Vital staining with indocyanine green: a review of the clinical and experimental studies relating to safety

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Abstract

Indocyanine green (ICG) is extremely effective when used as a vital stain during macular hole surgery. By staining the internal limiting membrane, ICG facilitates removal of this delicate and sometimes hard to visualize structure. There is, however, considerable debate regarding its safety. This review considers the clinical and experimental studies of ICG and a related agent, infracyanine green. Some clinical papers show visual field defects, reduced visual acuity, and persistence of ICG at the macula and optic nerve. Other clinical studies fail to demonstrate toxicity. The experimental studies are also conflicting, but there are emerging trends. These suggest that surgeons who continue to use ICG should use concentrations not greater than 0.05 mg/ml, in fluid-filled eyes, with short exposure times, iso-osmolar solutions, and avoid proximal or prolonged endoillumination of stained tissue. A smaller number of studies suggest that infracyanine green produces similar staining to ICG, and may possibly be safer, but there are too few well-designed studies to reach a conclusion. Although the use of ICG continues, on the balance of evidence, this review suggests that it has the potential to produce subtle visual damage.

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Keywords: indocyanine green; infracyanine green; toxicity; review; safety; macular hole

Introduction

Indocyanine green (ICG) is a tricarbo-cyanine dye (C₄₃H₄₇N₂O₆S₂Na) that binds avidly to

serum proteins, with peak absorption in the infrared spectrum (800 nm). The latter property underlies its application as a fluorophore in choroidal angiography. ICG can also act as an effective chromophore, staining the internal limiting membrane (ILM) of the retina green. By staining the ILM, ICG can help surgeons dissect this hard-to-visualise structure from the underlying retina during pars plana vitrectomy.

Initial enthusiasm for ICG as a vital stain was followed by reports showing possible toxicity, and a sometimes vociferous debate ensued regarding the safety, or otherwise, of ICG. Some clinicians have reported favourable surgical results using ICG, others identified adverse effects including reduced visual acuity (VA), optic disc changes, and visual field defects. Experimental studies have also shown potential toxicity, but not all studies concur, particularly when using lower concentrations of ICG.

This review appraises the clinical and experimental literature on ICG safety. Firstly, it considers the clinical series in which ICG was used, and then studies indicating that ICG can persist in the eye for an extended period of time. Next is a section dealing with surgical techniques that may minimize the risk of toxicity. Following this is an appraisal of the *in vivo* animal experiments, organ culture, cell culture, and histological studies. Infracyanine green, a closely related agent, is also evaluated, considering both clinical and experimental studies. Finally this article attempts to distil the key issues and draw some conclusions on the safety of ICG.

Materials and methods

Clinical and experimental articles were identified using a Biomed Medline and

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EMBASE database search (1997–2007 inclusive) using the following keywords and Mesh headings: indocyanine green, ICG, infracyanine green, toxicity, safety, macular hole, and vitrectomy. Articles in English, French, and German were included, but those appearing only as abstracts were excluded. Further articles were identified from the reference list of each article, and following peer review. Because of the paucity of randomized clinical trials and varying investigative methods, statistical meta-analysis was not deemed appropriate. Instead, all papers were reviewed and emerging trends were identified, with selected articles referenced in this summary paper.

Clinical studies

Background

There have been a considerable number of clinical studies of ICG-assisted peeling of the ILM in the past few years, mostly in the context of macular hole surgery. Authors in favour of ICG-assisted ILM peeling provide multiple justifications for its use. ICG can undoubtedly improve visualization of the ILM, and it may shorten operating times and thereby also reduce endoillumination of the fovea. Improved visualization of the ILM may facilitate less traumatic removal and damage to the inner retina. There are, however, many reports of toxicity, with multiple adverse events reported in association with ICG use (see summary below)—the difficulty is establishing a causal link between the use of ICG and visual damage.

Summary: adverse events reported in association with ICG use

- 1 Reduced VA
 - 2 Visual field defects
 - 3 Retinal pigment epithelium changes
 - 4 Reduced rate of macular hole closure
 - 5 Persistence of ICG in the retina and optic nerve
 - 6 Optic atrophy
 - 7 Altered surgical cleavage plane with ILM peeling
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Randomized clinical trials

Beutel *et al*¹ compared ILM peeling with trypan blue, an alternative vital stain, or ICG in macular hole surgery. The ICG concentration was 0.05 mg/ml (0.005%), with the dye washed out immediately after its application. Final ETDRS VA improved by two lines in both groups, without a significant difference. Compared with the trypan blue group, the ICG group had a larger number of central scotomas, as measured by micropertimetry. Macular hole closure rate was 84% and similar in both groups. The strengths of this study were the standardized examination of patients by a single examiner and a single surgeon. The weakness was the

low number of patients (19 in each group), and the concern therefore that it might not have been adequately powered to detect a subtle difference in VA.

Hillenkamp *et al*¹ undertook a randomized clinical trial of 60 patients undergoing vitrectomy and epiretinal membrane peel, comparing the outcome with and without ICG. Surgery was combined with cataract surgery and 1 mg/ml ICG was dissolved in glucose to avoid hypo-osmolality. Comparing both groups, there was no statistically significant difference in final VA, reduction in macular oedema using optical coherence tomography, or postoperative Amsler grid testing. The incidence of residual of recurrent epiretinal membrane was also similar but two patients in the ICG group were found to have visual field defects (a non-significant difference). On the basis of these findings, it was concluded that ICG did not produce manifest toxicity.

Kwok *et al*² compared visual outcome and macular hole closure rates at 1 year, between ICG-assisted ILM peeling and no ILM peeling, with a better outcome in the former group. As the surgical intervention (peel or no peel) also differed, this study cannot be used to conclude on the effect of ICG *per se*. It did, however, fail to show any evidence of toxicity. The same team³ also conducted a randomized clinical trial to determine the effective minimum concentration required to produce sufficient ILM staining. Among the three different concentrations studied (0.25, 0.5, and 1.25 mg/ml), the 1.25 mg/ml concentration produced the best ILM staining. The operating time was equivalent in the three groups and no toxicity was observed clinically or angiographically in any group.

Summary: randomized clinical trials

- 1 Two trials directly assessed the effect of ICG staining on visual outcome
 - 2 One trial showed a significant increase in central visual field defects with ICG
 - 3 Neither showed a deleterious effect on VA
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Non-randomized studies

Despite a plethora of non-randomized studies, there is still no clear consensus on the clinical safety of ICG macular staining (Tables 1 and 2). The key questions are related to the effect of ICG on visual outcome and whether or not ICG causes visual field defects.

Visual and anatomic outcome

Studies showing adverse visual and anatomic outcome

In a retrospective multicentre study of 1627 patients, the use of ICG was found to make macular hole closure significantly less likely (96 vs 92%).²¹ Further, in the holes

Table 1 Visual outcome in relation to ICG concentration and duration of exposure

<i>Authors</i>	<i>N (eyes)</i>	<i>Concentration (mg/ml)</i>	<i>Time (s)</i>	<i>≥ 2 lines improvement in VA (%)</i>
Da Mata <i>et al</i> ⁴	24	5	118–300	96
Da Mata <i>et al</i> ⁵	121	5	30	96
Kumar <i>et al</i> ⁶	18	5	120	50
Apostolopoulos <i>et al</i> ⁷	20	5	60	90
Lochead <i>et al</i> ⁸	34	5	10	82
Sheidow <i>et al</i> ⁹	35	4.2	120–300	71
Wolf <i>et al</i> ¹⁰	37	2.5	0 ^a	62
Das <i>et al</i> ¹¹	25	2.5	NS	64
Lai and Williams ¹²	59	1.25	20	73
Kadonosono <i>et al</i> ¹³	13	0.6	30	85
Ando <i>et al</i> ¹⁴	16	0.5	10	100
Kwok <i>et al</i> ²	18	0.25–1.25	30	67
Kwok <i>et al</i> ¹⁵	9	0.25	30	78

Abbreviations: ICG = indocyanine green; NS = not specified; VA = visual acuity.

Table includes non-randomized studies of macular hole surgery that specifically reported the number of eyes with a VA improving by two lines or greater (see also Figure 1).

^aImmediate washout after introduction of the dye.

Table 2 Visual field testing in patients exposed to ICG during macular hole surgery (non-randomized studies)

<i>Adverse effects</i>				<i>No adverse effects</i>		
<i>Authors</i>	<i>N (eyes)</i>	<i>Concentration (mg/ml)</i>	<i>VF defect (%)</i>	<i>Authors</i>	<i>N (eyes)</i>	<i>Concentration (mg/ml)</i>
Kanda <i>et al</i> ¹⁶	4	5 ^a	25	Kanda <i>et al</i> ¹⁶	6	2.5
Kanda <i>et al</i> ¹⁶	12	5 ^b	100			
Nagai <i>et al</i> ¹⁷	35	5	20			
Tsuiki <i>et al</i> ¹⁸	96	2.5	18			
Gass <i>et al</i> ¹⁹	18	0.5	50	Ando <i>et al</i> ¹⁴	16	0.5
Haritoglou <i>et al</i> ²⁰	20	0.5	70			

Abbreviations: ICG = indocyanine green; VF = visual field.

^aImmediate washout of ICG.

^bA 3-min exposure of ICG.

that did close, visual outcome was significantly worse if ICG was used: 67% of the no-ICG group obtained two logMAR lines of improvement compared with 56% in the ICG group. This study was designed to compare ILM peeling with no peeling, rather than to look for ICG toxicity. ICG use was not standardized, and the exposure time and ICG concentration were not recorded. In addition, its retrospective nature means that confounding is a possibility. Nonetheless, this large and well-conducted analysis presents important findings that raise concerns about the efficacy and safety of ICG.

Nagai *et al*¹⁷ compared ILM-stained *vs* non-stained ILM in a retrospective study involving 35 eyes (17 unstained and 18 stained). The ICG concentration was 0.5 mg/ml followed by immediate rinsing of the dye. They found RPE atrophy in six stained eyes *vs* two in the unstained group. Furthermore, retinoschisis (in three patients) and visual defects (in seven patients) were found only in the ICG group. ICG has a maximal absorption at 800 nm, which overlaps with the emission

spectrum of the most commonly used halogen light sources. It was speculated that this might be the reason for the posterior RPE atrophy. RPE changes were also described by Engelbrecht *et al*²² in macular hole surgery with 0.1 mg/ml ICG staining. It should be noted that RPE changes have been described in macular hole surgery without ILM removal, before the introduction of ICG as a vital stain.²³

Studies showing favourable visual and anatomic outcome

In direct contrast to studies showing toxicity, several studies comparing ILM peel with and without ICG have shown better visual and anatomic outcome with ICG, despite using a range of concentrations and exposure times. Ando *et al*¹⁴ revisited their findings of visual field loss and worse visual outcome with 5 mg/ml ICG by decreasing the dye concentration and exposure time. Surgeries were performed with the same technique and the same surgeon, the only change being reduced ICG concentration (0.5 mg/ml) and exposure times (less than 10 s). The ILM could still be visualized after one or two

intravitreal ICG injections, but there were no visual field defects and no RPE changes. Macular hole closure was achieved in 93.5% and mean visual outcome improved significantly to 12/20.¹⁴

Lochhead⁸ compared ILM stained with 5 mg/ml and no dye and reported a higher rate of macular hole closure in the stained group (91.2 vs 73.5%). This was associated with a significant difference in visual outcome: logMar acuity improved above baseline in 53% of the no-ICG group, compared with 82% of the ICG group. In a recent study, Mavrofrides *et al*²⁴ did not report any toxicity in patients receiving 2.5 mg/ml ICG. The macular hole closure rate was 87% with ICG vs 83% without ICG. After a follow-up of 8 and 9 months, median best corrected VA was 20/70 and 20/80 in the ICG and no-ICG groups, respectively. Da Mata *et al*⁵ reported excellent visual outcomes in a large series (121 eyes) of patients undergoing macular holes surgery using 5 mg/ml, with follow-up averaging 2 years, and with no ICG toxicity detected.

When considering the retrospective studies that specifically reported the percentage of eyes improving by greater than two lines of VA, there was no clear relationship between the final visual outcome and concentration or duration of dye exposure (Table 1 and Figure 1).

Visual field defects

Studies showing visual field defects

A number of authors have reported visual field defects in patients undergoing ICG staining.²⁵ Tsui *et al*¹⁸ found a significantly increased incidence of nasal and temporal defects in the left and right eye, respectively—the authors suggested that this was related to direct

illumination of ILM stained with 0.25 mg/ml ILM. Visual field defects could not be found when using an ICG concentration of 0.125 mg/ml. Kanda *et al*¹⁶ corroborated this finding; they showed that all eyes exposed to 5 mg/ml ICG for 3 min developed a visual field defect. The defects were only found in 25% of patients if immediate washout was performed and in no patients if 2.5 mg/ml was used. Nasal field defects were more common than temporal or concentric defects, and there were corresponding areas of optic disc pallor. Ando *et al*¹⁴ also found visual field defects, mainly nasally in patients receiving 0.5 mg/ml ICG compared to no stain or no peeling of the ILM.

Of concern, Gass *et al*¹⁹ found visual field defects despite using a very low (0.05 mg/ml) concentration of ICG. Another study showed that retinal nerve fibre layer thicknesses reduced in eyes with visual field defects after ICG-assisted vitrectomy for macular holes.²⁶ The authors suggested that the postoperative visual field defects might have occurred secondary to ICG-mediated damage to the retinal nerve fibre layer.

Studies failing to show visual field defects

Several of the studies that tested for visual field defects failed to demonstrate ICG-mediated damage, with concentrations ranging from 0.05 to 0.25%. Authors performed ICG washout immediately after or up to 15 s following intravitreal ICG injection.^{14,16,27} There are also many reports of visual field defects occurring before the introduction of ICG, with several possible aetiologies including retinal dehydration, elevated intraocular pressure, and direct trauma to the retinal nerve fibre layer or optic nerve.^{28–38}

Surgery for epiretinal membrane and macular pucker

In surgery for diabetic macular oedema, macular pucker, and epiretinal membrane, the retina remains intact, unlike macular hole surgery, where there is a direct communication between the dye and the RPE and inner retinal elements. This might be expected to reduce the volume and concentration of dye reaching inner retinal elements, and some studies suggest that ICG is cleared more quickly from the eye in this setting.³⁹ It is also interesting that the randomised study of epiretinal membrane surgery by Hillenkamp *et al*⁴⁰ did not find visual field defects associated with ICG use, whereas a randomised study of macular hole surgery did.¹

The molecular weight of ICG is 775, and experimental studies show that molecules of this size pass rapidly across the retina.⁴¹ Therefore, while an intact retina has the potential to reduce the level of dye exposure, it may not exclude it altogether. Further, if visual field defects occur secondary to retinal nerve fibre layer damage, as

Figure 1 Compares visual acuity (VA) outcome in non-randomized studies that detailed the percentage of eyes improving by two lines or greater (see also Table 1). A similar result was obtained when comparing VA and duration of dye exposure.

suggested by some reports,^{26,42} then a full-thickness defect may be less important. Haritoglou *et al*⁴³ compared two consecutive series of patients undergoing macular pucker surgery and found that eyes with ICG had a significantly worse visual outcome than those without ICG. The authors used the same solution of dye (5 mg/ml, 275 mOsm) in a second series of patients undergoing macular hole surgery²⁰ and interestingly, the rate of visual field defects was identical in both macular hole and macular pucker cases (7 of 20 patients). Taken together, these two papers suggest that an intact retina does not protect against visual field defects.

Surgery for diabetic macular oedema

In addition to the presence of an intact retina, it might be expected that the metabolic changes associated with diabetes would have some effect on ICG safety. There are three reports of ICG use in patients undergoing surgery for diabetic macular oedema. Two showed a favourable safety profile,^{44,45} whereas one showed visual field defects,⁴² making it difficult to draw a conclusion.

Electrophysiology

A prospective study analysed visual outcome and multifocal electroretinograms (mfERG) in eyes undergoing macular hole surgery with and without staining with 5 mg/ml ICG. The unstained group had a better final VA, greater visual improvement, and a significantly greater improvement in the mfERG response.⁴⁶ The significantly better outcomes without staining may suggest dye toxicity and the authors advocated the use of other dyes to assist ILM peeling.

Horio and Horiguchi⁴⁷ also compared ILM peeling with and without ICG. They obtained significantly better visual outcomes without staining. MfERG did not show significant differences between both groups. The electrophysiological findings were interpreted as showing ICG-mediated damage to the ganglion cells and their axons, but the authors did not undertake visual field testing.

Summary: electrophysiology

- 1 One study showed ICG-assisted surgery adversely affected the multifocal electroretinogram, whereas another failed to show a significant effect
-

Persistence of ICG

Clinical studies have firmly established that ICG can persist for many months or possibly years after surgery.⁴⁸ Many studies have shown persistence of the dye over approximately 6–7 months in patients undergoing

surgery for macular hole,^{39,49,50} with highly sensitive tests such as Heidelberg retina angiography, able to detect low levels of staining for up to 3 years.⁵¹ Hyperfluorescence is observed around the macular hole, at the optic disc, in the optic nerve fibres, and some authors found that it may be especially intense in the area along the vascular arcade.⁵¹ Over time, fluorescence appears to migrate from the macula towards the optic nerve, probably along the optic nerve fibres.⁵² Interestingly, areas of RPE atrophy lack hyperfluorescence, suggesting that the integrity of the RPE influences dye uptake or clearance.⁵³ One study suggested that diabetes may slow dye elimination.⁵⁴

Importantly, a causal link, or even an association between persistent staining and toxicity has not been established. Indeed, many eyes with persistent staining, even in association with RPE changes, have shown favourable visual outcomes.⁴⁹

Summary: persistence of ICG

- 1 Clinical studies show that ICG can persist in the fundus for many months
 - 2 Over time, residual ICG appears to move from the macula to the optic nerve
 - 3 A causal link between persistent staining and toxicity has not been established
-

Surgical techniques to minimize ICG toxicity

Owing to concerns about ICG toxicity, several surgical techniques have been developed to protect the fovea. Facino *et al*⁵⁵ added a small bubble of perfluorocarbon to protect myopic macular holes with retinal detachment, before injecting ICG. The aim was to prevent contact between the dye and the fovea, and the authors reported good anatomic and functional results in three cases. Olson *et al*⁵⁶ also obtained favourable results using the same technique in patients with idiopathic macular holes.

Two studies^{57,58} injected autologous whole blood over the fovea, to prevent ICG contact, with favourable visual and anatomic outcome in a total of 51 patients. No RPE changes were observed. Saito and Iida⁵⁹ used viscoelastic as a barrier and also reported favourable outcomes.

Nakamura *et al*⁶⁰ used autologous serum to wash out ICG at the end of the surgery. ICG was detected for 2.4 months after surgery with serum compared to 6.1 months without.

Summary: techniques to minimize ICG contact with the fovea

- 1 Agents placed on fovea to act as a barrier
 - Perfluorocarbon
 - Autologous whole blood
 - Viscoelastic
 - 2 Vitreous washout with serum
-

Summary of ICG clinical studies

There are, at present, only two randomized clinical trials comparing surgery with and without ICG. One found a significant increase in visual field defects¹ and neither found any effect on acuity.⁶¹ A large non-randomized study found that ICG was associated with lower rates of anatomic success, and worse acuity in those eyes with closed holes.²¹ The large number of non-randomized, observational studies are all potentially subject to selection bias, publication bias, and confounding, and many were not adequately powered. Their findings differ widely, but the most common observation appears to be visual field defects.

Experimental studies

Animal studies

Several reports^{49,62–64} evaluated the potential toxicity of ICG, at different concentrations, injected into the subretinal space in animal models. The rationale for this approach is to simulate the contact that occurs between ICG and the RPE and photoreceptors when the dye is applied to full-thickness macular holes.

Subretinal injections of ICG⁶⁵ using concentrations at or above 1.25 mg/ml in rabbits have been reported to produce apoptosis and degenerative changes of the photoreceptors and RPE cells after a 3-day exposure. After a 4-week exposure, there was destruction of the outer retina and RPE in a dose-dependent manner. Hypo-osmolar ICG induced more damage to the retina than iso-osmolar solutions. As might be expected, subretinal ICG appears to be more damaging than intravitreal ICG.⁶⁵

Yip *et al*⁶⁶ performed an intravitreal injection of 1.0 mg/ml ICG in rat eyes with or without illumination from a standard endoillumination light pipe. Eyes with ICG and illumination showed a significant reduction in retinal ganglion cell density and increased outer nuclear layer thickness compared with negative controls, or eyes exposed to ICG, but not illumination.⁶⁷

A series of studies in unvitrectomized rabbit and rat eyes showed that damage increases in relation to the concentration of intravitreal ICG, with electrophysiological changes occurring at lower concentrations than structural changes.^{68–70}

A series of studies by different authors suggest that vitrectomy reduces ICG toxicity.^{68,71,72} These studies do not determine how this might occur, but one hypothesis is that vitrectomy enhances dye elimination. These three studies also give some indication of the lower threshold for toxicity in the vitrectomized rabbit eye. Following intravitreal injection of ICG, electrophysiological changes appear to occur with concentrations >0.1 mg/ml,

whereas structural changes occur above 0.25 mg/ml, although with somewhat different methodology.

Other animal studies have investigated the persistence of ICG in the retina and the optic nerve after intravitreal injection. Cekiç *et al*⁷³ investigated the migration of ICG into the optic nerve after intravitreal injection in rabbits, with ICG found to have migrated into the intraorbital portion of the optic nerve, and persisting for 1 month. Paques⁷⁴ injected ICG in both the vitreous cavity and the lateral geniculate nucleus of unvitrectomized rats. Similar to Cekiç's findings, ICG was present in the ganglion cells and the optic nerve. Furthermore, retrograde labelling of the retina was demonstrated with the presence of ICG vesicles inside ganglion cells. Despite this, no toxic effect on ganglion cells was noted.

Czajka *et al*⁷⁵ injected 0.5 and 1 mg/ml ICG in vitrectomized pigs either in fluid-filled or air-filled eyes. Fundoscopy showed more severe RPE atrophy in the animals with air-filled eyes than fluid-filled eyes. Outer retinal degeneration was more pronounced in the air-filled group. No inner retinal degeneration could be found in either group.

Gandorfer *et al*⁷⁶ looked at the toxicity of ICG in the cat retina. ICG staining of the ILM followed by illumination caused severe inner retinal damage. ICG without illumination resulted in focal ILM detachments associated with tearing of Müller cell endfeet.

In summary, animal studies do show some evidence of toxicity. Even low concentration (0.1 mg/ml) ICG can produce electrophysiological alterations of the b-wave, suggesting Müller cell dysfunction. At higher doses, starting from 2.5 mg/ml, ICG can produce both structural and functional alterations of the retina in several species. Outer retina and RPE were significantly affected in several studies. Long illumination and dye exposure, absence of vitrectomy, an air-filled vitreous, and a central retinal location all predisposed to damage. Animal models have also verified the clinical observation that ICG can persist in the eye for an extended period of time.

Summary: animal studies

- 1 Studies have been conducted in rat, rabbit, cat, and pig
 - 2 The risk of toxicity increases with concentrations >0.1 mg/ml
 - 3 ERG changes occur at lower concentrations than structural changes
 - 4 Vitrectomy appears to have a protective effect
-

Organ culture studies

Saikia *et al*⁷⁷ used a porcine organ culture exposed to iso-osmolar 0.1 and 1% ICG for 1 and 30 min, with and without illumination. There was no increase in TUNEL-positive cells using 0.1% ICG for 1 min, but

moderate apoptosis in retina exposed to the 1% solution for a 1-min, or with a 30-min exposure to 0.1% ICG. Severe apoptosis was found after a 30-min exposure to 1% ICG. Concomitant application of light did not influence the degree of apoptosis. No signs of cell necrosis were found.

Cell culture studies

Cell cultures have been used extensively to investigate the safety of ICG. Studies have been reported on human RPE (ARPE-19), choroidal endothelial cells, Müller cells, and retinal ganglion cells from both human and rat.

As with the animal studies, some cell culture experiments suggest that the functional changes may occur at lower concentrations than structural changes.⁷⁸ Sippy *et al*⁷⁹ studied the effect of ICG (1 mg/ml) with or without light on human RPE cell cultures. RPE cells exposed to ICG showed no histological or ultrastructural changes at this concentration. However, those exposed to ICG alone or ICG plus light demonstrated a significant decrease in mitochondrial enzyme activity.

Murata *et al*⁸⁰ studied the toxicity of 0.5 and 0.05 mg/ml ICG on cultured retinal ganglion cells. At 0.5 mg/ml, there was a significant decrease in the total number of cells and of resazurin metabolic activity. Bcl-2 expression (a marker of apoptosis) increased with ICG concentrations > 0.05 mg/ml, but at 0.05 mg/ml, there was no toxicity.

Yam *et al*⁸¹ studied the effect of light and two concentrations (0.25 and 2.5 mg/ml) of ICG on human RPE cells in culture. ICG was applied for 1 min, rinsed, and then cells were exposed to a light source (400–800 nm). ICG at 0.25 mg/ml and without illumination appeared relatively safe. At 2.5 mg/ml, a reduction in cell viability occurred, with considerable cell shrinkage without light exposure. The combination of ICG (2.5 mg/ml) and light exposure resulted in the upregulation of apoptosis-related genes (p53, bax and protein p21).

Stalmans *et al*⁸² reported that ICG toxicity was related to the hypo-osmolality of the solvent solution rather than ICG itself. At that time, the most commonly used ICG solution was hypo-osmolar, and the authors found that this induced RPE apoptosis, while infracyanine green diluted in 5% glucose produces an iso-osmolar solution and this did not affect RPE viability.

Rezai *et al*⁸³ looked specifically at the rate of apoptosis among human RPE cell cultures exposed to different concentrations of ICG (1–25 mg/ml). All concentrations produced RPE apoptosis, with damage occurring in a dose-dependant manner. This occurred in the absence of light exposure and independent of the osmolality of the solution.

Jackson *et al*⁸⁴ attempted to validate previous reports and study the interaction of osmolality, concentration, and light exposure on RPE and Müller cells. In general, Müller cells appeared more vulnerable than RPE cells. Neither RPE cells nor Müller cells showed toxicity with a 0.5 mg/ml solution of ICG, but 1 mg/ml ICG produced toxicity. Brief exposure to hypo-osmolality alone did not produce toxicity, even with saline solutions as low as 181 mOsm, but when low osmolality and ICG were combined, this produced cell damage, as did concomitant light exposure.

Worryingly, Hsu *et al*⁸⁵ reported that very low concentrations of ICG (0.01 mg/ml) were capable to produce a relatively toxic effect on cultured human RPE cells in the absence of illumination, despite using an iso-osmolar solution.

An interesting study by Lai *et al*⁸⁶ investigated the protective effect of whole blood and plasma on RPE cultures exposed to increasing concentrations of ICG (0.05–5 mg/ml) and halogen light exposure for 20 min. A mitochondrial dehydrogenase assay failed to show any reduction in cell viability, when these agents were added.

Mennel *et al*⁷⁸ assessed the outer blood–retinal-barrier by measuring the transepithelial electrical resistance of RPE cells in culture. Human RPE cells and choroidal endothelial cells were cultured in monolayers on semipermeable membranes representing an *in vitro* model of the outer blood–retinal barrier. They modelled fluid-filled and air-filled eyes by adding ICG solution to culture media or cell monolayers, respectively. In the fluid-filled model, transepithelial resistance remained intact at all ICG concentrations, whereas in the air-filled model, 0.5 and 5 mg/ml ICG produced a significant decrease in transepithelial electrical resistance. Transmission electron microscopy did not show any morphological change except when using 5 mg/ml ICG in the air-filled model.

Recently Kunikata *et al*⁸⁶ reported a protective role of low temperature (4°C) in cultured ARPE-19 cells exposed to ICG over a range of concentrations. At 37°C, cell viability was reduced with concentrations > 0.25 mg/ml; at 4°C this safety threshold increased, with no toxicity below 5 mg/ml.

Three reports by Jin *et al*⁸⁷, Iriyama *et al*⁸⁸ and Iriyama *et al*⁸⁹ investigated the potential toxicity of ICG at the retinal ganglion cell layer. They found that cultured retinal ganglion cells exposed to ICG for 10 s to 30 min showed almost no damage. By contrast, longer exposure to ICG resulted in cell loss in a dose-dependent manner, with effects measurable up to 14 days after exposure to ICG and light.

Some studies identified experimental artefacts that have the potential to produce false measures of toxicity. The mitochondrial enzyme assay used by Sippy *et al*⁷⁹

and many other researchers assesses the conversion of an optically clear precursor to a blue formazan reaction product, produced by the mitochondria of healthy cells. Residual staining of the cell monolayer by dyes may interfere with the optical measurement of this reaction product. This produces a concentration-dependent, falsely low measure of cell viability, unless corrections are made for baseline staining.⁸⁴ Further, Ikagawa *et al*⁹⁰ found that ICG could damage RPE cell cultures by altering nutrient molecules in the culture media. Over a 12-h exposure, ICG resulted in serum molecules becomes insoluble, with resultant adverse effects on cell survival.

In summary, these cell culture studies show that, under certain conditions, ICG has the potential to produce toxicity in RPE cells, glia, and retinal ganglion cells. While not all studies concur, the trends emerging are that the threshold for ICG toxicity is reduced with longer exposure, higher concentration, light exposure, concurrent air exposure, and hypo-osmolality.

From this, it follows that surgeons using ICG should select a concentration below 0.5 mg/ml, preferably below 0.05 mg/ml. Exposure time should be the minimum required to achieve staining, and the endoilluminator should be kept away from the fovea as much as possible. Instilling ICG in a fluid-filled eye may be preferable to an air-filled eye: this is easier if Hartman's solution is used as the infusate, as this has a lower density than most balanced saline solutions (BSS), encouraging ICG to settle over the macula under gravity. Hypo-osmolar solutions should be avoided altogether—this is possible if glucose is used as the diluent rather than distilled water. A potential protective effect of whole blood applied to the macula, and the use of a cooled infusate have both been identified in culture, but neither has been tested in an adequate number of patients to draw a conclusion. Caution also needs to be exercised in translating findings in avascular, often immortalized, single-cell lines, to the situation *in vivo*, where non-replicating, mature cells exist in a multicellular environment. More so, as some studies show that mitochondrial assays need careful modification to avoid inaccuracy, and other studies show potential experimental artefact, with ICG-mediated damage to nutrient molecules in the culture media.

Summary: cell culture studies

- 1 ICG has been tested using retinal pigment epithelium, choroidal endothelial cells, Müller cells, and retinal ganglion cells
 - 2 Toxicity increases with increasing concentration and exposure time
 - 3 Few studies show toxicity at or below 0.05 mg/ml
 - 4 Hypo-osmolality and concurrent light exposure may increase toxicity
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Histology and cadaveric studies

Histological analysis of human retinal and epiretinal tissue has provided useful information on potential ICG toxicity, although the authors' conclusions differ in many respects. Analysis has been completed on eye bank eyes, and on tissue excised during vitrectomy. The key questions to emerge are whether ICG acts as a photosensitizer, damages tissue, and alters the surgical cleavage plane such that deeper retinal elements are removed with the ILM.

Burk *et al*⁹¹ performed morphological analysis of the ILM in 11 donor eyes. Eyes underwent an open-sky vitrectomy followed by a 5-min exposure to 5 mg/ml ICG. Macroscopically, the ILM was well stained with this exposure routine. Tissue was then removed for light and electron microscopy. All specimens contained ILM, without retinal or glial cell elements. There was a clear distinction between the ILM and the retina, suggesting ICG did not alter the cleavage plane.

Kwok *et al*¹⁵ obtained six complete histological ICG-stained ILM specimens from 10 macular hole surgeries. Two of six ILM specimens had a small amount of epiretinal membrane. Myofibrocytes were noted in the ILM, either scattered or as a single layer, in three cases.

Drs Gandorfer, Haritoglou, and Kampik in Munich have provided a large number of papers on ICG use, including the earliest articles raising concern about toxicity, with many clinical and experimental studies since. Initially, Gandorfer *et al*⁹² showed in patients with macular hole (five patients) or diffuse macular oedema (five patients) that ICG was capable of staining the ILM after vitrectomy and cortex removal. Light and transmission electron microscopy revealed that the ILM was effectively stained and removed in all cases.

Haritoglou *et al*²⁰ analysed the ultrastructure of excised ILM by light and transmission electron microscopy in 20 eyes that had undergone macular hole surgery with ICG staining. They found that ICG-stained specimens had cellular elements resembling the plasma membrane of Müller cells, and other undetermined retinal elements adherent to the retinal side of the ILM. The authors contended that the presence of these deeper retinal elements suggests that ICG alters the surgical cleavage plane deep to the ILM, with associated retinal damage. Gandorfer *et al*⁹³ reported similar findings in 10 ILM specimens removed after ICG staining during surgery for idiopathic macular hole.

The same team⁹⁴ reported on the ultrastructure of human donor eyes exposed to 5 mg/ml ICG, and different wavelength light. Electron microscopy showed severe retinal damage to the inner retina, including loss

of the ILM, cellular disorganization, and fragmentation of the cytoplasm at wavelengths beyond 620 nm. ICG staining alone, or in combination with wavelengths of 380–620 nm, produced rupture of Müller cells with detachment of the ILM. They concluded that ICG alone could induce debridement of Müller cell components, and that the exposure to light, especially wavelengths above 620 nm, induced phototoxic damage to the inner retina.

When other authors attempted to validate these findings in freshly enucleated pig eyes, they failed to show equivalent results.⁹⁵ However, La Heij *et al*⁹⁶ did find extraneous tissue in their analysis of ILM specimens removed after ICG-assisted peeling.

Immunohistochemistry revealed ILM positivity for GFAP (a glial marker) in 8 of 10 specimens. Two other specimens were positive for S-100, indicating the presence of neural cells or ganglion cells.

Haritoglou *et al*⁹⁷ used reflection spectroscopy to study the absorption spectrum of ICG adsorbed onto the retinal surface of cadaver eyes. Compared to ICG in saline or water, the absorption profile showed less variation in relation to concentration and time, with twin peaks at 736 and 800 nm persisting over the 13-day experiment. The question that remains unanswered is how the natural light that reaches the macula interacts with residual ICG.

In conclusion, some histological studies suggest that ICG-assisted peeling of the ILM may result in morphological changes to the ILM and the underlying retina. Additionally, damage may be aggravated by light exposure, particularly longer wavelength light that overlaps with the absorption spectrum of ICG. ICG may also alter the cleavage plane, such that the ILM is removed with adherent cellular constituents. It should be noted, however, that other authors have shown cellular constituents associated with ILM removal, in the absence of ICG. Further, some studies have failed to demonstrate ultrastructural damage of excised ILM, and it is not certain that all damage relates to ICG *per se*. On balance, ICG appears to be damaging, but experimental artefact may be a factor.

Summary: histological studies

- 1 Light and electron microscopy and immunohistochemistry have been used to study excised surgical specimens and cadaver eyes exposed to ICG
- 2 ICG may alter the cleavage plane, with deeper retinal elements adherent to the peeled ILM
- 3 ICG combined with long wavelength light (>620 nm) may increase cell damage
- 4 The relevance of cadaver eyes to the *in vivo* use of ICG is not known
- 5 It is not certain that all the changes observed in surgical specimens were caused by ICG

Summary of experimental studies

Authors have utilized a wide range of methodologies to investigate the safety of ICG as a vital stain. These have included cell culture, analysis of excised specimens, mock surgery in enucleated eyes, and *in vivo* animal experiments. The findings differ and no model perfectly simulates the clinical application of ICG in humans, that is, brief delivery to a vascularized, immunologically active, multicellular environment in predominantly mature patients. Some data suggest that ICG is safe in the concentrations used clinically, whereas others show ICG producing profound cellular damage. Further, some studies show quite different results using very similar methodology. Reading the experimental literature as a whole, there are more studies showing toxicity than safety, but publication bias cannot be excluded.

Infracyanine green

Background

Infracyanine green is a proprietary solution containing the same fluorophore found in ICG. It differs from ICG in two respects. First, infracyanine green is iodine free. Second, infracyanine green tends to precipitate in water, and therefore it is provided with 5% glucose as the diluent. Infracyanine green prepared in this glucose diluent is near to iso-osmolar (294–314 mOsm), relative to intraocular tissue, whereas ICG prepared in water is hypo-osmolar. As the studies of ICG showed that its low osmolarity might be at least partly responsible for toxicity, it has been suggested that infracyanine green may be a safer alternative.

Clinical studies

At the time of writing, there were no randomized clinical studies of infracyanine green. The use of infracyanine green (0.5–2.0 mg/ml) has, however, been reported in prospective observational studies of macular hole, epiretinal membrane, and macular oedema,^{98–102} and in several retrospective studies of macular hole surgery.^{102–106}

Most studies show a favourable anatomic and visual outcome with no obvious toxicity. In contrast, one observational series suggested that infracyanine green might be associated with an adverse outcome. This compared the visual outcome in 29 patients undergoing the removal of the ILM or epiretinal membranes with infracyanine green to 14 patients in whom trypan blue was used instead.¹⁰⁷ Compared to the preoperative status, only the trypan blue group showed a significant improvement in acuity, with no reduction in the central visual field. In contrast, the infracyanine group showed a

non-significant improvement in acuity, but a significant attenuation of central visual fields. The authors concluded that the central visual field may be a more sensitive measure of damage from infracyanine green than acuity. Of note, however, there was no significant difference in visual outcome when directly comparing trypan blue and infracyanine green groups.

Experimental studies

Human RPE cell culture has been extensively used to investigate the safety of infracyanine green.^{82,84,108,109} Mature confluent RPE monolayers appear to be more resistant to potential toxicity than younger cells. This may be of relevance, as in adult human eyes, RPE cells exist as a monolayer of mature non-dividing cells. Iso-osmolar infracyanine green dissolved in glucose solution, at a concentration below 5 mg/ml and with exposure times ranging from 2 min to 6 days did not alter RPE cell survival in culture.¹⁰⁸ Jackson *et al*⁸⁴ exposed both RPE and Müller cell monolayers to a range of concentrations of infracyanine green, up to 5 mg/ml. RPE cells failed to show significant damage, but Müller cells did show damage with a 5 mg/ml solution. When exposure to infracyanine green was combined with a 5-min illumination by a vitrectomy endolight powered by a xenon light source, there was some additional damage in RPE cells, but not relative to an active control (light alone). Müller cells did not show phototoxicity.

Finally, Stalmans *et al*⁸² exposed RPE cell cultures to different solutions for 20 min. These solutions included BSS, ICG in BSS, glucose 5% alone, and infracyanine green in glucose 5%. The main differences between the solutions were their osmolarity. Hypo-osmolar solutions with or without indocyanine resulted in higher cell death. Iso-osmolar solutions (including those with infracyanine green) did not alter RPE cell survival rate. They concluded that the toxicity of ICG on RPE cells is probably related to the hypo-osmolarity of the solvent and may be avoided by using infracyanine green dissolved in glucose 5%.

Taken together, these studies suggest that mature RPE cell culture monolayers do not show toxicity when exposed to low concentration infracyanine with a short exposure time. However, high concentration exposure, combined with low osmolarity or bright endoillumination may increase the risk of damage. It is also important to note the cautionary comments given in the section on ICG, regarding the relevance of cell culture to the clinical environment.

Haritoglou *et al*¹¹⁰ injected postmortem human eyes with either iso-osmolar infracyanine green dissolved in glucose solution (SERB, France), or ICG in saline solution (ICG Pulsion, Germany). Eyes were then illuminated

with a commonly used vitrectomy light source, and compared with unilluminated controls. Electron microscopy revealed disorganization of the inner retina and complete loss of ILM after the application of both ICG solutions and illumination.¹¹⁰ In contrast, no abnormalities were found after the application of both dyes without illumination and in unstained control specimens. Despite the hypothesis that the glucose solvent might favourably alter the light-absorbing properties of the dye and reduce the potential for photosensitivity, this was not found to be the case. Again, as with ICG, the validity of using enucleated human eyes to model *in vivo* toxicity is not known.

As with ICG, immunohistochemistry of tissue removed during infracyanine green-assisted surgery has shown Müller cell footplates, neural cells, and ganglion cells, but whether this can be attributed to alteration in the cleavage plane, or a normal consequence of tissue excision is not known.^{96–111} Despite the presence of various cell types in excised specimens, no clinical evidence of dye-related toxicity was reported in a study by Ullern *et al*.¹⁰² The specimens removed in macular hole surgery disclosed that few cells, were very thin, and were scarcely positive for vimentin using immunohistochemistry, suggesting that the surgical cleavage plane was not altered.¹⁰³

Summary: infracyanine green

- 1 Infracyanine green contains the same dye as ICG
 - 2 Infracyanine green is iodine free and comes packaged with glucose as the diluent—this produces an iso-osmolar solution
 - 3 ICG contains iodine and comes packaged with water as the diluent—this produces a hypo-osmolar solution (ICG can, however, be dissolved in glucose)
 - 4 Most clinical and experimental reports show a favourable outcome
 - 5 There are fewer studies of infracyanine green than ICG
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Conclusion

Given that infracyanine green contains the same fluorophore as ICG, it is perhaps unsurprising that many of the controversies run parallel. The key issue is related to whether or not its iso-osmolarity confers a safety benefit. Unfortunately, the experimental evidence points in different directions and randomized trials have not been completed. However, as a general statement, the proportion of clinical studies showing safety appears to be higher than with ICG, although with a much smaller evidence base. Further, at least some studies show ICG toxicity is aggravated, or perhaps mediated by hypo-osmolarity, so there is an argument for using infracyanine green instead of ICG dissolved in water.

Now that many surgeons dissolve ICG in BSS or glucose, it is not certain that this theoretical advantage remains.

Conclusion

As with many surgical techniques and devices, ICG's use as a vital stain was not subjected to the rigorous and formalized safety testing that's required for novel pharmaceutical agents. Rather, its introduction most likely reflects its availability and familiarity to ophthalmologists, rather than a systematic assessment^{94,95} of the many hundreds of dyes available and their individual merits.

After initial enthusiasm, many clinicians discontinued the use of ICG as an intraoperative vital stain because of concerns about toxicity. There are, however, many who continue to use it without manifest toxicity. It seems, therefore, that if ICG is toxic, the effects are subtle.

Assuming that any ICG toxicity is subtle, it may be difficult to detect for several reasons. Firstly, most clinical reports are imperfect. Only two randomized clinical trial directly assessed ICG toxicity. The first showed increased visual field defects with micropertimetry and this, in itself, raises concerns, but it may not have been sufficiently powered to detect subtle differences in VA.¹ The second randomized trial of 60 patients found two visual field defects in the ICG group with no significant difference in VA.⁶¹ With so few randomized clinical trials, it is extremely difficult to draw a conclusion on ICG safety. Most other studies were small observational series, often without a comparator. Secondly, many of the component steps of vitrectomy can damage VA, or cause visual field defects, and these may confound analysis. Thirdly, an underlying disease such as macular hole or epiretinal membrane may mean that postoperative visual function is impaired and subtle defects easily missed.

Like the clinical studies, it is difficult to reach a firm conclusion when analysing the experimental research on ICG toxicity. It is impossible to develop a perfect experimental model, as few cell culture or animal models can fully replicate the clinical application of ICG in humans. Nonetheless, these studies do point to emerging trends, many of which are consistent with our understanding of toxicology in general. These studies show that toxicity may be aggravated by solutions that contain a high concentration of ICG, low osmolarity, and long exposure times. Endoillumination may also aggravate cell damage, possibly by direct interaction with ICG, which may act as a photosensitizer. While intraoperative endoillumination was not standardized across studies, it is reasonable to mitigate the risk of light-mediated damage by reducing not only the duration of macular illumination but also the power setting. Increasing the distance from the retina is likely to

be beneficial, with halogen light sources offering a theoretically lower risk than xenon units.

In the absence of robust clinical or experimental evidence, clinicians might reasonably continue to use ICG, but the method of application should take account of the evidence that exists. They should, therefore, use low concentration solutions, such as 0.05 mg/ml, in fluid-filled eyes, and use diluents such as glucose to prepare an iso-osmolar solution. Brief exposure, ideally 10 s or less, reduces the risk of damage, as may thorough rinsing from the eye. It would also be prudent to avoid bright, proximal illumination with the endolight, when ICG remains visible on the surface of the retina. Infracyanine green may be safer than ICG and stains the ILM as effectively.

An alternative analysis of the literature is to accept that even if the studies are imperfect, there is an increasing body of evidence to show that the safety margin of ICG is narrow and toxicity is a distinct possibility. At the very least, patients should be warned of this. Now that other vital stains are readily available, it could be argued that the use of ICG is inappropriate.

Summary: the evidence base is uncertain, but on the balance of evidence the following appear likely:

- 1 ICG use is associated with visual field defects
 - 2 ICG may adversely affect the VA in some patients
 - 3 Low levels of ICG may persist in the eye for months
 - 4 ICG toxicity can be minimized by using the following:
 - Fluid-filled eyes
 - Iso-osmolar solutions
 - Concentration of 0.05 mg/ml or less
 - Exposure times of 10 s or less
 - Thoroughly rinsing ICG from the eye
 - Avoiding proximal or prolonged endoillumination of stained tissue
 - 5 Now that other dyes are available, the use of ICG may not be appropriate
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