

Safety Testing of Indocyanine Green in an Ex Vivo Porcine Retina Model

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PURPOSE. To assess retinal toxicity of indocyanine green (ICG) in a porcine ex vivo perfusion organ culture model, and to measure intraretinal penetration of ICG applied to the retinal surface.

METHODS. The retinal surface of fresh porcine retinal tissue was exposed to ICG 0.1% and 1% dissolved in glucose 5% for 1 and 30 minutes with and without concomitant illumination. Specimens were then kept in perfusion organ culture for 24 hours before examination by light microscopy and the TUNEL technique. Tissue samples treated with DNase served as positive controls, and samples exposed to saline served as negative controls. Fluorescence microscopy was used to localize ICG at 1 minute, 60 minutes, 2 hours, and 3 hours after a 1-minute exposure of the retinal surface to ICG 1%.

RESULTS. No increase in TUNEL-positive cells was observed after exposure to ICG 0.1% for 1 minute. Moderate apoptosis was found after 1-minute exposure to ICG 1% and 30-minute exposure to ICG 0.1%, and severe apoptosis was found after 30-minute exposure to ICG 1%. Concomitant application of light did not influence the degree of apoptosis. No signs of cell necrosis were found. After 1-minute exposure of the retinal surface, ICG 1% gradually penetrated the entire retina.

CONCLUSIONS. ICG induced apoptosis but not necrosis in all nuclear retinal layers in a dose-dependent manner. Brief exposure to ICG 0.1% for 1 minute and illumination for 3 minutes simulated the intraoperative use of ICG. No retinal apoptosis or necrosis was observed. ICG briefly applied to the retinal surface gradually penetrated the entire retina. (*Invest Ophthalmol Vis Sci.* 2006;47:4998-5003) DOI:10.1167/iovs.05-1665

Indocyanine green (ICG) has been used to stain and visualize epiretinal membranes and the internal limiting membrane (ILM) to facilitate the delicate surgical maneuver of their removal in epiretinal membrane¹⁻³ and macular hole surgery.⁴⁻⁶ Several authors have reported good functional outcome using ICG-assisted vitrectomy,^{5,7-10} whereas some authors have re-

ported less favorable results in visual acuity^{1,11,12} and significant visual field defects when intraocular ICG was used.^{1,11-13} Because of this controversy, a possible toxic effect of ICG on the retina has become the subject of an ongoing debate.^{14,15}

Several authors have investigated ICG toxicity using in vitro,¹⁶⁻²⁰ in vivo,²¹⁻²³ and ex vivo models,^{24,25} and reports also show dose-, light-, and exposure time-dependent toxicity in ganglion cells,¹⁶ Müller cells,¹⁷ and retinal pigment epithelial (RPE) cells.¹⁷⁻²⁰

ICG toxicity after macular surgery might be expected to depend on the concentration of the ICG solution,^{16,17} its osmolarity,²⁰ the length of time before ICG is removed from the eye,²¹ and whether ICG is injected into an air-filled or a fluid-filled eye (with less toxicity in fluid-filled eyes).²² A wavelength-dependent photocytotoxic effect of ICG irradiated with a diode laser has been described.^{26,27} Hence, retinal toxicity in macular surgery may also depend on the emission spectrum and the energy density of the light source.

However, all quoted models have significant limitations: in vitro cell culture studies have demonstrated a cytotoxic effect of ICG on Müller cells, ganglion cells, and RPE cells, but cell culture experiments do not simulate the complex multicellular environment of the retina. Furthermore, not all retinal neurosensory cell types can be tested in cell culture.

Enaida et al.²¹ found direct dose-dependent functional and morphologic damage of the retina after intravitreal application of ICG in an in vivo rat model. The doses used in this study were within or below the range (0.05-5.0 mg/eye) of clinical application, but, in contrast to clinical practice, where the ICG solution is removed within seconds to 1 minute, ICG was left in the eye for 10 days.²¹ Czajka et al.²² investigated the effect of epiretinally and subretinally applied ICG in an in vivo porcine vitrectomy model, and Maia et al.²³ used an in vivo rabbit vitrectomy model. Both groups observed no retinal damage when ICG was applied to the retinal surface at a 0.5% concentration in a fluid-filled eye, but a potential morphologic toxic effect was investigated only by light microscopy²² or by light microscopy and electron microscopy²³ and could therefore not be definitively excluded by the applied methods.

Previous ex vivo studies using whole human²⁴ and porcine²⁵ retinas have reported contradictory results. Tissue preparations were evaluated by light microscopy immediately after exposure to ICG. Gandorfer et al.²⁴ found severe damage of the inner retina of human donor eyes after exposure to ICG 0.05% and exposure to light from a standard surgical light pipe, whereas Grisanti et al.²⁵ did not find any disruption of the retinal cytoarchitecture in fresh porcine eyes under otherwise similar experimental conditions. Both groups used light microscopy and electron microscopy²⁴ to look for retinal damage and light microscopy²⁵ after exposure to ICG. Neither group tested cell viability.

Therefore, we sought to undertake safety testing of ICG using an established porcine ex vivo perfusion organ culture model and exposure routines that simulated the surgical use of ICG.

The first aim of the present study was to investigate a possible induction of apoptosis by clinically used ICG solutions, with and without concomitant application of light from

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a standard surgical light pipe in whole retina preparations. An organ culture model was used to allow time for a possible induction of apoptosis.

The second aim was to determine which retinal layers might be exposed to ICG after macular surgery by measuring the depth of penetration of a common clinically used ICG solution briefly applied to the retinal surface.

MATERIALS AND METHODS

Tissue Preparation

Porcine eyes were obtained from a local abattoir and transported to the laboratory on ice within 30 to 60 minutes of death. Globes were dissected under sterile conditions as described by Kobuch et al. (manuscript submitted). The anterior portion of the eye was carefully removed by a circumferential incision at the pars plana, and the cornea, lens, and iris were discarded. The anterior vitreous was engaged with dry filter paper and gently detached from the retinal surface and also discarded. A 1.5 × 1.5-cm quadratic full-thickness sample was cut from the macular area with a sterile scalpel, and the retina-RPE-Bruch membrane-choroid complex was gently teased from the sclera under a dissecting microscope.

Application of ICG

The retinal surface was exposed to ICG (Pulsion, Munich, Germany) under balanced salt solution (BSS; Alcon Laboratories, Fort Worth, TX) for 1 minute or 30 minutes before removal by irrigation with BSS. In the control group, the retina was exposed to BSS only. ICG was dissolved in glucose 5% at a concentration of 0.1% or 1%. The osmolarity of this solution was 296 mOsm, as measured with an osmometer (Knauer, Berlin, Germany).

Perfusion Organ Culture

Perfusion organ culture was performed as previously described (Kobuch et al., manuscript submitted).²⁸ Tissue culture was initiated within 2 hours of death. Retina-RPE-choroid tissue samples were mounted in tissue carriers (Minucells and Minutissue; Bad Abbach, Germany) and placed in gradient containers (Minucells and Minutissue) on a thermo plate (MEDAX, Kiel, Germany) with a coverlid that maintained a constant temperature of 37°C within the gradient container. Apical (retinal side) and basal (choroidal side) compartments of the gradient containers were thus separated by the tissue sample. Both compartments were continuously perfused with Dulbecco modified Eagle medium (DMEM; Sigma-Aldrich, Seelze, Germany) supplemented with 15% porcine serum, 2.5% HEPES-buffer solution, and 1% penicillin/streptomycin (Gibco Life Technologies, Eggenstein, Germany). The medium was pumped through 1-mm inner diameter thin, gas-permeable silicone tubes, which allowed the continuous exchange of atmospheric gases. The medium was transported at a rate of 1 mL/h driven by a peristaltic pump (IPC N8; Ismatec, Wertheim, Germany). Tissue samples were kept in culture for 24 hours.

Illumination

Simultaneously with the application of ICG, the retinal surface was illuminated for 3 minutes with a standard surgical 24-W metal halide light pipe with an interposed UV filter (type 1266-XII; D.O.R.C., Zuidland, The Netherlands). The fiberoptic was placed 8 mm above the retinal surface of the tissue sample at a slightly oblique angle, simulating the intraoperative condition as described by Gandorfer et al.²⁴ The emission spectrum of metal halide surgical light sources²⁹ and the absorption spectrum of ICG¹⁰ overlapped.

Histology and TUNEL Test

Tissue samples were fixed in neutral-buffered formalin for 24 hours and then were embedded in paraffin. Sections 2- to 5- μ m thick were cut, deparaffinized in xylol, rehydrated, and mounted on microscope

TABLE 1. Grading of Apoptosis

Grade	No. Apoptotic Cells
0	0
1 (mild)	1-5
2 (moderate)	5-10
3 (severe)	>10

Apoptosis was graded by counting TUNEL-positive cells in three representative fields of vision at ×40 magnification in histologic sections under a light microscope, as described.³⁰

slides (SuperFrost Plus; Menzel-Gläser, Braunschweig, Germany). One section of each sample was stained with hematoxylin and eosin for histology. Sections were permeabilized with freshly prepared 0.1% Triton X-100 solution containing 0.1% sodium citrate and treated with 3% hydrogen peroxide in ethanol for 20 minutes to block endogenous peroxidase. Detection of apoptotic cell death was performed in situ using the TUNEL test according to the manufacturer's protocol (Cell Death Detection Kit; Roche, Penzberg, Germany). Positive controls incubated with 3, 300, and 3000 U DNase I in TdT buffer before incubation with terminal transferase and biotinylated nucleotides showed TUNEL-positive cells. The number of TUNEL-positive cells increased with the concentration of DNase. Sections were counterstained with hematoxylin, mounted, and viewed under a light microscope (Axiotech; Zeiss, Jena, Germany). Apoptosis was identified by shrunken, highly condensed, or sometimes fragmented cell nuclei. The degree of apoptosis was graded by two independent masked investigators (PS, TM) on a scale of 0 to 3 (Table 1) through quantification of the number of TUNEL-positive cells in three representative fields of vision at ×40 magnification in each histologic section, as described by Buggage et al.³⁰

Evaluation of Cell Necrosis

Cell necrosis was evaluated under a light microscope (Axiotech; Zeiss) according to standard histologic criteria such as death of cell groups, swelling of cells, and cell lysis.³¹ The presence of an inflammatory response is another criterion for cell necrosis³¹ that cannot be studied in an ex vivo model.

Treatment Groups

Tissue samples were divided into the following groups (*n* = 8 each): group A: ICG 0.1%, exposure 1 minute, light exposure 3 minutes; group B: ICG 1%, exposure 1 minute, light exposure 3 minutes; group C: BSS, exposure 1 minute, light exposure 3 minutes (control); group D: ICG 0.1%, exposure 30 minutes, light exposure 3 minutes; group E: ICG 1%, exposure 30 minutes, light exposure 3 minutes; group F: BSS, exposure 30 minutes, light exposure 3 minutes (control); group G: ICG 0.1%, exposure 1 minute, no light exposure; group H: ICG 1%, exposure 1 minute, no light exposure; group I: BSS, exposure 1 minute, no light exposure (control).

Retinal Penetration of ICG

Twelve tissue samples were exposed to ICG 1% for 1 minute, as described, and were kept in oxygenated DMEM in a refrigerator at a temperature of 4°C to minimize postmortem changes. At 10 minutes, 60 minutes, 2 hours, and 3 hours after exposure to ICG, tissue samples were embedded in tissue freezing medium (Jung; Leica Instruments, Nussloch, Germany) and were frozen in liquid nitrogen. Frozen tissue sections were cut at a thickness of 6 μ m and mounted on slides (SuperFrost Plus; Menzel-Gläser). As described previously,³² tissue sections were immediately examined by fluorescence microscopy (Axiotech; Zeiss) equipped with an appropriate dual-filter set for excitation and emission of near infrared fluorescence (λ_{ex} , 730 - 750 nm; λ_{em} , >780 nm; Omega Optical, Brattleboro, VT). An HBO/100-W mercury lamp was used for excitation. For each sample, autofluorescence was

TABLE 2. TUNEL

Group*	A	B	C	D	E	F	G	H	I
No. TUNEL-positive nuclei	0.25 ± 0.46	4.6 ± 1.1	0.25 ± 0.46	8.4 ± 1.7	11.0 ± 1.9	0.38 ± 1.7	0.63 ± 1.1	4.8 ± 0.9	0.5 ± 0.76
Grading	0	1	0	2	3	0	0	1	0

Apoptotic cell death was detected with the use of TUNEL. Mean number of TUNEL-positive nuclei in each group and the corresponding apoptosis grading (Table 1). Group A: ICG 0.1%, exposure 1 minute, light exposure 3 minutes. Group B: ICG 1%, exposure 1 minute, light exposure 3 minutes. Group C: BSS, exposure 1 minute, light exposure 3 minutes (control). Group D: ICG 0.1%, exposure 30 minutes, light exposure 3 minutes. Group E: ICG 1%, exposure 30 minutes, light exposure 3 minutes. Group F: BSS, exposure 30 minutes, light exposure 3 minutes (control). Group G: ICG 0.1%, exposure 1 minute, no light exposure. Group H: ICG 1%, exposure 1 minute, no light exposure. Group I: BSS, exposure 1 minute, no light exposure (control).

* $n = 8$ tissue samples in each group. Values are mean \pm SD.

subtracted from the mean fluorescence intensity of the positive samples. Histograms of the line profile were calculated using an image processing program (Image-Pro Plus; Media Cybernetics, Silver Spring, MD).

RESULTS

Only mild (grade 0) TUNEL labeling was observed in retinal sections after exposure to ICG 0.1% for 1 minute and in the control sections (groups A, C, F, G, D). Grades 1 and 2 apoptosis were found in the ganglion cell layer and the inner and outer nuclear layers after 30-minute exposure to ICG 0.1% and 1-minute exposure to ICG 1% (groups B, D, H). Grade 3 apoptosis was found in the ganglion cell layer and the inner and outer nuclear layers after 30-minute exposure to ICG 1% (group E; Table 2, Fig. 1).

Statistical comparison of the groups showed no significant difference between groups A, C, F, G, and I ($P > 0.05$; Mann-Whitney U test). We found significant differences between groups B and D ($P = 0.001$; Mann-Whitney U test), B

and E ($P < 0.001$; Mann-Whitney U test), and D and E ($P = 0.005$; Mann-Whitney U test), but not between groups B and H ($P = 0.88$; Mann-Whitney U test).

No light microscopic signs of cell necrosis were found. Light microscopy showed a well-preserved retinal cytoarchitecture in all sections. The ILM was in place in all sections.

Fluorescence microscopy at different time points after 1-minute exposure of the retinal surface to ICG showed that in our model ICG gradually stained the entire retina within 3 hours. Line profiles of the fluorescence intensity showed that the concentration of ICG in the retinal layers increased and then decreased as ICG diffused through the tissue (Fig. 2).

DISCUSSION

Fluorescence microscopy experiments studying the transretinal diffusion of ICG demonstrated that even brief exposure to low-concentration ICG produced staining in all retinal layers. This gradual retinal penetration was presumably driven by the

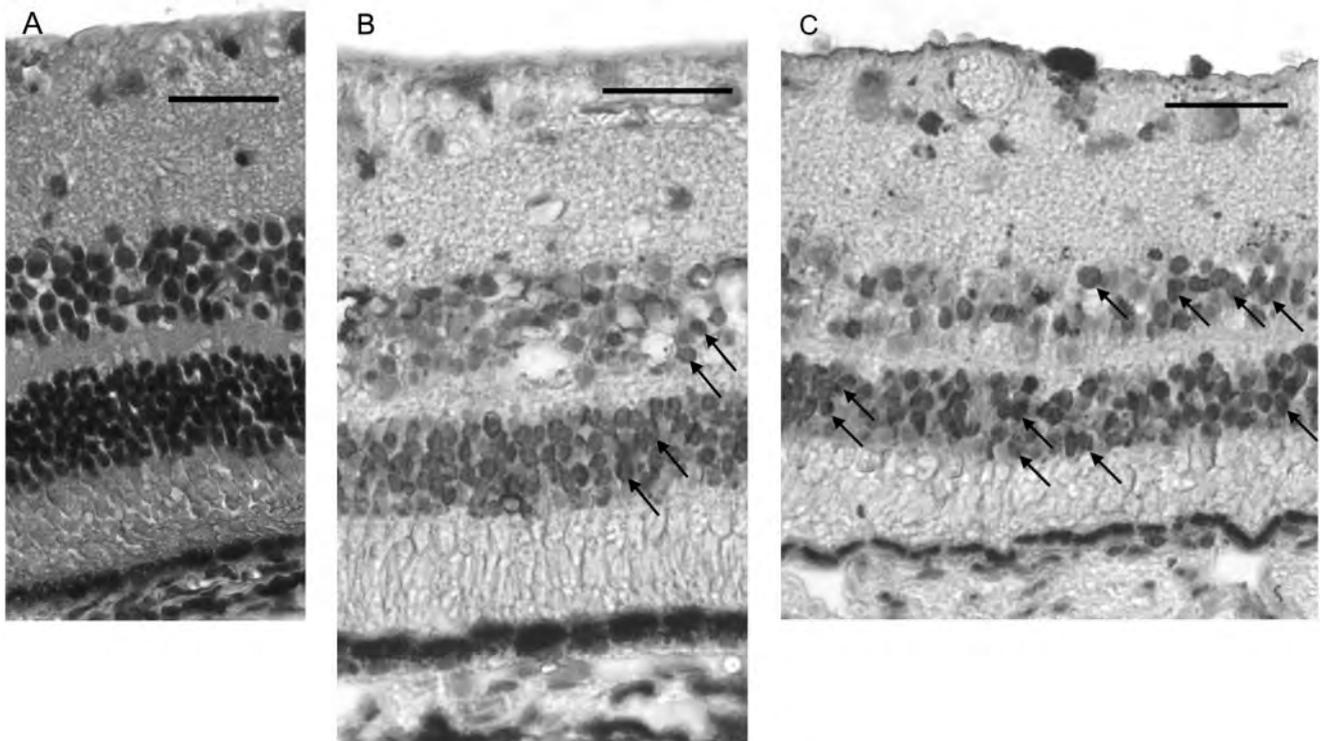


FIGURE 1. TUNEL test. Light photomicrographs of porcine retina processed with the TUNEL technique and counterstained with hematoxylin after 24-hour perfusion in organ culture. Arrows: TUNEL-positive nuclei. (A) No or mild apoptosis (grade 0) as seen in groups A, C, F, G, and I. (B) Mild to moderate apoptosis (grades 1-2) as seen in groups B, D, and H. (C) Severe apoptosis (grade 3) as seen in group E. Scale bar: 50 μ m.

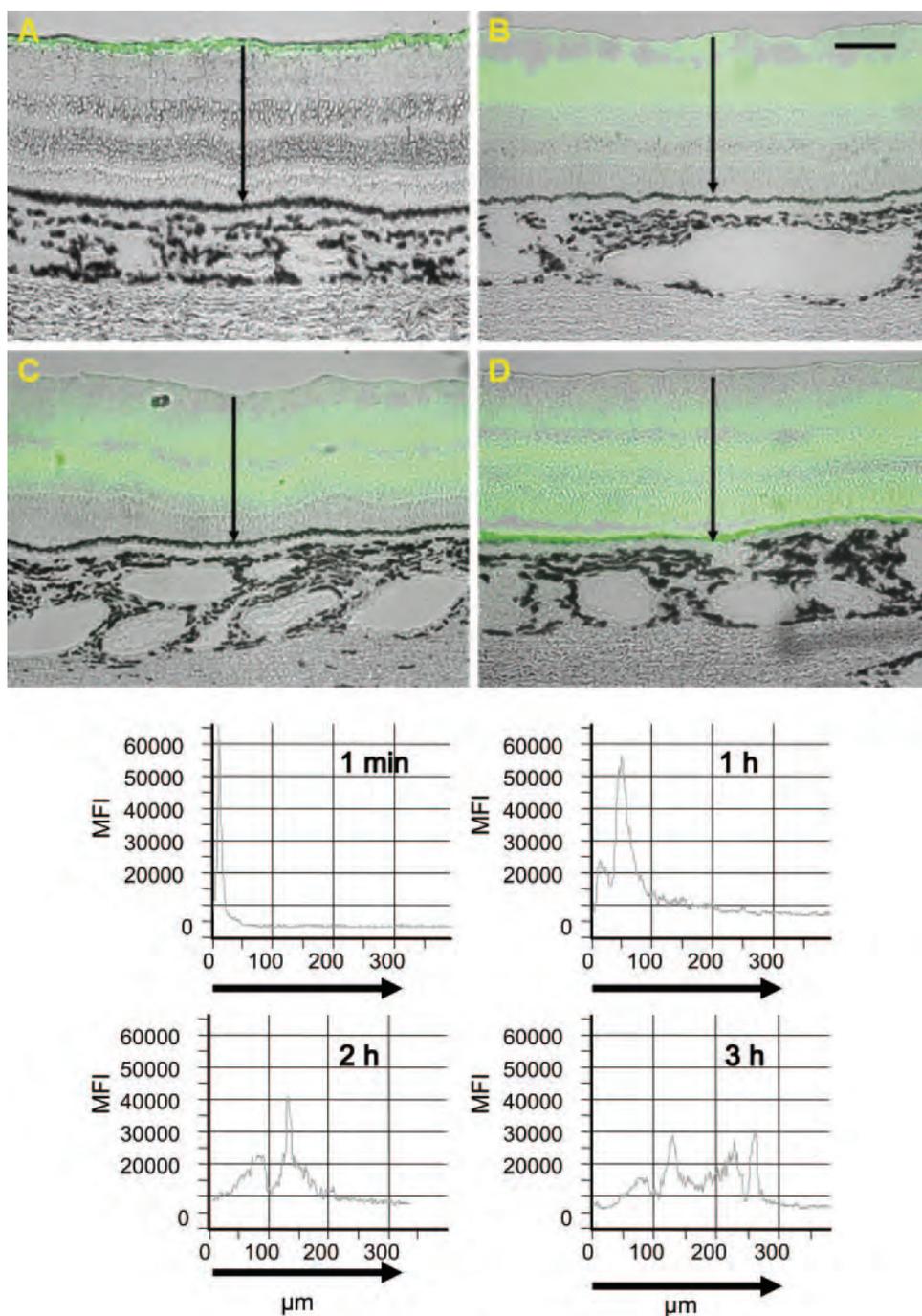


FIGURE 2. Intraretinal penetration of epiretinally applied ICG. Fluorescence photomicrographs of frozen tissue sections showing complete intraretinal penetration of an ICG 1% solution applied to the inner retinal surface for 1 minute, then rinsed free with BSS. Distribution of the mean fluorescence intensity (MFI) of ICG is shown as line profiles (along the arrows; $n = 3$ each): (A) 1 minute after exposure, (B) 60 minutes after exposure, (C) 2 hours after exposure, (D) 3 hours after exposure. Scale bar: 100 μm .

concentration gradient within the tissue and possibly by gravity. Transretinal diffusion of ICG is as expected given that ICG has a molecular weight of 775 Da, and this is well below the porcine retinal molecular weight exclusion limit of 60,000 Da.³³ Conversely, ICG is an amphiphilic agent^{34,35} that binds avidly to the ILM, and this might be expected to limit its diffusion through the retina. However, ICG angiography leads to choroidal extravascular staining despite 98% of molecules being plasma bound within the choriocapillaris.³⁶ A similar state of flux might exist in the retina, with some nonbound molecules free to diffuse beyond the ILM, but the exact pharmacodynamics remain to be elucidated.

The diffusion of ICG into the entire retina implies that the RPE may be affected not only during macular hole surgery, when the dye is in direct contact with the RPE through a

full-thickness retinal hole, but also during epiretinal membrane surgery, when ICG is applied to the intact retinal surface. When the ILM is quickly removed after the application of ICG in macular surgery, a certain fraction of the dye may be removed with it and thereby kept from diffusing into deeper retinal layers. However, clinical observations have shown that ICG fluorescence persists after ICG-assisted epiretinal membrane surgery,^{37,38} even within the area of ILM removal.³⁷ Fundus fluorescence from residual ICG has been detected for up to 9 months after epiretinal membrane surgery.³⁸

Paques et al.³⁹ injected ICG into the vitreous cavity of rabbits and observed long-term staining of the visual pathway through anterograde axonal transport. It is possible that ICG may be toxic to the neuronal tissue of the visual pathway because neurotoxic effects have been described in single my-

elinated and unmyelinated dorsal root neurons after intrathecal ICG injection in rats.⁴⁰

In contrast to the findings of Gandorfer et al.²⁴ but in accordance with those of Grisanti et al.,²⁵ we did not find a disruption of the retinal cytoarchitecture after exposure to ICG. In the present study, ICG induced apoptosis in all retinal nuclear layers in a dose-dependent manner. This finding is in accordance with the findings of cell culture studies using Müller cells^{17,41} or RPE cells^{17,19,42} but with the advantage that the present study used whole retinas and thereby investigated all retinal cell types.

ICG has been shown to be toxic to RPE cells in a dose-dependent manner by functioning as a precipitating factor when dissolved in salt solution.¹⁸ This finding is in accordance with that of others, who found the degree of toxicity is modulated by sodium in the solvent as the removal of sodium from the solvent reduces toxicity.⁴³ The toxicity of ICG solutions is also modulated by the osmolarity of the solvent.²⁰

The present study did not reveal any ICG-mediated phototoxicity, though ICG is known to act as a photosensitizer. Its absorption spectrum exhibits a strong absorption band around 800 nm, and a photo-oxidative effect can be triggered with a diode laser emitting at 805 nm.^{26,27} However, ICG is a comparatively weak photosensitizer because the yields of triplet formation appear low compared with other photosensitizers used in photodynamic therapy.²⁶ Overlap occurs between the absorption spectrum of ICG and the emission of commonly used surgical light sources,^{10,29} but this does not imply the occurrence of a photodynamic effect because such an effect is related to the amount of ICG and the energy density of the illumination.^{26,27} In view of the low concentrations of ICG now used in surgery and the fact that endoillumination is certainly of lower energy density than a diode laser, it is possible that this may not be sufficient to produce phototoxicity. This may explain the lack of phototoxicity observed in our study. It does not, however, preclude ICG-mediated phototoxicity in vivo because ambient illumination greater than 400 nm will be transmitted through the human cornea and lens to the retina, and this may interact with persistent ocular ICG. An alternative explanation is that the methodology used to detect apoptosis in this experiment was not sufficiently sensitive to detect subtle retinal damage.

In summary, the results of the present study add to the ongoing debate^{14,15} about the safety of ICG. ICG briefly applied to the retinal surface eventually penetrated the entire retina. ICG induced apoptosis but not necrosis in all nuclear retinal layers in a dose-dependent manner. One-minute exposure to ICG 0.1% with 3-minute illumination stimulated the intraoperative use of this vital stain and did not induce retinal apoptosis or necrosis. However, the safety margin was narrow; therefore, ICG and other dyes should be used cautiously. Future studies should explore the potential surgical usefulness of alternative vital stains with improved safety profiles, as recently initiated by Jackson et al.⁴⁴ and Haritoglou et al.⁴⁵

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