

# Edaravone Prevents Retinal Degeneration in Adult Mice Following Optic Nerve Injury

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**PURPOSE.** To assess the therapeutic potential of edaravone, a free radical scavenger that is used for the treatment of acute brain infarction and amyotrophic lateral sclerosis, in a mouse model of optic nerve injury (ONI).

**METHODS.** Two microliters of edaravone (7.2 mM) or vehicle were injected intraocularly 3 minutes after ONI. Optical coherence tomography, retrograde labeling of retinal ganglion cells (RGCs), histopathology, and immunohistochemical analyses of phosphorylated apoptosis signal-regulating kinase-1 (ASK1) and p38 mitogen-activated protein kinase (MAPK) in the retina were performed after ONI. Reactive oxygen species (ROS) levels were assessed with a CellROX Green Reagent.

**RESULTS.** Edaravone ameliorated ONI-induced ROS production, RGC death, and inner retinal degeneration. Also, activation of the ASK1-p38 MAPK pathway that induces RGC death following ONI was suppressed with edaravone treatment.

**CONCLUSIONS.** The results of this study suggest that intraocular administration of edaravone may be a useful treatment for posttraumatic complications.

**Keywords:** edaravone, oxidative stress, neuroprotection, ASK1, retinal ganglion cell

Traumatic optic neuropathy is a common clinical problem that occurs in 0.5% to 5% of patients with closed head injury.<sup>1</sup> Damage to the optic nerve induces secondary swelling within the optic canal, accompanied by subsequent retinal ganglion cell (RGC) loss and optic nerve atrophy.<sup>2</sup> Although no large natural history or randomized controlled trials have been published, corticosteroid therapy and optic canal decompression surgery are not considered to be effective for patients with traumatic optic neuropathy.<sup>3</sup> Research into finding therapeutic targets for treatment of traumatic optic neuropathy indicated that neuroprotection might be an effective strategy and studies using an optic nerve injury (ONI) model in rodents have provided useful information. For example, neurotrophins, such as brain-derived neurotrophic factor and ciliary neurotrophic factor, protect retinal RGCs in an ONI model.<sup>4–6</sup> Also, inhibitors of glutamate receptors, tumor necrosis factor receptors, and nitric oxide synthase may be effective for RGC protection.<sup>7–11</sup> The ONI model mimics some aspects of glaucoma, including RGC death induced by oxidative stress, and therefore, it is also a useful animal model for glaucoma.

Edaravone is a free radical scavenger that has been used clinically to treat acute brain infarction and amyotrophic lateral sclerosis (ALS). Edaravone quenches hydroxyl radicals (OH) and inhibits lipid peroxidation dependent and independent of OH.<sup>12,13</sup> In an in vitro cell culture study using the RGC-5 to study the neurobiology of RGCs, edaravone scavenged the intracellular OH, superoxide anion (O<sub>2</sub><sup>-</sup>), and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) while demonstrating the strongest scavenging activity against OH.<sup>14</sup> Recent studies have shown that oxidative stress plays an important role in many ocular diseases including glaucoma.<sup>15–18</sup> Interestingly, edaravone attenuates retinal ischemia/reperfusion injury in rats<sup>19</sup> and retinal damage in

experimental rats with glaucoma showing high intraocular pressure.<sup>20</sup> These findings suggest a possibility that edaravone protects RGCs by scavenging reactive oxygen species (ROS). In the present study, we examined the effects of intraocular injection of edaravone on RGC protection following ONI.

## MATERIALS AND METHODS

### Mice and ONI

Experiments were performed using 8- to 10-week-old C57BL/6J mice (CLEA Japan, Tokyo, Japan) in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were anesthetized with sodium pentobarbital before ONI. Optic nerves were exposed intraorbitally and crushed at approximately 0.5 to 1.0 mm from the posterior pole of the eyeball with fine surgical forceps for 5 seconds.<sup>21,22</sup> Two microliters of edaravone (7.2 mM; Mitsubishi Tanabe Pharma Co., Osaka, Japan) or vehicle (phosphate-buffered saline [PBS]) were injected intraocularly to control mice or 3 minutes after ONI. The mice were euthanized at 7 days after the injection. Once the surgery was completed, the mice were assigned identification numbers randomly so that the nature of treatment (vehicle or edaravone) was masked to evaluators of the results.

### Imaging Acquisition of Spectral-Domain Optical Coherence Tomography (SD-OCT)

SD-OCT (RS-3000; Nidek, Aichi, Japan) examinations were performed at just before and 7 days after ONI as previously



reported.<sup>22-24</sup> Briefly, a 60-D adapter lens was placed on the objective lens of the Multiline optical coherence tomography (OCT) to focus on the mouse retina. All the images were location matched manually, scanning vertically through the center of the optic nerve head at a three-disc diameter length above the optic nerve head. The average thickness of the ganglion cell complex (GCC; between the internal limiting membrane and the interface of the inner plexiform layer and the inner nuclear layer) was measured. In this study, the maximum number of B-scans set by the manufacturer (50 for line scans) was used for averaging.

### Histological and Morphometric Studies

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital and perfused transcardially with saline, followed by Zamboni's fixative (2% paraformaldehyde and 15% picric acid in 0.1 M phosphate buffer). Eyes were removed, postfixed, and processed as previously reported.<sup>25,26</sup> Briefly, eyes were embedded in paraffin wax, and sagittal sections through the optic nerve at the thickness of 7  $\mu\text{m}$  were collected and stained with hematoxylin and eosin. The number of neurons in the ganglion cell layer (GCL) of the retina was counted from one ora serrata through the optic nerve to the other ora serrata. In the same section, the thickness of the inner retinal layer (IRL; between the internal limiting membrane and the interface of the outer plexiform layer with the outer nuclear layer) was measured.

### Retrograde Labeling

Mice were deeply anesthetized with isoflurane (Intervet, Tokyo, Japan), placed on a stereotaxic frame, and injected with 2  $\mu\text{L}$  1% Fluoro-Gold (FG; Fluorochrome, LLC, Denver, CO, USA) dissolved in PBS into the superior colliculus.<sup>24,27</sup> Ten days later, the ONI procedure was performed and 17 days after FG application, mice were anesthetized, eyes were enucleated, and retinas were isolated for whole mount preparation. Retinas were fixed in Zamboni's fixative for 20 minutes, mounted on a glass slide with a mounting medium (Vectashield; Vector Laboratories, Burlingame, CA, USA), and the RGC density was examined with a fluorescent microscope. One central (0.1 mm from the optic disc) and one peripheral (1.5 mm from the optic disc) areas (0.04 mm<sup>2</sup>) per quadrant of each retina were chosen. FG-labeled cells were counted, and the mean number of RGCs per square millimeter was calculated.

### Immunohistochemistry

Mice were perfused with Zamboni's fixative at 6 hours after ONI. Eyes were enucleated, postfixed in Zamboni's fixative for 1 hour and then transferred into a sucrose buffer (30% sucrose in a 0.1 M phosphate buffer) for cryoprotection. Retinal cryostat sections of 10- $\mu\text{m}$  thickness were prepared and examined by immunostaining using a phospho-apoptosis signal-regulating kinase-1 (ASK1) rabbit polyclonal antibody (Bioss, Boston, MA, USA) and phospho-p38 mouse monoclonal antibody (Promega, Madison, WI, USA). Images of both phosphoproteins were acquired with the same settings, including the exposure time, to quantify the intensity at the GCL, and analyzed using the National Institutes of Health (NIH) Image program (ImageJ 1.50c4; NIH, Bethesda, MD, USA).

### Detection of Oxidative Stress Using CellROX Green Reagent

The oxidative stress level was detected using CellROX Green Reagent, which is a novel fluorogenic probe for measuring

oxidative stress in cells, including RGCs.<sup>28</sup> Mice were killed by cervical dislocation at 6 hours after ONI and eyes were enucleated. Retinas were isolated for a whole-mount preparation in cold PBS. Isolated retinas were immersed in a 5- $\mu\text{M}$  concentration of the CellROX Green Reagent (Life Technologies, Rockville, MD, USA) at 4°C for 45 minutes with agitation. After washing with PBS, retinas were fixed with Zamboni's fixative at 4°C for 30 minutes and flat mounted onto glass slides. For detection of ROS, images were observed and captured using an Olympus BX51 Microscope (Olympus, Tokyo, Japan). Images were acquired with the same settings, including the exposure time, to quantify the intensity of the CellROX Green Reagent, and analyzed using the NIH Image program (ImageJ 1.50c4).

### Statistics

Data are presented as means  $\pm$  SEM. When statistical analyses were performed, the 1-way ANOVA followed by the Tukey-Kramer post hoc test was used.  $P < 0.05$  was regarded as statistically significant. JMP version 12.2.0 (SAS Institute, Inc., Cary, NC, USA) was used for the statistical analyses.

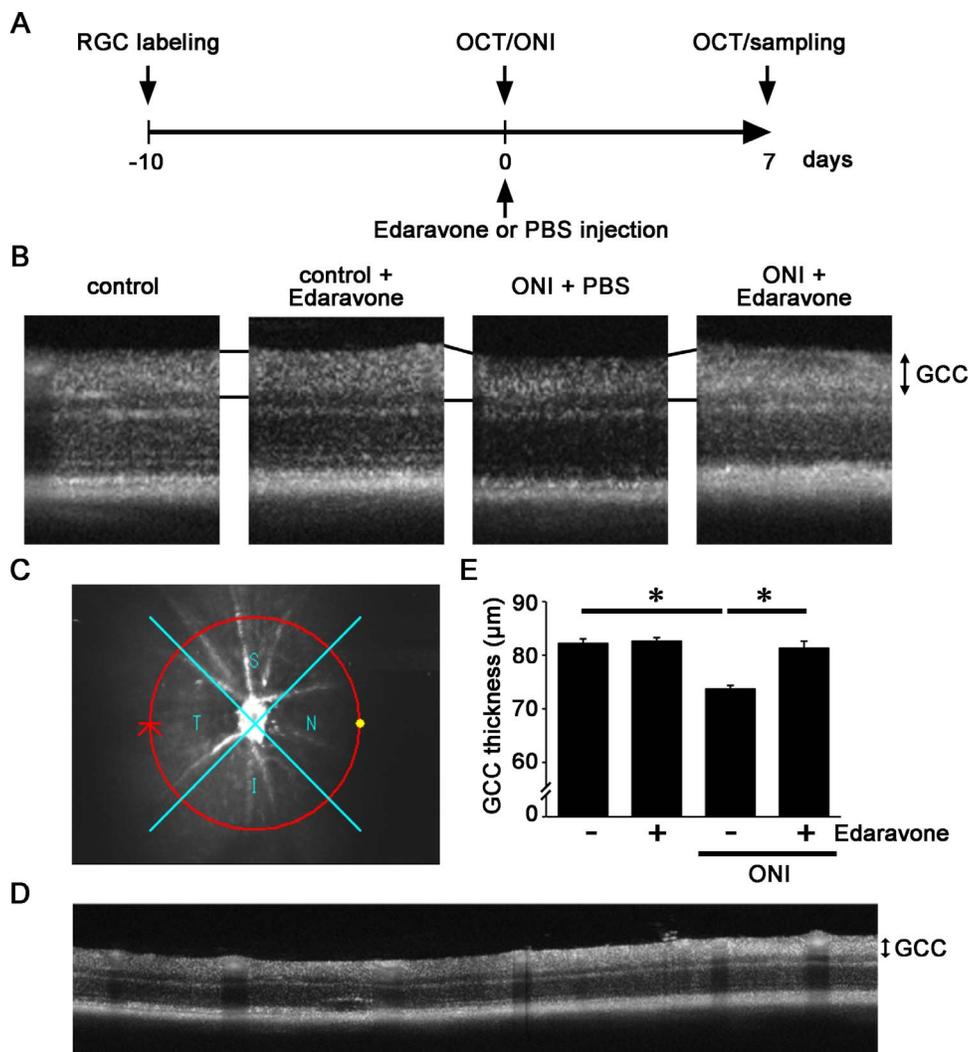
## RESULTS

### Edaravone Protects Retinal Neurons After ONI

To investigate whether edaravone prevents retinal degeneration, we administered edaravone or PBS intraocularly to adult mice 3 minutes after ONI (Fig. 1A). We visualized retinal layers in living mice using SD-OCT, a noninvasive imaging technique that can be used to acquire cross-sectional tomographic images of the retina in vivo.<sup>22-24</sup> The average thickness of the GCC, which includes the nerve fiber layer, GCL, and the inner plexiform layer, was markedly greater in edaravone-treated mice compared with PBS-treated mice (Fig. 1B). For quantitative analysis, GCC was measured by scanning the retina in a circle centering around the optic nerve disk (Fig. 1C), and the average GCC thickness was determined from acquired images (Fig. 1D). GCC thickness was significantly reduced in PBS-treated mice ( $73.8 \pm 0.5 \mu\text{m}$ ,  $n = 6$ ;  $P < 0.01$ ) compared with control mice ( $82.2 \pm 0.9 \mu\text{m}$ ,  $n = 6$ ), but edaravone significantly suppressed the thinning of the GCC ( $81.3 \pm 1.3 \mu\text{m}$ ,  $n = 6$ ;  $P = 0.92$ ) (Fig. 1E). In addition, edaravone showed no toxic effects in control mice ( $82.6 \pm 0.7 \mu\text{m}$ ,  $n = 6$ ;  $P = 0.99$ ) (Figs. 1B, 1E).

We also analyzed the histopathology of the retina before and after ONI (Fig. 2A). ONI induced severe RGC loss in PBS-treated mice ( $290 \pm 77$  cells/section,  $n = 6$ ;  $P < 0.01$ ) compared with control mice ( $563 \pm 31$  cells/section,  $n = 6$ ), but the number of surviving neurons in the GCL was significantly higher in edaravone-treated mice ( $445 \pm 64$  cells/section,  $n = 6$ ;  $P < 0.05$ ) (Figs. 2A, 2B). Also, the thickness of the IRL was significantly greater in edaravone-treated mice ( $111 \pm 6 \mu\text{m}$ ,  $n = 6$ ;  $P < 0.05$ ) compared with PBS-treated mice ( $92 \pm 2 \mu\text{m}$ ,  $n = 6$ ) (Figs. 2A, 2C), which are consistent with the results from the OCT (Fig. 1E). Edaravone showed no toxic effects in control mice ( $555 \pm 20$  cells/section,  $n = 6$ ;  $P = 0.99$  and  $121 \pm 4 \mu\text{m}$ ,  $n = 6$ ;  $P = 0.58$ ) (Fig. 2). These data indicate that edaravone treatment prevents retinal degeneration following ONI.

Because the GCL contains cell types other than RGCs including displaced amacrine cells,<sup>29</sup> we next performed retrograde labeling of RGCs with FG and determined the effect of edaravone on RGC survival. Consistent with the results of cell counting in the GCL (Fig. 2B), the RGC number



**FIGURE 1.** In vivo imaging of the retina in control and edaravone-treated mice. (A) Experimental protocols. Two microliters of edaravone (7.2 mM) or PBS were injected intracocularly into control mice or 3 minutes after ONI. The mice were euthanized at 7 days after the injection. (B) OCT cross-sectional images of retinas. (C) An image of a circle centered around the optic disc. (D) An OCT circular scan image captured from (C). (E) Longitudinal evaluation of the GCC thickness by a circular scan. The data are presented as mean  $\pm$  SEM of six retinas for each experiment. \* $P < 0.05$ .

in edaravone-treated mice ( $2168 \pm 87$  cells/mm<sup>2</sup>,  $n = 6$ ;  $P < 0.01$ ) was significantly increased compared with PBS-treated mice ( $1466 \pm 49$  cells/mm<sup>2</sup>,  $n = 6$ ) in the central retina (Fig. 3). In addition, the RGC number in edaravone-treated mice ( $1782 \pm 62$  cells/mm<sup>2</sup>,  $n = 6$ ;  $P < 0.01$ ) was significantly increased compared with PBS-treated mice ( $1118 \pm 48$  cells/mm<sup>2</sup>,  $n = 6$ ) in the peripheral retina (Fig. 3B). These data demonstrate that edaravone prevents RGC death all across the retina following ONI.

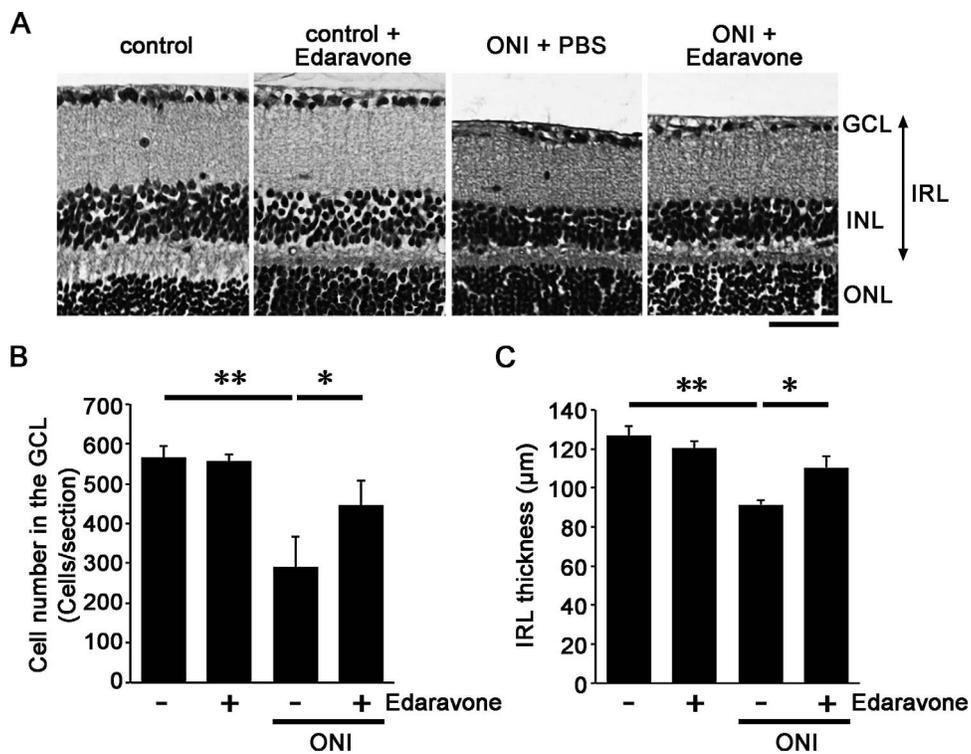
#### Effects of Edaravone on ONI-Induced RGC Death Signaling

We previously reported that activation of the ASK1-p38 mitogen-activated protein kinase (MAPK) pathway was detected at 3, 4, and 6 hours after ONI and involved in ONI-induced RGC death.<sup>22,30</sup> We, therefore, examined the effect of edaravone on ONI-induced activation of the ASK1-p38 MAPK signaling at 6 hours after ONI. Immunohistochemical analysis revealed that ONI induces expression of phosphorylated

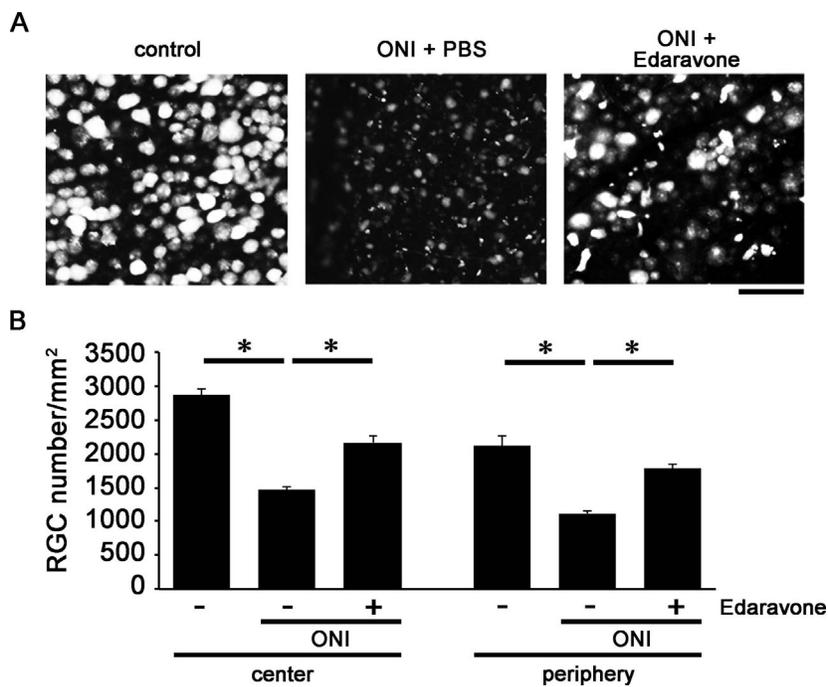
(activated) ASK1 ( $1.86 \pm 0.13$ -fold,  $n = 6$ ;  $P < 0.05$ ) and p38 MAPK ( $2.34 \pm 0.17$ -fold,  $n = 6$ ;  $P < 0.05$ ) primarily in the GCL (Fig. 4). Intraocular injection of edaravone significantly suppressed the expression levels of both phosphorylated ASK1 ( $1.20 \pm 0.07$ -fold,  $n = 6$ ;  $P < 0.05$ ) and p38 MAPK ( $1.43 \pm 0.26$ -fold,  $n = 6$ ;  $P < 0.05$ ) (Fig. 4). These results suggest that edaravone prevents RGC degeneration by suppressing the activation of the ASK1-p38 MAPK pathway.

#### Edaravone Suppresses ROS Production Following ONI

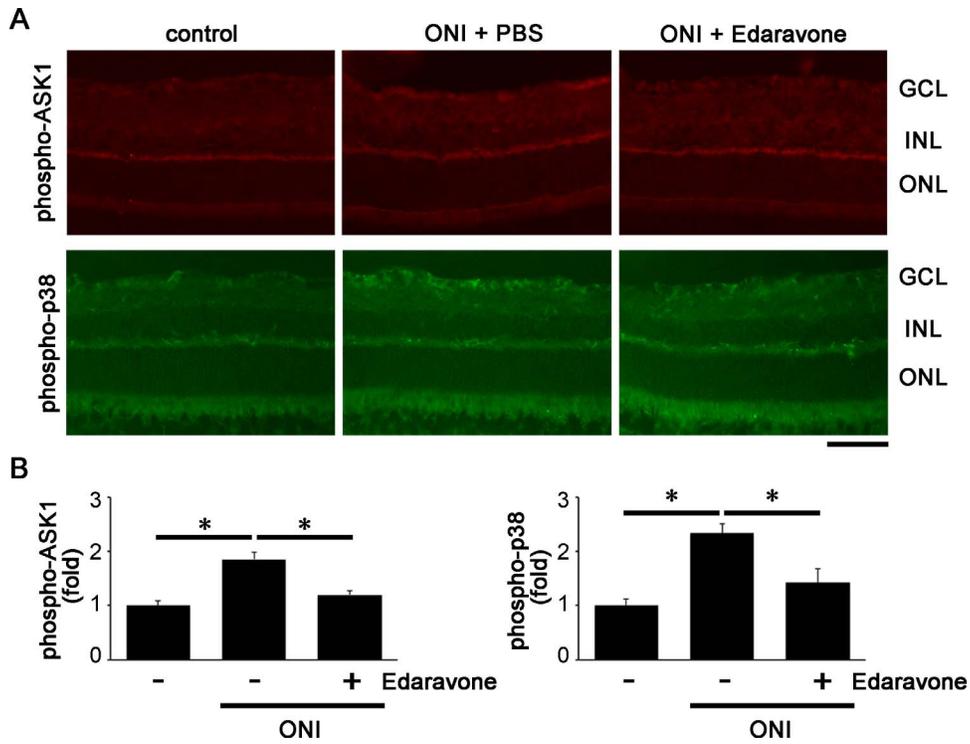
Previous studies have shown that production of ROS after ONI occurs primarily in RGCs.<sup>28,31</sup> To examine the effect of edaravone on ONI-induced ROS production, we performed staining of ROS using CellROX green reagent<sup>31</sup> in the whole-mount retina. Because ROS is a powerful activator of ASK1-p38 MAPK signaling, we also performed CellROX green reagent analyses at 6 hours after ONI. ONI induced ROS production in the PBS-treated retinas ( $3.25 \pm 0.37$ -fold,  $n = 6$ ;  $P < 0.05$ ) compared with the control retinas ( $1.00 \pm 0.28$ -



**FIGURE 2.** Effects of edaravone on retinal degeneration following ONI. (A) Retinal sections stained with hematoxylin and eosin in control and edaravone-treated mice. Scale bar: 50 μm. (B, C) Quantification of the cell number in the GCL (B) and IRL thickness (C) in PBS- and edaravone-treated mice. The data are presented as mean ± SEM of six retinas for each experiment. \**P* < 0.05, \*\**P* < 0.01. INL, inner nuclear layer; ONL, outer nuclear layer.



**FIGURE 3.** Effects of edaravone on RGC death following ONI. (A) Representative images of retrograde-labeled RGCs in the central retina. Scale bar: 50 μm. (B) Quantitative analyses of RGCs in the central and peripheral areas of the retina. The data are presented as mean ± SEM of six retinas for each experiment. \**P* < 0.05.

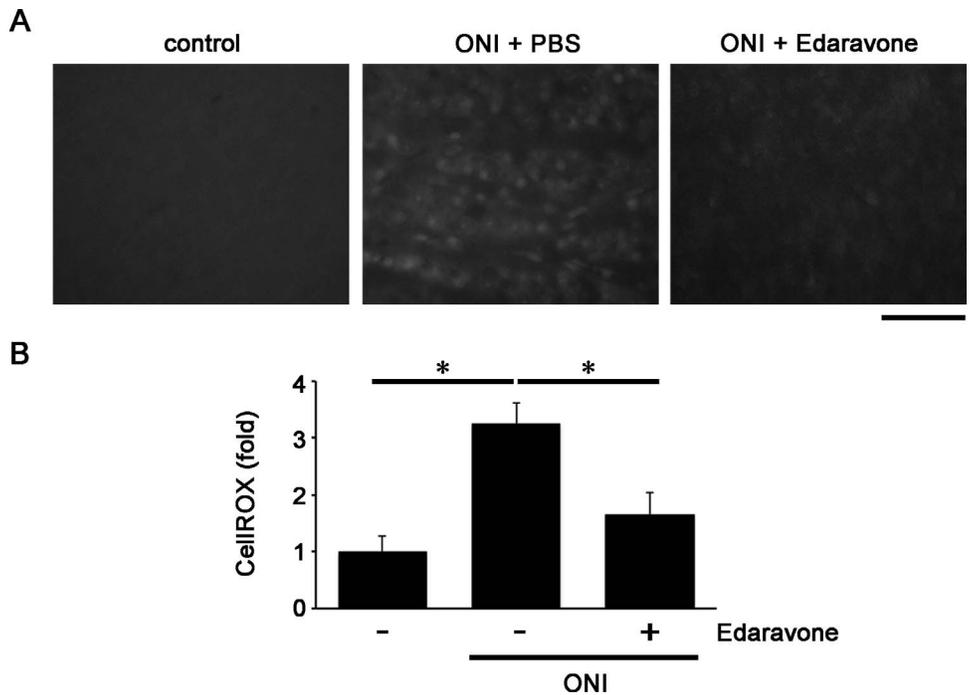


**FIGURE 4.** Effects of edaravone on ONI-induced activation of the ASK1-p38 pathway. (A) Representative images of immunostaining (phospho-ASK1 and phospho-p38). Scale bar: 50  $\mu$ m. (B) Quantitative analyses of (A). The data are presented as mean  $\pm$  SEM of six retinas for each experiment. \* $P$  < 0.05.

fold,  $n = 6$ ), but edaravone clearly suppressed the ROS production ( $1.66 \pm 0.39$ -fold,  $n = 6$ ;  $P < 0.05$ ) (Fig. 5). These results suggest that edaravone prevents RGC death by suppressing cell death pathways through the inhibition of ROS production after ONI.

**DISCUSSION**

In this study, we reported that intraocular injection of edaravone exerts neuroprotective effects in an ONI model. Sequential in vivo retinal imaging revealed that post-ONI



**FIGURE 5.** Effects of edaravone on ONI-induced oxidative stress. (A) Representative images of CellROX green reagent staining. Scale bar: 50  $\mu$ m. (B) Quantitative analyses of (A). The data are presented as mean  $\pm$  SEM of six retinas for each experiment. \* $P$  < 0.05.

treatment with edaravone was effective for maintaining the retinal structure. We also demonstrated that edaravone suppressed the production of ROS and stress-induced ASK1-p38 MAPK signaling, which leads to RGC survival following ONI. The ASK1-p38 MAPK pathway is activated in response to multiple types of stress and implicated in diseases such as cancer, Alzheimer's disease, and multiple sclerosis.<sup>32,33</sup> This signaling pathway also plays a role in RGC death and optic nerve degeneration under various conditions.<sup>26,34</sup> Post-ONI treatment with a p38 MAPK inhibitor injected into the eyeball was effective for RGC protection.<sup>22</sup> Our results suggest that edaravone stimulates neuroprotection and may be useful for the treatment of posttraumatic complications. Further studies are required to examine its long-term effects.

Oxidative stress is thought to be an important risk factor in human glaucoma.<sup>15-18</sup> Based on our results, it is plausible that edaravone suppressed several ONI-induced RGC death signals including the ASK1-p38 MAPK pathway by suppressing oxidative stress in RGCs. We recently reported that oral treatment of spermidine, a polyamine compound found in ribosomes and living tissues, has strong antioxidative effects and suppresses RGC death in mouse models of ONI<sup>30</sup> and normal tension glaucoma.<sup>35</sup> Similarly, an antioxidant,  $\alpha$ -lipoic acid, delivered through the diet protects RGCs in DBA/2J mice, an animal model that recapitulates the slow, progressive nature of human glaucoma.<sup>36</sup> Therefore, drugs with antioxidant properties, including edaravone, are good candidates for the treatment of glaucoma and optic neuropathy. Intraocular injection of edaravone plus oral spermidine and  $\alpha$ -lipoic acid may have synergistic effects on RGC protection.

Recent studies have indicated that development of new uses for existing drugs can be extremely beneficial to the healthcare industry.<sup>37</sup> Consequently, drug repurposing is emerging across various fields, including the central nervous system. We recently reported that valproic acid (VPA) protects RGCs from glutamate neurotoxicity in a mouse model of normal tension glaucoma.<sup>24,38</sup> VPA is a short chain fatty acid that has been used clinically worldwide for treatment of epilepsy since the 1970s. VPA has been shown to exert antioxidant properties in the brain following ischemia/reperfusion injury and in motor neurons following spinal cord injury.<sup>39,40</sup> Recently, free radicals have been investigated as an emerging therapeutic target for the treatment of neurological disorders including Alzheimer's disease and ALS,<sup>41</sup> as well as ocular diseases such as glaucoma, diabetic retinopathy, and age-related macular degeneration.<sup>42-44</sup> Thus, VPA, as well as edaravone, seems to be a suitable candidate for drug repurposing for neurotrauma. We also showed that administration of edaravone was effective even after ONI, strongly suggesting the possibility that edaravone is a potential drug for treatment of optic neuropathy. Although further in vivo studies are required, our findings raise intriguing possibilities for the management of ONI by existing drugs such as edaravone, VPA, and spermidine in combination with inhibitors of the ASK1-p38 MAPK pathway.<sup>22,34</sup>

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