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News & Views

Does Edaravone (MCI-186) Act as an Antioxidant and a Neuroprotector in Experimental Traumatic Brain Injury?

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ABSTRACT

Edaravone (MCI-186) is a novel synthetic free radical scavenger intended to have neuroprotective effect against ischemic insult. It is currently used on patients with cerebral infarction. Here, we note beneficial pharmaceutical effects of edaravone in rat experimental traumatic brain injury. Under specific experimental conditions, edaravone minimized traumatic brain injury by functioning as a synthetic antioxidant. Clinical trials testing the efficacy of edaravone are warranted. *Antioxid. Redox Signal.* 8, 281–287 .

TRAUMATIC BRAIN INJURY AND OXIDATIVE INJURY

Traumatic brain injury (TBI) is a critical condition in the field of emergency medicine. In the United States, about 40% of acute trauma deaths are due to head injury (17), whereas in Japan, sudden death by accident, including TBI, is the third major cause of death, with the mortality of severe TBI being 51% (20). The pathophysiologic profile of TBI is varied and complicated. However, one of the neurotoxic factors thought to be involved is oxidative stress, including the generation of reactive oxygen species (ROS) (6, 15, 21, 25). A large number of studies have reported that oxidative stress, which generates ROS and as a consequence induces lipid peroxidation, plays a key role in the development of TBI (22, 24, 25). Consequently, one of the keys to managing TBI may be the ability to control lipid peroxidation (4, 10, 13, 29). Animal experiments have supported the notion that free radical scavengers and antioxidants dramatically reduce cerebral ischemic damage (5, 9, 16).

EDARAVONE AND NEUROPROTECTION

Edaravone (MCI-186), a novel free radical scavenger, was developed to prevent lipid peroxidation under neurologic conditions (27, 30), and it is currently the only such drug approved for use on cerebral infarction patients, having been shown to improve the functional outcome in comparison to a placebo after ischemic stroke (8). Because ischemic damage contributes to the complicated pathophysiology of TBI, edaravone was therefore considered potentially to have a therapeutic effect in such cases. The administration of edaravone (3 mg/kg, i.v.) has recently been reported to reduce the water content of injured brain tissue in a rat model of controlled cortical impact injury (19). However, it remains to be determined whether it exerts a protective effect in TBI. To elucidate this point, we investigated the neuroprotective effect of edaravone (3 mg/kg, i.v) in a rat TBI model (cryoinjury model). We also analyzed its effect on alkoxyl radicals (RO), the serum hydroperoxide (ROOH) levels, and serum antioxidant potency by assaying the jugular blood.

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Edaravone reduces injured brain areas after TBI

First we investigated the neuroprotective effect of edaravone in a rat experimental TBI model. Forty-eight hours after trauma, the injured brain areas were measured using TTC staining. Images of the anterior surface of TTC-stained coronal sections are shown in Fig. 1. The damaged area in the group administered edaravone (3 mg/kg, i.v.) (5.0 ± 2.1 mm^2; $n = 9$) was significantly smaller than that of the control (saline-administered) group (7.8 ± 3.6 mm^2; $n = 8$; $p < 0.05$) (Fig. 1B).

Edaravone is direct RO• and ROO• scavengers in vitro

Edaravone has direct scavenging activity in relation to hydroxyl (HO•) and nitric oxide (NO) radicals, but has no effect on the superoxide radical ($O_2^{\bullet-}$) (23, 27). However, direct RO• and ROO• scavenging activities of edaravone had not been proven. Recently we reported direct RO• scavenging activity of edaravone (7). RO• was generated *in vitro* by mixing methemoglobin (MetHb) with *t*-BuOOH (tert-butyl hydroperoxide). Edaravone (400 μ*M*) completely absorbed the RO•. Electron spin resonance (ESR) spin-trapping analysis could also detect the formation of ROO• adducts resulting from mixing MetHb with *tert*-BuOOH *in vitro* (Fig. 2A).

Edaravone (1 m*M*) was able to scavenge completely the ROO• produced by *tert*-BuOOH (Fig. 2B), and the ability of edaravone to scavenge ROO• *in vitro* was stronger than that of 1 m*M* α-tocopherol (Fig. 2C).

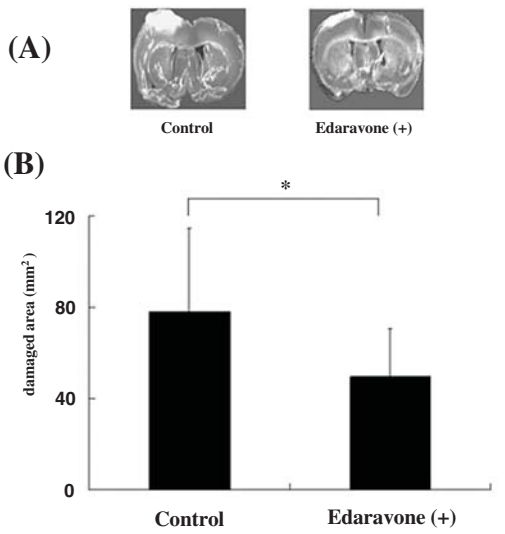

FIG. 1. Neuroprotective effect of edaravone in rat TBI model. TTC-stained coronal brain sections 48 h after TBI in the control group and the edaravone-administered groups (**A**). The damaged area in the edaravone-treated group (5.0 ± 2.1 mm^2; $n = 9$) was significantly smaller than that in the control group (7.8 ± 3.6 mm^2; $n = 8$; $p < 0.05$). All values represent the mean ± SEM (**B**).

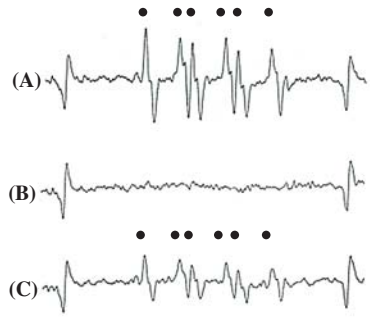

FIG. 2. *In vitro* ROO• scavenging activity of edaravone. The ESR spectra of the ROO• levels generated by mixing MetHb and *tert*-BuOOH *in vitro* are shown. The ESR spectrums of the mixture of untreated (**A**), DMSO control) or treating it with 1 m*M* edaravone (**B**) and 1 m*M* α-tocopherol (**C**). Complete scavenging of ROO• adducts was observed in the presence of edaravone. •, ROO• spin adduct.

EDARAVONE SUPPRESSED JUGULAR BLOOD ALKOXYL RADICAL (RO•) INTENSITIES AND SERUM HYDROPEROXIDE (ROOH) CONCENTRATIONS AFTER TBI

The study of free radicals in blood is currently severely hampered by technical difficulties in their detection. Here, we used an *ex vivo* ESR method with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin trap to investigate whether edaravone can scavenge OR•. To determine whether ESR spin-trapping analysis could also detect RO• in rat blood, RO• were artificially generated by the addition of *tert*-BuOOH. Spin adducts were not detected if *tert*-BuOOH had not been added to the blood (Fig. 3A) but were efficiently detected in oxidized rat blood (Fig. 3B).

The ESR spectra at 0, 6, 12, and 24 h after TBI are shown in Fig. 4. The ESR spectra at 0 h failed to show RO• spin adducts in either group (Fig. 4A). RO• spin adducts were observed in both groups at 24 h, as illustrated in Fig. 4B and C. Higher RO• spin adducts were seen 24 h after TBI in the control group than in the edaravone-administered group (Fig. 4B and C). The radical adducts were chemically identified to be RO• by adding either dimethyl sulfoxide or catalase in the ESR experiments (data not shown). A composite computer simulation of the RO• adducts was performed for the detection of ROS.

The RO• intensities after TBI are shown in Fig. 5. At 24 h after TBI, RO• intensity in the control group was significantly higher than that in the edaravone-administered group [223.8 ± 17.9 *vs.* 78.9 ± 58.6 (RI), $p = 0.0028$].

The serum hydroperoxide concentrations were also evaluated using the free radical electron evaluator (FREE; Health & Diagnostic Limited Co., Naples, Italy), and they are shown in Fig. 6 (2). After TBI, the hydroperoxide concentration gradually increased in both groups. However, the level in the edaravone-administered group remained significantly lower

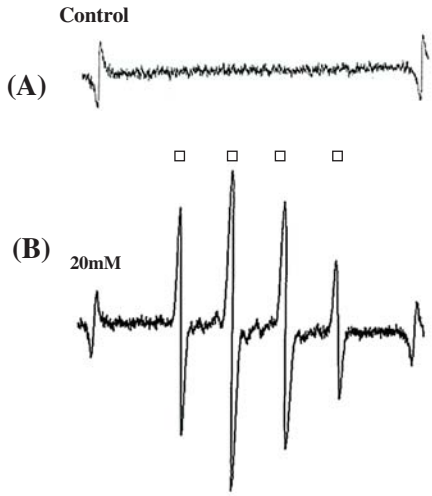

FIG. 3. *In vitro* RO• generation of rat blood by oxidative stress. An ESR spin-trapping analysis can detect RO• in rat blood. The rat blood was mixed with the oxidant *tert*-BuOOH and then was subjected to an ESR spin-trapping analysis. The ESR spectrum of the untreated blood is shown (**A**), thus demonstrating no spin adducts. In addition, the ESR spectrum of the blood treated with *tert*-BuOOH (**B**) is shown, thus revealing the presence of RO• spin adducts. □, RO• spin adduct.

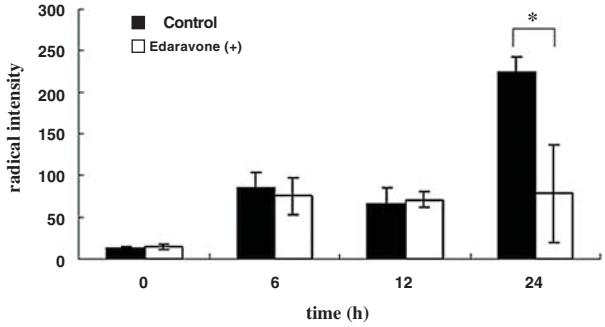

FIG. 5. Changes in the blood RO• intensity after TBI. The RO• intensity was detectable at 6, 12, and 24 h after TBI. No significant difference was observed between the groups at 6 and 12 h. However, at 24 h, the RO• intensity of the control group was significantly higher than that of the edaravone-administered group [223.8 ± 17.9 *vs.* 78.9 ± 58.6 (RI); p = 0.0028].

than that in the control group at both 6 h (186.4 ± 15.5 *vs.* 216.3 ± 15.5 Carr U/L; p = 0.012) and 12 h (193.6 ± 15.8 *vs.* 247.5 ± 23.3 Carr U/L; p = 0.0022) after TBI ($p > 0.05$). No significant difference was noted at 24 h (341.4 ± 54.3 *vs.* 348.75 ± 36.2 Carr U/L; p = 0.4119).

EDARAVONE INFLUENCES BOTH THE JUGULAR SERUM BIOANTIOXIDANT POTENCY AND THE JUGULAR SERUM α-TOCOPHEROL CONCENTRATIONS AFTER TBI

To evaluate the oxidant/antioxidant balance after TBI, the serum bioantioxidant potency (BAP) was measured using the FREE (2). Antioxidant potencies at 0, 6, 12, and 24 h after TBI are illustrated in Fig. 7. The time course of serum antioxidant potency was very different from that of the serum hydroperoxide response. In the control group, the serum antioxidant potency was the highest 6 h after TBI, but it decreased by 12 h. In contrast, the serum antioxidant potency gradually

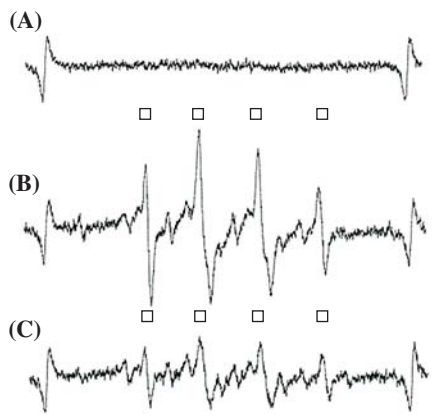

FIG. 4. Effect of edaravone treatment on RO• intensities in the jugular venous blood. The ESR spectrum at 0 h after TBI (**A**). The ESR spectra in the control group (**B**) and the edaravone-administered groups (**C**) 24 h after TBI. High RO• intensity was observed in the untreated situation (**B**). □, RO• spin adduct.

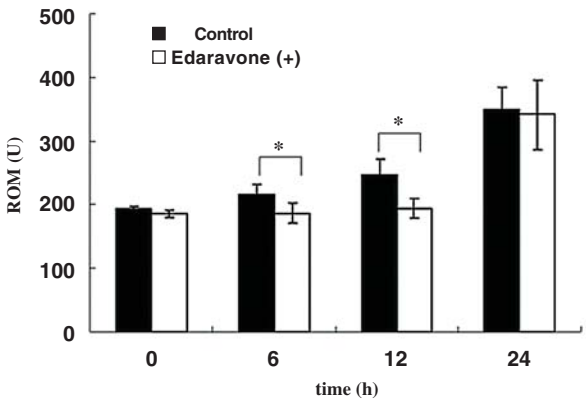

FIG. 6. The time courses of the serum hydroperoxide (ROOH) levels after TBI. The serum hydroperoxide (ROOH) concentrations gradually increased in both groups. However, in the edaravone-administered group, the concentration was significantly lower than that in the control group 6 h (186.4 ± 15.5 *vs.* 216.3 ± 15.5 Carr U/L; p = 0.012) and 12 h (193.6 ± 15.8 *vs.* 247.5 ± 23.3 Carr U/L; p = 0.0022) after TBI.

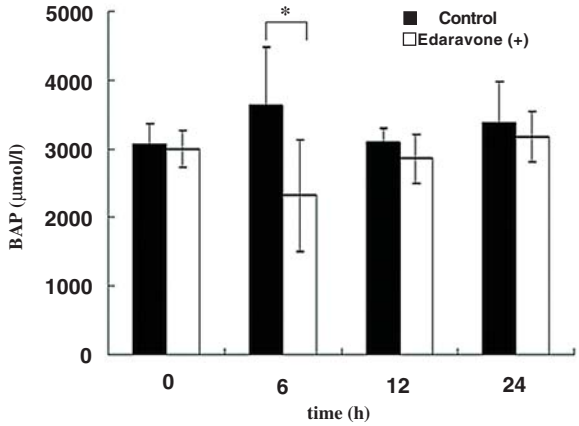

FIG. 7. Changes in the serum bioantioxidant potentials after TBI. The serum bioantioxidant potency (BAP) was highest at 6 h, but it decreased by 12 h in the control group. Although the serum BAP gradually increased over time in the edaravone-administered group, it was still significantly lower than that of the control group at 6 h (3,633.6 ± 845.3 *vs.* 2,320.1 ± 814.1 μ*M*; $p = 0.0250$).

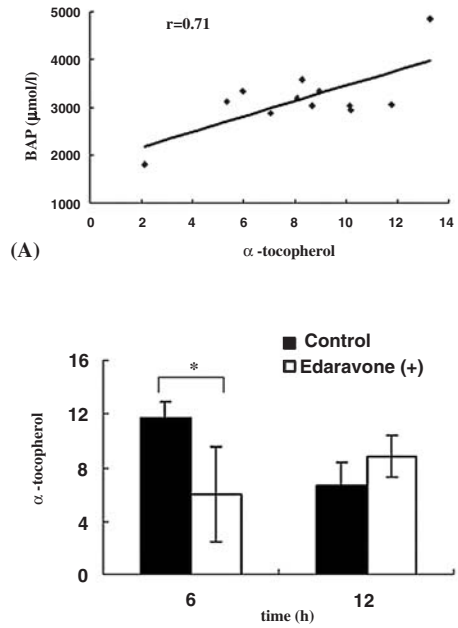

FIG. 8. Relation between the serum bioantioxidant potency (BAP) and the serum α-tocopherol (a-TOC) concentrations (A). The serum α-TOC concentrations at 6 and 12 h after TBI (**B**). A strong correlation was observed between the (α-TOC) concentration and BAP (**B**). The results of all samples are shown. $R = 0.71$, Pearson correlation coefficient. The serum α-TOC concentration at 6 h was significantly higher in the control group than that in the edaravone-administered group (11.71 ± 1.57 *vs.* 6.04 ± 3.52 μ*M*; $p = 0.0315$), whereas at 12 h, it was lower (6.64 ± 1.77 *vs.* 8.86 ± 1.16 μ*M*; $p = 0.0723$). The time course of the changes in the α-TOC concentration after TBI was similar to that of BAP.

increased in the edaravone-administered group. When comparing the two groups, the potency in the control group at 6 h was significantly higher than that in the edaravone-administered group (3,633.6 ± 845.3 *vs.* 2,320.1 ± 8,141 μ*M*; $p = 0.0250$). No differences were noted at 12 or 24 h (3,091.4 ± 202.2 *vs.* 2,851.6 ± 360.8 μ*M*; $p = 0.1384$, 3,378.0 ± 598.0 *vs.* 3,175.3 ±372.9 μ*M*; $p = 0.2755$).

To evaluate the antioxidant potency results in more detail, the serum α-tocopherol concentrations were measured in the same samples at 6 and 12 h after TBI. The serum antioxidant potency correlated closely with the plasma α-tocopherol concentrations ($r = 0.71$), with dynamic changes also being observed in the latter after TBI (Fig. 8A). The serum α-tocopherol concentration at 6 h was significantly higher in the control group than in the edaravone-administered group (11.71 ± 1.57 *vs.* 6.04 ± 3.52 μ*M*; $p = 0.0315$) (Fig. 8B). However, to our surprise, this situation was reversed at 12 h, with the concentration being lower in the control group (6.64 ± 1.77 *vs.* 8.86 ± 1.16 μ*M*; $p = 0.0723$). The time course of changes in the α-tocopherol concentration after TBI was similar to that of the antioxidant potency.

CONCLUSIONS AND OPEN QUESTIONS

One of the outcomes of this study was the fact that edaravone, a free radical scavenger, demonstrates a neuroprotective and antioxidative effect in a rat TBI model. Another aim of this work was to determine the direct involvement of free radicals and the kinetics of ROM and antioxidant potency after TBI. Our results suggest that oxidative stress and lipid peroxidation are strongly correlated with the pathophysiology of brain injury. Finally, to evaluate the changes in the oxidant/antioxidant balance after TBI, the antioxidant potencies and α-tocopherol concentrations were investigated, thus revealing both fluctuations in the former and the contributory influence of α-tocopherol.

Edaravone has previously been shown to display the scavenging activity for $HO^{\bullet}$, $RO^{\bullet}$, and NO radicals but not for the superoxide radical ($O_2^{\bullet -}$) (7, 23, 27). Our present data extended these findings by demonstrating the potent $ROO^{\bullet}$ scavenging ability of this drug, which exceeds that of α-tocopherol. In the present study, we used jugular blood to evaluate the oxidative stress caused by neuronal injury. The ability to monitor the free radical levels directly, which has previously been problematic, would provide clinicians with a great deal of vital pathophysiologic information. Our findings show that the $RO^{\bullet}$ levels in human blood can be assayed using the *ex vivo* ESR spin-trap method. We recently developed this novel method to analyze and monitor directly the $RO^{\bullet}$ levels in human blood and have previously shown that this method detects the $RO^{\bullet}$ levels in the blood from healthy volunteers (the average levels were 31.9 ± 19.5) (27). Our findings show these average $RO^{\bullet}$ levels of the healthy controls to be significantly lower than those of the patients in this series (71.1 ± 6.5). Moreover, we have found the $RO^{\bullet}$ levels in the arterial blood of TBI patients to be significantly lower than the levels in their jugular venous blood (18). In the experimental cryoinjury of the rat, the $RO^{\bullet}$ levels in the jugular blood have also

been shown to be significantly higher than the levels in their arterial blood (data not shown). Our findings support the notion that the RO• level in the jugular blood is useful for evaluating the oxidative damage of the brain. In a clinical setting, edaravone also has been shown to reduce the jugular blood RO• intensity in TBI patients (7). Many studies on the strong neuroprotective effect of edaravone in brain ischemia have been reported (27, 30). Edaravone has been shown to prevent vascular endothelial cell injury and to ameliorate neuronal damage (26). With an *in vivo* ESR method, the administration of intravenous edaravone (3 mg/kg) immediately after reperfusion also was shown to suppress the cerebrovascular ROS generation in a rat transient middle cerebral artery occlusion model, thereby decreasing the size of the cerebral infarction, and blocking brain edema by inhibiting lipid peroxidation, which is reportedly one of the major causes of cerebral ischemic damage (30). Edaravone treatment significantly inhibits the c-Jun NH_2-terminal kinase (JNK) pathway (28), and it not only exerts a neuroprotective effect but also plays a role in various other organ diseases (1, 11). Despite the large number of previous reports, however, little has been published on the effects of edaravone on TBI, although Nakamura *et al*. (19) reported it to reduce brain edema 180 min after injury. In the present study, we observed a 35.9% reduction in the damaged area 2 days after TBI.

A large number of studies have reported that oxidative stress, which generates ROS and, as a consequence, induces lipid peroxidation, plays a key role in the development of TBI. First, oxidative stress has been shown to participate in both the primary and secondary damage that follows acute TBI (12, 15, 25). Second, ROS have been reported to play important roles in the development of various neuronal conditions (15, 16). Third, oxidative stress has been shown to induce lipid peroxidation and brain damage, and finally, lipid peroxidation has an established link with TBI (25). In the present study, the elevated RO• intensities and hydroperoxide concentrations were demonstrated in jugular venous blood after TBI. The sources of free radical generation, whether in the injured brain or endothelial cells, were not identified in this report. However, our data provide direct evidence that TBI causes oxidative stress and lipid peroxidation, whereas edaravone ameliorates this effect. Moreover, further investigations regarding the ideal dosage and method of medication also are needed.

In contrast to the study of oxidative stress, the bioactivities of antioxidants in various pathologic conditions have not yet been thoroughly discussed. In the present study, the antioxidant potencies changed dramatically after TBI, an outcome that could be influenced by the administration of edaravone. These data may indicate that the antioxidant potency is influenced by the consumption and production of antioxidants during oxidative injury in the rat.

Many different endogenous antioxidants exist, one of the strongest of which is α-tocopherol. In the rat TBI model, an α-tocopherol derivative has been shown to suppress brain edema, thereby exerting a protective effect (10). α-Tocopherol–supplemented rats display more protection against stroke-induced injury than do matched controls. Such protection is associated with a lower c-Src activation and 12-lipoxygenase (12-Lox) phosphorylation at the stroke site (14). The close correlation between the antioxidant potency and the α-tocopherol levels in the present study supports the concept that α-tocopherol plays an important role in the balance between oxidant and antioxidant level in the pathophysiologic state associated with TBI. Conversely, the changes of other antioxidants in TBI remain unclear.

This study provides the first evidence that edaravone can exert neuroprotective and antiradical effects after TBI in the rat. Given that lipid peroxidation plays an important role in neurotrauma, the investigation of free radical generation, as well as determining the antioxidant abilities and the effects of antioxidants, including α-tocopherol, may be important for evaluating the overall balance of oxidative stress after acute brain injury.

ABBREVIATIONS

ESR, electron spin resonance; HO•, hydroxyl radical; NO, nitric oxide radicals; $O_2^{\bullet -}$, superoxide radical; RO•, alkoxyl radical; ROO•, peroxyl radical; ROS, reactive oxygen species; TBI, traumatic brain injury.

APPENDIX

Notes

1.Animals and surgical procedure

Male Sprague–Dawley rats (250–300 g) obtained from the Saitama Experimental Animal Center (Saitama, Japan) were housed in a temperature- (22°C) and light-controlled room (lights on at 0600 and lights off at 1800) and supplied with standard laboratory chow and tap water. All experiments were carried out in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of Showa University (06044).

Animals were anesthetized with a 50-mg/kg i.p. dose of sodium pentobarbital and mounted in a stereotaxic frame. The core temperature was continuously monitored with a flexible rectal thermometer and maintained at 37°C. A midline scalp incision was made to expose the skull, after which a 5 × 5-mm craniectomy was performed in the right parietal region between the coronary and lambdoid sutures. A sterile metal rod measuring 4 mm in diameter, which is a device for inducing cold injury, was chilled in liquid nitrogen, and then it was applied to the surface of the parietal cortex for 1 min. After a 10-min injury period, edaravone (3 mg/kg, i.v.) was administered via the right femoral artery in six rats (edaravone-administered group). A control group of six animals was similarly treated with saline.

2. Measurement of the area of brain damage

Forty-eight hours after brain injury, the rats were again anesthetized with sodium pentobarbital (50 mg/kg), after which their brains were removed and sliced into 2-mm coronal sections. These sections were then stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Wako, Tokyo, Japan) at 37°C for 30 min. Photographs were taken of the anterior surface of each section, and the area of injury was delineated based on the relative lack of staining. The damaged areas were measured using the Canbas X software program in the slices involving the greatest injury.

3. Assay to determine the direct ROO• scavenging activity of edaravone *in vitro*

To investigate whether edaravone has a direct peroxyl radical (ROO•) scavenging activity, the ROO•-scavenging activity of edaravone (1 m*M*) and α-tocopherol (1 m*M*) was measured *in vitro* by

ESR. ROO• were produced by the reaction of methemoglobin (MetHb) and *tert*-BuOOH. The 200-µl reaction solution consisted of 20 µl of 1 mg/ml MetHb in 0.1 *M* phosphate buffer (PB; pH 7.4), 60 µl of 0.1 *M* PB, 20 µl of 1 m*M* DETAPAC, 30 µl of 92 m*M* DMPO, 20 µl of edaravone or α-tocopherol sample in DMSO, and 50 µl of 600 m*M* *tert*-BuOOH. One minute after mixing the solution, we determined the radical intensity using an ESR spectrometer (JEOL JES RE1X, X-band, 100-KHz modulation frequency). The instrument settings were as follows: center field, 335.5 ± 5 mT; microwave power, 12 mW; modulation amplitude, 0.1 mT; gain, 5 × 100.

4. Blood sampling

We previously reported that the radical intensity of jugular venous blood is higher than that of arterial blood in TBI patients (7, 18). Moreover, the hydroperoxide concentration in the serum collected from the rat jugular vein is higher than that in the arterial blood. Based on these results, blood samples (500 µl at each point) were taken from the jugular vein under anesthesia with 2% sevoflurane (n = 4), at 0, 6, 12, and 24 h after injury. The collected serum samples were put into siliconized microcentrifuge tubes (0.5 ml), immediately frozen in liquid nitrogen, and then were stored in a −80°C freezer.

5. *In vitro* alkoxyl radical (RO•) production in rat blood by oxidative stress

Alkoxyl radicals (RO•) were generated in rat blood (n = 3) by mixing 100 µl of fresh blood and *tert*-butyl hydroperoxide (*tert*-BuOOH) (final concentration, 6 m*M*). The fresh blood samples were mixing with a 9.2 *M* solution of the spin trap 5,5-dimethyl-l•pyroline-l•oxide (DMPO) (Dojin Ltd., Kumamoto, Japan) by vortexing for 10 s, and storing the mixture in liquid nitrogen. These samples were dissolved and mixed with *tert*-BuOOH. Within 4 min of thawing, the RO• intensities were measured using an ESR spectrometer. The ESR spectra were recorded at room temperature (23°C) with the following spectrometer settings: center field, 335.0 ± 5.0 mT; microwave power, 16 mW; modulation amplitude, 0.1 mT; gain, 630; time constant, 0.1 s; scanning time, 2 min.

6. Blood alkoxyl radical (RO•) intensities after TBI

To analyze blood RO•, whole blood samples (100 µl) at each time point were immediately mixed with DMPO and analyzed within 30 min. The ESR spectra were recorded at room temperature (23°C) with the same settings as outlined earlier. The radical intensity (RI) was defined as the ratio of the signal intensity of the first peak of the alkoxyl-DMPO spin adduct to that of MnO (7, 18).

7. Assays of reactive oxygen (ROOH) metabolites

The generation of free radicals was evaluated in each plasma sample by the colorimetric determination of reactive oxygen metabolites (d-ROM test) using the free radical electron evaluator (FREE; Health & Diagnostic Limited Co.) (2). This method makes it possible to estimate the total amount of hydroperoxide present in a 20-µl sample using a spectrophotometric procedure. In brief, the samples described later were dissolved in acetate buffer (pH 4.8) with $FeCl_2$ at 37°C. They were then mixed gently, followed by the addition of 20 µl of chromogenic mixture including aromatic alkyl-amine. After incubation for 5 min at 37°C, the resultant pink aromatic derivative was measured at 546 nm according to the following reactions:

$$\text{R-OOH} + Fe^{2+} \rightarrow \text{R-O}^{\bullet} + OH^{-} + Fe^{3+} \quad (1)$$

$$\text{R-OOH} + Fe^{3+} \rightarrow \text{R-OO}^{\bullet} + H^{+} + Fe^{2+} \quad (2)$$

$$\text{R-O}^{\bullet} + \text{A-NH}_2 \rightarrow \text{RO-} + (\text{A-NH}_2^{\bullet})^{+} \text{ (for alkoxyl radicals)} \quad (3)$$

$$\text{R-OO}^{\bullet} + \text{A-NH}_2 \rightarrow \text{ROO}^{-} + (\text{A-NH}_2^{\bullet})^{+} \text{ (for peroxyl radicals)} \quad (4)$$

R-OOH, hydroperoxides; R-O•, alkoxyl radicals; OH^-, hydroxyl radical; A-NH2, aromatic amine; $(A\text{-}NH2^{\bullet})^+$, pink aromatic derivative; R-OO•, peroxyl radicals.

The results were expressed in conventional arbitrary units, known as Carr units. The value of 1 Carr unit is equal to a concentration of 0.08 mg/dl of hydrogen peroxide. Within-run variations were >2.6%, and between-run variations, <4.6%.

8. Assay of antioxidant potency (bioantioxidant potency)

The serum antioxidant abilities were measured with the BAP (bioantioxidant potency) test performed with FREE (2). The underlying principle of this test is similar to that of the well-known FRAP test, which measures the ferric reducing activity of plasma (3).

Ten microliters of plasma was dissolved in a colored solution, prepared previously by mixing $FeCl_3$ with a thiocyanate derivative. After a short incubation (5 min) at 37°C, the solution loses its color, with the intensity of this chromatic change being directly proportional to the ability of plasma to reduce, during the incubation, ferric ions to ferrous ions, according to the following reactions:

$$FeCl_3 + \text{AT (uncolored)} \rightarrow FeCl_3 - \text{AT (colored)}$$

$$FeCl_3 - \text{AT (colored)} + \text{BP (e-)} \rightarrow FeCl_2 + \text{AT (uncolored)} + \text{BP}$$

$FeCl_3$, ferric chloride; AT (uncolored), a thiocyanate derivative (uncolored); $FeCl_3$ AT (colored), the colored complex of ferric chloride with the thiocyanate derivative; BP (e-), a molecule of the plasma barrier with reducing/electron donating/antioxidant activity against ferric ions; BP, the oxidized form of BP (e-); $FeCl_2$, the ferrous chloride obtained by the reducing activity of BP (e-).

A photometric readout was used to assess the intensity of decoloration. A lyophilized human control serum with known antioxidant activity (µ*M*) was used periodically to calibrate the FREE system.

9. α-Tocopherol measurements with a coulometric array HPLC analysis

The serum levels of α-tocopherol and retinol were measured by a coulometric array HPLC analysis (ESA, Inc., MA). Standard chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO). A 0.2-ml volume of serum was vortexed (1 min) with 0.2 ml diluent and 10 µl of 10 µg/ml retinyl acetate as the internal standard; 1.0 ml of hexane was added, and the resulting mixture was vortexed (10 min) and centrifuged. Supernatant (0.8 ml) was withdrawn, and the sample was reextracted with an additional 1.0 ml of hexane. The combined extracts were evaporated under N_2, the residue was dissolved in 0.2 ml diluent, and 10 µl was analyzed.

A 150 × 3.0-mm i.d., 3 µm, C18 column (MD-150, ESA, Inc.) was kept at 37°C. Mobile phase A was methanol/water/buffer (1.0 *M* ammonium acetate adjusted to pH 4.4 with acetic acid) (90:8:2) (vol/vol/vol), and mobile phase B was methanol/*n*-propanol/buffer (78:20:2) (vol/vol/vol). A 32-min chromatographic run consisted of an 11-min linear gradient from 0 to 80% B, followed by a 10-min linear gradient to 100% B and a 7-min hold at 100% B before returning to the initial conditions for 4 min.

10. Statistical analysis

All data were expressed as the mean ± SEM. A statistical comparison of damaged brain areas was made using Student's t test for unpaired samples. Other statistical comparisons were made using Bonferroni's multiple t test. A value of $p < 0.05$ was considered to be statistically significant.

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