Small temperature variations alter edaravone-induced neuroprotection of cortical cultures exposed to prolonged hypoxic episodes

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Background. Edaravone, a free radical scavenger, has been shown to be neuroprotective in vivo and in vitro. However, the impact of small temperature variations on its neuroprotective actions remains unknown.

Methods. We examined the degree of neuroprotection conferred by various concentrations of edaravone on cortical cultures exposed to prolonged hypoxia (24 h) under three conditions: mild hypothermia (32°C), normothermia (37°C), and mild hyperthermia (39°C). The survival of cortical neurones from E16 Wistar rats (SR) was evaluated using photomicrographs taken before and after exposure to hypoxia.

Results. The mean survival of neurones exposed to hypoxia at normothermia was 14.7 (SEM 1.8)%. The addition of 50 μM edaravone significantly improved the mean survival to 40.5 (4.7)%. This improvement was noted at higher doses of edaravone (5 μM ≤) but not at lower doses (<500 nM). With mild hypothermia and prolonged hypoxia without edaravone, neuroprotection was significantly improved with a mean survival of 63.0 (5.2)%. This neuroprotective effect was not enhanced with the addition of edaravone, even at the highest dose. Hypoxia-induced neurotoxicity was aggravated by mild hyperthermia as reflected by a mean survival of 9.1 (2.1)%. However, higher concentrations of edaravone inhibited the deleterious effect of mild hyperthermia, thereby demonstrating a significant neuroprotective effect. The survival of neurones subjected to both hyperthermia and edaravone was the same as that of neurones exposed to normothermia and edaravone.

Conclusions. Temperature is a potential factor in determining whether edaravone confers a neuroprotective effect when applied during prolonged hypoxic insults.

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Acute hypoxic injury to the central nervous system (CNS) triggers a cascade of biochemical events that provoke prolonged secondary injury to the surrounding tissues. Even small variations in the CNS temperature are known to critically affect the neurological and histological outcome of hypoxic injury. The use of hypothermia is a proven potent neuroprotective tool for improving the outcome of cerebral ischaemia. The neuroprotection in the CNS conferred by hypothermia is attributed to a reduction in the cerebral metabolic rate (CMR), decreased release of neurotransmitters, attenuation of N-methyl-d-aspartate (NMDA) receptor activity, reduction in intracellular calcium influx, decreased lipid peroxidation, and reduced production of reactive oxygen species (ROS). We previously reported that mild (32°C) and moderate (27°C) hypothermia conferred as much neuroprotection as deep (22°C) hypothermia in primary cultured neurones when subjected to long-lasting hypoxia. In addition, profound (17°C) hypothermia does not offer any greater protection to the neuronal culture compared with that offered by mild, moderate, or deep hypothermia during hypoxic insults for 24 or 48 h.

In contrast to hypothermia, hyperthermia has been shown to increase the vulnerability of certain neurones to infarction. For example, in a model of hypoxic insult to rat
hippocampal slices, hyperthermia significantly worsened the electrophysiological recovery after 3 min of hypoxia exposure and accelerated ATP depletion. There are many temperature-dependent processes associated with neuronal injury such as the release of glutamate from synaptic vesicles, the intracellular accumulation of Ca\(^{2+}\), modulation of calcium-calmodulin-dependent protein kinase, the production of free radicals, and Zn\(^{2+}\) translocation from neuronal terminals into postsynaptic neurones.

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a potent synthetic free-radical scavenger, has been used during the early phase of an acute stroke in order to rescue the ischaemic penumbra in the brain tissue. Edaravone has the ability to eradicate free radicals including hydroxyl radicals. This eradication results in edaravone’s antioxidant activities. Several in vivo and in vitro studies have revealed that the administration of edaravone may possibly lessen cerebral damage resulting from ischaemic insults. These studies have indicated the efficacy of edaravone in diminishing oedema formation, in inhibiting oxidative tissue damage, and in inhibiting ROS generation, thereby delaying the development of cerebral infarcts. Moreover, studies indicate that edaravone improves one’s functional outcome after an acute ischaemic stroke. However, little is known about the sensitivity of edaravone-induced neuroprotection to temperature during hypoxia. Therefore, the object of the present study is to explore whether small variations in temperature would influence the neuroprotective effect of edaravone on cortical cultures exposed to prolonged hypoxic episodes.

**Methods**

**Chemical reagents**

The chemicals used in this study were obtained from the following sources: Dulbecco’s modified Eagle’s medium (DMEM) from Nissui Pharmaceutical (Tokyo, Japan); 5-fluoro-2'-deoxyuridine (5-FU), poly-l-lysine, streptomycin, penicillin, and anti-microtubule-associated protein 2 (MAP2) from Sigma–Aldrich (St Louis, MO, USA); trypsin from ICN Biochemicals (Costa Mesa, CA, USA); horse serum (HS) from Gibco BRL (Carlsbad, CA, USA); and anti-glial fibrillary acidic protein (GFAP) antibody from Dako (Carpinteria, CA, USA). Edaravone was kindly provided by Tanabe Mitsubishi Pharm Corporation (Osaka, Japan).

**Cell culture**

All animals were treated in strict accordance with the institutional and NIH guidelines for the care and treatment of laboratory animals. The study protocol was approved by the Animal Care Committee at the Osaka University Graduate School of Medicine.

Primary cultures of cortical neurones were prepared as described previously. Briefly, rat fetuses were removed at embryonic day 16 from anaesthetized pregnant Wistar rats, which were obtained from Nihon SLC (Hamamatsu, Japan). The fetal rat brains were examined under a microscope. Cerebral cortical neurones were treated with 0.25% trypsin at 37°C for 20 min and triturated with a Pasteur pipette. Dispersed cells were diluted to a concentration of 1.0 × 10^6 cells ml\(^{-1}\) in DMEM, which contained 8% FCS and 4% HS, 50 μg ml\(^{-1}\) streptomycin, and 50 IU ml\(^{-1}\) penicillin. This suspension was placed on a poly-l-lysine-coated 35 mm diameter tissue culture dish (1.5 ml per well) with a 2 mm grid (Nunc, Naperville, IL, USA) used to observe the same neurones in a given area over time. After 4 days in culture, cells were treated with 5 μg ml\(^{-1}\) of 5-FU for 3 days to prevent proliferation of non-neuronal cells. The neurones were maintained in DMEM containing 8% FCS and 4% HS in an atmosphere of 5% CO\(_2\) and 95% air, and under 100% humidity at a temperature of 37°C. The medium was changed twice weekly. All subsequent experiments were carried out after 13–14 days in culture.

**Immunohistochemical assessment**

In order to confirm the purity of the neuronal culture, cells were immunostained with anti-MAP2 or anti-GFAP antibody before and after the experiment. More than 97% of the cells expressed MAP2 and less than 2% expressed GFAP regardless of the duration of the experiments. This demonstrated that most of the cells in our cultures were neurones.

**Cytotoxicity**

Neurotoxicity was investigated using Shibuta’s model, as described previously. Before each experiment, the culture medium was replaced with fresh medium but lacking FCS and HS. A hypoxic atmosphere was maintained in a special anoxic incubator (ASTEC water jacket type multi-gas incubator, APM-30D; Fukuoka, Japan). In this incubator, the ambient temperature and concentration of oxygen could be precisely controlled. Before the experiments, we used a gas analyser (ABL 620, Radiometer Copenhagen Trading Co., Denmark) to measure changes in the partial pressure of oxygen in the medium. The oxygen concentration was then adjusted in the incubator to ensure that the medium was maintained in a hypoxic atmosphere. During the hypoxic periods, neurones were maintained in an atmosphere consisting of 5% CO\(_2\) and 94–95% nitrogen under 100% humidity. Cultured neurones were exposed to hypoxic insults without reoxygenation after the hypoxic insults and survival was evaluated. Experiments were conducted by examining the effects of three different temperatures and various concentrations of edaravone. The survival of neurones was analysed after a 24-h period of hypoxia. Assessment of survival was carried out at the end of each experiment.
The experimental groups were as follows: Group A—normothermia, 37°C; Group B—mild hypothermia, 32°C; Group C—mild hyperthermia, 39°C; Group D—normothermia (37°C) with edaravone at five concentrations ranging from 5 nM to 50 μM; Group E—mild hypothermia (32°C) with edaravone at five concentrations ranging from 5 nM to 50 μM; Group F—mild hyperthermia (39°C) tested with edaravone at five concentrations ranging from 5 nM to 50 μM.

There were 18 groups altogether from which 185 cortical culture dishes were derived. In clinical practice, hypothermia is commonly divided into four groups: (i) mild hypothermia (32–35°C); (ii) moderate hypothermia (26–31°C); (iii) deep hypothermia (20–25°C); and (iv) profound hypothermia (14–19°C). Polderman concluded that, on the basis of available evidence, patients should be cooled to 32–34°C. Therefore, we chose 32°C as the lowest temperature that we could clinically use for hypothermic therapy. On the other hand, 39°C is a level typical for febrile adults or neonates.

As control conditions, we investigated neuronal survival in 50 cortical culture dishes at three different temperatures (37°C, 32°C, 39°C) exposed to a normoxic atmosphere for a 24-h period with and without 50 μM edaravone.

We used the Shibuta’s model, which was described in our previous studies, to evaluate cytotoxicity using a photomicroscopy system (Axiovert 25, Carl Zeiss, Germany; Canon EOS10D, Japan). The traditional assay of lactate dehydrogenase release is a well-established method to investigate cell viability. However, our model of counting cells with microphotographs is the most cost-effective method of determining cell viability in our institution. Three or four photomicrographs were made of each well shortly before exposing the cells to hypoxia and at the end of the experiment. The grid arrangement of the dish helped in determining the specific location of each well. The cells were exposed to 0.4% trypan blue with phosphate-buffered saline to stain non-viable cells and photomicrographs were again taken at the exact area as performed before the experiment. Viable neurones were readily distinguished from non-viable neurones. Non-viable neurones were either stained with trypan blue or disappeared from the culture dish, whereas viable neurones remained unstained. Approximately 500–1000 viable neurones 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 inter-observer variability. Survival rates were calculated using the following formula: 100 × the number of unstained cells at the end of the experiment divided by the total number of whole cells shortly before the experiment per grid area.

Statistical analysis
The results are expressed as the percentages of the mean and the standard error of the mean (SEM). The differences between the means were assessed using analysis of variance followed by Fisher’s protected least significant difference (PLSD) test with P-values <0.05 considered significant.

Results
In Group A, the mean survival of the neurones was 14.7 (SEM, 1.8)% after exposure to 24 h of hypoxia at normothermia without the administration of edaravone. In Group D, the survival did not improve considerably when hypoxic neurones exposed to normothermia were administered low concentrations (5, 50, 500 nM) of edaravone. However, significant neuroprotection was achieved in this group at the higher concentrations (5 and 50 μM) of edaravone. Neuronal survival in Group D at the higher concentrations was 26.7 (4.7)% at 5 μM and 40.5 (4.7)% at 50 μM. These are statistically significant differences (P＜0.01) from Group A, as shown in Figs 1 and 2.

As a control condition, cortical neurones were exposed to normoxia in a normothermic condition for 24 h with and without 50 μM edaravone. The survival of cells exposed to edaravone was approximately 96%, indicating it had no lethal effect.

Fig 1 The survival of cultured neurones exposed to hypoxia at different concentrations of edaravone and temperatures. Each bar represents the mean (SEM) percentage. The higher concentrations of edaravone (≥5 μM), but not the lower concentrations (≤500 nM), improve the survival of neurones at both normothermia (37°C) and hyperthermia (39°C) after exposure to prolonged hypoxia. With mild hypothermia (32°C) and prolonged hypoxia, survival significantly improved, but this neuroprotective effect was not enhanced with the addition of edaravone, even at the highest concentration. The differences between the means were assessed using analysis of variance followed by Fisher’s PLSD test with P-values <0.05 considered significant. *P<0.05 indicates a significant difference from having no application of edaravone at the same temperature.
In Group B, a significant neuroprotection was achieved with a 24 h period of hypoxia at mild hypothermia, even without edaravone administration. The mean survival was 63.0 (5.2)%. This was a significant difference ($P < 0.01$) from Group A, as shown in Figs 1 and 3.

The SR of neurones exposed to mild hypothermia (32°C) and various concentrations of edaravone was analysed in Group E. As shown in Figs 1 and 3, edaravone did not impact the neuroprotective effect of mild hypothermia during 24 h of hypoxia. The survival of neurones exposed to various concentrations (5 nM–50 μM) of edaravone and mild hypothermia were 58–68% in all groups. No significant intergroup differences were observed.

As a control condition, cortical neurones were exposed to mild hypothermia with normoxic conditions with and without edaravone. The survival of cells was approximately 95% independent of edaravone. There were no deleterious effects seen in neuronal survival.

In Group C, mean survival was extremely low at 9.1 (2.1)% after exposure to 24 h of hypoxia with mild hyperthermia and without the administration of edaravone. This was a significant difference ($P < 0.05$) from Group A, as shown in Figs 1 and 4. In Group F, neuronal survival at mild hyperthermia did not improve considerably when the neurones were exposed to low concentrations (5, 50, and 500 nM) of edaravone. At concentrations of 5 nM and 500 nM, the mean survival with mild hyperthermia were significantly lower ($P < 0.05$) compared with survival with normothermia (i.e. Group A). However, significant neuroprotection of hypoxic neurones under mild hyperthermia was achieved at higher concentrations (5 and 50 μM) of edaravone. In this case, mean survival at 5 μM was 24.5 (3.3)% and at 50 μM was 39.5 (3.5)%. These were significantly different ($P < 0.01$) from the survival in Group C, as shown in Figs 1 and 4. No significant differences were seen in the survival of neurones in Group D and Group F when exposed to higher concentrations of edaravone.

As a control condition, cortical neurones were exposed to mild hyperthermia at normoxia both with and without edaravone. The survival of cells was approximately 92%, which was independent of the administration of edaravone. There was no significant difference between the survival of neurones exposed to normoxia with either normothermia or hyperthermia.
Discussion

These results demonstrate that a small variation in the temperature of cultured neurones can affect whether edaravone confers neuroprotection when utilized during prolonged hypoxia. In our in vitro experiments, the neuroprotective effect of mild hypothermia was greater than the neuroprotective effect of edaravone used at clinical concentrations and independent of edaravone.

On the other hand, hypoxia-induced neurotoxicity was aggravated by mild hyperthermia. However, edaravone used at clinical concentrations conferred a significant neuroprotective effect; suppressed the deleterious effect of mild hyperthermia, and maintained the survival of neurones at the same level as with normothermia. These experimental findings might be clinically important as stroke patients typically experience a range of brain and body temperatures.

A limitation of this experiment is that the neuroprotective effect of cortical neurones in vitro may not necessarily correlate with their neuroprotective effect in vivo. Another limitation is that the response of rat fetal neurones to experimental conditions does not always mirror the response of adult neurones under the same conditions. Cultures of embryonic and fetal mammalian CNS neurones have been used in many experiments because of their reliability. However, the in vitro maintenance of neurones of adult mammals has hitherto been largely unsuccessful.

Edaravone, a potent free-radical scavenger, has been used in clinical practice during the early phase of acute stroke to reduce neuronal damage. Acute traumatic, ischaemic, or hypoxic injuries to the CNS provoke a cascade of biochemical events that result in a prolonged secondary injury to neurones adjacent to the site of the local injury. Excessive stimulation of excitatory amino acid receptors in these pathological conditions might trigger the production of free radicals such as superoxide anion (O$_2^-$). Superoxide is rapidly converted to a hydroxyl radical (OH$^-$) in the presence of Fe$^{2+}$. Moreover, superoxide reacts with nitric oxide to produce peroxynitrite (ONOO$^-$). Peroxynitrite is protonated to form peroxynitrous acid, an unstable species. In shock, inflammation, oxidative stress, and the formation of nitric oxide (NO), ONOO$^-$, and superoxide radicals could produce neuronal cellular damage. Therefore, scavenging these free radicals would attenuate neuronal injury and improve the outcome of cerebral ischaemia.

Previous studies have shown that edaravone can eradicate the hydroxyl radical (OH$^-$) and inhibit −OH$^-$-dependent and −OH$^-$-independent lipid peroxidation. In addition, edaravone inhibits both water-soluble and lipid-soluble peroxyl radical-induced peroxidation systems, non-enzymatic lipid peroxidation, and lipoxygenase and NO-induced activation of mitogen-activated protein (MAP) kinases. Other mechanisms by which edaravone might offer neuroprotective effects are by reducing Ca$^{2+}$ overload, regulating NO synthase (NOS), and improving cerebral blood flow by upregulating eNOS expression and downregulating nNOS and iNOS expression.

In the present study, high doses of edaravone (5 and 50 μM) appreciably improved the mean survival of hypoxic cortical neurones cultured at normothermia, demonstrating a neuroprotective effect against hypoxic insult. The concentrations of edaravone used in this study ranged between 5 nM and 50 μM; these concentrations are comparable with the serum concentrations observed in patients who receive edaravone i.v. for the treatment of acute embolic strokes.

It is well known that neuroprotective mechanisms of hypothermia include reductions in CMR, excitatory neurotransmitter release, and intracellular Ca$^{2+}$ influx. The degree of neuroprotection that mild hypothermia confers against hypoxia has been extensively studied. In this study, considerable neuroprotection was elicited at mild hypothermia. This finding is compatible with many previous in vivo and in vitro studies demonstrating the extremely deleterious effects of normothermia compared with that of hypothermia during hypoxic insults. We have previously demonstrated the advantages of mild hypothermia in reducing neuronal death; we also suggested that hypothermic injury associated with profound hypothermia might possibly be caused by enhanced glutamate- and NMDA-induced excitotoxicity. Moreover, compared with profound and deep hypothermia, mild hypothermia could easily be administered in patients as it has fewer adverse effects. Therefore, we performed these experiments at 32°C, as the lowest temperature that we could clinically use for hypothermic therapy, instead of at deep and profound hypothermia.

Although a number of reports have validated the neuroprotective benefits of mild hypothermia, a fair number of studies have exhibited contradictory findings as well. Our results clearly indicate that cooling to mild hypothermia (32°C) confers considerable protection to neurones exposed to prolonged hypoxia.

We have previously shown using the in vitro studies that the combined use of anaesthetic drugs and hypothermia enhances neuroprotection, more than what would occur if either were applied individually during prolonged hypoxia. Moreover, we have demonstrated that maximal neuroprotection during exposure to 24 h of hypoxia was achieved with a clinically relevant combination of hypothermia (32°C) and thiopental. The survival of cultured neurones subjected to this combination was approximately 90% and was significantly higher than the survival offered by either of these two agents alone. Similarly, we expected that the combined administration of mild hypothermia and edaravone would show additive neuroprotective effects as the neuroprotective mechanism of each is independent of the other. Referring to the administration of edaravone, a few reports have disclosed adverse effects in patients such as acute renal failure and fulminate
hepatitis. In consideration of these adverse effects, the use of edaravone in extremely high doses might not be clinically useful.\textsuperscript{35, 36}

The pattern for thiopental-induced neuroprotection was similar to that of edaravone at the same temperature. We hypothesize that the combined use of mild hypothermia and low-dose edaravone might confer considerable neuroprotection without causing undesirable effects. Surprisingly, the current study failed to demonstrate an additional increase in cortical neuronal survival with mild hypothermia even at the highest dose of edaravone. It is difficult to determine why—in contrast to thiopental—edaravone failed to enhance the neuroprotection conferred by mild hypothermia. One reason might be that, in order to elicit neuroprotective effects as reported by Wu and colleagues,\textsuperscript{24} and Ikegami and colleagues,\textsuperscript{37} edaravone might be needed at higher concentrations \textit{in vitro} than is appropriate for use clinically. Based on the present findings, the neuroprotective effect of mild hypothermia is much greater than that of edaravone, and neuroprotection by edaravone is too small to augment the protective effects of mild hypothermia. Another reason might be that thiopental has several mechanisms for neuroprotection—other than as a free-radical scavenger—such as a reduction in CMR, Na\textsuperscript{+} channel blockade, glutamate receptor blockade, inhibition of Ca\textsuperscript{2+} influx, or potentiation of GABAergic activity.\textsuperscript{38–40}

The cerebral cortex is a brain region that is very sensitive to temperature manipulation.\textsuperscript{41} Our results also show that the survival of neurons after exposure to hypoxia at mild hyperthermia and without the administration of edaravone (39°C) was significantly lower than that of neurons exposed to these same conditions at normothermia. Temperature-dependent neuronal death has been attributed to the release of glutamate, intracellular accumulation of Ca\textsuperscript{2+}, Zn\textsuperscript{2+} translocation, and the production of free radicals.\textsuperscript{39}

In our experiments, edaravone administered during mild hyperthermia and at higher doses of 5 and 50 μM markedly improved the survival of cultured cortical neurons, and at normothermia. These experimental findings may be clinically important as stroke patients might present with variable body temperatures.

To our knowledge, this is the first report that compares the protective effect of different temperatures at various concentrations of edaravone on hypoxia-induced neurotoxicity in cultured cortical neurons. Although edaravone did not demonstrate an enhanced neuroprotective effect at mild hypothermia, this novel free-radical scavenger showed significant neuroprotection at both normothermia and mild hyperthermia. The induction of hypothermia requires special equipment\textsuperscript{42} and a time frame to attain a particular hypothermic temperature. In contrast, edaravone can be administered quite easily and has a relatively wider therapeutic time window. Zhang and colleagues\textsuperscript{22} reported that even though edaravone administration was delayed until 6 h after reperfusion, it nevertheless significantly improved neurological function and reduced the infarct volume. Initial therapy with edaravone might extend the therapeutic window until mild hypothermia can be administered. In conclusion, the temperature of the CNS is a potential factor in determining whether edaravone confers a neuroprotective effect when applied during prolonged hypoxic insult.

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