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Protective effects of the free radical scavenger edaravone against glutamate neurotoxicity in nearly pure neuronal culture

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Abstract
Purpose. Edaravone, a free radical scavenger, has shown neuroprotection in both animals and humans. To evaluate the mechanism of this protection, we examined the effect of edaravone on neurons themselves against glutamate neurotoxicity.
Methods. Neurons were collected from 18-day fetal rat brains and a culture of almost pure neurons was obtained after 14-day culture. The neurons were exposed to 50 μM glutamate for 10 min, followed by normal culture for 24 h. Edaravone was added to the medium during the glutamate insult (prophylactic effect) or after the insult (treatment effect). First, the cell survival rate was measured by staining with trypan blue. Second, the cells were stained with 6-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate, di-(acetoxymethyl ester) (C-DCDHF-DA) and the relative amount of reactive oxygen species (ROS) was measured by flow cytometry. Third, the cells were stained with Hoechst 33342 and propidium iodide and the numbers of apoptotic and necrotic cells were counted.
Results. A dose-dependent prophylactic effect was observed and the cell survival rate in 500 μM edaravone was significantly higher than that without it. However, there was no treatment effect beyond 2 h after the insult. The amount of ROS under 500 μM edaravone at 4 h after the glutamate insult was significantly lower than the control amount. Necrosis, but not apoptosis, was significantly inhibited by edaravone.
Conclusion. Edaravone mainly showed a prophylactic effect on neurons against glutamate neurotoxicity, possibly through the inhibition of necrosis via the suppression of ROS production. However, for a protective effect, a higher, supraclinical concentration was required, compared to the concentrations producing a protective effect in glial and endothelial cells in previous studies.

Key words Cerebral ischemia · Edaravone · Reactive oxygen species · Glutamate · Neuronal culture · Cell death · Excitotoxicity

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Introduction

Although many pharmacological agents, including anesthetic agents, have been tested for neuroprotective effects, to date this has not yielded sufficient results in the clinical setting [1,2]. However, edaravone (Mitsubishi Tanabe Pharma, Tokyo, Japan), a free radical scavenger available in Japan since 2001, has been reported to have a neuroprotective effect in a rodent model of the forebrain [3] and in focal ischemia [4,5]. Moreover, several clinical studies have demonstrated the efficacy of this drug in patients with acute brain infarction [6,7], acute lacunar infarction [8], and carotid endarterectomy [9]. It has been suggested that the mechanisms of the protective effect involve inhibition of the activation of microglia [10,11] and astrocytes [10], and protection against endothelial cell injury [12], as well as the inhibition of cerebral edema [13]. Regarding the effect on the neurons themselves, it has been reported that cultured hippocampal neurons and neuronal PC 12 cells treated with edaravone were protected against glucose-oxygen deprivation [14,15]. However, as far as we know, there have been no reports about the effect of edaravone on neurons themselves against glutamate neurotoxicity, i. e., excitotoxicity. In this study, accordingly, we evaluated whether edaravone had a neuroprotective effect against glutamate neurotoxicity by using a nearly pure neuronal culture.

Materials and methods

Nearly pure neuronal culture

With institutional approval for animal care and use, a nearly pure neuronal culture was prepared from the cortices of 18-day-old Wistar rat fetuses. The cortices were digested with trypsin (Invitrogen, Carlsbad, CA, USA) and DNase I (Sigma-Aldrich, St. Louis, MO,

USA), followed by mechanical dissociation. Cortical cells were seeded at a density of 3000 cells/mm^2 in poly-L-lysine-coated plastic plates (BD Bioscience, Franklin Lakes, NJ, USA). The cells were grown in neurobasal medium (Invitrogen) with B27 minus AO (Invitrogen) and N2 (Invitrogen) supplements and L-glutamine (Sigma-Aldrich). Serum-free neurobasal medium was optimized for cell survival and neurite outgrowth of hippocampal neurons and the almost complete absence of glial cells, without reagent to inhibit them [16]. Cultures were kept at 37ºC and 5% CO2 in a humidified incubator. The medium was changed every third day and the cells were grown for 14 days.

As a pilot study, the proportion of neurons and glial cells was evaluated after 14 days of culture by immunocytochemistry with the following antibodies: microtubule-associated protein-2 (MAP-2) for neurons [17] and glial fibrillary acidic protein (GFAP) for glial cells. GFAP antibody was produced according to a method reported previously [18,19]. Evaluation using a fluorescence microscope (BX5; Olympus, Tokyo, Japan) revealed that the proportion of neurons was more than 90% (Fig. 1). Accordingly, we confirmed that a nearly pure neuronal culture was established in our culture system.

Glutamate insult and application of edaravone (Fig. 2)

After 14 days of culture, 50 µM glutamate was added to the medium for 10 min, followed by return to the normal culture for 24 h. To evaluate the prophylactic effect, three different doses of edaravone (10, 100, and 500 µM) or saline were added to the same sister dishes from 30 min before the glutamate insult until the end of the glutamate exposure. To evaluate the treatment effect, 500 µM edaravone or saline was added to the medium of the same sister dishes 2, 6, or 12 h after the end of the glutamate insult.

Cell viability

To evaluate the cell viability 24 h after the end of the glutamate insult (Fig. 2), we used the method of Shibuta

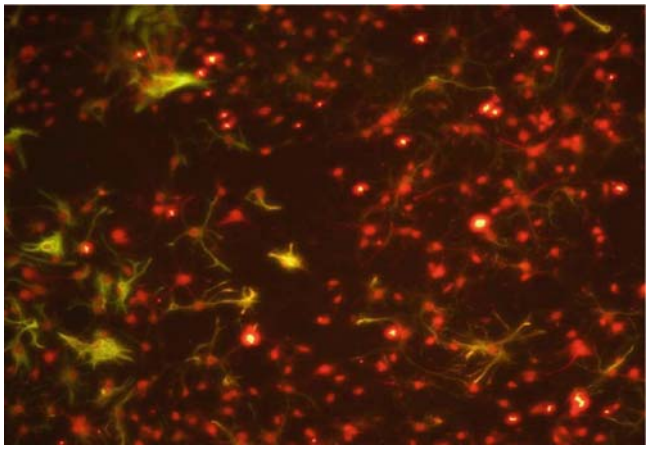

Fig. 1. Proportion of neurons and glial cells evaluated by immunocytochemistry with microtubule-associated protein-2 (MAP-2) and glial fibrillary acidic protein (GFAP). GFAP antibody was produced in rabbit and guinea pig, using glutathione S-transferase fusion protein carrying C-terminal 66 amino acids of mouse GFP (GenBank, AF332061). *Red*, neurons; *green*, glial cells. The proportion of red cells (neurons) was more than 90%

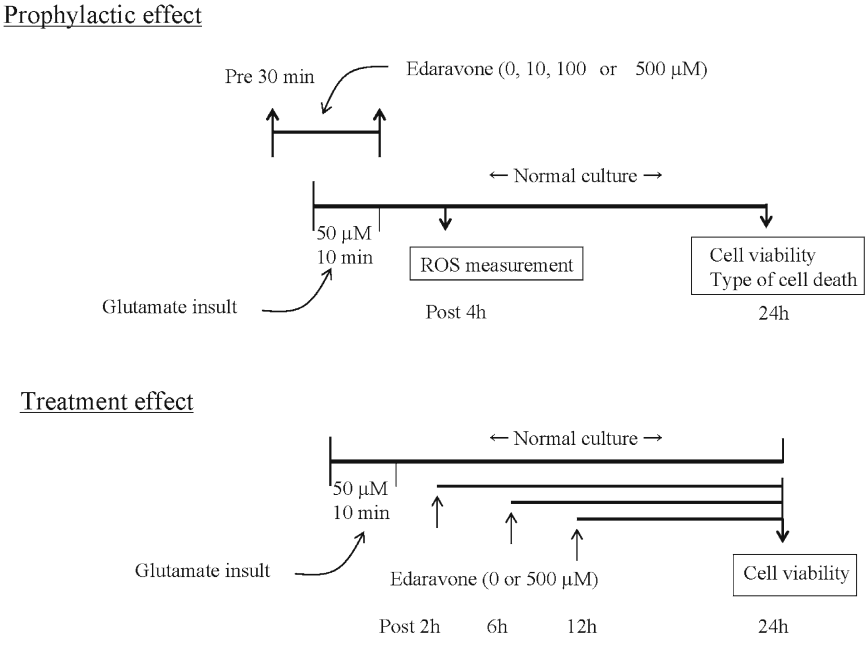

Fig. 2. Experimental protocol. *Pre* and *Post* indicate before and after the end of glutamate insult, respectively. Measurements of the items in the *open rectangles* were performed at the indicated times. In the measurement of reactive oxygen species (*ROS*) and the type of cell death, 10 and 100 µM edaravone concentrations were not used

et al. [20,21]. Photomicrographs of three or four areas within the dish were taken shortly before the exposure to glutamate. At the end of the protocols to determine the prophylactic or treatment effect (24 h after the end of the glutamate insult), the cells were exposed to 0.4% trypan blue with phosphate-buffered saline (PBS; Invitrogen) to stain nonviable cells, and photomicrographs were taken again in the same areas as those before the exposure. Approximately 1000 viable neurons per culture dish were subjected to manual counting. A second observer, blinded to the arrangement of photographs, study design, and treatment protocol, replicated all manual counts to ensure count accuracy and minimal interobserver variability. Survival rates were calculated by the following formula: 100 × (number of nonstained cells detected at the end of the experiments / total number of cells detected shortly before the exposure).

Measurement of relative amount of reactive oxygen species (ROS)

We measured the relative amount of ROS by using 6-carboxy-2′, 7′-dichlorodihydrofluorescein diacetate, di-(acetoxymethyl ester) (C-DCDHF-DA; Invitrogen) [22,23]. C-DCDHF-DA can be used as an indicator of ROS because its fluorescence intensity increases significantly when it is oxidized by ROS [22]. Four hours after the end of 50-μM glutamate exposure for 10 min under 500 μM of edaravone or saline (Fig. 2), C-DCDHF-DA was added, to a final concentration of 10 μM, for 30 min at 37ºC. The cells were collected by pipetting without washing and then analyzed by flow cytometry (FACS Caliber; Becton Dickinson, San Jose, CA, USA). The number of cellular events observed was 1000. As a control, we measured the ROS level of the neurons 4 h after the medium change without glutamate exposure or the application of edaravone.

Percentages of apoptotic and necrotic cells

The relative frequencies of necrotic and apoptotic cells were examined 24 h after 50-μM glutamate exposure for 10 min under 500 μM of edaravone or saline (Fig. 2) by the method of Shimizu et al. [24]. The cells were stained for 30 min at 37ºC with Hoechst 33342 (Sigma-Aldrich) and propidium iodide (Sigma-Aldrich). Then they were analyzed under a nonconfocal fluorescence microscope (Axiophoto; Zeiss, Jena, Germany) with excitation at 360 nm. Quantitative analysis, counting about 1000 cells, was performed by a second observer blinded to the arrangement of photographs, study design, and treatment protocol. Necrotic cells were regarded as those with round red nuclei, whereas apoptotic cells had fragmented nuclei, regardless of their color. The percentage of each cell type was calculated by the following formula: 100 × (number of necrotic or apoptotic cells / total number of cells, including live ones).

Statistics

Values are expressed as means ± SD. To compare the prophylactic dose-dependent effects of edaravone on cell viability and the relative amount of ROS, one-factor analysis of variance (ANOVA) was used. When a significant difference was seen, post-hoc analysis was performed with the Tukey-Kramer test. In other measurements, the paired *t*-test was used to compare cell viability and percentages of cell death types between cells with and without edaravone. Statistical significance was assumed when $P < 0.05$.

Results

Cell viability prophylactic effect

Each dish of the same sister culture was assigned to one of three different doses of edaravone, or saline. The cell viability in each dish was measured 24 h after 50-μM glutamate exposure for 10 min. Measurement was performed eight times.

Cell viability without edaravone was 49.0 ± 7.8%. Application of edaravone from 30 min before the glutamate insult to the end of exposure increased cell viability in a dose-dependent fashion (Fig. 3). There was a

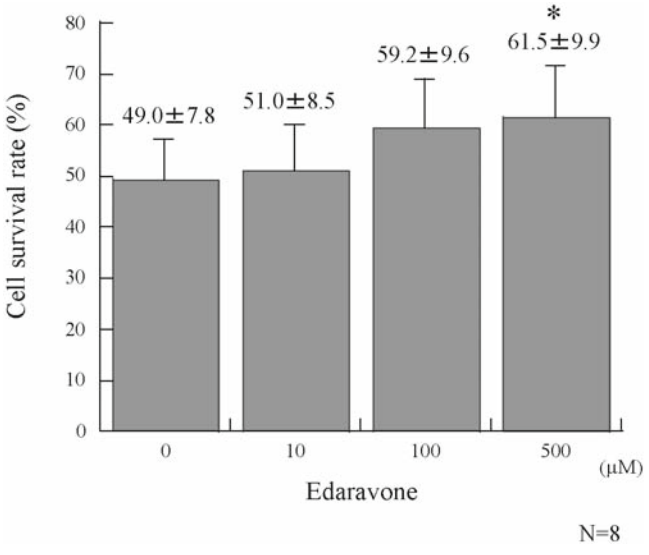

Fig. 3. Prophylactic effect of edaravone against glutamate insult. Cell viability was evaluated 24 h after 50-μM glutamate exposure for 10 min in the same sister dishes. Values are expressed as means ± SD. *P* values in one-factor analysis of variance (ANOVA) were 0.02. **P* < 0.05 versus the control without edaravone by the Tukey-Kramer test

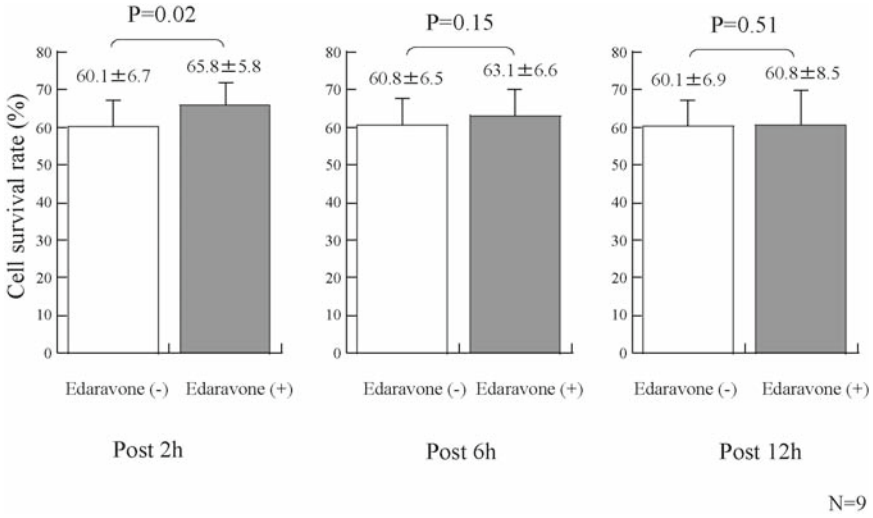

Fig. 4. Treatment effect of edaravone against the glutamate insult. Edaravone 500 μM or saline was added to dishes of the same sister culture 2, 6, or 12 h (*Post 2 h*, *6 h*, or *12 h*) after the end of the 50-μM glutamate exposure for 10 min. Cell viability was compared 24 h after the glutamate insult between the paired dishes with and without 500 μM edaravone and measurement was performed nine times. Values are expressed as means ± SD. *P* values were obtained by paired *t*-test comparing dishes with and without 500 μM edaravone

significant difference in one-factor ANOVA, and the cell viability with 500 μM edaravone was significantly higher than that without edaravone.

Treatment effect

Edaravone 500 μM or saline was added to each dish of the same sister culture 2, 6, or 12 h (post 2 h, 6 h or 12 h) after the end of the 50-μM glutamate exposure for 10 min. Cell viability was compared 24 h after the glutamate insult between the paired dishes with or without 500 μM edaravone, and measurement was performed nine times. When 500-μM edaravone exposure was started 2 h after the glutamate insult, the protective effect still existed (Fig. 4). However, a significant protective effect was not seen after 6 or 12 h.

Measurement of ROS (Fig. 5)

Edaravone 500 μM or saline was added to each dish of the same sister culture and the relative amount of ROS was measured 4 h after 50-μM glutamate exposure for 10 min. As a control, the relative amount of ROS was measured 4 h after medium change in the same sister dish without the application of edaravone or glutamate exposure. The measurement was performed eight times.

One-factor ANOVA showed a significant difference; compared to the control, the relative amount of ROS was significantly increased 4 h after the glutamate insult without edaravone. The amount was significantly lowered by the application of 500 μM edaravone, although the increase was not completely blocked compared to the control.

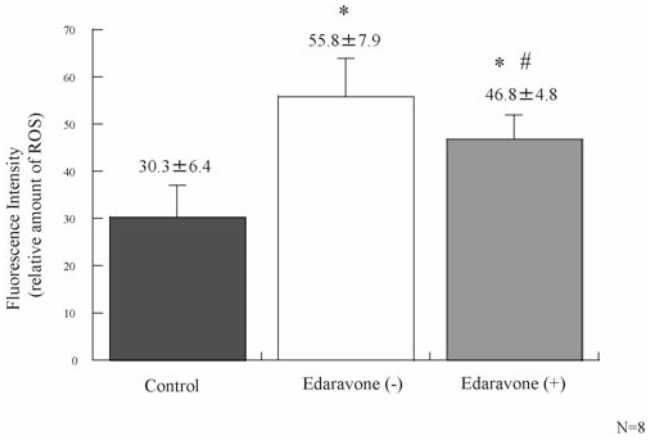

Fig. 5. Measurement of relative amount of ROS. The relative amount of ROS was measured in the same sister dishes 4 h after 50-μM glutamate exposure for 10 min with or without 500 μM edaravone and 4 h after medium change without glutamate and edaravone as a control. The measurement was performed eight times. Values are expressed as means ± SD. *P* values in one-factor ANOVA were below 0.01. $^{*}P < 0.05$ against the control; $^{\#}P < 0.05$ against edaravone (–) by the Tukey-Kramer test

Percentages of apoptotic and necrotic cells (Fig. 6)

Edaravone 500 μM or saline was added to each dish of the same sister culture. The relative frequencies of necrotic and apoptotic cells were examined 24 h after 50-μM glutamate exposure for 10 min and the measurement was performed eight times.

The percentage of necrotic cells with 500 μM edaravone was slightly but significantly lower than that of the control without edaravone. On the other hand, there was no significant difference in the percentage of

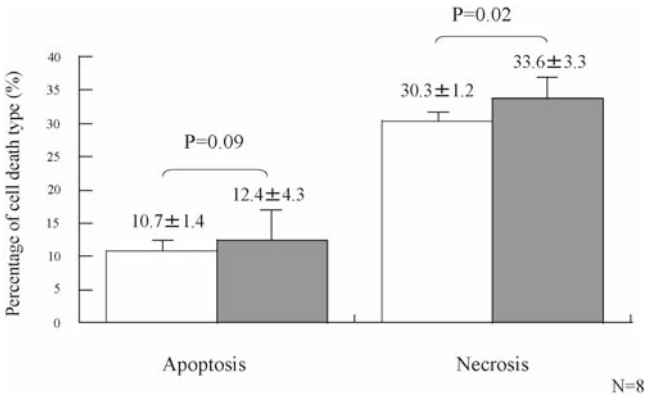

Fig. 6. Percentages of apoptotic and necrotic cells. The relative frequencies of necrotic and apoptotic cells were examined 24 h after 50-μM glutamate exposure for 10 min between the paired sister dishes with and without 500 μM edaravone, and measurement was performed eight times. Values are expressed as means ± SD. *P* values were obtained by paired *t*-test, xcomparing cultures with and without 500 μM edaravone for each cell type. *White bars*, edaravone (+); *gray bars*, edaravone (–)

apoptotic cells between cultures with and without edaravone.

Discussion

Perioperative neuroprotection for cerebral ischemia still remains an issue of major concern for anesthesiologists. Various approaches have been investigated, but almost all the pharmacological strategies have resulted in failure in the clinical setting. However, edaravone showed neuroprotection for patients in various clinical settings of cerebrovascular disease [6–9]. The mechanism of the protective effect is uncertain, including the effects on neurons themselves. Accordingly, we evaluated the protective effect of edaravone on neurons against glutamate neurotoxicity by using a nearly pure neuronal culture.

It is widely accepted that the excitatory amino acid neurotransmitter glutamate has neurotoxicity [25]. A quantitative dose-toxicity study suggested that the 50% effective dose (ED50) was 50–100 μM for 5-min exposure to glutamate in a cortical cell culture [26]. In our study, cell viability 24 h after 50-μM glutamate exposure for 10 min was also about 50%. Under such an insult, edaravone showed a dose-dependent prophylactic effect. This protective effect persisted when edaravone was applied until 2 h after the insult. However, there was no protective effect after that. Accordingly, our study indicated that edaravone showed mainly a prophylactic and partly a treatment effect against glutamate neurotoxicity.

In our study, the amount of ROS was significantly lower 4 h after glutamate exposure with edaravone than without it. It has been suggested that, in glutamate neurotoxicity, calcium-induced activation of one member of the cystein protease family converts xanthine dehydrogenase into xanthine oxidase, thus allowing for the production of superoxide anions and then hydroxyl radicals (OH) and that this leads to the general enhancement of oxidative stress and radical-mediated mitochondrial injury [27]. It has also been reported that ROS production increases after 4-h exposure to 50-μM glutamate in rat cortical neurons [28]. And in an in vitro study to examine the generation of OH by the reaction of hydrogen peroxide and iron, edaravone was shown to inhibit this ROS production [29]. An in vivo study also indirectly indicated that edaravone diminished the increase of OH in the hippocampus after rat forebrain ischemia [3]. Accordingly, our study suggested that edaravone inhibited the production of OH induced by glutamate neurotoxicity.

It has been reported that *N*-methyl-D-aspartate (NMDA)- or glutamate-mediated neuronal cell death is predominantly necrotic, probably with passive injury to cytoplasmic organelles, collapse of internal homeostasis, and uncontrolled Ca^{2+} overload [30,31]. Indeed, in our study, the percentage of necrotic cells was more than 30%, whereas the percentage of apoptotic cells was about 10%. And the necrosis induced by glutamate exposure was slightly but significantly inhibited by edaravone in our study. The mechanism of this action was unclear; however, one study showed that edaravone inhibited neuronal necrosis as well as apoptosis after hypoxic-ischemic insult in the immature rat brain by inhibiting mitochondrial injury [32]. On the other hand, it was reported that apoptosis was mainly induced by oxygen-glucose deprivation [33,34]. In addition, one study showed that the percentage of apoptotic cells induced by oxygen-glucose deprivation was significantly inhibited by edaravone [14]. These findings suggest that ROS production may be one of the key factors that determine neuronal viability in both the necrotic pathway induced by glutamate neurotoxicity and the apoptotic pathway induced by oxygen-glucose deprivation.

In our study, the high concentration of edaravone (500 μM) had a significant protective effect against glutamate neurotoxicity. Neuronal apoptosis induced by oxygen-glucose deprivation was inhibited by more than 100 μM edaravone [14]. On the other hand, in a coculture of neuronal cells and microglia, 10 μM edaravone (about the clinical concentration) significantly inhibited the neuronal injury induced by activated microglia [11]. The same amount of edaravone inhibited the production of nitric oxide and ROS produced by activated microglia. In a study using cultured bovine aortic endo-

thelial cells, 10 μM edaravone showed marked protection against endothelial cell death caused by 15-hydroperoxyeicosatetraenoic acid (HPETE). In these studies the significant protective effect of edaravone for glial and endothelial cells appeared at a lower concentration than the protective effect for neurons. Accordingly, the dominant target cells of edaravone in the whole brain may be glial and/or endothelial cells rather than neurons.

The present study was a basic study to evaluate the effect of edaravone on neurons themselves. In the clinical setting, it is improbable that the concentration of edaravone would reach 500 μM. Accordingly, there have been no reports about the effect of such an extremely high dose of edaravone on humans. However, one previous study evaluated single high-dose toxicity by using mice, rats, and dogs [35]. In that study, more than 400 $mg \cdot kg^{-1}$ edaravone was administered intravenously (i.v.). The usual clinical dosage for cerebral infarction is 60 mg (about 1 $mg \cdot kg^{-1}$) i.v. per day for 14 days. At the high dose, mice and rats showed toxic signs such as bradypnea, nictation, lacrimation, salivation, and sedation. The 50% lethal dose (LD50) was around 600–800 $mg \cdot kg^{-1}$ i.v. The cause of death was respiratory distress and/or inhibition of cardiac function. In dogs, toxic changes were sedation, nictation, incomplete eyelid closure, sneezing, tachypnea, emesis, and ataxic gait. The approximate lethal dose was about 600 $mg \cdot kg^{-1}$ i.v. The cause of death was a decrease in blood pressure, anemia, and/or renal infarction.

In conclusion, a protective effect of edaravone against glutamate neurotoxicity in neurons was seen by using a nearly pure neuronal culture. It was suggested that this effect resulted from the inhibition of necrosis through the suppression of ROS production. However, the significance of this direct neuronal protection afforded by edaravone needs to be further evaluated in the whole brain, because, in other studies, protective effects for glial and endothelial cells were seen with lower clinical concentrations of edaravone.

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