In Vivo Toxicity Study of Rhodamine 6G in the Rat Retina

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Purpose. To investigate the intraocular effect of rhodamine 6G (R6G) on retinal structures and function in an in vivo rat model and to develop an in vivo method for accurate evaluation of new dyes for intraocular surgery.

Methods. R6G in physiologic saline solution (PSS) was injected into the vitreous of adult Brown Norway rats at concentrations of 0.0002%, 0.002%, 0.02%, 0.2%, and 0.5%. Control animals received only PSS. Retinal toxicity was assessed by retinal ganglion cell (RGC) counts, light microscopy 7 days later, photopic electroretinography (ERG), and measurement of scotopic sensitivity and recovery of dark adaptation 48 hours and 7 days after intravitreous injection.

Results. R6G at concentrations of 0.2% and 0.5% led to a dose-dependent loss of RGC. The most significant loss occurred at 0.5%. Lower concentrations (0.0002%, 0.002%, and 0.02%) produced no statistically significant retinal ganglion cell loss. Analysis of the eyes by light microscopy showed no structural changes in the central retina, although injections of 0.5% R6G were followed by impressive degenerative changes adjacent to the injection sites. ERGs showed no effects of the highest R6G concentration on rods, kinetics of rhodopsin recovery after bleaching, or cone-driven responses.

Conclusions. R6G can be safely injected in doses of up to 0.02% in rats, but has a toxic effect on retinal ganglion cells at higher concentrations. Accumulation of R6G may be a problem at higher concentrations, particularly at the injection site. (*Invest Ophthalmol Vis Sci.* 2008;49:2120–2126) DOI:10.1167/iovs.07-1476

Vital dyes are commonly used to assist in ophthalmic surgery in the anterior and posterior segments. Especially in capsulorrhexis or dye-assisted vitrectomy in macular surgery, dyes are valuable tools for better intraoperative visualization of the lens capsule and the vitreoretinal interface.

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Two dyes in particular are presently useful for intraocular surgery: trypan blue and indocyanine green (ICG).[1–3] However, concerns still exist about the long-term safety and possible adverse effects of both. Toxic effects of trypan blue have been found in animal models, although no adverse effects have been described in humans.[4–6] Adverse effects of ICG have been found by some groups in in vitro, in vivo and clinical studies,[7–10] but not by others.[11–13] In particular, safety margins with ICG and the pathomechanisms of potential ICG toxicity are not completely understood.

The ongoing controversy about the toxicity of dyes presently used in intraocular surgery indicates a need for alternative dyes with equal or better staining properties. This in turn indicates a need for thoroughly designed in vitro and in vivo toxicity studies before such dyes can be used clinically. It is noteworthy that no such studies were performed before ICG was introduced into vitreoretinal surgery in humans.

Recently, several research groups have attempted to explore these issues by investigating the staining properties and toxicity of different dyes, particularly those with high photochemical stability. For example, Haritoglou et al.[14] have developed an in vitro method of testing, evaluating, and comparing new dyes for intraocular surgery and have also tested some of the most promising dyes morphologically, but not functionally, in an animal model.[15]

One dye that has been found to be very effective in staining both the lens capsule and internal limiting membrane is rhodamine 6G (R6G).[14] However, this dye also showed adverse effects in the in vitro studies of Haritoglou et al.[14] Therefore, it was of interest for us to determine whether these effects would also appear in vivo. In addition, our goal was to establish a reliable in vivo method of evaluating dyes, both morphologically and functionally, as a prelude to future trials in larger animals and in humans.

Materials and Methods

Animals

Adult male Brown Norway rats (Charles River, Sulzfeld, Germany) with a body weight of 125 to 150 g were used. The rats were housed in a 12-hour light–dark cycle. All experiments were performed in compliance with guidelines for animal care in the European Community and those of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Rhodamine Preparation

R6G (Sigma-Aldrich, St. Louis, MO) was dissolved in physiologic saline solution (PSS) (BSS, Alcon Laboratory, Fort Worth, TX) to a concentration of 0.5% and diluted to 0.2%, 0.02%, 0.002%, and 0.0002%. The osmolarity of PSS was 307 mOsM.[16] No difference in osmolarity was measured for PSS-diluted R6G.

Intravitreous Injections

The rats were anesthetized with an intraperitoneal injection of chloral hydrate (7%, 6 mL/kg body weight). Intravitreous injections were

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performed as described previously.[17] Two microliters of the different concentrations of R6G (0.0002%–0.5%) were injected into the posterior side of the globe. The final intraocular concentrations of R6G were 0.00002%, 0.0002%, 0.002%, 0.02%, and 0.05% (given an estimated intravitreous volume of 20 μL per rat eye). The contralateral eyes served as control eyes and were injected with 2 μL of PSS.

Retrograde Labeling and Quantification of Retinal Ganglion Cells

Retinal ganglion cell (RGC) survival was assessed as described previously.[18] Five days after intraocular injection, the animals were anesthetized deeply, and a total of 7 μL fluorescent dye (hydroxystilbamidine methanesulfonate; FluoroGold; Invitrogen-Molecular Probes, Eugene, OR) was applied to each superior colliculus. Retinal flatmounts were prepared 2 days later, as described previously.[15] RGC counts were performed in 12 distinct areas of 62,500 μm^2. Images were coded and analyzed in a masked fashion. The labeled cells were defined as surviving. Counts are expressed in terms of cell density (cells per square millimeter).

Statistical analysis was performed with a Kruskal-Wallis nonparametric ANOVA followed by the Dunn post hoc test. Differences were considered significant at $P < 0.05$.

Histology

After the rats were killed, their eyes were immediately immersion fixed in Ito's solution. The eyes were bisected into nasal and temporal segments, and the specimens were postfixed in 1% osmium tetroxide, rinsed in cacodylate buffer, dehydrated in an ascending series of alcohols, and embedded in araldite. Semithin sections were cut along a superior–inferior plane, stained with toluidine blue, and investigated by light microscopy. In addition, sections were obtained through the injection site of the R6G.

The total thickness of the neural retina and the thicknesses of the different retinal layers were quantitatively evaluated in the central retina. The measuring field was defined by a distance of 200 μm from the optic nerve head rim, and single-thickness measurements were obtained within the next 300 μm peripherally. Because there was no significant difference between the superior and inferior regions, the data were pooled for statistical analysis with the Wilcoxon test (α = 0.01).

Electroretinography

Electrophysiological measurements were performed after intravitreous injection of the highest concentration of R6G (0.5%). Measurements were performed 48 hours and 7 days after intravitreous injections in five rats. In addition, 13 rats were measured in the same protocol and served as the control group.

Experimental Procedure. After 12 hours of dark adaptation (overnight), the rats were anesthetized with a combination of ketamine (100 mg/kg) and xylazine (5 mg/kg) injected intraperitoneally. An SC injection of the same combination (one third of the initial doses) was performed 1 hour after the initial injection, to guarantee full anesthesia

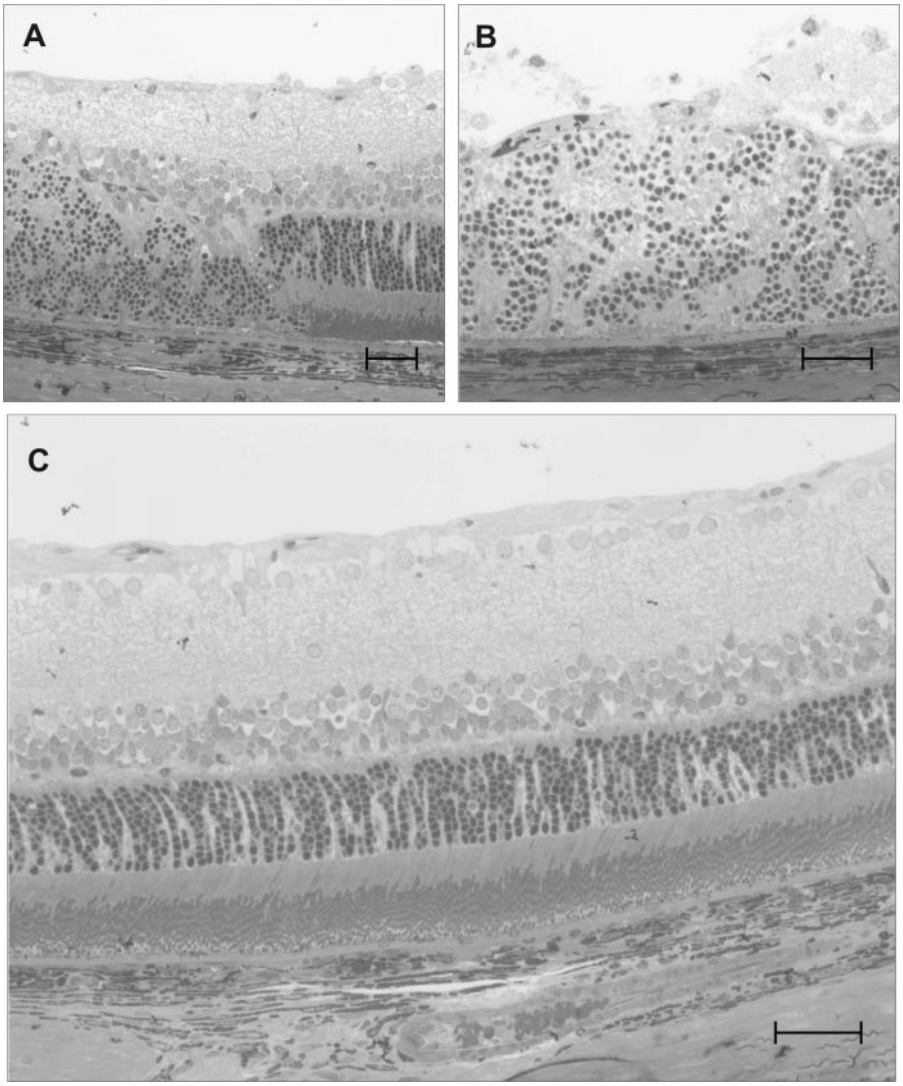

FIGURE 1. Focal retinal changes after intravitreous injection of 0.5% R6G (**A**, **B**) and normal appearance in the central region (**C**). Scale bar, 25 μm.

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TABLE 1. Quantification of the Central Retina

	Total	NFL	GCL	IPL	INL	OPL	ONL	IS	OS
PSS	192 ± 8	6.7 ± 1	13 ± 2	50 ± 12	28 ± 2	6.8 ± 1.0	39 ± 2	13 ± 2	24 ± 2
0.0002%	189 ± 11	10 ± 2	16 ± 3	48 ± 7	30 ± 3	6.5 ± 0.7	40 ± 3	15 ± 2	23 ± 1
0.002%	183 ± 10	10 ± 3	13 ± 3	49 ± 5	27 ± 4	6.3 ± 0.7	42 ± 3	15 ± 1	23 ± 3
0.02%	177 ± 9	8.3 ± 3	13 ± 3	49 ± 4	26 ± 3	5.4 ± 0.3	37 ± 1	13 ± 1	22 ± 1
0.2%	180 ± 11	9.7 ± 2	15 ± 2	47 ± 5	25 ± 5	6.2 ± 1.0	38 ± 3	14 ± 1	23 ± 3
0.5%	188 ± 6	6.8 ± 1	14 ± 3	53 ± 6	29 ± 2	6.4 ± 1.1	38 ± 3	15 ± 1	23 ± 1

Data are expressed as mean micrometers ± SD. There was no significant difference between any of the layers and the PSS-treated control. 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, photo receptor inner segment; PR-OS, photo receptor outer segment.

through the entire experiment (roughly 2 hours). Pupils were fully dilated with 1 drop of tropicamide 10 minutes before ERG started. The animals were kept on a warming plate to maintain core body temperature at 36°C. Custom-made contact lenses suited with a gold wire on the interior surface were used as active electrodes; one SC needle between the eyes was used as the reference and one in the tail as the ground. Anesthesia, electrode placement, and animal positioning were performed under dim red light. ERG measurement started 10 minutes after the red light had been turned off.

ERG Protocol. ERGs were recorded and stored for offline analysis (Espion; Diagnosys LLC, Littleton, MA). The recorded signals were filtered online with a band-pass filter from 0.03 to 500 Hz. Electrode impedance was less than 10 kΩ at 25 Hz (manufacturer's recommendation). Eyes were stimulated with a Ganzfeld stimulator (ColorDome; Diagnosys LLC).

The ERG protocol consisted of the following steps: (1) evaluation of dark-adapted scotopic sensitivity, (2) recovery of b- and a-wave amplitudes after exposure to a bleaching light, and (3) cone response under photopic conditions.

Dark-Adapted Scotopic Sensitivity and Oscillatory Potentials. Stimuli of increasing luminance (4 ms duration) in nine steps (0.0000003–0.3 cd-s/m^2) were used to elicit responses that ranged from a baseline (b-wave amplitude undetectable) to a saturated b-wave response. For the first four steps (0.0000003–0.00001 cd-s/m^2), there were 15 responses on average, with an interstimulus interval (ISI) of 10 seconds. For the next four steps (0.00003–0.001 cd-s/m^2) there was an average of five responses with an ISI of 25 seconds, and for the last three steps (0.003 to 0.3 cd-s/m^2), there was an average of two responses with an ISI of 40 seconds. A single flash was then used to elicit the mixed (rod+cone) maximum ERG response with a stimulus luminance of 10 cd-s/m^2 and a duration of 4 ms.

A hyperbolic saturation model[19] was created for the interrelationship between b-wave amplitude and luminance (I) to derive three parameters: V_{max}, saturated b-wave amplitude; k, luminance necessary for b-wave to reach one half of V_{max} (the semisaturation point); and n, a one-dimensional parameter related to the linear phase slope

$$V(I) = \frac{V_{max} \times I^n}{I^n + k^n}.$$

TABLE 2. Quantification of Labeled RGCs 7 Days after Injection with Different Doses of R6G

	Total	Central Retina	Quadrants Not Including Injection Site
PSS	2269 ± 34	2707 ± 38	2177 ± 37
0.0002%	2199 ± 39	2479 ± 68	2135 ± 42
0.002%	2214 ± 30	2670 ± 66	2186 ± 49
0.02%	2190 ± 44	2648 ± 69	2233 ± 71
0.2%	1993 ± 45	2470 ± 65	2064 ± 65
0.5%	1296 ± 163	1710 ± 190	1932 ± 169

Data represent the mean cell counts per square micrometer ± SE.

OPs were obtained from the maximum dark-adapted response by means of fast Fourier transform (FFT), implemented as a band-pass frequency filter (75–300 Hz). The absolute value of the area under the curve for all OP wavelets was determined between the a- and b-wave implicit times.

Recovery of Rod b-Wave after Bleaching. A single flash (0.003 cd-s/m^2, 4-ms white 6500 K) was used to elicit the semisaturated rod b-wave before and during recovery after bleaching. Three results were measured in dark-adapted condition 1 minute before bleaching. Subsequently, bleaching was accomplished by exposure to a 400-cd/m^2 white background for 30 seconds (estimated to bleach 25% of rod rhodopsin content) in the Ganzfeld chamber. Recovery was monitored by recording responses every 2.5 minutes for 70 minutes after bleaching. b-Wave amplitude was normalized to the values of corresponding amplitudes recorded in the prebleaching phase. A quotient between normalized b-wave amplitude after treatment and at baseline was calculated intraindividually and plotted against time after bleaching.

Cone Response: Photopic ERG. Eyes were light-adapted for 10 minutes (25 cd/m^2, white 6500 K) for rod saturation. Light-adapted recordings were performed with a single flash and 20-Hz flicker with a stimulus luminance of 10 cd-s/m^2 (white 6500 K).

Statistical Analysis

An intraindividual quotient was calculated between results found in eyes injected with PSS and the eyes injected with R6G. This quotient was compared with quotients found between the right and left eyes of nontreated rats (control group) using one-way analysis of variance (ANOVA).

RESULTS

Histology

After injection of R6G into the rat vitreous, a dose-dependent reaction of the retina appeared at the injection site. An R6G concentration of 0.5% led to dramatic regional degeneration in all retinal layers: the outer segments of the photoreceptors were completely absent, and the outer and inner nuclear layers were displaced and partially mixed (Fig. 1A). In some places, the inner retinal layers were completely destroyed and detached into the vitreous (Fig. 1B). The affected areas were circular in shape, with a diameter of 3 to 4 mm. In contrast, the central retina around the optic nerve was not affected (Fig. 1C) and satisfied quantitative criteria for normal morphology of all retinal layers (Table 1).

Retinas treated with concentrations of 0.2% R6G also showed some of these reactive alterations, but not as prominently as in the 0.5% group. Areas with massive local degeneration were only approximately 500 μm in size. At lower concentrations of R6G (0.02%, 0.002%, and 0.0002%), the injection site showed only mild punctual retinal damage due to

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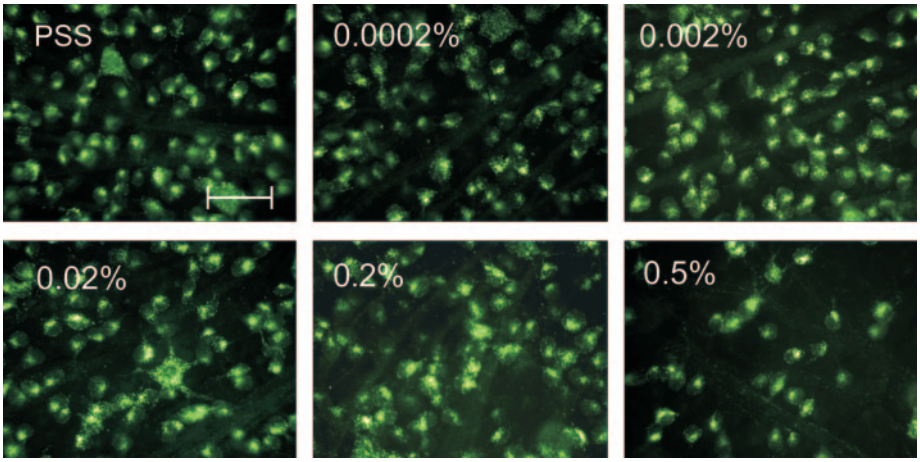

FIGURE 2. Wholemounts of rat retinas 7 days after intraocular injection with all tested concentrations of R6G. RGCs were back labeled by injection of hydroxystilbamidine. Note the diminished number of RGCs after 0.5% R6G. Scale bar, 50 μm.

the injection needle. Quantification of the retinal layers revealed normal values (Table 1).

RGC Counts

RGCs were counted 7 days after intravitreous injection of R6G in relation to the different concentrations and the control (Table 2; Figs. 2, 3). At concentrations of up to 0.02%, there was no significant difference in RGC counts compared with PSS-injected eyes. In contrast, R6G concentrations of 0.2% and 0.5% led to a significant reduction in RGC counts in 12 distinct areas in all four quadrants of the retina. This significant RGC loss was also detectable when only central areas of the retina were analyzed. To avoid a misleading reduction in RGC counts due to the massive morphologic changes at the respective injection sites, an additional analysis of only two anterior quadrants per retina lacking an injection site was performed. It, too, showed a significant loss of RGCs after injections with 0.5% R6G, but not with 0.2%. At concentrations up to 0.2%, there was no significant difference in RGC counts compared with PSS for the anterior quadrants.

Electroretinography

Dark-Adapted Scotopic Sensitivity. Examples of ERG responses and the relationships between dark-adapted b-wave amplitude and luminance are shown in Figure 4.

The mean ± SD V_{max} in the control group was 882.93 ± 198.13 μV in right eyes and 893.44 ± 190.81 μV in left eyes. In rats from the test group, it was 737.51 ± 76.49 μV in eyes injected with PSS and 794.66 ± 147.22 μV in eyes injected with R6G.

Similar results were found for parameter k of the Naka-Rushton function: k in right eyes of control group was -3.22 ± 0.25 $\log_{10}$ cd-s/m^2 and in left eyes was -3.23 ± 0.27 $\log_{10}$ cd-s/m^2. In the test group, k was -3.04 ± 0.12 $\log_{10}$ cd-s/m^2 in eyes injected with PSS and -3.09 ± 0.03 $\log_{10}$ cd-s/m^2 in eyes injected with R6G.

No statistically significant differences were found intraindividually for V_{max} or k (V_{max}: $P = 0.3520$; k: ANOVA $P = 0.6981$; Fig. 5).

b-Wave Recovery after Bleaching. The mean ± SD of the normalized b-wave amplitude (postbleaching/prebleaching; see the Methods section) 60 minutes after bleaching was 0.92 ± 0.12 in the right eyes and 0.92 ± 0.08 in the left eyes in the control group; in the test group, it was 0.99 ± 0.20 in the eyes injected with PSS and 1.04 ± 0.10 in the eyes injected with R6G.

Figure 6 shows examples of scotopic b-wave recovery after bleaching in one eye injected with PSS and the other eye of the same animal injected with R6G.

There was no statistically significant difference between the control group (right versus left eyes) and the test group (R6G versus PSS eyes) with the normalized b-wave amplitude at 60 minutes after bleaching (ANOVA $P = 0.3966$; Fig. 7).

Photopic ERG. No differences were found between the test (PSS versus R6G) and control groups in cone single-flash or 20-Hz flicker a- or b-wave amplitudes or implicit times (data not shown).

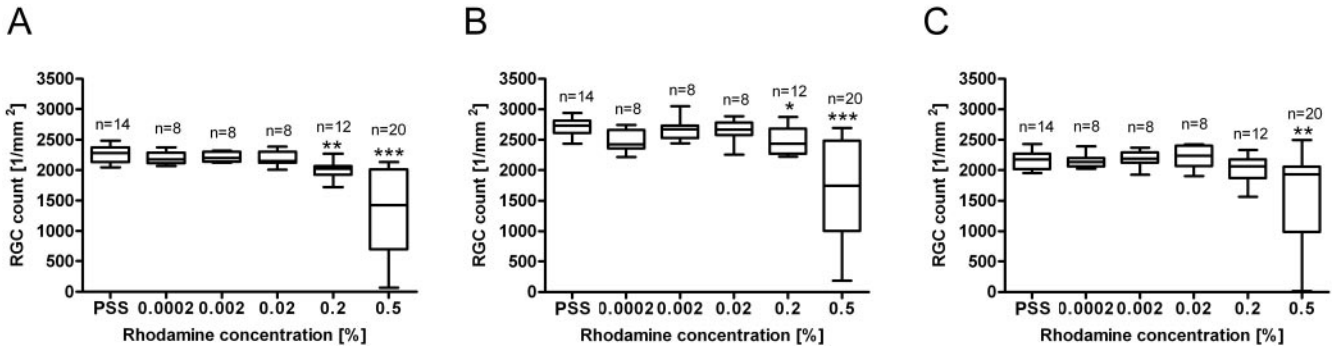

FIGURE 3. Density of labeled RGC 7 days after injection with different doses of R6G. (**A**) Counts obtained in 12 distinct areas of all four quadrants per retina. (**B**) Counts obtained in four distinct areas within the central part of the retina. (**C**) Counts obtained in six distinct areas of two quadrants not including the injection site. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. The *line* in the box represents the median observation, the *top* and *bottom* boundaries of the box represent the 25th and 75th percentiles, and the *whiskers* extend to the outermost data points.

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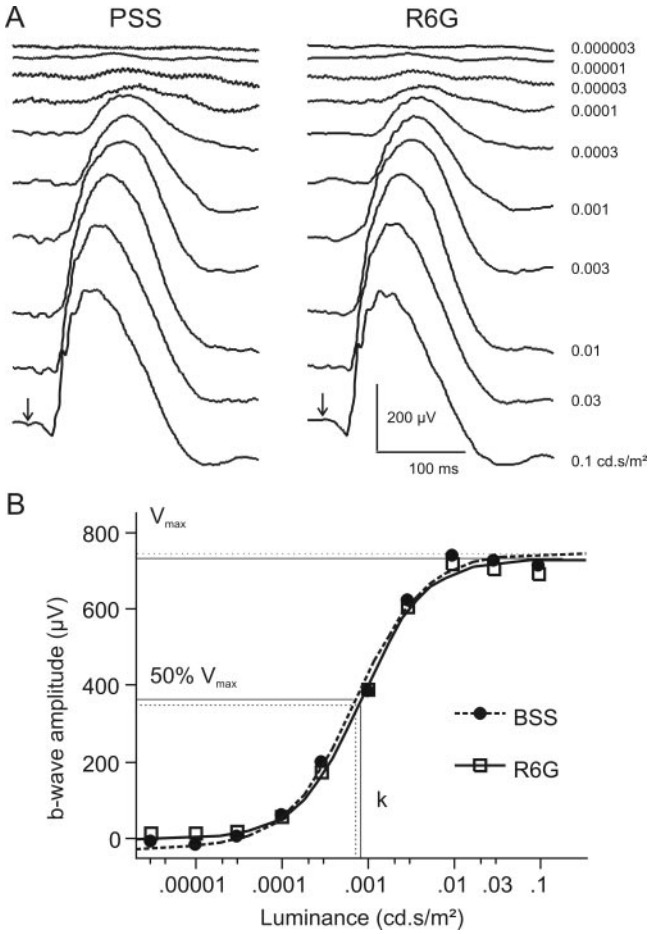

FIGURE 4. (**A**) Examples of ERG responses to nine flashes of increasing luminance (0.000003–0.1 cd-s/m^2) in dark-adapted condition of one eye injected with PSS (control) and one eye injected with R6G (0.5% dose). (**B**) Example of the interrelations between dark-adapted b-wave amplitude and luminance of these two eyes. (●) Amplitudes measured after PSS injections; (□) amplitudes measured after injections of R6G. Lines show the best fit with the Naka-Rushton function. *Dotted lines*: parameters V_{max} and k.

DISCUSSION

R6G has shown very good staining characteristics in the lens capsule, and good staining of the internal limiting membrane in previous experiments on enucleated porcine eyes.[14] In addition, R6G has good solubility in water and physiologic saline,[20]

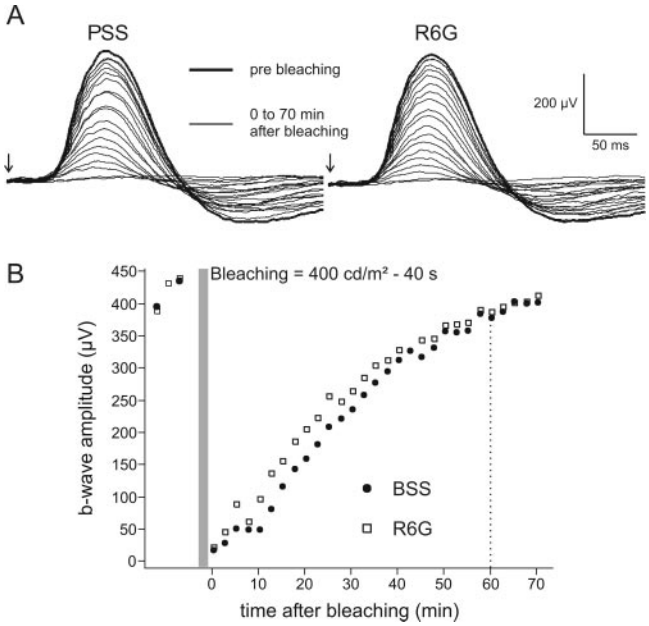

FIGURE 6. (**A**) Examples of ERG responses under dark-adaptation (prebleaching—*bold traces*) and during recovery after light exposure (400 cd/m^2 for 30 seconds just after bleaching to 70 minutes after bleaching—*thin traces*) of one eye injected with PSS (control) and one eye injected with 0.5% R6G. (**B**) Example of the interrelationships between b-wave amplitude and time in the dark after bleaching. *Dashed line*: the time point used for comparison between groups.

exhibits a high absorption coefficient, and has better photostability than ICG, thus theoretically making it a good candidate for vital dye staining. It is currently used, for example, to coat nanoparticles in intraocular delivery[21] and as an intraocular leukocyte marker.[22] It is also under consideration for use in intraocular surgery.

The intraocular toxicity of R6G has been studied in vitro in ARPE-19 cells by colorimetric testing, where it showed significant toxicity at doses of 0.2% and higher.[14] To our knowledge, however, no toxicity studies in vivo have yet been performed. The present study was therefore the first to investigate intraocular toxicity of the dye R6G in vivo.

The electrophysiological results of our study are of paramount interest for future testing of vital dyes, particularly in view of the possibility of vital dyes gaining access into the subretinal space during membrane peeling in intraocular surgery.[23] Some studies have suggested that dye may be transferred to the RPE along Müller cells, thus entering into Müller cell endfeet, despite an intact inner limiting membrane.[24]

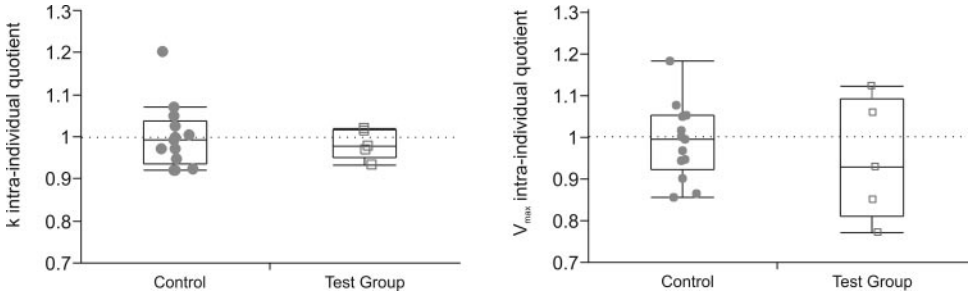

FIGURE 5. Distributions of intraindividual quotient (results found in left eyes divided by results found in right eyes) of k and V_{max} in the control group (n = 13; no treatment) and the test group (n = 6; right eyes injected with R6G and left eyes injected with PSS). A ratio of 1 indicates equal results in animals' left and right eyes. The box plots are as described in Figure 3: the *whiskers* extend to upper quartile +1.5*(interquartile range) and lower quartile −1.5*(interquartile range).

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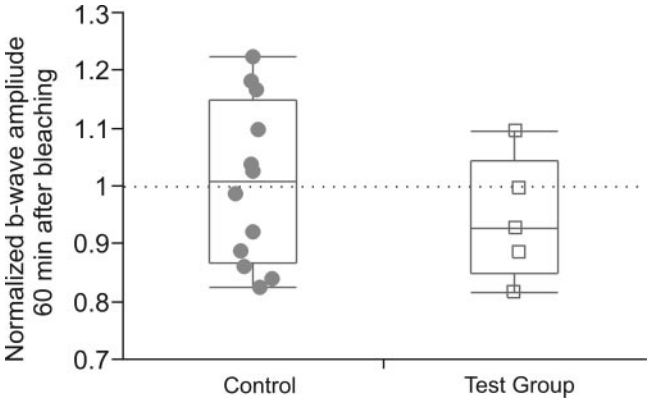

FIGURE 7. Distributions of intraindividual quotient of normalized b-wave amplitude at 60 min after bleaching in the control and test groups. Box and whisker plots are as described in Figure 5.

As in in vitro studies, intravitreous R6G at concentrations of 0.2% and above led to significant ganglion cell loss in the rat retina. However, the thickness of all retinal layers in the central retina was the same as in the control eyes at all tested concentrations of R6G after 7 days. Of interest, 0.5% of R6G resulted in intense local destruction at the respective injection sites in the retinal wholemount preparations, with a nearly total loss of labeled ganglion cells in this area.

This cell loss was also observed in retinal slices from the periphery and midperiphery, but the effect was not seen at lower concentrations. One may speculate that R6G in higher concentrations accumulates, perhaps due to incomplete distribution into the vitreous. Also, dye particles adhering to the outside surface of the injection cannula may be wiped off when it is removed after injection, thus constituting an additional factor (a problem that can also occur in vitreoretinal surgery).

It is already known that R6G can penetrate the sclera and thus directly approach the outer retina, so that functional testing of the outer retina is desirable. In contrast to ICG, which leads to significant but not progressive RGC loss at all tested concentrations from 0.0002% to 0.5%, R6G reduced the number of ganglion cells only at concentrations of 0.2% and 0.5%, and the reduction was progressive from 0.2% to 0.5%. Of importance, the results show not only a boost in R6G's toxic effect around the injection site, but also a significant loss of ganglion cells at a 0.5% concentration of R6G only in anterior retinal segments without injection sites and solely in the central retina. R6G at higher concentrations therefore clearly leads to a reduction of RGCs in the whole retina, not only at the injection sites.

The advantage of this study's approach over that of previous in vivo approaches is its combination of functional and histologic methods. ERG measurements showed no significant change at the highest tested concentration of R6G after 7 days. The leading edge of the dark-adapted b-wave is a measure of the extracellular field potential that primarily arises from depolarizing rod bipolar cells in response to dim flashes of light. The inclusion of progressive luminance steps for analysis of b-wave amplitude light dependency makes it possible to establish a model of amplitude versus luminance interrelationships with the classic Naka-Rushton hyperbolic function. This model yields two reliable markers for rod (dark-adapted) sensitivity: the parameters V_{max}, representing the saturated b-wave amplitude, and k, representing the luminance necessary for rods to achieve half of this amplitude. Thus, they provide information about rod sensitivity. Our data showed unchanged V_{max} and k 48 hours and 7 days after R6G injections at the highest tested concentrations, which in turn provides strong evidence of the nontoxicity of this compound along the rod pathway.

In addition, to investigate the integrity of the interaction between RPE and rod outer segments, we applied a protocol in which we observed the recovery of the scotopic b-wave amplitude in the dark after exposure to light. This exposure bleaches the rod photopigment rhodopsin. The recovery of rhodopsin content in rod outer segments depends on the functionality of the visual cycle (see Ref. 25 for a review). Our data showed no change in the kinetics of rhodopsin recovery after bleaching in eyes treated with R6G, suggesting that this dye did not interfere with the visual cycle and RPE functionality.

This finding agrees with histologic results in the central retina. The results of the present study also showed that locally confined adverse effects (e.g., at the injection site), may be missed by electrophysiological examination methods, at least with short time intervals. This in turn demonstrates the importance of systematic, complementary in vivo testing for maximum safety when transferring in vivo results in animal models to clinical applications in humans.

It must be kept in mind that animal in vitro experiments may fail to reflect important aspects of potential toxicity in clinical situations. Even with our improved in vivo setting, several systematic limitations must still be considered when interpreting the results. For one thing, the dye is introduced into the vitreous body of the rat eye without the removal of the vitreous. The situation is not the same in intraocular membrane peeling, in which dye is normally introduced into the air-filled vitreous cavity after vitrectomy. In clinical use, the local dose at the central retina may therefore be higher than in our animal model. Theoretically, effects similar to those at the injection sites in this study at higher concentrations may occur. In addition, the dye in this study remained in the vitreous cavity for 7 days, which by far exceeds the relevant time frame for intraocular surgery, in which dye is washed out by irrigation after approximately 1 minute. On the other hand, one may argue that a long-term toxicity assessment such as that described herein helps to predict the likelihood of toxic effects. A dye showing no adverse effects after 7 days is most likely not toxic after shorter exposure times in animals or after a period of 1 minute in clinical applications in humans.

In view of our results, we do not regard R6G as a candidate of first choice for intravitreous surgery. Nevertheless, it is likely that R6G can be safely used in concentrations up to 0.02%. Before clinical application, however, additional short-term exposure experiments for higher doses than 0.02% could provide additional safety. Since toxic effects of selected compounds have been described in pigmented but not in nonpigmented animals and vice versa,[26] it may also make sense, as a precaution, to test nonpigmented animals for toxic effects of R6G.

In summary, the search for new substances with improved chemical, physical, and spectral properties remains an important goal. To reach this goal, it is important to have reliable in vitro and in vivo testing procedures that help to assess potential toxic effects before their use in humans.

The experimental setting used in this study seems suitable for systematic in vivo testing of potential new dyes for intraocular surgery. It also presents an important step forward due to its functional testing approach. The study results are in good agreement with those of previous in vitro experiments with R6G. The in vivo approach presented herein therefore seems appropriate for evaluating the risks and benefits of new dyes for ocular surgery before their clinical application.

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