Protective effects of free radical scavengers on transient ischemia-induced retinal ganglion cell death

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Abstract

Objective: To evaluate the suppressive effect of two free radical scavengers, edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) and Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), on retinal ganglion cell death in transgenic Thy1-EGFP mice expressing enhanced green fluorescent protein in retinal ganglion cells.

Method: Retinal ischemia-reperfusion injury was performed on mice following intraperitoneal administration of edaravone or Trolox, and the number of retinal ganglion cells that survived ischemia/reperfusion was measured non-invasively over time using fluorescent fundus angiography. In addition, viable cells were counted in flat mount retinal specimens prepared from enucleated eyes.

Results: The fluorescence intensity at the fundus decreased steadily after transient ischemia/reperfusion. Administration of edaravone and Trolox suppressed this decrease by approximately 20% on day 7. These results were comparable with the reduction in the number of ganglion cells counted in flat mount retinal preparations.

Conclusion: Free radical scavengers effectively suppress ischemia-induced retinal ganglion cell death.

Keywords

Reactive oxygen spieces, Trolox, edaravone, RGC, glaucoma

Abbreviations

retinal ganglion cell, RGC; enhanced green fluorescent protein, EGFP; fluorescent angiography, FAG

1. Introduction

Glaucoma is a progressive optic neuropathy characterized by selective retinal ganglion cell (RGC) death. However, the number of effective anti-glaucoma agents is limited. Many aspects of the etiology and pathogenesis of glaucoma remain unclear, but several hypotheses have been proposed. In ischemia/reperfusion [1] and optic nerve crush [2] models, excessive glutamate release is neurotoxic and is thought to cause RGC death [3-4]. Failure of RGC retrograde axonal transport, which abolishes the influence of neurotrophic factors, can also produce RGC apoptosis [5]. In the central nervous system, free radicals and active oxygen species are critical for inducing and modulating neuronal necrosis. Thus, free radical scavengers have been clinically applied to reduce neuronal degeneration following ischemic damage, such as cerebral infarction [6-8]. In ophthalmologic animal research, free radical scavengers suppress transient ischemia-induced increases in active oxygen generation [9-10]. The transgenic Thy1-EGFP mouse strain, which possesses the Thy1 gene promoter linked to the enhanced green fluorescent protein (EGFP) gene, specifically labels RGCs with fluorescent marker [11]. Non-invasive mouse

fundus imaging techniques using scanning laser ophthalmoscopy [12] or fluorescent angiography (FAG) allow estimation of the time-dependent RGC survival rate based on fluorescence intensity. The present study investigated the effect of two free radical scavengers, edaravone and Trolox®, on neuronal cell death in a transgenic Thy1-EGFP mouse model of transient ischemia/reperfusion injury. RGC counts were measured both by histochemical evaluation of formalin-fixed retinal specimens and by FAG bio-imaging techniques.

2. Materials and Methods

2.1 Transgenic Thy1-EGFP Mouse

To specifically label RGCs, a gene delivery vector was engineered by introducing the EGFP gene downstream of the mouse Thy1.2 gene promoter [13]. The vector was microinjected into fertilized C57/BL6J mouse oocytes at a contract laboratory (Japan SLC, Inc., Hamamatsu, Japan). Genomic DNA

extracted from the tails of 6-week-old mouse pups was used for polymerase chain reaction (PCR)-based genotyping. RGC-specific EGFP expression was also confirmed by PCR. The PCR primers used for genotyping complemented either the Thy1.2 gene (forward primer: TCTGAGTGGCAAAGGACCTTAGG) or the EGFP gene (reverse primer: CGCTGAACTTGTGGCCGTTTACG).

2.2 Transient Retinal Ischemia

All animal handling and experimental procedures were performed in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research given by the Association for Research in Vision and Ophthalmology.

Mice were anesthetized with intraperitoneal pentobarbital (65 mg/kg body weight), and 0.5% oxybuprocaine hydrochloride ophthalmic solution was applied topically to anesthetize the cornea. The anterior chamber of one eye was cannulated with a 30-G needle connected to an irrigating solution container located 150 cm above the bench. This eye was irrigated for 45 minutes at an intraocular pressure of approximately 120 mm Hg [14,15]. The other eye was left

intact. During testing, animals were placed on a heating plate to minimize the phenobarbital-induced body temperature decrease and its potential neuroprotective effects.

2.3 Pharmacological Treatment

Twelve transgenic Thy1-EGFP mice were divided into three groups; one group received an intraperitoneal injection of edaravone, the second group received an intraperitoneal injection of Trolox and the third group served as the control group. Edaravone was dissolved in 1 M NaOH, adjusted to pH 7.4 with 1 M HCl, and diluted with saline to a final concentration of 3 mg/mL. Trolox was dissolved to dimethyl sulfoxide and diluted with saline to a final concentration of 3 mg/mL. Mice received an intraperitoneal injection of edaravone (3 mg/kg) or Trolox (3 mg/kg) 30 minutes prior to transient ischemic injury, as well as 1 and 5 days after the injury. FAG imaging of the retina was performed 4 and 7 days after the injury (see below). After completing FAG imaging on day 7, eyes were extirpated, the retina were dissected, and viable RGCs were counted for comparison with controls.

2.4 FAG Imaging

Under general pentobarbital (65 mg/kg) anesthesia, a single drop of 0.5% tropicamide hydrochloride (0.1 mL) was used to dilate each pupil. Fundus imaging was performed *in vivo* with a FAG camera (excitation, 488 nm; barrier filter, 515 nm). The plane of focus of the FAG camera was adjusted for each mouse using a 40-D fundoscopy lens (Nikon, Tokyo, Japan). The same spot was imaged prior to ischemic damage, as well as 4 and 7 days after reperfusion. The fluorescence intensity of the images was calculated for each of these time points [12], and differences in intensity were tested statistically using a one-way analysis of variance. The significance level was set at 5%.

2.5 Histologic Evaluation

Following completion of the above-mentioned FAG imaging procedures 7 days after ischemic damage, flat mount mouse retinal specimens were prepared [16]. Eyeballs were extirpated, and the posterior segments were

dissected and fixed with 2% paraformaldehyde in 0.1 M phosphate buffer for 30 minutes. The retinas were detached, incised radially in four places, flattened, and fixed again with 4% paraformaldehyde. A fluorescence microscope was used to take pictures of two microscopic fields surrounding the blind spot (total area: 0.32 mm^2), and the mean number of RGCs per unit area was calculated for each mouse. The number of RGCs in the injured eye was expressed relative to that in the control eye from the same mouse. Differences in the proportion of surviving RGCs were tested statistically using a one-way analysis of variance. The significance level was set at 5%.

3. Results

3.1 Effect of Free Radical Scavengers on Post-Ischemic RGC Death

This study evaluated the effect of the free radical scavengers, edaravone and Trolox on RGC death using a transgenic Thy1-EGFP mouse model of ischemia/reperfusion injury. Seven days after ischemic injury, RGCs

were reduced by 60% in flat mount retinal samples from untreated control mice. The proportion of dead RGCs was reduced to 40% by edaravone treatment (i.e. approximately a 20% reduction) and to 48% with Trolox. These results demonstrate that there was a larger proportion of viable RGCs in mice treated with free radical scavengers, suggesting that these agents have a neuroprotective effect. In this experiment, the effect of ischemic/reperfusion injury on RGCs was evaluated relative to the untreated control eye in the same mouse.(Fig.1)

3.2 Changes in Mouse Fundus FAG Images

FAG analysis and flat mount retinal specimens revealed a similar reduction in the proportion of viable RGCs. No spatial differentiation was observed in the retinal protective effect of these agents; both the FAG images and specimens revealed uniform suppression of changes in fluorescence intensity.(Fig.2)

The time course of changes in post-ischemic RGC fluorescence was followed *in vivo* in the same transgenic Thy1-EGFP mice using a FAG camera.

The pre-ischemic fluorescence intensity in each eye was used as a control for quantitative evaluation of fundus fluorescence.

Fundus fluorescence intensity in untreated mice fell to approximately 62% of pre-ischemia control levels 4 days after ischemia and to approximately 42% of control levels 7 days after ischemia. In edaravone-treated mice, fluorescence intensity was approximately 74% 4 days after ischemia and approximately 62% 7 days after ischemia. Fluorescence intensity was similar for the Trolox group: approximately 71% 4 days after ischemia and approximately 57% on day 7. Although treated and untreated groups commonly showed post-ischemic decreases in RGC fluorescence intensity, free radical scavenger–treated groups exhibited less of a decrease than the untreated group.(Fig.3)

4. Discussion

The transgenic Thy1-EGFP mice described in this work have fluorescently labeled RGCs, because the Thy1 gene confers RGC-specific expression of the EGFP gene located downstream [13]. Commonly used

methods for assessing the effects of ischemia/reperfusion and optic nerve crush on RGCs include retrograde labeling of RGCs and determination of Thy1 gene expression by reverse-transcription PCR and enzyme-linked immunosorbent assay. The use of transgenic Thy1-EGFP mice carrying genetically engineered fluorescent RGCs allowed direct and accurate counting of RGCs in flat mount retinal preparations without the addition of the above mentioned techniques. Moreover, in the present study, the fluorescence intensity of FAG fundus images was measured *in vivo* in individual mice over time in order to evaluate time-dependent, pathological changes in RGCs [15].

Active oxygen species have been implicated in delayed neuronal cell death following transient cerebral ischemia [16,17]. In particular, oxygen and hydroxyl radicals produced after ischemia/reperfusion are involved in neuronal cell death after cerebral infarction in the peri-ischemic region, which is also known as the ischemic penumbra [6, 18,19]. Edaravone, which has powerful scavenging activity against these toxic radicals, was approved in Japan in 2001 as a neuroprotective agent for cerebral ischemia (brand name: Radicut®) [8,11,19]. Trolox is a water-soluble derivative of vitamin E that has been used as a comparative standard for antioxidative capacity [20]. Both are lipid- and

water-soluble compounds. In a rat model of cerebral ischemia, edaravone reduced infarct size and vasogenic edema, when administered prior to, or within 24 hours of, the induction of ischemia [8, 19]. Moreover, pretreatment of Mongolian gerbils with Trolox (30 mg/kg) before global cerebral ischemia improves neurological symptoms and locomotor activity [20].

There is a marked post-ischemic increase in free radical concentrations in the rat retina [9, 10]. The results of the present study indicate that the loss of RGCs can be curbed by administering either of two free radical scavengers, edaravone or Trolox, before and after the induction of ocular ischemia. No significant difference was noted in the neuroprotective effect of these two agents. The experimental design of our study precludes accurate discrimination of whether the drug administration is effective before or after induction of ischemia. However, *in vivo* FAG evaluation of post-ischemia/reperfusion changes in mouse fundus fluorescence intensity suggests that suppression of RGC cell death can be observed before the administration of additional doses. Differences in the time course of changes in the fluorescence intensity(Fig.3) between treated and untreated groups are unlikely to be enhanced by additional doses. These observations support the idea that administration of free radical scavengers

before the induction of ischemia/reperfusion offers the most neuroprotection. The post-ischemic effect of edaravone has been documented in several cerebral ischemic models [8,19]. Thus, further research is needed to clarify the temporal characteristics of edavarone-mediated neuroprotection against RGC death.

Suppression of active oxygen species and other free radicals has been studied as a novel therapeutic target for curing neurodegenerative diseases. Free radical scavengers are being studied as possible agents for the treatment of ischemic RGC death and glaucoma [21,22]. Edaravone is the only free radical scavenging agent that is currently approved for clinical application in Japan [19]. A wide range of clinical information on edaravone, including pharmacokinetics and adverse drug reactions in humans, is available. The results of this study suggest a possible future role for edaravone in the treatment or prevention of neurodegenerative ophthalmic diseases.

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Figure Legends

Figure 1. The relative number of residual mouse RGCs in flat mount retinal specimens prepared 7 days after transient ischemic damage.

Both eyes were extirpated 7 days after transient ischemic damage. Flat mount retinal specimens were prepared, and the number of residual RGCs in the damaged eye and the intact eye were compared. In the control group, the number (± SD) of residual RGCs in the treated eye relative to that in the untreated eye was 40.7± 2.9%. For the edaravone and Trolox groups, the proportion was 60.2 ± 3.5% and 52.9 ± 4.2%, respectively, indicating that RGC death was suppressed. Values are means ± SD. *$P < 0.05$, one-way analysis of variance (n = 4 mice for each group).

Figure 2. Comparison of residual mouse RGCs by fluorescent angiography 7 days after transient ischemic damage.

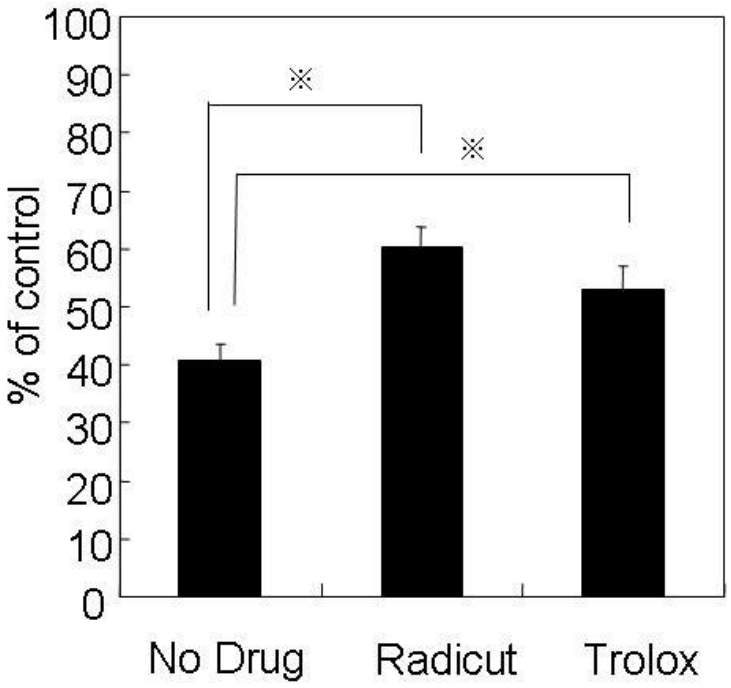
Fluorescent angiography images of the retina were recorded 7 days after transient ischemic damage, and the number of residual RGCs in the damaged eye and the intact eye were compared. In the control group, the number (± SD)

of residual RGCs relative to that observed pre-ischemia was 39.5 ± 3.4%. For the edaravone and Trolox groups, the proportion was 60.8 ± 4.5% and 55.6 ± 6.6%, respectively, indicating that RGC death was suppressed. Values are means ± SD. *P < 0.05 (n = 4 mice for each group).

Figure 3. The time course of changes in the fluorescence intensity of mouse fundus was measured by fluorescent angiography.

Fluorescence images of EGFP protein expressed in RGC were recorded on days 0, 4, and 7 using a FAG camera. The mean fluorescence intensity on days 4 and 7 were compared with that on day 0 (n = 4 mice for each group). Typical fluorescent angiography images of the fundus taken on days 0 and 7 are shown.

Figure 1


100
90
80
70
60
50
40
30
20
10
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% of control
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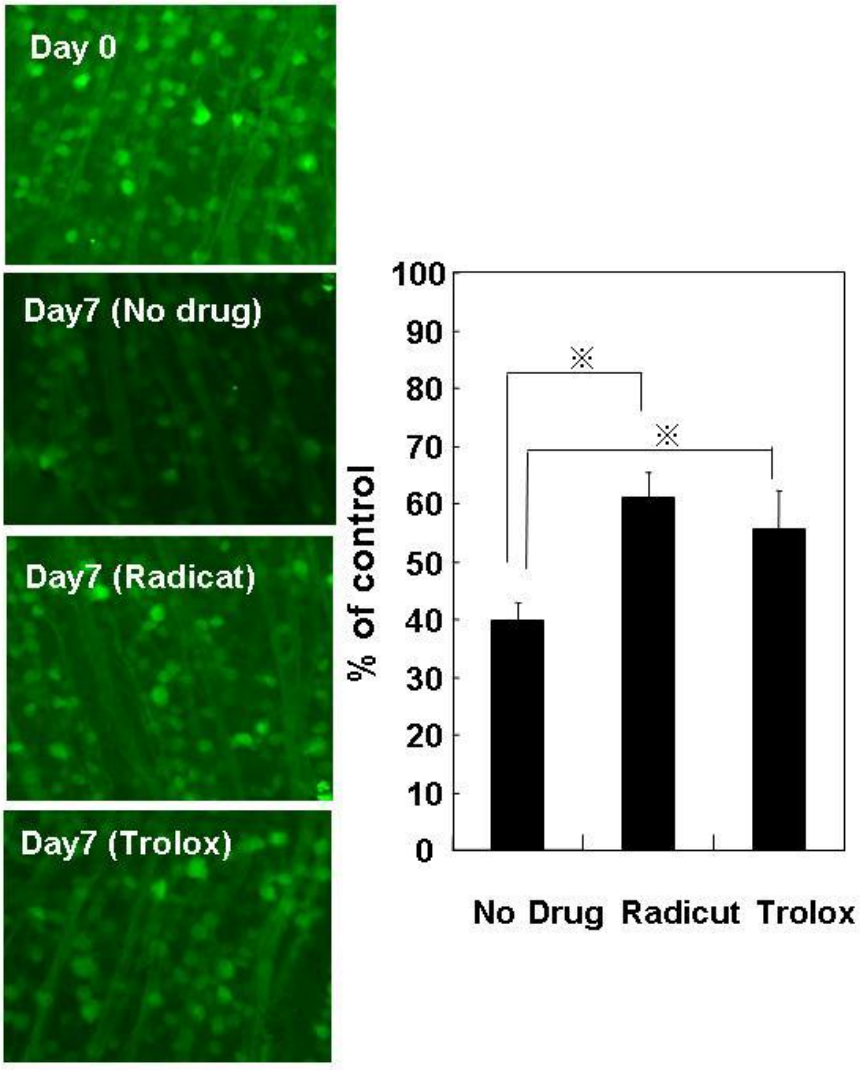
No Drug
Radicut
Trolox

Figure 2


Day 0
Day7 (No drug)
Day7 (Radicat)
Day7 (Trolox)
% of control
100
90
80
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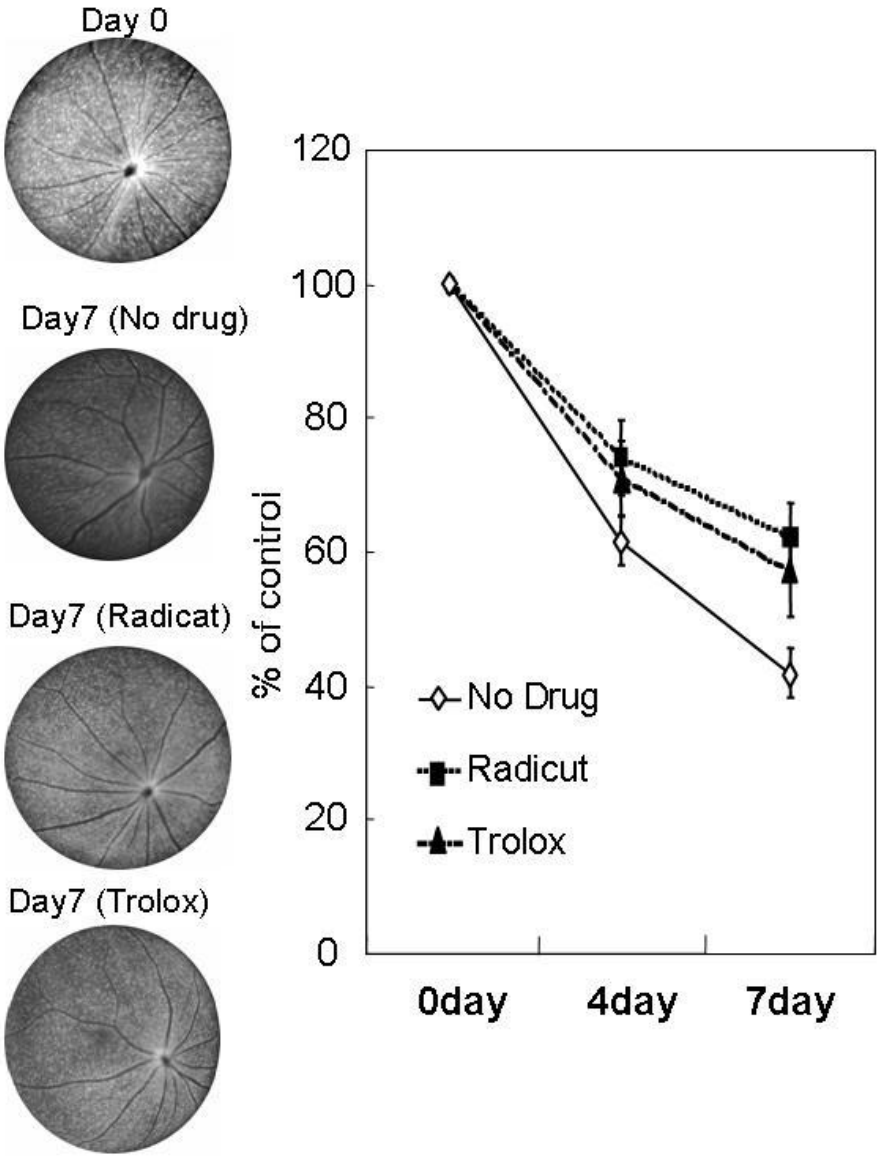
No Drug
Radicut
Trolox

Figure 3


Day 0
Day7 (No drug)
Day7 (Radicat)
Day7 (Trolox)
120
100
80
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% of control
No Drug
Radicut
Trolox
0day
4day
7day