc., Fort Worth, TX) to obtain concentrations of

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TABLE 1. Quantification of Central Retina Thickness

Dye	Total	NFL	IPL	INL	OPL	ONL	PR-IS	PR-OS
PSS control	194 ± 34	8 ± 3	53 ± 15	30 ± 4	7.8 ± 0.9	40 ± 11	17 ± 4	24 ± 2
LGSF 0.5%	185 ± 29	10 ± 1	45 ± 7	30 ± 3	6.7 ± 0.5	38 ± 7	18 ± 2	25 ± 2
LGSF 0.02%	234 ± 30	11 ± 1	57 ± 15	36 ± 4	8.2 ± 1	48 ± 4	22 ± 3	27 ± 2
BPB 0.5%	210 ± 14	12 ± 2	53 ± 4	35 ± 1	7.6 ± 1.1	45 ± 4	20 ± 2	24 ± 2
BPB 0.02%	238 ± 22	14 ± 2	57 ± 3	37 ± 4	7 ± 0.6	50 ± 7	23 ± 3	29 ± 3
CB 0.5%	235 ± 36	11 ± 1	58 ± 16	38 ± 1	7.5 ± 1.1	47 ± 6	21 ± 1	26 ± 2
CB 0.02%	214 ± 18	11 ± 2	52 ± 3	36 ± 2	7.5 ± 0.7	49 ± 2	22 ± 1	28 ± 3
E68 0.5%	186 ± 25	9 ± 2	45 ± 9	32 ± 4	7.3 ± 0.8	39 ± 5	15 ± 3	18 ± 3
E68 0.02%	210 ± 19	11 ± 2	53 ± 5	35 ± 3	7.3 ± 0.5	45 ± 3	21 ± 2	24 ± 1
E 68 0.002%	184 ± 44	7 ± 1	46 ± 10	28 ± 3	8.7 ± 1.1	40 ± 14	17 ± 1	23 ± 3
E68 0.0002%	199 ± 13	7 ± 1	49 ± 3	29 ± 1	6.8 ± 0.2	40 ± 2	17 ± 1	24 ± 2
ICG 0.5%	**148 ± 16**	**5 ± 2**	**35 ± 4**	29 ± 2	7.0 ± 0.9	34 ± 6	16 ± 2	18 ± 2
ICG 0.02%	179 ± 21	7 ± 2	42 ± 7	30 ± 2	6.8 ± 0.6	40 ± 7	17 ± 2	20 ± 3
ICG 0.002%	226 ± 12	8 ± 1	57 ± 6	38 ± 2	7.5 ± 0.6	49 ± 2	25 ± 2	28 ± 2
ICG 0.0002%	218 ± 27	10 ± 1	59 ± 18	35 ± 3	8.1 ± 1.1	48 ± 2	23 ± 3	27 ± 3

Data are mean micrometers ± SD. Total, whole thickness; NFL, nerve fiber layer, IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PR-IS, photoreceptor inner segment; PR-OS, photoreceptor outer segment. The only significant difference in any of the layers compared with the PSS-treated control was seen with 0.5% ICG treatment (total thickness, NFL and IPL, bold).

0.5% and 0.02%. Dry ICG powder was first dissolved with sterile water provided by the manufacturer, resulting in a 0.5% solution. The solution was used for injections and as a stock solution for further dilution with PSS to a concentration of 0.02%. E68 and ICG were also used in the additional concentrations of 0.002% and 0.0002%. The osmolarity of all dyes used was within a physiological range (approximately 300 mOsM) and was equal to that of PSS, which was used as the control.

Intravitreal Injection of Dyes

All experiments complied with the guidelines for animal care of the European Community and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Adult male Brown Norway rats were anesthetized with an intraperitoneal injection of chloral hydrate (6 mL of a 7% solution/kg body weight). Eyes were injected intravitreally using a heat-pulled glass capillary connected to a microsyringe (Drummond Scientific Co., Broomall, PA) under direct observation through the microscope. Animals with visible lens damage were excluded from the experiments and not included in the analysis. A single 2-μL injection of dye or PSS was given, with dye concentrations, as described earlier. Contralateral eyes served as control eyes and were randomly injected with PSS or left untreated.

Clinical Examination

To detect possible toxic or staining effects of dyes on the anterior segment of the eye (e.g., corneal opacification and/or cataract induction), all eyes were examined by slit lamp biomicroscopy by an observer unaware of the treatment 0, 1, 24, 48, and 168 hours after the intravitreal injections. Animals with cataracts were excluded from the experiment. At the same time points, pupils were dilated with 1 drop of a mixture of 1.7% tropicamide and 3.3% phenylephrine, and indirect ophthalmoscopy was performed to detect vitreous opacification and to verify retinal perfusion.

Histology

Eyes of adult Brown Norway rats were used for morphologic investigation. One eye per animal was treated as for the clinical examination with 0.5%, 0.02%, 0.002%, or 0.0002% E68, 0.5% or 0.02% BPB, 0.5% or 0.02% CB, 0.5% or 0.02% LGSF, and 0.5% or 0.02% ICG, or with the control PSS ($n = 3$ in each case). Additional animals were treated with injections of 0.002% or 0.0002% ICG into one eye (each $n = 2$).

Animals were killed with CO_2 7 days after dye or PSS injection. The eyes were immediately enucleated and immersion-fixed for at least 7 days in Ito's solution containing 2.5% glutaraldehyde, 2.5% paraformaldehyde (wt/vol; PFA), and 0.01% picric acid in 0.1 M cacodylate buffer (pH 7.2).

The eyes were bisected, separating the anterior and posterior segments at the equator. The posterior half was then bisected into nasal and temporal segments. The specimens were postfixed in 1% osmium tetroxide, rinsed in cacodylate buffer, dehydrated in an ascending series of alcohol solutions and embedded in araldite. Semithin sections were cut along a superior–inferior plane, stained with toluidine blue and investigated by light microscopy.

Ultra-thin sections of selected areas were stained with leaded citrate and uranyl acetate and viewed with a transmission electron microscope.

The total thickness of the neural retina and the thickness of the different retinal layers were quantitatively evaluated at the same location of the central retina in each eye. The measuring field was defined by a distance of 200 μm from the optic nerve head rim, and the single-thickness measurements (5–10 per eye) were obtained within the next 300 μm peripherally.

The number of leukocytes after treatment with E68 was counted in six visual fields of 400-μm length, and the number of cells per millimeter was estimated. Statistical analysis was performed with the Wilcoxon test (with $\alpha = 0.01$).

Quantification of RGCs

RGC survival was assessed as described previously.[17] Labeling was performed 5 days after intraocular injection. Animals were anesthetized deeply, and the fluorescent tracer hydroxystilbamidine methanesulfonate (Fluorogold; Invitrogen, Eugene, OR) was applied to each of the superior colliculi by stereotactic injection as described elsewhere.[17] Two days later, the animals were killed by chloral hydrate overdose. After enucleation, the retinas were dissected, flatmounted on cellulose nitrate filters (pore size 60 μm; Sartorius, Westbury, NY), and fixed in 2% PFA for 30 minutes. Labeled cells were defined as surviving. Observation was performed immediately under a fluorescence microscope, and counting was performed in 12 distinct areas of 62,500 μm^2 per retina. Images were obtained using a digital imaging system connected to the microscope (ImagePro; Media Cybernetics, Silver Spring, MD), coded, and analyzed semiautomatically in a masked fashion using a computer-assisted image-analysis system.[17]

Statistical analysis was performed using a paired Student's *t*-test to determine significant differences in RGC counts between groups. Differences were considered significant when $P < 0.05$ (Table 1). All values are expressed as the mean ± SEM.

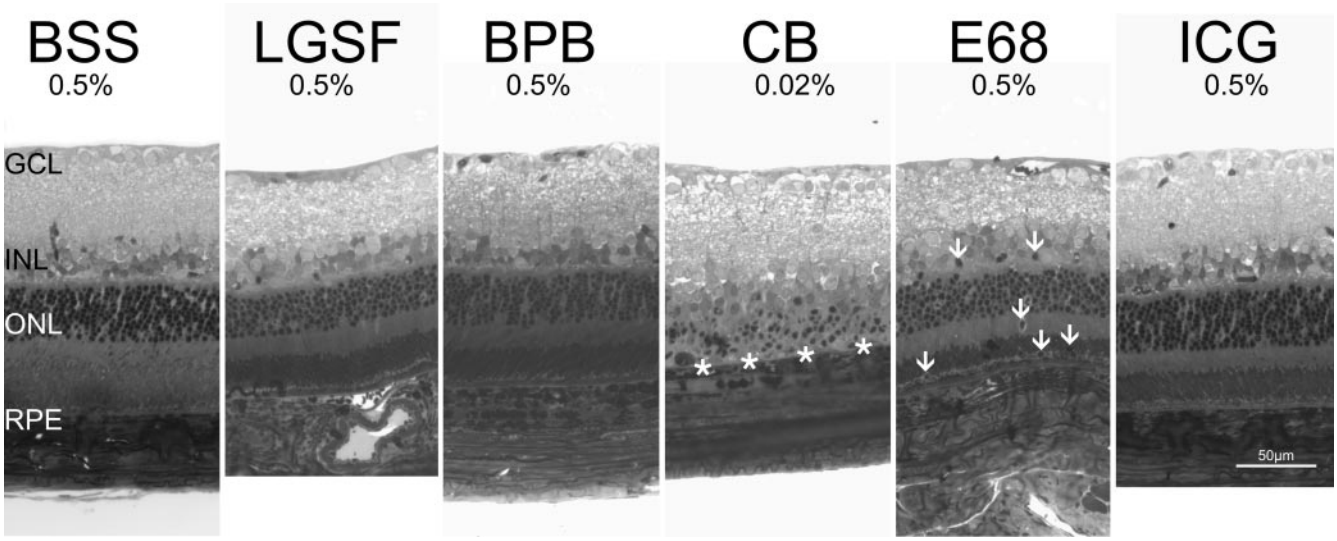

FIGURE 1. Representative micrographs of the midperipheral region of the retina. Eyes treated with PSS (control), LGSF and BPB showed normal retinal morphology even at the highest dosages applied. After treatment with CB there was a focal loss of photoreceptors (✶). Treatment with E68 resulted in numerous leukocytes (*arrows*). Although there were no substantial qualitative changes after treatment with ICG, there was measurable thinning (see Table 1).

RESULTS

Clinical Examination

Rats' eyes were injected intravitreally with either dye or PSS, without complications. No animal had to be excluded from further analysis due to difficulties connected with intravitreal drug administration. Animals injected with either E68 0.5% or ICG 0.5% showed a discrete staining of both cornea and lens in the respective color of the dye. This staining was also present to a clearly lesser extent in eyes injected with CB 0.5%. After injections with lower concentrations of these or other dyes, examination by slit lamp biomicroscopy showed no evidence of toxicity to the anterior segment of the eye such as corneal opacification or cataract induction. No visible inflammatory response in the form of vitreous opacification and/or retinal perfusion defects was seen with indirect ophthalmoscopy at any of the examination time points.

Histology

Qualitatively, the whole retina of eyes treated with BPB (0.5% and 0.02%), LGSF (0.5% and 0.02%), or the control PSS revealed normal morphology. The central retina also satisfied quantitative criteria for normal morphology (Table 1).

Treatment with CB resulted in a heterogeneous incidence of morphologic alterations. Of the three eyes treated with 0.5% CB, one eye showed no morphologic alterations, one eye showed a focal mild loss of photoreceptors and loss of cells in the ganglion cell layer, and one eye showed an increase in hyalocytes in the vitreous. Of the three eyes treated with 0.02% CB, two were without morphologic alterations, yet one eye showed focally complete outer retinal degeneration in the midperipheral region. Because this disease lay outside the region of quantification, the measurements of the central retina showed normal values (Table 1).

Treatment with E68 led to a consistently dose-dependent reaction. At a concentration of 0.5% E68, all eyes showed signs of inflammation with numerous leukocytes between the photoreceptor outer segments (mean number of leukocytes: 9.3 ± 1.8/mm). One eye also showed an accumulation of hyalocytes in the vitreous. The inflammation was pronounced in the middle and peripheral regions and less intense in the central region of the retina. Concentrations of 0.02% E68 still triggered leukocyte infiltration (mean number of leukocytes: 3.7 ±1.6/mm), but these were less numerous than in the group treated with 0.5% E68. At concentrations of 0.002% and 0.0002% E68, no morphologic alterations were noted anywhere in the retina.

All eyes treated with 0.5% ICG showed degenerative changes. Quantification revealed a significant thinning of the inner retinal layers compared with PSS control eyes. There were focal changes in the outer retina, located in the central and midperipheral regions. ICG 0.02% still resulted in focal changes in two of three eyes. However, quantification of the different layers showed no statistically significant decrease. No morphologic alterations of the retina were seen with lower concentrations of ICG (0.002% and 0.0002%).

Figure 1 shows retinal morphology after injections of the respective dyes or PSS.

TABLE 2. RGC Counts in Brown Norway Rats

Treatment	*n*	RGC	*P*
PSS	8	2490 ± 109	
LGSF 0.5%	3	2553 ± 37	0.7444
LGSF 0.02%	7	2574 ± 86	0.8941
CB 0.5%	8	2230 ± 91	0.0571
CB 0.02%	4	2558 ± 99	0.7026
BPB 0.5%	9	2385 ± 25	0.4605
BPB 0.02%	4	2658 ± 210	0.4481
E68 0.5%	7	1263 ± 195†	<0.0001***
E68 0.02%	8	2528 ± 93	0.7959
E68 0.002%	6	2468 ± 98	0.8881
ICG 0.5%	8	2197 ± 43	0.0254*
ICG 0.02%	8	2190 ± 56	0.0277*
ICG 0.002%	8	2141 ± 50	0.0116*
ICG 0.0002%	8	2172 ± 65	0.0407*

Data were collected 7 days after intravitreal injection of BSS or the various dyes. Retinal ganglion cells were identified by retrograde labeling and counted in a semiautomatic fashion (cells/mm^2, mean + SEM).

* $P < 0.05$, *** $P < 0.001$ vs. controls.

† Count questionable because of destruction of retina.

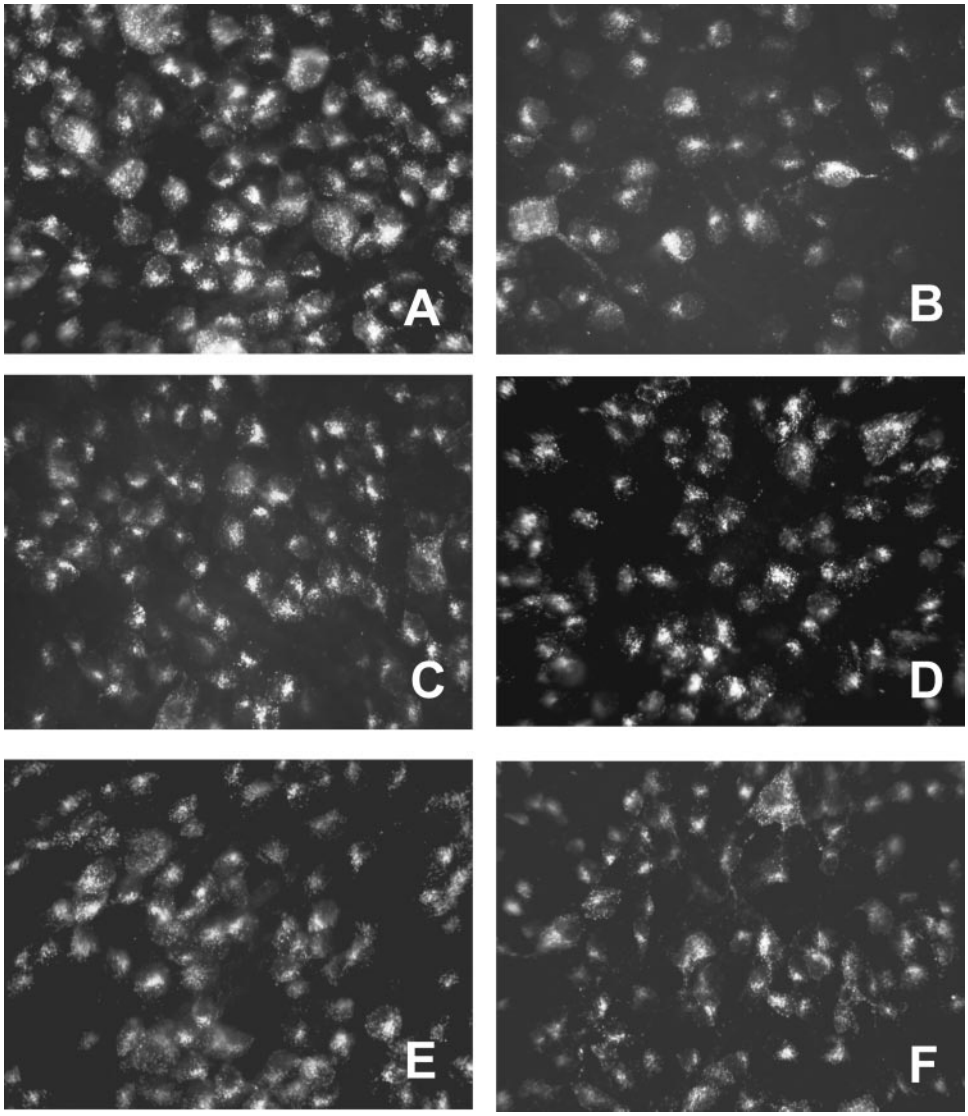

FIGURE 2. Wholemounts of rat retinas 7 days after intraocular injection. RGCs cells were back-labeled by injection of hydroxystilbamidine. (**A**) PSS, (**B**) E68 0.5%, (**C**) LGSF 0.5%, (**D**) ICG 0.5%, (**E**) BPB 0.5%, and (**F**) CB 0.5%. Note the diminished number of RGCs after 0.5% dilutions of E68, ICG, and CB (**B**, **D**, **F**), whereas the same concentration of LGSF and BPB (**C**, **E**) led to RGC counts comparable to those in PSS-treated retinas (**A**).

RGC Count

Seven days after intravitreal injections with E68 0.5% and ICG at all tested concentrations, a significant loss of RGC was observed compared with PSS-injected control eyes (Table 2). The most dramatic loss of ganglion cells was recorded after E68 0.5% injection, when the number of RGCs decreased to 1263 ± 195 cells/mm^2 (mean ± SEM; $n = 7$, $P < 0.0001$). A less pronounced, but still significant, loss of RGCs was seen after injections with ICG at 0.5% (2197 ± 43; $n = 8$; $P = 0.0254$), 0.02% (2190 ± 56; $n = 8$; $P = 0.0277$), 0.002% (2141 ± 50; $n = 8$; $P = 0.0116$), and 0.0002% (2172 ± 65; $n = 8$; $P = 0.0407$). Figure 2 shows RGC counts after injections with 0.5% solutions of the respective dyes or of PSS.

At the same time point, injections with lower concentrations of E68 or other dyes did not lead to statistically significant RGC loss (see Table 2). The PSS injection itself did not influence RGC survival compared with untreated eyes (data not shown).

DISCUSSION

The present investigation represents the first in vivo toxicity evaluation of a variety of potential new dyes for intraocular surgery. Before the in vivo application was performed as described herein, the biocompatibility of the dyes was thoroughly evaluated in an ex vivo experimental setting,[14] in different cell culture models. The staining characteristics were investigated in extracted human lens capsules and epiretinal membranes and were also tested on the lens capsule in situ in porcine eyes.[14] During short-term in vivo investigations in porcine eyes, three novel dyes (CB, BPB, and E68) revealed satisfactory staining characteristics[18] and therefore also appeared to be applicable in humans. In contrast, light-green SF (LGSF) yellowish stained neither the retinal surface nor the lens capsule. It should be noted that the in vivo experiments in porcine eyes were designed to describe the staining effect, not for reliably evaluating dye-related toxicity in vivo. This toxicity was the subject of the present investigation performed in rat eyes. The experimental setting and the method used to count RGCs are very well established approaches for checking potential toxic effects on the retina.[16,19–24] Besides the four novel dyes, ICG was also evaluated as a reference and PSS served as the control.

We were able to demonstrate that two of the four new dyes tested did not produce a histologically detectable toxic effect on the retina in our in vivo rat model, even after a very long exposure time. However, the other two dyes as well as the commonly used ICG produced histologic changes in the form

of mild focal loss of photoreceptors and loss of cells in the ganglion cell layer 7 days after intraocular injections.

Several aspects must be considered when interpreting our findings: First, the dye was injected into the vitreous cavity without the vitreous having been removed. In humans, the dye will most probably be injected into the air-filled vitreous cavity after vitrectomy. Therefore, in clinical use, their local retinal dose may be higher than that found in the present experimental setup. Thus, there may be toxicity in the clinical situation that is not observed in our animal model. Second, the dye remained within the eye for a prolonged period (7 days) without any further dilution, which could cause aggregation or coagulation at the highest dye concentration used.

This experimental protocol exceeds the usual timeframe relevant for intravitreal surgery and therefore does not mimic the intraoperative situation in humans, where the dye will be applied for approximately 1 minute and then washed out completely by irrigation with PSS. Nevertheless, the approach as performed here in the rat eye appeared to be a very useful and reasonable method for evaluating potential toxic effects, which should become apparent 7 days after injection. In other words, one might reasonably speculate that a dye that has not affected the intraocular structures after a period of 7 days is not likely to have a histological effect on the human retina and other structures after a period of 1 minute. However, one always has always to keep in mind that toxicity studies in animals may still not reflect all aspects of potential toxicity in a clinical situation. This applies especially to the situation in which the results of functional testing are not yet available.

It is interesting to note that all concentrations of ICG led to a significant decrease in the number of RGCs, even if the reduction was not as pronounced as after E68 at a concentration of 0.5%. Because some groups have reported potential adverse effects of ICG on the functional outcome of macular surgery, there has been intense discussion on whether the use of ICG causes toxic side effects. Several publications have discussed potential dye-related toxicity, whereas other investigators have observed no negative effects attributable to ICG. However, although a number of hypotheses have been advanced, the underlying mechanisms of action and safety margins of ICG are not yet fully understood. One interesting aspect may be the known photochemical properties of ICG,[25] which could contribute to ICG-related toxicity.[15,16] In light of this information, one of the most important criteria for the selection of dyes for the present investigation was high photochemical and dark stability. It should also be remembered that no ex vivo or in vivo animal studies were performed before the use of ICG in humans, and so this still represents an off-label use. In our view, a careful step-by-step approach seems mandatory when investigating new dyes for intraocular surgery.

In summary, we were able to demonstrate that two dyes tested in the present study did not lead to detectable toxic effects in the rat eye, even after prolonged presence within the eye and an observation period of 7 days. Other novel dyes as well as ICG showed toxic effects, such as histologic alteration of the retina and loss of RGCs. On the basis of the results obtained in the present study and our previous investigations using both ex vivo and in vivo experimental approaches, we conclude that some of the novel dyes, especially BPB, provide excellent staining characteristics and biocompatibility. BPB will now be the subject of further carefully monitored in vivo investigations in humans (e.g., in patients with traction maculopathies including macular holes and macular pucker).

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