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Original Article

Edaravone alleviates brain-to-heart signaling after ischemia and reperfusion injury in aged rats

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Abstract: *Aims:* The present study was to reveal the molecular mechanism of the protective effect of edaravone, a novel drug with antioxidant properties, against oxidative damage in brain-to-heart signaling triggered by ischemia-reperfusion (I/R) injury to the brain by using an animal model of bilateral carotid artery occlusion (BCAO). *Methods:* Male Sprague-Dawley rats were divided into sham group, vehicle group, and edaravone group. Rat model of BCAO was constructed. Glutathione peroxidase (GSHPx) activity was determined. Enzyme-linked immunosorbent assay was used to determine the level of protein carbonyl. Western blotting was performed to measure inducible nitric oxide synthase (iNOS) level. Terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay was performed to study histopathologic damages. *Results:* In the brain, reperfusion decreased the level of GSHPx and significantly increased protein carbonyl level, iNOS level and the percentage of TUNEL-positive nuclei. Expression of iNOS that was decreased after drug treatment was accompanied with a decrease in the percentage of TUNEL-positive nuclei. c-Jun N-terminal kinase (JNK) activity was significantly correlated with the percentage of TUNEL-positive nuclei. In the heart, no significant change in the activity of GSHPx was observed. However, there was a mild decrease in protein carbonyl level and increase in iNOS expression. Mitogen-activated protein kinase (MAPK) activity in the heart was different from that in brain tissue, with significant activation of p38 MAPK. These results were further complimented by a slight increase in the percentage of TUNEL-positive cells in heart sections. The above-mentioned events were reversed by treatment with edaravone (3 mg/kg, i.v.). *Conclusion:* The present study demonstrates that edaravone ameliorates oxidative damage in brain-to-heart signaling after cerebral ischemia-reperfusion injury by modulating MAPK activity and normalizing oxidative stress in aged rats.

Keywords: Brain-to-heart signaling, cerebral ischemia-reperfusion, edaravone, mitogen-activated protein kinase, aged rats

Introduction

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a potent free radical scavenger, has being used in the management of acute ischemic stroke [1, 2]. Edaravone has potent hydroxyl radical scavenging properties and reduces nitric oxide production in a dose-dependent manner [3]. Its ability to prevent lipid peroxidation is comparable to that of ascorbic acid and tocopherol [4]. Furthermore, it has recently been reported to confer protection against ischemia-induced neuronal damage in brains of neonatal rats [5, 6]. However, the effect of edaravone on mitogen-activated protein kinase (MAPK) pathway after global cerebral ischemia is still obscure. In the present study, we investigate the effect of edaravone on ischemia-reperfusion (I/R) injury-induced neuronal damage in aged rats with a focus on MAPK family members c-Jun NH_2-terminal kinase (JNK), extracellular regulated kinase 1/2 (ERK1/2) and p38 MAPK.

A devastating consequence of tissue reperfusion is the development of damages in organs

that are not involved in the initial ischemia insult. Experimental data and clinical experience show that brain injury is often accompanied by a secondary injury in the heart [7]. Brain ischemia induces an increase in protein carbonyl level. Free iron then catalyzes the formation of semiquinones and free radicals, which may act in turn as mediators of the brain-to-heart signaling. In a previous study, we have found that 45 min reperfusion of the brain after 85 min ischemia simultaneously triggers accumulation of protein carbonyl and changes in MAPKs in the brain and heart of rats. In the present study, we focused our interest on the mechanism of brain-to-heart signaling by using free radical scavenger, edaravone, as a tool that may provide valuable information about possible participation of MAPK pathways signaling in this process.

Materials and methods

Animals

A total of 60 male Sprague-Dawley rats (age, 20 months; weight, 500-540 g; Charles River Japan Inc., Kanagawa, Japan) were used in the present study. The rats were divided into three groups: sham group (without any treatment), vehicle group (rats were treated with saline after bilateral carotid artery occlusion (BCAO)), and edaravone group (edaravone was dissolved in saline and administered intravenously at a dose of 1.5 mg/kg at 5 min and 35 min after BCAO). All animal experiments were conducted according to the ethical guidelines of Xinjiang Medical University.

BCAO

Experimental model was adopted according to the description by Ichikawa *et al.* [8]. During surgical procedure, body temperature of animal was maintained at 37.5°C with a heating pad (Model: TP-401, Gaymar Industries Inc., NY, USA). Anesthesia was maintained by pentobarbital. Cerebral ischemia was produced by the occlusion of both right and left common carotid arteries exposed through a middle skin incision using aneurysm clips for 85 min. Later, cerebral ischemia was confirmed by the change in color of the eyeball from red to white. At the end of the ischemic period, carotid arteries were declamped to allow blood reperfusion for 45 min. All rats were decapitated under anesthesia and then the whole brain was excised and used for analysis.

Glutathione peroxidase (GSHPx) activity measurement

GSHPx activity was determined according to the method reported by Wendel *et al.* [9]. Briefly, an aliquot of brain homogenate (0.4 mg protein) in 0.05 M phosphate buffer containing 1.15% (w/v) KCl was mixed in a microplate with 230 µL of coupling solution (containing 33.6 mg disodium EDTA, 6.5 mg NaN_3, 30.7 mg reduced glutathione, 16.7 mg NADPH and 100 units of glutathione reductase in 100 ml of 50 mM Tris-HCl pH 7.6). The volume was then adjusted to 260 µL with 0.05 M phosphate buffer. Kinetic decay of NADPH fluorescence (excitation wavelength, 355 nm; emission wavelength, 465 nm) was measured after addition of 40 mL of 1 mM H_2O_2 as the substrate using a microplate spectrophotometer (Labsystem Fluoroskan Ascent CF, Osaka, Japan).

Enzyme-linked immunosorbent assay (ELISA)

The level of protein carbonyl in brain and heart tissue homogenate was measured by ELISA [10]. Briefly, bovine serum albumin (BSA) oxidized by $CuSO_4/H_2O_2$ (3 mM/5 mM) was used as the protein carbonyl standard. Carbonyl content of oxidized BSA was determined by colorimetric method reported previously [11]. Tissue homogenate was centrifuged at 3,500 rpm for 10 min to remove the debris. Then, supernatant was diluted 2 fold with phosphate-buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 2.9 g $Na_2HPO_4{\cdot}12H_2O$, and 0.2 g KH_2PO_4 in 1000 ml distilled water, pH 7.4) and incubated with 10% streptomycin sulfate (9:1, v/w) for 15 min at 4°C. After centrifugation at 10,000 rpm for 10 min, protein concentration in the supernatant was measured by BCA method using BSA as standard, and protein concentration was adjusted to 1 mg/ml with PBS. The samples (500 µL) were reacted with 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.5 N HCl (100 µL) at room temperature for 1 h. Proteins in samples and oxidized BSA were precipitated with 20% trichloroacetic acid (TCA, 500 µL) and protein concentration was measured again and adjusted to 4 µg/100 µl. Standard curve was prepared using oxidized BSA diluted with 40 µg/ml BSA at a defined ratio (0-40%). Aliquots (100 µL) of test samples and standards (4 µg

of protein) were loaded into a 96-well immunoplate and incubated overnight at 4°C. The plate was washed with PBS containing 0.1% Tween 20 (PBST) and then incubated with blocking buffer (1% BSA in PBST) for 2 h at room temperature. The samples were further incubated with mouse anti-dinitrophenyl (DNP) IgE primary antibody (Sigma-Aldrich, St. Louis, MO, USA) for 4 h at 37°C, washed with PBST and then incubated with rat anti-mouse IgE secondary antibody (Southern Biotechnology Associates Inc., Birmingham, AL, USA) for 1 hour at 37°C. Peroxidase reaction was performed by addition of 100 µl of 3,3',5,5'-tetramethyl benzidine (Sigma-Aldrich, St. Louis, MO, USA) and stopped by 100 µl H_2SO_4 (0.18 M). Absorbance was measured at 450 nm using a microplate reader (Model 550; Bio-Rad, Hercules, CA, USA).

Western blotting

Brain and heart tissue samples homogenized in lysis buffer (50 mM Tris HCl (pH 7.4), 200 mM NaCl, 20 mM NaF, 1.0 mM Na_3VO_4, and 1.0 mM DTT). In order to study the role of the MAPK pathway and inducible nitric oxide synthase (iNOS) in global cerebral I/R injury, rat brain and heart homogenates were examined with antibodies that are highly specific for dual phosphorylated active forms of JNK, ERK, and p38 MAPK, as well as iNOS. MAPK activation was quantified by normalizing phospho-MAPK expression level to total MAPK expression in the same sample, whereas iNOS expression was normalized to GAPDH expression in the same sample. MAPK activation in sham group was taken as 1 arbitrary unit. Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose filters. Filters were blocked with 5% non-fat dried milk in TBST (20 mM Tris, pH 7.6, 137 mM NaCl, and 0.5% Tween 20) for 1 h at room temperature. Anti-phospho JNK rabbit polyclonal antibody, anti-JNK rabbit polyclonal antibody, anti-phospho ERK1/2 mouse monoclonal antibody, anti-ERK1/2 rabbit polyclonal antibody, anti-phospho p38 MAPK rabbit polyclonal antibody, anti-p38 MAPK rabbit polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA), anti-iNOS mouse monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA), and anti-GAPDH goat polyclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA) were used with a dilution of 1:1000. After incubation with primary antibodies, bound antibody was visualized with horseradish peroxidase-coupled secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA) and chemiluminescence developing agents (ECL Plus, Amersham, Piscataway, NJ, USA).

Terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay

Paraffin-embedded sections of brain and heart tissues were deparaffinized and dehydrated in a descending alcohol series and then incubated in proteinase K (20 mg/ml), washed with PBS, incubated with 3% H_2O_2 for 5 min, and washed with PBS again. TUNEL assay was performed as specified by the kit (Takara, Shiga, Japan). Sections were mounted and examined using light microscopy. For each animal, five sections were scored regionally for TUNEL-positive cells located in both cerebral hemispheres.

Statistical analysis

All statistics were calculated using SPSS 21.0 and plots were drawn using Prism 6.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed as standard error of the mean (SEM). Differences between groups were analyzed using one-way analysis of variance (ANOVA). The general linear model was used for repeated measures. $P < 0.05$ was considered statistically significant (two sided test, $a = 0.05$).

Results

I/R injury decreases GSHPx activity but increases carbonyl and iNOS levels, and edaravone treatment tends to correct this change by I/R injury

To study the protective effect of edaravone on cerebral oxidative damage induced by I/R in rats, GSHPx enzyme activity, protein carbonyl level and iNOS level were investigated. In the brain, I/R injury reduced GSHPx activity by 66 ± 4% when compared to sham group ($P < 0.05$). Of note, the level of GSHPx activity was almost restored to baseline level after treatment with edaravone ($P < 0.05$, **Figure 1A**). In the heart, reduction of GSHPx activity in vehicle group was not significantly different compared to sham group (**Figure 1B**). Moreover, the level of protein carbonyl, a marker of protein oxidation, in brain homogenate in the model of I/R injury after BCAO was significantly higher than that in

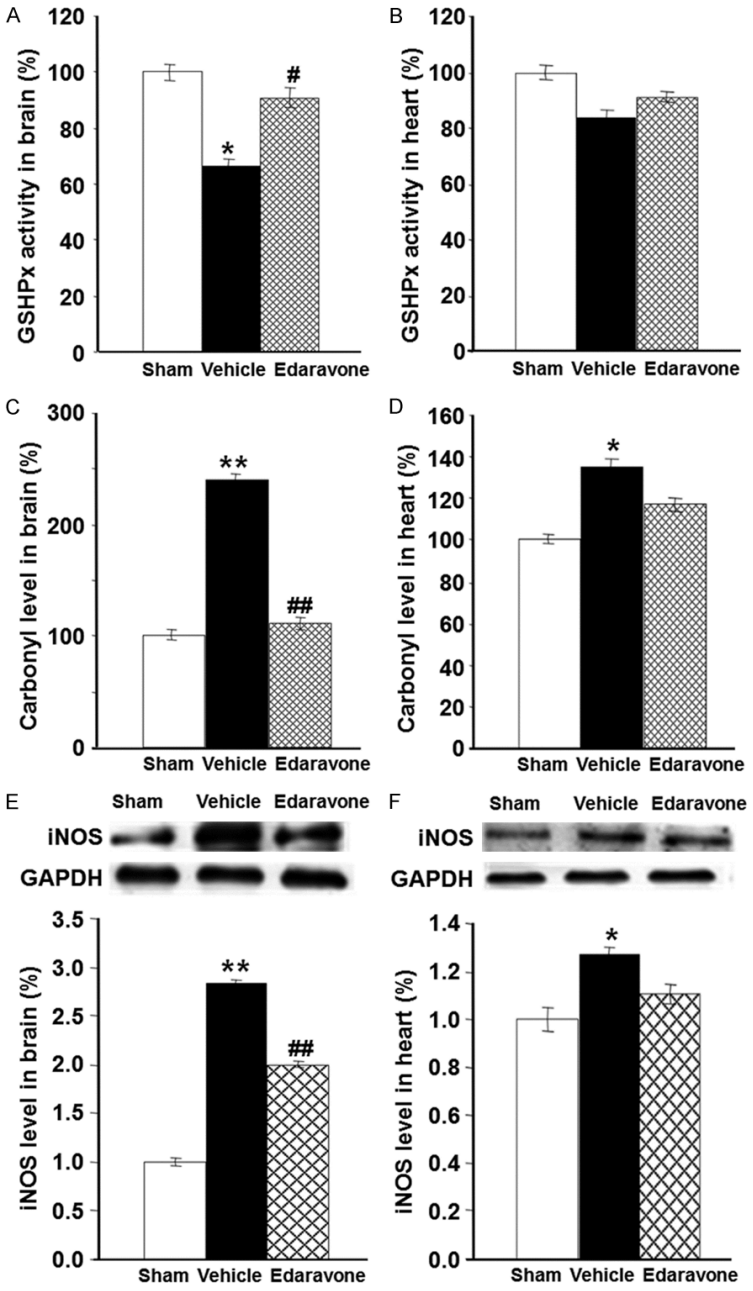

Figure 1. Effects of I/R and edaravone on oxidative stress variables. (A, B) GSHPx activity in (A) brain and (B) heart determined by the method reported by Wendel et al. [9]. (C, D) Protein carbonyl contents in (C) brain and (D) heart measured by ELISA. (E, F) iNOS expression in (E) brain and (F) heart measured by Western blotting. I, sham group; II, vehicle group; III, edaravone group. Each rat was tested in triplicate. The data are shown as means ± SEM of five animals per group. $*P < 0.05$ and $**P < 0.01$ compared with sham group; $\#P < 0.05$ and $\#\#P < 0.01$ compared with vehicle group.

sham group (P < 0.01), and treatment with edaravone (3 mg/kg) significantly decreased the level of protein carbonyl compared with vehicle group (P < 0.01, **Figure 1C**). Similarly, the level of protein carbonyl in vehicle group was significantly higher than that in sham group (P < 0.05), but edaravone treatment did not significantly reduce the level of protein carbonyl compared with vehicle group (P > 0.05, **Figure 1D**). Western blotting analysis showed that iNOS level in the brain of vehicle group was significantly higher than that in sham group (P < 0.01), and edaravone treatment significantly decreased the level of iNOS compared with that in vehicle group (P < 0.01, **Figure 1E**). In addition, the level of iNOS in the heart from vehicle group was significantly higher than that from sham group (P < 0.05), but edaravone failed to significantly decrease the level of iNOS compared with vehicle group (P > 0.05, **Figure 1F**). These results suggest that I/R injury decreases GSHPx activity but increases carbonyl and iNOS levels, and edaravone treatment tends to correct this change by I/R injury.

The p38 MAPK and JNK pathways play regulatory roles in I/R injury-induced neuronal damages, and edaravone potentially alleviates the injuries

To understand the mechanism of action of edaravone on I/R injury-induced neuronal damage in aged rats, we studied the activation of p38 MAPK, JNK and ERK1/2 after BCAO for 85 min followed by 45 min of reperfusion in rats. I/R injury slightly induced brain activation of p38 MAPK in vehicle group (P > 0.05), while edaravone treatment slightly reduced the activation of p38 MAPK compared with vehicle group (P > 0.05, **Figure 2A**). By contrast, I/R injury significantly enhanced p38 MAPK activation in the heart compared with sham group (P < 0.05), while treatment with edara-

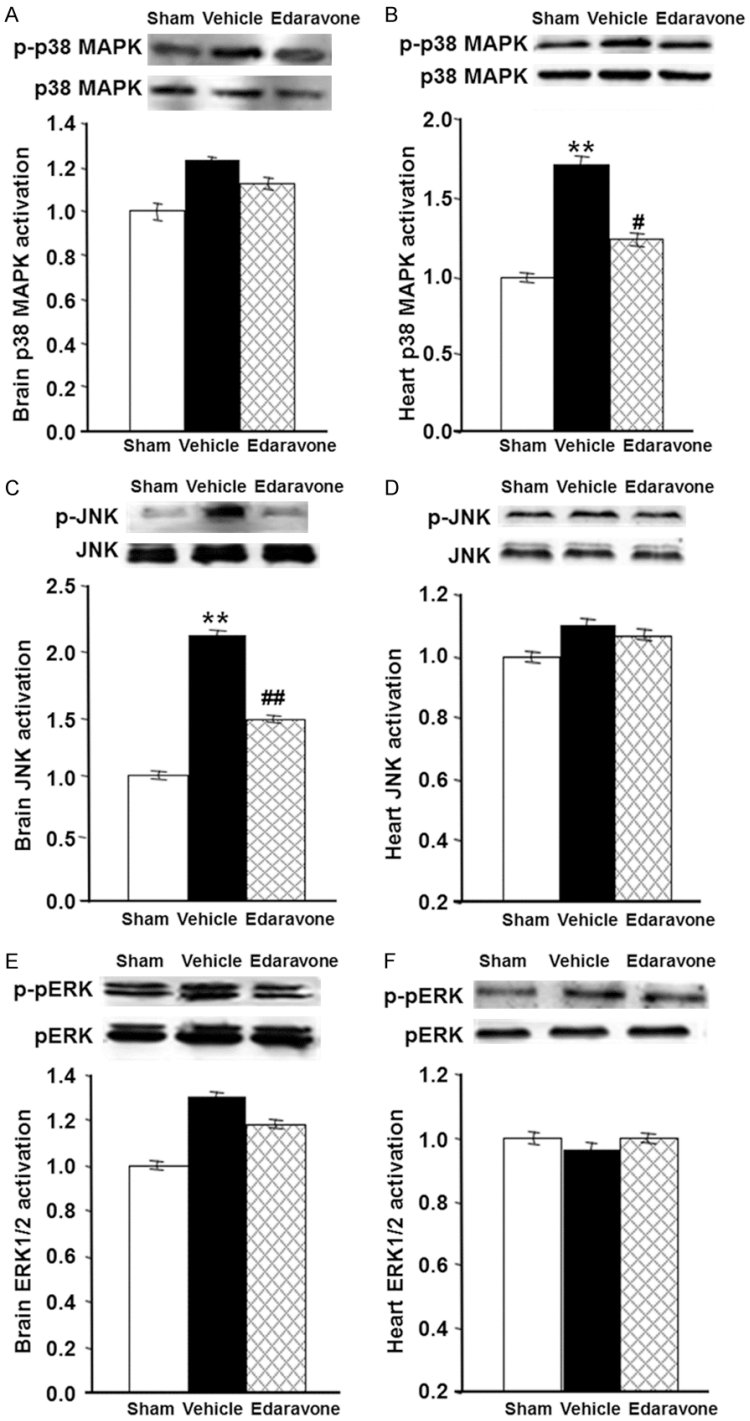

Figure 2. Effects of I/R and edaravone on MAPK activity in rats. Western blotting was used to examine (A, B) p38 MAPK activation in (A) brain and (B) heart, (C-D) JNK activation in (C) brain and (D) heart, and (E-F) ERK1/2 activation in (E) brain and (F) heart. I, sham group; II, vehicle group; III, edaravone group. Each rat was tested in triplicate. The data are shown as means ± SEM of five animals per group. $^{**}P < 0.01$ compared with sham group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with vehicle group.

vone significantly decreased p38 MAPK activation in the heart compared with vehicle group ($P < 0.05$, **Figure 2B**). Moreover, JNK activation in the brain from vehicle group was significantly higher than that in sham group ($P < 0.01$), while treatment with edaravone significantly decreased JNK activation compared with vehicle group ($P < 0.01$, **Figure 2C**). However, JNK activation in the heart from vehicle group was not significantly higher than that in sham group ($P > 0.05$), and treatment with edaravone failed to significantly reduce JNK activation compared with vehicle group (**Figure 2D**). In the brain, ERK1/2 activation was significantly increased in vehicle group as compared to sham group ($P < 0.05$), but treatment with edaravone did not significantly decrease ERK1/2 activation compared with vehicle group ($P > 0.05$, **Figure 2E**). However, ERK1/2 activation in the heart from vehicle group was not significantly different from that in sham group ($P > 0.05$), and treatment with edaravone failed to alter ERK1/2 activation in vehicle group ($P > 0.05$, **Figure 2F**). These results indicate that the p38 MAPK and JNK pathways play regulatory roles in I/R injury-induced neuronal damages, and edaravone potentially alleviates the injuries.

JNK activation and p38 MAPK activation are positively correlated with the percentage of TUNEL-positive cells in the brain and heart of rats with neuronal damage after BCAO

To examine neuronal damage after BCAO, TUNEL assay was performed. Histopathologic changes were observed in brain and heart of all ischemia-lesioned animals, and treatment with edaravone reversed the changes (**Figure 3A**). Quantification showed that the percentage of TUNEL-positive cells in the brain or heart from vehicle group was significantly increased compared

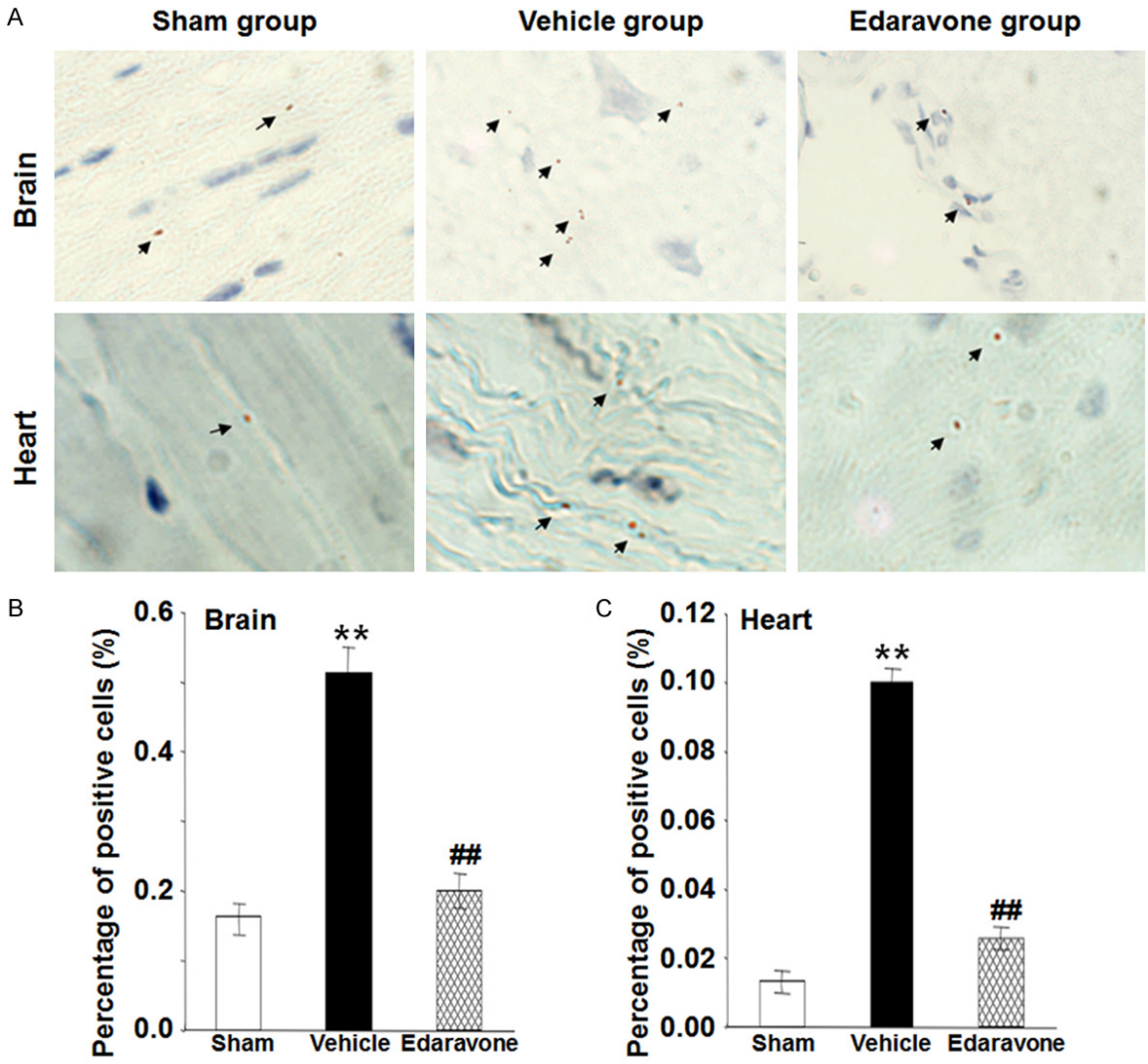

Figure 3. Effects of I/R and edaravone on histologic features of brain and heart of rats with neuronal damage after BCAO. (A) Representative photomicrographs of rat brain and heart section with TUNEL-positive insets (TUNEL staining, ×400). (B, C) Percentage of TUNEL-positive cells in (B) brain and (C) heart. I, sham group; II, vehicle group; III, edaravone group. The data are shown as means ± SEM (n = 5). $^{**}P < 0.01$ compared with sham group; $^{\#\#}P < 0.01$ compared with vehicle group.

with sham group ($P < 0.01$). In addition, treatment with edaravone significantly decreased the percentage of TUNEL-positive cells in the brain or heart compared with vehicle group ($P < 0.01$) (**Figure 3B** and **3C**). In the brain, a significant positive correlation was found between the activation of JNK and total percentage of TUNEL-positive cells induced by I/R injury ($P < 0.01$, **Figure 4A**). In the heart, a positive correlation was also discovered between the activation of p38 MAPK and total percentage of TUNEL-positive cells induced by I/R injury ($P < 0.01$, **Figure 4B**). The results suggest that JNK activation and p38 MAPK activation are positively correlated with the percentage of TUNEL-positive cells in the brain and heart of rats with neuronal damage after BCAO.

Discussion

The results of the present study demonstrated that free radical scavenger MCI-186 prevents brain and myocardial injury due to brain reperfusion by inhibiting JNK and p38 MAPK pathway signaling. Therefore, MAPK caspase cascade activation that is responsible for pathological apoptosis may be inhibited [12]. It has been suggested that the protective effect of

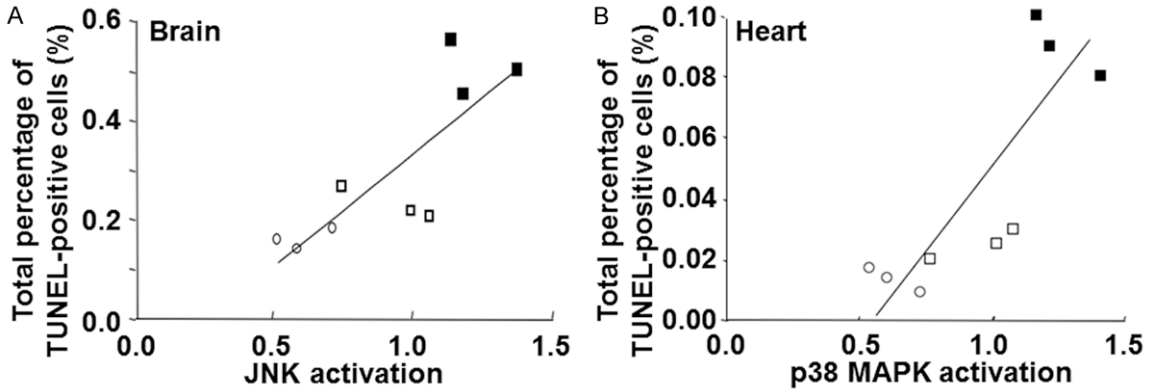

Figure 4. Quantification and correlation of total percentage of TUNEL-positive cells with (A) JNK activation in the brain and (B) p38 MAPK activation in the heart of rats with neuronal damage after BCAO.

edaravone on I/R-induced injury of sympathetic neurons might be mainly due to the ability of this drug in inhibiting the propagation of lipid peroxidation by interaction with free oxygen radicals [13]. Because free oxygen radicals may participate in damage of nervous tissue in processes such as stroke and cerebral ischemia, great effort has been made to protect nervous tissue against these radicals using pharmacological methods [14]. It is known that injury of the brain is often accompanied by a secondary injury of the heart. In our previous study, we observed that 45 min reperfusion of the brain after 85 min ischemia increases protein carbonyl content in the heart. Our result in the present study indicates that the brain-to-heart transmitted effects that are expressed in percentage of postischemia value are reperfusion-induced elevation in GSHPx level and protein carbonyl content in the heart. This finding seems to indicate that the mechanism of brain-to-heart signaling observed after ischemia event and postischemia reperfusion in the brain is not mediated via direct involvement of reactive oxygen species [15]. In addition, edaravone exerts a more specific antioxidant effect in the brain than in the heart.

In conclusion, edaravone treatment significantly inhibits neuronal damage by regulating oxidative stress and MAPK pathway signaling after I/R injury.

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Disclosure of conflict of interest

None.

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