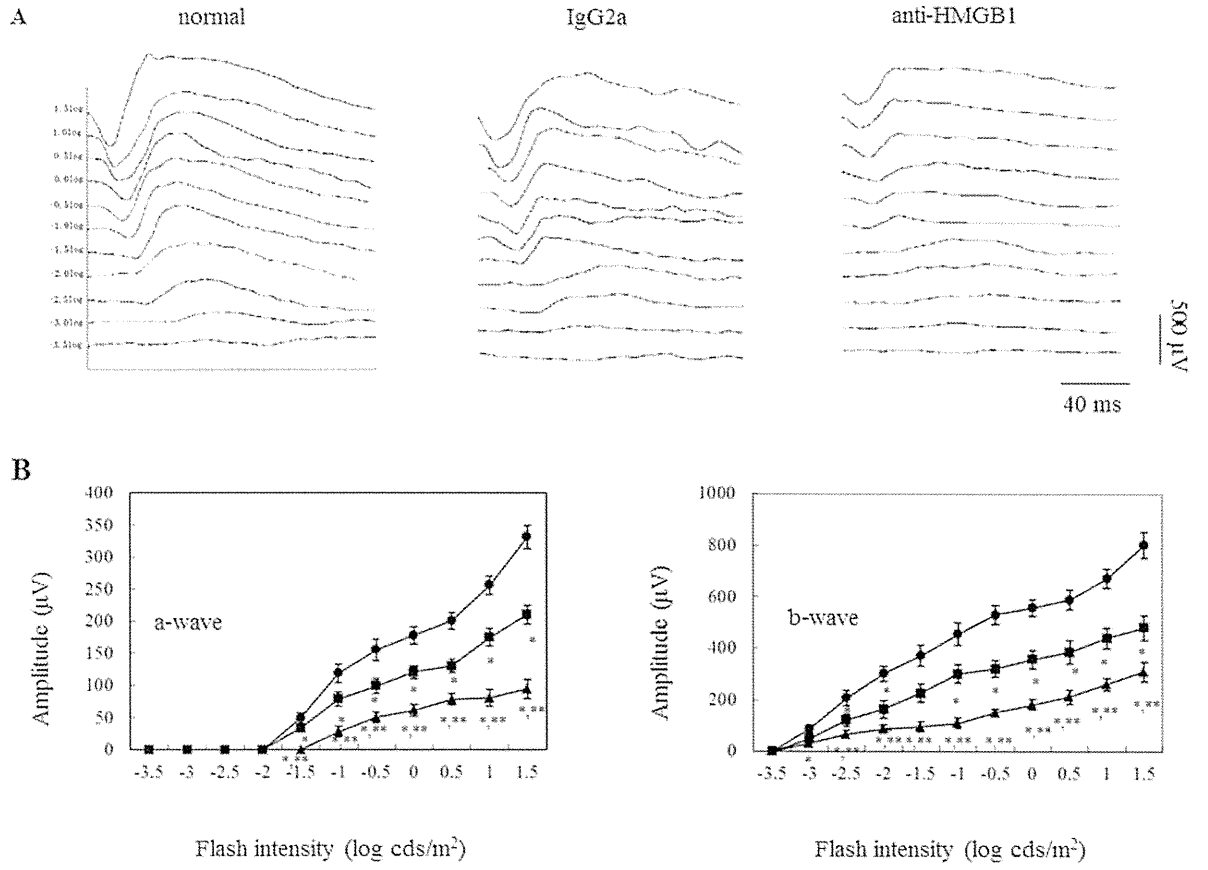

FIGURE 5 (A) Representative scotopic ERGs at baseline and at 7 days after ischemia when treated with control mAb or anti-HMGB1 mAb. (B) Amplitudes for a- and b-waves plotted as a function of flash intensity. Pretreatment with anti-HMGB1 mAb markedly reduced the amplitudes. Results are expressed as the mean ± SD. •: normal, ▪: IgG2a, ▲: anti-HMGB1. *$p<0.05$ versus normal retina. **$p<0.05$ versus ischemic retina with IgG2a.

ROS ACTIVATION BY ISCHEMIA

We used DHE staining to test whether ROS were enhanced by treatment with 200 μg anti-HMGB1 mAb. DHE specifically reacts with intracellular O_2^-, a ROS, and is converted to the red fluorescent compound ethidium in nuclei. In the post-ischemic retina, DHE fluorescence was clearly up-regulated in the retinal neuronal cells, and this up-regulation was efficiently enhanced by anti-HMGB1 mAb (Figure 10A–C). Figure 9D shows the quantified specific retinal DHE fluorescence. The mean ROS activation was significantly increased by treatment with anti-HMGB1 mAb ($n=4$, each group).

DISCUSSION

This study shows that, compared to the IgG2a treatment, pretreatment with anti-HMGB1 mAb significantly enhanced the ischemic injury of the retina. The results also showed that there was expression of HMGB1 mAb in the retina after ischemia-reperfusion injury.

A recent study showed that HMGB1 inhibited glial glutamate transport by GLAST in mouse gliosomes and suggested that HMGB1 can increase extracellular glutamate levels in ischemic brain.[21] We previously reported that anti-HMGB1 mAb suppressed ischemia-reperfusion-induced brain injury in a transient middle cerebral artery occlusion model in rats.[17] Based on these findings, we predicted that neutralizing mAb could be used to inhibit HMGB1 activity, and thus to significantly decrease the progression of the retinal ischemia-reperfusion injury. However, use of the neutralizing anti-HMGB1 mAb treatment in the present study remarkably increased the retinal damage following ischemia-reperfusion. This was due to an increased production of ROS caused by the anti-HMGB1 mAb treatment. Therefore, it might be possible that elevated levels of HMGB1 had neuroprotective effects against retinal ischemia- reperfusion injury. It has also been reported that treatment with anti-HMGB1 mAb increased ischemia-reperfusion

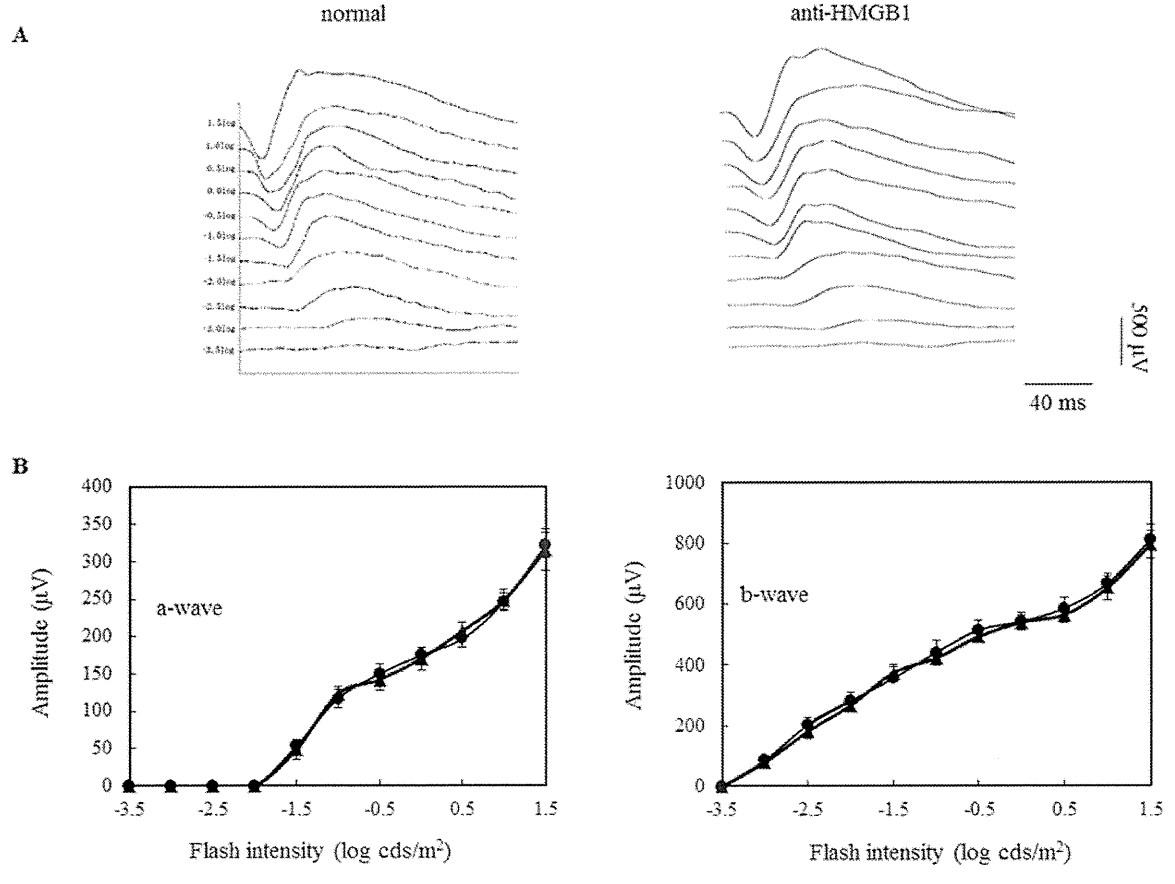

FIGURE 6 (A) Representative scotopic ERGs 7 days after treatment with anti-HMGB1. (B) Amplitudes for a- and b-waves plotted as a function of flash intensity. Results are expressed as the mean ± SD. •: normal, ▲: anti-HMGB1.

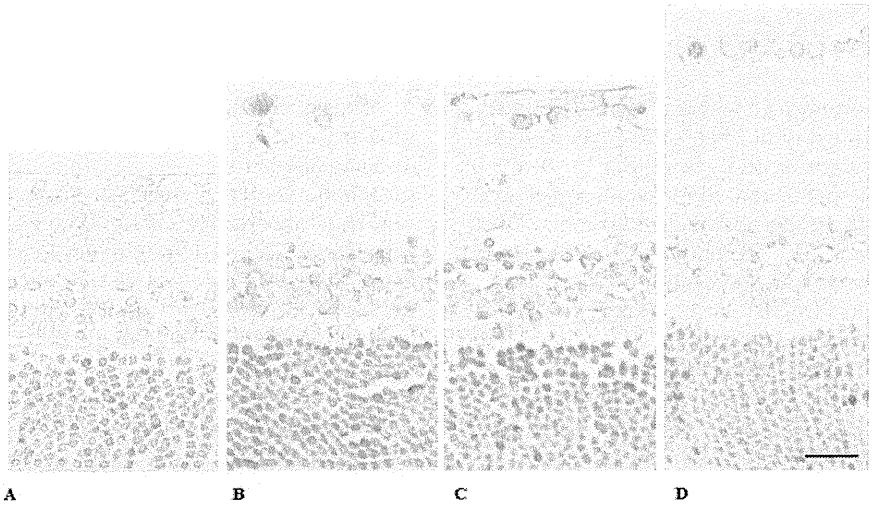

FIGURE 7 Immunohistochemical staining of HMGB1 expression in the retina. Retinal sections from normal animals (A) or 6 h (B), 12 h (C), or 24 h (D) after ischemia. Scale bar = 20 μm.

injury in the rat heart.[22] Therefore, it appears that the effect of anti-HMGB-1 mAb depends on the organ involved.

When the IOP is increased, glutamate is released from the retina during and after the ischemia.[3,23,24] The major causes of the cell death that occur after activation

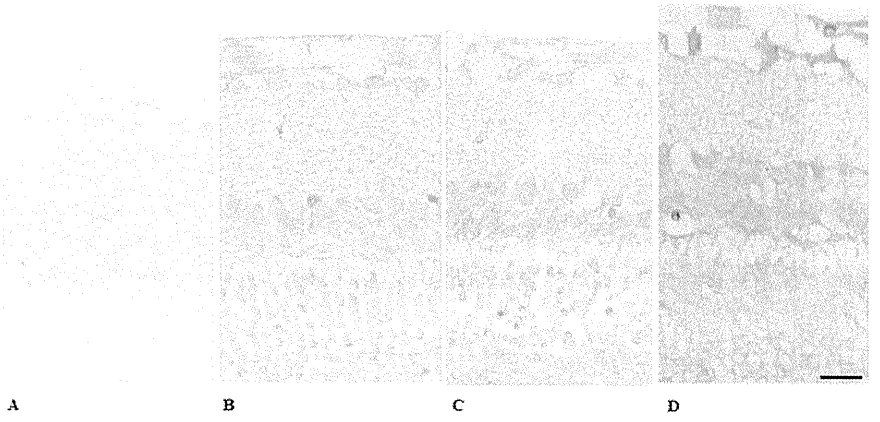

FIGURE 8 The effect of anti-HMGB1 mAb on endogenous HMGB1 expression. Intraperitoneal injection of anti-HMGB1 mAb was administered 30 min before ischemia. Retinal sections from normal animals (A) or 6 h (B), 12 h (C), or 24 h (D) after ischemia. Scale bar = 20 μm.

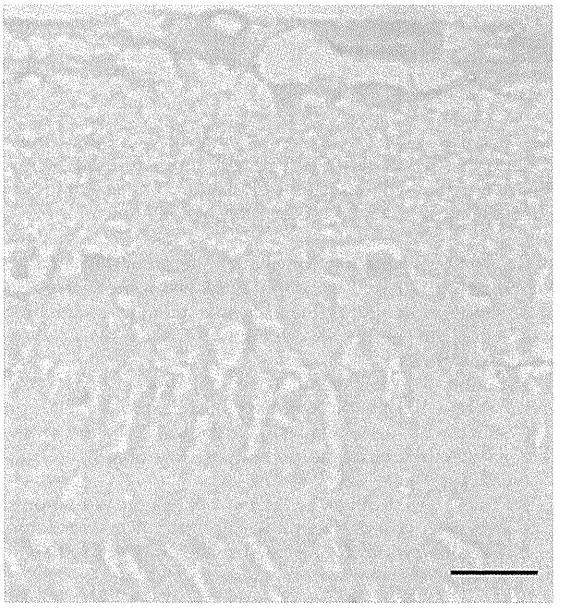

FIGURE 9 The direct effect of anti-HMGB1 mAb on endogenous HMGB1 expression in the normal retina. Scale bar = 20 μm.

of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors are related to the influx of calcium into the cells and the generation of free radicals.[25] Excessive accumulation of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) can have a wide range of detrimental effects, including inhibition of mitochondrial function, reduction of cellular ATP levels, enhancement of ROS production, and activation of cellular proteases and nitric oxide (NO) synthase.[26] Since production of ROS was increased by anti-HMGB1 mAb treatment in the current study, anti-HMGB1 mAb played a large deleterious role in the resultant ischemia-reperfusion injury. In the present study, there was an increase in the HMGB1 level in the retina after the ischemia-reperfusion injury. These results suggest that endogenous HMGB1 released from retinal cells may modulate ischemia-reperfusion injury in the retina. Therefore, the anti-HMGB1 mAb treatment increased the delayed neuronal death.

We evaluated the functional retinal damage after ischemia-reperfusion injury by measuring the ERG a- and b-wave amplitudes. The b-wave of the ERG has been identified as a particularly sensitive index of retinal ischemia both in humans[25] and in experimental models of retinal ischemia in vitro.[27] After the anti-HMGB1 mAb treatment, there was a decrease in the thickness of ONL following ischemia-reperfusion, with the a-wave of the ERG also lower than that noted in the eyes treated with IgG2a. There was a good correlation between the ERG for both a- and b-waves and the histological results. It has been reported that administration of pentobarbital enhance the a- and b-wave of the ERG.[28,29] Under the anesthesia, many factors indirectly affecting the retinal activity could not be completely excluded.

It has been reported in previous studies that HMGB1 is expressed in GCL, INL, ONL, the inner and outer segments of photoreceptors, and in the retinal pigment epithelial cells in normal retina.[30,31] However, the current immunohistochemical study showed that HMGB1 was present in the ONL in normal retinas, which may be due to the use of different antibodies in the various studies (monoclonal antibody vs. polyclonal antibody).

HMGB1 passively released from necrotic cells.[32] Cell death was frequently observed in both the GCL and the INL after 3 h of ischemia-reperfusion.[33] In the present study, we demonstrated that immunostaining for HMGB1 in the post-ischemic retina was detected in not only the ONL but also in the INL and GCL. HMGB1 may play a key role in the protection of retinal injury after ischemia-reperfusion.

The current study showed, for the first time, that treatment with anti-HMGB1 mAb increased ischemia-reperfusion injury in the rat retina. Further investigations are needed to clarify the mechanism of anti-HMGB1 mAb in retinal ischemia-reperfusion

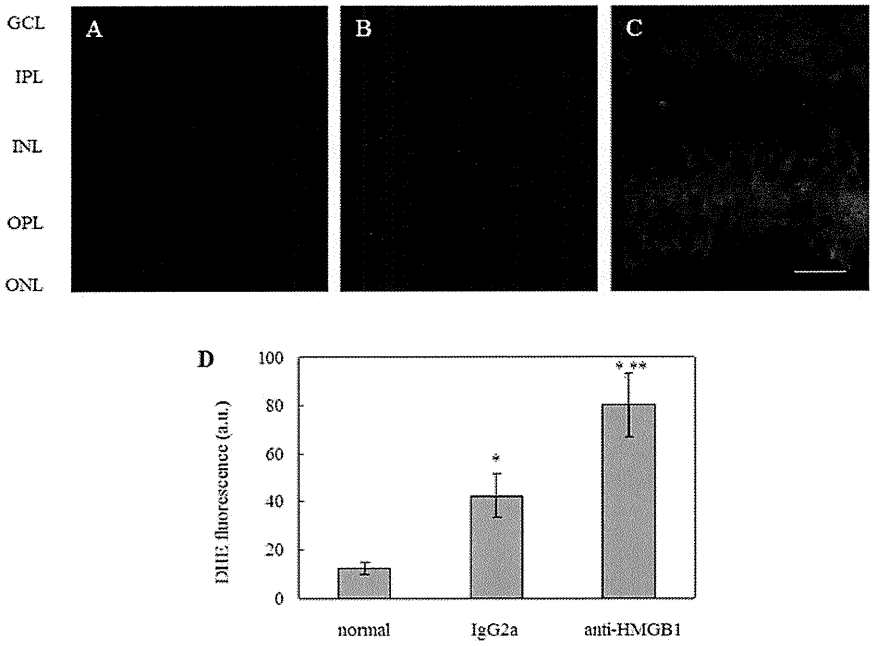

FIGURE 10 Effect of anti-HMGB1 mAb pretreatment on the release of ROS. The use of DHE to detect ROS indicated up-regulation of retinal neuronal cells in the retina after ischemia (IgG2a (B) as compared to normal retina (A)). Pretreatment with anti-HMGB1 mAb enhanced the level of ROS (C). (D) Quantified specific retinal DHE fluorescence is expressed for sections in arbitrary units (AU) for each group. Data express the mean ± SD; $*p < 0.05$ normal retina. $**p < 0.05$ versus ischemic retina with IgG2a. Scale bar = 20 μm.

injury. Additionally, anti-HMGB1 mAb function needs be further explored, as this could potentially lead to the development of neuroprotective therapeutic strategies for acute retinal ischemic disorders.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research from The Ministry of Education, Culture, Sports, Science, and Technology of Japan (20592078).

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES

[1] Tsujikawa A, Ogura Y, Hiroshiba N, Miyamoto K, Kiryu J, Honda Y. Tacrolimus (FK506) attenuates leukocyte accumulation after transient retinal ischemia. *Stroke*. 1998;29:1431–1437; discussion 1437–1438.

[2] Tsujikawa A, Ogura Y, Hiroshiba N, Miyamoto K, Kiryu J, Tojo SJ, Miyasaka M, Honda Y. Retinal ischemia-reperfusion injury attenuated by blocking of adhesion molecules of vascular endothelium. *Invest Ophthalmol Vis Sci*. 1999;40:1183–1190.

[3] Hirooka K, Miyamoto O, Jinming P, Du Y, Itano T, Baba T, Tokuda M, Shiraga F. Neuroprotective effects of D-allose against retinal ischemia-reperfusion injury. *Invest Ophthalmol Vis Sci*. 2006;47:1653–1657.

[4] Iwama D, Miyamoto K, Miyahara S, Tamura H, Tsujikawa A, Yamashiro K, Kiryu J, Yoshimura N. Neuroprotective effect of cilostazol against retinal ischemic damage via inhibition of leukocyte-endothelial cell interactions. *J Thromb Haemost*. 2007;5:818–825.

[5] Sakamoto K, Kawakami T, Shimada M, Yamaguchi A, Kuwagata M, Saito M, Nakahara T, Ishii K. Histological protection by cilnidipine, a dual L/N-type $Ca^{(2+)}$ channel blocker, against neurotoxicity induced by ischemia-reperfusion in rat retina. *Exp Eye Res*. 2009;88:974–982.

[6] Büchi ER. Cell death in the rat retina after a pressure-induced ischemia-reperfusion insult: An electron microscopic study. I: Ganglion cell layer and inner nuclear layer. *Exp Eye Res*. 1992;55:605–613.

[7] Nitatori T, Sato N, Waguri S, Karasawa Y, Araki H, Shibanai K, Kominami E, Uchiyama Y. Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. *J Neurosci*. 1995;15:1001–1011.

[8] Rosenbaum DM, Rosenbaum PS, Gupta A, Michaelson MD, Hall DH, Kessler JA. Retinal ischemia leads to apoptosis which is ameliorated by aurintricarboxylic acid. *Vision Res*. 1997;37:3445–3451.

[9] Halliwell B, Gutteridge JM. Free Radicals in Biology and Medicine. Oxford, UK: Clarendon Press; 1985.

[10] Goodwin GH, Sanders C, Johns EW. A new group of chromatin-associated proteins with a high content of acidic and basic amino acids. *Eur J Biochem*. 1973;38:14–19.

[11] Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): Nuclear weapon in the immune arsenal. *Nat Rev Immunol*. 2005;5:331–342.

[12] Calogero S, Grassi F, Aguzzi A, Voigtländer T, Ferrier P, Ferrari S, Bianchi ME. The lack of chromosomal protein Hmgb1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice. *Nat Genet*. 1999;22:276–280.

[13] Hoppe G, Rayborn ME, Sears JE. Diurnal rhythm of the chromatin protein Hmgb1 in rat photoreceptors is under circadian regulation. *J Comp Neurol*. 2007;501:219–230.
[14] Goldstein RS, Gallowitsch-Puerta M, Yang L, Rosas-Ballina M, Huston JM, Czura CJ, Lee DC, Ward MF, Bruchfeld AN. Elevated high-mobility group box 1 levels in patients with cerebral and myocardial ischemia. *Shock*. 2006;25:571–574.
[15] Kim JB, Sig Choi J, Yu YM, Nam K, Piao CS, Kim SW, Lee MH, Han PL, Park JS, Leem JK. HMGB1, a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation in the postischemic brain. *J Neurosci*. 2006;26:6413– 6421.
[16] Faraco G, Fossati S, Bianchi ME, Patrone M, Pedrazzi M, Sparator B. Moroni F, Chiarugi A. High mobility group box 1 protein is released by neural cells upon different stresses and worsens ischemic neurodegeneration *in vitro and in vivo J Neurochem*. 2007;103:590–603.
[17] Liu K, Mori S, Takahashi HK, Tomono Y, Wake H, Kanke T, Sato Y, Hiraga N, Adachi N, Yoshino T, Nishibori M. Anti-high mobility group box 1 monoclonal antibody ameliorates brain infarction induced by transient ischemia in rats. *FASEB J*. 2007;21:3904–3916.
[18] Kim JB, Lim CM, Yu YK, Lee JK. Induction and subcellular localization of high-mobility group box-1 (HMGB1) in the postischemic rat brain. *J Neurosci Res*. 2008;86:1125–1131.
[19] Muhammad S, Barakat W, Stoyanov S, Murikinati S, Yang H, Tracey KJ, Bendszus M, Rossetti G., Nawroth PP, Bierhaus A, Schwaninger M. The HMGB1 receptor RAGE mediates ischemic brain damage. *J Neurosci*. 2008;28:12023–12031.
[20] Qiu J, Nishimura M, Wang Y, Sims JR, Qiu S, Savitz SI, Salamone S, Moskowitz MA. Early release of HMGB-1 from neurons after the onset of brain ischemia. *J Cereb Blood Flow Metab*. 2008;28:927–938.
[21] Pedrazzi M, Raiteri L, Bonanno G, et al. Stimulation of excitatory amino acid release from adult mouse brain glia subcellular particles by high mobility group box 1 protein. *J Neurochem* 2006;99:827–838.
[22] OozawaS, Mori S, Kanke T, Takahashi H, Liu K, Tomono Y, Asanima M, Miyazaki I, Nishibori M, Sano S. Effect of HMGB1 on ischemia-reperfusion injury in the rat heart. *Circ J*. 2008;72:1178–1184.
[23] Louzada-Junior P, Dias JJ, Santos WF, Lachat JJ, Bradford HF, Coutinho-Netto J. Glutamate release in experimental ischaemia of the retina: An approach using microdialysis. *J Neurochem*. 1992;59:358–363.
[24] Adachi K, Kashii S, Masai H, Ueda M, Morizane C, Kaneda K, Kume T, Akaike A, Honda Y. Mechanism of the pathogenesis of glutamate neurotoxicity in retinal ischemia. *Graefes Arch Clin Exp Ophthalmol*. 1998;236:766–774.
[25] Osborne NN, Ugarte M, Chao M, Chidlow G., Bae JH, Wood JP, Nash MS. Neuroprotection in relation to retinal ischemia and relevance of glaucoma. *Surv Ophthalmol*. 1999;43:S102–S128.
[26] Coleman K, Fitzgerald D, Eustace P, Bouchier-Hayes D. Electroretinography, retinal ischemia and carotid artery disease. *Eur J Vasc Surg*. 1990;4:569–573.
[27] Zager E, Ames A 3rd. Reduction of cellular energy requirements. Screening for agents that may protect against CNS ischemia. *J Neurosurg*. 1998;69:568–579
[28] Jacobson JH, Gestring GF. Centrifugal influence upon the electroretinogram. *AMA Arch Ophthalmol*. 1958;60:295–302.
[29] Noell WK. Differentiation, metabolic organization, and viability of the visual cell. *AMA Arch Ophthalmol*. 1958;60:702–733.
[30] Arimura N, KI-I Y, Hashiguchi T, Kawahara K, Biswas KK, Nakamura M, Sonoda Y, Yamakiri K, Okubo A, Sakamoto T, Maruyama I. Intraocular expression and release of high-mobility group box 1 protein in retinal detachment. *Lab Invest*. 2009;89: 278–289.
[31] Watanabe T, Keino H, Sato Y, Kudo A, Kawakami H, Okada AA. High mobility group box protein-1 in experimental autoimmune uveoretinitis. *Invest Ophthalmol Vis Sci*. 2009;50:2283–2290.
[32] Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature*. 2002;418:191–195.
[33] Büchi ER. Cell death in the rat retina after a pressure-induced ischemia-reperfusion insult: an electron microscopic study. I. ganglion cell layer and inner nuclear layer. *Exp Eye Res*. 1992;55:605–613.

Jpn J Ophthalmol (2011) 55:294–300
DOI 10.1007/s10384-011-0012-8

LABORATORY INVESTIGATION

D-Allose as ischemic retina injury inhibitor during rabbit vitrectomy

Masanori Mizote · Kazuyuki Hirooka · Kouki Fukuda · Takehiro Nakamura · Toshifumi Itano · Fumio Shiraga

Received: 19 April 2010 / Accepted: 16 November 2010 / Published online: 13 May 2011

Abstract
Purpose To investigate the protective effects of D-allose, a rare sugar, on pressure-induced ischemia during vitrectomy in the rabbit eye.
Methods The rabbits underwent pars plana vitrectomy, and continuous intraocular irrigation at a perfusion pressure of 140 mmHg was performed for 45 min. Intraocular pressure was regulated by adjusting the height of a bottle of balanced saline solution containing D-allose. Morphometric studies were performed to study the effects of D-allose on the histological changes induced by ischemia in the rabbit retina. Electroretinograms (ERGs) were taken before and 1 and 7 days after vitrectomy. Nitroblue tetrazolium was used as an index of superoxide anion ($O_2^{\cdot-}$) generation. Data were analyzed by use of the unpaired Student's *t* test.
Results Seven days after ischemia, significant reductions in both number of ganglion cells and the thickness of the inner plexiform layer were observed. D-Allose significantly inhibited ischemic injury of the inner retina ($P < 0.05$). On postoperative day 7, amplitudes of ERG b-waves were significantly lower in the control group than in the D-allose group ($P < 0.05$). D-Allose suppressed the production of $O_2^{\cdot-}$.
Conclusions Intraocular irrigation with D-allose during vitrectomy may protect the retina against ischemia-induced damage.

Keywords D-Allose · Neuroprotection · Retinal ischemia · Superoxide anion · Vitrectomy

Introduction

Rare sugars are monosaccharides that exist in nature in limited quantities only. Whereas naturally abundant monosaccharides such as D-glucose and D-fructose are few in number, more than 50 kinds of rare sugars have been identified. D-Allose is a rare aldo-hexose sugar produced from D-psicose by enzymes from microorganisms [1]. Murata et al. [2] examined the scavenging activity of D-allose using electron spin resonance. They showed that D-allose inhibits the production of reactive oxygen species (ROS) in a dose-dependent manner. Furthermore, we recently reported that D-allose might protect neurons against retinal ischemia–reperfusion injury by reducing extracellular glutamate and attenuating oxidative stress [3].

Improvements in the techniques of pars plana vitrectomy (PPV) have resulted in better prognosis for vision-threatening eye diseases such as macular hole, epiretinal membrane, retinal detachment, and proliferative diabetic retinopathy (PDR). However, optic nerve atrophy after successful PPV for diabetic retinopathy is one of the most serious complications and can lead to blindness [4]. Because the extent of autoregulation in the retina and optic nerve head is limited, high infusion pressures during

M. Mizote (✉)
Department of Ophthalmology, Kagawa Prefectural Central Hospital, 5-4-16 Ban, Takamatu, Kagawa 760-8557, Japan
e-mail: m-mizote@chp-kagawa.jp

K. Hirooka · K. Fukuda · F. Shiraga
Department of Ophthalmology, Kagawa University Faculty of Medicine, 1750-1 Ikenobe, Miki, Kagawa 761-0793, Japan

T. Nakamura · T. Itano
Department of Neurobiology, Kagawa University Faculty of Medicine, 1750-1 Ikenobe, Miki, Kagawa 761-0793, Japan

vitreous surgery may cause retinal ischemia and lead to damage of the retina and optic nerve.

During or after ischemia, ROS such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical (OH^-) can be produced in large quantities and act as cytotoxic metabolites. ROS have been implicated in the process of apoptosis, because treatment of cells with ROS can result in this form of cell death, whereas application of antioxidants can prevent it under some conditions [5, 6]. ROS scavengers have been shown to be neuroprotective against ischemia in acute experimental models [7].

The purpose of this study was to investigate the protective effects of D-allose on pressure-induced ischemia during vitrectomy in the rabbit eye.

Materials and methods

Animals

Female New Zealand White rabbits, weighing 2.0–2.4 kg, were obtained from Kitayama Labs KK (Nagano, Japan). The rabbits were anesthetized by intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). Animal care and experiments followed the standard guidelines for animal experimentation of Kagawa University Faculty of Medicine and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were divided into two groups according to the intraocular irrigating solutions they received: the vehicle group, using a balanced saline solution (BSS) alone, and the D-allose group, using BSS + D-allose (2%).

Vitrectomy and subsequent vitreous perfusion

A 250-ml bottle of intraocular irrigating solution (BSS; Alcon Laboratories, Fort Worth, TX, USA) was suspended approximately 100 cm above the eye level of each rabbit, and connected to a 20-gauge infusion cannula through a 200-cm-long tube. The infusion cannula was inserted through the sclerotomy in the inferonasal quadrant 3 mm posterior to the limbus, and sutured in place. A vitreous cutter (MVS XX; Alcon) was placed through the sclerotomy in the supratemporal quadrant, and vitrectomy was then performed for 10 min. The vitreous cavity was irrigated during vitrectomy either with BSS alone or with BSS + D-allose. Adequate illumination was provided by a paraxial light operation microscope without intraocular fiberoptic illumination. If retinal tear or retinal detachment occurred during vitrectomy, those animals were excluded from the experiment. After completion of the vitrectomy, the cutter was removed and the sclerotomy wound was tightly sutured using a 7-0 vicryl. Intraocular pressure (IOP) was raised to 140 mmHg for 45 m by elevating the solution bottle. Retinal ischemia was confirmed by whitening of the fundus. Rectal temperature was monitored throughout the surgery. Because body temperature during vitrectomy in acute ischemic eyes may affect ischemia-induced retinal damage [8, 9], rectal and tympanic temperature was maintained at approximately 37°C by use of a feedback-controlled heating pad (BRC, Nagoya, Japan) during the operation. After restoration of blood flow, the temperature was continuously maintained at 37°C. Eyes were treated topically with levofloxacin (Santen Pharmaceutical, Osaka, Japan) after surgery to prevent postoperative infection.

Electroretinograms (ERGs)

Before surgery, ERG responses were measured after 20 min of dark adaptation using a recording device (Mayo, Aichi, Japan). The procedure was repeated on postoperative days 1 and 7. ERGs were recorded by positioning the rabbit in a box and placing a xenon lamp 15 cm in front of the eye. A flash of maximum intensity 20 J provided photostimulation. A contact lens electrode was placed on the cornea, and a reference electrode subcutaneously at the vertex. ERGs were taken of both eyes for each animal. The a- and b-wave amplitudes of each operated eye were shown as a percentage of those in the pre-operated eye.

Histological examination

For histological examination, the rabbits were anesthetized by intraperitoneal injection of ketamine and xylazine 1 week after ischemia and perfused intracardially with phosphate-buffered saline (PBS), followed by perfusion with 4% paraformaldehyde in PBS. Eyes were removed and embedded in paraffin, and thin sections (5-μm thickness) were cut using a microtome. Each eye was mounted on a silane-coated glass slide and then stained with hematoxylin and eosin (HE).

Morphometric analysis was performed to quantify ischemic injury. The sections for analysis were selected randomly for each eye. A microscopic image of each section within 0.5–1 mm of the optic disc was scanned. In each computer image, the thickness of the inner plexiform layer (IPL) and inner nuclear layer (INL) at the tire frame were measured. Finally, in each eye, the thicknesses of the IPL and INL were obtained as the mean values of five measurements. For each animal, these values for the right eye were normalized to those for the intact left eye and shown as a percentage.

Retrograde labeling of retinal ganglion cells (RGCs)

Seven days before the rabbits were killed, the optic nerves were exposed by lateral orbitotomy. Using a Hamilton syringe, 10 μl of a 0.1% fluorescent dye (Fast Blue; Polysciences, Warrington, PA, USA) was injected into the optic nerve 2 mm behind the eyeball. Care was taken not to injure any blood vessels, especially the ophthalmic artery that enters the sclera from the ventral margin of the optic nerve. After the surgery the eyes were treated topically with levofloxacin.

Assessment of RGC survival

Animals were killed by an overdose of ketamine and xylazine 1 week after the fast blue application. Whole, flat-mounted retinas were then assayed for the retinal ganglion cell density. The rabbit eyes were enucleated and fixed in 4% paraformaldehyde for 10 h at room temperature. After removal of the anterior segments, the resulting posterior eyecups were left in place. Subsequently, 4 radial cuts were made in the periphery of each eyecup, with the retina then carefully separated from the retinal pigment epithelium. To prepare the flat mounts, the retina was dissociated from the underlying structures, flattened by making 4 radial cuts, and then spread on a gelatin-coated glass slide. Labeled RGCs were visualized under a fluorescence microscope (Olympus BX-51/DP70; Olympus, Tokyo, Japan) by using a filter set (excitation filter 330–385 nm; barrier filter 420 nm; WU; Olympus). Fluorescence-labeled RGCs were counted in 12 microscopic fields of retinal tissue from 2 regions in each quadrant at 2 different eccentricities, central and peripheral. We counted the RGCs in each eye by using Image-Pro Plus software (Version 4.0; Media Cybernetics, The Imaging Expert, Bethesda, MD, USA). Cell counts were conducted by the same person in a masked fashion, with the identity of the original retinas unknown to the investigator until all cell counts from all the different groups were completed. Changes in the densities of the RGCs were expressed as the RGC survival percentage, which was based on comparison of the surgical and contralateral control eyes. The specimens that were compared came from different retinal regions of the same animal.

O_2^- analysis

O_2^- generation was determined by measuring the reduction of nitroblue tetrazolium (NBT) to a diformazan precipitate as previously described [10]. NBT (50 mg/ml; Research Organics, Cleveland, OH, USA) was dissolved in dimethylformamide (DMF) and distilled BSS. The final DMF concentration was 10%. Reduction was detected by intravitreal injection of 5-μl NBT solution in the period after vitrectomy before ischemia. For observation of the diformazan precipitate, a microscope (S5; Carl Zeiss, München, Germany) was used with a 3CCD digital camera (MKC-507; Ikegami Tsushinki, Tokyo, Japan) and recorded using a DVD recorder.

Statistical analysis

Image analysis was performed with Image-Pro Plus software (The Imaging Expert) to assess O_2^- generation area density. We determined the total blue-stained area indicated O_2^- generation area. Evaluation by Image-Pro Plus analysis of photographs was performed blind by two researchers not directly involved in this study and the generation area was automatically produced by the software. All statistical values are presented as mean ± standard deviation (SD). Data were analyzed by use of the unpaired Student's t test. Values of $p < 0.05$ were considered statistically significant.

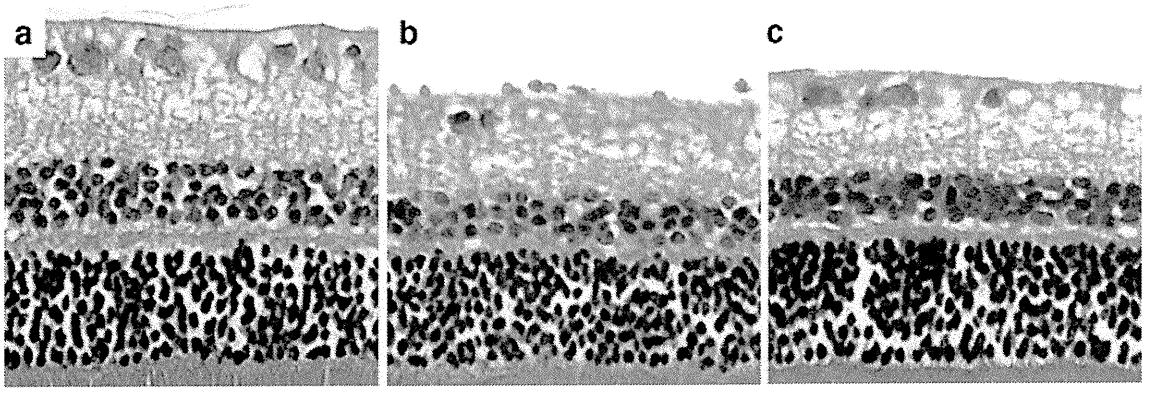

Fig. 1 Light micrographs of a cross-section of **a** hematoxylin and eosin histology through normal rabbit retina and **b** 7 days after ischemia with balanced salt solution (BSS) alone, or **c** BSS + D-allose. Cell loss in the ganglion cell layer (GCL) and reduced inner nuclear layer (INL) thickness were ameliorated in the D-allose group. *Bar* 10 μm

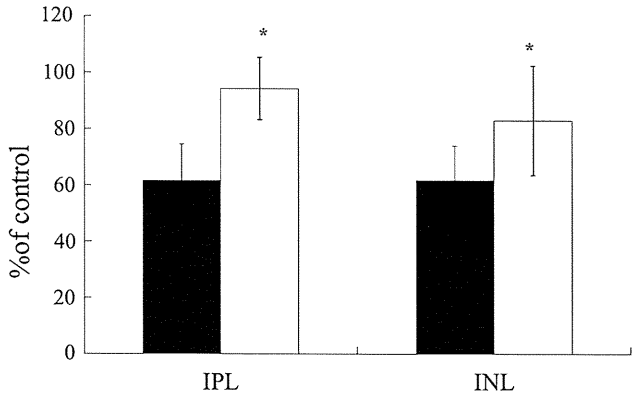

Fig. 2 Percentage changes relative to control values in the thicknesses of the IPL and INL 7 days after ischemia with BSS alone and BSS + D-allose. *Black bar* D-allose group, *white bar* vehicle group. Administration of 2% D-allose significantly prevented the reduction in the number of cells in the GCL and the thickness of the INL. Results are expressed as mean ± standard deviation (SD). $^*P < 0.05$

Results

Histological change in the retina after ischemia with and without D-allose

Figure 1a shows a normal retina. Light-microscopic photographs were taken 7 days after ischemia (Fig. 1b, c). The retina of the untreated eye in the animals, was used as control. In the vehicle group, significant reductions in the thickness of the INL were observed. The thickness of the IPL was 61.6 ± 13.0% that of the control and the thickness of the INL was reduced to 61.8 ± 12.2% that of the control ($n = 6$; Fig. 2). In the D-allose group, the thickness of the IPL was 94.2 ± 11.1% that of the control and the thickness of the INL was 82.9 ± 19.4% that of the control ($n = 5$; Fig. 2). Reduced INL thickness was ameliorated in the D-allose group.

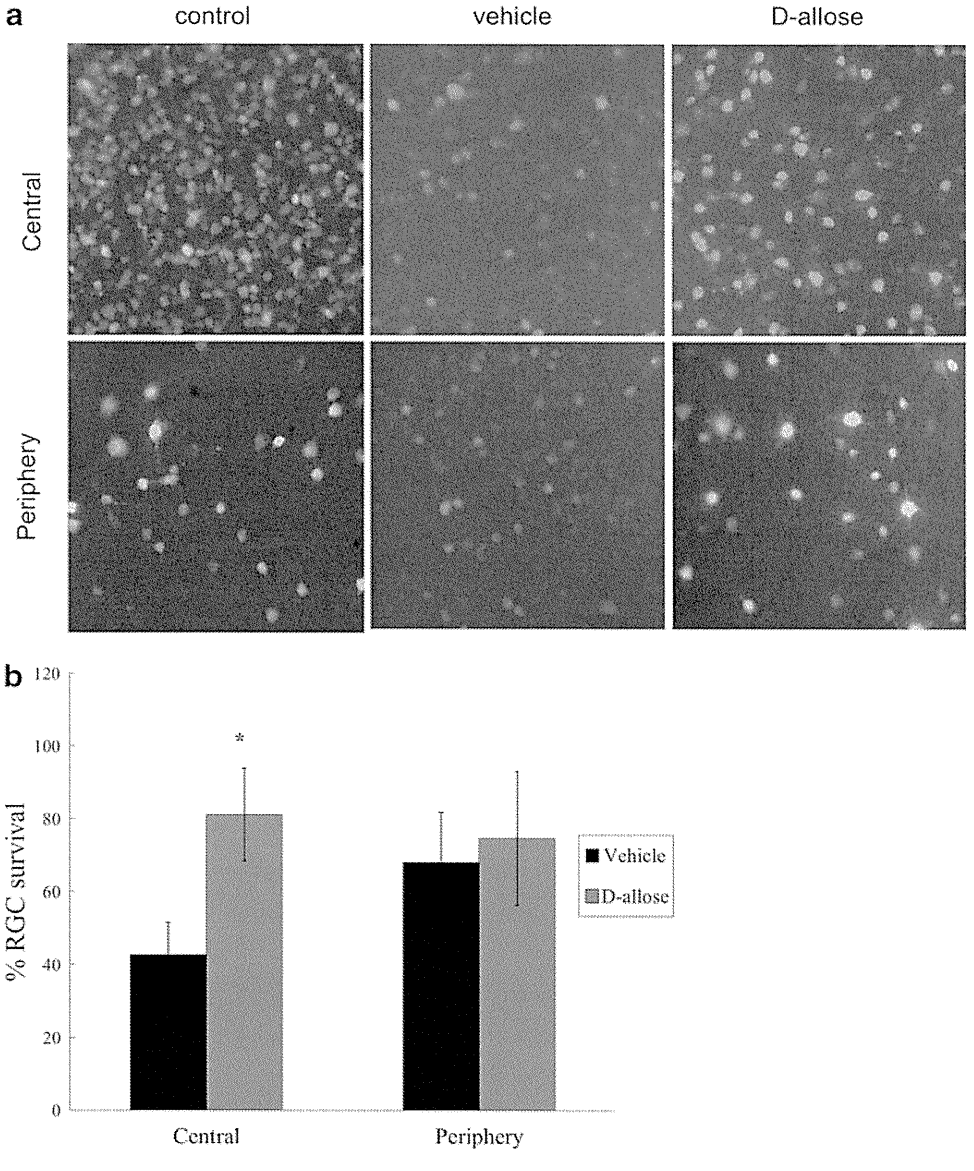

Fig. 3 Effects of D-allose on ischemia-induced retinal ganglion cell (RGC) death. **a** Retrograde labeling of RGCs in nonischemic eyes, and **b** 7 days after ischemic injury treated with vehicle or D-allose. *Bar* 100 μm. RGCs were counted in the central and peripheral areas. *Gray bar* D-allose group, *black bar* vehicle group. Graph depicts the mean ± SD for three rabbits treated with vehicle and three rabbits treated with D-allose. $^*P < 0.05$

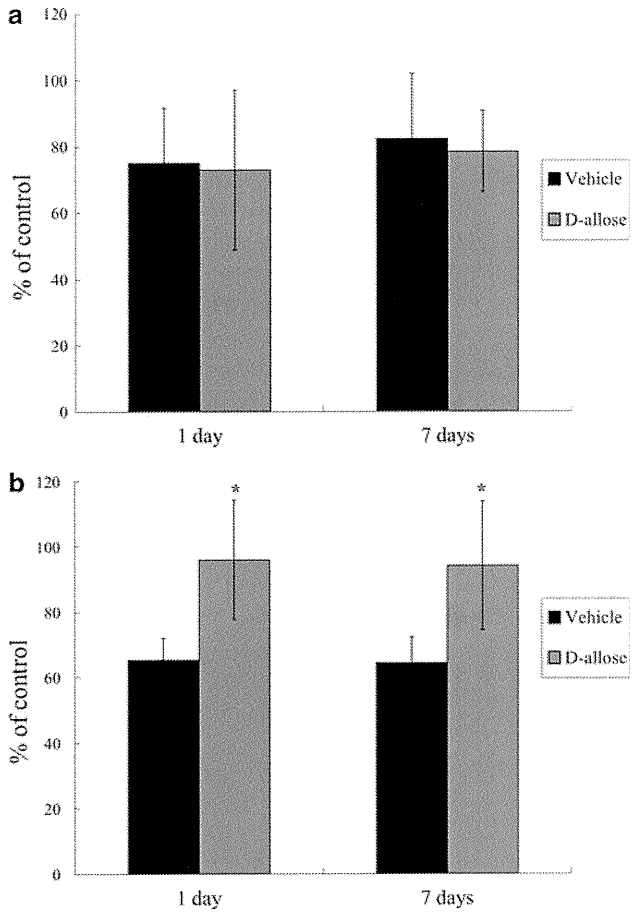

Fig. 4 **a** On postoperative day 7, the a-wave amplitude was 78.4 ± 12.3% in the D-allose group and 82.3 ± 19.6% in the vehicle group. *Gray bar* D-allose group, *black bar* vehicle group. **b** The b-wave amplitudes were 94.1 ± 19.8 and 64.3 ± 7.9%, respectively. On postoperative days 1 and 7, the mean amplitude of the b-wave for eyes treated with D-allose was significantly higher than for those treated with vehicle. *Gray bar* D-allose group, *black bar* vehicle group. Results are expressed as mean ± SD. $^*P < 0.05$

Survival of RGCs

Figure 3a shows representative results of RGC labeling for both vehicle and D-allose-treated rabbits. RGC survival in the central retinas of the eyes with ischemia was 42.8 ± 8.8% in the vehicle-treated group ($n = 4$) and 81.2 ± 12.7% in the D-allose-treated group ($n = 4$, $P = 0.01$; Fig. 3b). In the peripheral retina, RGC survival in eyes with ischemia was 68.0 ± 13.8% in the vehicle-treated group and 74.7 ± 18.3% in the D-allose-treated group ($P = 0.64$; Fig. 3b).

ERGs

The mean amplitudes of both the a-wave and b-wave are shown in Fig. 4. On postoperative day 7, the a-wave amplitude was 78.4 ± 12.3% in the D-allose group ($n = 5$) and 82.3 ± 19.6% in the vehicle group ($n = 5$; Fig. 4a). The b-wave amplitudes were 94.1 ± 19.8 and 64.3 ± 7.9%, respectively (Fig. 4b). The mean amplitude of the b-wave in eyes treated with D-allose was significantly higher than in those treated with vehicle. Likewise, no significant differences in the mean amplitude of a-waves were identified between the D-allose and vehicle groups. Both a-wave and b-wave amplitudes in the non-operated eyes were stable and essentially equal both before and after surgery.

Effects of D-allose on released O_2^-

Light-microscopic photographs were taken after treatment without D-allose, i.e., BSS alone (Fig. 5a), and with D-allose (Fig. 5b). Without D-allose treatment, the blue color was observed after ischemia and became stronger over time. However, in the presence of D-allose, the blue color

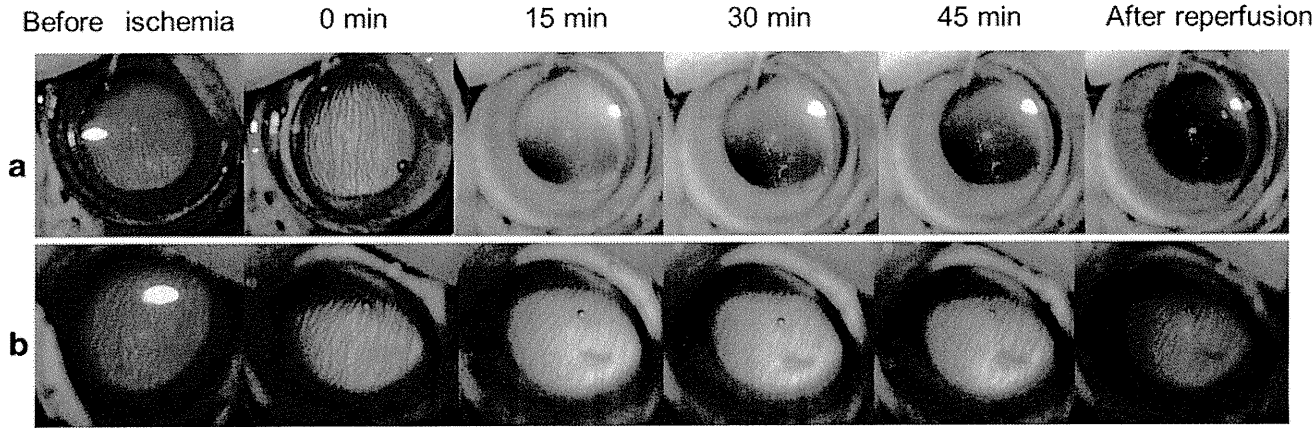

Fig. 5 Effects of D-allose on the release of superoxide anion (O_2^-). *Blue color* indicates release of O_2^-. Ischemia was induced for 45 min. Color photographs were taken before ischemia induction, then 0, 15, 30 and 45 min after starting ischemia, then immediately after reperfusion. **a** Vehicle group and **b** D-allose group

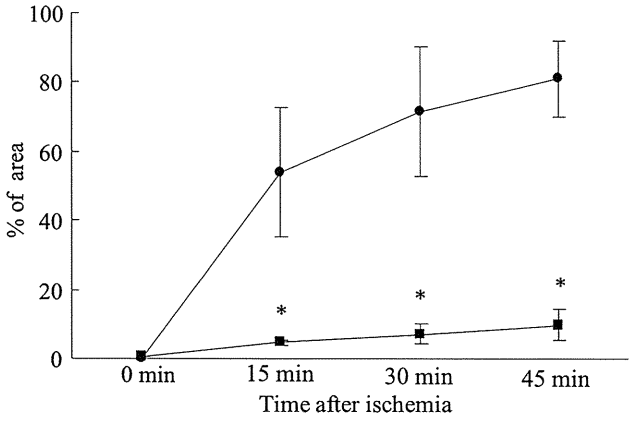

Fig. 6 Measured area O_2^- expression (%). *Filled squares* D-Allose group, *filled circles* vehicle group. Comparison of mean values of O_2^- expression between the vehicle group and the D-allose group at each time point. Mean O_2^- expression was significantly different 15, 30, and 45 min after ischemia. Data are mean ± SD. $^*P < 0.05$

decreased compared with that without D-allose. Compared with the peripheral area, the blue color intensity was stronger in the central area. Figure 6 shows results from quantification of the color levels expressed as a percentage change. Mean O_2^- expression was significantly different between the vehicle group and the D-allose group 15, 30, and 45 min after ischemia.

Discussion

These findings show that intraocular irrigation with D-allose during vitrectomy protects the morphology and function of the retina against ischemia injury.

Because D-allose may inhibit hexose transport [11], co-injection of 200 mg/kg glucose with 200 mg/kg D-allose has been shown to have no protective effect against retinal ischemia reperfusion injury [3]. BSS contains 5.11 mM glucose; i.e., approximately 0.1% glucose. Because 2% D-allose in BSS was used in this study, the glucose in BSS was not sufficient to abolish the protective effects of D-allose.

Both clinically and under experimental conditions, the functional status of the retina can be monitored continuously by recording ERGs. The b-wave of the ERG has been identified as a particularly sensitive index of retinal ischemia both in humans [12] and in experimental models of retinal ischemia in vitro [13]. Glutamate acts as a mediator of neuronal injury under ischemic conditions [14] and extracellular glutamate has been found to increase in ischemic eyes [3, 15, 16]. Reperfusion injury is thought to be mediated in part by relative hyperglycemia and high oxygen levels, leading to oxygen radical formation. D-Allose may be a down-regulation agent of hexose transport [11]. D-Allose could reduce the production of ROS by modulating the glycolytic response. Because D-allose can suppress glutamate release and the production of ROS after ischemia [3], D-allose protects both the morphology and function of the retina. Because there were no morphological changes in the outer retina in this study (data not shown), recovery of the a-wave amplitudes in the vehicle group was not suppressed. Therefore, we could not confirm any differences in the recovery of a-wave amplitudes between the vehicle and D-allose groups.

We recently reported that D-allose suppresses the production of H_2O_2 as determined by diaminobenzidine solution without hydrogen peroxide [3]. NBT is an electron acceptor that can be reduced by accepting electrons from various reductants, including superoxide [17] and other reductants, for example those generated from dehydrogenase systems [18, 19]. Studies have previously shown that NBT staining in normal rat retina is affected by inhibition of free radical-related enzyme systems, suggesting that NBT might be useful in the study of free radicals. During and after ischemic episodes, univalent reduction of oxygen in the mitochondrial respiratory chain is thought to be a major source of O_2^- [20]. O_2^- can be reduced to H_2O_2, a reaction catalyzed by superoxide dismutase (SOD) [7]. H_2O_2 has been identified as a potent inducer of apoptosis [21]. D-Allose may exert neuroprotective effects by reducing the production of not only H_2O_2, but also O_2^-.

Ocular perfusion pressure would be especially important in eyes with diabetes if, as has been suggested, autoregulation in the retina and optic nerve head is impaired [22, 23]. Because high infusion pressure during PPV is useful in preventing bleeding, we must also consider protecting the retina against damage caused by pressure-induced ischemia in cases of diabetic retinopathy [24]. Levels of glutamate potentially toxic to retinal ganglion cells have been found in the vitreous of patients with PDR [25]. This glutamate could then initiate a forward cascade of further neuronal ischemia.

Our findings suggest that intraocular irrigation with D-allose during vitrectomy may protect both the morphology and function of the retina against ischemia-induced damage.

Acknowledgments This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (20592078).

References

1. Bhuiyan SH, Itami Y, Yuhko R, Katayama T, Izumori K. D-Allose production from D-psicose using immobilized L-rhamnose isomerase. J Ferment Bioeng. 1998;85:540–2.
2. Murata A, Sekiya K, Watanabe Y, Yamaguchi F, Hatano N, Izumori K, et al. A novel inhibitory effect of D-allose on production of reactive oxygen species from neutrophils. J Biosci Bioeng. 2003;96:89–91.
3. Hirooka K, Miyamoto O, Jinming P, Du Y, Itano T, Baba T, et al. Neuroprotective effect of D-allose against retinal ischemia–reperfusion injury. Invest Ophthalmol Vis Sci. 2006;47:1653–7.

4. Seki M, Togashi H, Ando N. Optic nerve atrophy after vitrectomy for diabetic retinopathy: its systemic and local risk factors. Nippon Ganka Gakkai Zasshi. 2006;110:462–7.
5. Jacobson MD. Reactive oxygen species and programmed cell death. Trends Biochem Sci. 1996;21:83–6.
6. Kroemer G, Petit P, Zamzami N, Vayssière JL, Mignotte B. The biochemistry of programmed cell death. FASEB J. 1995;9:1277–87.
7. Bonne C, Muller A, Villain M. Free radicals in retinal ischemia. Gen Pharmacol. 1998;30:275–80.
8. Tamai K, Toumoto E, Majima A. Local hypothermia protects the retina from ischemic injury in vitrectomy. Br J Ophthalmol. 1997;81:789–94.
9. Traustason S, Eysteinsson T, Agnarsson BA, Stefánsson E. GABA agonists fail to protect the retina from ischemia–reperfusion injury. Exp Eye Res. 2009;88:361–6.
10. Digregorio KA, Cilento EV, Lantz RC. Measurement of superoxide release from single pulmonary alveolar macrophages. Am J Physiol. 1987;252:C677–83.
11. Ullrey DB, Kalckar HM. Search for cellular phosphorylation products of D-allose. Proc Natl Acad Sci USA. 1991;88:1504–5.
12. Coleman K, Fitzgerald D, Eustace, Bouchier-Hayes D, et al. Electroretinography, retinal ischemia and carotid artery disease. Eur J Vasc Surg. 1990;4:569–73.
13. Zager E, Ames A. Reduction of cellular energy requirements: screening for agents that may protect against CNS ischemia. J Neurosurg. 1988;69:568–79.
14. Choi DW. Glutamate neurotoxicity and diseases of the nervous system. Neuron. 1988;1:623–34.
15. Louzada-Junior P, Dias JJ, Santos WF, Lachat JJ, Bradford HF, Coutinho-Netto J. Glutamate release in experimental ischaemia of the retina: an approach using microdialysis. J Neurochem. 1992;59:358–63.
16. Adachi K, Kashii S, Masai H, Ueda M, Morizane C, Kaneda, et al. Mechanism of the pathogenesis of glutamate neurotoxicity in retinal ischemia. Graefes Arch Clin Exp Ophthalmol. 1998;236:766–74.
17. Auclair C, Voisin E. Nitroblue tetrazolium reduction. In: Greenwald RA, editor. Handbook of methods for oxygen radicals research. Boca Raton: CRC Press; 1988. p. 123–32.
18. Altman FP. Tetrazolium salts and formazans. Prog Histochem Cytochem. 1976;9:1–56.
19. Zhang H, Agardh E, Agardh CD. Nitro blue tetrazolium staining: a morphological demonstration of superoxide in the rat retina. Graefes Arch Ophthalmol. 1993;231:178–83.
20. Gonzalez-Flecha B, Boveris A. Mitochondrial sites of hydrogen peroxide production in reperfused rat kidney cortex. Biochim Biophys Acta. 1995;1243:361–6.
21. Hockenbery DM, Oltvai Z, Yin XM, Milliman CL, Korsmeyer SJ. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell. 1993;75:241–51.
22. Kohner EM, Patel V, Rassam SM. Role of blood flow and impaired autoregulation in the pathogenesis of diabetic retinopathy. Diabetes. 1995;44:603–6.
23. Arnold AC. Pathogenesis of nonarteritic anterior ischemic optic neuropathy. J Neuroophthalmol. 2003;23:157–63.
24. Ikushima M, Tano Y, Ikeda T, Sato Y. Hyper-infusion pressure for diabetic membrane dissection. Jpn J Ophthalmol. 1990;34:393–400.
25. Ambati J, Chalam KV, Chawla DK, D'Angio CT, Guillet EG, Rose SJ, et al. Elevated γ-aminobutyric acid, glutamate, and vascular endothelial growth factor levels in the vitreous of patients with proliferative diabetic retinopathy. Arch Ophthalmol. 1997;11:1161–6.

Morphologic and Functional Advantages of Macular Hole Surgery with Brilliant Blue G–Assisted Internal Limiting Membrane Peeling

Removal of the internal limiting membrane (ILM) is an effective additional treatment in macular hole (MH) surgery.[1–3] The transparency of the ILM requires high skill to peel the membrane. In 2000, a technique using indocyanine green (ICG) to stain and peel the ILM was reported.[4,5] However, some investigators[6,7] have reported retinal toxicity of the residual ICG. Other investigators have shown the toxicity of ICG to the retinal pigment epithelium in vitro[8–10] and in vivo.[11–13] These reports indicate that surgeons have to be very careful not to allow ICG to remain subretinally at the end of MH surgery because it can cause postoperative complications such as retinal pigment epithelial changes[14] and subsequent visual field loss.[15,16]

In 2006, Enaida et al[17] initially reported that brilliant blue G (BBG) stains the ILM while having low retinal toxicity in their morphologic study using electron microscopy. In rapid succession, they also reported the clinical possibility of using BBG for ILM staining and peeling in MH and epiretinal membrane cases with no adverse events.[18] Compared with ICG, the toxicity of BBG to cultured retinal ganglion cells was significantly lower based on evaluation of retinal ganglion cell apoptosis.[19] Ueno et al[20] injected ICG and BBG in clinical concentrations into the subretinal space of rats. They found that ICG caused retinal degeneration and retinal pigment epithelium cell atrophy, while BBG had no detectable toxic effects. After confirmation of the safety of BBG, Cervera et al[21,22] reported their experience with ILM peeling using BBG and concluded that dyeing with BBG appeared to be an interesting alternative to ICG.

From the *Department of Ophthalmology, Kagawa University Faculty of Medicine, Kagawa, Japan; †Department of Ophthalmology, Kyushu Medical Center, Fukuoka, Japan; and ‡Department of Ophthalmology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan.

The authors report no conflicts of interest.

Reprint requests: Kouki Fukuda, MD, Department of Ophthalmology, Kagawa University Faculty of Medicine, 1750-1 Ikenobe, Miki-cho, Kagawa 761-0793, Japan; e-mail: snipeman@med.kagawa-u.ac.jp

Much improvement in the resolution of optical coherence tomography has enabled us to observe microstructure of the macula in MHs before and after surgery. Recent studies have revealed the correlation between visual recovery and the presence of the inner and outer segments of the photoreceptor (IS/OS) junction after MH surgery.[23,24] The IS/OS junction can be observed in the normal eye as the continuous line located in the outer retina. Another investigator has reported the importance of the external limiting membrane (ELM) compared with the IS/OS in visual recovery after MH surgery.[25] Thus, continuity of the IS/OS junction and the ELM has been well known as an important factor for postoperative recovery of visual acuity. In the present study, the results, including macular microstructure and visual acuity, of MH surgery using BBG and ICG were compared.

Materials and Methods

This was a nonrandomized, retrospective, interventional case series. Between September 2007 and April 2009, 53 eyes of 53 consecutive patients with idiopathic full-thickness MH underwent MH surgery with ILM peeling using ICG (n = 22) (between September 2007 and August 2008) or BBG (n = 31) (between September 2008 and April 2009) at Kagawa University Hospital. In all patients, the surgery was performed as soon as possible after an initial visit to our hospital. Best-corrected visual acuity (BCVA), optical coherence tomography examination using spectral-domain optical coherence tomography (Carl Zeiss Meditec, Inc, Dublin, CA), and slit-lamp fundus examinations using a 78-diopter lens were performed before and 1, 3, and 6 months after surgery. Optical coherence tomography reading was performed by one of the authors (F.S.) in a masked fashion without knowledge of the staining dye used in ILM peeling or the surgical outcomes. The optical coherence tomography reader evaluated the ELM or IS/OS junction as reconstructed or restored when continuity of the ELM or the IS/OS line was observed at the fovea after

surgery. The presence or absence of continuity of the ELM or IS/OS line could be clearly determined (Figures 1 and 2).

All cases underwent 25-gauge, transconjunctival, sutureless vitrectomy. Cataract surgery was performed simultaneously in patients aged 50 years. After posterior vitreous detachment creation in eyes with Stage 2 or 3 holes and removal of the posterior hyaloid, 0.125% ICG or 0.25 mg/mL of BBG was sprayed onto the posterior retina around the MHs. The ICG solution (Ophthagreen, Santen Pharmaceutical Co Ltd, Osaka, Japan) was prepared at a concentration of 0.125% using dilution in BSS plus (Alcon Lab, Fort Worth, TX). The BBG solution (Coomassie BBG 250; Sigma–Aldrich, St. Louis, MO) was prepared at a concentration of 0.25 mg/mL using dilution in BSS plus. Three surgeons (K.F., F.S., and H.Y.) performed the MH surgeries using ICG (between September 2007 and August 2008) or BBG (between September 2008 and April 2009).

Immediately after the injection of both dyes, the dye solution in the vitreous cavity was aspirated using a vitreous cutter. The ILM was incised using a 25-gauge microvitreoretinal blade and carefully peeled from the underlying retina in a circumferential manner within about a 1.5-disk diameter radius around an MH, using a microforceps. If stain solution remaining within MHs was observed, it was aspirated with a soft-tipped needle. An air–fluid exchange was performed, and 20% sulfur hexafluoride was infused. Strict face-down positioning was maintained for 3 days after surgery. This study was approved by the Institutional Review Board of Kagawa University Faculty of Medicine.

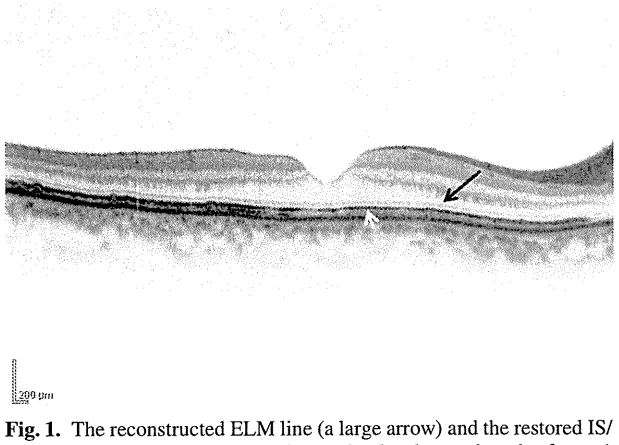

Fig. 1. The reconstructed ELM line (a large arrow) and the restored IS/OS junction line (a small arrow) are clearly observed at the fovea 1 month after surgery.

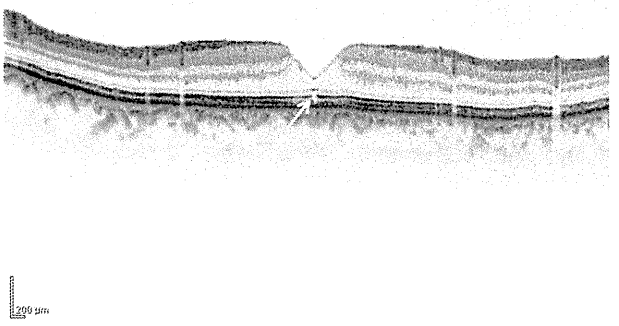

Fig. 2. The lack of continuity of the IS/OS junction line (an arrow) is observed. The IS/OS junction is not restored in this case.

Results

Baseline Characteristics

Baseline characteristics for all patients are shown in Table 1. The BBG group included 31 eyes of 31 patients (14 men and 17 women). Median age at the time of surgery was 67 years (range, 56–80 years). Stage 2, 3, and 4 MHs were present in 14, 13, and 4 eyes, respectively. Preoperative mean BCVA ± SD was 0.61 ± 0.29 logarithm of the minimal angle of resolution (logMAR). The ICG group included 22 eyes of 22 patients (12 men and 10 women). Median age at time of surgery was 68 years (range, 54–79 years). Stage 2, 3, and 4 MHs were present in 10, 8, and 4 eyes, respectively. Preoperative mean BCVA ± SD was 0.59 ± 0.27 logMAR. No significant differences were noted between the groups in age ($P = 0.59$, Mann–Whitney U test), sex ($P = 0.18$, Fisher exact probability test), disease duration ($P = 0.98$, Mann–Whitney U test), stage of MHs ($P = 0.84$, chi-square test), and preoperative mean logMAR visual acuity ($P = 0.77$, unpaired t-test).

Best-Corrected Visual Acuity Results and Macular Hole Closure Rates

Best-corrected visual acuity results and MH closure at 6 months after surgery are shown in Tables 2 and 3. In both BBG and ICG groups, the MH was successfully closed in all cases at 6 months postoperatively. Table 3 shows the visual results after surgery. In the BBG group, the mean BCVA ± SD improved significantly from 0.61 ± 0.29 logMAR preoperatively to 0.10 ± 0.20 logMAR at 6 months postoperatively ($P < 0.001$, paired t-test). Best-corrected visual acuity improved by ≥0.3 logMAR in 27 eyes (87%) and stabilized in 4 eyes (13%) at 6 months after surgery. Best-corrected visual acuity was 20/20 or better at 6 months after surgery in 20 of 31 eyes

Table 1. Baseline Characteristics

	BBG Group (n = 31)	ICG Group (n = 22)	*P*
Patient age (years)			
Median	67	68	0.59, Mann–Whitney *U* test
Range	56–80	54–79	
Disease duration (months)			
Median	3	3	0.98, Mann–Whitney *U* test
Range	1–8	1–8	
Stage (n)			
2	14	10	0.84, chi-square test
3	13	8	
4	4	4	
BCVA at baseline, logMAR	0.61 ± 0.29	0.59 ± 0.27	0.77, paired *t*-test

(65%). In the ICG group, the mean BCVA ± SD improved significantly from 0.59 ± 0.27 logMAR preoperatively to 0.14 ± 0.17 logMAR at 6 months postoperatively ($P < 0.001$, paired *t*-test). Best-corrected visual acuity improved by ≥0.3 logMAR in 18 eyes (82%) and stabilized in 4 eyes (18%) at 6 months after surgery. Best-corrected visual acuity was 20/20 or better at 6 months after surgery in 7 of 22 eyes (32%). No significant differences between the 2 groups were seen in mean BCVA ($P = 0.39$, unpaired *t*-test) and change in BCVA by ≥0.3 logMAR ($P = 0.71$, chi-square test) at 6 months after surgery. However, for a BCVA of 20/20 or better, the BBG group showed a significantly higher rate than the ICG group ($P = 0.03$, Fisher exact probability test). Figure 3 shows the changes in logMAR visual acuity of both groups.

Microstructural Results After Macular Hole Surgery

The ELM reconstruction rates at 1, 3, and 6 months after surgery were 65%, 90%, and 94%, respectively, in the BBG group and 68%, 91%, and 100%, respectively, in the ICG group (Table 2, Figure 4). The rates of IS/OS junction restoration at 1, 3, and 6 months after surgery were 32%, 61%, and 87%, respectively, in the BBG group, compared with 5%, 50%, and 91%, respectively, in the ICG group (Table 2, Figure 5). A significant difference in the rate of IS/OS junction restoration at 1 month after surgery was found between the 2 groups ($P = 0.02$, Fisher exact probability test; Table 2, Figure 5).

Proportion of Simultaneous Cataract Surgery and Adverse Effects

In the BBG group, 24 eyes (excluding 4 eyes that were pseudophakic before surgery) underwent combined phacoemulsification and posterior chamber intraocular lens implantation, because progression of nuclear sclerotic cataracts is not preventable in patients >50 years of age. In the 3 eyes without combined cataract surgery, cataract surgery was not performed after vitrectomy, and 28 eyes (90%) were pseudophakic at 6 months after surgery. For the 22 eyes in the ICG group, because 2 eyes were pseudophakic preoperatively and cataract surgery was combined in 18 eyes, 20 eyes (90%) were pseudophakic at 6 months after surgery. No significant difference in the proportion of pseudophakic eyes at 6 months after surgery between the 2 groups was identified.

No significant adverse effects related to either dye were observed in the present study. In both groups, neither retinal detachment nor MH reopening was observed.

Table 2. Best-Corrected Visual Acuity Results and MH Closure at 6 Months After Surgery

	BBG Group (n = 31)	ICG Group (n = 22)	*P*
Anatomical results			
MH closure, n (%)	31 (100)	22 (100)	—
Recovery of ELM line, n (%)			
At 1 month	20 (65)	15 (68)	1.00, Fisher exact probability test
At 3 months	28 (90)	20 (91)	1.00, Fisher exact probability test
At 6 months	29 (94)	22 (100)	0.51, Fisher exact probability test
Recovery of IS/OS line, n (%)			
At 1 month	10 (32)	1 (5)	0.02, Fisher exact probability test
At 3 months	19 (61)	11 (50)	0.57, Fisher exact probability test
At 6 months	27 (87)	20 (91)	1.00, Fisher exact probability test

Table 3. Visual Results After Surgery

	BBG Group (n = 31)	ICG Group (n = 22)	*P*
Visual results			
Mean BCVA at 6 months, logMAR	0.10 ± 0.20	0.14 ± 0.17	0.39, paired *t*-test
Changes in BCVA ≥0.3 logMAR, n (%)			
Improved	27 (87)	18 (82)	0.71, chi-square test
Stable	4 (13)	4 (18)	
Worsened	0 (0)	0 (0)	
Eyes with BCVA of 20/20 or better	20/31 (65)	7/22 (32)	0.03, Fisher exact probability test

Discussion

Indocyanine green is the first adjuvant clinically used to stain the ILM.[4] This procedure of staining the ILM has spread quickly and is still now performed by vitreoretinal surgeons around the world. However, several reports[8–10] have noted the retinal toxicity of ICG. Alternative stains have been tried to stain the ILM, such as infracyanine green, trypan blue, Patent blue, Bromophenol blue, and BBG.[17,26–29] Of these stains, BBG shows a high ability to stain the ILM and, more importantly, a low possibility of cytotoxicity.[17–20]

Internal limiting membrane peeling procedures with any stains have achieved almost 100% postoperative MH closure rates. In a previous report,[25] the ELM reconstruction rate was 80% at 3 months postoperatively. In the present study, almost 90% of ELMs were reconstructed at 3 months in both groups, and almost 100% of ELMs were reconstructed at 6 months postoperatively. There were no significant differences between the BBG and ICG groups in the ELM reconstruction rates. The rate of IS/OS junction restoration has been reported as 4% at 1 month after surgery.[30] The dye they used in their operation for ILM peeling was 0.25% ICG, and their result was very close to the results of the present study's ICG group. As with ELM reconstruction, the rates of IS/OS junction restoration increased to almost 90% with time. At 1 month postoperatively, the IS/OS junction had restored in 32% of the BBG group and 5% of the ICG group; the difference was significant ($P = 0.02$; Fisher exact probability test). Because of the features of MHs, direct exposure of the bare retinal pigment epithelium and retina inside MHs to dyes is unavoidable. Because ICG injected into the subretinal space induces retinal cell degeneration,[20] this lag in restoration might be reasonable. The postoperative microstructural change of the IS/OS junction in the present

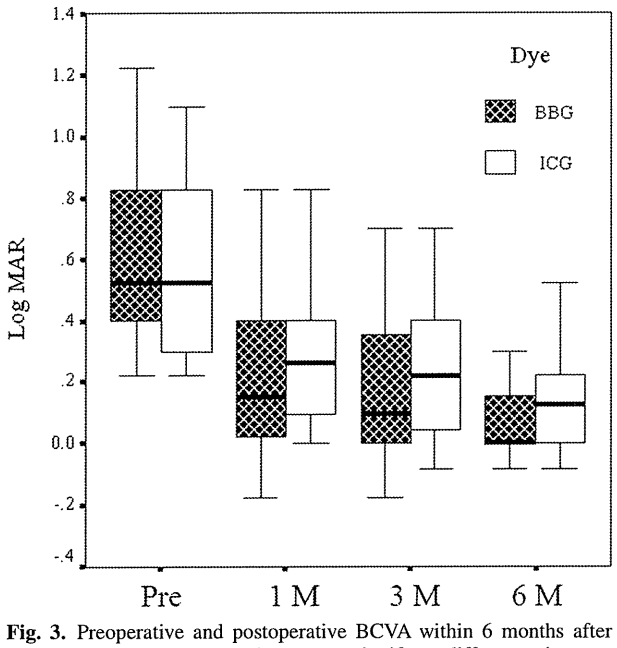

Fig. 3. Preoperative and postoperative BCVA within 6 months after MH surgery in both groups. There are no significant differences in mean logMAR BCVA between the two groups at any visit.

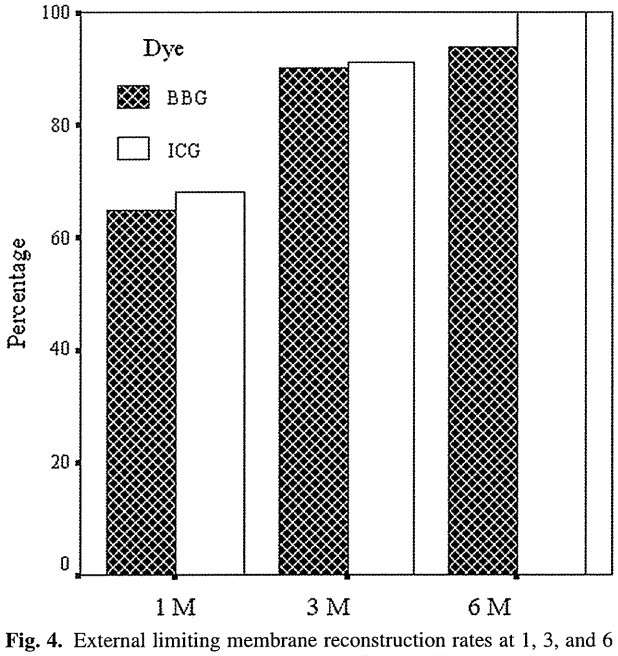

Fig. 4. External limiting membrane reconstruction rates at 1, 3, and 6 months after surgery. There are no significant differences in the rates between the two groups at any visit.

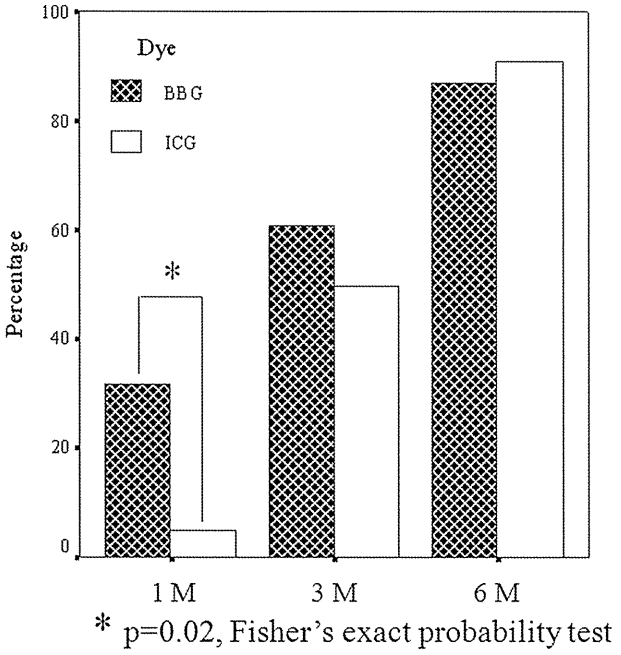

Fig. 5. The rates of IS/OS junction restoration at 1, 3, and 6 months after surgery. There are no significant differences in the rates between the 2 groups at 3 and 6 months postoperatively. The rate at 1 month after surgery is significantly higher in the BBG group than in the ICG group (P = 0.02; Fisher exact probability test).

study seems to indicate that BBG is more useful than ICG, though the mechanism of the restoration of the IS/OS junction is not well known.

Overall changes in BCVA in both groups were similar to those in previous reports about MH surgery. There were no significant differences between the two groups at any time points postoperatively. Although the lag in visual acuity improvement as expected by the morphologic restoration lag observed in the ICG group was not found in the current study, the rate of visual acuity of 20/20 or better at the final visit was significantly higher in the BBG group than that in the ICG group. This fact indicates that a restored IS/OS line, which indicates the presence of photoreceptors, may not work well at an early stage of restoration, so that the visual results at the 1-month visit did not show any significant difference between the 2 groups. Because the BBG group showed a significantly higher rate than the ICG group for a BCVA of 20/20 or better at the final visit, early restoration of the IS/OS junction can be important for the long-term visual outcome.

In contrast with earlier reports[19,20] confirming the safety of BBG, Yuen et al[31] reported the toxicity of BBG in an in vitro study. They evaluated the toxicity of several dyes including BBG and ICG using a human retinal pigment epithelial cell line (ARPE-19) and a murine retinal ganglion/Muller glial cell primary cell culture. A viability assay of ARPE-19 cells after 30 minutes of exposure to 4 different concentrations (10, 2.5, 0.25, and 0.125 mg/mL) of BBG was used. Every concentration of BBG resulted in a significantly lower viability than control, though every concentration (1, 0.5, 0.25, and 0.125 mg/ml) of ICG showed absolutely no toxicity in exactly the same study. As they noted in their report, 30-minute exposure is unlikely to occur in regular MH surgery, but it could occur in cases of MH with retinal detachment. In contrast, the influence of 0.25 mg/mL of BBG on cultured retinal ganglion cells was negligibly small after 30 minutes of exposure, and this result agrees with a similar previous report,[19] though the predetermined exposure time was shorter. Yuen et al[31] also studied a short exposure time of 3 minutes, and both dyes showed no toxicity in the concentrations used in the current study. All the cases in which we performed MH surgery with 0.25 mg/mL of BBG in the current study did not develop any adverse effects, such as retinal pigment epithelium atrophy inside MHs or retinal degeneration around MHs. Because a high dye concentration facilitates apoptosis of cultured retinal pigment epithelium within 72 hours, we infer that the 6-month observation period of the current study is long enough to conclude that 0.25 mg/mL of BBG has no toxicity.

In conclusion, BBG is useful as an adjuvant for easy ILM peeling in MH surgery. No apparent retinal toxicity was observed in both the ICG and BBG groups. The early restoration of the IS/OS junction observed in the BBG group seems important for a better long-term visual outcome. Further clinical investigations focused on the early restoration of the IS/OS junction observed in the BBG group are needed.

Key words: brilliant blue G, indocyanine green, internal limiting membrane, macular hole, vitrectomy, spectral-domain optical coherence tomography.

*KOUKI FUKUDA, MD,**
*FUMIO SHIRAGA, MD,**
*HIDETAKA YAMAJI, MD,**
*HIROYUKI NOMOTO, MD,**
*CHIEKO SHIRAGAMI, MD,**
HIROSHI ENAIDA, MD,†
TATSURO ISHIBASHI, MD†

References

1. Park DW, Sipperley JO, Sneed SR, et al. Macular hole surgery with internal-limiting membrane peeling and intravitreous air. Ophthalmology 1999;106:1392–1398.
2. Olsen TW, Sternberg P Jr, Capone A Jr, et al. Macular hole surgery using thrombin-activated fibrinogen and selective removal of the internal limiting membrane. Retina 1998;18: 322–329.

3. Brooks HL Jr. Macular hole surgery with and without internal limiting membrane peeling. Ophthalmology 2000; 107:1939–1949.
4. Kadonosono K, Itoh N, Uchio E, et al. Staining of internal limiting membrane in macular hole surgery. Arch Ophthalmol 2000;118:1116–1118.
5. Burk SE, Da Mata AP, Snyder ME, et al. Indocyanine green-assisted peeling of the retinal internal limiting membrane. Ophthalmology 2000;107:2010–2014.
6. Horiguchi M, Nagata S, Yamamoto N, et al. Kinetics of indocyanine green dye after intraocular surgeries using indocyanine green staining. Arch Ophthalmol 2003;121:327–331.
7. Tadayoni R, Paques M, Girmens JF, et al. Persistence of fundus fluorescence after use of indocyanine green for macular surgery. Ophthalmology 2003;110:604–608.
8. 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; 132:433–435.
9. Ho JD, Tsai RJ, Chen SN, Chen HC. Cytotoxicity of indocyanine green on retinal pigment epithelium: implications for macular hole surgery. Arch Ophthalmol 2003;121:1423–1429.
10. Rezai KA, Farrokh-Siar L, Ernest JT, et al. Indocyanine green induces apoptosis in human retinal pigment epithelial cells. Am J Ophthalmol 2004;137:931–933.
11. Enaida H, Sakamoto T, Hisatomi T, et al. Morphological and functional damage of the retina caused by intravitreous indocyanine green in rat eyes. Graefes Arch Clin Exp Ophthalmol 2002;240:209–213.
12. Iriyama A, Uchida S, Yanagi Y, et al. Effects of indocyanine green on retinal ganglion cells. Invest Ophthalmol Vis Sci 2004;45:943–947.
13. Maia M, Margalit E, Lakhanpal R, et al. Effects of intravitreal indocyanine green injection in rabbits. Retina 2004;24:69–79.
14. Engelbrecht NE, Freeman J, Sternberg P Jr, 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.
15. Gandorfer A, Haritoglou C, Gass CA, et al. Indocyanine green-assisted peeling of the internal limiting membrane may cause retinal damage. Am J Ophthalmol 2001;132:431–433.
16. Kanda S, Uemura A, Yamashita T, et al. Visual field defects after intravitreous administration of indocyanine green in macular hole surgery. Arch Ophthalmol 2004;122:1447–1451.
17. Enaida H, Hisatomi T, Goto Y, et al. Preclinical investigation of internal limiting membrane staining and peeling using intravitreal brilliant blue G. Retina 2006;26:623–630.
18. Enaida H, Hisatomi T, Hata Y, et al. Brilliant blue G selectively stains the internal limiting membrane/brilliant blue G-assisted membrane peeling. Retina 2006;26:631–636.
19. Kawahara S, Hata Y, Miura M, et al. Intracellular events in retinal glial cells exposed to ICG and BBG. Invest Ophthalmol Vis Sci 2007;48:4426–4432.
20. Ueno A, Hisatomi T, Enaida H, et al. Biocompatibility of brilliant blue G in a rat model of subretinal injection. Retina 2007;27:499–504.
21. Cervera E, Díaz-Llopis M, Salom D, Udaondo P. High dose intravitreal brilliant blue G. Arch Soc Esp Oftalmol 2007;82:473.
22. Cervera E, Díaz-Llopis M, Salom D, et al. Internal limiting membrane staining using intravitreal brilliant blue G: good help for vitreo-retinal surgeon in training. Arch Soc Esp Oftalmol 2007;82:71–72.
23. Baba T, Yamamoto S, Arai M, et al. Correlation of visual recovery and presence of photoreceptor inner/outer segment junction in optical coherence images after successful macular hole repair. Retina 2008;28:453–458.
24. Inoue M, Watanabe Y, Arakawa A, et al. Spectral-domain optical coherence tomography images of inner/outer segment junctions and macular hole surgery outcomes. Graefes Arch Clin Exp Ophthalmol 2009;247:325–330.
25. Wakabayashi T, Fujiwara M, Sakaguchi H, et al. Foveal microstructure and visual acuity in surgically closed macular holes: spectral-domain optical coherence tomographic analysis. Ophthalmology 2010;11:1815–1824.
26. Van De Moere A, Stalmans P. Anatomical and visual outcome of macular hole surgery with infracyanine green-assisted peeling of the internal limiting membrane, endodrainage, and silicone oil tamponade. Am J Ophthalmol 2003;136:879–887.
27. Feron EJ, Veckeneer M, Parys-Van Ginderdeuren R, et al. Trypan blue staining of epiretinal membranes in proliferative vitreoretinopathy. Arch Ophthalmol 2002;120:141–144.
28. Mennel S, Meer CH, Tietjen A, et al. Patent blue: a novel vital dye in vitreoretinal surgery. Ophthalmologica 2006;220: 190–193.
29. Haritoglou C, Schumann RG, Strauss R, et al. Vitreoretinal surgery using bromphenol blue as a vital stain: evaluation of staining characteristics in humans. Br J Ophthalmol 2007;91:1125–1128.
30. Sano M, Shimoda Y, Hashimoto H, Kishi S. Restored photoreceptor outer segment and visual recovery after macular hole closure. Am J Ophthalmol 2009;147:313–318.
31. Yuen D, Gonder J, Proulx A, et al. Comparison of the in vitro safety of intraocular dyes using two retinal cell lines: a focus on brilliant blue G and indocyanine green. Am J Ophthalmol 2009;147:251–259.

JOURNAL OF OCULAR PHARMACOLOGY AND THERAPEUTICS
Volume 27, Number 5, 2011

DOI: 10.1089/jop.2011.0020

Efficacy and Safety of Switching from Topical Latanoprost to Bimatoprost in Patients with Normal-Tension Glaucoma

Shino Sato,[1] Kazuyuki Hirooka,[1] Tetsuya Baba,[1] Masanori Mizote,[1] Takashi Fujimura,[2] Kaori Tenkumo,[2] Hirokazu Ueda,[3] and Fumio Shiraga[1]

Abstract

Purpose: The aim of this study was to evaluate the efficacy and safety of bimatoprost in Japanese patients with normal-tension glaucoma (NTG) who showed insufficient response to latanoprost.
Methods: A prospective, nonrandomized study was conducted in patients with NTG, with ≤20% intraocular pressure (IOP) decrease from pretreatment baseline with latanoprost monotherapy who had been switched to bimatoprost. The IOP was measured at 4, 8, and 12 weeks after the switch to bimatoprost. In 12 weeks after the switch to bimatoprost, efficacy and safety were evaluated.
Results: Postswitch to bimatoprost, IOP was significantly reduced at every visit. Bimatoprost produced significantly greater mean% IOP reduction rate from pretreatment than that of latanoprost at week 12 ($P<0.01$). There was a significant correlation between% IOP reduction of bimatoprost and that of latanoprost (Pearson $r^2=0.374$; $P=0.007$). No significant difference was observed in the mean scores of conjunctival hyperemia and corneal epithelial disorder between bimatoprost-treated eyes and latanoprost-treated eyes.
Conclusions: Significant additional IOP lowering was achieved by switching to bimatoprost in Japanese patients with NTG with insufficient response to latanoprost. Bimatoprost treatment was safe and well tolerated.

Introduction

PROSTAGLANDIN ANALOGS have gained widespread clinical use for treatment of glaucoma because of their efficacy at lowering intraocular pressure (IOP).[1–3] Latanoprost is a prodrug of the naturally occurring prostaglandin (PG) $F_{2\alpha}$ and is endowed with a strong IOP-reducing effect.[4–6] Bimatoprost is an analog of $PGF_{2\alpha}$-1-ethanolamide (prostamide $F_{2\alpha}$). Prostamides are derived from an endocannabinoid anandamide by COX-2,[7] and have pharmacological and biochemical properties distinct from PG $F_{2\alpha}$.[7,8] Similar to $PGF_{2\alpha}$ analogs, the IOP lowering mechanism of bimatoprost is likely to be attributed to the increase in uveoscleral outflow,[9] which is associated with extracellular matrix remodeling.[10] In addition, in subjects with ocular hypertension (OH) and glaucoma, the increase of both the pressure-sensitive (trabecular) outflow and the pressure-insensitive (uveoscleral) outflow by bimatoprost could be ascribed to the changes in the trabecular meshwork or in the sclera, or both.[9,11–13] Although the pharmacological mechanisms of actions of latanoprost and bimatoprost have been thought to be similar, there is a possibility that with patients for whom 1 agent is neither fully effective nor tolerable, another agent may be useful.[14]

The Tajimi study, which is one of the largest glaucoma epidemiology studies in Japan, showed that the glaucoma prevalence rate in Japanese older than 40 years of age is 5.0%, and the rate of open-angle glaucoma is 3.9%.[15,16] The study also reported that almost 90% of the open-angle glaucoma consisted of normal-tension glaucoma (NTG). The NTG is a clinical entity characterized by glaucomatous optic nerve damage and visual field defects with an IOP in the statistically normal range. The IOP is, however, a part of the pathogenic process in NTG, and IOP lowering is effective in reducing the progression of glaucomatous damage.[17] Although latanoprost is commonly used as first-line therapy in the treatment of NTG, there are some cases that show insufficient response to latanoprost.[18]

The purpose of this study was to evaluate the efficacy and safety of bimatoprost in eyes with insufficient response to latanoprost in Japanese patients with NTG.

[1]Department of Ophthalmology, Kagawa University Faculty of Medicine, Miki, Kagawa, Japan.
[2]Department of Ophthalmology, Social Insurance Ritsurin Hospital, Takamatsu, Kagawa, Japan.
[3]Ueda Eye Clinic, Ayauta, Kagawa, Japan.

Methods

This clinical trial was conducted at the following 3 investigational sites. December 2009 to December 2010: Department of Ophthalmology, Kagawa University Faculty of Medicine, Social Insurance Ritsurin Hospital, and Ueda Eye Clinic (Kagawa, Japan). All the aspects of the study were in compliance with the Declaration of Helsinki, and all the patients gave their consent on being sufficiently informed by an investigator.

Examinations of visual acuity, refraction, both central and peripheral fields, slit-lamp examination, and gonioscopy were performed on all the patients. The eligibility criteria were age ≥20 years; bilateral or unilateral NTG: glaucomatous optic disc abnormalities and corresponding glaucomatous visual field defects, normal open angle, and IOP (measured using Goldmann applanation tonometer) of 21 mmHg or lower without medication; ≤20% IOP decrease from pretreatment baseline at least 12 weeks of treatment with latanoprost 0.005% (Xalatan®; Pfizer, New York, NY) monotherapy. Exclusion criteria were the subjects being with active ocular diseases in either eye except glaucoma; with retinal disease that has a potential risk of progression; with experience of ocular surgery or lazer treatment; with regimen for systemic or local administration of steroid during this study; with corneal disease in either eye that poses a problem for veracious IOP measurement.

A total of 18 patients who fit the study criteria were enrolled in this study. The study consisted of 4 scheduled visits over 12 weeks (day 0 and weeks 4, 8, and 12). At day 0 (preswitch), eligible patients who had used latanoprost 0.005% were switched to bimatoprost 0.03% (Lumigan®; Allergan, Inc., Irvine, CA) treatment. The administration time of bimatoprost had been set to just around the same time before administration of latanoprost.

Measurements of IOP, best-correlated visual acuity, and biomicroscopic examinations were conducted at each visit. The IOP was measured at the same time period during the administration of latanoprost with Goldmann applanation tonometer by using the same procedure at all centers. The outcome due to primary efficacy was the main change in IOP at week 12 from preswitch.

Biomicroscopy was performed by using a slit-lamp examination without pupil dilation. The examination included an assessment of the lid/lashes, conjunctiva, anterior chamber, cornea, iris, and lens. Conjunctival hyperemia was assessed by a single observer by using a 5-point hyperemia grading scale using 5 different photographs for hyperemia matching: 0=none, 0.5=trace, 1=mild, 2=moderate, and 3=severe. Corneal epithelial disorders were recorded by using an A (area) D (density) grading scale by a slit-lamp examination.[19]

The study outcome for efficacy was based on the conditions of the patients' eyes with the higher IOP at the eligibility visit. If IOP was same in both eyes, we analyzed the right eye. Descriptive statistics for mean IOP, mean IOP change, and% IOP change from pretreatment were calculated. Statistical significance was assessed by using paired t test. The degrees of conjunctival hyperemia and corneal epithelial disorder were analyzed by using an averaged score of both eyes' values. Evaluation of the degrees of conjunctival hyperemia and corneal epithelial disorder was analyzed by using a Wilcoxon signed-rank test. The correlation mean%

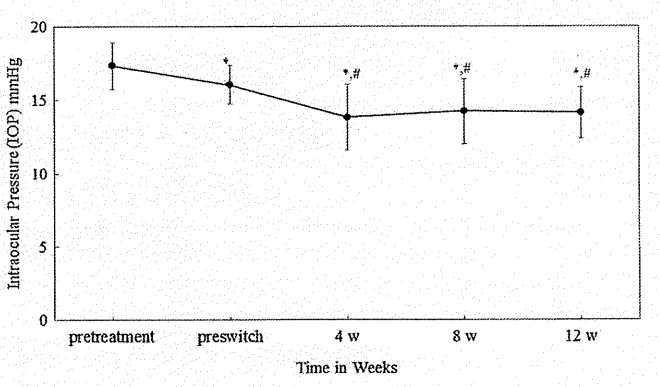

FIG. 1. Reduction in mean IOP after a switch to bimatoprost. Data express the mean±SD. $^{*}P<0.05$ versus pretreatment (paired t test). $^{\#}P<0.05$ versus preswitch (paired t test). IOP, intraocular pressure; SD, standard deviation.

IOP change from pretreatment between eyes treated with latanoprost and eyes treated with bimatoprost was analyzed by using a Pearson's correlation coefficient test. All the statistical analyses were performed by using SPSS for Windows, Version 11.5 (SPSS, Inc., Chicago, IL). A P value of 0.05 or less was considered statistically significant. Data are presented as mean±standard deviation.

Results

There were 4 men and 14 women (mean age, 68.2±15.3 years), who had the mean refractive error of −2.3±4.9 diopters. All subjects completed the study. No significant changes in visual acuity were detected throughout follow-up (data not shown).

The IOP data were as follows: pretreatment=17.3±1.6 mm Hg; preswitch=16.0±1.3 mm Hg; 12 weeks=14.1±1.7 mm Hg. At week 12, IOP was significantly lower than both the pretreatment IOP ($P<0.01$) and the preswitch IOP ($P<0.01$) (Fig. 1). Although the mean% IOP reduction from pretreatment to preswitch (latanoprost) was −7.5%±5.6%, the mean% IOP reduction from pretreatment to 12 weeks (bimatoprost) was −18.7%±8.9% (Fig. 2). At week 12, 7 patients showed ≥20% IOP decrease, and 2 patients showed ≥30% IOP decrease from pretreatment (Fig. 3). There was a

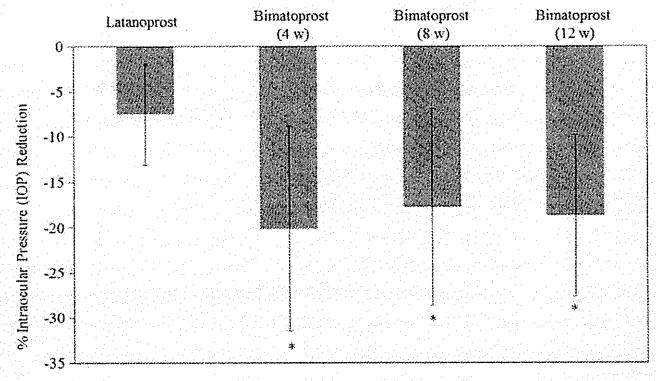

FIG. 2. Mean% IOP reduction from pretreatment to preswitch (Latanoprost) and at week 4, 8, and 12 (Bimatoprost). The mean% IOP reduction rate of bimatoprost was significantly greater than that of latanoprost ($^{*}P<0.01$, paired t test). Data express the mean±SD.