SCIENTIFIC REPORT

Removal of sodium from the solvent reduces retinal pigment epithelium toxicity caused by indocyanine green: implications for macular hole surgery

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Backgrounds/aims: Staining of internal limiting membrane with indocyanine green (ICG) has been reported to be associated with postoperative atrophic retinal pigment epithelium (RPE) change. Here the authors examined whether removing sodium from the solvent reduces ICG induced RPE cytotoxicity.

Methods: Human RPE cells were exposed to ICG (0.25 and 0.025 mg/ml) reconstituted with balanced salt solution (BSS) or Na^+ free BSS. Light microscopy, trypan blue dye exclusion, acridine orange/ethidium bromide staining, and DNA electrophoresis were used to evaluate the cytotoxic effects of ICG. ICG uptake was measured by optical absorption at 790 nm.

Results: Sodium removal reduced the ICG induced changes in cell morphology and improved the RPE cell viability. When RPE cells were incubated for 4 hours in 0.25 mg/ml ICG dissolved in BSS and sodium free BSS, 86.3% (SD 6.7%) and 2.4% (1.1%) of the cells were stained with trypan blue, respectively. After ICG treatment, RPE dies mainly through a necrotic mechanism. ICG uptake by RPE was also reduced with sodium removal.

Conclusions: ICG induced cytotoxicity in cultured human RPE was reduced with removal of sodium from the solvent. This reconstitution method may provide a safer intravitreal use of ICG in macular hole surgery.

Staining the internal limiting membrane (ILM) with indocyanine green (ICG) facilitates ILM peeling during macular hole surgery.[1 2] However, there have been studies describing ICG associated adverse effects. Gandorfer *et al*[3] reported less favourable visual outcomes. Similarly, Engelbrecht *et al*[4] observed a high incidence of unusual atrophic retinal pigment epithelium (RPE) changes at the site of the previous macular hole and its surrounding subretinal fluid after ICG assisted ILM peeling. Haritoglou and coworkers[5] reported no statistically significant improvement of postoperative visual acuity. In an in vitro study, Sippy *et al*[6] reported a significant reduction in mitochondrial dehydrogenase activity in cultured RPE after ICG exposure. ICG is an organic anion[7]; after intravenous injection, it is rapidly cleared from the circulation by the liver.[8] The uptake of organic anions by hepatocytes involves both sodium (Na^+) dependent and independent transport systems.[9] In this study, we explored the Na^+ dependence of ICG mediated RPE toxicity. We demonstrated that Na^+ removal from the solvent was associated with reduced ICG uptake and reduced RPE cytotoxicity. This finding may be useful in preventing ICG associated intraocular toxicity.

MATERIALS AND METHODS

Cell culture and ICG preparation

Human RPE cells (ARPE-19) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The RPE cells were cultured in DMEM /F12 medium (1:1) containing 10% fetal bovine serum.

ICG stock solution was prepared by completely dissolving 25 mg of sterile ICG powder (Daiichi Pharmaceutical, Japan) in 0.5 ml sterile distilled water. The desired ICG concentrations were prepared by diluting the stock solution with the appropriate volume of BSS (BSS Plus; Alcon Laboratories). The ICG concentrations tested were 0.25 mg/ml and 0.025 mg/ml (304 mOsm/kg and 305 mOsm/kg, respectively).

For the Na^+ free BSS solution, we prepared a solution with the same constituents as BSS except that NaCl, Na_2HPO_4, and $NaHCO_3$ in the BSS were replaced with 17.06 g/l choline chloride, 0.52 g/l K_2HPO_4, and 2.50 g/l $KHCO_3$, respectively (to achieve the same molar concentration of the replaced chemicals). The pH was adjusted to 7.4 with HCl/KOH. The osmolarity of this solution was 296 mOsm/kg, as measured by an auto-osmometer. ICG was also dissolved in Na^+ free BSS. The preparation method was identical to that described previously. The ICG concentrations tested were 0.25 mg/ml and 0.025 mg/ml (295 mOsm/kg and 296 mOsm/kg, respectively).

Cell viability evaluation (trypan blue exclusion)

RPE cells were seeded into wells of the six well culture plates. After reaching total confluence, the culture medium was removed and the cells were rinsed with PBS. Various concentrations of ICG solution (in BSS or Na^+ free BSS) were added and the cells were incubated for the predetermined period (5 minutes to 4 hours) in the dark. BSS or Na^+ free BSS alone was added to the control wells. The cells were rinsed with PBS and 0.2% trypan blue was added for 5 minutes. Dead cells (those stained with trypan blue) were counted. Results were compared using Z test.

Acridine orange/ethidium bromide staining

RPE cells were cultured in four well chamber slides and treated with ICG for the specified periods after reaching total confluence. After rinsing with PBS, cultures were treated with acridine orange (100 μg/ml) plus ethidium bromide (100 μg/ml) and evaluated immediately under an epifluorescence microscope.

Abbreviations: ICG, indocyanine green; ILM, internal limiting membrane; RPE, retinal pigment epithelium

DNA electrophoresis

RPE cells were cultured in T75 flasks. After reaching total confluence, RPE cells were treated with 0.25 mg/ml ICG in BSS for the specified incubation periods. DNA was extracted with QIAamp DNA Mini Kit (Qiagen Inc, Valencia, CA, USA) as in the manufacturer's instructions. A volume of 10 μg DNA was loaded per slot on 2% agarose gel for electrophoresis. The DNA was visualised by staining with ethidium bromide.

ICG uptake assay

The ICG uptake was measured by utilising the optical absorption of ICG at 790 nm.[10] RPE cells were seeded into wells of the six well culture plates. After reaching total confluence, the RPE cells were treated with 0.25 mg/ml ICG (in BSS or Na^+ free BSS) for 30 minutes at 37℃ in the dark. After rinsing with PBS, the cells in each well were lysed with 0.8 ml of 0.1 N NaOH. The optical absorption of the lysate was measured at 790 nm using a microplate reader. The actual ICG concentration in the cell lysate was calculated using a calibration curve.

RESULTS

ICG induced changes in cellular morphology were reduced by Na^+ removal

After a 30 minute treatment with 0.25 mg/ml ICG in BSS, some RPE cells appeared shrunken, leaving gaps between the cells (which were totally confluent before ICG treatment) (fig 1A). Some cells contained fine vesicles in their cytoplasm. When the ICG exposure was extended to 2 hours, more cells appeared shrunken (fig 1B). With a more prolonged exposure (4 hours), many cells underwent lysis: the cellular outline was poorly defined, and only the nucleus was discernible (fig 1C).

Minor morphological changes were observed when RPE were exposed to 0.25 mg/ml ICG in Na^+ free BSS (fig 1D, E). After a 4 hour incubation with ICG in Na^+ free BSS, some cells appeared slightly shrunken; however, no lysed cells with exposed nuclei were observed (fig 1F). Removing Na^+ from the solvent also reduced the 0.025 mg/ml ICG induced morphological changes in cultured RPE. No morphological changes were evident in control cultures (in BSS or Na^+ free BSS only).

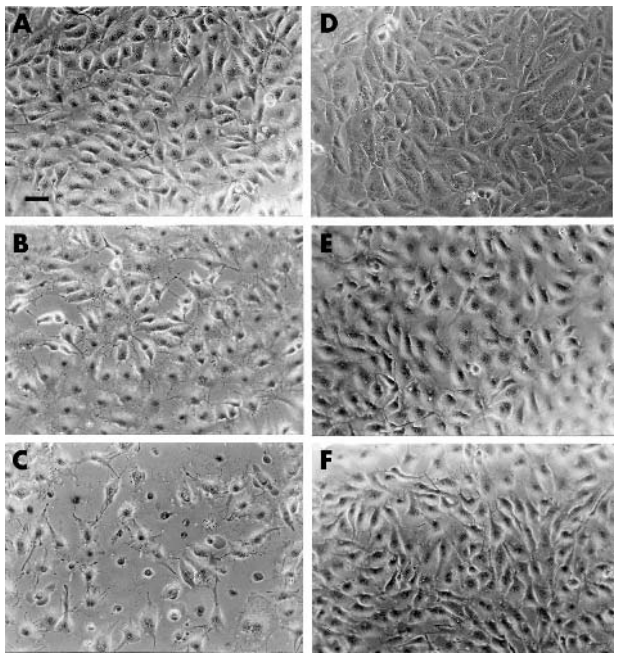

Figure 1 Cultured human RPE cells were exposed to ICG dissolved in BSS or Na^+ free BSS. (A) After a 30 minute exposure to 0.25 mg/ml ICG (in BSS), some RPE cells appeared shrunken, leaving gaps between the RPE cells (which were totally confluent before ICG treatment). (B) After a 2 hour exposure to 0.25 mg/ml ICG (in BSS), more RPE cells had shrunk. (C) Many RPE cells lysed after a 4 hour exposure to 0.25 mg/ml ICG (in BSS); the cellular outline was poorly defined, and only the nucleus was discernible. These nuclei were stained green by ICG. (D) After a 30 minute exposure to 0.25 mg/ml ICG dissolved in Na^+ free BSS, most cells were normal. (E) After a 2 hour exposure to 0.25 mg/ml ICG (in Na^+ free BSS), some cells appeared slightly shrunken while most cells retained their normal size and morphology. (F) Some RPE cells appeared somewhat shrunken after a 4 hour exposure to 0.25 mg/ml ICG (in Na^+ free BSS); however, no cellular lysis was noted (scale bar: 50 μm).

Na^+ removal improved RPE cell viability after ICG treatment

Viability in cultured RPE was evaluated by trypan blue, which assesses the integrity of the cell membrane. Exposure to BSS only or Na^+ free BSS for 4 hours did not compromise RPE viability. The number of trypan blue (+) cells (dead cells) increased with exposure time to 0.25 mg/ml ICG in BSS. For the same ICG concentration and exposure duration, fewer cells were stained blue when the solvent was Na^+ free BSS (fig 2). This difference was more obvious as the exposure time increased.

Acridine orange/ethidium bromide staining

Acridine orange/ethidium bromide staining allowed identification of live, early apoptotic, late apoptotic, and necrotic cells.[11] RPE incubated in BSS or Na^+ free BSS for 4 hours exhibited green fluorescence in the cytoplasm with no orange fluorescence in the nuclei; they remained viable. After a 30 minute incubation with 0.25 mg/ml ICG (in BSS), some nuclei were orange fluorescent with ethidium bromide stain, indicating compromised membrane integrity. These cells also exhibited decreased green cytoplasmic fluorescence (fig 3A). With a longer exposure time, more RPE had orange fluorescent nuclei (fig 3B). These orange fluorescent nuclei showed normal nuclear architecture. There were no signs of apoptosis, such as condensed and fragmented nuclei. At this concentration, ICG apparently caused cell death by a necrotic mechanism. After ICG exposure for 4 hours, there were more

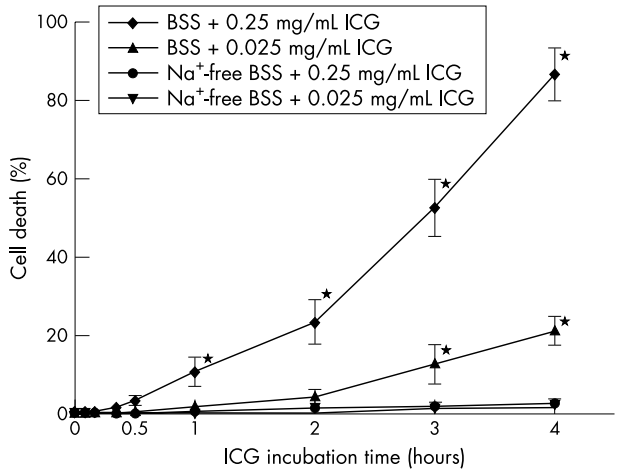

Figure 2 Viability of RPE cells after ICG exposure was estimated by trypan blue dye exclusion. Cells stained with trypan blue represented dead cells with compromised membrane integrity. Data represent the percentage of trypan blue positive cells (mean percentage (SE)) and were obtained from at least five independent experiments. (*Statistically significant difference between BSS medium and Na^+ free BSS medium for the same ICG concentration; $p<0.05$.)

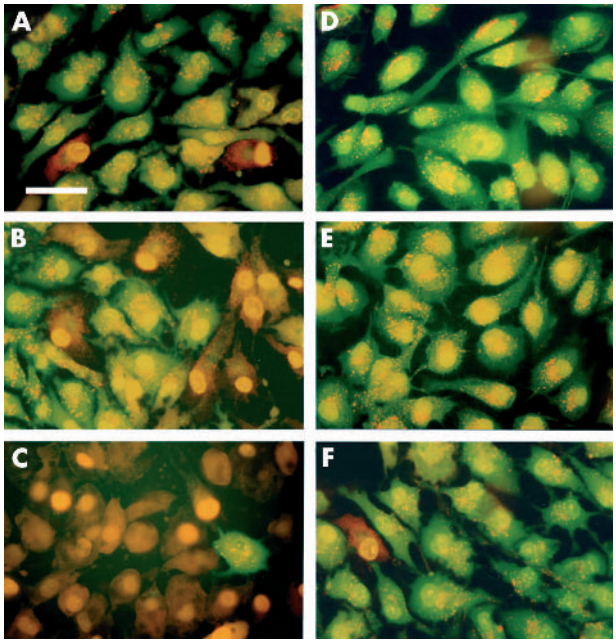

Figure 3 RPE cells were stained with acridine orange/ethidium bromide to determine viability after incubation with ICG. (A) After a 30 minute exposure to 0.25 mg/ml ICG dissolved in BSS, some cells had compromised membrane integrity and showed the orange fluorescence of ethidium bromide. Punctate orange red fluorescence in the cytoplasm represented lysosomes stained by acridine orange. (B) After a 2 hour exposure to 0.25 mg/ml ICG (in BSS), more cells had compromised membranes and orange fluorescent nuclei. (C) Many RPE cells incubated with 0.25 mg/ml ICG (in BSS) for 4 hours exhibited the orange fluorescence of ethidium bromide. The orange fluorescent nuclei showed normal nuclear architecture. There were no condensation or fragmentation of the nuclei. (D) After a 30 minute exposure to 0.25 mg/ml ICG dissolved in Na^+ free BSS, all cells were viable and exhibited green fluorescence. (E, F) RPE cells were incubated with 0.25 mg/ml ICG (in Na^+ free BSS) for 2 and 4 hours, respectively. Few cells with orange fluorescent nuclei were found; most cells remained viable (scale bar: 50 μm).

orange fluorescent cells with normal nuclear structure. These cells were dead and necrotic (fig 3C).

When incubated for 30 minutes with 0.25 mg/ml ICG in Na^+ free BSS, almost all RPE showed green fluorescence and remained viable (fig 3D). As the exposure time increased, only a small number of RPE cells had orange fluorescent nuclei. Most cells were still alive (green fluorescent) after a 4 hour exposure (fig 3E, F).

DNA electrophoresis revealed that RPE died mainly through a necrotic mechanism

DNA was extracted from RPE treated with 0.25 mg/ml ICG in BSS for various time periods. Agarose gel electrophoresis showed no laddering of DNA (fig 4). This indicated that RPE cells died mainly through a necrotic mechanism at this concentration of ICG.

Eliminating Na^+ in the solvent reduced ICG uptake by RPE cells

ICG uptake by RPE was 5.16 (1.29) $\mu g/10^5$ cells for RPE incubated with 0.25 mg/ml ICG in BSS for 30 minutes at 37°C. This value was significantly decreased ($p = 0.022$; *t* test) to 1.08 ± 0.19 $\mu g/10^5$ cells for RPE incubated with 0.25 mg/ml ICG in Na^+ free BSS.

DISCUSSION

In this study, we utilised a novel method for reconstituting ICG that reduced its toxicity in RPE. This method was

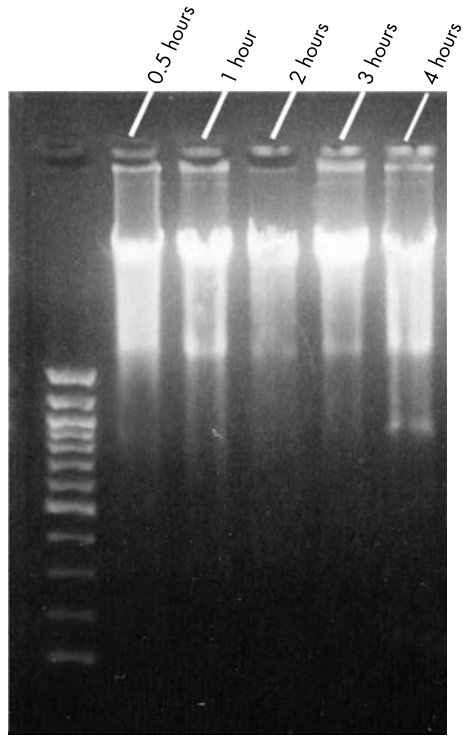

Figure 4 DNA was extracted from the RPE treated with 0.25 mg/ml ICG in BSS for the specified periods. DNA was analysed by 2% agarose gel electrophoresis in the presence of ethidium bromide staining. There was no laddering of extracted DNA. A 100 bp DNA ladder was used as a marker (the leftmost lane).

associated with decreased ICG uptake into RPE. Besides, we demonstrated that ICG solubilised in BSS induced RPE cell death mainly through a necrotic mechanism.

The causes of the atrophic RPE changes[4] and the poorer visual outcome[3–5] after ICG assisted ILM peeling are not yet clear. However, these observations could possibly be attributed to the cytotoxic effects of ICG. When ICG is used in macular hole surgery, it has direct access to the bare RPE at the base of the macular hole. The RPE can take up ICG, as demonstrated in this study. Although ICG is applied for periods ranging from 30 seconds to 5 minutes and removed by washing,[1 2 12–16] residual ICG may remain in the eye for a prolonged period[17–19] (even up to 8 months). Therefore, the exposure durations used in this study (from 5 minutes to 4 hours) were clinically relevant.

ICG is most often administered intravenously and has a long history of safety.[20 21] It is taken up exclusively by hepatocytes after intravenous injection and is rapidly cleared from the circulation.[8] When administered intravitreally, however, ICG must have a longer half life owing to the slow turnover rate in the vitreous cavity. This was verified in several reports[17–19] describing persistent ICG fluorescence after intraocular ICG administration. Therefore, the safety of ICG, as assessed from intravenous studies, may not be relevant to intravitreal applications.

In this study, we demonstrated that removal of Na^+ from the solvent reduces ICG induced RPE toxicity and the underlying mechanism may involve reduced uptake of ICG. The actual mechanism through which ICG is taken up by RPE remains unclear. Recently, an organic anion transporter, organic anion transporting polypeptide 2 (OATP2) was found in the rat RPE.[22] This transporter mediated transport of many endogenous and exogenous amphiphilic compounds in a Na^+ independent manner.[9] However, in our study, ICG uptake by

RPE was partially Na^+ dependent. One possible explanation is that another transporter(s), which is Na^+ dependent, is present on RPE cells and is responsible for the Na^+ dependent portion of ICG transport. Another possibility is that the ICG uptake is coupled to a secondary active Na^+ involving co-transporter, which is driven by Na^+ gradient, just like the cholate uptake in hepatocyte.[23]

It has been suggested that the toxic effects of ICG on RPE is related to the hypo-osmolarity of the solvent.[24] In our study, the osmolarities of the 0.25 mg/ml ICG solutions were 304 mOsm/kg (in BSS) and 295 mOsm/kg (in Na^+ free BSS). These osmolarities were only 1 mOsm/kg less than the pure solvent (305 mOsm/kg for BSS and 296 mOsm/kg for Na^+ free BSS). Therefore, the ICG induced RPE cytotoxicity cannot be explained by the hypo-osmolarity of the ICG solution.[25]

ICG staining can be a valuable tool in macular hole surgery; however, no standardised reconstitution method exists for its intravitreal use. In this study, we demonstrated that removal of Na^+ from the solvent reduces the ICG induced toxicity on cultured human RPE. Further in vivo studies are required to examine if safer intravitreal ICG use and efficient ILM dissection can be achieved with this approach.

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REFERENCES

1 **Kadonosono K**, Itoh N, Uchio E, *et al*. Staining of internal limiting membrane in macular hole surgery. *Arch Ophthalmol* 2000;**118**:1116–18.
2 **Da Mata AP**, Burk SE, Reimann CD, *et al*. Indocyanine green-assisted peeling of the retinal internal limiting membrane during vitrectomy surgery for macular hole repair. *Ophthalmology* 2001;**108**:1187–92.
3 **Gandorfer A**, Haritoglou C, Gass CA, *et al*. Indocyanine green-assisted peeling of internal limiting membrane may cause retinal damage. *Am J Ophthalmol* 2001;**132**:431–3.
4 **Engelbrecht NE**, Freeman J, Sternberg P, *et al*. Retinal pigment epithelial changes after macular hole surgery with indocyanine green-assisted internal limiting membrane peeling. *Am J Ophthalmol* 2002;**133**:89–94.
5 **Haritoglou C**, Gandorfer A, Gass CA, *et al*. Indocyanine green-assisted peeling of the internal limiting membrane in macular hole surgery affects visual outcome: a clinicopathologic correlation. *Am J Ophthalmol* 2002;**134**:836–41.
6 **Sippy BD**, Engelbrecht NE, Hubbard GB, *et al*. Indocyanine green effect on cultured human retinal pigment epithelial cells: implication for macular hole surgery. *Am J Ophthalmol* 2001;**132**:433–5.
7 **Devoiselle JM**, Mordon S, Soulie S, *et al*. Fluorescence properties of indocyanine green/part 1: in vitro study with micelles and liposomes. In: Lakowicz JR, Thompson JB, eds. *Advances in fluorescence sensing technology*, III 1997. Bellingham, CA: SPIE, 1997:530–7.
8 **Desmettre T**, Devoisselle JM, Mordon S. Fluorescence properties and metabolic features of indocyanine green (ICG) as related to angiography. *Surv Ophthalmol* 2000;**45**:15–27.
9 **Meier PJ**. Molecular mechnisms of hepatic bile salt transport from sinusoidal blood into bile. *Am J Physiol* 1995;**269**:G801–12.
10 **Fickweiler S**, Szeimies RM, Baumler W, *et al*. Indocyanine green: Intracellular uptake and phototherapeutic effects in vitro. *J Photochem Photobiol B* 1997;**38**:178–83.
11 **Amarante-Mendes GP**, Bossy-Wezel E, Brunner T, *et al*. Apoptosis assay. In: Spector DL, Goldman RD, Leinwand LA, eds. *Cells—a laboratory manual*. New York: Cold Spring Harbor Laboratory Press, 1998:15.1–15.24.
12 **Burk SE**, Da Mata AP, Snyder ME, *et al*. Indocyanine green-assisted peeling of the retinal internal limiting membrane. *Ophthalmology* 2000;**107**:2010–4.
13 **Gandorfer A**, Messmer EM, Ulbig MW, *et al*. Indocyanine green selectively stains the internal limiting membrane. *Am J Ophthalmol* 2001;**131**:387–8.
14 **Kwok AK**, Li WW, Pang CP, *et al*. Indocyanine green staining and removal of internal limiting membrane in macular hole surgery: histology and outcome. *Am J Ophthalmol* 2001;**132**:178–83.
15 **Kwok AK**, Lai TY, Man-Chan W, *et al*. Indocyanine green assisted retinal internal limiting membrane removal in stage 3 or 4 macular hole surgery. *Br J Ophthalmol* 2003;**87**:71–4.
16 **Weinberger AW**, Schlossmacher B, Dahlke C, *et al*. Indocyanine-green-assisted internal limiting membrane peeling in macular hole surgery—a follow-up study. *Graefes Arch Clin Exp Ophthalmol* 2002;**240**:913–17.
17 **Weinberger AW**, Kirchohof B, Mazinani BE, *et al*. Persistent indocyanine green (ICG) fluorescence 6 weeks after intraocular ICG administration for macular hole surgery. *Graefes Arch Clin Exp Ophthalmol* 2001;**239**:388–90.
18 **Spaide RF**. Persistent intraocular indocyanine green staining after macular hole surgery. *Retina* 2002;**22**:637–9.
19 **Tadayoni R**, Paques M, Girmens JF, *et al*. Persistence of fundus fluorescence after use of indocyanine green for macular surgery. *Ophthalmology* 2003;**110**:604–8.
20 **Hope-Ross M**, Yannuzzi LA, Gragoudas ES, *et al*. Adverse reactions due to indocyanine green. *Ophthalmology* 1994;**101**:529–33.
21 **Lutty GA**. The acute intravenous toxicity of biological stains, dyes, and other fluorescent substances. *Toxicol Appl Pharmacol* 1978;**44**:225–49.
22 **Gao B**, Wenzel A, Grimm C, *et al*. Localization of organic anion transport protein 2 in the apical region of rat retinal pigment epithelium. *Invest Ophthalmol Vis Sci* 2002;**43**:510–14.
23 **Boelsterli UA**, Zimmerli B, Meier PJ. Identification and characterization of a basolateral dicarboxylate/cholate antiport system in rat hepatocytes. *Am J Physiol* 1995;**268**:G797–G805.
24 **Stalmans P**, Van Aken EH, Veckeneer M, *et al*. Toxic effect of indocyanine green on retinal pigment epithelium related to osmotic effects of the solvent. *Am J Ophthalmol* 2002;**134**:282–5.
25 **Ho JD**, Tsai RJF, Chen SN, *et al*. Toxic effect of indocyanine green on retinal pigment epithelium related to osmotic effects of the solvent (Correspondence). *Am J Ophthalmol* 2003;**135**:258.