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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.

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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.

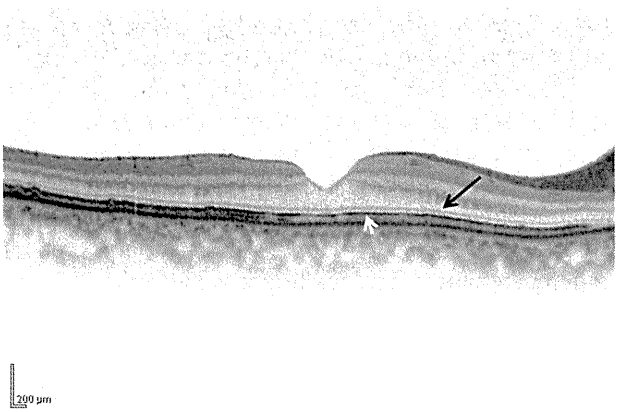

Fig. 1. The reconstructed ELM line (a large arrow) and the restored IS/OS junction line (a small arrow) are clearly observed at the fovea 1 month after surgery.

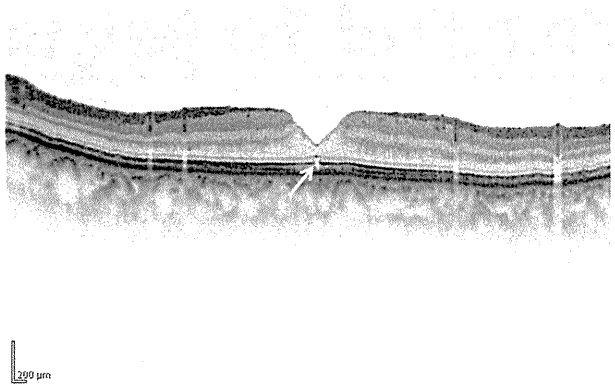

Fig. 2. The lack of continuity of the IS/OS junction line (an arrow) is observed. The IS/OS junction is not restored in this case.

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

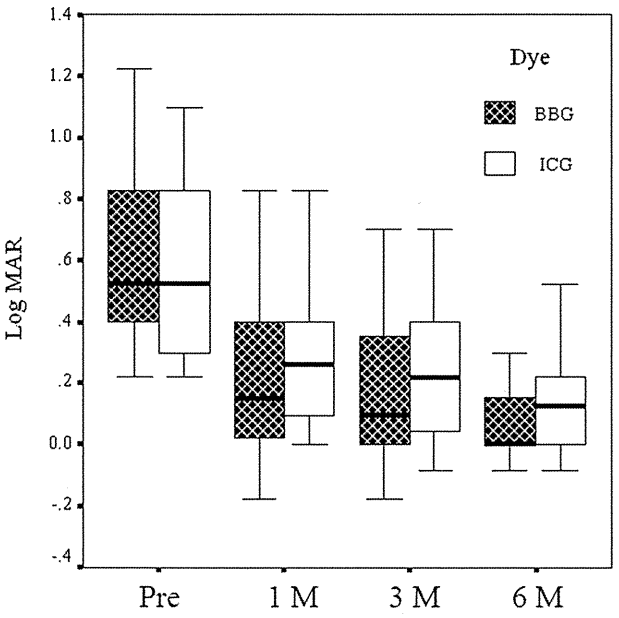

Fig. 3. Preoperative and postoperative BCVA within 6 months after MH surgery in both groups. There are no significant differences in mean logMAR BCVA between the two groups at any visit.

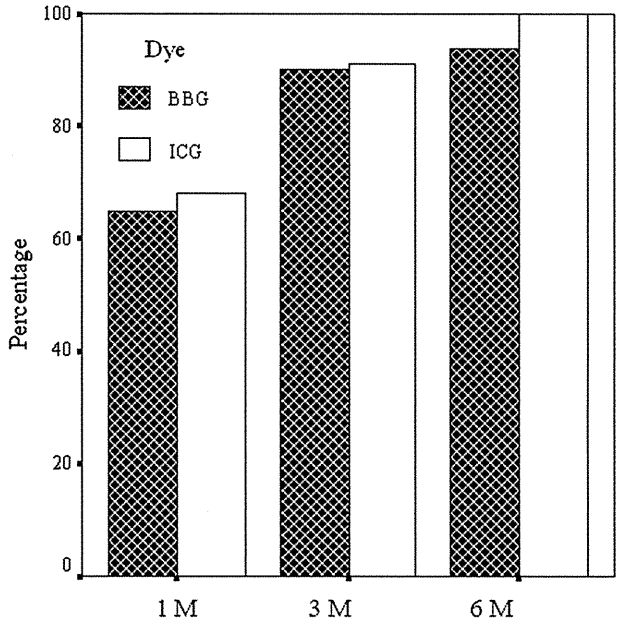

Fig. 4. External limiting membrane reconstruction rates at 1, 3, and 6 months after surgery. There are no significant differences in the rates between the two groups at any visit.

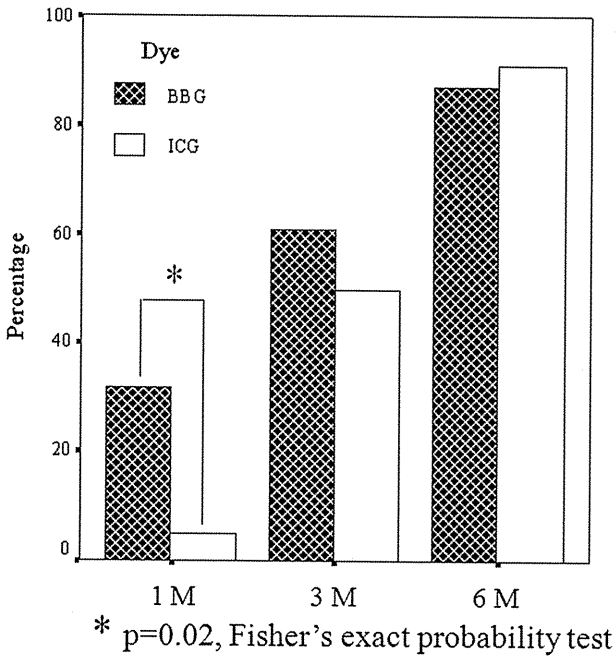

Fig. 5. The rates of IS/OS junction restoration at 1, 3, and 6 months after surgery. There are no significant differences in the rates between the 2 groups at 3 and 6 months postoperatively. The rate at 1 month after surgery is significantly higher in the BBG group than in the ICG group ($P = 0.02$;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

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Edaravone, a free radical scavenger, attenuates behavioral deficits following transient forebrain ischemia by inhibiting oxidative damage in gerbils

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ABSTRACT

The present study investigates the neurological protective effects of edaravone against global brain ischemia. Gerbils were treated with edaravone (3 mg/kg; i.p.) 30 min before transient forebrain ischemia, which was induced by occluding the bilateral common carotid artery for 5 min. The effects of edaravone were examined by measuring neuronal damage and behavioral deficits. Hexanoyl-lysine adduct (HEL) and 8-hydroxy-2′-deoxyguanosine (8-OHdG), oxidative stress markers, were also examined to assess the anti-oxidative effects of edaravone. Edaravone treatment significantly inhibited both lipid and DNA oxidative damage 72 h after ischemia, and decreased neuronal damage. Edaravone also significantly reduced the locomotor activity deficit 72 h after ischemia and improved memory impairment. These findings suggest that edaravone inhibits oxidative stress and attenuates neuronal damage induced by transient forebrain ischemia in gerbils and which may contribute to improvements in behavioral deficits.

Cerebral ischemia results in irreversible brain damage with associated behavioral deficits, including motor and sensory symptoms, impaired spatial orientation, memory impairment [1,4]. Increased oxidative stress after brain ischemia has been considered a primary cause for brain injury due to free radical-induced lipid peroxidation, and oxidation of proteins and nucleic acids [20]. There has been much interest in the use of free radical scavengers as potential treatments for brain ischemia [6].

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a free radical scavenger, is currently used in the treatment of acute ischemic stroke as a neuroprotective reagent. In animal models, protective effects of edaravone in brain ischemia have been reported, including effects on free radicals, reduced infarct size and protection against neuronal cell death [10,21,22]. However, relatively little is known of the effects of edaravone on behavioral deficits after brain ischemia. The aims of the current study were to examine whether treatment with edaravone can reduce neuronal damage and behavioral deficits after transient forebrain ischemia in gerbils and to investigate mechanism of action.

A total of 39 adult male Mongolian gerbils (Kyudo, Saga, Japan) with a body weight of 60–80 g were used for this study. Animal protocols were approved by the Animal Committee of Kagawa University Faculty of Medicine. All the gerbils were allowed free access to food and water. The animals were anesthetized with sodium pentobarbital (30 mg/kg i.p.), after which transient global ischemia was induced for 5 min by occlusion of the bilateral common carotid arteries with micro-aneurysm clips (Sugita Clip, Mizuho, Nagoya, Japan) [12,14]. During the operation, rectal temperature was controlled at 37.0 °C using a feedback-controlled heating pad (CMA, Stockholm, Sweden) to prevent hypothermia. After recirculation, the temperature was maintained at 37.0 °C for 30 min. Sham animals underwent the same operation except for occlusion of the carotid arteries.

This study was performed in three parts. In the first part, we evaluated the effect of edaravone on ischemic damage by histological analysis. Nine gerbils were divided into three groups randomly as sham, ischemia and ischemia + edaravone treatment ($n = 3$ per group). Edaravone (3 mg/kg) was administered intraperitoneally 30 min before brain ischemia, and then continued twice per day. Gerbils were anesthetized and sacrificed for histology 3 days after

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ischemia. The second part investigated the effect of edaravone on behavioral deficits after ischemia. Twelve gerbils in the same three groups ($n=4$ per group) underwent activity tests at 3 days after ischemia and a 8-arm radial maze test 7 days after ischemia. In the third part, we examined the anti-oxidative effects of edaravone by examining HEL and 8-OHdG, two oxidative stress markers, 72 h after transient cerebral ischemia. A total of 18 gerbils in sham, ischemia and ischemia + edaravone groups ($n=6$ per group) were used.

For the histological examinations, we examined neuronal cell damage in CA1 area of hippocampus using hematoxylin and eosin (H&E) staining and deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) as a marker of cell death [14]. We also evaluated oxidative injury in the CA1 region by immunohistochemistry using 8-OHdG antibody [19]. Gerbils were anesthetized with sodium pentobarbital (50 mg/kg i.p.) at 72 h after ischemia, and transcardially perfused with 4% phosphate-buffered paraformaldehyde after flushing with 0.1 M phosphate-buffered saline. Brains were removed, post-fixed at 4 °C in the same fixative overnight, and then transferred into 25% sucrose for 2 days. The sucrose immersed brains were embedded with OCT compound (Sakura Finetek USA, Torrance, CA) and sectioned at a thickness of 14 μm on a cryostat as previous described [14]. The sections in the CA1 area at roughly 1.7 mm posterior to the bregma from each animal were used for analysis, and the pyramidal cell density (cells/mm) in the CA1 area was counted after H&E staining [12].

Apoptotic cells were detected using the TUNEL method with a commercial apoptosis detection kit (S7101; Temecula, CA, USA) according to the manufacturer's instructions.

Additionally, 8-OHdG immunostaining was performed to investigate the oxidative injury to the CA1 neuronal cells. Sections were first incubated in PBS containing 0.3% Triton X-100 for 15 min. After rinsing three times in 0.01 M PBS (pH 7.4), sections were blocked with 5% albumin solution for 30 min and then incubated with anti-8-OHdG monoclonal antibody (5 μg/ml; Cosmobio Co., Ltd, Tokyo, Japan) at 4 °C overnight. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide for 20 min at the beginning of the second day. After rinsing in PBS three times, the sections were incubated with biotinylated anti-mouse IgG (Vector Laboratories) for 90 min and continued with 90 min incubation of avidin-biotin horseradish peroxidase (Vector Laboratories) at room temperature. Sections were then reacted with diaminobenzidine (DAB) peroxidase substrate for 5 min. All sections were dehydrated, mounted and evaluated by light microscopy.

To investigate lipid oxidative damage, the level of HEL, a marker for early lipid peroxidation, was determined with a competitive enzyme-linked immunosorbent assay (ELISA) kit (Cosmobio Co., Ltd). Hippocampus from each gerbil was homogenized in protein lysate, and then centrifuged (12,000 × g) for 15 min at 4 °C. The supernatants were gently obtained and used to measure HEL concentration according to the manufacturer's recommendations.

To examine oxidative DNA damage, the gerbils were anesthetized (50 mg/kg; sodium pentobarbital i.p.) and sacrificed 72 h after ischemia. The bilateral hippocampal region from each gerbil was obtained and homogenized for DNA extraction using a DNA isolation kit (Dojindo Molecular Technologies, Kumamoto, Japan) as described previously [12]. 8-OHdG levels in each sample were determined with an ELISA kit (Japan Institute for the Control of Aging, Shizuoka, Japan), which can measure even very low levels of 8-OHdG. Optical density was measured at 450 nm. The data, expressed as pg of 8-OHdG per μg of DNA, were calculated on the basis of a linear calibration curve generated with 8-OHdG standard solutions.

To investigate functional outcome, locomotor activity and 8-arm radial maze tests were used. Locomotor activity of the animal in a transparent cage was measured hourly for 24 h and then from 48 h to 72 h after ischemia with photo beam interruption sensors (LOCOMO LS-8, Melquest, Toyama, Japan). The number of beam breaks was evaluated as locomotor activity [13]. An 8-arm radial maze was used to test memory function after ischemia as described previously [14]. In brief, a small food pellet was placed at the end of each arm (25 cm long, 6 cm high, 6 cm wide) of the maze device, and the animal was placed in the central part of the maze (22 cm in diameter). Gerbils enter the arms to eat the food, and an error was noted if the animal entered an arm that it had visited before. We started the test 7 days after ischemia and tested for 4 consecutive days, with 1 trial conducted per day ($n=4$ per group). The number of errors was recorded. The 8-arm radial maze trial was scored by investigators who were blinded to the treatment condition.

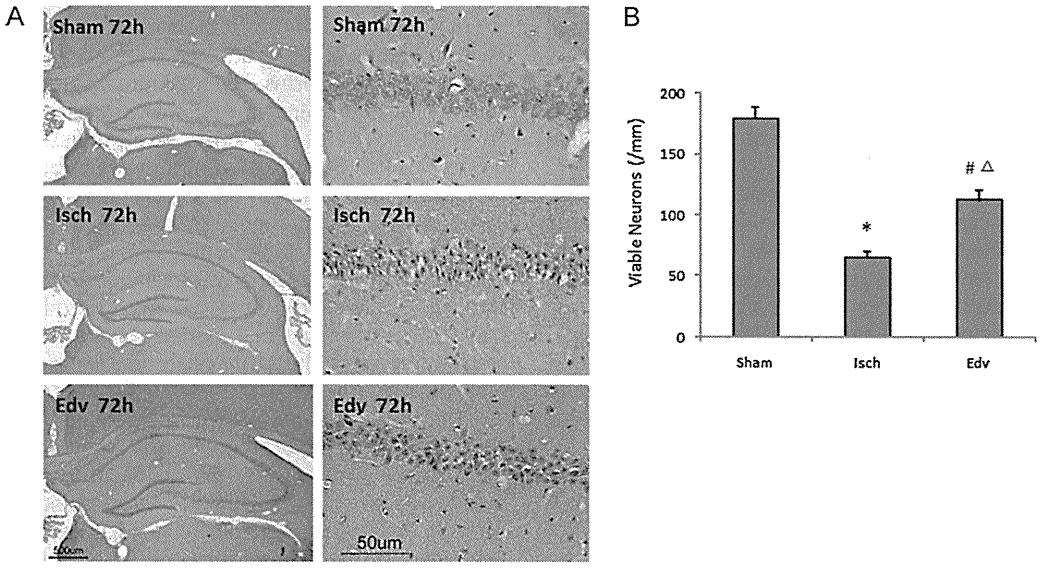

Fig. 1. (A) Hematoxylin and eosin staining in the hippocampal CA1 area. Top pictures are from a section 72 h after sham-operation, the middle pictures are from a section 72 h after ischemia, and the bottom pictures are from the sections 72 h after ischemia with edaravone (Edv) treatment. The pictures in each group are shown at both low (left panel) and high magnification (right panel). (B) Viable neurons per millimeter of the hippocampal CA1 area. $n=3$ per group. *$P<0.05$, compared with the sham group; #$P<0.05$, compared with the sham group; Δ$P<0.05$, compared with the ischemia group.

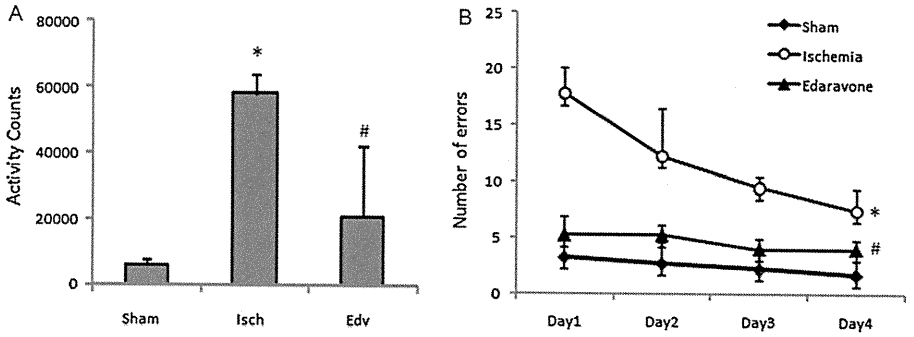

Fig. 2. Edaravone (Edv) treatment ameliorated ischemia (Isch)-induced behavioral deficits. (A) The outcome of activity test; $n=4$ per group. *$P<0.05$, compared with the sham group; #$P<0.05$, compared with the ischemia group. (B) Outcome in the 8-arm radial maze trial; $n=4$ per group. *$P<0.05$, compared with the sham group; #$P<0.05$, compared with the ischemia group. The number of errors in the ischemia group was significantly increased in comparison with those in both sham and edaravone groups.

Data are expressed as mean ± SD. The differences in the histological data, ELISA data and locomotor activity data were analyzed for significance by one-way ANOVA followed by Tukey's post hoc test. Two-way repeated ANOVA was used to compute the test of 8-arm radial maze, and the differences among the groups were evaluated for significance using Tukey's post hoc test.

H&E staining was performed to examine brain histological changes in hippocampal CA1 area 3 days after transient forebrain ischemia in gerbils (Fig. 1). In the ischemia group, marked neuronal damage in CA1 pyramidal neurons was observed at 72 h after ischemia, with significant cell shrinkage and minimal cytoplasm. These neuropathological signs were suppressed by treatment with edaravone (Fig. 1A). The effect was quantified by counting CA1 cell numbers in the sham, ischemia and ischemia + edaravone treated groups. Edavarone significantly reduced neuronal cell death (Fig. 1B).

Edaravone treatment also ameliorated behavioral deficits. The outcome of the activity test is shown in Fig. 2A. The activity counts of the ischemia group significantly increased compared with the sham group 3 days after ischemia ($P<0.05$), and the activity counts of the ischemia + edaravone group were decreased in comparison with the ischemia group ($P<0.05$).

The number of errors recorded in the 8-arm radial maze is shown in Fig. 2B. The number of errors tended to decrease with time in all groups. The gerbils in ischemia group significantly increased the number of errors one week after ischemia compared to the sham group ($P<0.05$), and the gerbils in edaravone treated group had a decreased number of errors compared with the untreated ischemia group ($P<0.05$).

We examined whether edaravone reduced free radical-mediated damage after transient forebrain ischemia by assessing lipid peroxidation by HEL. Ischemia increased HEL levels compared to the sham group and edaravone treatment significantly decreased HEL levels ($P<0.05$). Indeed, there was no difference between the ischemia + edaravone group and the sham group (Fig. 3). Similarly, oxidation of DNA was assessed by immunohistochemistry of 8-OHdG in the hippocampal CA1 region (Fig. 4A). Three days after ischemia, 8-OHdG immunoreactivity and TUNEL positive cells were elevated in the CA1 region of the ischemia group, but 8-OHdG immunoreactivity and TUNEL positive cells were decreased in the ischemia + edaravone group. Furthermore, quantification of 8-OHdG levels in the CA1 region by ELISA showed markedly increased levels after ischemia, levels which were reduced with edaravone treatment ($P<0.05$; Fig. 4B).

The present study demonstrated that edaravone decreased the oxidative stress, attenuated the neuronal damage induced by transient forebrain ischemia in gerbils and improved behavioral performance.

Ischemia and reperfusion injury is known to produce free radicals, which can cause widespread damage to cellular components such as lipids, proteins and DNA, leading to subsequent necrosis or apoptosis [6,20]. Edaravone is an effective free radical scavenger and its protective role against brain ischemia has been linked to that property [9,21]. In this study, ischemia and reperfusion injury induced an increase in 8-OHdG immunoreactivity and TUNEL-positive cells in the hippocampal CA1 area, indicating oxidative damage to nuclear DNA and neuronal apoptosis [11]. Our results indicate that oxidative stress is involved in the ischemia-mediated apoptotic cell death mechanisms [2,16,22]. Meanwhile, edaravone can ameliorate both oxidative and apoptotic neuronal death induced by transient global ischemia to the gerbils.

Multiple interactive mechanisms (e.g. free radicals and glutamate) contribute to neuronal death after transient forebrain ischemia in the gerbil. While there is a short-term burst of free radical production upon reperfusion in this model, there is also long-term oxidative stress [5,17]. By using pretreatment and dosing with edaravone for 72 h, the aim of this study was to protect from both acute and chronic oxidative stress but it should be noted that edaravone given after ischemia can still induce protection [15]. Edaravone was given every 12 h based on current medical practice in Japan and prior animal stroke studies [23]. Edaravone does, though, have a short plasma half-life and it is possible that more frequent administration would produce greater protection.

Although a number of studies have reported effects of edaravone on oxidative stress and delayed neuronal death after brain ischemia, few studies have focused on behavioral deficits. As a common outcome of ischemic brain injury, alleviating neurological deficits is important for the treatment of brain ischemia. Our present study examined the effect of edaravone on behavioral deficits after ischemia using locomotor activity and 8-arm radial maze tests. Locomotor activity can easily be assessed by placing the animals in a transparent cage equipped with infrared beams. It

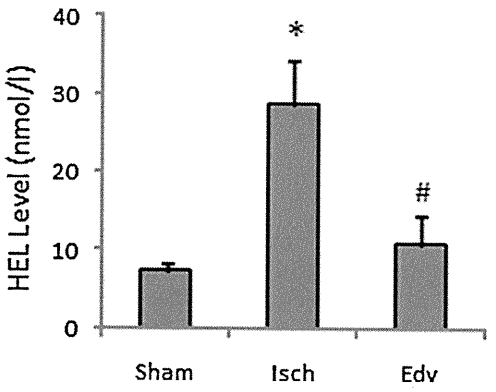

Fig. 3. Hippocampal HEL levels measured by ELISA in sham-operated, ischemia (Isch) or ischemia with edaravone (Edv) groups; $n=3$ per group. *$P<0.05$ compared to sham group; #$P<0.05$ compared to ischemia group.

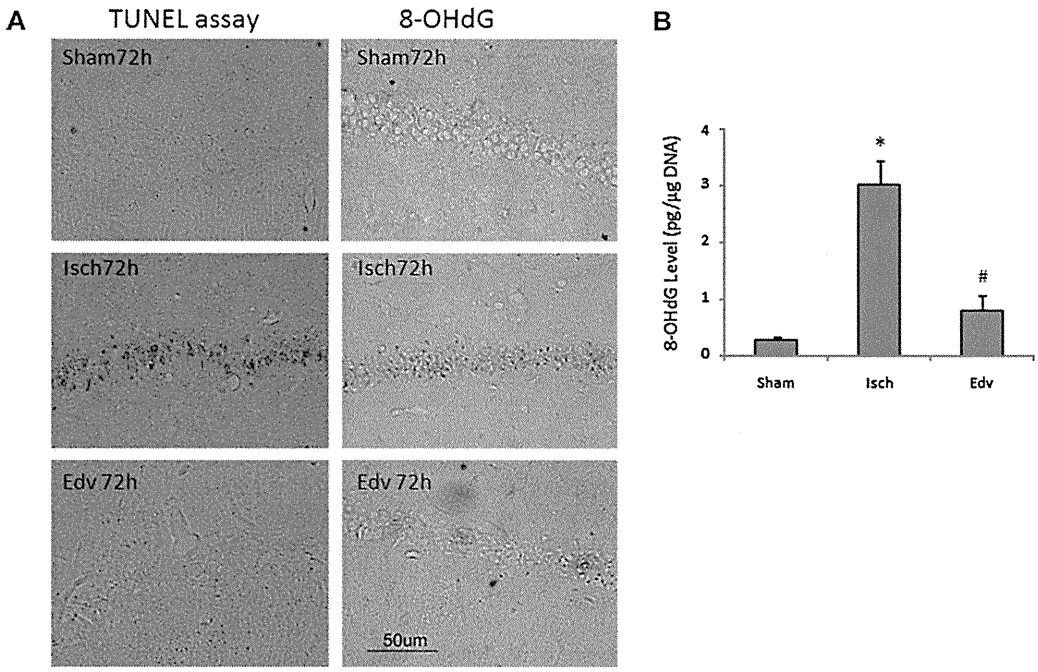

Fig. 4. Effect of edaravone on cell death and DNA oxidative damage following ischemia. (A) TUNEL and immunohistochemical staining for 8-OHdG in the hippocampal CA1 region in sham-operated gerbils (top panels), 72 h after ischemia (Isch; middle panels) and 72 h after ischemia with edaravone treatment (Edv, lower panels). Micrographs are at high magnification (400×). Note that TUNEL positive cells and 8-OHdG immunoreactivity were significantly elevated in the hippocampal CA1 region in the ischemia group and only weakly detected in that region in the edaravone-treated group. (B) 8-OHdG levels in the hippocampus of the three groups measured by ELISA; $n = 3$ per group. $^*P < 0.05$ compared with the sham group; $\#P < 0.05$ compared with ischemia group.

has been reported that brain ischemia increases locomotor activity [3,13]. The hyperactivity following ischemia might be attributed to hippocampal neuronal damage. Some even found a correlation between locomotor hyperactivity and hippocampal damage [7]. Although the neuronal damage in striatum may also be one reason to explain the locomotor hyperactivity following ischemia as it is involved in locomotor control [18], our present study revealed that edaravone decreased ischemia-induced hyperactivity and reduced hippocampal neuronal damage at the third day after transient forebrain ischemia, supporting the assumption that the hippocampal neuronal damage may be one reason for the hyperactivity after ischemia [7].

Recently, Jiao et al. reported that edaravone improved long-term cognitive dysfunction in a rat middle cerebral artery occlusion model tested with the Morris water maze task [8]. In our present study, we tested the effect of edaravone on memory impairment using 8-arm radial maze task and demonstrated that edaravone provides a protective role against learning and memory dysfunction after transient forebrain ischemia.

In conclusion, our findings suggest that edaravone treatment can reduce oxidative DNA damage and subsequent cell death, and may also be beneficial for the improvement of neurological deficits accompanying ischemic injury.

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Activation of the aldosterone/mineralocorticoid receptor system and protective effects of mineralocorticoid receptor antagonism in retinal ischemia-reperfusion injury

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ABSTRACT

The purpose of this project was to investigate the effects of the mineralocorticoid receptor antagonist against retinal ischemia-reperfusion injury and identify the aldosterone/mineralocorticoid receptor (MR) system in the rat retina. Retinal ischemia was induced by increasing intraocular pressure to 130 mmHg. Rats were treated with the angiotensin II type 1 receptor (AT1-R) antagonist (candesartan), MR antagonist (spironolactone), or aldosterone. Retinal damage was evaluated at 7 days after the ischemia by measuring the retinal thickness and the number of retinal ganglion cells. Pretreatment with candesartan, spironolactone, or candesartan and spironolactone significantly inhibited retinal ischemic injury. However, there was no protective effect against retinal ischemia-reperfusion injury provided by the combined aldosterone with candesartan treatment. Additionally, pretreatment with aldosterone alone also did not provide any neuroprotective effects against retinal ischemia-reperfusion injury. When rats were treated via local administration of aldosterone in the absence of ischemia, the number of retinal ganglion cells decreased while the retinal thickness remained unchanged. The present findings demonstrated the existence of a local aldosterone/MR system in the retina. Our results also demonstrated that an MR antagonist can attenuate subsequent ischemic damage in the rat retina.

The systemic renin-angiotensin-aldosterone system (RAAS) is known to play an important role in both the blood pressure and electrolyte homeostasis. In recent years, the local renin-angiotensin system (RAS) has been identified in several organs, including the heart, adrenal gland, ovary, thymus and eye (Jurklies et al., 1994, 1995; Pepperell et al., 1995; Wagner et al., 1996; Kohler et al., 1997; Rong et al., 1999, 2001; De Mello and Danser, 2000; Senanayake et al., 2007; Downie et al., 2009; Fletcher et al., 2010; Milenkovic et al., 2010). In human eyes, the mRNA for renin, angiotensin-converting enzyme (ACE), and angiotensinogen have been isolated. In addition, current data suggest that in humans there is local production of these constituents (Wagner et al., 1996; Senanayake et al., 2007; Fletcher et al., 2010). In several animal models, it has been reported that angiotensin II (Ang II) receptors were identified in the retina (Murata et al., 1997; Otani et al., 1998; Downie et al., 2009; Yang et al., 2009; Fukuda et al., 2010), and that Ang II was locally produced in the ocular tissue by a process that was independent of the blood-borne Ang II (Danser et al., 1994; Kohler et al., 1997). Although many studies have investigated the role of Ang II, relatively little attention has been paid to the role of aldosterone.

Aldosterone is a steroid hormone that elicits its effects by binding to the mineralocorticoid receptor (MR). It is released in response to a variety of stimuli, including Ang II. In the stroke-prone spontaneously hypertensive rat, which is a genetic model of spontaneous hypertension, it has been demonstrated that administration of either spironolactone (Rocha et al., 1998) or an ACE inhibitor (Stier et al., 1989, 1991; Rocha et al., 1999) greatly attenuates renal and cerebral vascular damage (MacLeod et al., 1997; Rocha et al., 1999). Likewise, in the remnant kidney hypertensive rat, administration of aldosterone was shown to reverse the renal protection that occurred after blockade of the RAAS by a combined ACE inhibition/Ang II type 1 receptor (AT1–R) antagonist treatment (Greene et al., 1996). Due to this phenomenon,

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which is referred to as "aldosterone escape" (Sato and Saruta, 2001), aldosterone may be present after such treatments and be able to influence the pathology independent of Ang II (Funck et al., 1997), thereby potentiating the actions of Ang II via the activation of the AT1-R and ACE (Robert et al., 1999; Harada et al., 2001).

We recently reported that both the ACE inhibitor and the AT1-R antagonist had a neuroprotective effect against retinal ischemia-reperfusion injury (Fukuda et al., 2010). The purpose of the present study was to investigate whether the MR-aldosterone system exists within the retina and whether it can influence retinal cell death in a rat retinal ischemia-reperfusion injury model.

1. Material and methods

1.1. Animals

Male Sprague–Dawley rats weighing 200–250 g were obtained from Charles River Japan (Yokohama, Japan). Rats were permitted free access to standard rat food (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. Animal care and all experiments were conducted in accordance with the approved standard guidelines for animal experimentation of the Kagawa University Faculty of Medicine, and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

1.2. Drugs

Aldosterone and the MR antagonist, spironolactone, were obtained from Sigma–Aldrich (St. Louis, MO) and the AT1-R antagonist, candesartan, was obtained from TRC (North York, Canada). Aldosterone and candesartan were dissolved in dimethyl sulfoxide (DMSO) to produce stock solutions, which were then diluted to the final required concentrations. The final DMSO concentration never exceeded 5%. Candesartan (1 mg/kg) was administered intraperitoneally 30 min before induction of the ischemia. Based on previously reported methodologies, (Weber et al., 1993; Young et al.,

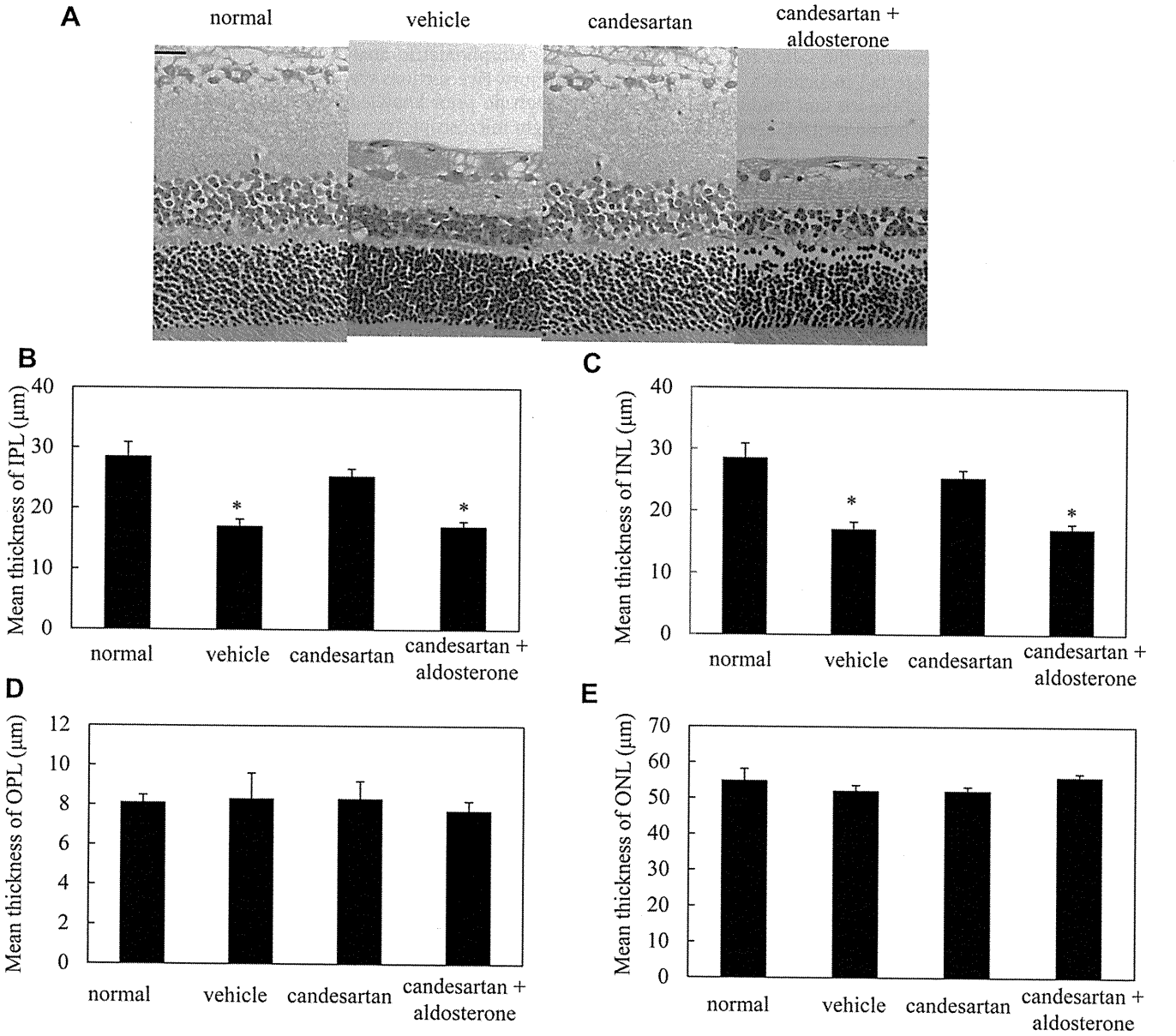

Fig. 1. Change in mean thickness of the inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), and outer nuclear layer (ONL) at 7 days after ischemia in the presence of vehicle, 1 mg/kg candesartan, or 1 mg/kg candesartan + 80 μg/kg/day aldosterone. Results are expressed as the mean ± SEM ($n = 5$ in each group). *$P < 0.05$ versus normal (Dunnett's multiple comparison test). Scale bar, 10 μm.

1995) aldosterone was either administered by a subcutaneous osmotic minipump (80 μg/kg/day) (Alzet model 1007D, Alza Corporation, Mountain View, CA) 1 day before the ischemia or was injected into the vitreous space (80 μg/kg) 30 min prior to the ischemia. The minipumps were implanted subcutaneously into the mid-scapular region. Spironolactone was dissolved in carboxymethyl cellulose (CMC) to produce the stock solution, which was then orally administered to animals on a daily basis via the use of feeding needles. The final CMC concentration never exceeded 0.5%. As a control, animals were treated with vehicle (distilled water, 5% DMSO in PBS, or 0.5% CMC in PBS).

1.3. Ischemia

Rats were anesthetized using an intraperitoneal injection of 50 mg/kg pentobarbital sodium (Abbott, Abbott Park, IL), and 0.4% oxybuprocaine hydrochloride, which was administered topically. The anterior chamber of the right eye was cannulated with a 27-gauge infusion needle connected to a reservoir containing normal saline. The intraocular pressure (IOP) was raised to 130 mmHg for 45 min by elevating the saline reservoir. Only the right eye of each rat was subjected to ischemia. Retinal ischemia was indicated by whitening of the iris and fundus. The left eye of each rat served as the nonischemic control. Rectal and tympanic temperatures were maintained at approximately 37 °C, using a feedback-controlled heating pad (BRC, Nagoya, Japan) during the operation. After restoration of blood flow, temperature was continually maintained at 37 °C.

1.4. Histological examination

For the histological examination, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) at 7 days after ischemia and then perfused intracardially with phosphate-buffered saline (PBS), followed by perfusion with 4% paraformaldehyde in PBS. The anterior segments, including the lens, were removed. The posterior eyecups were embedded in paraffin, and thin sections (5-μm thickness) were cut using a microtome. The sections were carefully cut to include the full length from superior to inferior along the vertical meridian through the optic nerve head. Each eye was mounted on a silane-coated glass slide and then stained with hematoxylin and eosin (HE). Scleral thickness was measured to confirm that the sections were not oblique sections.

Morphometric analysis was performed to quantify ischemic injury. Five sections were randomly selected in each eye. A person with no prior knowledge of the treatments performed all of the light microscopic (magnification; 10 × 100; Olympus BX-51, Tokyo,

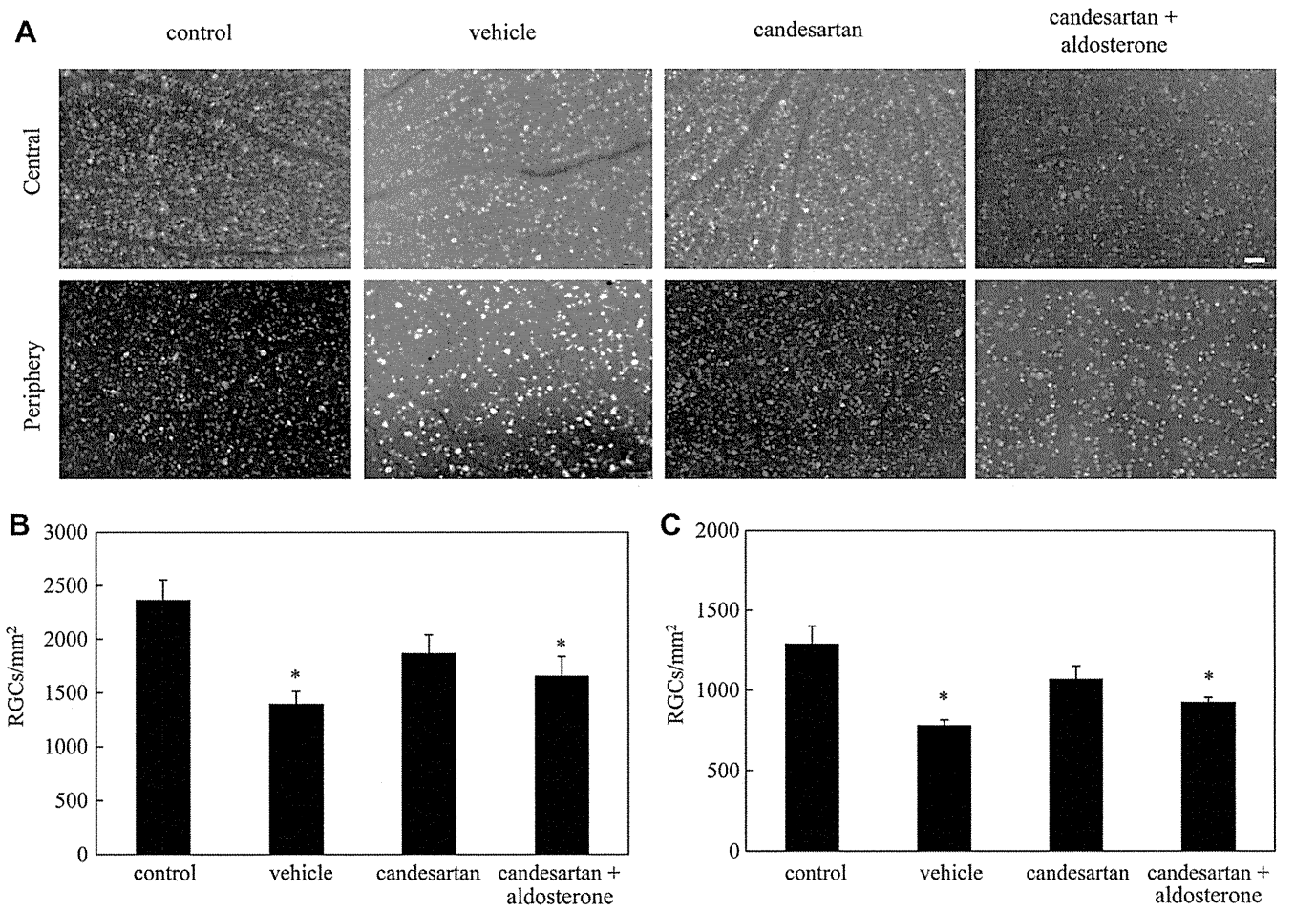

Fig. 2. Effect of candesartan + aldosterone on ischemia-induced retinal ganglion cell death. (A) Retrograde labeling of retinal ganglion cells (RGCs) in nonischemic eyes and at 7 days after ischemic injury after the administration of vehicle, candesartan, or candesartan + aldosterone. Micrographs of the central and peripheral areas were taken approximately 1 and 4 mm from the optic nerve head. Scale bar, 100 μm. RGCs were counted in the central (B) and peripheral (C) areas at approximately 1 and 4 mm from the optic nerve head. Graph depicts the mean ± SEM for the vehicle-treated animals with or without ischemia, candesartan, or candesartan + aldosterone ($n = 4$ in each group). *$P < 0.05$ versus control (Dunnett's multiple comparison test).

Japan) examinations. A microscopic image of each section within 0.5–1 mm superior of the optic disc was scanned. In each computer image, the thickness of the inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL) and outer nuclear layer (ONL) were measured.

1.5. Retrograde labeling of retinal ganglion cells

At 7 days prior to sacrifice, hydroxystilbamidine (Molecular Probes Inc., Eugene, OR) was injected bilaterally into the superior colliculi of anesthetized rats. The skull was exposed and kept dry and clean. After identifying and marking the bregma, a small window was drilled in the scalp in both the right and left hemispheres. The windows were drilled to a depth of 3.6 mm from the surface of the skull and located at 6.8 mm behind the bregma on the anteroposterior axis, and 1.5 mm lateral to the midline. Using a Hamilton syringe, 1.5 μl of 2% hydroxystilbamidine was slowly injected into the bilateral superior colliculi. After suturing the skin over the wound, antibiotic ointment was applied.

1.6. Tissue preparation and assessment of RGC survival

Animals were sacrificed using an overdose of Nembutal at 1 week after 2% hydroxystilbamidine (Molecular Probes Inc.) application. Whole, flat-mounted retinas were then assayed for retinal ganglion cell density. Rat eyes were enucleated and fixed in 4% paraformaldehyde for 10 h at room temperature. After removal of the anterior segments, the resultant posterior eyecups were left in place. Subsequently, four 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 four radial cuts, and then spread on a gelatin-coated glass slide. Labeled retinal ganglion cells (RGCs) were visualized under a fluorescence

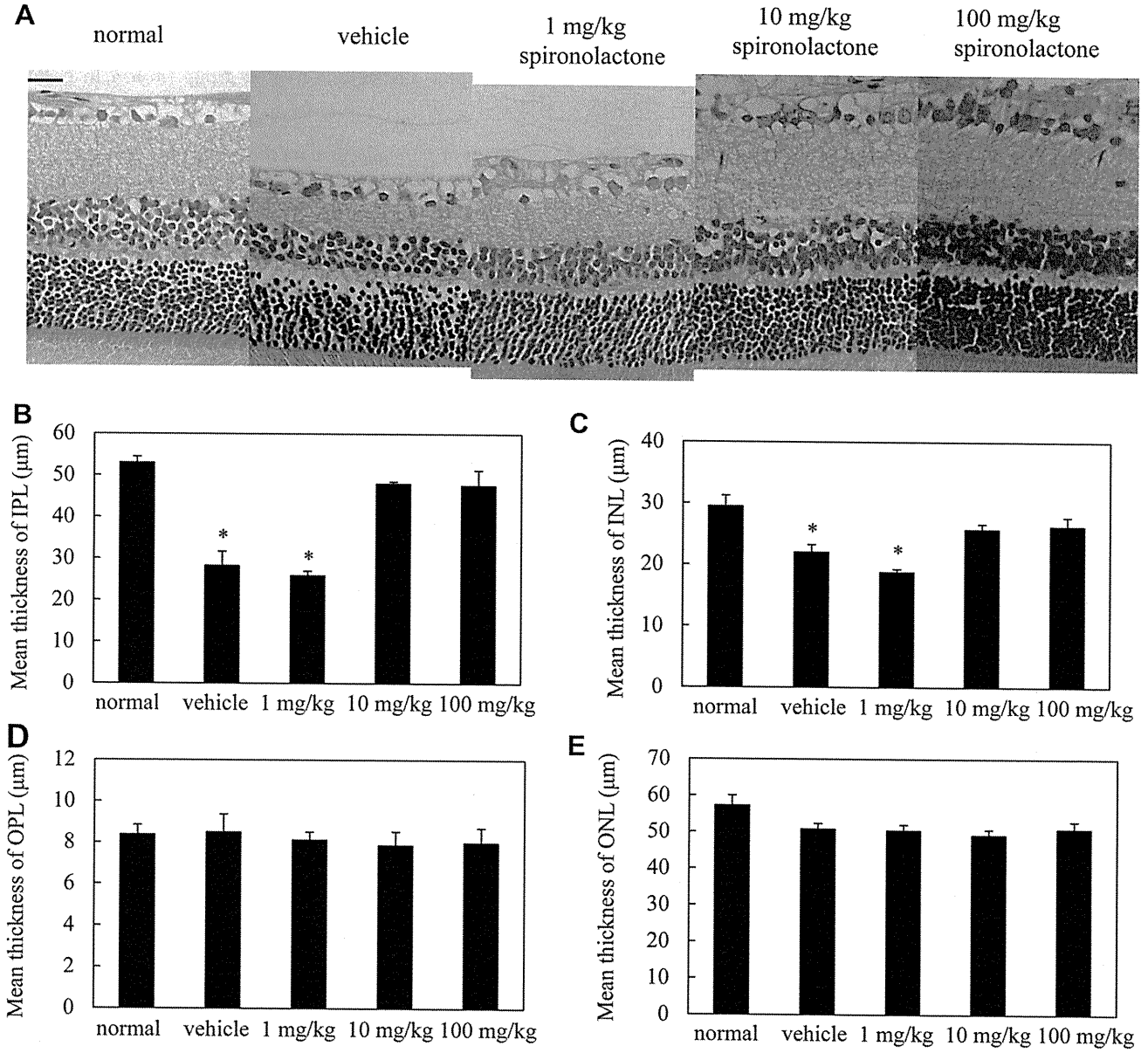

Fig. 3. Change in mean thickness of the IPL, INL, OPL, and ONL at 7 days after ischemia without spironolactone or with 1, 10 and 100 mg/kg spironolactone. Data express the mean ± SEM ($n = 4$ in each group). *$P < 0.05$ versus normal (Dunnett's multiple comparison test). Scale bar, 10 μm.

microscope (Olympus BX-51/DP70, Olympus, Tokyo, Japan) with an ultraviolet filter (blue-violet: 395–440 nm). Fluorescence-labeled RGCs were counted in 12 microscopic fields of retinal tissue from two regions in each quadrant at two different eccentricities, 1 mm (central) and 4 mm (peripheral) away from the optic disc. Image-Pro Plus software (Version 4.0, Media Cybernetics, Bethesda, MD) was used to count the total number of RGCs in each eye.

1.7. Statistical analysis

All data are presented as the mean ± SEM. Data were analyzed using an independent Student's *t*-test or Dunnett's multiple comparison test, as appropriate. Statistical analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL). A P value of less than 0.05 was considered statistically significant.

2. Results

2.1. Histologic changes in the retina after ischemia with aldosterone

Thicknesses in the normal retina were 48.0 ± 2.5 μm for IPL, 27.2 ± 2.3 μm for INL, 8.2 ± 0.4 μm for OPL, and 54.8 ± 2.7 μm for ONL ($n = 5$) (Fig. 1). Thickness measurements in animals pretreated with distilled water were 27.6 ± 2.7 μm ($P < 0.001$) for IPL, 17.0 ± 0.9 μm ($P < 0.001$) for INL, 8.4 ± 0.5 μm ($P = 0.980$) for OPL, and 51.6 ± 1.3 μm ($P = 0.405$) for ONL ($n = 5$) (Fig. 1). Thicknesses in animals pretreated with 1 mg/kg candesartan were 41.8 ± 1.8 μm ($P = 0.129$) for IPL, 25.4 ± 1.0 μm ($P = 0.681$) for INL, 8.3 ± 0.7 μm ($P = 0.999$) for OPL, and 51.8 ± 1.0 μm ($P = 0.454$) for ONL ($n = 5$). However, in animals pretreated with 1 mg/kg candesartan and 80 μg/kg/day aldosterone, the thicknesses were 24.5 ± 1.0 μm ($P < 0.001$) for IPL, 17.0 ± 0.7 μm ($P < 0.001$) for INL, 7.8 ± 0.2 ($P = 0.760$) for OPL, and 55.0 ± 1.1 μm ($P > 0.999$) for ONL ($n = 5$). Thus, treatments combining aldosterone with candesartan provided no protective effect against retinal ischemia-reperfusion injury.

2.2. Effect of aldosterone on RGC survival

Fig. 2A shows representative results for the RGC labeling in the vehicle-, in the candesartan-, and in the candesartan and aldosterone-treated rats. While RGC death was mild in the candesartan-treated rats, treatments that also included aldosterone blocked the neuroprotective effects of the candesartan. In the central retina, the number of RGCs in the eyes with ischemia were 1394 ± 120 in the vehicle-treated group ($P = 0.005$), 1865 ± 176 ($P = 0.145$) in the candesartan-treated group, and 1652 ± 187 ($P = 0.031$) in the candesartan plus aldosterone-treated group ($n = 4$ in each group) (Fig. 2B). The number of RGCs in the peripheral retinas of the eyes with ischemia were 781 ± 36 in the vehicle-treated group ($P = 0.001$), 1069 ± 83 ($P = 0.137$) in the candesartan-treated group, and 925 ± 31 ($P = 0.012$) in the candesartan plus aldosterone-treated group ($n = 4$ in each group) (Fig. 2C).

2.3. Effect of spironolactone on the retina after ischemia

Thicknesses in the normal retina were 53.0 ± 1.5 μm for IPL, 29.5 ± 1.8 μm for INL, 8.4 ± 0.5 μm for OPL, and 56.0 ± 3.2 μm for ONL ($n = 4$) (Fig. 3). Thicknesses in the animals orally administered

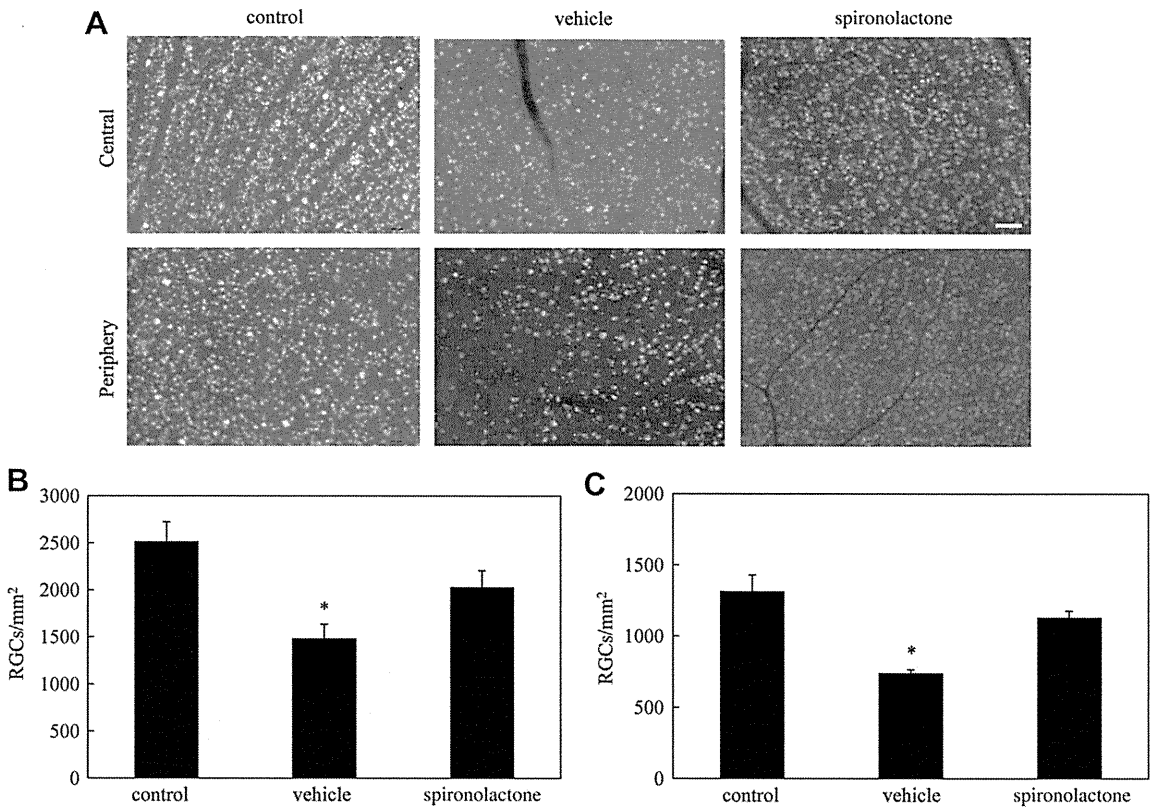

Fig. 4. Effect of spironolactone on ischemia-induced retinal ganglion cell death. (A) Retrograde labeling of RGCs in nonischemic eyes and at 7 days after ischemic injury after administration of vehicle or 10 mg/kg spironolactone. Micrographs of the central and peripheral areas were taken approximately 1 and 4 mm from the optic nerve head. Scale bar, 100 μm. RGCs were counted in the central (B) and peripheral (C) areas at approximately 1 and 4 mm from the optic nerve head. Graph depicts the mean ± SEM for the vehicle-treated animals with or without ischemia or spironolactone ($n = 4$ in each group). *$P < 0.05$ versus control (Dunnett's multiple comparison test).

1, 10, or 100 mg/kg/day dose of spironolactone were 25.9 ± 1.1 μm ($P < 0.001$), 48.0 ± 0.5 μm ($P = 0.403$) and 47.6 ± 3.6 μm ($P = 0.344$) for IPL, 18.8 ± 0.5 μm ($P < 0.001$), 25.8 ± 0.9 μm ($P = 0.16$) and 26.3 ± 1.5 μm ($P = 0.253$) for INL, 8.1 ± 0.4 μm ($P = 0.996$), 7.9 ± 0.7 μm ($P = 0.948$) and 8.0 ± 0.7 μm ($P = 0.981$) for OPL, and 50.4 ± 1.6 μm ($P = 0.211$), 50.1 ± 1.5 μm ($P = 0.101$) and 50.8 ± 2.0 μm ($P = 0.259$) for ONL, respectively ($n = 4$ in each group) (Fig. 3).

2.4. Effect of spironolactone on RGC survival

Fig. 4A shows representative results of the RGC labeling in both the vehicle- and the spironolactone (10 mg/kg/day)-treated rats. As seen in Fig. 4B, the number of RGCs in the central retinas of the eyes with ischemia were 1480 ± 156 in the vehicle-treated group ($P = 0.006$) and 2025 ± 179 ($P = 0.159$) in the spironolactone-treated group. In the peripheral retina, the number of RGCs in the eyes with ischemia were 738 ± 28 in the vehicle-treated group ($P = 0.001$) and 1128 ± 48 ($P = 0.188$) in the spironolactone-treated group ($n = 4$ in each group).

2.5. Renin-angiotensin-aldosterone system in the retina

As shown in Fig. 5, there was no protective effect against retinal ischemic damage after pretreatment with aldosterone ($n = 4$). However, a protective effect against retinal ischemic damage was seen after pretreatment with spironolactone and candesartan (Fig. 5) ($n = 4$).

2.6. Effect of local administration of aldosterone

Although treatment with local administration of aldosterone without ischemia did not affect the retinal thickness in the normal rat, a significant decrease in the number of RGCs was observed (Fig. 6) ($n = 4$). Results for treatment with local administration of aldosterone 30 min before ischemia were similar to those found after systemic administration of aldosterone (Fig. 6) ($n = 4$). No protective effects against retinal ischemia-reperfusion injury were noted after the treatments that combined local administration of aldosterone with candesartan (Fig. 6) ($n = 5$).

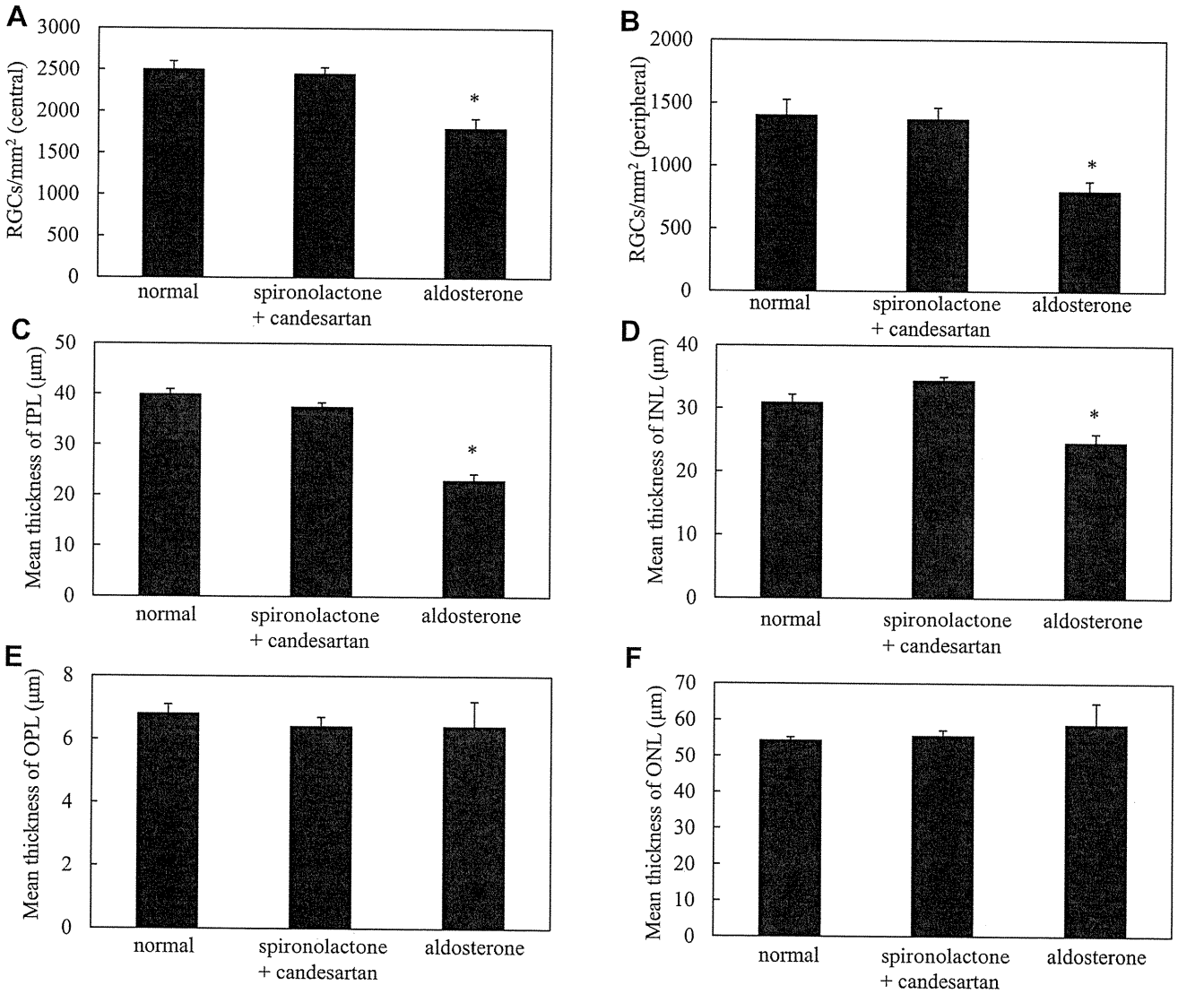

Fig. 5. RGC survival and change in mean thickness of the IPL, INL, OPL, and ONL at 7 days after ischemia and treatments with either aldosterone, or spironolactone and candesartan. Data express the mean ± SEM ($n = 4$ in each group). *$P < 0.05$ versus normal (Dunnett's multiple comparison test).

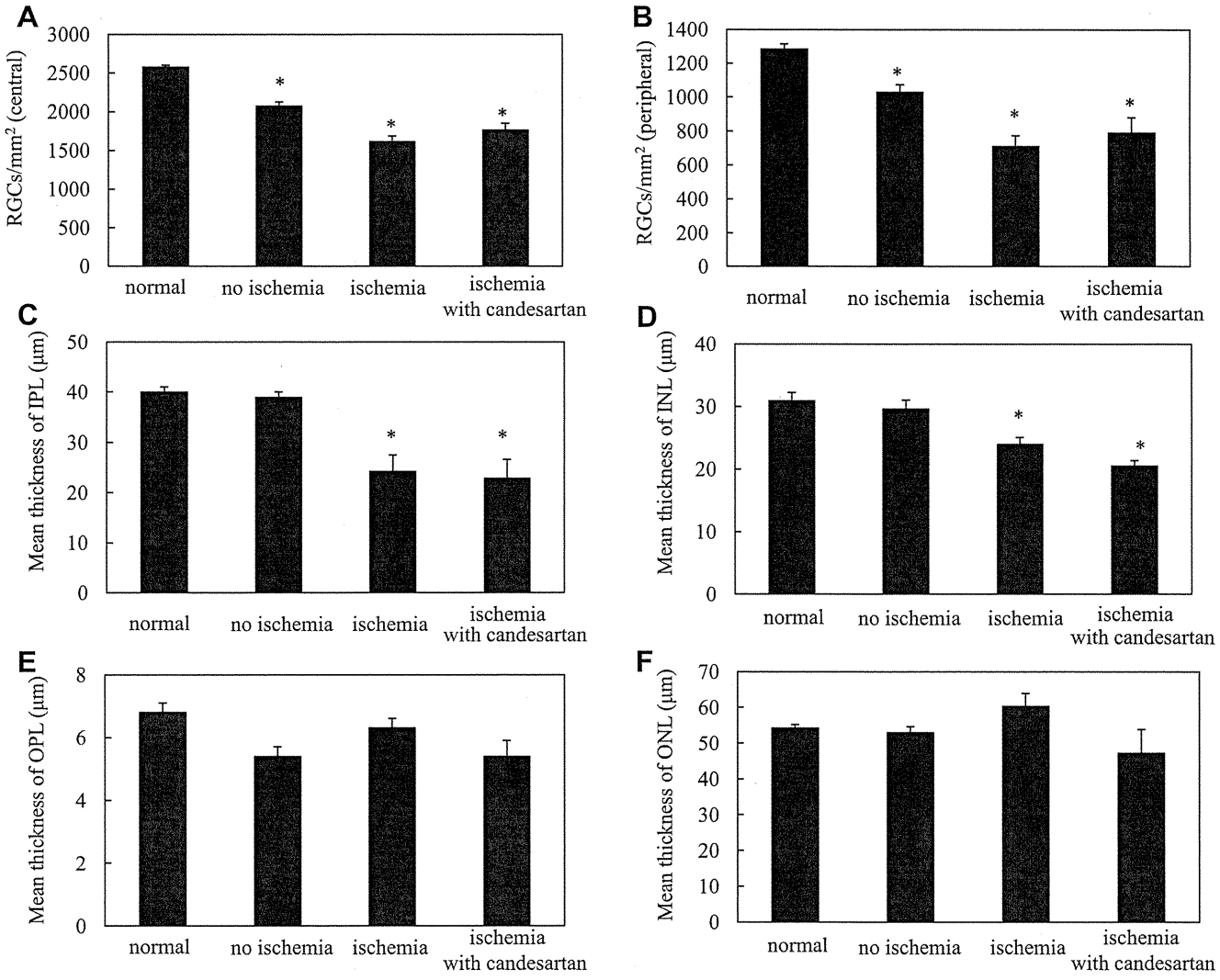

Fig. 6. RGC survival and change in mean thickness of the IPL, INL, OPL, and ONL at 7 days after local administration of aldosterone. Data express the mean ± SEM. *$P < 0.05$ versus normal (Dunnett's multiple comparison test).

3. Discussion

The present study demonstrated that the protective effect of candesartan was lost when retinal ischemic rats were administered aldosterone. Similarly, aldosterone antagonism with spironolactone also decreased the retinal ischemia-reperfusion injury.

In a recent study that used a rat model of oxygen-induced retinopathy, it was shown that the eye expresses MRs and that these receptors are involved in retinal vascular pathology (Wilkinson-Berka et al., 2009). In addition, the authors also noted that MR antagonism led to a reduction of the pathological angiogenesis normally associated with the inflammation and oxidative stress in this rat model (Wilkinson-Berka et al., 2009). Therefore, it is conceivable that the beneficial effects on the neurologic outcome of ischemia-reperfusion injury that are observed after spironolactone administration may be related to MR inhibition.

Aldosterone synthase is the rate-limiting enzyme in aldosterone production. In the adrenal gland and cardiovascular tissues, Ang II stimulates aldosterone synthase via the AT1-R (Rogacz et al., 1990) and AT1-R blocker (ARB), which causes a reduction in the aldosterone-related pathology (Harada et al., 2001; Fukuda et al., 2010). A previous study has reported finding enhanced MR signaling in the kidney and heart (Williams et al., 2004). Additionally, this study also demonstrated that eplerenone, which is an aldosterone antagonist, was able to dramatically retard the progression of renal and cardiac diseases. Thus, as was pointed out by the authors of this previous study, plasma renin–aldosterone profiles are not always predictive of the antihypertensive efficacy of MR blockade. Overall, these findings raise the possibility that molecules other than circulating aldosterone may be involved in the activation of the MR.

We also have recently reported that candesartan, which is an ARB, had a neuroprotective effect against retinal ischemia-reperfusion injury because of its ability to reduce oxidative stress (Fukuda et al., 2010). When aldosterone was administered to rats receiving candesartan, there was a complete reversal of the RAAS suppression-induced neuroprotective effect against the retinal ischemia-reperfusion injury. In addition, we have also demonstrated that spironolactone had a neuroprotective effect against retinal ischemia-reperfusion injury. When taken together, these findings suggest that MR and aldosterone are able to influence ischemic damage in the retina and thus, these MR antagonists could potentially be used for therapeutic application in cases of retinal ischemic damage.

AT1-R is known to be expressed primarily by RGCs, including Müller cells and astrocytes (Downie et al., 2009). We previously reported that AT1-R was present in RGCs and the INL of the ischemic retina (Fukuda et al., 2010). Additionally, ARBs reduce aldosterone synthase mRNA in oxygen-induced retinopathy (Wilkinson-Berka et al., 2009). This indicates that aldosterone synthase should be produced by AT1-R-positive cells. It has also been demonstrated that MRs are present in RGCs, the INL, retinal pigment epithelium, and in the vasculature (Wilkinson-Berka et al., 2009). Since a decrease occurred in both the INL thickness and the number of RGCs after ischemic-reperfusion injury, we assumed that this could be caused by the death of AT1-R- or MR-positive cells. In the present or previous (Fukuda et al., 2010) study, we showed that systemic administration of candesartan was able to inhibit this retinal ischemic damage. While it is possible that plasma candesartan might reach the inner retina and play a part in this inhibition, as of yet, we do not know if the plasma Ang II is also capable of reaching the inner retina. Future studies that definitively clarify the mechanism of this inhibition will need to be conducted.

The present study demonstrated that a local aldosterone/MR system exists within the retina and thus, MR blockade might be of potential therapeutic benefit in retinal ischemic insult cases.

Acknowledgments

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