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Effects of Edaravone on Hippocampal Antioxidants in EL Mice

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Background: The role of oxidative stress in susceptibility to seizures has been the focus of several recent studies. The aim of the present study was to evaluate the antiepileptic effects of the free radical scavenger edaravone on EL mice, a strain that is highly susceptible to convulsive seizures.

Methods: EL mice were treated intraperitoneally with edaravone or saline for 1 week. The levels of reduced glutathione (GSH), oxidized glutathione (GSSG) and 3 isozymes of superoxide dismutase (SOD) (cytoplasmic copper- and zinc-containing SOD, extracellular SOD, and mitochondrial manganese-containing SOD) were measured in the hippocampus, and electroencephalograms (EEGs) were used to evaluate seizure sensitivity.

Results: Hippocampal levels of GSSG were lower in the edaravone group than in the untreated control group, and the GSH/GSSG ratio, Cu/Zn-SOD, and EC-SOD activities were higher in the edaravone group. Edaravone shortened the duration of interictal spike discharges and clinically suppressed epileptic seizures.

Conclusion: Edaravone increases antioxidant potency and reduces seizure susceptibility in EL mice, making it a promising novel antiepileptic agent. (J Nippon Med Sch 2016; 83: 100–106)

Key words: EL mice, glutathione, antioxidant, edaravone, electroencephalogram

Introduction

Epilepsy is one of the most common neurological disorders in humans. It is characterized by recurrent spontaneous seizures, either convulsive or nonconvulsive, and by synchronized abnormal electrical activities arising from a group of cerebral neurons or neuronal networks[1–3]. The pathophysiological mechanisms of epilepsy are not yet fully understood, but recent studies have focused on the role of oxidative stress in epileptic seizures.

Oxidative reactions are maintained by complex systems composed of various antioxidants, including reduced glutathione (GSH) and superoxide dismutase (SOD). Of these, GSH is an important constituent of the cellular antioxidant defense mechanism; it reacts nonenzymatically with a variety of different free radicals[4–7] to change to an oxidized form, oxidized glutathione (GSSG), and the GSH/GSSG ratio serves as a sensitive index of oxidative stress[8,9]. In addtion, GSH has been shown to protect neurons against glutamate-induced cytotoxicity, ischemic damage, and aging[10,11]. Three SOD isozymes are also essentially involved in cellular defense against the oxidative processes occurring along with the generation of superoxide anion radicals[12]: extracellular SOD (EC-SOD), cytoplasmic copper- and zinc-containing SOD (Cu/Zn-SOD), and mitochondrial manganese-containing SOD (Mn-SOD).

The EL mouse is an inbred mutant strain of the DDY mouse and is susceptible to convulsive seizures. The strain was developed in 1954[13] and was established with electroencephalography (EEG) as an authentic epileptic mutant in 1976[14]. Studies of EL mice show that decreased antioxidant protection or excessive free-radical formation is associated with a redox shift to an oxidized state[15–17] and elevated extracellular glutamate concentration

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caused by dysfunction of excitatory amino acid transporters[18,19].

Edaravone (MCI-186; 3-methyl-1-phenyl-2-pyrazolin-5-one) is a scavenger of low-molecular-weight free radicals which readily crosses the blood-brain barrier[20] and exerts inhibitory effects on both water-soluble and lipid-soluble peroxyl radical-induced peroxidation systems[21–23]. Recent studies indicate that edaravone prevents kainic acid-induced neuronal death[24] and hippocampal damage after pilocarpine-induced status epilepticus[25]; it also delays the development of amygdala kindling in rats[26]. However, no previous studies have investigated the antiepileptic effects of edaravone in EL mice. In the present study, we investigated the antioxidant potency of edaravone and used EEGs to assess changes in seizure susceptibility.

Materials and Methods

Edaravone

Edaravone was kindly supplied by Mitsubishi Tanabe Pharma Corporation (Osaka, Japan). For the present study, the drug (30 mg) was dissolved in 0.5 mL of 1 M NaOH and 8 mL of distilled water and titrated to pH 7.0 with 1 M HCl. The concentration was adjusted to 0.3 mg/mL or 3 mg/mL in 0.9% saline solution.

Animals

We used male EL mice (30–50 g) aged 90 days from an established epileptic strain maintained at Tokyo Metropolitan University Graduate School of Human Health Science by sister-brother inbreeding. The mice were housed in a conventional animal room at 22℃ ± 1℃ under a 12-hour: 12-hour light-dark cycle with humidity maintained at 60%. They were fed a commercial diet and given water ad libitum.

All procedures on mice were approved by the Laboratory Animals Committee of Nippon Medical School (Ethics Number 27-070) and conformed to National Institutes of Health guidelines for the ethical use of animals in scientific research. All efforts were made to minimize stress and the number of animals necessary to produce reliable data.

GSH and GSSG Assay

Nine mice were divided into 3 groups and were given 0.1 mL saline (controls) or 0.1 mL edaravone (1 or 10 mg/kg/day) intraperitoneally for 1 week. They were then decapitated, and their brains were removed and placed on ice. The hippocampus was separated and weighed. Both GSH and GSSG were analyzed with a GSSG/GSH Quantification Kit (Dojindo Laboratories, Kumamoto, Japan). The hippocampus was homogenized (10% w/v) in 5% sulfosalicylic acid and centrifuged at 8,000 g for 10 minutes at 4℃. The supernatant was collected and diluted 20 times for GSH analysis, and 5 times for GSSG analysis. Samples were incubated at 37℃ for 10 min, and absorbance was then measured at 405 nm with a microplate reader.

SOD Activity Assay

Other mice were prepared in the same way as for the GSH and GSSG assay, except that there was only one edaravone group, the members of which received 10 mg/kg/day. The SOD activity was assayed with a SOD assay kit-WST (Dojindo Laboratories). The hippocampus was homogenized (10% w/v) in 10 mM Tris-HCl buffer (pH 7.4) with 0.25 M sucrose and 1 mM EDTA, and centrifuged at 10,000 g for 60 min at 4℃. The supernatant was used for SOD activity analysis.

Anticonvulsive Effect of Edaravone

Once a week from the age of 28 days, mouse was taken carefully from its home cage, placed in a box (30×20×10 cm), and observed for 3 min. It was then tossed 50 times to a height of 10 cm to induce seizures, the grades of which were determined according to the classifications of Ito and Tsuda[27] and of Koide et al.[28]: grade 0, no seizures with 50 tossing stimulations; grade 1, abortive seizures within 50 tossing stimulations; grade 2, abortive seizures within 25 tossing stimulations; grade 3, tonic-clonic seizures within 50 tossing stimulations; and grade 4, tonic-clonic seizures within 25 tossing stimulations. Abortive seizures are characterized by immobility with fixed staring[28]. Six mice with grade-4 seizures were used in the present study. These mice were intraperitoneally administered with edaravone (10 mg/kg/day) on 7 consecutive days. After intraperitoneal injections of edaravone for 7 consecutive days, they were subjected to tossing stimulation and the severity of the resulting seizures was determined.

EEG Preparation and Testing

Electrodes were implanted as previously described (**Fig. 1**)[29]. Briefly, mice were anesthetized with ketamine and xylazine, and 4 burr holes were created symmetrically anterior to the coronal and lambdoid sutures for epidural electrodes consisting of 1-mm-diameter silver balls covered with epoxy resin. The dural attachment was 0.2 mm in diameter at the tip. A reference electrode was placed on the nasal bone, and a ground electrode was placed subcutaneously near the rump. An 8-pin header was used to connect the electrodes to an EEG device (EEG-8310; Nihon Kohden, Tokyo, Japan) with a band-pass filter (120 Hz low-pass and 50 Hz band elimi-

nation filter) at a time constant of 0.1 second. The EEGs were sampled with an A-D converter at 200 Hz and recorded with a digital data recorder (DR-M3b; TEAC, Tokyo, Japan).

Five mice were used for EEG recording. The EEGs were recorded before edaravone treatment and after intraperitoneal administration of edaravone (10 mg/kg/day) for 7 consecutive days. The EEG recording was performed for more than 1 hour per day. During the recording, the mice went through several sleep/wake cycles. They were considered asleep when consciousness level decreased substantially, and they remained still in a supine position with their eyes closed, showing diminished response to environmental stimuli[30]. The EEG waves were analyzed with the Pc-Wave Form software (DEICY, Tokyo, Japan). The duration time and the frequency of interictal spike discharges were determined by manual observations in the raw EEGs of 1-hour length. The total duration time of interictal spike discharges was calculated by multiplying the mean duration of interictal spike discharges per hour times the frequency of spike discharges per hour.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM), and the statistical significance of differences was determined with analysis of variance and Student's *t*-test.

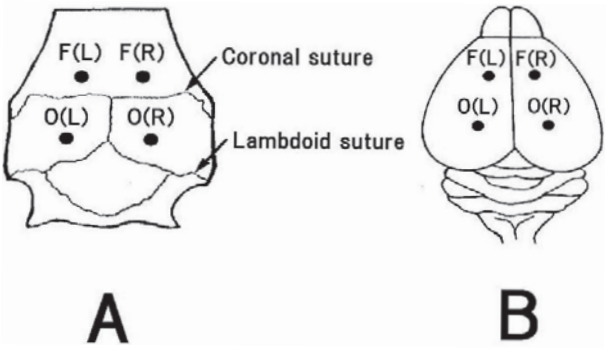

Fig. 1 **A**: Locations of burr holes in the skull **B**: Locations of electrodes on the brain
F: frontal, O: occipital, R: right, L: left

Results

Evaluation of Hippocampal GSH and GSSG Levels

The GSH levels did not vary according to edaravone dosage (untreated, 3.07 ± 0.05 μmol/g; 1 mg/kg/day edaravone, 3.16 ± 0.05 μmol/g; and 10 mg/kg/day edaravone, 3.06 ± 0.03 μmol/g) (**Fig. 2A**). Hippocampal GSSG levels in the mice treated with edaravone (1 mg/kg/day, 0.018 ± 0.002 μmol/g; 10 mg/kg/day, 0.013 ± 0.001 μmol/g) were dose-dependently decreased, and levels in mice receiving 10 mg/kg/day edaravone were significantly lower than levels in control saline-treated mice (0.023 ± 0.002 μmol/g, $p<0.01$) (**Fig. 2B**). The GSH/GSSG ratio was dose-dependently increased by treatment with edaravone (1 mg/kg/day, 176.41 ± 20.77; 10 mg/kg/day,

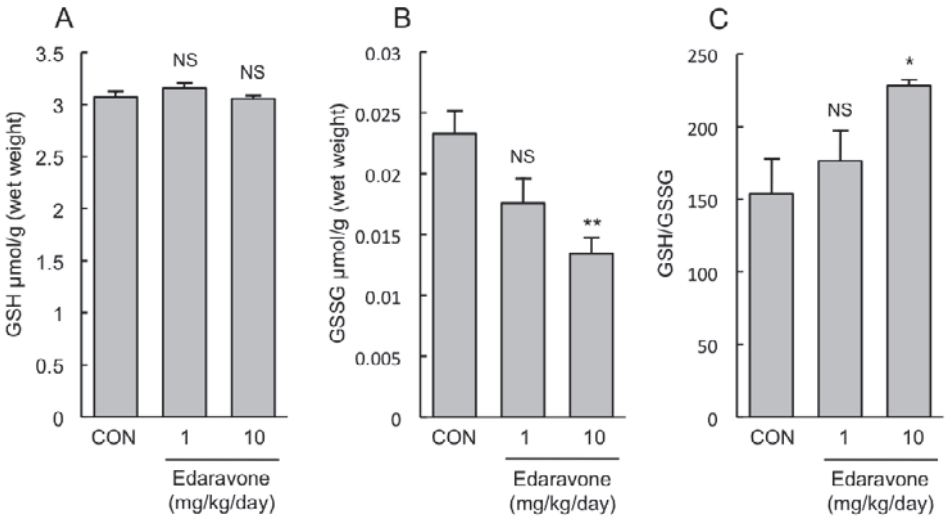

Fig. 2 Effects of edaravone on reduced glutathione (GSH) (A), oxidized glutathione (GSSG) (B), and GSH/GSSG (C) in hippocampal tissue of EL mice
The mice were intraperitoneally administered with saline (controls: CON) or edaravone (1 or 10 mg/kg/day) for 1 week. Values are expressed as mean±SEM (n=3). The statistical significance of differences was determined by analysis of variance (*$p<0.05$, **$p<0.01$; NS: not significant vs. controls).

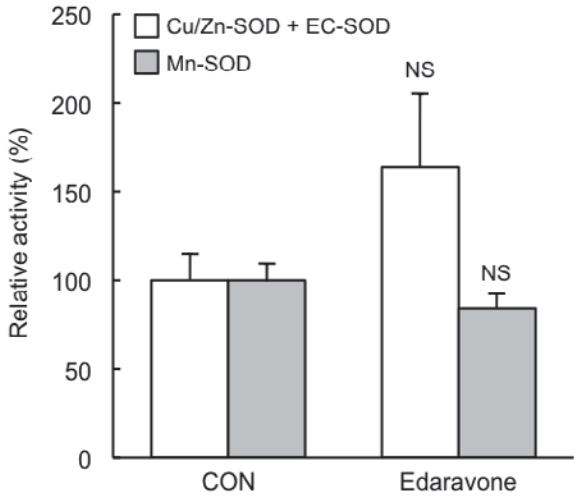

Fig. 3 Effects of edaravone on extracellular superoxide dismutase (EC-SOD), cytoplasmic copper- and zinc-containing superoxide dismutase (Cu/Zn-SOD), and mitochondrial manganese-containing superoxide dismutase (Mn-SOD) activities in hippocampal tissue of EL mice
The mice received intraperitoneally administered saline (control: CON) or edaravone (10 mg/kg/day) for 1 week. Values are expressed as mean±SEM (n=3). The statistical significance of differences was determined with Student's *t*-test (NS: not significant).

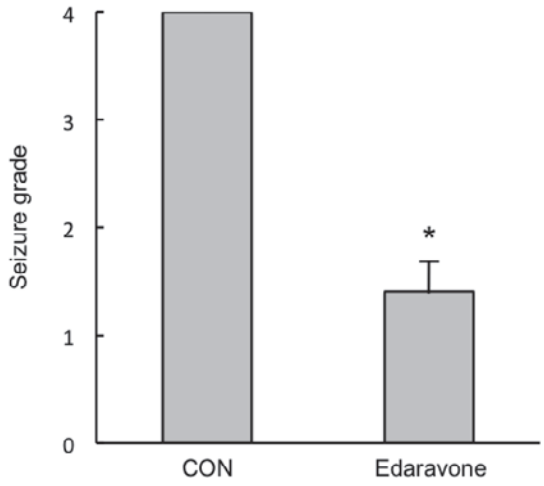

Fig. 4 Effect of edaravone on seizure grades in EL mice
Seizure grades were evaluated following intraperitoneal administration of edaravone (10 mg/kg/day) for 7 consecutive days. The edaravone-untreated EL mice (control: CON) had grade-4 seizures. Values are expressed as means±SEM (n=6). The statistical significance of differences was determined with Student's *t*-test (*<0.05).

228.18 ± 4.38) and was significantly increased with treatment of 10 mg/kg/day over that without treatment with edaravone (153.83 ± 23.96; p<0.05) (**Fig. 2C**).

Evaluation of Hippocampal SOD Activity

Hippocampal EC-SOD and Cu/Zn-SOD activities were higher in the edaravone-treated mice (163.78% ± 40.66%) than in saline-treated control mice (100% ± 15.19%), but the increase was not statistically significant. No difference in Mn-SOD activity was observed between the control mice (100 ± 9.45%) and the edaravone-treated mice (84.37% ± 8.05%) (**Fig. 3**).

Effect of Edaravone on Seizure Grades

Seizure grades in the edaravone-treated mice were 1.4 ± 0.28 and indicated that edaravone significantly suppressed convulsive seizures (p<0.05) (**Fig. 4**).

EEG Changes

The duration of interictal spike discharges was significantly shorter after treatment with edaravone (1.00 ± 0.03 seconds) than before (1.78 ± 0.07 seconds) (**Fig. 5, 6A**), but there was no statistical difference in the frequency of spike discharges (**Fig. 6B**). The mean total duration time of interictal spike discharges after treatment showed a decreasing trend, but the difference was not statistically significant (**Fig. 6C**).

Discussion

The present study suggests that edaravone decreases seizure susceptibility by regulating oxidative stress in the hippocampus of EL mice.

Compared with nonepileptic DDY mice, EL mice have lower GSH levels and higher GSSG levels in the hippocampus[17]. Edaravone protects against various free radicals[21], so reduced GSSG levels indicate a direct effect of edaravone on free radicals as a substitute for GSH. However, edaravone had no effect on the hippocampal GSH levels of the EL mice we used in our study, so it is possible that the GSH reacted with proteins containing redox-sensitive thiols: it is known that GSH reversibly modifies many proteins containing redox-sensitive thiols during basal or mild oxidative stress. Because GSH is abundant within cells, sulfur redox status plays an important role in the regulation of cell functions, including signaling, growth, survival, and cell death. Therefore, the disruption of sulfur redox status is linked to abnormal cell functioning, aging, and many diseases, including arthritis, cancer, cardiovascular disorders, diabetes, and neurodegenerative diseases[31]. Reversible thiol modifications, including intramolecular disulfides, intermolecular disulfides, protein sulfenic acids, and protein-glutathione mixed disulfides, protect proteins from irreversible oxidation and allow for a return to normal function after the oxidant challenge has subsided[32].

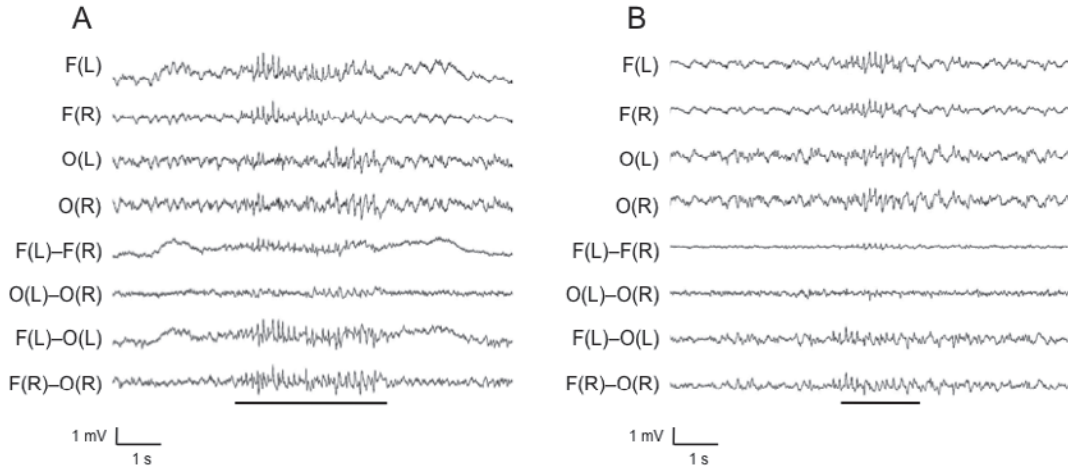

Fig. 5 Electroencephalograms (EEGs) showing spike discharges recorded with 4 electrodes in a sleeping EL mouse
The EEGs were recorded before edaravone treatment and after intraperitoneal administration of edaravone (10 mg/kg/day) for 7 consecutive days. **A**: untreated state; **B**: after edaravone administration

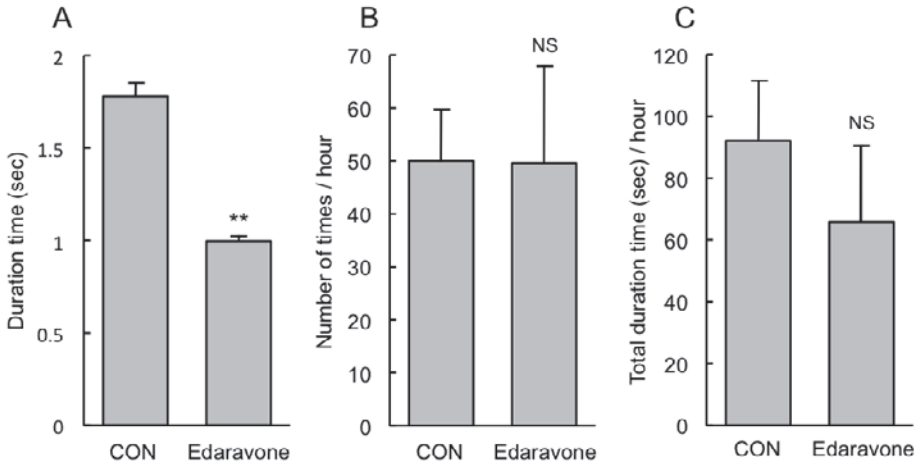

Fig. 6 Comparison of the average duration of interictal spike discharges (A), the frequency of interictal spike discharges per hour of EEG recording (B), and total duration of interictal spike discharges per hour (C)
Control mice (CON) were untreated. Values are expressed as means±SEM (n=5). The statistical significance of differences was determined with Student's *t*-test (**<0.01, NS: not significant).

A recent study suggests that micromolar concentrations of extracellular GSH affect the regulation of seizure susceptibility[33], and another indicates that seizure susceptibility is related to reversible thiol modifications[34]. Thus, the reversible thiol modifications due to GSH appear to regulate seizure susceptibility.

The extracellular glutamate concentrations and the oxidation of glutamate transporters increase in the brains of EL mice during epileptogenesis[17,18]. This dysfunction of glutamate transporters in EL mice is related to neuronal excitability. Several isoforms of glutamate transporters have redox-sensitive thiols that are vulnerable to biological oxidants and are inhibited by antioxidants such as GSH via direct action on the transporter proteins[35]. We observed increased GSH/GSSG ratios induced by edaravone, indicating reduced oxidative stress in the hippocampal tissues of EL mice. Therefore, we propose that edaravone modulates hippocampal neurotransmission by protecting glutamate transporters from oxidation.

Overexpression of Cu/Zn-SOD in transgenic mice exerts a major effect on neuronal excitability in the hippocampus[36]. The Cu/Zn-SOD activity in EL mice de-

creases as epileptogenesis progresses[15]. These effects suggest that Cu/Zn-SOD activities are associated with the regulation of neuronal excitability, and our present findings indicate that edaravone increases hippocampal Cu/Zn-SOD activities and decreases seizure susceptibility in EL mice. Therefore, we believe that the reversible thiol modifications due to GSH and the increasing Cu/Zn-SOD activities reduce the seizure susceptibility of EL mice.

Our results suggest that edaravone has potent antiepileptic effects on EL mice. Further studies are needed to clarify whether edaravone affects seizure susceptibility at a genetic level or via neurotransmitters, such as glutamate and gamma-aminobutyric acid.

Conflict of Interest: The authors declare no conflict of interest.

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