Staining Ability and Biocompatibility of Brilliant Blue G

Preclinical Study of Brilliant Blue G as an Adjunct for Capsular Staining

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Objective: To evaluate the effectiveness and biocompatibility of brilliant blue G (BBG) for capsular visualization for continuous curvilinear capsulorrhexis.

Methods: The capsular staining ability of BBG was evaluated at graded concentrations of 10.0, 1.0, 0.5, 0.25, 0.1, and 0.01 mg/mL in enucleated pig's eyes. The biocompatibility of BBG was assessed in rat's eyes for 2 months. The eyes were analyzed using light, fluorescence, transmission electron, and scanning electron microscopy. TUNEL (terminal deoxynucleotidyl transferase–mediated biotin–deoxyuridine triphosphate nick-end labeling) was used to detect apoptotic cells, and endothelial cell counts were analyzed using scanning electron microscopy. The results were compared using indocyanine green and trypan blue.

Results: The BBG improved capsular visualization, and a complete capsulorrhexis could be performed. In the rat model, no apparent toxic effect was observed using biomicroscopy during 2 months. Histologically, BBG showed satisfactory biocompatibility. Apoptotic cell death of the endothelial cells was detected in only the trypan blue group. In contrast to BBG, indocyanine green and trypan blue showed degeneration of corneal endothelial cells using transmission and scanning electron microscopy.

Conclusion: The BBG contributed to better capsular visualization and caused no apparent complications to the corneal endothelium.

Clinical Relevance: The BBG is effective and safe capsular staining for continuous curvilinear capsulorrhexis.

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CREATING A CONTINUOUS curvilinear capsulorrhexis (CCC) in an eye with a white mature cataract can be challenging because it is difficult to distinguish the anterior capsule from the underlying white cortex. Poor visualization of the capsule tends to result in an incomplete or inadequate CCC, which could cause a subsequent capsular tear, vitreous loss, and intraocular lens dislocation. To alleviate this problem, the intraocular administration of dyes for anterior capsule staining to aid in the performance of CCC in eyes with cataracts in which there is poor or no red reflex has become increasingly popular.1,2 Dada et al3 demonstrated that capsular staining facilitates CCC even in immature cataracts and could be a useful adjunct for trainee surgeons. Trypan blue (TB) (0.1% and 0.06%) has already been introduced for capsular staining and has been found to have no apparent toxic effects in vivo.2,4 However, TB has been reported to be toxic to corneal endothelium in vitro in severe conditions.5 Another dye, indocyanine green (ICG), is also frequently used for capsular staining.6,7 McEnerney and Peyman8 described the use of ICG for cell counts in rabbit corneal endothelium and suggested that the dye did not damage living endothelium. However, our group9 reported the potential toxic effects of ICG to retinal cells in 2002, and recently the toxic effects of ICG have been reported in retinal pigment epithelium, ganglion cells, and photoreceptors.10,11 Both TB and ICG, therefore, have been shown to have shortcomings regarding capsular staining. What is now sought is an ideal dye, possessing satisfactory staining ability yet with minimal toxic effects, that can be used to facilitate the successful creation of CCC. In the present study, we investigate a new dye, brilliant blue G (BBG), which stains the anterior capsule at a lower concentration than the other dyes in common use while showing minimal toxic effects. This experimental study reports the effectiveness and biocompatibility of BBG in capsular staining.

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All procedures conformed to the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines for animal care at Kyushu University.

CAPSULAR STAINING ABILITY OF BBG

Pig’s eyes were obtained from a local slaughterhouse and transported to the laboratory on ice. The extraocular muscles and other connective tissues were carefully cut off, and the eyes were placed on the operation chamber. Brilliant blue G is a blue dye (color index No. 42655, C₃H₆N₂O₅S₂Na, and molecular weight 854.0) that is also known as acid blue 90 and Coomasie BBG. The dye has been used for protein staining in biological fields because it binds nonspecifically to almost all proteins. The dye is also often used for gel electrophoresis. The pharmacologic function of the dye remains unconfirmed. There are no reports on the medical use of this dye, but there is a long history of biological use in which no apparent toxic effects have been reported. To our knowledge, this is the first article to examine the toxic effects of BBG used for medical and ophthalmic purposes. The BBG solution was prepared using the following method. Twenty milligrams of BBG (Coomassie Brilliant Blue G 250; Sigma–Aldrich Corp, St Louis, Mo) was dissolved in 10 mL of intracocular irrigating solution (Opeguard-MA; Senju Pharmaceutical Co Ltd, Osaka, Japan) and sterilized using a syringe filter (Minisart; Sartorius AG, Goettingen, Germany). The capsular staining effected by BBG was then examined under the surgical microscope as follows. The anterior chamber was entered through the clear cornea using a 26-gauge needle mounted on a syringe. The BBG (10, 1, 0.5, 0.25, 0.1, and 0.01 mg/mL) was injected onto the anterior capsule through a 26-gauge needle introduced through the same entry. The anterior chamber was immediately irrigated using enhanced balanced salt solution (BSS) (BSS Plus; Santen Pharmaceutical Co Ltd, Osaka, Japan) so that the excessive dye was easily washed out. To deepen the anterior chamber, 1% sodium hyaluronate (Healon; Pharmacia, Uppsala, Sweden) was injected. A CCC was initiated by preparing a small triangular anterior capsular flap using a bent 26-gauge disposable needle mounted on a viscoelastic syringe. The CCC was completed using a cystotome or capsulorrhexis forceps.

BIOCOMPATIBILITY OF BBG

Brown Norway rats (Kyudo, Fukuoka), at postnatal 8 week, were studied in the following manner. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital, and their pupils were dilated with topical 1% tropicamide and 2.5% phenylephrine hydrochloride. The right eye of each rat was examined in the following experiments (N=54). The anterior chamber was entered through the clear cornea using a 30-gauge needle mounted on a syringe, and a single anterior chamber injection was then performed with each dye—BBG (10, 1, 0.5, 0.25, 0.1, and 0.01 mg/mL), ICG (5 mg/mL), and TB (1 mg/mL)—and the control Opeguard-MA solution (n=6 for each). The mean osmolarity of each solution is given in the Table. Osmolarity of Each Dye Solution.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration, mg/mL</th>
<th>Osmolarity, mOsm/kg H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBG</td>
<td>10</td>
<td>310</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>299</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>298</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>298</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>298</td>
</tr>
<tr>
<td>ICG</td>
<td>5</td>
<td>271</td>
</tr>
<tr>
<td>TB</td>
<td>1</td>
<td>316</td>
</tr>
<tr>
<td>Control</td>
<td>(vehicle)</td>
<td>NA</td>
</tr>
</tbody>
</table>

Abbreviations: BBG, brilliant blue G; ICG, indocyanine green; NA, not applicable; and TB, trypan blue.

Light Microscopy

The eyes were fixed in 4% paraformaldehyde and cut in half, embedded in paraffin, deparaffinized in xylene, rehydrated in ethanol, and washed in phosphate-buffered saline (PBS). The 4-µm-thick sections were stained by hematoxylin-eosin and observed by light microscopy (3 per dye, a total of 27 eyes).

Terminal Deoxynucleotidyl Transferase–Mediated Biotin–Deoxyuridine Triphosphate Nick-End Labeling

TUNEL (terminal deoxynucleotidyl transferase–mediated biotin–deoxyuridine triphosphate nick-end labeling) was used to detect apoptotic cell death. Four-micron-thick sections were made from samples fixed in 4% paraformaldehyde and embedded in paraffin. The TUNEL staining was performed on these sections using a fluorescein direct in situ apoptosis detection kit (ApopTag; Intergen Co, New York, NY) according to the manufacturer’s protocols. The sections were then co-stained with propidium iodide (Molecular Probes, Eugene, Ore), thus allowing observation of the cell nuclei by using a fluorescence microscope (Olympus, Tokyo, Japan). Ten sections for each eye specimen were randomly selected and observed (3 per dye, a total of 27 eyes).

Transmission Electron Microscopy

Enucleated eyes and their anterior segments were fixed in 1% glutaraldehyde and 1% paraformaldehyde in PBS (3 per dye, a total of 27 eyes). The anterior segment was then cut in half. The specimens were postfixed in veronal acetate buffer osmium tetroxide (2%), dehydrated in ethanol and water, and embedded in Epon. From these, ultrathin sections were cut and mounted on copper grids. The specimens were observed by using an electron microscope (JEM-100CX; JEOL, Tokyo).

Scanning Electron Microscopy

Enucleated eyes and their anterior segments were fixed in 1% glutaraldehyde and 1% paraformaldehyde in PBS (3 per dye, a total of 27 eyes). The anterior segment was then cut in half. The specimens were postfixed in veronal acetate buffer osmium tetroxide (2%) and dehydrated in ethanol and water. The specimens were then saturated in t-butyl alcohol and dried using a critical point dryer (Eiko, Tokyo). The specimens were then placed on stubs by means of self-adhering carbon tabs and were sputtered with 20-nm-thick gold using an argon plasma.
coater (Eiko). Finally, the endothelial surface of the cornea was studied using a scanning electron microscope (JEM-840; JEOL). A 1-mm² area was analyzed using scanning electron microscopy to find the endothelial cell count, the results of which are given as mean±SD (3 per dye).

**RESULTS**

**CAPSULAR STAINING BY BBG**

The capsular staining ability of BBG was assessed in pig’s eyes using graded concentrations of the dye. The dye stained the anterior capsule homogenously, and the edge of the CCC could be clearly observed under the surgical microscope. The staining deepened as the concentration of the dye increased (from 0.25 to 1 mg/mL) (Figure 1), and the minimal concentration needed to produce high-quality staining with clear visualization was found to be 0.25 mg/mL. Lower concentrations of 0.1 and 0.01 mg/mL of BBG did not provide adequate capsular staining. The side port was also stained by the anterior chamber injection.

**BIOCOMPATIBILITY OF BBG**

**Light Microscopy**

Sections of the cornea stained with hematoxylin-eosin showed no remarkable changes in any group (BBG, ICG, and TB) (data not shown). In the light microscopic examination, no signs of endothelial cell loss or corneal edema were observed. The lamellar collagen layers, stromal cells, and epithelial cell layer were well preserved. No inflammatory cell infiltration was observed in any corneal layers.

**Apoptotic Cell Death Detected Using TUNEL**

In all the groups, apoptotic cell death of the corneal epithelium due to physiologic turnover was observed occasionally (Figure 2). In the BBG groups, no TUNEL staining was observed in the corneal stromal cells, endothelium, ciliary body, and lens cells (Figure 2). In the ICG group, no apparent apoptotic cell death was detected in the 5-mg/mL concentration. In the TB group, apoptotic cell death of the corneal endothelium was detected in 2% of all endothelial cells. The other cells in the TB group did not undergo apoptotic cell death.

**Transmission Electron Microscopy**

In the BBG groups, the ultrastructure of the corneal cells and collagen cellular matrix was well preserved in the highest concentration (10 mg/mL) (Figure 3). In the corneal endothelium, cellular membranes, well-defined nuclei, and cytoplasmic organelia showed no degenerative changes (Figure 3). The ICG group showed well-preserved structure of endothelial cells, but some endothelial cells demonstrated signs of mitochondrial swelling (Figure 3). The TB group showed cyst formation in the endothelial cell layer due to separation between the cells and occasional degeneration of the corneal endothelium in a patchy manner (Figure 3).

**Scanning Electron Microscopy**

In the BBG-exposed corneas, scanning electron microscopy showed normal cells similar to those in the controls. The scanning electron microscopic image demonstrated a normal hexagonal endothelial cell sheet with intact borders and no endothelial swelling (Figure 4). The ICG group also showed some cellular swelling in the corneal endothelial sheet. Occasionally, degenerated endothelial cells were observed to have broken free from their original location. In the TB group, endothelial cell shrinkage was recognized in the central area of the cornea, which led to endothelial cell loss in a sporadic manner. The mean corneal endothelial cell counts were 7728±268/mm² in the control group, 7642±317/mm² in the BBG groups, 7501±230/mm² in the ICG group, and 7030±246/mm² in the TB group.

**COMMENT**

To our knowledge, this is the first study of staining by the new dye BBG. Many dyes have previously been...
examined for their staining ability of the anterior capsule and toxic effects in vitro and in vivo. However few have been used to stain the anterior capsule in human eyes. Those used to date include TB, ICG, gentian violet, fluorescein, and methylene blue. These dyes can be used to stain the capsule from above, under an air bubble, or by intracameral subcapsular injection, and ICG and TB are now commonly used to stain the anterior capsule with no apparent toxic effects under normal conditions. Nevertheless, we demonstrate that BBG has better staining ability and biocompatibility than either ICG or TB.

**STAINING ABILITY AND EASE OF HANDLING OF BBG**

In the present study, BBG revealed positive blue staining of the anterior capsule, providing a striking contrast to the white lens cortex and red retinal reflex. Under the surgical microscope, BBG blue staining provided a better contrast than ICG green and TB blue staining. In addition, satisfactory staining was obtained at the lower concentration of 0.25 mg/mL of BBG compared with 5 mg/mL of ICG and 1 mg/mL of TB.

In terms of handling, BBG also has advantages over ICG and TB. Indocyanine green is packaged as lyophilized powder and will not dissolve properly in BSS alone; ICG first has to be diluted in 0.5 mL of aqueous solvent before adding 4.5 mL of BSS. Furthermore, if the aqueous solvent, namely, the distilled water vehicle, is injected into the anterior chamber without being diluted with BSS, corneal edema can result with or without ICG. In addition, in practical terms, all reconstituted ICG must be used on the same day, and it is more expensive than the alternative dye (TB). In contrast, TB can easily be diluted in BSS, although, owing to its low affinity to the anterior capsule, it is beneficial to undertake fluid-air exchange of the anterior chamber before effecting capsular staining. Thus, after air injection into the anterior chamber, TB can be injected drop by drop under the air bubble. On the other hand, BBG was easily diluted in BSS alone, and satisfactory capsular staining was obtained by a single injection into the anterior chamber without the need for fluid-air exchange. The capsular staining was obtained immediately after the injection, and the excessive dye was easily washed out with BSS irrigation.

Thus, the results of this study show that BBG has effective staining ability combined with ease of handling. Brilliant blue G provided better staining at lower concentrations than either ICG or TB in a clinically relevant model for the study of capsular staining in pig’s eyes. The minimal concentration of BBG needed to demonstrate favorable staining with clear visualization was 0.25 mg/mL. Furthermore, BBG could easily be dissolved directly in the irrigation solution.

**BIOCOMPATIBILITY OF BBG**

Indocyanine green has been reported to possess good biocompatibility with the anterior chamber when injected under normal conditions. However, it has also been reported that the intravitreous injection of ICG could cause retinal degeneration. In addition, ICG has recently been reported to cause retinal pigment epithelial atrophy, photoreceptor apoptosis, glial cells, and retinal ganglion cell loss. The adverse clinical effects of visual field loss, possibly related to the use of ICG in vitrectomy, have also been reported. In severe conditions, our rat experimental model showed that ICG also caused swelling of the cytoplasmic organella (mitochondria) and cellular degeneration. Trypan blue has a long history of safety in ophthalmologic use.
Tailed examinations have reported fewer toxic effects of TB than ICG in glial cells and retinal pigment epithelium cell cultures. However, TB has also been reported to possess potential toxicity when introduced into cultured retinal pigment epithelium. In the present study, scanning electron microscopy showed corneal endothelial shrinkage in a sporadic manner as a result of the injection of TB into the anterior chamber. This could be due to the toxicity of TB or to the high osmolarity of the TB solution. Although some previous studies reported on the safety of ICG and TB in endothelial cells, under the more severe experimental conditions of the present study, the potential toxicity of the dyes was revealed. In contrast to ICG and TB, the anterior chamber injection of BBG showed no remarkable toxic effects histologically, even at 10 mg/mL. Functionally, the cornea did not show edema, and it remained transparent throughout the observation period. Apoptotic cell death observed by TUNEL also confirmed the lower toxicity of BBG compared with TB. With high affinity and biocompatibility with the anterior chamber, BBG is, therefore, a good candidate for a capsular staining dye.

The osmolarity of the solution is an important factor for the toxicity for cell survival. We, therefore, tested the osmolarity of each solution (Table 1). Indocyanine green has a much lower osmolarity than the control be-
cause of the aqueous solvent used in the dilution, whereas the osmolarity of TB is higher than that of the control. In contrast, BBG (0.25 mg/mL) has an almost identical osmolarity to the control.

The mean corneal endothelial cell counts were 7728±268/mm² in the control group, 7642±317/mm² in the BBG group, 7501±230/mm² in the ICG group, and 7030±246/mm² in the TB group. Thus, although BBG showed marginally less toxicity, there was no significant difference across the 4 groups. In rat’s eyes, corneal endothelial cells are known to have a higher proliferative capacity than in human endothelial cells. Our endothelial cell count of rat’s eyes is suggestive for clinical use; however, the data do not apply to human endothelial cell counts. Although the corneal endothelial cell count did not change in the observation period in the present study, further research is now needed in human clinical studies.

In conclusion, the results of this study show high biocompatibility of BBG for use in capsular staining. Brilliant blue G demonstrated better-preserved morphologic features of corneal endothelial cells using transmission and scanning electron microscopy than ICG and TB. Also, TUNEL confirmed the lower toxicity of BBG vs TB. Because rat corneal endothelial cells have higher proliferative capacity than human endothelial cells, the rat data do not apply to human eyes, and further detailed studies are needed in human clinical studies. In addition, the BBG solution has an osmolarity similar to that of the physiologic aqueous humor. Thus, BBG is a good alternative dye for capsular staining, with superior staining ability and biocompatibility.

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