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Morphological and functional damage of the retina caused by intravitreous indocyanine green in rat eyes

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Abstract *Background:* This study was designed to investigate the influence of intravitreal indocyanine green (ICG) on retinal morphology and function. *Methods:* Brown Norway rats eyes (*n*=24) were vitrectomized by the injection of 0.05 ml of 100% SF_6 gas. Two weeks later, ICG solution was injected into the vitreous cavity of vitrectomized eyes at a dose of 25 mg/ml, 2.5 mg/ml, 0.25 mg/ml or 0.025 mg/ml (0.05 ml/eye). Retinal toxicity was histologically assessed by light microscopy on day 10. The retinal function was also evaluated by electroretinography (ERG) in the low-dose groups (0.25 mg/ml and 0.025 mg/ml) after 10 days and again after 2 months,. Sham-operated eyes (SF_6 injected followed by 0.05 ml of BSS plus, *n*=6) were used as controls. *Results:* In the high-dose group (25 mg/ml ICG), the retinal structure was severely deformed and the retinal pigment epithelium partly disappeared. In eyes with 2.5 mg/ml ICG, the retinal structure was also affect-ed but less strongly so than with 25 mg/ml. No apparent pathologic change was observed in the low-dose groups (0.25 mg/ml or 0.025 mg/ml) by light microscopy. In contrast, 10 days later the amplitude of dark-adapted a- and b-waves of ERGs in the eyes of low-dose group rats were found to have decreased. In addition the light-adapted b-waves did not change significantly. These changes remained for 2 months. *Conclusion:* Even at a low dose (0.025 mg/ml), intravitreous ICG induced functional damage of the retina without any apparent morphological damage. This information should be taken into account when clinically administering ICG into the vitreous cavity.

Introduction

The removal of the internal limiting membrane (ILM) is one of the important developments of surgery for such vitreo-retinal diseases as macular hole [2, 12]. However, this technique is difficult to perform because of the poor visibility of the ILM. Recently Kadonosono et al. [8] and Burk et al. [3] described that improvements in ILM visibility thanks to indocyanine green (ICG) staining have now made the ILM peeling procedures much easier than before. Indeed, the results of macular hole surgery have improved [2, 3, 4, 8, 9]. As a result, this technique is now widely accepted by many surgeons [4, 9].

ICG has been used for evaluating the liver function in various diseases and the toxicity of intravenous ICG has been reported to be insignificant [14]. However, this does not necessarily mean that ICG is non-toxic to any cells, but rather that intravenous ICG is not toxic because it is excreted from the tissue immediately by binding to intravascular proteins.

In the ophthalmic field, McEnerney et al. described that the transient presence of ICG in the anterior chamber was not toxic to the corneal endothelium [11]. None-

theless, to the best of our knowledge, there has been no report on the effects of intravitreous ICG on retinal morphology and function. Since ICG is mainly used to stain the most important retinal area for vision, namely the macula, during pars plana vitrectomy and furthermore remains in the tissue for more than 6 weeks [1], it is important to maintain the vision of treated patients. In the present study, we attempted to elucidate the effects of intravitreal ICG on the retina by means of both morphological and functional analyses in a rat model.

Materials and methods

Animals and surgical procedures

The rats used in this experiment were prepared in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Brown Norway rats (male, 8 weeks old; Kyudo, Fukuoka, Japan) were used. The rats were anesthetized with intraperitoneally injected ketamine hydrochloride at a dose of 75 mg/kg body weight. Each eye was gas-vitrectomized using 0.05 ml of pure SF_6 gas by our previous method [13]. At 2 weeks after injection, 0.05 ml of ICG (Dai-ichi Seiyaku, Tokyo, Japan) solution was injected into the vitreous cavity of each vitrectomized eye under microscopic surgery. ICG solution was prepared in doses of 25 mg/ml, 2.5 mg/ml, 0.25 mg/ml and 0.025 mg/ml diluted with balanced salt solution (BSS plus, Santen, Osaka, Japan). The concentration was determined according to ICG solution used in a vitrectomy for humans (5 mg/ml) [4, 9]. Six eyes were included in each experimental group. Sham-operated eyes (SF_6 injected followed by 0.05 ml of BSS plus, n=6) were used as controls.

Light microscopy

Our preliminary study showed that the intravitreal ICG disappeared macroscopically within 10 days. Therefore, the eyes were enucleated and fixed in 10% paraformaldehyde on day 10. Whole eyes were cut approximately along the vertical meridian. Paraffin-embedded sections were stained using routine hematoxylin-eosin (HE) staining, and each section was examined by blinded observers.

Electroretinography

In our preliminary study, the eyes receiving a high dose of ICG (25 and 2.5 mg/ml ICG) showed a significant destruction of retinochoroidal tissue histologically. As a result, these eyes were also functionally damaged. Therefore, the eyes of both groups were excluded from the following functional analysis. The rats were kept in a dark room for at least one night, prepared under dim red illumination and anesthetized by intraperitoneal injection of 15 µl/g body weight of a saline solution containing ketamine (1 mg/ml), xylazine (0.4 mg/ml) and urethane (40 mg/ml). Electroretinography (ERG) was then performed as previously described [5, 6, 13].

Briefly, the pupils of the rats were maximally dilated. The cornea was anesthetized with 1% proparacaine HCl drops and then the rats were placed on a heating pad for the duration of the experiment. A wire electrode, coated with 1% methylcellulose, was placed over the cornea to record the ERGs. A similar wire electrode placed in the mouth served as reference electrode, while a needle electrode inserted into the tail was grounded. The responses were differentially amplified (0.8 to 1,200 Hz), averaged and stored using a PC 9801 computer. White (xenon) strobe flashes were presented in a Ganzfeld stimulator (VPA-10; Cadwell, Kennewick, Wash., USA) against an achromatic adapting field. Dark-adapted (rod-mediated) ERGs were recorded first to check the response stability at both intensities. Each rat was then adapted to dark background luminance for 20 min, a period sufficient to achieve a stable level of response. Thereafter, dark-adapted a (rod-mediated) and dark-adapted b (bipolar and Müller-mediated) ERGs were elicited at a flash luminance of 1.30 log cd s/m^2. The responses to five successive flashes at an interstimulus interval of 1 min were averaged to determine the dark-adapted responses. The rats were exposed to a white light-adapting field (1.50 log cd/m^2) for at least 25 min, and then light-adapted b (cone-mediated) ERGs were elicited at a flash luminance of 1.30 log cd s/m^2 (rod-desensitized condition in rats). The responses to 50 successive flashes made at 2 Hz were averaged.

Results

Light microscopic analysis

In the eyes with the highest dose of ICG (25 mg/ml), the normal structure of the retina was destroyed. Retinal pigment epithelial cells were lost in most areas

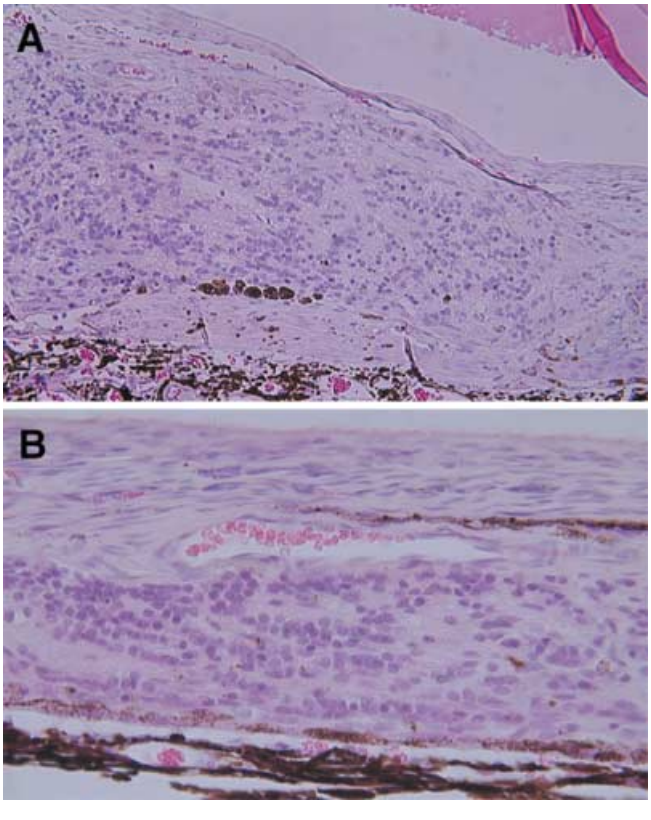

Fig. 1A, B Light micrographs of a rat eye with intravitreal ICG injection (25 mg/ml, 0.05 ml) at 10 days after injection. Loss of the retinal pigment epithelium, photoreceptors, and destruction of all layers of the retina at the posterior pole (**A**, original magnification ×200) and equator (**B**, original magnification ×400) were observed

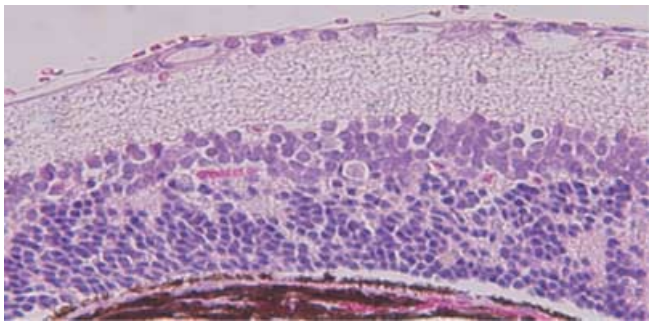

Fig. 2 Light micrograph of a rat eye with intravitreal ICG injection (2.5 mg/ml, 0.05 ml) at 10 days after injection. Loss of the photoreceptor outer segments and destruction of the inner layer of the retina are observed. These changes were not as severe as those seen in the high-dose group (25 mg/ml, 10 µl). Original magnification ×400

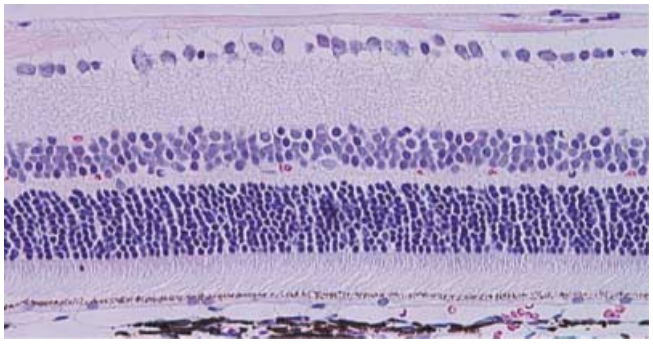

Fig. 3 Light micrograph of a rat eye with intravitreal ICG injection (0.25 mg/ml, 0.05 ml) at 10 days after injection. No remarkable changes in retinal structures were observed in the low-dose groups. Original magnification ×400

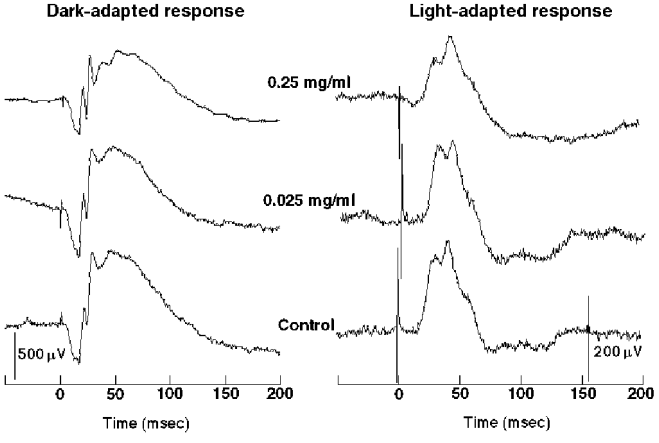

Fig. 4 *Left:* Electroretinogram traces showing the dose-dependent reduction of dark-adapted responses in comparison to that of the controls and intravitreal ICG injection group at 10 days after injection. *Right:* The light-adapted responses show no remarkable dose-dependent reduction

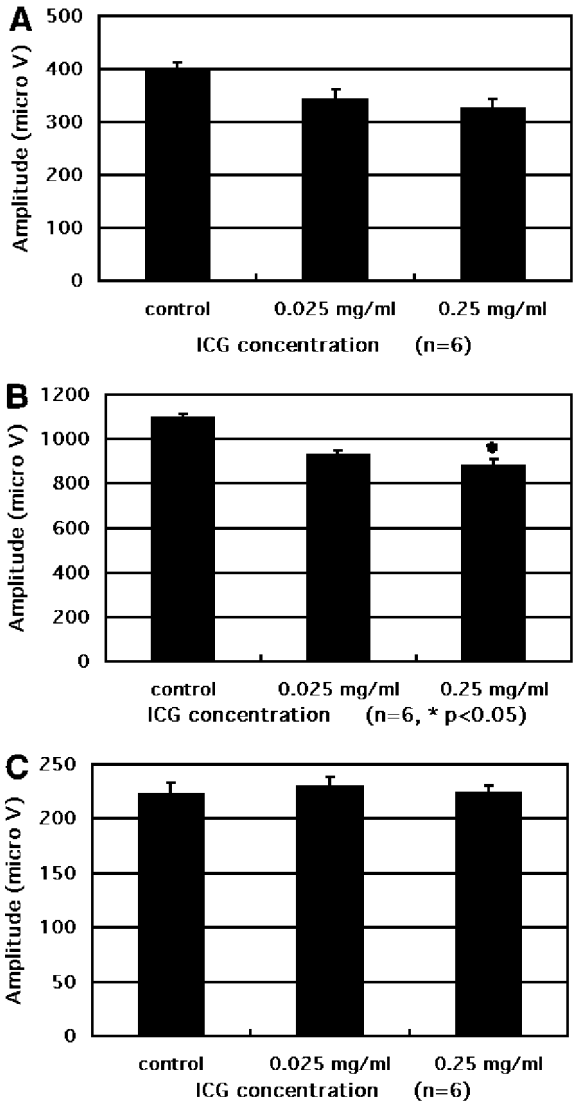

Fig. 5A–C The maximal amplitude of ERGs of the rat eye with intravitreal ICG injection at 10 days after injection. The maximal amplitude decreased in a dose dependent manner after ICG injection (**A** dark-adapted a-wave; **B** D dark-adapted b-wave; **P*<0.05). The amplitude of the light-adapted b-wave showed no remarkable reductions (**C**)

(Fig. 1). A significant number of mononuclear inflammatory cells was found in all the retinal layers. In the eyes with the second highest dose of ICG (2.5 mg/ml), the retinal structure was also destroyed, albeit much less than with the highest dose. The outer and inner segment were deformed. Many photoreceptor nuclei disappeared in some areas (Fig. 2). The normal structure of the retina was preserved in the eyes with low doses of ICG (0.25 and 0.025 mg/l) both in those enucleated on day 10 and in those enucleated at 2 months. In addition, no inflammatory cell infiltrate was observed (Fig. 3).

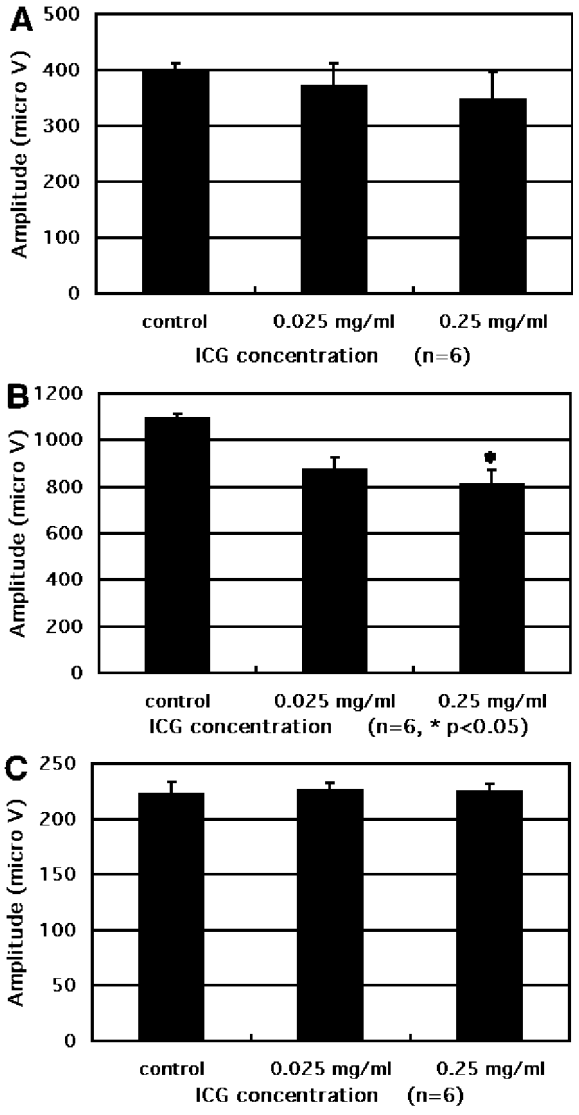

Fig. 6A–C The maximal amplitude of ERGs of the rat eye with intravitreal ICG injection at 2 months after injection. The amplitude decreased in a dose-dependent manner. (**A** dark-adapted a-wave; **B** dark-adapted b-wave; **P*<0.05). The amplitude of the light-adapted b-wave showed no remarkable reductions (**C**)

Electroretinography analysis

Since ERG showed damage in the eyes with high doses of ICG, we performed ERG analysis of the eyes with the low doses of ICG (0.25 and 0.025 mg/l) that had no apparent damage histologically. The eyes with cataract were excluded from these experiments.

Figure 4 represents the dark-and light-adapted ERG wave forms of the rats at 10 days. All waves were clearly recorded, and no differences were observed among the rats in the wave forms and implicit time. The amplitudes of dark-adapted responses obtained at the beginning of the experiments showed low variability across the rats.

Figures 5A and 6A demonstrate the amplitude of the dark-adapted a-wave (rod-mediated) at 10 days and 2 months. The amplitude of each wave decreased in a dose-dependent manner compared with the controls. Similar changes were obtained for dark-adapted b-waves (Figs. 5B, 6B). A maximum mean reduction of 19.7% was found in the 0.25 mg/ml injection group ($P<0.05$). At 2 months after injection, the reduction rate remained at 20.1% ($P<0.05$). Light-adapted a-wave (cone-mediated) ERGs demonstrated no remarkable reduction (Figs. 5C, 6C).

Discussion

ILM removal is an important development in the surgical approach to several vitreo-retinal diseases such as macular hole surgery [2, 12]. Recently Kadonosono et al. [8] and Burk et al. [3] described the usefulness of ICG for ILM staining, and this technique now enables surgeons to perform the ILM peeling procedures much more safely and easily. However, there is still concern about the potential toxicity of ICG on the retina from the beginning of this application [3, 8]. Nevertheless, there have been no reports evaluating the potential adverse effects of intravitreous ICG on retina.

In the present study, moderate to high doses (0.25 mg/ml and higher) of intravitreous ICG were found to cause significant morphological damage in the rat retina. In a previous study, more than 5 mg/ml ICG solution was toxic in cultured lens epithelial cells in vitro [7]. This toxic effect of ICG has also been reported in liver cells by inhibiting mitochondrial oxygen consumption [10]. As a result, the retinochoroidal damage of eyes with high doses of ICG was likely to be due to this direct toxic effect. The retina could be destroyed by ICG and thereafter be replaced by inflammatory cells and glial scar cells, as shown in a histological study. The present findings showed that the 0.25 mg/ml ICG solution is toxic to neural retina when it remains in the vitreous for 10 days.

It should be noted that retinal function was impaired even by the lowest dose of ICG (0.025 mg/ml), which did not cause any apparent morphological damage.

Since ICG is a dye which can block the light stimulant of ERG, the present decrease in ERG might be caused by this blocking effect of residual ICG in the vitreous. Such a mechanism is unlikely, however, due to the fact that 0.025 mg/ml of ICG was a clear solution.

Under these conditions, ERG analysis on day 10 showed that the amplitude of dark-adapted a- and b-waves decreased in a dose-dependent manner, although a statistically significant difference was found only in dark-adapted b-waves. In addition, these similar findings were also observed in the eyes at 2 months after injection, thus indicating these are irreversible changes and probably not non-specific blocking effects.

The primary site of the retina affected by the low dose of ICG was uncertain due to the fact that the ERG dark-adapted b-wave reflects the function not only of Müller cells but also of secondary neuron bipolar cells. In this way, the dark-adapted b-wave also reflected the changes of the a-wave-mediated primary neuron rod cells spread over a wide area of the retina, and the b-wave reflects the accumulative response of primary and secondary neurons. As a result the difference in b-waves reached statistical significance. Therefore, the results of ERG could not identify the site responsible for the present phenomenon. The morphological findings in the eyes treated with high doses of ICG indicate that ICG can affect every cell of the retina, and therefore all the cells might be nonspecifically affected by ICG.

The present study demonstrated that intravitreous ICG is toxic to the retina at high doses, and it decreased the ERG amplitude even at low doses (0.025 mg/ml).

The results of macular hole surgery have improved with ILM peeling achieved with the help of ICG staining and this technique is widely accepted by many surgeons [2, 3, 4, 8, 9]. We are not proposing that the use of ICG in vitreous surgery must be stopped; however, surgeons should be aware of the possible adverse effects of ICG on the patient's vision. Care should be taken when using intravitreous ICG.

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