Effect of Vital Dyes on Retinal Pigmented Epithelial Cell Viability and Apoptosis: Implications for Chromovitrectomy

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Key Words

Apoptosis · Cell viability · Retinal pigment epithelium · Vital dyes

Abstract

Purpose: To investigate the in vitro effect of vital dyes on toxicity and apoptosis in a human retinal pigment epithelial cell line. **Methods:** ARPE-19 cells were exposed to brilliant blue (BBG), Evans Blue (EB), bromophenol blue (BroB), indocyanine green (ICG), infracyanine green (IfCG), light green (LG), fast green (FG), indigo carmine (IC) and Congo red (CR). Balanced salt solution was used as the control. Five different concentrations and 2 exposure times were tested. Cell viability was determined by the MTS (1-solution methyl thiazolyl tetrazolium) assay and apoptosis by Bax expression on Western blot. **Results:** All dyes significantly reduced cell viability after 3 min of exposure at all concentrations ($p < 0.01$), except for BBG that was safe at concentrations up to 0.25 mg/ml and CR up to 0.05 mg/ml, while LG was safe at all concentrations. Toxicity was higher after 30 min of exposure. Expression of Bax was upregulated after all dye exposures, except BBG; ICG had the highest Bax expression ($p < 0.01$). **Conclusions:** Overall the safest dye was BBG followed by LG,

IfCG, FG, CR, IC, BroB, EB and ICG. ICG was toxic at all concentrations and exposure times tested. Moreover, BBG was the only dye that did not induce apoptosis in ARPE-19 cells.

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Introduction

Removal of the internal limiting membrane (ILM) has been an important maneuver for anatomical and functional success in macular hole and other macular surgeries [1–3]. However, due to its anatomical characteristics, the identification of the ILM during surgery is a difficult step in the surgical procedure. Therefore, the use of dyes to identify structures during vitreoretinal surgery, ‘chromovitrectomy’, has become a popular technique in recent years [4]. Indocyanine green (ICG) was the first dye to be used in macular surgery promoting a good contrast with retinal tissue and making ILM removal technically easier [5], although, in the past few years, several studies have

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demonstrated toxicity to the retinal pigment epithelium (RPE) and neurosensory retina, as well as cases of optic nerve atrophy, after the use of ICG [1, 6–9]. Other alternative dyes have emerged for staining the ILM with less toxicity.

Trypan blue demonstrated a lower toxicity profile to RPE cells and retinal tissue when compared to ICG, with an excellent affinity for epiretinal membranes, but it is not a good dye for acellular membranes, such as the ILM [7, 10, 11]. Brilliant blue G (BBG) has emerged as second-generation dye with an outstanding staining of the ILM [12–16]. Moreover, BBG has recently been released on the European market in a concentration of 0.25 mg/ml – Brilliant Peel™ (DORC, The Netherlands). This presentation of the dye was shown to provide a good staining capacity for the ILM and was not toxic in experimental studies and case series in humans [17]. However, BBG could induce RPE changes after accidental subretinal dye injection in humans [18–20].

Currently, ICG, trypan blue and BBG have been used in chromovitrectomy [4]. However, a dye with little toxicity and with a good affinity for the ILM is yet to be found. The aim of this study was to provide a detailed *in vitro* toxicity investigation of 7 dyes – Evans blue (EB), bromophenol blue (BroB), infracyanine green (IfCG), light green (LG), fast green (FG), indigo carmine (IC) and Congo red (CR) – and compare them to ICG and BBG, which are in clinical use. Five dye concentrations (1, 0.5, 0.25, 0.05 and 0.005 mg/ml) and 2 exposure times (3 and 30 min) were used.

The evaluation of apoptosis in retinal toxicity studies of dyes has become an important issue, since it was shown that residual ICG can be found months after surgery [21]. For this reason, in the present study we also evaluated the link between cell toxicity and apoptosis in ARPE-19 cells exposed to these vital dyes.

Methods

Compounds

The dyes ICG, LG, CR, FG, EB, BroB, IC and BBG and cell culture reagents were obtained from Sigma-Aldrich (Munich, Germany). IfCG was obtained from SERB (Paris, France). Balanced salt solution (BSS®) was obtained from Alcon Laboratories (Fort Worth, Tex., USA). The MTS Cell Titer 96 Aqueous One Solution Cell Proliferation Assay was purchased from Promega (Madison, Wisc., USA). A lactate dehydrogenase cytotoxicity assay kit was purchased from Abcam Inc. (Cambridge, Mass., USA). Primary antibody Bax was purchased from EMD Millipore Corporation (Billerica, Mass., USA). The secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif., USA).

Dye Preparation

Initially, 5 mg of each dye were measured using an analytical balance (Mettler-Toledo Inc., Columbus, Ohio, USA) and dissolved in sterile BSS to obtain stock solutions. ICG and IfCG required different methods of preparation. ICG was first diluted in distilled water and IfCG with 5% glucose solution as recommended by the manufacturer. Subsequent dilutions were performed with sterile BSS to obtain final concentrations of 1, 0.5, 0.25, 0.05 and 0.005 mg/ml. This serial dilution was made to evaluate a wide range of concentrations that could be present in the surgical field due to variations of dilution technique and dye injection (air- or fluid-filled vitreous cavity).

Afterwards, the pH and osmolarity of each dye solution were determined using a previously calibrated pH meter and osmometer (Advanced Instruments Inc., Norwood, Mass., USA). These measurements were made to minimize deleterious conditions related to unexpected variations of pH and osmolarity, which could have an influence on the toxicity found in the experiments.

Cell Viability Assay

All experiments were performed using the immortalized human retinal pigment cell line ARPE-19 (American Type Culture Collection, Manassas, Va., USA), a well-established model to test the safety of vital dyes in RPE cells. The rationale for using this cell line in dye toxicological studies is that during macular hole surgery the dye could come in direct contact with the RPE through the macular hole. Another reason is that the dye can penetrate through retinal layers and cause RPE toxicity as well. Therefore, a meticulous study of this cell layer is very important in these preclinical toxicity evaluations.

ARPE-19 cells were grown in Dulbecco's modified Eagle's medium/Ham's F-12 (Gibco, Carlsbad, Calif., USA; 1:1 vol/vol) medium supplemented with 10% fetal bovine serum, 1 mM L-glutamine, 100 µg/ml penicillin/streptomycin, and 0.348% Na₂CO₃ in a 5% CO₂ humidified air incubator at 37°C. Cells were used between passages 5 and 8. For experiments, cells were seeded at a concentration of 5×10^3 cells/well in 96-well, flat-bottomed tissue culture plates in 200 µl of culture medium and grown for 24 h before the experiments in a 5% CO₂ humidified incubator at 37°C. Subsequently, the cell culture medium was removed and the cells washed 3 times with BSS. Subsequently, cells were incubated with ICG, IfCG, FG, LG, BBG, BroB, EB, IC or CR (1, 0.5, 0.25, 0.05 and 0.005 mg/ml) for 3 or 30 min. After incubation, cells were washed 3 times with 200 µl of phosphate-buffered saline. The number of surviving cells was measured by cell count (Coulter ZI cell counter; Beckman Coulter, Hialeah, Fla., USA) and by the MTS (a tetrazolium salt) assay (Cell Titer 96 Aqueous One Solution kit; Promega). The results for MTS were obtained by measuring absorbance at 490 nm with an ELISA plate reader (Bio-Rad, Hercules, Calif., USA). All experiments were performed in quadruplicate and repeated 3 times.

The rationale of these 2 time exposures, 3 and 30 min, is to simulate an acute exposure to these dyes that occurs during vitreoretinal surgery (3 min) and also a prolonged exposure that might happen if the dye is not entirely washed out from the vitreous cavity (30 min).

Apoptosis Assay

Background

Recent studies have delineated one key mechanism responsible for initiating the executioner phase of apoptosis. It is widely recognized that apoptosis is mediated by a Bax cascade through mi-

Table 1. Effect of vital dyes on RPE cell viability (% of control, means $\pm$ SD) at different dye concentrations and exposure times

Exposure time	Dyes	Concentration				
		1 mg/ml	0.5 mg/ml	0.25 mg/ml	0.05 mg/ml	0.005 mg/ml
3 min	BSS	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0
	BBG	64.8 $\pm$ 3.6**	77.4 $\pm$ 4.5**	94.4 $\pm$ 4.9	94.9 $\pm$ 6.2	98.2 $\pm$ 6.5
	ICG	43.1 $\pm$ 3.3**	51.6 $\pm$ 3.5**	55.2 $\pm$ 2.7**	61.9 $\pm$ 4.2**	66.3 $\pm$ 3.9**
	IfCG	56.5 $\pm$ 3.5**	62.0 $\pm$ 2.9**	68.1 $\pm$ 2.9**	74.3 $\pm$ 3**	79.2 $\pm$ 3.5**
	LG	92.7 $\pm$ 6.8	93.6 $\pm$ 4.3	96.9 $\pm$ 4.2	97.7 $\pm$ 4.8	101.2 $\pm$ 5.1
	FG	69.2 $\pm$ 5.1**	69.8 $\pm$ 8.3**	77.4 $\pm$ 4.8**	79.7 $\pm$ 4.6**	80.1 $\pm$ 3.4**
	IC	47.3 $\pm$ 4.9**	53.0 $\pm$ 3.5**	56.4 $\pm$ 3.5**	59.7 $\pm$ 4.5**	66.6 $\pm$ 3.5**
	BroB	49.6 $\pm$ 3.1**	57.2 $\pm$ 3.7**	61.4 $\pm$ 2.4**	62.1 $\pm$ 4.9**	66.6 $\pm$ 5.3**
	EB	50.4 $\pm$ 3.6**	58.7 $\pm$ 6.3**	62.6 $\pm$ 7.2**	67.3 $\pm$ 8.1**	61.9 $\pm$ 5.3**
30 min	CR	55.8 $\pm$ 3.1**	64.3 $\pm$ 4.1**	73.7 $\pm$ 3.4**	89.1 $\pm$ 5	93.9 $\pm$ 6.7
	BSS	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0	100 $\pm$ 0
	BBG	62.3 $\pm$ 3.1**	72.8 $\pm$ 5.1**	81.6 $\pm$ 6.1**	87.4 $\pm$ 8.3*	94.2 $\pm$ 9.3
	ICG	33 $\pm$ 4.6**	31.4 $\pm$ 3.3**	33.5 $\pm$ 3.3**	37.2 $\pm$ 3.6**	37.2 $\pm$ 5**
	IfCG	52.4 $\pm$ 6.7**	52.3 $\pm$ 7.8**	54.6 $\pm$ 7.7**	53.4 $\pm$ 8**	48.5 $\pm$ 9.1**
	LG	39.4 $\pm$ 2.6**	44.9 $\pm$ 4.5**	47.1 $\pm$ 2.8**	45.5 $\pm$ 3.3**	46.7 $\pm$ 4**
	FG	46.3 $\pm$ 3.7**	51.1 $\pm$ 2.4**	44 $\pm$ 4.3**	41.3 $\pm$ 1.5**	56.1 $\pm$ 2**
	IC	42.7 $\pm$ 2.4**	44.3 $\pm$ 3.3**	49.2 $\pm$ 2**	50.4 $\pm$ 3.1**	53.7 $\pm$ 3.1**
	BroB	37.2 $\pm$ 5.1**	51.6 $\pm$ 2.5**	56.9 $\pm$ 4.2**	57.3 $\pm$ 6.2**	60.4 $\pm$ 2**
	EB	39.9 $\pm$ 6.2**	52.2 $\pm$ 7**	55.4 $\pm$ 3.2**	60 $\pm$ 2.5**	58.6 $\pm$ 7.5**
	CR	39.9 $\pm$ 4.1**	42.5 $\pm$ 2**	49.9 $\pm$ 4.2**	52.3 $\pm$ 4**	59.9 $\pm$ 3.3**

Analysis of variance – Tukey test. The lower the values, the higher the dye toxicity. Values are means of at least 3 independent experiments. With regard to the p values presented in this table, there was a significant difference between the dyes with all concentrations and exposure times (* $p < 0.05$, ** $p < 0.01$) when compared with nontreated (BSS) control cells.

tochondrial stress [22]. Bax is a proapoptotic Bcl-2 family protein that resides in the cytosol and translocates to mitochondria upon induction of apoptosis [23]. Recently, Bax has been shown to induce cytochrome c release and caspase activation in vivo and in vitro [24, 25].

Some of the tested dyes were really toxic to RPE cells and were excluded from the apoptosis assay. We therefore examined the expression of proapoptotic protein, Bax by ICG, IfCG, FG, LG and BBG in ARPE-19 cells exposed to 0.05 mg/ml dyes for 3 min, which are the concentration and time mostly used in vitreoretinal surgery.

Western Blot Analysis

After treatment, lysates of ARPE-19 cells were obtained, and total protein was extracted in protein lysis buffer M-PER (Pierce, Rockford, Ill., USA) and quantified by a detergent-compatible protein assay (Bio-Rad). Protein extracts (20–40 μ g) were denatured in Laemmli's sample buffer, followed by boiling for 5 min, and then resolved on a 4–20% Tris-glycine gel. After electrophoresis (120 V for 2 h), proteins were transferred in 1 $\times$ transfer buffer [25 mmol/l Tris, 192 mmol/l glycine, 0.1% sodium dodecyl sulfate, and 20% methanol (pH 8.4)] to a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Biosciences, Piscata-

way, N.J., USA) using constant current (350 mA for 45 min). Membranes were blocked in 5% nonfat dry milk-Tris-buffered saline (TBS) solution for 1 h at room temperature. Blots were incubated overnight at 4°C with primary antibodies against Bax, cytochrome c, caspase-9, Bcl-2, and GAPDH. Membranes were washed 3 times with TBS-Tween 20, then incubated with horseradish peroxidase-linked donkey anti-rabbit antibody or donkey anti-mouse antibody for 2 h at room temperature, and finally washed 3 times in TBS-Tween 20. Immunoreactive bands were determined by exposing the nitrocellulose blots to a chemiluminescent solution and exposing to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y., USA). Three independent experiments were performed in triplicate.

Statistical Analyses

All experiments were performed 3 times, showing reproducible results. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad, La Jolla, Calif., USA). Data are expressed as means $\pm$ SEM of the percentage of cell viability/toxicity with respect to control. Statistical comparisons were performed using 1-way analysis of variance followed by the Tukey post hoc test for multiple comparisons. Values of $p < 0.05$ were considered statistically significant.

Fig. 1. Effect of different doses of ICG, IfCG, FG and LG on ARPE-19 cell viability assessed by the MTS cell assay. Cells were exposed to dyes for 3 min. Bars correspond to means of 3 independent experiments. Data are expressed as percentage of control, and the results shown are means $\pm$ SEM of 3 independent experiments run in triplicate. ** $p < 0.01$: statistical significance for comparison with the control (BSS solution).

Fig. 2. Effect of different doses of IC, BroB, EB, CR and BBG on ARPE-19 cell viability assessed by the MTS cell assay. Cells were exposed to dyes for 3 min. Bars correspond to means of 3 independent experiments. Data are expressed as percentage of control, and the results shown are means $\pm$ SEM of 3 independent experiments run in triplicate. ** $p < 0.01$: statistical significance for comparison with the control (BSS solution).

Results

Effect of Tested Dyes on Cell Viability

In order to determine the range of dye toxicity, the cells were treated with 9 different dyes, in 5 concentrations (1, 0.5, 0.25, 0.05, and 0.005 mg/ml) for 3 or 30 min, and the cell toxicity was determined by the MTS reduction assay and cell count.

As shown in figures 1 and 2, all concentrations tested for ICG, IfCG and FG during 3 min of exposure signifi-

cantly decreased cell viability when compared to control (BSS). This effect was dose-dependent (table 1). However, for LG no toxicity was observed at 3 min. Moreover, treatment with these dyes for 30 min dramatically reduced the viability when compared to control (fig. 3, table 1). ICG (1 mg/ml) for 3 min decreased cell viability by approximately 57% ($43.1 \pm 3.3\%$, $p < 0.01$) whereas 0.5 mg/ml and 0.25 mg/ml ICG decreased cell viability by approximately 49% ($51.6 \pm 3.5\%$, $p < 0.01$; $55.2 \pm 2.7\%$, $p < 0.01$) and concentrations of 0.050 and 0.005 mg/ml by approxi-

Fig. 3. Effect of different doses of ICG, IfCG, FG and LG on ARPE-19 cell viability assessed by the MTS cell assay. Cells were exposed to dyes for 30 min. Bars correspond to means of 3 independent experiments. Data are expressed as percentage of control, and the results shown are means $\pm$ SEM of 3 independent experiments run in triplicate. ** $p < 0.01$: statistical significance for comparison with the control (BSS solution).

Fig. 4. Effect of different doses of IC, BroB, EB, CR and BBG on ARPE-19 cell viability assessed by the MTS cell assay. Cells were exposed to dyes for 30 min. Bars correspond to means of 3 independent experiments. Data are expressed as percentage of control, and the results shown are means $\pm$ SEM of 3 independent experiments run in triplicate. * $p < 0.05$, ** $p < 0.01$: statistical significance for comparison with the control (BSS solution).

mately 38% ($61.9 \pm 4.2\%$, $p < 0.01$; $66.3 \pm 3.9\%$, $p < 0.01$; fig. 1). However, after 30 min of treatment the decrease in cell viability was about 70% for all the assayed doses (fig. 3, table 1).

Exposure for 3 min to 1 and 0.5 mg/ml BBG showed a statistically significant reduction in cell viability of approximately 35 and 23%, respectively ($64.8 \pm 3.6\%$, $p < 0.01$; $77.4 \pm 4.5\%$, $p < 0.01$). However, doses of 0.25, 0.05 and 0.005 mg/ml did not modify cell viability (fig. 2, table 1). When cells were exposed to this dye for 30 min, cell

viability decreased by approximately 37 and 27% at concentrations of 1–0.05 mg/ml, respectively ($62.3 \pm 3.1\%$, $p < 0.01$; $72.8 \pm 5.1\%$, $p < 0.01$) and by about 18 and 13% ($81.6 \pm 6.1\%$, $p < 0.01$; $87.4 \pm 8.3\%$, $p < 0.05$) at concentrations of 0.25 and 0.05 mg/ml, respectively (fig. 4, table 1). BBG (0.005 mg/ml) did not modify cell viability.

Our data also show that IC, BroB and EB for 3 min induced a decrease in cell viability at all tested doses. On the other hand, CR induced a decrease in RPE cell viability just in doses higher than 0.05 mg/ml ($55.7 \pm 5.81\%$, $p <$

Fig. 5. Combined results of the effects of vital dyes on ARPE-19 cells. The effects of dyes on cell viability were combined for each concentration and compared between 3 and 30 min of exposure. Bars correspond to means of 3 independent experiments. Data are expressed as percentage of control, and the results shown are means $\pm$ SEM of 3 independent experiments run in triplicate. ** $p < 0.01$ for comparison with the control.

Fig. 6. Combined results of effects of vital dyes on ARPE-19 cell viability. The effects of dyes on cell viability were combined for all concentrations and both exposure times and the dyes compared. Bars correspond to means of 3 independent experiments. Data are expressed as percentage of control, and the results shown are means $\pm$ SEM of 3 independent experiments run in triplicate. * $p < 0.05$, ** $p < 0.01$: statistical significance for comparison with the control (BSS solution).

0.01; $64.2 \pm 8.1\%$, $p < 0.01$; $73.7 \pm 6.7\%$, $p < 0.01$), whereas it had no effect at concentrations of 0.05 and 0.005 mg/ml (fig. 3, table 1). However, 30 min of exposure to IC, BroB, EB and CR reduced cell viability significantly in all tested concentrations (fig. 4, table 1).

When exposure time was evaluated as an isolated factor, it was shown that all concentrations at 30 min induced higher toxicity when compared with 3 min of exposure (fig. 5).

With regard to the dye itself, when all values were analyzed independently of concentration or exposure time, all dyes induced a reduction of RPE cell viability except BBG. The order of cell viability reduction (lower to higher) is: BBG, LG, IfCG, FG, CR, IC, BroB, EB and ICG (fig. 6). Comparable results were observed for cell viability measured by cell count (data not shown).

Expression of Bax by Vital Dyes in ARPE-19 Cells

The dysregulated expression of Bax by RPE may be involved in the cell susceptibility to apoptosis [26]. Therefore, based on the observations obtained for cell viability, it was explored whether the tested dyes induce apoptosis through Bax overexpression in ARPE-19 cells. With the use of Western blot analysis, the expression of Bax in ARPE-19 cells treated with or without 0.05 mg/ml ICG, IfCG, LG, FG, BBG and CR for 3 min was investigated. Our results revealed that ICG robustly increases Bax ex-

pression at protein levels (383.1 ± 2.38 , $p < 0.01$; fig. 7). The other dyes LG, FG, IfCG and CR also induced an overexpression of Bax by 2, 2.18, 2.07 and 2.17 times, respectively ($p < 0.05$; fig. 7). BBG revealed a slight increase in Bax expression, but not significantly when compared to untreated cells (fig. 7).

Discussion

In recent years the use of vital dyes in vitrectomy, i.e. chromovitrectomy, has become the standard method to facilitate intraoperative surgical procedures such as ILM or epiretinal membrane peeling. The two dyes available for chromovitrectomy, ICG and trypan blue, may lead to complications in macular surgery [27–31]. BBG has been used as an alternative and safer dye, but even so they may migrate to the subretinal space and produce alterations in the RPE and campimetric and papillary defects. The ideal, nontoxic dye is yet to be determined. Therefore, in this study, a detailed in vitro toxicity investigation of 9 dyes – ICG, IfCG, LG, FG, BBG, BroB, EB, IC and CR – was done.

Five different dye concentrations (1, 0.5, 0.25, 0.05 and 0.005 mg/ml) and 2 exposure times (3 and 30 min) were selected to simulate the possible dye dilutions and injec-

tion techniques that can be performed by surgeons during chromovitrectomy [27].

ICG is a tricarboxyanine anionic vital dye with a molecular formula of $C_{43}H_{47}N_2NaO_6S_2$ and a mass of 775 kDa [32]. ICG adheres well to the extracellular matrix components of the ILM, such as collagen type 4, laminin, and fibronectin [4]. Wollensak and Engels [33] showed, in a porcine model, that ICG with light exposure produces a significant increase in biomechanical stiffness, thereby facilitating ILM peeling. Following the publication of Kadonosono et al. [34] of ICG use in macular hole surgery, many authors have reported easier and less traumatic ICG-guided peeling with good clinical results. Controversial publications have shown ICG-related toxicity such as perimetric defects, vision loss, optic nerve atrophy and RPE lesions. The present study reinforces the toxic profile of ICG showing a reduction of RPE cell viability in all tested concentrations and exposure times. More recently some papers have been dedicated to reduce the ICG-related toxicity with free radical scavengers (tempol), less concentration, short time exposure, or iodine-free solution such as IfCG [35–37].

IfCG also binds acellular membranes, such as the ILM, with a high affinity [38]. Differently from ICG, IfCG is an iodine-free solution and should be dissolved with 5% glucose solution generating an iso-osmotic solution (294–314 mosm); both characteristics that make IfCG a safer dye. Several experimental and clinical studies reported positive results with little retinal toxicity in concentrations lower than 0.05% [6, 37, 39, 40]. Indeed, the present study shows that IfCG had a safer profile when compared to ICG, but still reduced RPE cell viability in all tested concentrations and exposure times.

BroB, also named tetrabromophenolsulfone phthalain, is a hydroxytriarylmethane color marker dye with a molecular weight of 670 kDa, and has been proposed as an alternative biostain for chromovitrectomy. An experimental in vitro study showed that BroB can stain the ILM and did not induce RPE cell toxicity at concentrations of 0.2 and 0.02% [41, 42]. Moreover in vivo studies in rodent and porcine eyes demonstrated that BroB at concentrations of 0.5 and 0.02% promoted less toxicity when compared to LG, Chicago blue and E68 [42, 43]. Clinically BroB 0.2% was used in patients with epiretinal membrane with good staining affinity and no side effects [44]. The present paper shows that in a wide range of concentrations and exposure times BroB may produce RPE toxicity. A similar effect was also observed in our previous in vivo study where BroB induced significant

Fig. 7. Bax protein expression in ARPE-19 cells following treatment with vital dyes. Confluent ARPE-19 cells were treated with 0.05 mg/ml BBG, LG, ICG, FG, IfCG and CR for 3 min. Total proteins were extracted to assess Bax protein expression by Western blot. GAPDH was used as the internal control. Representative Western blot gel (a) of the proapoptotic protein Bax. The numbers to the left are molecular weights in kilodaltons. b Average densitometry results from 3 independent experiments. Data are means $\pm$ SE and represent the average results of 3 independent experiments run in duplicate. * $p < 0.05$, ** $p < 0.01$: statistical significance versus control.

retina toxicity after intravitreal injections in rabbits [18]. Moreover, its clinical use in macular hole surgery should be investigated with caution.

LG and FG are anionic amino triarylmethanes with a molecular weight of 792 and 809 kDa, respectively. In 1939, Sorsby [45] first applied FG in vitreoretinal surgery for retinal break identification after intravenous injection. In vitro and in vivo studies showed that FG did not induce remarkable retinal toxicity [18, 46]. With regard to LG, Haritoglou et al. [42] found in their in vitro study no toxic effect on ARPE-19 and primary RPE cell lines. Our group also showed that no remarkable histological and functional retinal abnormalities were observed in a rabbit model with LG [18]. The present study showed that FG was toxic to the ARPE-19 cell line, but in contrast LG did not induce reduction in cell viability in all con-

centrations after 3 min of exposure. However, after 30 min, both FG and LG were toxic in all tested concentrations.

Azo dyes constitute a group of dyes containing an azo chemical group linked to benzene, naphthalene, or aromatic heterocyclic rings. EB and CR are part of this group with a molecular weight of 960 and 696 kDa. 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 [47]. 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 the ILM [46]. In contrast, an *in vivo* investigation showed that EB caused severe functional and morphological retinal toxicity at the higher dose of 0.5% [18]. The current study showed that EB could reduce RPE cell viability in all experiments conducted. Based on these observations, EB at least at high doses should not be considered for human application in chromovitrectomy. CR on the other hand did not induce RPE cell toxicity in concentrations up to 0.05 mg/ml. Besides a safer profile, CR is not a good candidate for ILM staining due to its poor contrast with the red fundus background color.

IC, with 466 kDa, is part of the thiazine dyes, which are small and cationic molecules containing a chromophore called thiazinium. Our group was the first to evaluate IC for vitreoretinal surgery, where it showed overall good safety about the retina [18]. However, the present *in vitro* analysis showed that IC might be toxic to RPE cells in all tested concentrations and exposure times. Such findings may indicate that IC and possibly other thiazine dyes may be useful in chromovitrectomy, but staining affinity issues and avoiding the contact with RPE cells should be considered.

BBG is a synthetic triarylmethane, in the same group as BroB, and has a molecular weight of 854 kDa. The safety profile of BBG was investigated by Enaida and Ishibashi [48], and later evaluated by other groups in preclinical and clinical experiments. BBG emerged as the first real safe alternative for ICG and IfCG in chromovitrectomy due to its remarkable affinity for the ILM, and no significant toxicity findings have been reported in uneventful retinal surgeries. The current study showed that BBG is safe to RPE cells up to 0.25 mg/ml after 3 min and up to 0.005 mg/ml after 30 min of exposure.

Based in our viability data and since it was shown that residual ICG can be found months after surgery, the eval-

uation of apoptosis in retinal toxicity studies of this dye has become an important issue [49]. Therefore, the present study also evaluated the link between dye exposure and apoptosis in RPE cells by studying Bax protein, which is involved in controlling apoptosis events [50]. The present study shows that ICG remarkably induced Bax expression after 3 min of exposure at 0.05 mg/ml, a common concentration still in use by some surgeons. This upregulation of Bax protein was also noticed after exposure to LG, FG, IfCG and CR (fig. 7). In contrast, BBG had Bax expression similar to control, BSS. Balaiya et al. [37] also showed similar results, where ICG but not BBG induces apoptosis in retinal cells.

One limitation of this study is that the results of this paper should not be extrapolated to entire retina toxicity induced by the dye exposure. The present paper evaluated dye toxicity and induction apoptosis only in an immortalized RPE human cell line (ARPE-19). Besides this drawback, this study design is necessary since direct dye-RPE contact may occur during macular hole surgery and it may induce RPE defects and vision loss.

In summary, the safety profiles of 9 different dyes were compared. The safest dye with regard to RPE cells was BBG followed by LG, IfCG, FG, CR, IC, BroB, EB and ICG. Moreover ICG had the highest Bax expression, indicative of apoptosis cascade activation. On the other hand, BBG was the safest dye and the only one that did not induce apoptosis in our experiments. Based on the literature and supported by our results, BBG seems to be the best alternative for ICG in vitreoretinal surgery. Indeed, it has been postulated that BBG could have a protective effect on retinal tissue, but it should be further investigated [51].

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Disclosure Statement

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