Vital Dyes and Light Sources for Chromovitrectomy: Comparative Assessment of Osmolarity, pH, and Spectrophotometry

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Purpose. To investigate the in vitro pH, osmolarity, spectral, and photostability properties of nine vital dyes for vitreoretinal surgery.

Methods. Nine dyes—indocyanine green (ICG), trypan blue (TB), brilliant blue (BriB), bromophenol blue (BroB), Congo red (CR), light green (LG), fast green (FG), indigo carmine (IC) and Evans blue (EB)— diluted in three solvents (saline solution, glucose 5%, and water) were tested for osmolarity and pH. Spectrophotometry was used to determine absorbance properties of 27 solutions. Irradiance emission spectra of seven endoillumination light sources and fiber-optics were compared with dye absorbance curves.

Results. Dye osmolarity in saline solution and glucose 5% varied widely (257–385 mOsm) and was lower (0–54 mOsm) when dyes were dissolved in water. Dyes diluted in three solvents showed pH values varying from 2.6 to 9.85. ICG, LG, TB, BroB, CR, and IC demonstrated different absorbances, depending on the solvent. BriB and FG showed similar absorbance curves with different solvents. Spectrophometric analysis showed that all dyes except ICG had remarkable spectral overlap with the light sources. Among endoillumination fiber-optics, overlap was greatest with dual-output illumination with an integrated laser pathway and least with a mercury vapor lamp.

Conclusions. Vital dyes showed variable osmolarity and pH, which also depended on the solvent used. Interaction of light from endoillumination source and vital dye may increase or decrease the risk for toxicity, making appropriate selection of both a desirable way to minimize the risk for phototoxic effects. (*Invest Ophthalmol Vis Sci.* 2009;50:385–391) DOI: 10.1167/iovs.08-2285

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Presented at the annual meeting of the Association for Research in Vision and Ophthalmology, Fort Lauderdale, Florida, April 2008.

Supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo and the Pan-American Association of Ophthalmology/Pan-American Ophthalmological Foundation.

Submitted for publication May 12, 2008; revised July 27, 2008; accepted October 6, 2008.

Disclosure: **E.F. Costa**, None; **E.B. Rodrigues**, None; **M.E. Farah**, None; **E. Dib**, None; **F. Penha**, None; **O. Magalhães, Jr**, None; **B.A. Furlani**, None; **A.A.S. Lima Filho**, None; **A. de Miranda**, None; **M. Maia**, None

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Chromovitrectomy enables the staining of intraocular vitreoretinal membranes and tissues, thereby allowing their surgical removal.[1] Current commercially available vital dyes for chromovitrectomy include indocyanine green (ICG) and trypan blue (TB; Ophthalmos, São Paulo, Brazil). Nonetheless, animal experiments and clinical data have demonstrated evidence of retinal toxicity associated with ICG and TB application. Recently, some research groups released their preliminary data on novel dyes for chromovitrectomy such as brilliant blue (BriB; Merck, Darmstadt, Germany) and bromophenol blue (BroB; Sigma-Aldrich, Munich, Germany). Various theories, in addition to the chemical influence of the dye itself, explain retinal injury induced by vital dyes during chromovitrectomy. One possible mechanism to consider is phototoxicity because light emission by the intraoperative light pipe may be absorbed by the dye-stained retina. Haritoglou et al.[2] demonstrated that the ICG absorbance spectrum overlaps partly the emission spectrum of one type of halogen light source and that ICG absorbance varies with solvent and osmolarity. Consecutive experiments with two different light sources showed histologic and functional damage to the retina after ICG exposure. In addition to potential phototoxicity, different osmolarities of dye solutions have been shown to exert direct retinal damage in animal studies. However, more comprehensive studies are lacking, including complete biochemical and spectrophotometric analyses of each vital dye prepared in different solvents and the determination of spectral overlap with several light sources used in chromovitrectomy.

The aim of this study was to investigate several in vitro aspects of vital dyes with regard to pH, osmolarity, spectrophotometric properties, and photostability. A detailed evaluation was performed of nine vital dyes used for chromovitrectomy: ICG, TB, BriB, BroB, Congo red (CR; Merck), light green (LG; Merck), fast green (FG; Merck), indigo carmine (IC; Merck), and Evans blue (EB; Merck). Before and after photostability measurements, pH and osmolarity were determined for dyes dissolved in three solvents— physiologic saline (PSS), glucose 5%, and water—and spectrophotometry was used to examine the absorbance of the nine dyes in these three solutions. Absorbance spectra were compared with the irradiance emission spectrum of seven endoillumination fiber-optics: high-brightness illuminator (Xenon 20G; Alcon Fort Worth, TX), dual-output halogen illumination (Accurus H3; Alcon), halogen (Grieshaber GLS; GLS Corp., McHenry, IL), dual-output metal halide illumination (Millenium; Bausch and Lomb, Rochester, NY), dual 150-W halogen source (metal halide; DORC, Zuidland, Netherlands), dual-output illumination with an integrated laser pathway (Photon Xenon; Synergetics Photon, Fort Collins, CO), and mercury vapor lamp (Photon 2; Synergetics).

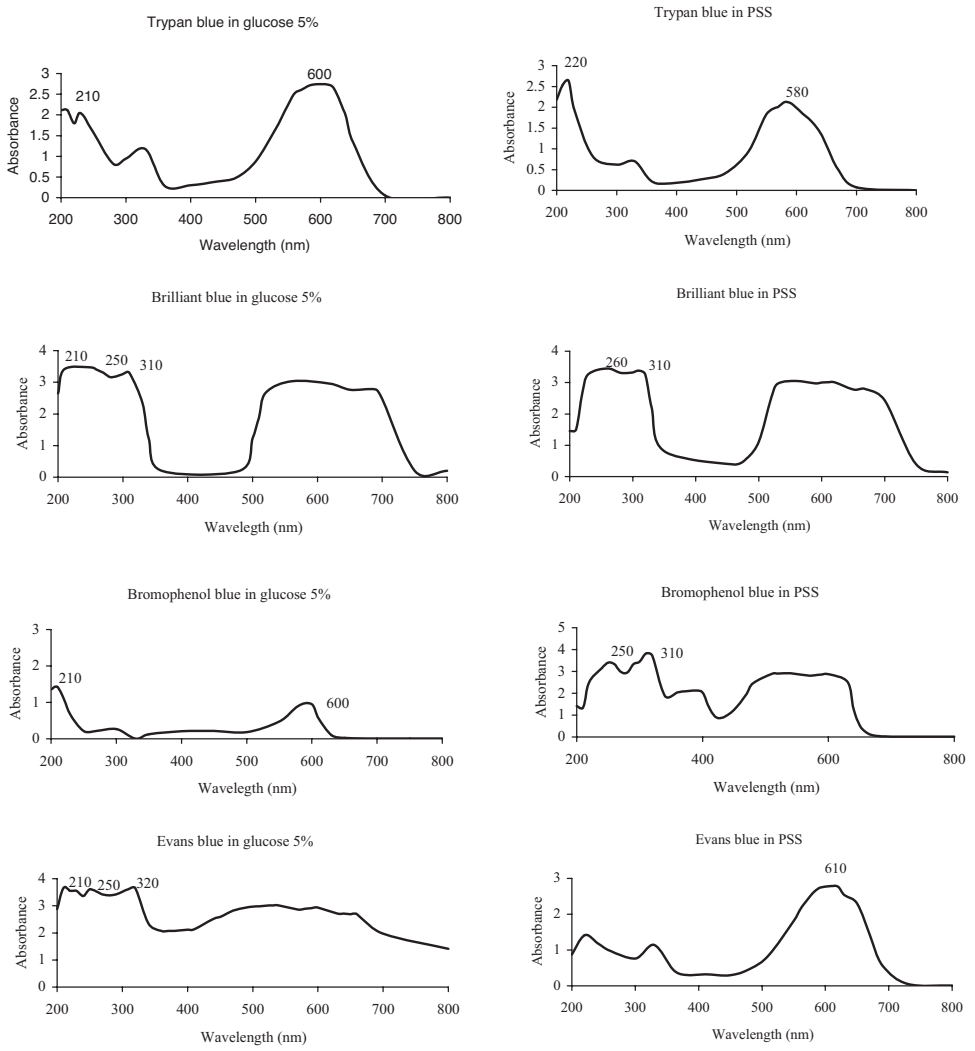

FIGURE 1. Absorbance spectra of blue dyes diluted in glucose 5% and PSS.

METHODS

Dye Dilution

A total of 0.5 mg of each dye (LG, BriB, FG, CR, EB, IC, ICG, TB, and BroB) was weighed with an analytical balance (Mettler-Toledo Inc., Columbus, OH) and was diluted in 10 mL PSS, glucose 5%, or distilled water to obtain a concentration of 0.005%. The mixture was shaken for 5 minutes and sonicated (Ultrasound Ultracleaner; Unique Ind., Idaiatuba, Brazil) to obtain a homogeneous solution.

Osmolarity, pH, and Photostability Analysis

The pH of the nine vital dyes was determined with a pH meter (Quimis, Diadema, Brazil), and osmolarity was measured with an osmometer (Advanced Instruments Inc., Norwood, MA). Samples of dye solutions were exposed to 1.2 million lux/h in a photostability chamber equipped with an ultraviolet lamp with output of at least 200 W/m^2 to allow direct comparison between the original fresh solution and the exposed product. Samples were exposed in the photostability chamber, positioned 7 inches away from the lateral walls, for 11 to 15 days; 2% quinine and a standard control were treated at the same time. Samples containing the solutions covered by aluminum foil were used as negative controls for evaluation of temperature changes inside the chamber. Eleven days after exposure, three samples of dye solutions and the tubes of 2% quinine and the standard control were removed daily for evaluation; the dye solutions were considered ready for removal and analysis once the difference in absorbance between the dye samples and standard control were less than 0.9. After the photostability measurements, pH and osmolarity were reassessed.

Light Absorption Properties and Emission Spectrum of Fiber-optic Light Sources

The 0.005% concentration was selected based on previous experiments by others.[2,3] Haritoglou et al.[3] examined the light absorbance characteristics of ICG at three concentrations: 0.005%, 0.001%, and 0.00025%. However, with appropriate selective retinal application of vital dyes, little or no diffusion occurred in the vitreous cavity, implying that concentrations less than 0.005% may not be encountered in the surgical setting. Light absorbance was measured in 0.005% solutions with PSS, water, or glucose 5% as the solvent immediately after preparation with a spectrometer (Spectronic GENESYS 5; Milton Roy, Ivyland, PA) between 190 and 1000 nm. Light absorbance curves were generated using PSS, glucose 5%, and water as the control to obtain absolute values. All measurements were repeated twice, and the samples were analyzed within 30 minutes To analyze and compare the absorbance spectra, absorbance data were collected and 27 curves were constructed (Figs. 1–3).

Overlap of Spectral Irradiance of Vitrectomy Light Sources and Absorbance Spectra of Dyes

For further analysis, the peaks of light absorbance of each of the 27 curves were compared with each of the seven light-emission spectra of

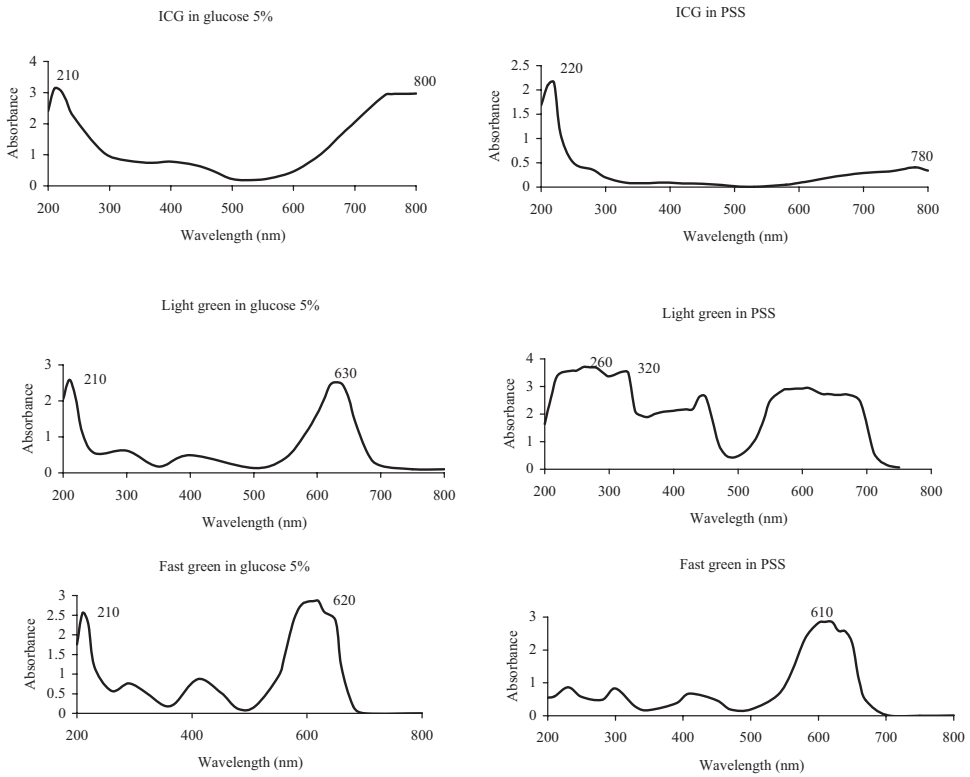

FIGURE 2. Absorbance spectra of green dyes diluted in glucose 5% and PSS.

the fiber-optic light sources (Figs. 4, 5). The light sources were high-brightness illuminator (Xenon 20G; Alcon), dual-output halogen illumination (Accurus H3; Alcon), halogen (Grieshaber GLS; GLS Corp.), dual-output metal halide illumination (Bausch and Lomb Millenium), dual 150-W halogen source (metal halide; DORC), dual-output illumination with an integrated laser pathway (Photon Xenon), and mercury vapor lamp (Photon 2; Synergetics).

An intersection curve was drawn based on the light absorbance spectrum of the dye diluted in water, glucose, and PSS and on the light emission spectrum of the fiber-optic light source. Intersection curves (total, 189) were built corresponding to the overlap among the nine dyes in three solutions and the seven fiber-optic light sources. The area under the overlap curve was calculated with a statistical analysis program (NCSS, Kaysville, UT). Differences were evaluated using the Kruskal-Wallis and Mann-Whitney U tests. $P < 0.05$ was considered statistically significant.

RESULTS

Osmolarity and pH

The osmolarity of all nine dyes was very low (0–54 mOsm) when the dyes were diluted in water, whereas PSS and glucose caused small but clinically relevant changes in osmolarity and pH. The osmolarity and pH of the nine dyes in PSS and glucose 5% ranged within the following values for the blue dyes: TB, 287–332 mOsm (pH, 6.12–7.84); BriB, 267–350 mOsm (pH, 5.15–7.12); BroB, 257–329 mOsm (pH, 2.6–6.71); EB, 291–345 mOsm (pH, 4.7–9.32). The other dyes showed the following ranges: ICG, 288–338 mOsm (pH, 4.83–7.48); LG, 288–325 mOsm (pH, 3.4–6.92); FG, 290–344 mOsm (pH, 3.82–7.12); CR, 291–385 mOsm (pH, 6.74–9.85); and IC, 291–333 mOsm (pH, 3.3–7.17). All the values are summarized in Table 1. No

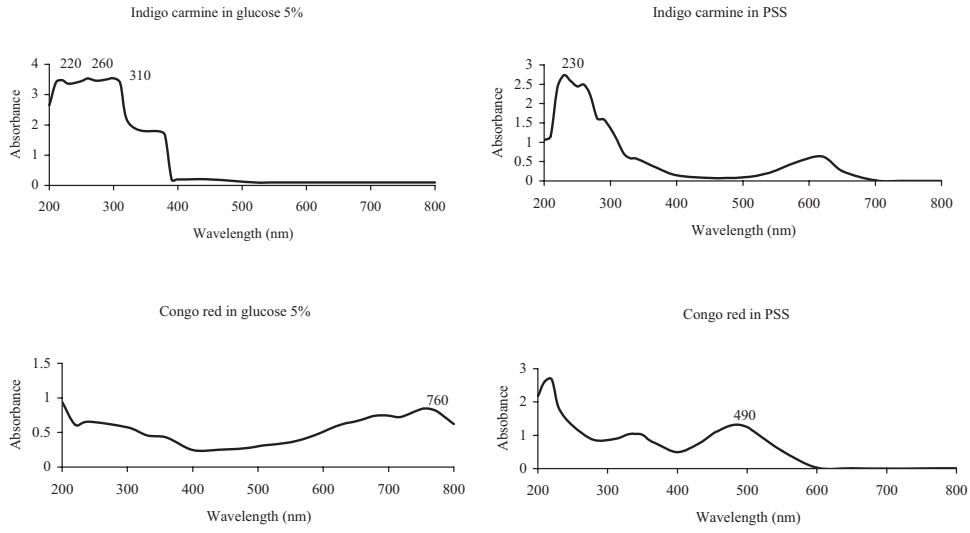

FIGURE 3. Indigo carmine and Congo red absorbance spectra in glucose 5% and PSS.

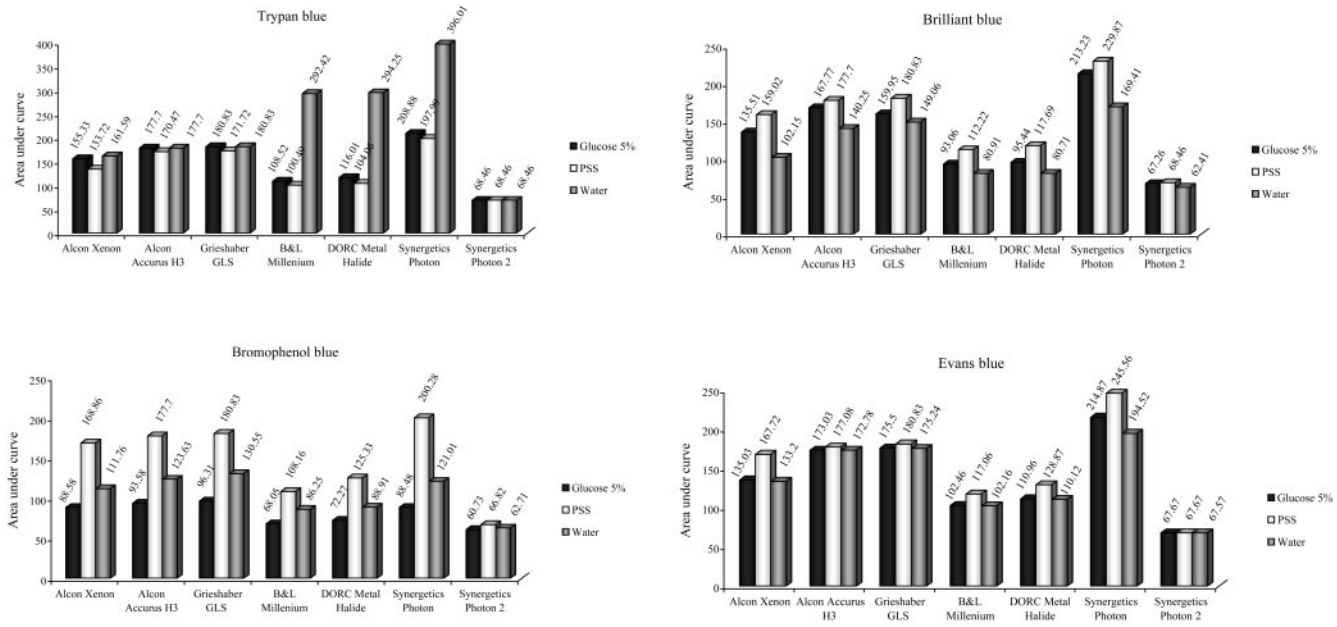

FIGURE 4. Overlap area between absorbance spectrum of blue dyes for chromovitrectomy and emission spectrum of vitrectomy light sources.

significant difference was found in terms of osmolarity after the photostability assay. The greatest change was an increase of 12% in the osmolarity of 0.5 mg/mL BriB prepared in glucose 5%. On the other hand, the pH values showed great variability; 5 mg/mL LG diluted in glucose 5% demonstrated an increase of 74%, and 5 mg/mL TB diluted in glucose 5% had a decrease of 39% after the photostability assay (Table 1).

Absorption Properties of Vital Dyes

Except for IC, which had a single peak between 220 and 310 nm, all dyes diluted in PSS, glucose 5%, and water at a concentration of 0.005% showed an absorbance spectrum with two maxima. The highest value was obtained in glucose 5% solution, and all curves returned to zero at 450 nm.

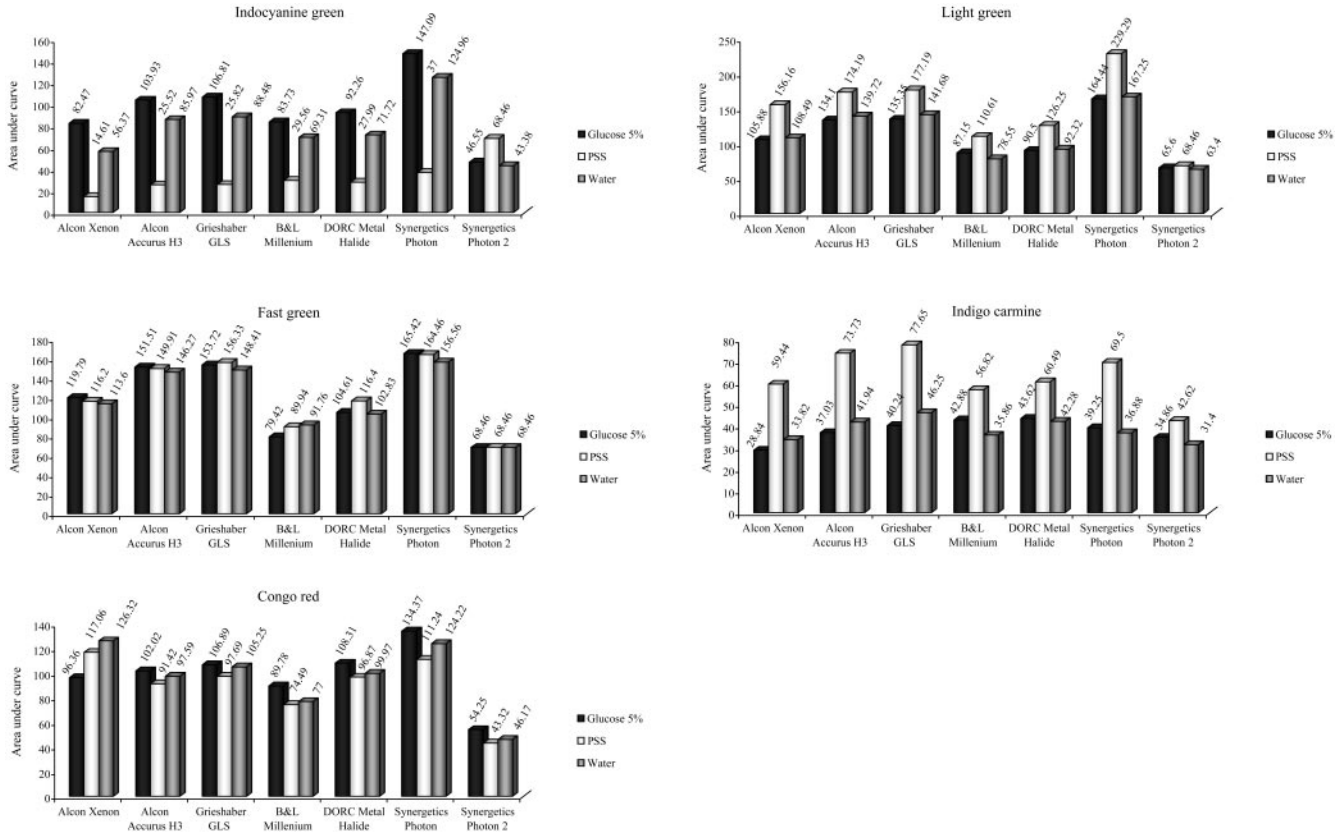

FIGURE 5. Overlap area between absorbance spectrum of green dyes, indigo carmine, and Congo red for chromovitrectomy and emission spectrum of vitrectomy light sources.

TABLE 1. Osmolarity and pH Results of Vital Dyes Diluted in Glucose 5% and PSS before and after Photostability Analysis

Vital Dye	Concentration (mg/mL)	Solvent	Before Photostability Analysis		After Photostability Analysis	
			pH	Osmolarity (mOsm)	pH	Osmolarity (mOsm)
Indocyanine green	5	Glucose 5%	5.27	294	4.83	300
		PSS	7.48	314	6.64	322
	0.5	Glucose 5%	6.64	288	6.48	289
		PSS	7.15	314	7.00	320
Trypan blue	5	Glucose 5%	6.86	304	6.12	287
		PSS	7.84	323	7.42	332
	0.5	Glucose 5%	7.32	290	6.78	290
		PSS	7.24	309	7.26	317
Brilliant blue	5	Glucose 5%	5.77	302	5.62	325
		PSS	6.64	339	6.77	350
	0.5	Glucose 5%	6.04	267	5.15	299
		PSS	7.02	311	7.12	324
Bromophenol blue	5	Glucose 5%	2.60	270	2.74	285
		PSS	5.00	315	5.07	328
	0.5	Glucose 5%	3.67	264	3.77	257
		PSS	6.34	308	6.40	320
Evans blue	5	Glucose 5%	7.43	301	4.70	312
		PSS	7.95	310	7.77	335
	0.5	Glucose 5%	6.00	291	4.35	299
		BSS	7.24	317	7.32	324
Light green	5	Glucose 5%	3.95	288	3.40	292
		PSS	5.97	325	6.53	320
	0.5	Glucose 5%	3.97	307	3.77	293
		PSS	6.82	312	6.80	323
Fast green	5	Glucose 5%	3.96	303	3.91	309
		PSS	6.79	326	6.80	344
	0.5	Glucose 5%	4.17	290	3.82	294
		PSS	7.07	311	7.12	319
Indigo carmine	5	Glucose 5%	4.01	299	3.30	303
		PSS	7.04	323	6.30	348
	0.5	Glucose 5%	3.83	291	3.33	296
		PSS	7.12	308	6.65	323
Congo red	5	Glucose 5%	8.87	323	7.07	329
		PSS	8.07	362	8.23	367
	0.5	Glucose 5%	6.74	291	4.68	295
		PSS	7.31	315	7.46	324

Among the blue dyes, TB showed a double-peak curve with one peak between 210 and 220 nm and a second between 580 and 600 nm, returning to zero at 750 nm. The lowest peak was obtained when TB was dissolved in PSS. For BroB, the first peak was between 200 and 210 nm, and the second was between 590 and 600 nm; however, in the PSS solution, a different result was seen. The first peak was at 250 nm and the second was at 310 nm, where the peaks were closer than in the other solutions. BriB absorbance spectrum was similar for the solutions in glucose 5% and PSS and also displayed two close peaks, one at 250 nm and the other at 310 nm. The two absorbance peaks were distant in water solution because one peak was at 200 nm and the other was at 580 nm. EB also showed different results in one of the solutions; when the solutions were prepared in water and PSS, the peaks were 200 nm and 610 nm, respectively, and in glucose 5% the second peak was observed at 310 nm (Fig. 1).

With regard to the green dyes, ICG showed the first peak between 210 and 220 nm and the second peak between 780 and 800 nm. The highest peak was obtained with ICG prepared in glucose 5%. FG showed the first peak at 210 nm and the second at 610 nm for all solutions. LG showed similar results with either glucose 5% or water; one peak was observed at 210 nm and the other between 620 and 630 nm. In PSS solution, the peaks were observed at 260 nm and 610 nm. For CR, the first peak varied between 200 and 240 nm in all solutions and the second between 490 and 500 nm when dissolved in PSS or water. CR with glucose 5% provided a second peak at 760 nm (Figs. 2, 3).

Overlap with the Fiber-optic Light Source

Intersection curves were built between the nine dyes with two different solvents (glucose 5% and PSS) and the seven light sources (Figs. 4, 5). In analyzing PSS and glucose solution of each dye, no significant difference was found in the overlap curves ($P = 0.8012$). Among all dyes, IC and ICG had the lowest overlap areas matched to the light sources ($P < 0.0001$), though no difference was found between those two dyes ($P = 0.1627$). IC dissolved in glucose 5% was the dye with the least overlap compared with the other dyes for all light source curves. However, the solution of ICG in PSS showed the lowest values of overlap among all staining agents. No significant difference was found between TB, BriB, BroB, FG, LG, and EB except with CR, which showed smaller overlap areas than TB and EB ($P < 0.0001$). Considering the light sources, mercury vapor lamp (Photon 2; Synergetics) had the lowest overlap values with all dyes ($P < 0.0001$). Dual-output metal halide illumination (Bausch and Lomb Millenium) also showed significantly lower values ($P < 0.0001$) when compared with halogen (Grieshaber GLS; GLS Corp.) and integrated laser pathway (Photon Xenon; Synergetics Photon). Integrated laser pathway (Photon Xenon) was the light source with the largest overlap.

Discussion

Chromovitrectomy has improved surgical techniques by allowing better identification of preretinal membranes, vitreous, and other intraocular tissues.[4] However, animal experiments and clinical data have demonstrated evidence of retinal toxicity, depending on the dosage of intraoperative dye applied.[2,5–7] Although various theories explain the retinal damage induced by vital dyes, one possible mechanism is photochemical damage because light emission by intraoperative endoillumination may be absorbed by the dye-stained retina. To help elucidate the importance of each mechanism, we investigated several in vitro aspects of vital dyes with regard to pH, osmolarity, spectral properties, and photostability.

The harmful effect to the retina by different types of solutions with nonphysiological osmolarity has been well demonstrated in preliminary animal studies. Small changes of 25-mOsm increments have caused injurious effects on electrical parameters in chick chorioretinal tissue.[8] Subretinal rabbit toxicity induced by hypo-osmolar solutions of ICG or TB were more severe than that caused by iso-osmolar solutions.[9] On the other hand, a hyperosmotic solution injected into the rabbit and primate vitreous caused retinal detachment and loss of RPE cells.[10] In our present investigation of three solvents, the use of water as a solvent produced by far the most hypotonic and alkaline dye solutions, whereas vital dyes dissolved in PSS (319 mOsm) resulted in slightly greater hyperosmolar solutions than glucose 5% (292 mOsm) for all dyes. The osmolarity of dye solutions dissolved in PSS and glucose 5% showed values as low as 257 (BroB) to as high as 385 (CR) (Table 1). Clinically, careful selection of the best solvents for vital dyes for chromovitrectomy should be performed, especially regarding those agents provided as a powder for reconstitution by the surgeon in the operating room, such as ICG.

Previous laboratory investigations have shown that various changes in pH from solutions applied to the retina may cause toxicity.[11–13] In the retina, pH changes were found to strongly affect the retinal component of the c-wave and b-wave in the perfused cat eye model.[14,15] In addition, a complex interaction may occur between acid-base disturbances and light on the retina because photoreceptor light response may induce changes in pH through alterations in subretinal K^+ levels.[16,17] In this study, detailed pH evaluation of nine vital dyes prepared in three solvents revealed that the pH of the vital dye solutions varied greatly, from 2.6 to 9.85. Interestingly, even with current vital dyes such as ICG and TB, pH values as low as 3.88 and 4.1 have been encountered when diluted in glucose 5%. Thus, during vitreoretinal surgery, the recently proposed preparation of TB and ICG solutions in glucose 5% or 10% must be viewed with caution to avoid severe retinal acid-base disturbance.[1,18]

Intraoperative retinal phototoxicity from endoilluminator light sources has been an important concern for more than three decades.[19,20] Light-induced retinal toxicity by the endoilluminator is dependent on factors such as the duration of use, type, power, and wavelength of light source. The hazard of light radiation increases with decreasing wavelength because stronger phototoxic effects are mediated by ultraviolet rays and blue light, as shown in experimental studies.[21] Endoilluminators with wavelengths greater than 400 nm have been shown to induce phototoxic effects mainly in RPE cells, whereas exposure to light below 400 nm also causes photoreceptor damage.[22,23] Such information is important with the introduction of the wide variety of new light source devices for endoilluminators in vitreoretinal surgery, including metal halide, xenon, and mercury light sources developed for small-gauge vitrectomy. For instance, xenon light has a wavelength between 420 and 700 nm with a peak at 450 nm, in contrast to the spectral irradiance of halogen light with a peak at 650 nm. Some mechanisms involved in retinal damage by light include thermal damage, perturbation of metabolic pathways, formation of a toxic photoproduct, and the photosensitizing or photo-oxidative reactions of natural pigments, photosensitizers, or dyes; various combinations of mechanisms may be involved as well.

Vital dyes are small chemical substances that pass freely through retinal tissue and may play a role in or exacerbate retinal phototoxicity from intraoperative light exposure. Photosensitizing dyes could enhance phototoxicity by increasing levels of free radicals, creating a photoproduct that could be harmful to retinal cells and shifting light absorbance from one site of the retina to another. In this regard, the dye on the retinal surface could increase the risk for phototoxicity to the neuroretina for light greater than 450 nm, which would not occur without dyes. In addition, dyes in the subretinal space may exacerbate damage to the RPE after exposure to various wavelengths of light. Clinically, consecutive experiments have shown histologic and functional damage to the retina after light plus ICG exposure in comparison to light without dye application.[1,2,7,24,25]

To analyze the risk for dye-induced light damage, the spectral overlap between light source emission and vital dye absorbance should be known. Thus far, ICG is the only dye whose light absorbance properties have been studied. Haritoglou et al.[2] proposed that a shift in the absorbance band of ICG during vitreoretinal surgery induces photosensitizing effects on the retinal surface. They demonstrated that the absorbance spectrum of ICG overlaps partially with the emission spectrum of one type of halogen light source and that ICG absorbance varies, depending on solvent and osmolarity. Notably, ICG in glucose 5% shifts the absorbance bands to higher wavelengths compared with ICG prepared in PSS, thereby decreasing the risk for spectral overlap with the light emission source.[3] Nevertheless, a decrease in pH as a result of such solution may increase the risk for cell damage through other mechanisms. Kadonosono et al.,[26] on the other hand, measured the absorbance coefficients at short wavelengths with a light source for vitreous surgery and observed no difference between ICG solution and PSS.

In the present study, we evaluated the absorbance spectra of nine vital dyes for chromovitrectomy and found that most of them had two absorbance peaks, the first below 400 nm and the other in the visible light range from 400 to 700 nm (Figs. 1–3). Absorbance spectra also varied greatly with the solvent used. In addition, we performed a detailed graphical analysis to determine the degree of overlap between the absorbance spectrum of vital dyes and irradiance emission spectrum of seven light sources available for chromovitrectomy. From a surgery perspective, among all light sources analyzed in our study, the greatest overlap was found with integrated laser pathway (Photon Xenon; Synergetics Photon) and halogen (Grieshaber GLS; GLS Corp.), and the least overlap was found with mercury vapor lamp (Photon 2; Synergetics). The lowest overlap values among the dyes were observed with ICG prepared in PSS, followed by IC, which showed low values for all three solvents compared with other dyes.

In summary, our study examined the properties of dyes currently in use and new candidate dyes for chromovitrectomy, regarding osmolarity, pH, absorbance, and interaction with vitrectomy light sources. We showed that pH and osmolarity are important variables that may influence the safety of the retina with the use of intravitreally applied substances. Although many uncontrolled intraoperative factors may influence pH and osmolarity, we determined the optimal biochemical profile of the vital stains to decrease the risks of surgery. Intraoperative light exposure should be carefully observed during surgery because spectral overlap between the absor-

bance of vital dyes and the endoillumination emission spectrum could cause or enhance retinal toxicity. Further in vitro and in vivo studies are required to elucidate the effect of the interaction between dye and light on retinal cells.

Acknowledgment

The authors thank the staff at Ophthalmos Laboratories, São Paulo, Brazil.

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