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LABORATORY STUDY

The structural effect of intravitreal Brilliant blue G and Indocyanine green in rats eyes

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Abstract

Purpose **To compare the potential retinal toxicity of two commercially Brilliant blue G dyes (Brilliant Peel and Ocublue Plus) and Indocyanine green (ICG) at usual clinical concentration.**

Methods **Brilliant Peel 0.025% ($n=9$), Ocublue Plus 0.025% ($n=9$), and ICG 0.05% ($n=9$) were injected intravitreally into Sprague–Dawley rat left eyes with balanced salt solution injected in the contralateral eyes as control. Evaluation of the effect of the dyes on retinal architecture was done by histological analysis of neurosensory retinal thickness and retinal ganglion cell (RGC) counts 7 days after intravitreal injection. Paired *t*-test was done to detect the presence of biologically significant thinning in neurosensory retina and five retinal layers for each dye (paired *t*-tests). One-way ANOVA and Tukey's Honestly Significant Difference test were used to assess whether different dyes caused significant thinning in mean neurosensory retinal thickness and reduction of mean RGC density.**

Results **Eyes treated with ICG had significantly thinner mean total neurosensory retinal thickness compared with the control eyes (P-value $=0.01$), followed by those treated with Ocublue Plus (P-value $=0.03$). Brilliant Peel did not cause significant thinning in any of the five retinal layers (all P-values >0.05). No significant difference in mean thinning of the total retinal thickness was detected between dyes (P-value $=0.11$). The mean thickness of the photoreceptor outer segment and outer plexiform layers were significantly reduced in ICG-injected eyes when compared with the control eyes (P-value $=0.02$). No significant difference in mean thinning between the three dyes was detected at all five retinal layers using one-way ANOVA (all P-values >0.35). RGC density was significantly reduced for ICG (P-value $=0.01$) but only marginally for Ocublue Plus (P-value $=0.05$). No significant reduction in RGC density was observed for Brilliant Peel (P-value $=0.2$).**

Conclusion **Intravitreal Brilliant Peel is safe to rats retina. The retinal thinning and reduction in RGC density induced by Ocublue Plus requires further studies to determine the safety profile of this product. Potential retinal toxicity is seen with ICG 0.05%.**

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Keywords: Brilliant blue G; Brilliant Peel; intravitreal; Indocyanine green; Ocublue Plus; retinal ganglion cells

Introduction

The advances in surgical technique have allowed macular hole to be treated effectively and removal of internal limiting membrane (ILM) is the most important step in optimizing the outcome of this condition.[1,2] The ILM is a thin transparent membrane, which is difficult to visualize during vitreoretinal surgery.[3] Therefore, the use of Indocyanine green (ICG) and more recently Brilliant blue G (BBG) as vital dyes to stain the ILM has become very popular. Recent studies have reported that BBG is similar to ICG in the case of ILM peeling during macular hole surgery.[4]

ICG is approved for intravenous use while its intravitreal application represents an off-label use. It can be found under different brands such as ICG-Pulsion (Pulsion Medical Systems, Feldkirchen, Germany; 25 and 50 mg vials); ICV Indocianina Verde (Ophthalmos, Sao Paulo, Brazil; 5, 25, and 50 mg vials); Diagnogreen (Daiichi Sankyo, Tokyo, Japan; 25 mg vial);

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IC-Green (Akorn Inc., Lake Forrest, IL, USA; 25 mg vial) and Aurogreen (Aurolab, Madurai, Tamil Nadu, India; 25 mg vials). It has been used for almost a decade because it exhibits a preferential affinity to ILM and facilitates its removal.[5] Although its staining characteristics are undisputed, conflicting reports regarding its complications and functional outcome at 0.05% concentration have been documented in clinical studies.[6,7,8,9,10,11]

BBG has been approved by the European Union for commercial use as Brilliant Peel (Fluoron/Geuder, Heidelberg, Germany). The other commercially available BBG in the market is Ocublue Plus (Aurolab). The BBG concentration in Brilliant Peel and Ocublue Plus is 0.025% and 0.05% w/v, respectively. In our study, the BBG concentration for Brilliant Peel and Ocublue Plus was standardized to 0.025% because at present only Brilliant Peel has been approved by the European Union to be used commercially and the approved concentration of BBG by EU is 0.025%.

Preclinical and clinical studies using Brilliant Peel have shown that it is non-toxic to rats and human retinas.[4,12,13,14,15] Ocublue Plus has already been widely used clinically but its complications are unknown as there has not been any documented preclinical and clinical studies using it.

The purpose of this study is to compare the potential retinal toxicity of Brilliant Peel, Ocublue Plus, and ICG at the usual clinical concentration in a well-established *in vivo* animal experimental setting using histologic evaluation of the retina and retinal ganglion cell (RGC) quantification.[13]

Materials and methods

Animal

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. Eight-week-old adult female Sprague–Dawley rats with body weight 200–250 g were housed in the animal laboratory under a 12-h light–dark cycle. Each of the three treatment groups: Brilliant Peel (BBG 0.025%, 306 mOsm), Ocublue Plus (BBG 0.025%, 208 mOsm), and ICG (0.05%, 290 mOsm) received nine experimental rats. A subset of the rats in each of the three treatment groups (Brilliant Peel, $n=6$; Ocublue Plus, $n=6$; and ICG, $n=5$) were randomly picked for retrograde labeling and quantification of RGCs. The remaining rats were used for investigation of retina histology.

Intravitreal injection

Adult female Sprague–Dawley rats with body weight 200–250 g were anesthetized with an intraperitoneal injection of chloral hydrate (6 ml of a 7% solution/kg body weight). Then, using a Hamilton microsyringe (Drummond Scientific, Broomall, PA, USA) under direct observation through the microscope, 2 μl of dye was injected intravitreally into the left eye of each rat. This was done at a distance of 1.5 mm from the limbus to avoid damaging the lens. The dye was not washed out after the intravitreal injection. The contralateral right eyes that served as control eyes were injected with 2 μl of balanced salt solutions. Animals with any type of eye disease were excluded from the experiment.

Retrograde labeling and quantification of RGCs

RGC survival was assessed as described previously.[16] The rats were anesthetized with an intraperitoneal injection of chloral hydrate (6 ml of a 7% solution/kg body weight) and were then placed into a stereotaxic frame (TSE Systems, Bad Homburg, Germany).

The head fur was shaved from the eye to the ear level, which was then disinfected with 10% povidone iodine solution followed by 70% alcohol. Using a blade size 15, a longitudinal incision was made over the shaved area skin measuring about 2 cm. The skin was reflected and the periosteum removed to expose the skull and the sutures. Hemostasis was secured with cotton buds. The bregma that is the intersection point between the coronal and sagittal sutures was identified. The location of the superior colliculus was located 6 mm posterior to the bregma and 1.5 mm lateral to the sagittal sutures on either side. These locations were then drilled using a stereotaxic driller (KOPF, Lidingö, Sweden, 323 IN) on each side of the sagittal sutures.

Two microliters of 2% Fluorogold (Fluorogold; Fluorochrome, Inc., Denver, CO, USA) diluted with sterile water was injected into both superior colliculi, 4 mm from the skull over a period of 2 min using a 5 μl syringe (Hamilton, Reno, NV, USA).[16] Then, the skin was closed with Ethilon 6-0 sutures and topical antibiotic cream was applied to the wound.

The rats were killed by chloral hydrate overdose 2 days later. The cornea was marked at the 12 o'clock position for orientation of the retinal quadrants before the eyes were enucleated. After enucleation, the eyes were fixed in 2% PFA for 1 h. Then, the retinas were dissected and flat mounted on gelatin coated slides with cover slips over the retinas.

Observation was performed immediately under a fluorescence microscope (Nikon Eclipse E400, Kawasaki, Japan) attached with Fluorogold filters (Chroma Tech Corp., Bellows Falls, VT, USA). The RGC was counted in 12 distinct areas of 62 500 μm^2 per retina. Images were obtained using a digital imaging system connected to the microscope, coded, and analyzed semiautomatically in a

masked manner by an independent observer using a computer-assisted image-analysis system (ImagePro 7.0; Media Cybernetics, Silver Spring, MD, USA). The labeled cells were defined as surviving and counts were expressed as cell density (cells per square millimeter).

Histology

The rats were killed with chloral hydrate overdose 7 days after the intravitreal dye injection. The eyes were immediately enucleated and immersion-fixed for 7 days in Ito's solution containing 2.5 glutaraldehyde, 2.5% paraformaldehyde (wt/vol; PFA), and 0.01% picric acid in 0.1 M cacodylate buffer (pH 7.2).

The eyes were bisected at the equator to separate the anterior and posterior segments. The posterior half was then bisected into nasal and temporal segments. The specimens were postfixed in 1% osmium tetroxide, rinsed in cacodylate buffer, dehydrated in an ascending series of alcohol solutions, and embedded in araldite. Semithin sections (4 mm $\times$ 1 mm) 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 five different retinal layers: photoreceptor outer segment (PROS), photoreceptor inner segment, outer nuclear layer, outer plexiform layer (OPL), and inner nuclear layer (INL) were quantitatively evaluated at the superior and inferior quadrants of the central retina in each eye. The measuring field was defined by a distance of 200 μm from the optic nerve head rim and the single thickness measurements (5–10 per eye) were obtained within the next 300 μm peripherally.[13]

Statement of ethics

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during this research.

Statistical analyses

For each dye treatment, we first computed the difference in total thickness of the neural retina and the thickness of each of the five retinal layers between the treatment and control (treatment – control) eyes at the superior and inferior quadrants. We then combined information from both quadrants by taking the average of these two values. Paired *t*-test was used to test for mean thinning and also dye toxicity for RGC density. For testing overall equality of the mean thinning as well as mean of the difference of average RGC between the three dye treatments, one-way ANOVA was performed. A significant result (P-value <0.05) was followed-up with Tukey's Honestly Significant Difference (HSD) test, which we used to assess the statistical significance of each pairwise comparison.[17] All statistical analyses were done using R version 2.13.1 (http://cran.r-project.org/bin/windows/base/old/2.13.1/).

Results

Histological analysis

ANOVA result indicated that the dyes did not appear to have significantly different mean change in thickness between themselves (P-value $=0.11$). The mean of total retinal thickness of the treatment eye for all dyes was generally less than the control's (Figure 1). Against the control eye, ICG induced the largest magnitude of thinning of the mean total retinal thickness ($-13\,\mu$m; 95% CI: (-20, -6); P-value $=0.01$), followed by Ocublue Plus (–8 μm; 95% CI: (-15, -2); P-value $=0.03$), and Brilliant Peel ($-7\,\mu$m; 95% CI: (-13, 0); P-value $=0.05$).

Table 1 shows the estimated amount of mean difference of five retinal segments between treatment and control eyes, for each dye treatment. For Brilliant Peel and Ocublue Plus, no statistically significant thinning was observed at all layers. For ICG, significant thinning was observed at the PROS ($-2.5\,\mu$m) and OPL ($-1.6\,\mu$m). One-way ANOVA failed to detect significant differences in mean thinning between the dyes (P-value >0.35 for all five retinal segments).

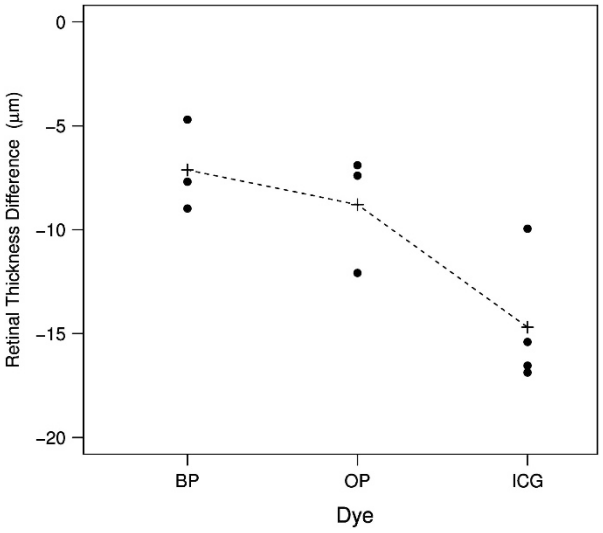

Figure 1 Distribution of difference in retinal thickness between control and treatment eyes (treatment – control) at the superior and inferior quadrants of the retina, according to dye treatment. Within each dye treatment, each dot represents difference between treatment and control eyes from one rat. The mean of each dye treatment is indicated by a cross. BP, Brilliant Peel; OP, Ocublue Plus.

Table 1 Difference of mean thickness between control and treatment (control–treatment) for each of the five retinal layers, with corresponding 95% CI given

Treatment	*Different retinal layers*				
	PROS	*PRIS*	*ONL*	*OPL*	*INL*
Brilliant Peel	−0.8 (−6.2, 4.5)	−0.9 (−5.8, 4.0)	−1.4 (−6.5, 3.7)	−0.1 (−4.2, 4.3)	−1.9 (−4.7, 0.9)
Ocublue Plus	−2.4 (−5.3, 0.5)	−1.4 (−6.3, 3.6)	−2.6 (−5.4, 0.3)	−0.6 (−2.6, 1.5)	−1.5 (−8.4, 5.3)
ICG	−2.2[a] (−3.8, −0.6)	−2.0 (−6.6, 2.7)	−1.0 (−4.9, 2.9)	−1.2[a] (−2.1, −0.3)	−2.8 (−6.4, 0.8)

Abbreviations: ICG, Indocyanine green; INL, inner nuclear layer; ONL, outer nuclear layer; OPL, outer plexiform layer; PROS, photoreceptor outer segment; PRIS, photoreceptor inner segment.
[a]Significant *P*-value of 0.02.

Figure 2 shows that Brilliant Peel and BSS-injected eyes presented normal retinal morphology with no apparent signs of inflammation or degeneration throughout the central and peripheral retina. Ocublue Plus-injected eyes showed thinning of the retina but normal retinal morphology with no apparent signs of inflammation. ICG-injected eyes showed global thinning of central retina when compared with the control group. There were no apparent signs of inflammation between the photoreceptors layer.

RGC count

Figure 3 shows whole mounts with Fluorogold-labeled RGC photographed 7 days after injections with Brilliant Peel, Ocublue Plus, ICG, and BSS. Figure 4 shows the distribution of difference in mean RGC density between the control and treatment eyes within each dye treatment. There was no statistically significant difference in the mean of average RGC density between control and treatment eyes (treatment – control) for the Brilliant Peel treatment (difference in mean = −3 cells/mm^2; 95% CI: (−8, 2); *P*-value = 0.2). For Ocublue Plus, the result was equivocal because a borderline *P*-value was observed (difference in mean = −8 cells/mm^2; 95% CI: (−16, 0); *P*-value = 0.05). For the ICG treatment, a large difference of −17 cells/mm^2 was detected (95% CI: (−28, −6); *P*-value = 0.01).

One-way ANOVA results indicated that at least one of the dye treatments had a significantly different mean RGC density (*P*-value = 0.02). Tukey's HSD test identified the comparison between ICG and Brilliant Peel as being statistically significant; their estimated difference in mean RGC density was −14 cells/mm^2 (95% family-wise CI: (−26, −3); *P*-value = 0.02). This magnitude of difference is large and hence biologically significant. For the ICG–Ocublue comparison, the estimated difference in mean RGC density was −9 cells/mm^2 (95% family-wise CI: (−21, 2); *P*-value = 0.12); for the Brilliant Peel–Ocublue Plus comparison, it was 5 cells/mm^2 (95% family-wise CI: (−6, 16); *P*-value = 0.49).

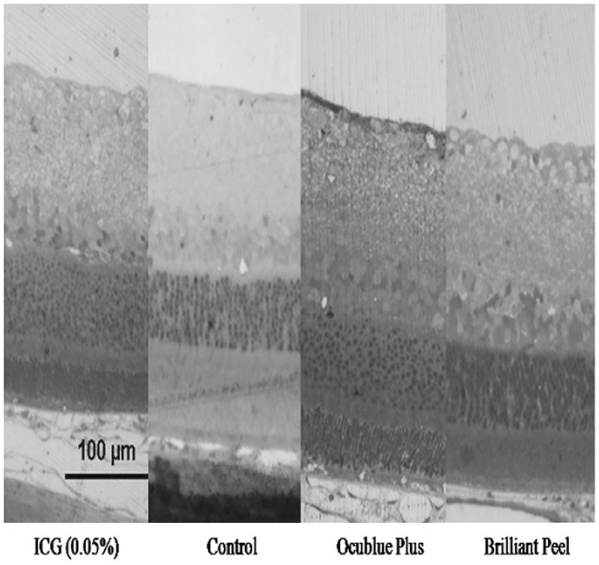

Figure 2 Representative micrographs of the central region of the retina. Scale bar: 100 μm.

Discussion

This is a pilot study comparing Brilliant Peel, Ocublue Plus, and ICG on retinal toxicity in an *in vivo* rat model. The result of this study in the Brilliant Peel (0.025% BBG) group is comparable to other *in vivo* animal[13,14] and *in vitro*[18] studies. Moreover from various human studies, Brilliant Peel did not demonstrate adverse effects or retinal toxicity in patients undergoing macular hole surgery. Their results showed improved visual acuity in 56–85% of patients.[4,10,15]

Ocublue Plus has only been recently used clinically as an adjuvant in macular hole surgery. Its complications are unknown as there have not been any documented preclinical and clinical studies using it. Therefore, our rat model represents the first *in vivo* animal evaluation of retinal toxicity comparing Ocublue Plus with Brilliant Peel to determine its toxicity to the retina. The result of this study showed that Ocublue Plus (0.025% BBG) did not cause significant decrease in mean RGC density when compared with the control group. In addition,

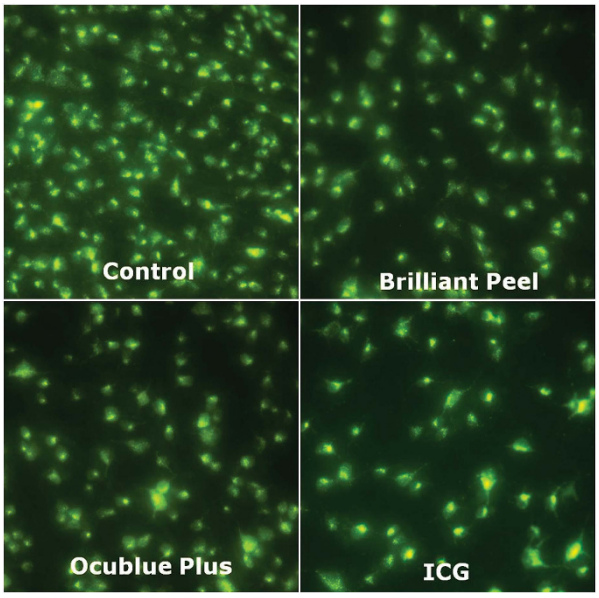

Figure 3 RGCs of rat retinas 7 days after intravitreal injection.

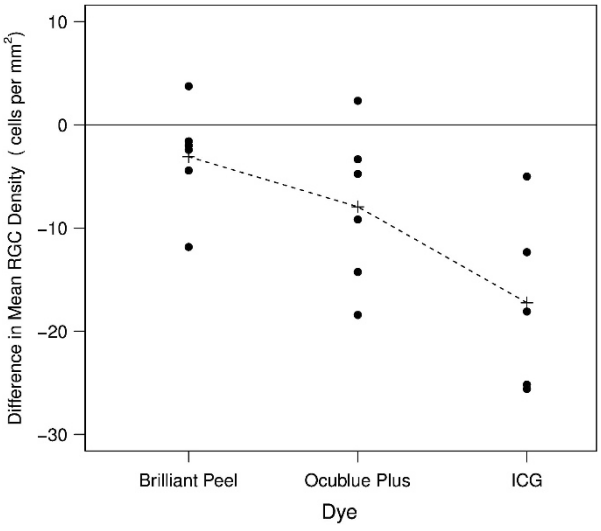

Figure 4 Distribution of difference in mean RGC density between treatment and control eyes (treatment – control) in each dye treatment. The mean value of each dye treatment is indicated by a cross.

Tukey's HSD test did not detect any significant difference in mean RGC density difference between Ocublue Plus and Brilliant Peel (*P*-value = 0.49).

We also did not detect any significant thinning in all five retinal layers for Ocublue Plus (all *P*-values > 0.05). However, the reduction in mean total neurosensory retinal thickness induced by Ocublue Plus was significantly greater than that of Brilliant Peel when compared with their controls. We postulate that the difference in the magnitude of mean retinal thickness reduction between Ocublue Plus and Brilliant Peel when compared with their controls was due to the difference of osmolarity between them (Ocublue Plus 208 mOsm, Brilliant Peel 306 mOsm). Although there seemed to be significant thinning of total retinal thickness against the control group ($-8\,\mu$m; *P*-value = 0.03) for Ocublue Plus, the magnitude of thinning was close to that observed in Brilliant Peel ($-7\,\mu$m). Moreover, significant mean thinning of total neurosensory retina layer in Ocublue Plus and Brilliant Peel was not detected by ANOVA (*P*-value = 0.11).

Rodrigues and Meyer[11] postulated that a concentration of 0.5 mg/ml (0.05%) or less of ICG should be used during vitreoretinal surgery. However, our results show that ICG 0.05% is potentially toxic to the rats retina because it caused significant thinning to the total neurosensory retina and reduction in the RGC densities when compared with the control group. Specifically, ICG preferentially affects the PROS and OPL layers. Our result concurs with the study by Creuzot-Garcher *et al*[14] where they reported a 65% reduction in the a-wave (which corresponds to the photoreceptor layer) and 63% reduction in the b-wave (the majority of which is contributed by the INL layer) 1 month after injection of ICG 0.05%. Furthermore, a clinical study by Gass *et al*[6] using ICG 0.05% for 1 min during vitrectomy for macular hole surgery reported visual field defects in 50% of patients.

Although it is reasonable to expect that ICG and Brilliant Peel should differ significantly in mean change of retinal thickness, on account of ICG's well-known toxicity and Brilliant Peel's relative safety, we did not manage to show this. Tukey's HSD test for pairwise comparisons of dye treatments showed that mean change in thickness between ICG and Brilliant Peel comparison differed somewhat substantially ($-7\,\mu$m; 95% family-wise CI: (−15, 2); *P*-value = 0.11), but was short of achieving the desired statistical significance.

The exact mechanisms underlying ICG toxicity are not fully understood. Direct toxicity, upregulation of apoptosis-related genes,[19] hypoosmolarity of the solvent,[20] intraoperative retinal illumination,[21,22] and the role of sodium were hypothesized as possible factors that could increase retinal ICG toxicity.[23,24]

There are several limitations in our study. Fluorogold has been used for decades as the standard dye for retrograde labeling of RGCs.[16] However, this method alone does not permit to differentiate between viable and dead cells, because dying cells only avoid being counted once they have undergone complete microglial phagocytosis. Grieshaber *et al*[25] demonstrated that staining of fluorogold pre-labeled RGCs with calcein–acetoxymethylester was able to differentiate between viable and dead RGC. Thus, the amount of viable RGC in our study could be underestimated by retrograde labeling of fluorogold only. Electroretinogram or visual evoked potential were not used to assess the retinal

function after intravitreal injection in the rats eyes. The experimental procedure does not fit in with usual human intraocular surgery situation where endoocular illumination is used and the dye is in contact with the retina for <1 min.

It is important to note that our *in vivo* animal model study does not resemble the exact clinical situation during vitreoretinal surgery. First, the dye was injected into the vitreous cavity of the rat without the vitreous having been removed. In human eyes, the dye is injected into the fluid or air-filled vitreous cavity after vitrectomy. Therefore, a higher dose of dye may be in contact with the retina during vitreoretinal surgery. Thus, there theoretically may be toxicity in the clinical situation that is not observed in our animal model.[26]

The dye remained within the rat eyes for 7 days, which exceeded the usual timeframe relevant for vitreoretinal surgery and therefore does not mimic the intraoperative situation in humans where the dye is applied for approximately 1 min and then washed out completely by irrigation with BSS. In human vitreoretinal surgery, the dye is left in the human eye for <1 min before it is being washed out. Therefore, it seems reasonable to assume that a dye that has not affected the intraocular structures after a period of 7 days is unlikely to have a histological and functional effect on the human retina after a period of 1 min.[26]

In conclusion, we demonstrated that intravitreal Brilliant Peel is safe to rats retina. Ocublue Plus caused thinning to the total neurosensory retina and reduction of the RGC density. These findings therefore, require further studies to determine the safety profile of this product. Our results showed that ICG at 0.05% is potentially toxic to rats retina.

Summary

What was known before

- Brilliant Peel did not cause significant effect on retinal morphology and reduction in RGCs in rats retina.
- Brilliant Peel did not cause retinal toxicity in clinical studies.
- Conflict of data regarding preclinical and clinical studies using ICG at 0.05%.
- No preclinical and clinical studies have been carried out using Ocublue Plus.

What this study adds

- Brilliant Peel and Ocublue Plus did not cause significant reduction in RGCs in rats retina.
- ICG 0.05% caused significant thinning to the total neurosensory retina and reduction in the RGC densities.
- The findings of Ocublue Plus require further studies to determine the safety profile of this product.
- ICG is potentially toxic at 0.05%.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Author contributions

YLO and MN carried out the experiment. TFK was involved in the data analysis and manuscript preparation. YLO, MN, and KCSF were involved in experimental design, sample collection, grant and ethics applications, and manuscript preparation.

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