Reduction of Indocyanine Green–Associated Photosensitizing Toxicity in Retinal Pigment Epithelium by Sodium Elimination

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Objective: To determine if eliminating sodium affects indocyanine green (ICG) photosensitizing toxicity and uptake in cultured human retinal pigment epithelial (RPE) cells.

Methods: Cultured human RPE cells were exposed to ICG (2.5 mg/mL) in balanced salt solution and sodium-free balanced salt solution for 2 minutes. Afterwards, ICG was removed, and the cells were irradiated with a light beam (4×10^4 lux) for 40 minutes. Toxicity was monitored using light microscopy, calcein AM–ethidium homodimer 1 staining, trypan blue exclusion test, and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium viability assay. Indocyanine green uptake was measured by optical absorption at 790 nm.

Results: Photoreactive changes occurred in RPE cells exposed to ICG and light. These changes included cell shrinkage, cell death, pyknotic nuclei, reduced viability, and reduced mitochondrial dehydrogenase activity. These changes were less severe when ICG was dissolved in sodium-free balanced salt solution. In addition, ICG uptake was reduced when the solvent was sodium-free balanced salt solution.

Conclusion: Indocyanine green and intense light exposure in RPE cells caused photosensitizing toxicity that was reduced when sodium in the solvent was eliminated and replaced with other cations.

Clinical Relevance: Eliminating sodium from the solvent reduced ICG uptake into RPE and its associated photosensitizing toxicity. This reconstitution method of ICG may be helpful for safer intravitreal ICG use in macular hole surgery.

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PEELING OF THE INTERNAL LIMiting membrane (ILM) to eliminate tangential traction force improves the anatomical closure rate and functional outcomes after macular hole surgery,[1-4] although some investigators hold a different opinion.[5-7] However, if ILM removal is attempted during macular hole surgery, its visualization may be difficult. Staining of the ILM with indocyanine green (ICG) to enhance its visibility[8-13] has been proposed. The procedure involves direct application of ICG to the inner surface of the retina in the macular area. No adverse effects of ICG were reported in these studies. The concentration of ICG used in these reports ranged from 0.6 to 5.0 mg/mL, and ICG was left in the vitreous cavity from 30 seconds to 5 minutes.

Other investigators, however, have described ICG-associated adverse effects. For example, Gandorfer et al[14] reported less favorable visual outcomes when 5 mg/mL of ICG was used and drained immediately after injection. Similarly, Engelbrecht et al[15] observed a high incidence of unusual atrophic retinal pigment epithelial (RPE) changes at the site of the previous macular hole and its surrounding subretinal fluid after using 1 mg/mL of ICG for 0.5 to 2.5 minutes. The median preoperative best-corrected visual acuity was 20/200, while the median postoperative best-corrected visual acuity was 20/400. The macular hole was closed in 86% of eyes. Haritoglou and coworkers[16] reported no statistically significant improvement in postoperative visual acuity after ICG-assisted ILM peeling. In an in vitro study, Sippy et al[17] reported a significant reduction in mitochondrial dehydrogenase activity in cultured RPE cells exposed to ICG alone or ICG plus light. In a recent study,[18] ICG plus intense light exposure caused cell cycle arrest and apoptosis in cultured human RPE. Because intraoperative endoillumination with a high

level of light intensity is used during macular hole surgery with ICG-assisted ILM peeling, photosensitizing toxicity to RPE is possible.

Indocyanine green is an organic anion with amphophilic (ie, hydrophilic and hydrophobic) properties.[19] It is most often administered intravenously. After intravenous injection, it is rapidly cleared from the circulation by the liver and eliminated in bile. The uptake of organic anions by hepatocytes involves sodium-dependent and independent transport systems.[20] Indocyanine green is also taken up by keratinocytes[21] and cultured aortic endothelial cells.[22] In an attempt to reduce ICG uptake into RPE cells and its associated photosensitizing toxicity, we replaced sodium in the solvent with other cations.

METHODS

CELL CULTURE AND ICG PREPARATION

Human RPE cells (ARPE-19) were obtained (American Type Culture Collection, Manassas, Va). This cell line is not transformed and has structural and functional properties characteristic of RPE cells in vivo.[23] The RPE cells were cultured in DMEM/F12 medium (1:1) containing 10% fetal bovine serum (Invitrogen Corp, Grand Island, NY). The following substances were added: 0.01 g/L of transferrin, 0.01 g/L of insulin, 0.91 g/L of sodium bicarbonate, 100 U/mL of penicillin G potassium, 0.1 mg/mL of streptomycin sulfate, 5.0 mg/mL of gentamicin sulfate, 3.58 g/L of HEPES, and 1.75 g/L of D-glucose. The cells were cultured at 37°C in 5% carbon dioxide.

Indocyanine green was prepared by completely dissolving 25.0 mg of sterile ICG powder (containing iodide) (Daiichi Pharmaceutical, Tokyo, Japan) in 0.5 mL of sterile distilled water. To achieve a final ICG concentration of 2.5 mg/mL (289.8 mOsm/kg), 9.5 mL of balanced salt solution (BSS Plus; Alcon Laboratories, Fort Worth, Tex) was added.

The sodium-free BSS solution was prepared in the laboratory with the same constituents as BSS, except that sodium chloride, sodium phosphate dibasic, and sodium bicarbonate were replaced with 17.06 g/L of choline chloride, 0.52 g/L of potassium phosphate dibasic, and 2.50 g/L of potassium bicarbonate, respectively, to achieve the same molar concentration of the replaced chemicals. The pH was adjusted to 7.4 with hydrochloric acid/potassium hydroxide (potassium hydroxide was used instead of sodium hydroxide to keep the solution sodium-free). The solution was then sterilized by using a disposable sterilizing filter (0.22-mm-diameter polyethersulfone bottle top filter; Corning Costar, Corning, NY). The osmolarity of this solution was 296 mOsm/kg, as measured by an autoosmometer (Osmostat, model OM-6020; Daiichi Kagaku Co, Kyoto, Japan). Indocyanine green was also dissolved in sodium-free BSS to achieve a final concentration of 2.5 mg/mL (281.2 mOsm/kg).

ICG TREATMENT AND LIGHT EXPOSURE

Retinal pigment epithelial cells were seeded into the wells of chamber slides or culture plates in appropriate density and volume. Retinal pigment epithelial cells were grown to total or 70% confluence (depending on the assay) for ICG treatment and light illumination. After removal of culture medium, the cells were rinsed with prewarmed (37°C) BSS or sodium-free BSS (the same as the ICG solvent used in ICG preparation). Indocyanine green (2.5 mg/mL, prewarmed to 37°C) in BSS or sodium-free BSS was then added and incubated for 2 minutes at 37°C in the dark. Balanced salt solution or sodium-free BSS alone was added to the control wells. The RPE cells were then washed with BSS or sodium-free BSS. The cells were then placed in BSS and subjected to intense light exposure of a surgical microscope. The light intensity was adjusted so that the illuminance at the level of RPE cells was 4×10^4 lux (comparable to the illuminance from an endoillumination probe, as measured by a lux meter; Lutron, Coopersburg, Pa). The cells were exposed to the light for 40 minutes. After illumination, the RPE cells were washed with BSS, culture medium was added, and the cultures were maintained at 37°C with 5% carbon dioxide in the dark for predetermined periods.

MORPHOLOGIC EVALUATION AND CALCEIN AM–ETHIDIUM HOMODIMER 1 STAINING

Retinal pigment epithelial cells were cultured in 2-well chamber slides. Each well was seeded with 1.0 mL of cell suspension (10^5 cells/mL). After reaching total confluence, RPE cells were treated with ICG and light. Phase-contrast microscopy was used to observe the morphologic changes. For calcein AM–ethidium homodimer 1 staining, the culture medium was removed at appropriate periods after the ICG and light treatment. The samples were rinsed with PBS and then incubated with a solution containing 2-μM calcein AM and 4-μM ethidium homodimer 1 (Molecular Probes, Eugene, Ore) for 45 minutes. The cells were evaluated under an epifluorescence microscope.

VIABLE CELL NUMBER DETERMINATION

Cells (5×10^4 in 0.5 mL) were seeded into each well of the 24-well plates. After reaching 70% confluence (to avoid contact inhibition during the observation period of 48 hours), the cells were treated with ICG incubation followed by illumination. The number of viable cells was determined at 2, 6, 12, 24, and 48 hours after the treatment. The RPE cells were trypsinized and stained with trypan blue (0.2%). Cells that excluded trypan blue were considered viable and counted with a hemocytometer.

MTS VIABILITY ASSAY

Cell viability was assessed by MTS colorimetric assay (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) (Promega Corporation, Madison, Wis). This quantitative assay detects living but not dead cells.[24] The absorbance at 490 nm (test wavelength) and at 650 nm (reference wavelength) was measured using an enzyme-linked immunosorbent assay microplate reader (VERSAmax; Molecular Devices, Sunnyvale, Calif); wells containing culture medium but no cells served as blanks. In this experiment, 10^4 cells in 100 μL of culture medium were seeded into each well of a 96-well plate. After achieving 70% confluence, the cells were treated with ICG and light. After incubation for 2, 6, 12, 24, and 48 hours, the wells were washed, and 100 μL of culture medium and 20 μL of MTS were added to each well. After a 2-hour incubation at 37°C, the absorbance at 490 nm was measured.

ICG UPTAKE ASSAY

The ICG uptake was measured by using the optical absorption of ICG at 790 nm.[21] The absorption spectrum of ICG in 0.1N sodium hydroxide (the medium used to lyse the RPE cells) was measured with a spectrophotometer (DU800 UV/Visible; Beckman Coulter, Fullerton, Calif) to ensure a high absorption coefficient at 790 nm in different ICG concentrations. For the ICG uptake assay, 1.5×10^5 cells in 1.5 mL of culture medium were seeded into each well of the 6-well culture plates. After reach-

ing total confluence, the cells were rinsed 3 times with prewarmed (37°C) BSS or sodium-free BSS (the same as the ICG solvent used in ICG preparation). Indocyanine green (2.5 mg/mL, prewarmed to 37°C) in BSS or sodium-free BSS was then added and incubated for 2 minutes at 37°C in the dark. The reactions were then terminated by aspiration of the ICG solution, followed by 4 rapid washings of the RPE cells with ice-cold BSS or sodium-free BSS. This washing procedure removed more than 99% of extracellular substrate, with minimal loss of cell-associated substrate.[25] The cells in each well were then lysed with 0.8 mL of 0.1N sodium hydroxide. The optical absorption of the lysate containing ICG was measured at 790 nm. The actual ICG concentration in the cell lysate was calculated using a calibration curve. To create the calibration curve, appropriate amounts of ICG were dissolved in 0.1N sodium hydroxide to make solutions of known ICG concentrations. The optical absorption at 790 nm for each ICG concentration was measured, and the calibration curve was created by linear regression analysis.

RESULTS

REDUCTION OF ICG AND LIGHT EXPOSURE TOXICITY BY SODIUM ELIMINATION

Cultured human RPE cells showed morphologic changes 2 hours after treatment with 2.5 mg/mL of ICG in BSS for 2 minutes and subsequent exposure to light for 40 minutes. These cells assumed a heterogeneous appearance. Some cells became round. Some cells were reduced in size (**Figure 1**A). Some cells detached from the bottom of the culture well and floated in the medium, leaving space between the cells that attached on the bottom of the culture well. The extent of these changes increased 6 hours after treatment, and more cells assumed a shrunken appearance (Figure 1B). After exposure to 2.5 mg/mL of ICG in sodium-free BSS for 2 minutes followed by illumination for 40 minutes, some cells became round 2 hours after the treatments. Many cells were somewhat reduced in size, and a few cells detached from the bottom of the culture well, producing gaps between the cells (which were totally confluent before treatment). However, these changes were less severe than previously observed (Figure 1C). These changes were slightly more evident 6 hours after treatment; more cells became round (Figure 1D). However, the changes observed after exposure to ICG in sodium-free BSS were less severe than those produced by ICG in BSS. As a control, RPE cells exposed to light showed no morphologic abnormality (Figure 1E).

CALCEIN AM–ETHIDIUM HOMODIMER 1 STAINING

To investigate ICG-associated photosensitizing toxicity, RPE cells were stained with 2 µM of calcein AM and 4 µM of ethidium homodimer 1. Live cells are identified by the presence of ubiquitous intracellular esterase activity, which converts the nonfluorescent, cell-permeant calcein AM to green-fluorescent calcein, which is retained within live cells. Ethidium homodimer 1 enters the cells with compromised membranes and, on binding to nucleic acid, provides a red fluorescence in dead cells.[26]

Some cells had shrunken and red-fluorescent nuclei with reduced intracytoplasmic green fluorescence 2 hours after treatment with 2.5 mg/mL of ICG in BSS for 2 minutes and subsequent exposure to light for 40 minutes. Their cell membrane integrity was compromised, and intracytoplasmic esterase activity was reduced. These cells were dead. Other cells showed intense green fluorescence in the cytoplasm, indicating they were viable (**Figure 2**A). More RPE cells showed red-fluorescent nuclei 6 hours after treatment. The green fluorescence was faint or absent in the cytoplasm of these cells containing red-fluorescent nuclei (Figure 2B).

A smaller fraction of cells treated with 2.5 mg/mL of ICG in sodium-free BSS for 2 minutes followed by illumination for 40 minutes contained condensed and red-fluorescent nuclei, compared with cells treated with ICG in BSS. Most cells remained green-fluorescent 2 hours after treatment (Figure 2C). Six hours after treatment, there was a slight increase in the number of cells with red-fluorescent nuclei (Figure 2D). However, the ratio of cells with red-fluorescent nuclei was much less compared with treatment with ICG dissolved in BSS. Retinal pigment epithelial cells exposed only to illumination for 40 minutes showed intense green fluorescence in the cytoplasm (Figure 2E), as did cells exposed only to 2.5 mg/mL of ICG (in BSS or sodium-free BSS) for 2 minutes (data not shown).

VIABLE CELL NUMBER DETERMINATION

The viable cell number of RPE cells was reduced by treatment with 2.5 mg/mL of ICG (in BSS and sodium-free BSS) for 2 minutes plus intense light illumination for 40 minutes (**Figure 3**A). It was reduced to a greater extent when 2.5 mg/mL of ICG was dissolved in BSS than in sodium-free BSS. After light exposure, the number of viable cells in the ICG BSS group was one fourth to one third of that in the control group (no ICG and no light exposure). In contrast, this ratio was around three fourths for the ICG sodium-free BSS group. Cell viability was significantly reduced in the ICG BSS group, compared with the ICG sodium-free BSS group, at each time point. Viability was not affected by illumination treatment for 40 minutes only or incubation in 2.5 mg/mL of ICG (in BSS or sodium-free BSS) for 2 minutes without illumination.

MTS VIABILITY ASSAY

Enzymatic integrity in cultured human RPE cells was evaluated with an MTS colorimetric assay. The MTS tetrazolium compound is reduced to a colored formazan product by a nicotinamide adenine dinucleotide phosphate–dependent or nicotinamide adenine dinucleotide–dependent dehydrogenase in metabolically active cells.[27] The formazan was quantitated with an enzyme-linked immunosorbent assay microplate reader at 490 nm. Indocyanine green (2.5 mg/mL) incubation for 2 minutes followed by light treatment for 40 minutes significantly reduced dehydrogenase activity in the ICG BSS and ICG

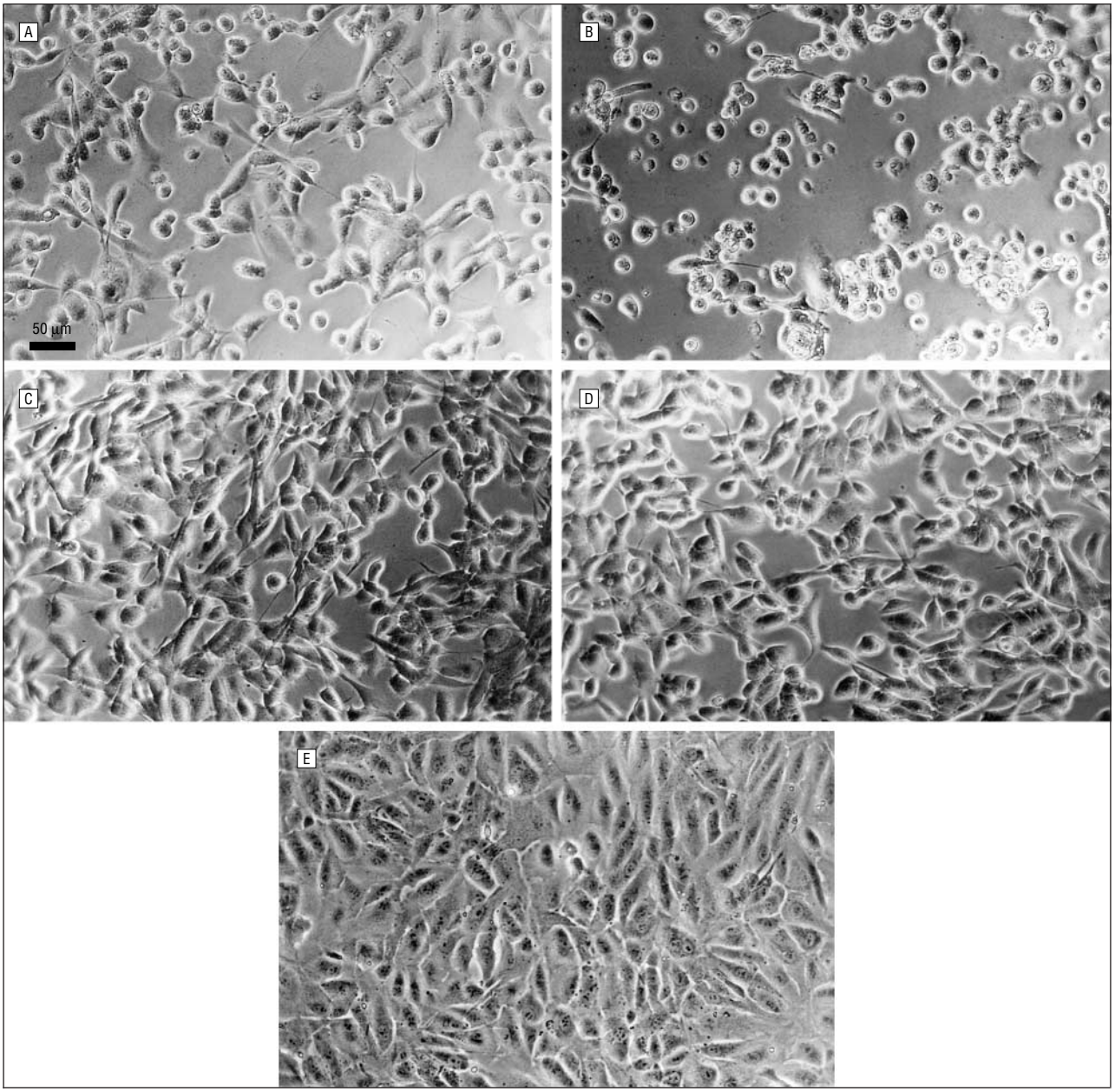

Figure 1. Phase-contrast micrographs of retinal pigment epithelial (RPE) cells treated with 2.5 mg/mL of indocyanine green and subsequent exposure to light. A, Two hours after treatment in balanced salt solution (BSS). B, Six hours after treatment in BSS. C, Two hours after treatment in sodium-free BSS. D, Six hours after treatment in sodium-free BSS. A few more cells became round, and some cells appeared shrunken. E, Six hours after 40 minutes of illumination. Control RPE cells revealed no morphologic abnormalities.

sodium-free BSS groups (Figure 3B). Treatment with ICG in BSS produced a greater photosensitizing toxic effect than ICG in sodium-free BSS. Exposure to 2.5 mg/mL of ICG for 2 minutes or light for 40 minutes alone did not affect enzymatic activity.

SODIUM ELIMINATION AND REDUCED ICG UPTAKE BY RPE CELLS

Indocyanine green dissolved in 0.1N sodium hydroxide had high absorption at 790 nm at different ICG concentrations (**Figure 4**). We measured the amount of ICG uptake by RPE cells in different solvents to elucidate the mechanism by which sodium affected ICG-associated photosensitizing toxicity. A calibration curve was created to correlate the ICG concentration with optical absorption at 790 nm. The mean±SE ICG uptake by RPE was 1.81±0.24 µg/10^5 cells for RPE cells incubated with 2.5 mg/mL of ICG dissolved in BSS for 2 minutes at 37°C. This value was significantly decreased (P=.01) to a mean±SE of 1.05±0.17 µg/10^5 cells for RPE cells incubated for 2 minutes with 2.5 mg/mL of ICG dissolved in sodium-free BSS (**Figure 5**).

COMMENT

In our study, incubation in 2.5 mg/mL of ICG (in BSS) for 2 minutes followed by 40 minutes of illumination pro-

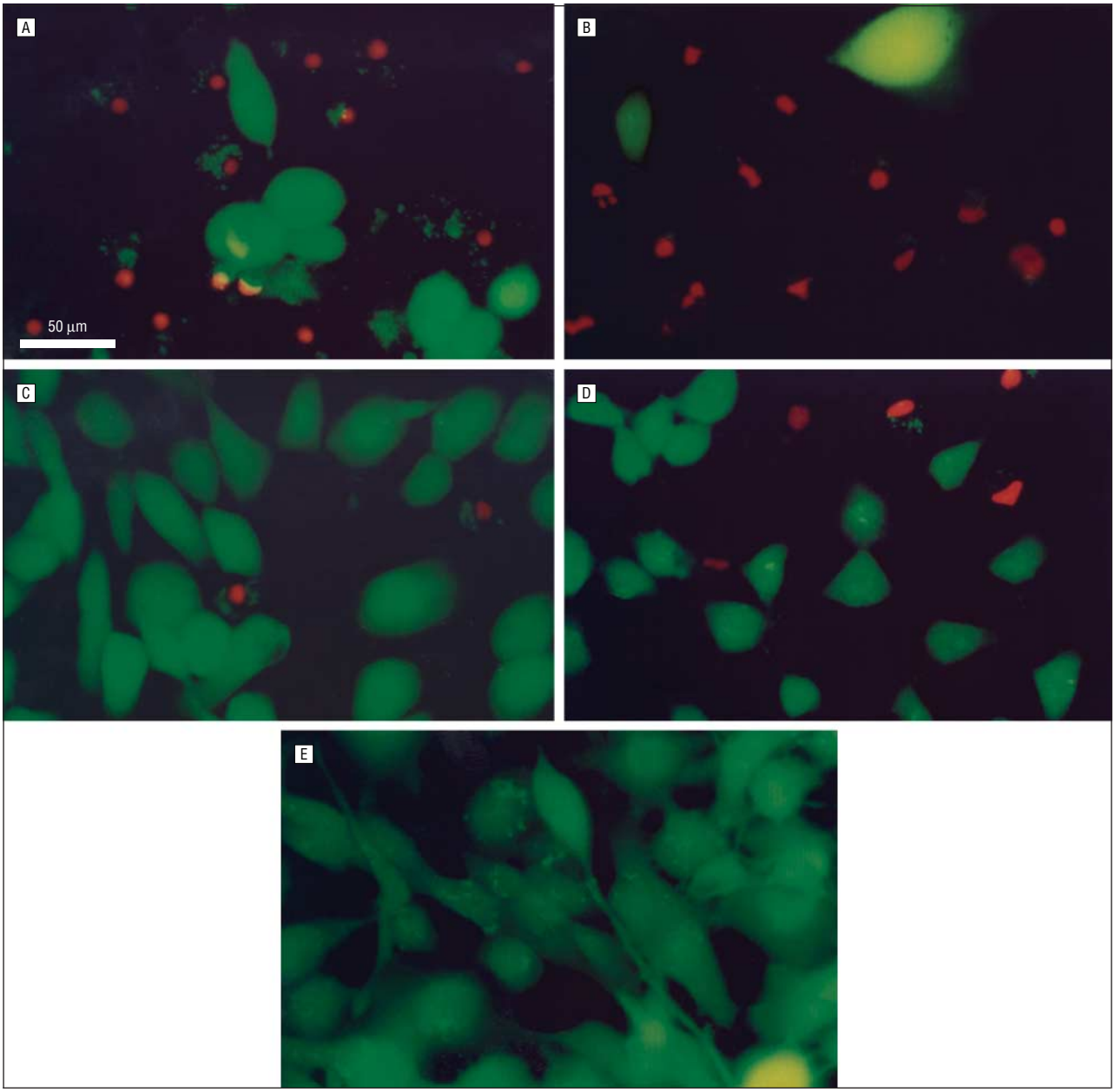

Figure 2. Calcein AM–ethidium homodimer 1 staining after treatment with 2.5 mg/mL of indocyanine green and subsequent exposure to light. A, Two hours after treatment in balanced salt solution (BSS). Some cells exhibited shrunken and red-fluorescent nuclei. These cells had reduced intracytoplasmic green fluorescence of esterase activity. They had compromised cell membrane integrity and were dead. B, Six hours after treatment in BSS. C, Two hours after treatment in sodium-free BSS. Only a small number of cells contained red-fluorescent nuclei. Most cells had intense intracytoplasmic green fluorescence, indicating they were still viable. D, Six hours after treatment in sodium-free BSS. E, Six hours after 40 minutes of illumination. Control retinal pigment epithelial cells showed intense green fluorescence of esterase activity, indicating their viability.

duced toxicity in cultured human RPE cells. This ICG-associated photosensitizing toxicity was reduced by replacing sodium in the ICG solvent with other cations. Reduced toxicity was associated with decreased ICG uptake into RPE cells.

The cause of the postoperative atrophic RPE changes[15] and the poorer visual outcome[14-16] after ICG-assisted ILM peeling is not yet clear. These observations may be due to cytotoxic (including photosensitizing toxicity) effects of ICG to RPE cells. When ICG is used to stain the ILM during macular hole surgery, ICG has direct access to the bare RPE at the base of the macular hole. Although ICG is applied for brief periods up to 5 minutes[5-13,15,16] and then washed out, ICG can be taken up by the RPE cells during this application, as we demonstrated. Furthermore, residual ICG may remain in the eye for prolonged periods that can extend up to 8 months.[28-31] Endoillumination with a high level of light intensity is used during macular hole surgery, and photosensitizing damage to RPE cells can occur.

Although ICG is a commonly used dye with a long history of safety and low toxicity,[32,33] it is most often administered intravenously. Indocyanine green is an organic anion that is taken up by liver cells after intravenous injection and rapidly cleared from the circulation via bile secretion. The clearance of ICG in blood is bi-

phasic, with a rapid initial phase (half-life, 3-4 minutes) and a secondary phase (half-life, >1 hour).[34] Because the barrier surrounding the vitreous compartment is tight,[35] the pharmacokinetics of intravenous and intravitreal ICG are probably different. In fact, scanning laser ophthalmoscopy revealed ICG fluorescence in the posterior pole of patients with macular holes, and it remained for several months postoperatively.[28-31] Besides, the ICG uptake rate by RPE was higher than that in other cells, such as skin keratinocyte, as evidenced by comparing our results of ICG uptake assay with those of others.[21]

than ICG BSS, if ICG concentrations were the same. Conversely, ICG sodium-free BSS showed less photosensitizing toxic effects on RPE cells, as demonstrated in this study. Therefore, the reduction in photosensitizing damage of ICG sodium-free BSS compared with ICG BSS was not due to the difference in the absorption qualities of these 2 solutions.

In this study, RPE cells were illuminated for 40 minutes. We chose this illumination duration because it demonstrated the distinction of the photosensitizing toxic effect on RPE cells between ICG BSS and ICG sodium-free BSS. In fact, we observed this difference in the morphologic structure of cells and cell nuclei when the light exposure duration was 25 minutes, but to a lesser extent and in a smaller number of cells. The results in this study emphasize that ICG dissolved in sodium-free BSS had a higher safety margin than ICG dissolved in BSS in terms of photosensitizing toxicity. That is, using sodium-free BSS as a solvent for ICG reduced the photosensitizing damage in RPE cells, even when the light exposure duration was prolonged to 40 minutes. Clinically, removing the ILM is a technically challenging procedure, especially for inexperienced surgeons. It is not uncommon for an inexperienced surgeon to take as long as 40 minutes to complete the ILM peeling procedure, even when ICG staining is used. In addition, there are other procedures to perform after ILM removal is completed. These include fluid-air exchange, air-gas exchange with sulfur hexafluoride or perfluoropropane, and closure of the sclerotomy and peritomy. All these procedures are performed under light illumination (from an endoillumination probe or surgical microscope).

Cytotoxicity of ICG may contribute to the clinically observed RPE changes after ICG-assisted ILM peeling, because ICG has been shown to decrease mitochondrial enzyme activity in RPE cells in vitro[17] and cause mitochondrial toxicity.[38] In addition, intravitreally administered ICG may be retained in the eye for a prolonged period.[28-31] Prolonged exposure to ICG, even at a low concentration, can lead to cytotoxicity in RPE cells.[39] However, because sodium removal can reduce ICG uptake, we believe that sodium removal would also reduce the cytotoxicity induced by prolonged ICG exposure.

It has been suggested that the toxic effects of ICG on RPE may be related to the hypoosmolarity of the solvent.[40] In our study, the osmolarities of the solutions containing 2.5 mg/mL of ICG were 289.8 mOsm/kg in BSS and 281.9 mOsm/kg in sodium-free BSS. To examine the effects of a hypoosmotic medium on RPE cells, we diluted BSS and sodium-free BSS with sterile distilled water to osmolarities of 289.8 mOsm/kg and 281.9 mOsm/kg, respectively. Exposure to diluted BSS or sodium-free BSS alone or followed by light exposure caused no alteration in RPE cell morphologic structure, calcein AM–ethidium homodimer 1 staining, or viable cell number counting (data not shown). Therefore, a change in osmolarity is probably not responsible for the ICG-induced RPE toxicity observed in vitro and clinically.[41]

A recent study[42] describes adverse effects on functional outcome of ICG-assisted macular pucker surgery, in which there were more cellular debris and inner processes of Müller cells in the removed specimens after ICG application during surgery. Epiretinal cells had ruptured and lost their cellular integrity. In macular pucker surgery, there is no direct contact of the RPE and the ICG solution. Therefore, ICG may be toxic to retinal cells other than RPE cells, and this toxicity may also contribute to the less favorable functional outcome observed after ICG-assisted macular hole surgery.

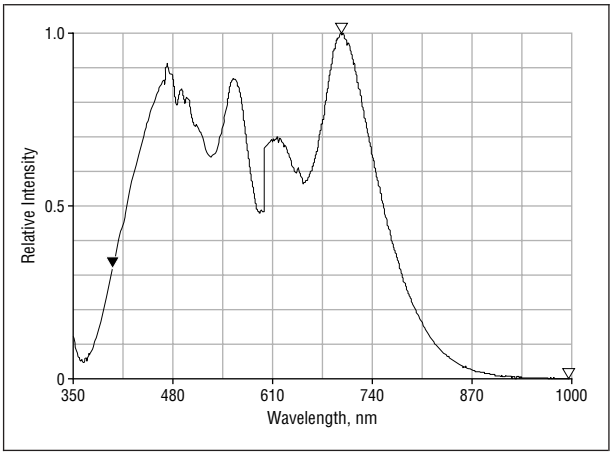

Figure 6. Emission spectrum (380-800 nm) of the light source used in this study.

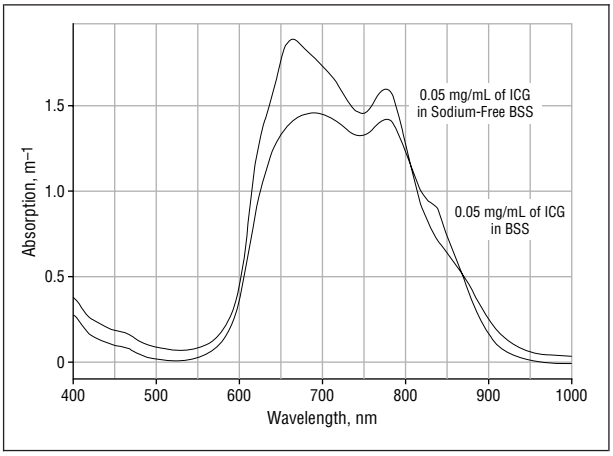

Figure 7. Absorption spectra of indocyanine green (ICG) sodium-free balanced salt solution (BSS) and ICG BSS (both containing 0.05 mg/mL of ICG).

The sodium-free BSS used in this study was prepared in the laboratory. To our knowledge, it is not available commercially. There are potential concerns about quality control and safety for this laboratory-prepared solution. These concerns include stability of osmolarity and pH, sterility, and shelf life of the solution. These concerns should be fully addressed before this solution could be used in human surgery.

Indocyanine green staining of the ILM facilitates its identification and removal during macular hole surgery. Indocyanine green staining can be a valuable tool; however, no standardized procedure that specifies reconstitution method, concentration, volume, and incubation time exists for the intravitreal use of ICG. In this study, we demonstrated that replacement of sodium in the solvent reduces the ICG-associated photosensitizing damage on cultured human RPE. Further in vivo studies are required to examine if this reconstitution method

can provide a safer intravitreal ICG use in macular hole surgery.

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Dr Ho had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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