

An in vivo evaluation of Brilliant Blue G in animals and humans

M Remy,¹ S Thaler,² R G Schumann,¹ C A May,³ M Fiedorowicz,^{2,4} F Schuettauf,² M Grüterich,¹ S G Priglinger,¹ M M Nentwich,¹ A Kampik,¹ C Haritoglou¹

¹ Department of Ophthalmology, Ludwig-Maximilians-University, Munich, Germany; ² Center for Ophthalmology, University Eye Hospital, Tübingen, Germany; ³ Department of Anatomy, Technische Universität, Dresden, Germany; ⁴ Department of Experimental Pharmacology, PAS Medical Research Center, Warsaw, Poland

Correspondence to: Dr C Haritoglou, Department of Ophthalmology, Ludwig-Maximilians-University, Mathildenstr. 8, 80336 Munich, Germany; christos.haritoglou@med.uni-muenchen.de

MR and ST contributed equally to this work.

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ABSTRACT

Background/Aims: To evaluate the retinal toxicity of Brilliant Blue G (BBG) following intravitreal injection in rat eyes and examine the biocompatibility and the staining properties in humans.

Methods: BBG was injected into the 11 rat eyes to evaluate toxic effects with balanced salt solution (BSS) serving as control. Retinal toxicity was assessed by retinal ganglion cell (RGC) counts and by light microscopy 7 days later. In addition, BBG was applied during vitrectomy for macular hole (MH) (n = 15) or epiretinal membranes (ERM) (n = 3) in a prospective, non-comparative consecutive series of patients. Before and after surgery, all patients underwent a complete clinical examination including measurement of best corrected visual acuity (VA) and intraocular pressure, perimetry, fundus photography and optical coherence tomography. Patients were seen 1 day before surgery and then in approximately four weeks intervals.

Results: No significant reduction in RGC numbers and no morphological alterations were noted. A sufficient staining of the internal limiting membrane (ILM) was seen in patients with MH, while the staining pattern in ERM cases was patchy, indicating that parts of the ILM were peeled off along with the ERM in a variable extent. All MHs could be closed successfully. VA improved in 10 eyes (56%; 8/15 MH patients, 2/3 ERM patients), was unchanged in four eyes (22%; all MH patients) and was reduced in four eyes (22%; 3/15 MH, 1/3 ERM). No toxic effects attributable to the dye were noted during patient follow-up. The ultrastructure of tissue harvested during surgery was unremarkable.

Conclusion: Brilliant Blue provides a sufficient and selective staining of the ILM. No retinal toxicity or adverse effects related to the dye were observed in animal and human studies. The long-term safety of this novel dye will have to be evaluated in larger patient series and a longer follow-up.

Vital dyes have become very popular to visualise barely visible structures such as the internal limiting membrane (ILM) during macular hole (MH) surgery using Indocyanine Green (ICG),¹ epiretinal membranes (ERM) using Trypan Blue (TB)² and the vitreous, which can be visualised using Bromophenol Blue.³ The main advantages of ICG were the good contrast provided and its selective staining properties. However, at present the “off label” intravitreal application of ICG has become a subject of ongoing discussion, as toxic effects of the dye leading to functional impairment of the patients cannot be ruled out.^{4–7} TB appears to be safe for intraocular application, but provides a far less pronounced staining of the ILM and lacks the

selective staining properties of ICG and requires a fluid–air exchange to achieve high enough concentration on the retinal surface. The latter may be overcome by special TB preparations.⁸ Recently, Enaida and co-workers introduced Brilliant Blue G (BBG) as an alternative for ILM peeling.^{9–11} The purpose of this investigation was to assess potential retinal toxicity of BBG in a well-established¹² animal model and to evaluate the staining properties and safety profile in a prospective series of patients undergoing MH and ERM surgery.

MATERIALS AND METHODS

Animal study

Animals

All experiments were performed in accordance with guidelines for animal care in the European Community and those of the Association for Research in Vision and Ophthalmology. Male Brown Norway rats (Charles River, Sulzfeld, Germany) with a body weight of 120–160 g were used. Rats were housed under a 12 h light–dark cycle.

Intravitreal dye injections

Eleven adult male Brown Norway rats were anaesthetised with an intraperitoneal injection of chloral hydrate (7%, 6 ml/kg body weight). Intravitreal BBG (Brilliant Peel; Fluoron GmbH, Neu-Ulm, Germany/306 mOsm/kg H₂O; pH = 7.52) injections were performed using a heat-pulled glass capillary connected to a microsyringe (Drummond Scientific, Broomall, PA), under direct observation through the microscope. Eyes with lens damage were excluded from the experiments and not used thereafter. Single injections of 2 µl of BBG (0.25%) were performed, reaching a final intraocular BBG concentration of 0.025% (estimated for an average rat eye intravitreal volume of 20 µl). Contralateral eyes served as control eyes and were injected with balanced salt solution (BSS).

Retrograde labelling and quantification of retinal ganglion cells (RGC)

RGC survival was assessed as described previously.¹² In brief, anaesthetised rats were placed into a stereotaxic frame (TSE systems, Bad Homburg, Germany). A portion of the skull and the meninges were removed, exposing the neocortex overlying the tectum on the side contralateral of the lesioned eye. Three injections of the fluorescent tracer Fluorogold (Hydroxystilbamidine methanesulfonate, Molecular Probes, Eugene, OR) were made over a period of

2 min each targeting all layers of both superior colliculi. Two days later, the animals were sacrificed with a choral hydrate overdose. Eyes were enucleated, retinas dissected, flat-mounted, and fixed in 3% paraformaldehyde (PFA) for 30 min. Visualisation was performed immediately under fluorescent microscope. Before enucleation the cornea was marked at the 12 o'clock position for orientation in retinal quadrants. The RGC-counting was carried out in 12 distinct areas of 62 500 μm^2 . Images obtained using a digital imaging system (ImagePro 3.0, Media Cybernetics, Silver Spring, MD) connected to a microscope were coded and analysed in a masked fashion. Labelled cells were defined as surviving. Counts are expressed as the cell density (cells per square millimetre).

Statistical analysis was performed using a Student t test. Differences were considered significant when $p < 0.05$.

Histology

After sacrifice, the eyes of three animals were immediately immersion-fixed in Ito's solution (2.5% glutaraldehyde, 2.5% paraformaldehyde, 10.1% picric acid in 0.1 M cacodylate buffer, pH 7.2). The eyes were bisected into nasal and temporal segments, and the specimens 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.

The total thickness of the neural retina and the thickness 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 the single-thickness measurements were obtained within the next 300 μm peripherally. Since there was no statistic difference between the superior and inferior region, the data were pooled for statistical analysis using the Wilcoxon test ($p = 0.01$).

Clinical investigation in humans

Dye application and surgery

The study was approved by the local Institutional review board (IRB) and ethics committee. In a prospective, interventional, non-comparative clinical case series, eyes from consecutive patients with a MH, 13 idiopathic, one myopic and one traumatic MH ($n = 15$) or epiretinal membranes (ERM, $n = 3$) were included between April and September 2007. Macular holes were clinically classified according to Gass.¹³ Concomitant retinal diseases, such as very mild non-proliferative diabetic retinopathy in one patient or mild changes attributed to age-related macular degeneration as seen in three patients, were accepted if the visual acuity (VA) of the non-study eye was not affected. All patients underwent standard 20-gauge three-port pars plana vitrectomy as described earlier.⁶ BBG solution (0.5 mL, 0.25%, Brilliant Peel; Fluoron GmbH, Neu-Ulm, Germany) was injected into the fluid filled globe over the macular area and washed out after a few seconds by irrigation. In patients with ERM, the dye was injected following the removal of the visible part of the ERM. The stained ILM was then grabbed using an end-gripping forceps and peeled off. A 15% hexafluoroethane (C_2F_6) gas mixture was used as a tamponade in MH cases, and patients were encouraged to keep a prone position for 5 days. Tissue removed during surgery was prepared for ultrastructural analysis as described previously.¹⁴ All surgeries were performed by a single surgeon (CH), who also graded the staining of the ILM during surgery as good, weak, variable or absent.

Clinical examination

Before and after surgery, all patients underwent a complete clinical examination including measurement of best corrected VA and intraocular pressure (IOP), colour and red-free fundus photography and optical coherence tomography (OCT) (Stratus OCT, Carl Zeiss Meditec, Jena, Germany). With respect to our previous observations⁶ of peripheral visual field defects, we preferred manual Goldmann perimetry for the assessment of the visual field before and after surgery. However, if during follow-up a complete Goldmann perimetry could not be obtained, we performed an automated perimetry for the 30° visual field, completed by an evaluation of the periphery by manual Goldmann perimetry.

Multifocal electroretinography (MERG) was performed in randomly selected cases ($n = 14$). Patients were seen 1 day before surgery and then in approximately 4-week intervals by one of the authors (MR). While the clinical examination, fundus photography and OCT were performed at each follow-up visit, perimetry and MERG were performed at the first postoperative examination.

Exclusion criteria were ocular diseases such as glaucoma, ischaemic optic neuropathy, any intraocular surgery within the last 60 days of the study eye, age less than 18 years and participation in other clinical investigations.

The treated eye, adverse events as well as the patient's lens status (phakic or pseudophakic), gender, age and treated eye were recorded. OCT was used to support the clinical diagnosis of a MH and evaluate the anatomic configuration of the macular area postoperatively and to measure retinal thickness (μm) in patients with ERMs.

Statistical analysis of pre- and postoperative VA was performed using a two-tailed Wilcoxon signed ranks test for two related samples. Differences were considered significant when $p < 0.05$. Patients' characteristics are summarised in table 1.

RESULTS

Animal study

RGC numbers

Whole mounts with Fluorogold-labelled retinal ganglion cells were photographed 7 days after injections of BBG and injections of balanced salt solution as controls (fig 1). Retinal ganglion cells numbers were then counted (fig 2); numbers are expressed as cell densities. In BBG injected eyes (2129 (61) cells/ mm^2 , mean (SEM), $n = 11$) no statistically significant differences in RGC densities were observed compared with BSS injected eyes (2202 (85), $n = 5$, $p = 0.43$).

Histological evaluation

BBG- and BSS-injected eyes showed normal retinal morphology with no signs of inflammation or degeneration throughout the central and peripheral retina. Quantification of the central retinal layers revealed normal values in the BBG-treated eyes and no statistical differences (table 2).

Clinical investigation in humans

We included 13 women and five men with a mean age of 67 years (SD 10) years in this investigation. We treated 10 right and eight left eyes. The mean follow-up up was 8 (3) weeks (range 4–14 weeks).

Over all patients, the mean VA before surgery was 20/80 (20/200) SD (range from 20/400 to 20/40) and increased not significantly to 20/60 (20/100) SD (range from 20/250 to 20/25)

Table 1 Clinical data of all patients

Case no.	Age (years)	Sex	Eye	Diagnosis	Follow-up (weeks)	VA		Lens status		ERM: retinal thickness in OCT (μm)		MH in OCT (Post)	Visual-field defect	Adverse events	ILM staining
						Pre	Post	Pre	Post	Pre	Post				
1	70	F	R	MH Stage 3	5	20/100	20/100	Phakic	Phakic			Closed	None	None	Weak
2	75	F	R	MH Stage 4	5	20/400	20/200	Phakic	Phakic			Closed	None	None	Good
3	66	F	R	MH Stage 2	7	20/50	20/30	Pseudo-phakic	Pseudo-phakic			Closed	None	None	Good
4	79	M	L	MH Stage 2	5	20/40	20/80	Phakic	Phakic			Closed	None	None	Good
5	41	F	R	MH Stage 4 High Myopia	6	20/60	20/100	Phakic	Phakic			Closed	None	None	Good
6	46	M	L	Traumatic MH	6	20/200	20/200	Phakic	Phakic			Closed rim still visible	None	None	Good
7	68	F	R	MH Stage 2	7	20/60	20/50	Pseudo-phakic	Pseudo-phakic			Closed	None	None	Good
8	67	F	R	MH Stage 3	12	20/100	20/50	Phakic	Phakic			Closed	None	None	Good
9	62	F	L	MH Stage 4	14	20/60	20/60	Phakic	Phakic			Closed	None	None	Good
10	74	F	R	MH Stage 3 ARMD	9	20/100	20/100	Phakic	Pseudo-phakic			Closed	None	None	Good
11	76	F	L	MH Stage 3 DR, ARMD	10	20/200	20/200	Pseudo-phakic	Pseudo-phakic			Closed	None	None	Good
12	77	M	L	MH Stage 2	6	20/60	20/25	Phakic	Pseudo-phakic			Closed	None	None	Good
13	72	F	R	MH Stage 4 ARMD	8	20/100	20/60	Phakic	Phakic			Closed	None	None	Good
14	71	F	L	MH Stage 4	8	20/200	20/250	Phakic	Pseudo-phakic			Closed	None	None	Good
15	65	F	R	MH Stage 3	4	20/200	20/40	Phakic	Pseudo-phakic			Closed	None	None	Good
16	72	M	R	ERM	14	20/50	20/30	Pseudo-phakic	Pseudo-phakic	360	327		None	None	Variable
17	63	F	L	ERM	12	20/50	20/30	Phakic	Pseudo-phakic	336	378		None	None	Variable
18	73	M	L	ERM	8	20/50	20/100	Phakic	Phakic	331	360		None	None	Variable

ARMD, age-related macular degeneration; DR diabetic retinopathy; ERM, epiretinal membrane; F, female; L, left; M, male; ME, macular oedema; MH, macular hole; Post, postoperative; Pre, preoperative; R, right; VA, visual acuity.

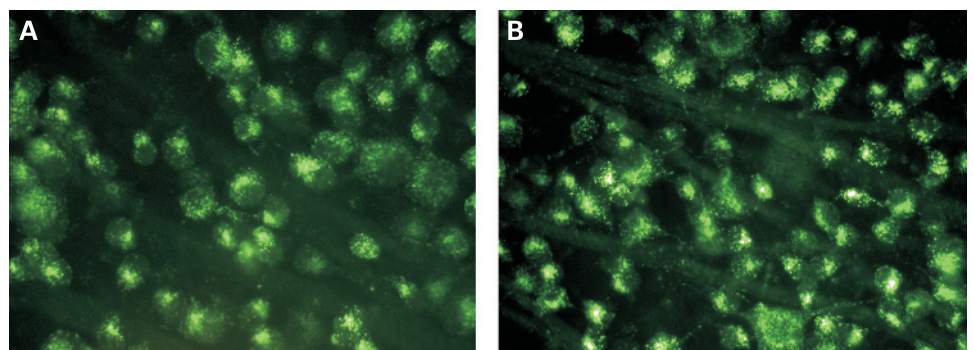
after surgery ($p = 0,094$). Looking at the group of MH patients, the mean VA improved not significantly ($p = 0,090$) from 20/100 (20/200) SD (range from 20/400 to 20/40) to 20/60 (20/100) SD (range from 20/250 to 20/25). VA improved in 10 eyes (56%, 8/15 MH patients, 2/3 ERM patients), was unchanged in four eyes (22%, all MH patients) and decreased in four eyes (28%, 3/15 MH, 1/3 ERM). In MH patients, the lack of visual improvement was partially related to the pathogenesis of the MH such as myopic and traumatic MH (patient 5 and 6) or may be attributed to concomitant diseases such as diabetic retinopathy and age-related macular degeneration (ARMD) (patients 10 and 11) that might have had an influence on the development of visual acuity. Two of the ERM patients (patients 16 and 17) developed a macular oedema seen shortly after surgery with an increase in retinal thickness to 412 μm

and 630 μm respectively and a visual acuity of 20/40 and 20/500. They were therefore treated by retrobulbar steroid injection, which led to a significant improvement of both retinal thickness (327 μm and 378 μm) and visual acuity (20/30 each) (table 1). The IOP was in normal range at any time.

In five eyes, surgery had been performed as a combined procedure of vitrectomy and cataract extraction with posterior chamber lens implantation. Nine eyes were pseudophakic at the last follow-up visit.

All MHs were completely closed as shown by optic coherence tomography or clinical investigation after the first surgical intervention. In one patient (patient 6) with a large traumatic MH, the rim of the was postoperatively attached but still visible.

Figure 1 Fluorogold-labelled retinal ganglion cells photographed 7 days after injections of Brilliant Blue G (A) and injections of balanced salt solution (B) as controls.



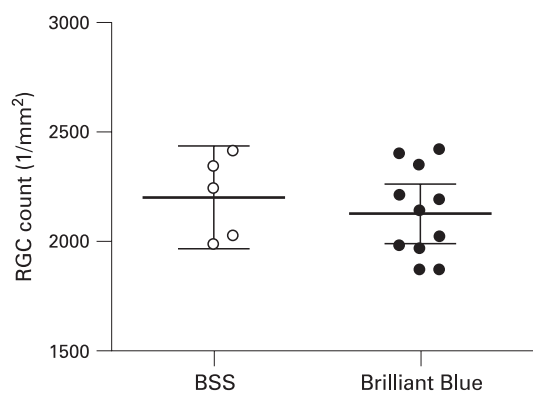


Figure 2 Retinal ganglion cell (RGC) numbers counted and numbers expressed as cell densities. In Brilliant Blue G-injected eyes (2129 (61) cells/mm², mean (SEM), n = 11) no statistically significant differences in retinal ganglion cell densities were observed compared with balanced salt solution (BSS)-injected eyes (2202 (85), n = 5, p = 0.43).

We did not observe any visual field defects or any abnormalities in the other functional tests such as MERG that might indicate toxic effects of the dye. Fundus photographs did not show any abnormal alterations of the retinal pigment epithelium or defects of the nerve fibre layer seen in red-free images. There were no systemic side effects.

Staining of the ILM during MH surgery was successful in 15 cases and allowed for an easy and controlled removal of the ILM with no haemorrhages of the retinal surface indicating surgical trauma (fig 3). In patients with ERMs, the staining of the ILM was variable. This was most likely attributed to the fact that the dyes had been injected after removal of the ERM, indicating that the ILM is peeled off along with the ERM in a variable extent. Transmission electron microscopy confirmed the presence of the ILM in all processed specimens (n = 18). The complete data on our patients are presented in table 1.

Ultrastructure of removed membranes

The ultrastructural analysis revealed fragments of the ILM in all specimens. There was a variable amount of epiretinal cellular proliferation seen at the vitreal side of the ILM without any cellular damage. The nuclei and cytoplasmic elements such as mitochondria or golgi complexes were well preserved. At the retinal side of the ILM, no relevant amount of retinal debris was seen (fig 4)

DISCUSSION

There is common agreement that toxic effects of any new dye suggested to assist intraocular surgery need to be carefully ruled out prior to its routine use in humans. Shortly after ICG had been recommended for ILM peeling,³ a less favourable functional outcome and a high incidence of peripheral visual field defects^{6 15 16} led to a still ongoing discussion on potential toxic effects of ICG which may be related to its photosensitising properties.^{17 18} The safety margin of ICG is unclear, and some

authors did not observe any dye-related complications.^{19 20} A new and promising dye to visualise the ILM may be BBG.^{10 11} First, experimental and clinical data, obtained in animals and humans, addressed the toxicity of BBG following subretinal injection⁹ and revealed a good biocompatibility of BBG in contrast to control dyes including ICG and TB. An experimental study exposing glial cells to ICG and BBG disclosed ICG toxicity as seen by the induction of apoptosis, involving induction of the caspase cascade through p38 mitogen-activated protein kinase phosphorylation while BBG did not cause apoptosis and therefore could be considered a safer adjuvant.²¹ In our own study, the dye was injected intravitreally and therefore had contact to the retinal surface but not the subretinal space as in previous investigations.⁹ The analysis of retinal ganglion cell numbers showed that intravitreal BBG did not lead to a significant change in RGC numbers in rat retina *in vivo*. It is important to note that our experimental setting to assess retinal toxicity does not resemble the situation in vitreoretina surgery exactly. First, the dye is injected into the vitreous gel, which is usually removed in human eyes before applying the dye during vitreoretinal surgery, which could lead to a higher local dose of the dye during vitreoretinal surgery. Second, the exposure time of 7 days in the rat eye is much longer than the intended application in humans, where the dye is washed out immediately by irrigation. However, we chose this setting, as it seems reasonable to assume that a dye leading to no visible toxic effect after 1 week will not show such an effect after 1 minute as well. Although the extended exposure time as chosen here offers additional information on the safety margin of BBG, one has to keep in mind that toxicity studies in animals may not resemble all possible toxic effects in a clinical situation in humans. Therefore, long-term side effects seen in humans during prospective clinical trials cannot be ruled out.

Following the positive results of our investigation in the animal, we decided to use the dye in humans as well. BBG was applied to assist MH surgery and surgery for epiretinal membranes. In MH surgery, the dye provided an excellent contrast of the ILM and allowed for a complete removal of the ILM. We did not observe any functional deficits in terms of visual field defects in our patient as seen following the use of ICG.⁶ No abnormalities of the RPE were seen clinically and in fundus photographs, although the dye may very well have come into contact with the RPE through the MH. The closure rate was comparable with our results obtained without dye assistance.²² VA improvement in MH patients was less pronounced compared with our previous studies where we observed a mean VA of 20/40 postoperatively.²² From our point of view, this is related to the relatively short follow-up in some patients, patient selection including concomitant diseases such as high myopia, trauma, ARMD or DR and the lens status. Despite the relatively short follow-up in some cases, conclusions on the safety of BBG can be drawn as in previous investigations on ICG adverse events such as visual field defects becoming apparent already at the first follow-up visit.⁶ Of note, we did not observe any defects of the nerve fibre layer seen in red-free

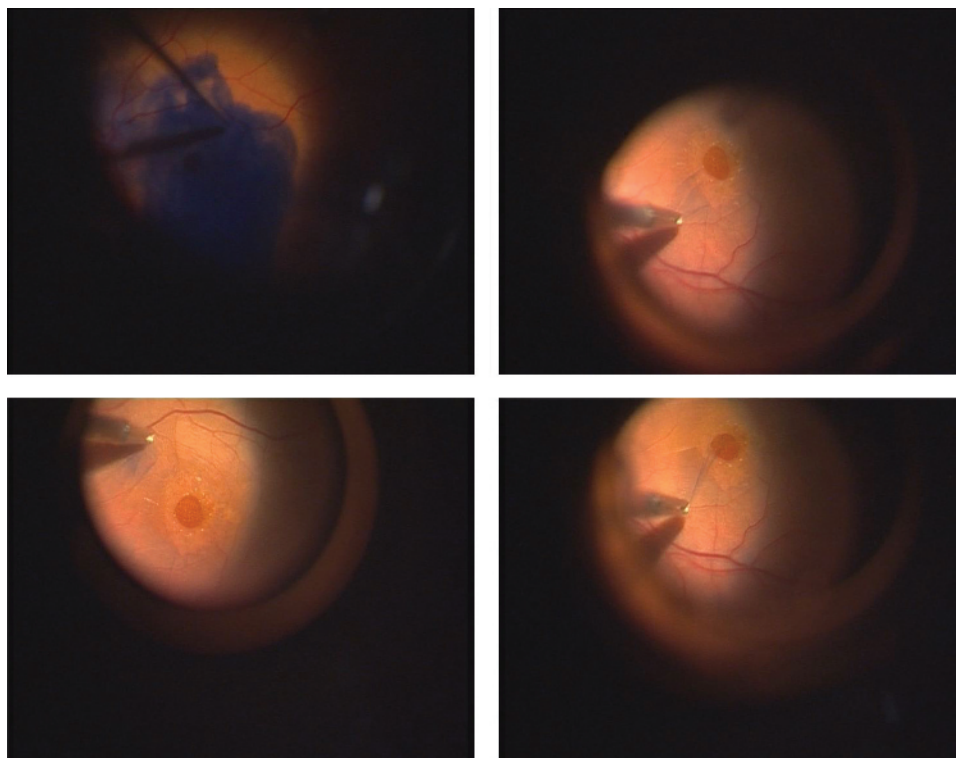
Table 2 Quantification of the central retinal layers

	Total (µm)	NFL (µm)	GCL (µm)	IPL (µm)	INL (rows)	OPL (µm)	ONL (rows)	PR-IS (µm)	PR-OS (µm)
OD, mean (SD)	223 (7)	8.5 (2.9)	12.6 (2.4)	67 (7)	5.2 (0.4)	6.4 (0.9)	9.8 (0.4)	17 (3)	26 (3)
OS, mean (SD)	216 (8)	8.9 (1.4)	13.2 (2.5)	64 (4)	5.2 (0.4)	7.5 (0.9)	9.8 (0.4)	15 (3)	24 (2)

Quantification of the central retina in Brilliant Blue G (OS) and BSS (OD, control) injected eyes.

GCL, ganglion-cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; NFL, nerve-fibre layer; ONL, outer nuclear layer; OPL, outer plexiform layer; PR-IS, photo receptor inner segment; PR-OS, photoreceptor outer segment; Total, whole thickness.

Figure 3 Peeling of the Brilliant Blue G-stained internal limiting membrane in a patient with a traumatic macular hole (patient 6).



fundus photographs as described in previous publications on ILM peeling without any dye.²³ These asymptomatic defects had been additionally confirmed by microperimetry as central visual field defects and were very likely correlated to a mechanical, focal trauma of the innermost retinal layers by ILM peeling. One may conclude therefore that BBG-assisted ILM peeling is less traumatic to the nerve fibre layer.

During surgery for epiretinal membranes BBG was injected following the removal of the visible epiretinal membrane using an end-gripping forceps in order to visualise fragments of the ILM

which were not removed along with the epiretinal membrane. As a consequence, the staining pattern was not as homogenous as seen during MH surgery, but provided a more patchy aspect. As during MH surgery, the ILM could be easily removed without damage to the underlying retina and with no toxic effects of the dye seen postoperatively. However, during all surgical procedures, the surgeon noted an increased rigidity of the stained ILM compared with previous surgeries without BBG application.

Our data underline the good biocompatibility of BBG and its applicability and safety for the use in humans. BBG provides a sufficient and selective staining of the ILM. No retinal toxicity related to BBG was observed in our animal study and our short-term clinical investigation in humans. As mentioned earlier, we feel that it is of great importance to collect as much safety data as possible on a new dye suggested for intraocular surgery in humans. The long-term safety of this novel dye will have to be evaluated in larger patient series and a longer follow-up.

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Competing interests: None.

Ethics approval: The study was approved by the local Institutional review board (IRB) and ethics committee.



Figure 4 Ultrastructure of the internal limiting membrane removed during Brilliant Blue G-assisted macular hole surgery. Note the absence of retinal debris at the retinal (rough) surface of the internal limiting membrane. Magnification 1800 \times .

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