

# Indocyanine Green Reduces the Viability of Human Lens Epithelial Cells and Promotes Cytolysis: An Ex Vivo Study

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**Received:** August 11, 2021

**Accepted:** October 29, 2021

**Published:** November 24, 2021

**Keywords:** posterior capsule opacification; indocyanine green; lens epithelial cells; electron microscopy

**Citation:** He ZF, Zhao YD, Zhang S, Chen FF, Liu YJ, Zhang WW, Xie ZG. Indocyanine green reduces the viability of human lens epithelial cells and promotes cytolysis: An ex vivo study. *Transl Vis Sci Technol.* 2021;10(13):30.  
<https://doi.org/10.1167/tvst.10.13.30>

**Purpose:** To investigate the effects of indocyanine green (ICG) solution on the viability and cytolysis of human lens epithelial cells ex vivo.

**Methods:** A total of 200 pieces of anterior capsules were obtained during cataract surgery, and 110 pieces of the anterior capsules were randomly divided into five groups and treated by immersion in different concentrations of ICG solution. The remaining 90 anterior capsules were also divided into five groups and treated with a combination of drug immersion and washing in balanced salt solution. Electron microscopy and trypan blue and eosin stains were used to analyze the cells. Percentage of dead, shedding, or living lens epithelial cells was estimated and used to demonstrate effects of the ICG on viability and cytolysis.

**Results:** Compared with the control group, the percentage of dead and shedding lens epithelial cells increased while the percentage of living lens epithelial cells decreased in all the immersion groups. In the washing groups, the percentage of the living lens epithelial cells was  $63.42\% \pm 2.49\%$ ,  $54.04\% \pm 1.84\%$ ,  $43.51\% \pm 2.63\%$ ,  $29.21\% \pm 2.40\%$ , and  $15.73\% \pm 1.61\%$  for the five groups and reflected a concentration–effect relationship. Electron microscopy showed that the higher the concentration of the ICG solution, the more severe the destruction of the lens epithelial cell structure.

**Conclusions:** ICG could reduce the viability of the lens epithelial cells and promote cell cytolysis.

**Translational Relevance:** Our study showed that ICG could directly reduce the viability of the lens epithelial cells in a concentration-dependent fashion, which can theoretically reduce the incidence of posterior capsule opacification.

## Introduction

Phacoemulsification combined with intraocular lens (IOL) implantation is the most common surgical procedure for the treatment of cataracts. To ensure in-the-bag implantation, it is necessary to preserve the circular anterior and intact posterior capsules during the operation. Although the lens capsule is transparent, residual lens epithelial cells (LECs) often proliferate, migrate, and adopt epithelial–mesenchymal transition and adhesion postoperatively, resulting in anterior

or posterior capsule opacification and contraction.<sup>1</sup> Anterior capsule opacification reduces the diameter of the anterior capsule orifice and the equator of the capsular bag. This shrinkage leads to clinical symptoms such as vision loss.<sup>2</sup> On the other hand, posterior capsule opacification is one of the most common long-term complications of cataract surgery and is the main reason for compromised visual acuity. The incidence of posterior capsule opacification ranges between 11.8% and 50% in adults, and it is as high as 100% in children.<sup>3</sup> To date, there is no effective preventive method for posterior capsule opacification. To reduce the incidence

of lens capsule opacification, some experts have tried to improve the surgical methods, such as polishing the anterior capsule or changing the shape and material of the IOL.<sup>4,5</sup> However, the impact remains marginal as the opacification cannot be completely avoided.

In recent years, there have been numerous attempts to use drugs in the prevention of lens capsule opacification. Some anti-inflammatory or immunomodulatory drugs and antimetabolites have been shown to reduce the proliferation of LECs, inhibit the adhesion of LECs, and prevent the migration of the LECs to the posterior capsule.<sup>5,6</sup> However, the preventive and therapeutic effects of these drugs are mostly in experimental stages and have been associated with toxicity on intraocular tissues.

In ophthalmology, indocyanine green (ICG) is mainly used as a contrast medium in choroidal angiography. Besides, it is used to dye the anterior capsule for surgical excision of hypermature cataracts,<sup>7,8</sup> as well as stain and strip the internal limiting membrane in vitrectomy.<sup>9</sup> Recent studies have shown that the ICG could reduce the activity of the LECs through photosensitization.<sup>10-12</sup> In present study, we evaluated the direct effect of different concentrations of ICG solution on the viability and adhesion of the LECs, as well as the ultrastructure of the isolated human capsule.

## Materials and Methods

### Participants

A total of 200 pieces of lens anterior capsules were obtained during phacoemulsification between June 2019 and January 2020. Out of the total, 110 pieces were used for drug immersion, and 90 pieces underwent immersion combined with washing. We included patients with age-related cataract aged  $\geq 50$  years who agreed to participate in the study by giving signed informed consent. Those with pigment, shrinkage, or calcification on the anterior capsule as well as a history of intraocular surgery, ocular trauma, and related eye diseases with cataracts were excluded from the study. This study was approved by the Ethics Committee of the Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School.

### Preparation of Experimental Drugs

ICG powder (Dandong Medical Innovation Pharmaceutical Co., Ltd., Dandong, China) was stored at 4°C in darkness. The powder was mixed with 5% glucose solution (GS) and shook to complete dissolution of the particles. We then prepared 0.5%

(5 mg/mL) ICG solution, 1.5% (15 mg/mL) ICG solution, and 2.5% (25 mg/mL) ICG solution.

Trypan blue solution (Beijing Solarbio Technology Co., Ltd., Beijing, China) was stored at 4°C and mixed with equal volume of balanced salt solution (BSS) to make 0.2% trypan blue solution.

### Grouping and Drug Treatment

The 110 pieces of the anterior capsule were randomly divided into five groups: negative control group ( $n = 14$ ), no treatment; 5% GS group ( $n = 24$ ), immersion in 5% GS for 3 minutes; 0.5% ICG group ( $n = 24$ ), immersion in 0.5% ICG solution for 3 minutes; 1.5% ICG group ( $n = 24$ ), immersion in 1.5% ICG solution for 3 minutes; and 2.5% ICG group ( $n = 24$ ), immersion in 2.5% ICG solution for 3 minutes. The capsules of each group were put in the BSS and gently rinsed two times. In the negative control group, 10 pieces were stained with trypan blue and eosin. The other four groups had 20 pieces stained with trypan blue and eosin solution. In each group, two pieces were examined by both transmission electron microscope (TEM; H-7650 Transmission Electron Microscope; Hitachi, Tokyo, Japan) and scanning electron microscope (SEM; SU8010 Scanning Electron Microscope; Hitachi).

### Transmission Electron Microscope

After drug treatment, the capsule specimens were immediately fixed in 2.5% glutaraldehyde fixative in 0.1 M phosphate-buffered saline (PBS) at 4°C for 24 hours. The specimens were rinsed three times in PBS  $15 \times 3$  and fixed in 1% osmium tetroxide in water at 4°C for 1 hour. The specimens were further rinsed three times in PBS  $15 \times 3$ . The capsule specimens were then dehydrated in graded ethanol (50% to 100%) solution followed by 100% ethanol for 20 minutes. Finally, the specimens were treated with pure acetone for 20 minutes. The samples were placed in a mixture of acetone and embedding agent and then in pure embedding agent overnight.

Thereafter, the specimens were heated embedded in the pure Spurr resin at 70°C for 24 hours overnight to make resin blocks. Ultrathin sections (70 nm) were cut using a Leica, Wetzlar (Germany) ultra-cut microtome, and the sections were stained with uranyl acetate and lead citrate. The ultrathin sections were observed under the TEM, and digital images were captured by a side-mounted camera (Hitachi H-7650, CCD: Gatan 832).

## Scanning Electron Microscope

The former several steps were the same with the TEM specimen preparation until treating with 100% ethanol twice for 20 minutes. The dehydrated specimens were treated with a mixture of equal volumes of ethanol and isoamyl acetate for 30 minutes plus pure isoamyl acetate for 1 hour. The specimens underwent critical-point drying (Leica EM CPD300 Critical Point Dryer) and sputter-coating with gold (Hitachi MCI000 Ion Sputter), and then they were imaged using a SU8010 SEM.

## Capsule Staining Method

All the treated capsule specimens were stained with 0.2% trypan blue solution for 1 minute and then rinsed twice with BSS. Each piece of the specimen was air-dried on a glass slide. The dried capsules were fixed in 10% neutral formalin for 2 hours and then washed in distilled water for 2 minutes. The specimens were dried naturally and imaged with a digital microscope (inverted fluorescence microscope, Axio Cam ICc 5 Digital Microscope Imaging System; Carl Zeiss AG, Oberkochen, Germany). Finally, the capsule specimens were stained in eosin solution for 2 minutes, washed twice with distilled water, and air-dried.

## Drug Treatment and BSS Washing

Ninety pieces of the anterior capsules were randomly divided into five groups: negative control washing group ( $n = 10$ ), 5% GS washing group ( $n = 20$ ), 0.5% ICG washing group ( $n = 20$ ), 1.5% ICG washing group ( $n = 20$ ), and 2.5% ICG washing group ( $n = 20$ ). All the capsule specimens were immersed in relative drug solution for 3 minutes, followed by washing in 70-cm-high BSS (Sichuan Kelun Pharmaceutical Co., Ltd. Chengdu, China; simulation of the perfusion/aspiration operation during cataract surgery) for 1 minute. The subsequent staining method was similar to what has been previously described.

## Quantitative Analysis of the LECs

Imaging of the trypan blue staining (staining the nucleus of dead LECs blue) and eosin staining (staining the cytoplasm of LECs pink) of the LECs on the capsule in each group was performed using a light microscope (inverted fluorescence microscope, Axio Cam ICc 5 Digital Microscope Imaging System; Carl Zeiss AG) with a 50 $\times$  objective lens. The percentage of dead, shedding, or living LECs on the capsule was

calculated using the ImageJ software (version 1.8.0; National Institutes of Health, Bethesda, MD, USA).

## Statistical Analysis

All the data were analyzed using SPSS 23.0 (SPSS, Inc., Chicago, IL, USA) and expressed as mean  $\pm$  standard deviation. For multiple-group comparisons, one-way analysis of variance or Kruskal–Wallis nonparametric tests were applied. In contrast, the Least-Significant Difference (LSD) method was used for pairwise comparisons. A Student's  $t$ -test was used to compare the differences between the drug group and the combined with washing group in the same drug concentrations.  $P < 0.05$  was taken to define a statistically significant difference.

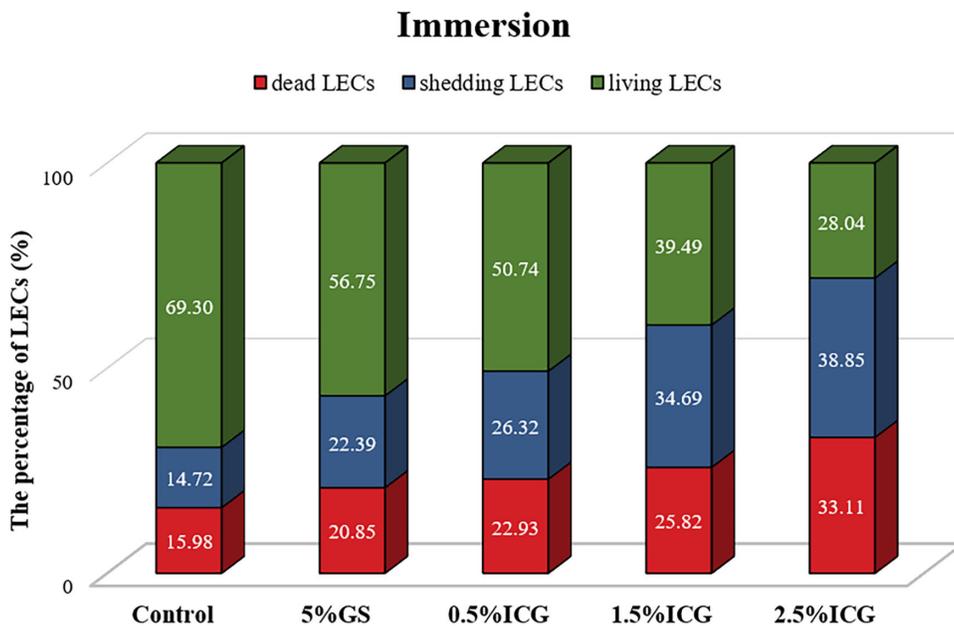
## Results

### Percentages of Dead, Shedding, or Living LECs in the Drug Immersion Group

Our analysis showed that the percentage of dead LECs on the capsule in the negative control group, 5% GS group, 0.5% ICG group, 1.5% ICG group, or 2.5% ICG group was  $15.98\% \pm 1.18\%$ ,  $20.85\% \pm 1.65\%$ ,  $22.93\% \pm 1.33\%$ ,  $25.82\% \pm 1.70\%$ , or  $33.11\% \pm 2.16\%$ , respectively. There were significant differences among the five groups ( $F = 11.548$ ,  $P = 0.000$ ). The percentage of dead LECs in the 0.5% ICG group, 1.5% ICG group, and 2.5% ICG group was significantly higher compared with the negative control group ( $P < 0.01$ ), while the percentage in the 2.5% ICG group was significantly higher compared with the 1.5% ICG group ( $P < 0.01$ ).

The percentage of shedding LECs on the capsule in the negative control group, 5% GS group, 0.5% ICG group, 1.5% ICG group, or 2.5% ICG group was  $14.72\% \pm 3.07\%$ ,  $22.39\% \pm 1.97\%$ ,  $26.32\% \pm 2.12\%$ ,  $34.69\% \pm 1.84\%$ , or  $38.85\% \pm 2.16\%$ , respectively. There were significant differences among the five groups ( $F = 36.786$ ,  $P = 0.000$ ). The percentage in the 0.5% ICG group, 1.5% ICG group, or 2.5% ICG group was higher than in the negative control group ( $P < 0.05$ ). Similarly, the 1.5% ICG group or 2.5% ICG group had a higher percentage compared with the 0.5% ICG group or 5% GS group ( $P < 0.05$ ).

The percentage of living LECs on the capsule in the negative control group, 5% GS group, 0.5% ICG group, 1.5% ICG group, or 2.5% ICG group was  $63.42\% \pm 2.49\%$ ,  $54.04\% \pm 1.84\%$ ,  $43.51\% \pm 2.63\%$ ,  $29.21\% \pm 2.40\%$ , or  $15.73\% \pm 1.61\%$ , respectively. Similarly, there were significant differences among the five groups ( $F$



**Figure 1.** The percentage of dead, shedding, or living LECs in the five groups of capsules after immersion for 3 minutes.

= 65.710,  $P = 0.000$ ). The 0.5% ICG group, 1.5% ICG group, or 2.5% ICG group had lower percentages in comparison with the 5% GS group ( $P < 0.01$ ). In addition, the percentage in the 2.5% ICG group was lower than that in the 0.5% ICG group and 1.5% ICG group ( $P < 0.01$ ), thus demonstrating a concentration–effect relationship (Fig. 1, Supplementary Fig. S1).

### Percentage of Dead, Shedding, or Living LECs in the Combined BSS Washing Group

Our data showed that combined BSS washing for 1 minute led to 17.51% ± 1.32%, 20.32% ± 0.97%, 25.77% ± 1.39%, 26.52% ± 1.75%, or 25.54% ± 3.37% dead LECs on the capsule in each group, respectively. The difference between the five groups was statistically significant ( $F = 13.059$ ,  $P = 0.011$ ). The percentage in the 0.5% ICG combined washing group, 1.5% ICG combined washing group, or the 2.5% ICG combined washing group was significantly higher compared with the negative control group ( $P < 0.05$ ).

The percentage of shedding LECs on the capsule in each group was 19.08% ± 2.40%, 25.64% ± 1.77%, 30.72% ± 1.95%, 44.27% ± 3.00%, or 58.73% ± 4.32%, respectively, with a statistically significant difference between the groups ( $F = 26.520$ ,  $P = 0.000$ ). The 0.5% ICG combined washing group, 1.5% ICG combined washing group, or the 2.5% ICG combined washing group had higher percentages than the negative control group ( $P < 0.05$ ). Both the 1.5% ICG combined

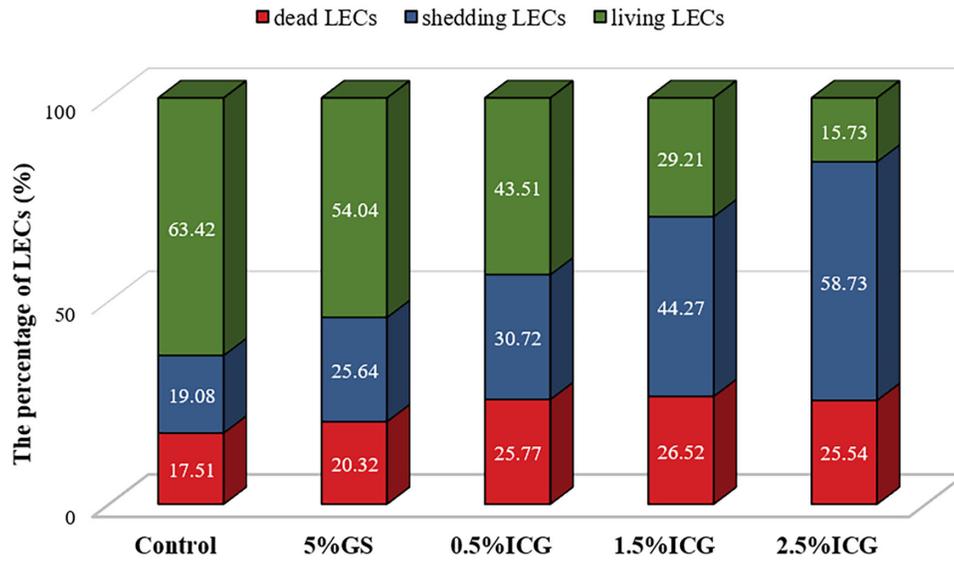
washing group and the 2.5% ICG combined washing group had higher percentages than the 0.5% ICG combined washing group ( $P < 0.01$ ).

On the other hand, the percentage of living LECs on the capsule in each group was 63.42% ± 2.49%, 54.04% ± 1.84%, 43.51% ± 2.63%, 29.21% ± 2.40%, or 15.73% ± 1.61%, and the differences among the five groups were significant ( $F = 65.710$ ,  $P = 0.000$ ). The percentages in the 0.5% ICG combined washing group, 1.5% ICG combined washing group, or 2.5% ICG combined washing group were lower than those in the 5% GS combined washing group ( $P < 0.01$ ). Similarly, the 2.5% ICG combined washing group had a lower percentage of living LECs compared with the 1.5% ICG combined washing group or the 0.5% ICG combined washing group ( $P < 0.01$ ), demonstrating a concentration–effect relationship (Fig. 2).

### Comparison of the Drug Immersion Group and Combined Washing Group

Our analysis showed that there was no statistically significant difference in the percentage of dead LECs between the drug immersion and combined washing groups ( $P > 0.05$ ). The percentage of shedding LECs in the 1.5% ICG combined washing group or 2.5% ICG combined washing group was higher compared with those of the simple immersion groups ( $P < 0.05$ ). In addition, there was no statistically significant difference in the percentage of the living LECs ( $P > 0.05$ ).

### Immersion combined washing



**Figure 2.** The percentage of dead, shedding, or living LECs in the five groups of capsules after washing with BSS for 1 minute.

However, the percentages of living LECs in the 0.5% ICG, 1.5% ICG, and 2.5% ICG combined washing groups were lower than those of the simple immersion groups ( $P < 0.05$ ).

#### TEM Results

The TEM data showed that the negative control group had intact and continuous nuclear membrane, uniform matrix in the cytoplasm, most round or rod-shaped mitochondria, clear and neatly arranged cristae, mostly round or oval nuclei shape, and clear nucleoli (Fig. 3A). The 5% GS group had most cells with mild edema but with intact mitochondria and nuclei (Fig. 3B). The 0.5% ICG group had gaps between the LECs as well as lapses between the cells and capsule. Few cells showed mild edema, while others had enlarged mitochondria with edema, showing vacuolar degeneration. In addition, most cell nuclei were irregularly shaped, had an intact nuclear membrane, and had an unclear and crumpled structure, with chromatin gathered into larger clumps (Fig. 3C). In the 1.5% ICG group, the connection between the LECs and the capsule was broken, along with irregular enlargement of the intercellular space and destroyed cell structures had severe vacuolar degeneration in the organelles. In addition, the mitochondria were swollen, with an enlarged and unclear mitochondrial structure. Most of the nuclear structures were distorted, with overflow nuclear contents (Fig. 3D). On the other hand, the 2.5% ICG group had an unidentified LEC structure and a

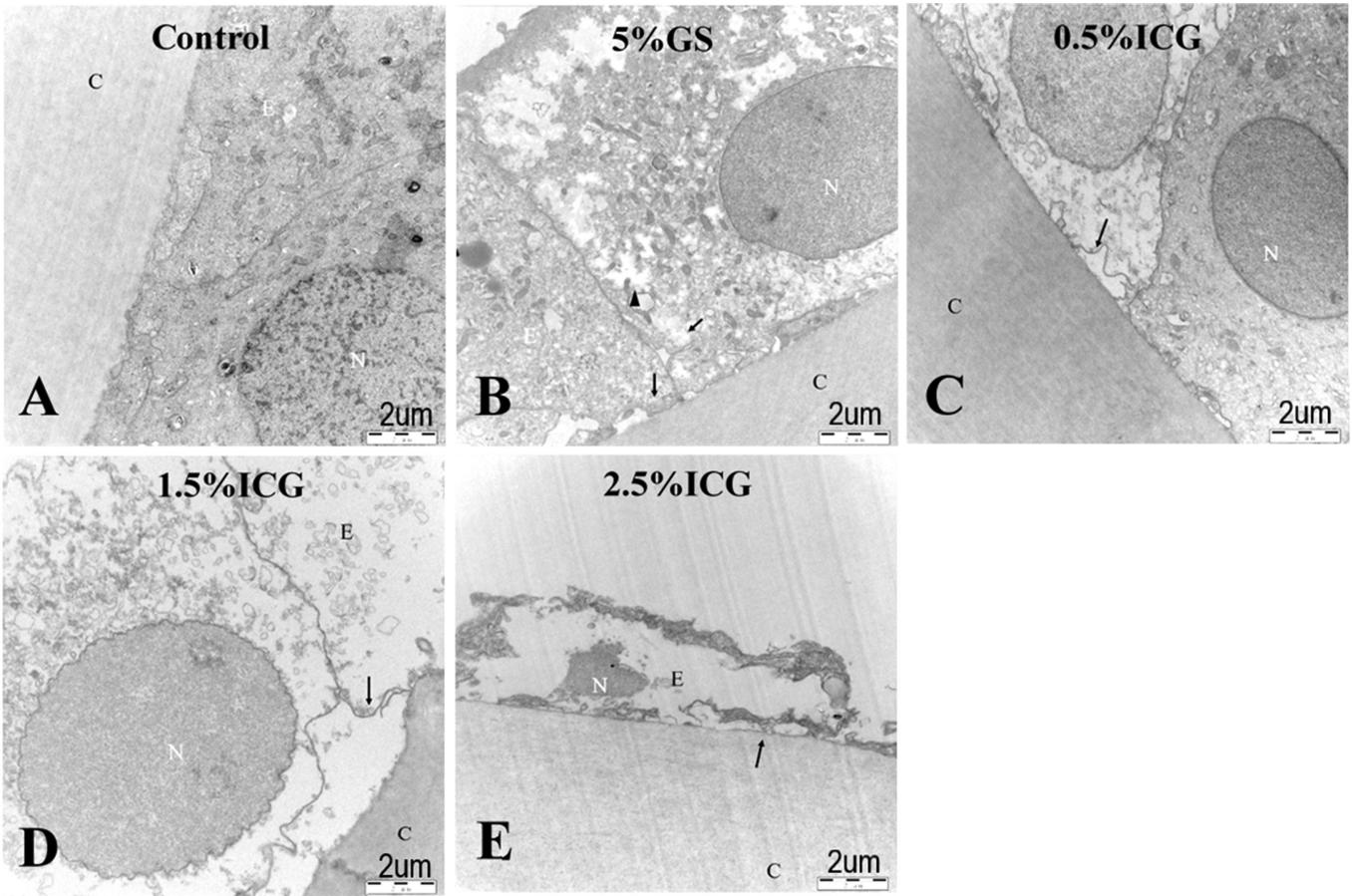
destroyed structure of the cell membrane with severe cytoplasmic extravasation. A large number of nuclear structures were destroyed with extravasated nuclear contents (Fig. 3E).

#### SEM Results

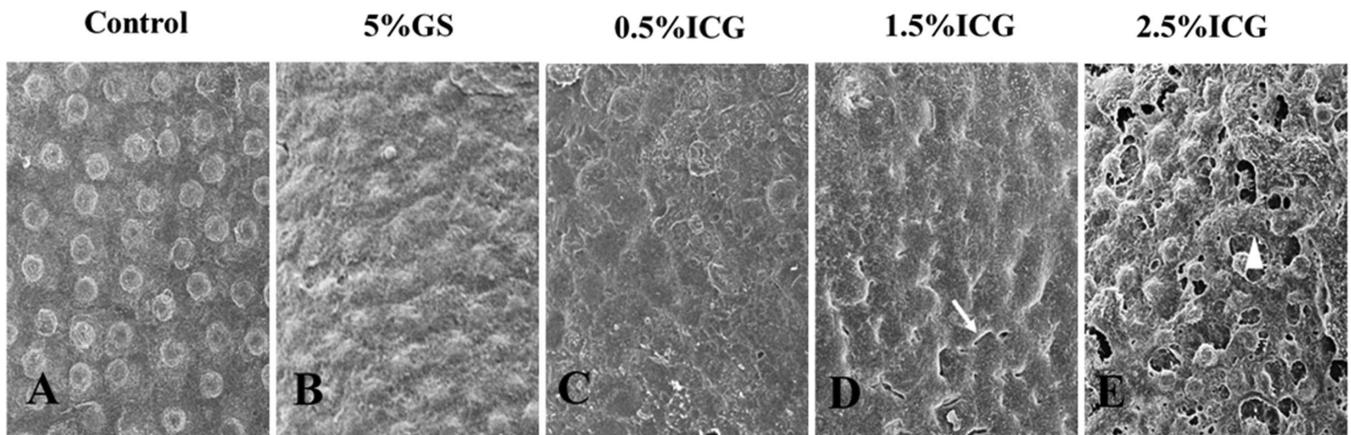
The SEM data showed that the negative control group had a relatively uniform arrangement of the LECs, with an undulated shape of a sand dune. The LECs were tightly connected, and the cell morphology had no obvious abnormalities. In the 5% GS group, there were gaps in a small number of intercellular areas, with no obvious abnormalities in cell morphology. The 0.5% ICG group had the gaps plus few shed cells, but the cell morphology was normal. In contrast, in the 1.5% ICG group, the connection between the LECs was relatively loose, with obvious gaps between some cells and partial cells shed. The lens capsule was exposed while some cell structures were destroyed. In the 2.5% ICG group, there was a loose connection between the LECs and a large gap between most cells. In addition, most of the cells were shed with more exposed lens capsules, and most of the cell structure was destroyed (Fig. 4).

### Discussion

ICG is a relatively nontoxic iodine-containing tricarboyanine dye. It is often used in cataract



**Figure 3.** TEM images of the five groups of the lens capsules after immersion for 3 minutes. (A) Part of the control epithelial cells showing the nucleus and lens capsule. (B) Part of the epithelial cell treated with 5% GS, showing most intact mitochondria and nuclei. (C) Part of the epithelial cell treated with 0.5% ICG, showing a gap between the LECs or a gap between the cells and the capsule or the vacuolated cytoplasm. (D) Part of the epithelial cell treated with 1.5% ICG, showing a broken connection between the LECs or between the cells and the capsule. (E) Part of the epithelial cell treated with 2.5% ICG, with an unidentified LEC structure. TEM images were taken using  $\times 12,000$  magnification. C, lens capsule; E, lens epithelial cells; N, nucleus. *Black arrows*: the gap between LECs or between cells and capsule. *Black triangle*: vacuoles in cytoplasm.



**Figure 4.** SEM images for the five groups of lens capsules after immersion for 3 minutes. The SEM images were taken using  $\times 1000$  magnification. *White arrow*: the gap between the LECs or between the cells and capsule. *White triangles*: shedding cells.

surgeries without red light reflection to increase the visibility of the anterior capsule and reduce the difficulty of capsulorhexis. Previous studies used a 0.1-mg/mL ICG solution to culture human LECs *in vitro* for 30 minutes and showed that ICG could reduce cell viability through inherent cytotoxicity.<sup>11</sup> At a 5-mg/mL concentration of ICG solution, only less than 5% of LECs survived. A higher than 0.5-mg/mL concentration of ICG solution led to cytotoxicity levels that masked the photosensitization effect.

In this study, we used different concentrations of the ICG solution to immerse the LECs on human lens capsule *ex vivo*. Our data showed that ICG could reduce the viability of the LECs and demonstrated a concentration–effect relationship, reduced the adhesion of the LECs, and promoted cytolysis. Ho<sup>13</sup> studied the toxicity of ICG on retinal pigment epithelium (RPE) cells and showed that after culturing RPE cells with 0.25 mg/mL ICG solution for more than 2 hours, cell shrinkage, lysis of many cells, and an unclear cell outline occurred. The author argued that the toxic effect of ICG on RPE was mediated by necrotic mechanisms. On the other hand, Abels et al.<sup>14</sup> suggested that since many cells could selectively transport and absorb ICG through organic anion transport peptides, the concentration of ICG in cells could reach several folds higher than the outside of the cell. Thus, with the increase in the ICG concentration and exposure time, there is corresponding increase in the toxicity. Our TEM results showed that ICG could destroy the structure of the LECs, thus reducing the viability of the LECs. We observed that the effect of ICG on the activity of LECs was also due to necrotic mechanisms. With the increasing ICG concentration, the transportation of ICG by LECs increases, thus exacerbating cell necrosis, leading to increased cell mortality.

Stalmans et al.<sup>15</sup> suggested that the toxic effect of ICG on RPE was related to the hypo-osmolarity of the solvent. The osmolarity of 5% GS was 308 mOsm/kg. In our study, the osmolarity of the 5-mg/mL ICG solution was 294 mOsm/kg<sup>16</sup> (in 5% GS), which was close to normal human aqueous humor<sup>17</sup> (290–295 mOsm/kg). Therefore, the ICG-induced cytotoxicity of the LECs cannot be a function of the hypo-osmolarity of the ICG solution.

ICG promoted the cytolysis of LECs from the lens capsule, as demonstrated by decreased cell adhesion. Gandorfer et al.<sup>18</sup> injected ICG solution into the vitreous cavity of cadaver eyes and showed that ICG could automatically make the internal limiting membrane separate from the retina. The separation interface of the internal limiting membrane was different from that which was separated in the inner layer of the retina. Moreover, it has been reported that application of the

ICG solution to human RPE cells and retinal glial cells led to different degrees of cell contraction, cell gap enlargement, and cell autonomy and detachment at the edge of the culture dish. The studies suggested that there was reduction of the adhesion of the cells after damage.<sup>19</sup> This observation was in parallel with our SEM results. Thus, we speculate that the higher the concentration of the ICG acting on the LECs, the more damage to the cells, which leads to an obvious decrease in adhesion and higher percentage of the capsule shedding area.

For better application in the phacoemulsification, we further used a 70-cm-high BSS to wash the capsule, to simulate the effect of the Irrigation/Aspiration (I/A) handle on the capsule LECs during aspiration. The results of the simulated I/A washing were similar to those of the immersion group. Under combined washing, the percentage of shedding LECs on the capsule was significantly higher compared with that in the drug-immersed group. Compared with the percentages of living LECs of the two groups at each concentration, it was shown that the percentage of living LECs in the combined washing group was lower, regardless of whether the difference was statistically significant. With the increase in the ICG concentration, there were obvious differences. This agrees with previous *in vitro* studies that showed that washing of the capsule could reduce the residual LECs on the canine capsule.<sup>20</sup> We therefore suggest that washing the capsule could use the force of water flow to increase the shedding LECs on the capsule and reduce the residual viable LECs on the capsule.

Unlike the drug immersion group, after 1 minute of combined washing, the dead LECs on the capsule showed a trend of first increasing and then decreasing. We speculate that this might have occurred because the concentration of ICG solution was diluted during the washing period, thereby reducing the cytotoxicity of ICG. As a result, there was a decrease in the percentage of dead LECs, particularly for higher concentrations of ICG.

Safety is one of the important factors to be considered in intraocular application of drugs to prevent capsular opacification. At present, 0.5% ICG solution is used for anterior capsule staining in cataract surgeries. This concentration of ICG solution can make the anterior capsule clear and cause less damage to other tissues in the anterior chamber as well.<sup>21</sup> However, the use of higher concentrations of ICG solution in the clinic might lead to tissue impairment. Therefore, when using a higher-solubility ICG solution, direct injection of the solution into the lens capsular bag after capsulorhexis might offer a solution. It can not only identify the target tissue but also

ensure the concentration of the solution. In addition, application of intraoperative viscoelastic might also increase the safety of other intraocular tissues.

This was an ex vivo study that evaluated isolated capsules from cataract patients. Since the LECs were out of the physiologic state, gentle manipulation of the capsule with platform forceps would inevitably affect the capsule. Moreover, mechanical damage increased the death and shedding of the LECs. Trypan blue could stain the nuclei of dead cells, so there was accuracy in the use of ImageJ analysis software to obtain the area of dead LECs, which was slightly higher than the real area of the dead LECs. Therefore, in-depth studies with large sample sizes are needed to determine the most suitable ICG concentration to prevent capsular opacification and minimize damage to other tissues and structures in the eye.

Taken together, our data demonstrated that ICG could reduce the viability of isolated human eye capsule LECs and presented a concentration–effect relationship. There was reduction of adhesion and promotion of cytolysis of the LECs. Thus, ICG might be an ideal drug for effective prevention of capsule opacity in clinical applications.

## Acknowledgments

The authors thank the patients who contributed to this study.

Disclosure: **Z.-F. He**, None; **Y.-D. Zhao**, None; **S. Zhang**, None; **F.-F. Chen**, None; **Y.-J. Liu**, None; **W.-W. Zhang**, None; **Z.-G. Xie**, None

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