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Involvement of illumination in indocyanine green toxicity after its washout in the *ex vivo* rat retina

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Abstract

Purpose—To elucidate the involvement of illumination in indocyanine green (ICG) retinal toxicity.

Methods—We incubated isolated rat retinas with or without illumination after exposure to 0.5 % ICG. We also examined whether a time lag following ICG exposure prior to illumination altered the damage. Toxicity was evaluated by histological and biochemical assays, including measurement of lactate dehydrogenase (LDH) release.

Results—Retinas fixed immediately after ICG exposure showed minimal morphological changes. However, illumination for 3 h at 34 °C starting after washout of ICG selectively damaged the outer nuclear layer. Retinas incubated for 3 h under the same condition in the dark showed preserved morphology but were damaged by subsequent illumination. When retinas were illuminated after washout of ICG at a lower temperature (30 °C), the damage was attenuated. Results obtained using LDH release were consistent with these morphological changes.

Conclusions—Incubating retinas in the dark and cooling after ICG exposure significantly inhibited retinal damage, suggesting that ICG interacts with illumination to induce retinal damage.

Summary Statement—Illumination after indocyanine green (ICG) exposure damaged the outer nuclear layer in isolated rat retinas. Incubating retinas in the dark or cooling after its exposure attenuated the damage, indicating phototoxic properties of ICG. Phototoxic damage developing after ICG exposure suggests the importance of protecting the retina from light during and after vitrectomy.

Keywords

cooling; illumination; indocyanine green; intraocular solution; isolated rat retinas; outer nuclear layer; phototoxicity; vital dye; vitreoretinal surgery

For half a century indocyanine green (ICG), a tricarboyanine dye, has been used for angiography to assess cardiac and liver function.^{1, 2} In ophthalmology, ICG has been used to evaluate the choroidal circulation³ and has played a significant role in the diagnosis of chorioretinal disorders.⁴⁻⁷ Because systemic adverse effects are rare,⁸ ICG is thought to be safe when injected intravenously. ICG is also useful in staining the internal limiting membrane (ILM) of the retina for visualization during vitrectomy⁹ and ICG is now routinely used in retinal surgeries for repair of macular holes^{9, 10} and in epiretinal membrane surgeries.¹¹ However, there remain clinical concerns about possible toxic effects of ICG on the retina following ICG-assisted vitrectomies. These toxic effects include poor functional outcomes,^{12, 13} visual field defects¹⁴⁻¹⁶ and retinal pigment epithelium degeneration.^{17, 18}

Several measures have been used during vitrectomy to minimize the toxic effects of ICG. Although use of lower concentrations of ICG^{19, 20} or irrigation immediately after ICG exposure^{21, 22} limit the toxic actions, adverse effects on the retina following ICG exposure remain common, suggesting that these simple measures do not successfully minimize the toxicity.^{13, 23} ICG exposure during vitrectomy must be followed by intensive intraocular illumination which itself has long been speculated to be responsible for poor outcomes following vitrectomy.^{24, 25} Thus, we hypothesized that one of the toxic actions of ICG results from an interaction with illumination during or after ICG exposure.

Phototoxicity in the retina is characterized by degeneration of photoreceptor cells in the outer nuclear layer (ONL).²⁶⁻²⁹ To assess layer-specific mechanisms involved in various forms of retinal injury, we have developed a mammalian *ex vivo* retinal preparation³⁰ that allows detailed morphological assessment of changes in specific cell layers. We previously used this preparation to examine interactions of a phototoxic drug with illumination in producing retinal damage,³¹ and have found that the preparation is useful for studying phototoxic interactions. Morphological assessment is critical for understanding the effects of phototoxicity and for characterizing the adverse effects of ICG.³² In the present study we used a rat retinal preparation and observed that ICG has interactions with intensive illumination in inducing retinal damage. To quantify retinal damage, we combined morphological assessments with a biochemical assay measuring lactate dehydrogenase (LDH) released from injured cells. We also examined whether changes in incubation temperature altered ICG-mediated phototoxicity.

Materials and Methods

Animal statement

All experimental protocols were approved by the Institutional Animal Studies Committee at Washington University School of Medicine. Every effort was made to minimize the number of animals used and their suffering in all experimental procedures.

Ex vivo rat retinal preparation

Male Sprague-Dawley rats obtained from Harlan (Indianapolis, IN, USA) at postnatal date (PND) 23 were reared with a cycle of 12 hours white light and 12 hours dim light. At PND 30 $\pm$ 2, rats were anesthetized with isoflurane and decapitated. Retinas were dissected using previously described methods.³⁰ After enucleation, the lens and vitreous were quickly removed at 4-6°C. The eye cap was cut into 3 pieces and then the retina was gently detached from the retinal pigment epithelium layer. The inferior portion of the isolated retina was discarded because of its lower sensitivity to light.³³ Following dissection, retinal segments were placed on nylon mesh in a beaker containing artificial cerebrospinal fluid (aCSF) and allowed to recover for one hour. The aCSF contained (in mM): 124 NaCl, 5 KCl, 2 MgSO₄, 2 CaCl₂, 1.25 NaH₂PO₄, 22 NaHCO₃, and 10 glucose and was bubbled continuously with 95% O₂ - 5% CO₂. We have reported that this aCSF is suitable as an incubating media for isolated rat retina.³⁴ The osmolarity and pH of aCSF were 287 mmol/kg H₂O and 7.32, respectively.

ICG exposure

Twenty-five milligram of ICG powder (Dai-ichi Pharmaceutical Co., Ltd., Tokyo, Japan) was dissolved in 1 ml distilled water, and 4.0 ml aCSF was added to make a final ICG concentration of 0.5%. The retinal tissues were exposed to 0.5% ICG or vehicle (control; distilled water: aCSF = 1:4) at room temperature (25°C) for 1 minute, then rinsed twice with fresh aCSF.

Light exposure

Retinal segments were transferred to glass vials (7 ml) filled with gassed aCSF. The retinal segments were exposed to white light or darkness during experiments as previously described.³¹ The glass vials were placed 5 cm from a light source (13 watts, model 13333; OTT-LITE Co., Tampa, FL, USA). The wavelength of this light ranges from 380 nm to 780 nm with a peak at 545 nm, providing 20,000 Lux to retinal segments. Each vial was gassed with 95% O₂ - 5%CO₂ every 15 min during incubation. The bath temperature was kept at 30°C or 34°C. Some samples were processed for light microscopy and morphological evaluation.

LDH measurements

Measurement of LDH activity released to the extracellular bathing media is a simple but quantitative method for neuronal cell injury.³⁵ As intracellular LDH is membrane-bound³⁶ and LDH release into extracellular space is slow relative to morphological damages,³⁷ LDH levels were determined using gentle brief sonication as we previously reported.³⁸ LDH activity was measured spectrophotometrically from 17 µl samples of incubating media. The activity of LDH was determined by the rate at which its substrate, pyruvate, is reduced to lactate, as monitored by diminished absorbance of the reduced form of nicotinamide adenine dinucleotide (NADH) at 340 nm (Ultrospec 2100 pro UV/Visible Spectrophotometer, Biochrom Ltd., Cambridge, UK). To control for differences in the size of individual retinal segments, LDH release into the medium was normalized to a percentage of the total LDH value obtained by sonication upon completion of an experiment (Model 250 Sonifier, Branson Ultrasonics Corp. CT, Danbury, USA). For cell lysis, 0.1% Triton X-100 was applied before sonication. There was no effect of 0.1% Triton X-100 on the absorption spectrum at 340 nm.

Histology

Retinal tissues were fixed in phosphate-buffered saline containing 1% paraformaldehyde and 1.5% glutaraldehyde overnight at 4°C. The fixed tissue was rinsed in 0.1% phosphate buffer, placed in 1% buffered osmium tetroxide for 60 minutes, dehydrated with alcohol and toluene, embedded in araldite, cut into sections 1 µm thick, stained with methylene blue and Azure B (Rowley Biochemical Institute, Danvers, MA, USA), and evaluated by light microscopy.

The degree of cellular damage in each sample was determined by the ratio of mean luminosity in the outer nuclear layer (ONL) compared with that in the inner plexiform layer (IPL). In order to confirm correlations between cell density and mean tissue luminosity in the ONL, we counted photoreceptor cells in the ONL in five randomly selected samples and found an inverse correlation ($r = -0.99$, $P < 0.01$). This was done using commercial software (Adobe Photoshop 8.0; Adobe Systems Inc., San Jose, CA, USA) in order to adjust for the variable intensity of staining among samples.

Chemicals

All chemical reagents except ICG were obtained from Sigma (St. Louis, MO, USA).

Statistical analysis

All quantitative results are expressed as mean $\pm$ standard error of the mean (SEM). Statistical differences between means were evaluated with Student's *t*-test using commercial software (SigmaStat 3.1.1; Systat Software inc., Richmond, CA, USA). If the samples were not drawn from normally distributed population with the same variance, the Mann-Whitney Rank Sum Test was applied. *P* values of < 0.05 were considered significant.

Results

Effects of illumination following ICG exposure on retinal damage

We first examined whether 0.5% ICG alone alters the histological integrity of isolated retinas. Consistent with prior reports,^{32, 39} we found that morphological changes immediately after brief (1 min) ICG exposures were minimal (Fig. 1A, $n = 5$, ICG 0 h). We subsequently illuminated isolated retinas at 34°C for 3 h starting immediately after washout of ICG. Retinal segments intensively illuminated for 3 h without ICG exposure did not show any morphological changes (Fig. 1A, $n = 5$, aCSF Light 34 °C 3 h). In contrast, retinas illuminated for 3 h after washout of ICG showed conspicuous changes in the ONL (Fig. 1A, $n = 5$, ICG Light 34 °C 3 h), characterized by pyknotic nuclei of the photoreceptor cells and disarrangement of the ONL columns. Damage in the outer limiting membrane (OLM) resulted in loss of photoreceptor cells from the ONL. These types of structural changes have been described previously as light-induced retinal damage in *in vivo* studies.²⁶⁻²⁸ On the other hand, cells in the ganglion cell layers (GCL) and in the inner nuclear layers (INL) showed no destructive changes. A mild spongiform appearance was observed in the IPL but was similar to that seen in retinas fixed immediately after ICG exposure. In contrast, retinas incubated for 3 h under the same conditions but kept in the dark after washout of ICG showed markedly less damage in the ONL (Fig. 1A, $n = 5$, ICG Dark 34 °C 3 h) and ONL columns were only minimally altered.

To quantify the morphological changes, we measured the ratio of tissue luminosity in the ONL to that in the IPL. Immediately following ICG exposure, the ONL luminosity ratio did not differ from controls (Fig. 1B, $n = 5$, $P = 0.24$, aCSF 0 h vs. ICG 0 h). However, there was a significant difference in the luminosity ratio between naïve retinas illuminated for 3 h and retinas incubated under same condition but with ICG exposure (Fig. 1B, $n = 5$, $P < 0.01$, aCSF Light 34 °C 3 h vs. ICG Light 34 °C 3 h). The luminosity ratio was significantly reduced in retinas maintained in the dark for 3 h following ICG washout (Fig. 1B, $n = 5$, $P < 0.01$, ICG Light 34 °C 3 h vs. ICG Dark 34 °C 3 h).

We previously observed that there is a good correlation between extracellular LDH levels and histological cellular damage in the *ex vivo* retina.^{38, 40} Consistent with the intact morphology observed after 3 h illumination in control retinas (Fig. 1A), we found no increase in LDH release from naïve retinas illuminated for 3 h compared with those sampled before illumination (Fig. 2, $n = 5-6$, $P = 0.76$, aCSF 0 h vs. aCSF Light 34 °C 3 h). Importantly, there was also no significant increase in LDH release from retinas sampled immediately after ICG exposure (Fig. 2, $P = 0.99$, aCSF 0 h vs. ICG 0 h). We did observe a significant increase in LDH release in retinas that had been exposed to ICG and subsequently illuminated for 3 h (Fig. 2, $P < 0.01$, aCSF 0 h vs. ICG Light 34 °C 3 h). This increase in LDH was markedly attenuated when retinas were incubated in the same fashion but kept in the dark (Fig. 2, $P < 0.01$, ICG Light 34 °C 3 h vs. ICG Dark 34 °C 3 h).

Retinas are damaged by illumination following incubation in the dark for 3h after ICG exposure

Based on these results, it appears that maintaining retinas in the dark following ICG exposure greatly improves morphological and biochemical outcomes. However, ICG may persist in retinal tissues beyond the initial exposure period and may have longer-lived photosensitizing effects. To test this, we incubated retinas for 3 h in the dark after washout of ICG and then illuminated for 2 h. In this condition, ONL architecture was severely damaged whereas other layers remained intact (Fig. 3A, $n = 3$, Additional 2 h with light). Furthermore, the ratio of tissue luminosity in the retinas additionally illuminated for 2 h was significantly greater than that in retinas incubated for the same period of time in the dark (Fig. 3B, $n = 3$, $P < 0.01$, additional 2 h with light vs. additional 2 h in the dark). No significant morphological changes

were detected in retinas incubated in the dark for 5 h in total following ICG exposure (Fig. 3A, $n = 3$, ICG (-) 5h with light). There was no difference in tissue luminosity between controls (illuminated for 5 h without ICG exposure) and samples incubated an additional 2 h in the dark (Fig. 3B, $n = 3$, $P = 0.53$, ICG (-) 5h with light vs. additional 2 h in the dark).

Effects of cooling on ICG-mediated retinal phototoxicity

To determine whether changes in incubation conditions affect ICG-mediated toxicity, we examined the effects of cooling retinas to 30°C during illumination for 3 h following ICG exposure. Retinas illuminated for 3 h incubated at 30°C without ICG exposure in the dark revealed no morphological changes (Fig. 4A, $n = 5$, aCSF Light 30 °C 3 h). Although retinas incubated under the same condition with ICG exposure showed some changes in the ONL (Fig. 4A, $n = 5$, ICG Light 30 °C 3 h), the damage was not as prominent as that observed in retinas incubated at 34°C (Fig. 4A, $n = 5$, ICG Light 34 °C 3 h). The loss of cells in the ONL was minimal and the morphology of the OLM was well preserved. There was a significant difference in the ratio of tissue luminosity between retinas illuminated for 3 h after ICG exposure at 30°C and retinas incubated under the same condition at 34°C (Fig. 4B, $n = 5$, $P < 0.01$, ICG Light 30 °C 3 h vs. ICG Light 34 °C 3 h).

Consistent with these histological results, LDH release from retinas illuminated for 3 h at 30°C after washout of ICG was significantly lower than that from retinas incubated under the same conditions at 34°C (Fig. 5, $n = 5$, $P < 0.01$, ICG Light 30 °C 3 h vs. ICG Light 34 °C 3 h).

Discussion

Prior studies have found that retinal phototoxicity is characterized by degeneration of photoreceptor cells in the ONL,²⁶⁻²⁹ but it is unknown whether visual problems associated with ICG reflect this type of injury. Using optical coherence tomography, Querques and co-workers⁴¹ observed atrophic changes in the ONL in a patient who had undergone ICG-assisted vitrectomy. Recently, Tsuiki et al.¹⁶ retrospectively analyzed 96 cases of macular hole surgeries using ICG-assisted visualization. They found that the left eyes of the patients likely had nasal field visual defects while the right eyes had a tendency to show temporal field visual defects in reverse. The authors noted that these visual defects may reflect the fact that right-handed surgeons typically hold endoscopic lights in their left hand and thus the left eye receives more intensive illumination in the temporal region during ILM peeling. These clinical observations suggest that illumination may play a significant role in ICG-mediated retinal toxicity.

In clinical uses, ICG injected into the vitreous cavity remains at the initial concentration because dilution in the cavity is limited following air-fluid exchange. Thus, we used 0.5 % ICG for our studies, a concentration that is at the upper limit of those used clinically.^{16, 41} We found that retinas fixed immediately after 0.5 % ICG exposure did not show any structural changes (Fig. 1A), similar to what has been reported by other investigators.^{32, 39} The lack of changes in retinal morphology is consistent with the lack of retinal damage using biochemical measurements of LDH release (Fig. 2). These observations suggest that ICG alone does not produce acute retinal damage when used at clinical concentrations.

In *in vivo* animal studies, morphological changes in the retina following intensive illumination are characterized by selective degeneration of neurons in the ONL.²⁶⁻²⁹ Similar degeneration is also observed in *ex vivo* retinal preparations. We previously found that isolated retinas exposed to ultraviolet B radiation exhibit phototoxicity that is limited to the ONL.⁴² In the present study, illumination for 3 h after ICG exposure induced remarkable morphological damage that was predominant in the ONL without affecting neurons in either the GCL or INL. Incubation in the dark prevented this damage, suggesting that the damage resulted from an

interaction of ICG exposure and illumination. Consistent with the morphological changes, we found a significant difference in LDH release between retinas incubated with illumination after ICG exposure and those incubated under same condition but kept in the dark (Fig. 2). Taken together, we find that ICG applied alone to the retina produces minimal damage but becomes toxic when the retina is intensively illuminated following ICG application.

Although our study indicates that the ICG induces phototoxic damage in the retina, clinical conclusions cannot be drawn directly from this *ex vivo* animal study. In *in vivo* animal studies, phototoxic damage in the retina is characterized by thinning of the ONL.²⁶⁻²⁹ However, such a finding has not yet been confirmed in clinical studies. In human donor post-mortem eyes, Gandorfer and colleagues reported that previous ICG treatment results in ILM detachment and disruption of Müller cells, and that, in a eyes from two donors, illumination with a light of wavelength over 620 nm added severe damage in the nerve fiber layer and ganglion cells.⁴³ However, damage in the ONL is not described in this report. In contrast, damage in the GCL is not clear in the present study. These discrepancies may be explained by difference in experimental protocols and time windows of observation. Further detailed observations in clinical settings are required to confirm the phototoxic properties of ICG.

Once injected into the vitreous cavity, it is difficult to completely remove ICG because it binds to laminin and type IV collagen on the retinal surface.⁴⁴ ICG also penetrates through all the sensory retinal layers to reach the retinal pigment layer.³⁹ Intravitreal remnants of ICG remain long after vitrectomy because of its slow metabolism in the vitreous cavity.⁴⁵⁻⁴⁸ Importantly, we observed that isolated retinas exposed to ICG were not damaged until illumination was delivered even if light exposure was delayed for three hours following ICG removal (Fig. 3). This strongly suggests that the toxic effects observed result from phototoxicity and not simply from ICG exposure alone. Furthermore, this suggests that in a clinical setting ICG that is trapped on the surface or in the sensory layers of the retina may become toxic or photosensitizing when intensive illumination is delivered during surgery even after washout of ICG. While our studies do not indicate how long retinas remain at risk following ICG exposure, our results suggest that ophthalmic surgeries with strong illumination soon after ICG-assisted vitrectomy was performed may result in unexpected retinal damage.

If intense illumination is responsible for ICG toxicity, then the temperature of the retina may be a critical issue because the development of phototoxicity is sensitive to changes in ambient temperature.^{27, 28, 49} Rinkoff et al.⁵⁰ examined the effects of intravitreal fluid temperature on rabbit eyes exposed to high intensity white light. They found that the damage threshold at 39°C was twice as high as that at 22°C. In the present study, lowering the temperature of the incubation media after ICG exposure significantly reduced damage (Fig. 4 and 5). The retinal protection by cooling again suggests that ICG has phototoxic properties. Irrigating the vitreous cavity with artificial intraocular solutions, which decrease the intraocular temperature elevated by illumination, may be beneficial for preventing ICG-mediated phototoxicity.

In addition to the phototoxic damage in the ONL, all retinas exposed to ICG showed a mild spongiform appearance in the IPL. It seems unlikely that this change results from a phototoxic reaction because similar histological effects are observed even when retinas are fixed immediately after ICG exposure and the changes are not altered by illumination. It is possible that these morphological effects reflect a direct action of ICG in the retina.

Multiple factors and processes are likely to contribute to ICG-mediated retinal toxicity including changes in osmolarity,⁵¹ apoptosis⁵² and cytotoxicity.⁵³ In this study, we used white light to induce damage because light sources used during vitrectomies (halogen, metal halide and xenon) have a wide spectrum ranging from 400 to 700 nm²⁵ and ICG retinal toxicity has been reported using each of these light sources (halogen,⁵⁴ metal halide,²⁴ xenon¹⁶). As

the absorption band of ICG is beyond 600 nm with two maxima at 700 and 780 nm,⁵⁵ filtering a certain spectrum of intraocular illumination may also represent a way to attenuate ICG-induced retinal phototoxicity.

In the present study we also showed that ICG-mediated toxicity becomes most clearly manifest following light exposure. Although the pathogenesis of drug-related phototoxicity is not fully understood, it is widely believed that reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and singlet oxygen, generated through photochemical reactions in the presence of a photosensitizer are responsible for inducing the damage.^{56, 57} Likewise, it is possible that ICG serves as a photosensitizer to generate ROS, which in turn result in retinal damage under illumination. Indeed, decomposition of ICG by illumination may be critical for its toxic actions. Engel et al.⁵⁸ recently proposed that singlet oxygen, generated by illumination of ICG, decomposes ICG. They also showed that decomposed ICG damages cultured retinal pigment epithelial cells even in the dark. Thus, it is also possible that decomposed ICG serves as a photosensitizer and contributes to damage in the retina under illumination. To determine the involvement of ROS in ICG-induced retinal toxicity it will be helpful to test antioxidants for their ability to inhibit ROS activity. It will also be important to examine the threshold of light intensity required to produce ICG retinal damage and to determine the wavelength of light that is responsible for the ICG-mediated retinal toxicity.

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Fig. 1. Effects of light on ICG retinal toxicity after ICG exposure and washout in an *ex vivo* rat retinal preparation.

A, Histological evaluation by microscopy. The first panel shows a control retinal segment immediately after exposure to vehicle (aCSF 0 h) and demonstrates normal appearing retinal morphology. A retinal segment fixed immediately after exposure to 0.5 % indocyanine green (ICG) shows no structural changes (ICG 0 h). Similarly, no morphological changes are seen in the retina incubated for 3 h with illumination after exposure to vehicle (aCSF Light 34 °C 3 h). A retina incubated at 34 °C with illumination for 3 h after exposure to 0.5 % ICG shows destructive changes selectively in the ONL (ICG Light 34 °C 3 h), whereas a retina incubated

under the same condition but kept in the dark showed little change in the ONL (ICG Dark 34 °C 3 h). Note that few changes in morphology were observed in both the INL and GCL regardless of ICG exposure. All retinas were stained with methylene blue and Azure B. Abbreviations: POS, photoreceptor outer segment; OLM, outer limiting membrane; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale: 50 μ m.

B, Histological evaluation by tissue luminosity. As a way to quantitate changes in retinal morphology, we measured the ratio of mean tissue luminosity in the ONL compared with that in the IPL in each retinal sample. The tissue luminosity ratio in the ONL immediately after ICG exposure (ICG 0 h) or after 3h incubation under illumination with exposure to vehicle (aCSF Light 34 °C 3h) did not differ from that of the control (aCSF 0 h) (n = 5). There was a significant difference in the luminosity ratio between naïve retinas illuminated for 3 h and retinas exposed to illumination following ICG treatment (n = 5, $*P < 0.01$, Student's *t*-test, aCSF Light 34 °C 3 h vs. ICG Light 34 °C 3 h). The tissue luminosity ratio in retinas incubated for 3 h after ICG exposure was significantly reduced when incubated in the dark. (n = 5, $*P < 0.01$, *t*-test, ICG Light 34 °C 3 h vs. ICG Dark 34 °C 3 h).

Fig. 2.

Effects of light on ICG retinal toxicity after ICG exposure and washout in an *ex vivo* rat retinal preparation: evaluation by LDH release. There was no significant increase in LDH release from retinas immediately after ICG exposure compared with control ($n = 5-6$, Student's *t*-test, aCSF 0 h vs. ICG 0 h). LDH release from samples illuminated for 3 h after washout of ICG showed a significant increase compared to samples illuminated for 3 h without ICG exposure ($n = 5$, $*P < 0.01$, *t*-test, aCSF Light 34 °C 3 h vs. ICG Light 34 °C 3 h). Incubation in the dark after ICG exposure markedly diminished LDH release ($n = 5$, $*P < 0.01$, *t*-test, ICG Light 34 °C 3 h vs. ICG Dark 34 °C 3 h).

Fig. 3.

Retinas maintained in the dark for 3 h after washout of ICG are damaged by subsequent illumination.

A, Histological evaluation by microscopy. A retina incubated in the dark for 3 h after ICG exposure followed by an additional 2 h incubation in the dark shows little morphological change ($n = 3$, additional 2 h in the dark). However, additional incubation for 2 h with intense illumination starting 3 h after ICG washout selectively damages the ONL ($n = 3$, Additional 2 h with light). A retina incubated for 5 h with illumination shows intact morphology throughout all layers ($n = 3$, ICG (-) 5 h with light). Abbreviations: POS, photoreceptor outer segment;

OLM, outer limiting membrane; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale: 50 μm .

B, Histological evaluation by tissue luminosity. There was no significant difference in tissue luminosity between controls (illuminated for 5 h without ICG exposure) and samples incubated an additional 2 h in the dark ($n = 3$, Student's t -test, ICG (-) 5 h with light vs. additional 2 h in the dark). However, when incubated an additional 2 h with illumination tissue luminosity in the ONL increased significantly compared to retinas incubated continuously in the dark ($n = 3$, $P < 0.01$, t -test, Additional 2 h with light vs. additional 2 h in the dark).

Fig. 4.
Effects of cooling on ICG retinal phototoxicity.

A, Histological evaluation by microscopy. No morphological changes are observed in a retina incubated at 30 °C for 3 h with illumination after exposure to vehicle (aCSF Light 30 °C 3 h). A retina incubated at 30 °C with illumination for 3 h after ICG exposure shows cellular loss in the ONL (ICG Light 30 °C 3 h), however, the changes are markedly diminished compared to a retina incubated under the same condition but kept at 34 °C (ICG Light 34 °C 3 h). A retina incubated at 34 °C in the Fig. 1A is shown again for comparison. All retinas were stained with methylene blue and Azure B. Abbreviations: POS, photoreceptor outer segment; OLM, outer

limiting membrane; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale: 50 μm .

B, Histological evaluation by tissue luminosity. The tissue luminosity ratio in retinas incubated at 30 °C with illumination for 3 h after ICG exposure (ICG Light 30 °C 3 h) showed a significant difference from that of retinas exposed to the same conditions but incubated at 34 °C ($n = 5$, $*P < 0.01$, Student's t -test, ICG Light 30 °C 3 h vs. ICG Light 34 °C 3 h).

Fig. 5.

Effects of cooling on ICG retinal phototoxicity: evaluation by LDH release. LDH release from retinas illuminated for 3 h after ICG exposure was significantly reduced when incubated at 30 °C compared to that from retinas incubated under the same conditions but at 34 °C ($n = 5$, $*P < 0.01$, Student's t -test, ICG Light 30 °C 3 h vs. ICG Light 34 °C 3 h). The data at 34 °C are shown for comparison and are the same as in Fig. 2.