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The free-radical scavenger edaravone rescues rats from cerebral infarction by attenuating the release of high-mobility group box-1 in neuronal cells

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d) Abbreviations: ANOVA, one-way analysis of variance; CBF, cerebral blood flow; Cmax, maximum plasma concentration; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; H_2O_2, hydrogen peroxide; HEPES, 4-(2-hydroxyethyl)-1 -piperazineethanesulfonic acid; HMGB1, high mobility group box-1; HRP, horseradish peroxidase; IgG, immunoglobulin G; JNK, Jun N-terminal kinase; LPS, Lipopolysaccharide; MABP, mean arterial blood pressure; MAPK, mitogen-activated protein kinase; MCAO, occlusion of the middle cerebral artery; MCI-186; edaravone; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5 diphenyltetrazolium bromide; NIH, National

Institutes of Health; NXY-059, disodium 2,4disulfophenyl-*N*-tert-butyl nitrone; OGD, Oxygen-glucose deprivation; PAGE, polyacrylamide gel electrophoresis; $PaCO_2$, partial pressure of carbon dioxide; PaO_2, partial pressure of oxygen; PBN, alpha-phenyl-*N*-tert-butyl nitrone; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline-0.02% Tween 20 PMSF, phenylmethylsulfonyl fluoride; rCBF, regional cerebral blood flow; RAGE, receptor for advanced glycation end products; ROS, reactive oxygen species; SD, standard deviation; SDS, sodium dodecyl sulfate; sh, short-hairpin; S-PBN, 2-sulfophenyl-*N*-tert-butyl nitrone; TBS, Tris-buffered saline; TBST, Tris-buffered saline containing 0.02% Tween 20; TNF, tumor necrosis factor; TTC, 2,3,5-triphenyl tetrazolium chloride

e) Neuropharmacology

Abstract

Edaravone, a potent free radical scavenger, is clinically used for the treatment of cerebral infarction in Japan. Here, we examined the effects of edaravone on the dynamics of high mobility group box-1 (HMGB1), which is a key mediator of ischemic-induced brain damage, during a 48 h post-ischemia/reperfusion period in rats and in oxygen-glucose deprived (OGD) PC12 cells. HMGB1 immunoreactivity was observed in both the cytoplasm and the periphery of cells in the cerebral infarction area 2 h after reperfusion. Intravenous administration of 3 and 6 mg/kg edaravone significantly inhibited nuclear translocation and HMGB1 release in the *penumbra* area, and respectively caused a 26.5±10.4 % and 43.8±0.5 % reduction of the total infarct area, at 24 h after reperfusion. Moreover, edaravone also decreased plasma HMGB1 levels. *In vitro*, edaravone dose-dependently (1-10 μM) suppressed OGD and hydrogen peroxide (H_2O_2)-induced HMGB1 release in PC12 cells. Furthermore, edaravone (3-30 μM) blocked HMGB1-triggered apoptosis in PC12 cells. Our findings suggest a novel neuroprotective mechanism for edaravone that abrogates the release of HMGB1.

Introduction

Oxygen free radicals are produced under various pathological conditions, including ischemia–reperfusion or stroke. They play a crucial role in brain ischemic injury by exacerbating membrane damage, leading to neuronal death. Therapeutic strategies have been developed to reduce free radical-induced damage processes in ischemic brain injury. Several compounds have been developed and some have also proceeded into clinical trials, including ebselen, tirilazad, NXY-059 and edaravone (Wang and Shuaib, 2007). However, the trial with ebselen and tirilazad in patients with stroke was recently terminated because there was no significant effect (van der Worp et al., 2002; Green and Shuaib, 2006). Recently, NXY-059 was found ineffective for the treatment of acute ischemic stroke within 6 h after the onset of symptoms in the Stroke-Acute Ischemic NXY Treatment II (SAINT II) trials (Shuaib et al., 2007).

Edaravone, which is a novel potent free radical scavenger, has been widely used for the treatment of acute cerebral infarction in Japan since 2001. Edaravone can quench hydroxyl radicals ($^{\bullet}$OH) and inhibit both $^{\bullet}$OH-dependent and $^{\bullet}$OH-independent lipid peroxidation (Watanabe et al., 2004). A clinical trial showed that the use of edaravone alone within 72 h after the onset of a stroke significantly reduced the infarct volume, producing sustained benefits during a 3-month follow-up period (The Edaravone Acute Brain Infarction Study Group, 2003; Zhang et al., 2005), while clinical treatment within 24 h of stroke onset has been used for patients with lacunae, large-artery atherosclerosis, and cardioembolism cerebral infarctions. Although its neuroprotective role has been widely investigated, no direct mechanism has been proposed for the effect of edaravone on hypoxia-related lethal systemic inflammatory mediators in the brain.

High mobility group box-1 (HMGB1) is a non-histone nuclear protein of 30 kD that is expressed in all eukaryotic cells. HMGB1 appears to play two distinct roles. Its first role is as a regulator of transcription in the maintenance of DNA function. Its second role is as a potent proinflammatory and cytotoxic cytokine, contributing to the development of disseminated intravascular coagulation and organ failure (Ito et al., 2007). Neutralization of HMGB1 function, even well after the onset of infection, was shown to rescue mice from lethal sepsis (Wang et al., 1999). HMGB1 translocates to the outside of the nucleus in all eukaryotic cells upon necrosis, and is released from macrophages through activation by lipopolysaccharide (LPS) and tumor necrosis factor (TNF)-α. It is also released from cultured macrophages, monocytes, and neutrophils following hydrogen peroxide (H_2O_2) exposure, from neuronal cells after ischemia/reperfusion, and it is known to exacerbate brain tissue damage in rats (Kim et al., 2006; Tang et al., 2006). Elevated levels of HMGB1 in the serum of human patients with cerebral infarction have also been reported (Goldstein et al., 2006).

To the best of our knowledge, no reports have so far demonstrated a role for HMGB1 in the neuroprotective effect of edaravone on reactive oxygen species (ROS)-mediated ischemic injury. In the present study, we therefore investigated the hypothesis that edaravone plays a neuroprotective role through the abrogation of HMGB1 release triggered by H_2O_2 or OGD, and ameliorates HMGB1-induced apoptosis in neuronal cells.

Methods

Materials

Bovine HMGB1 and anti-HMGB1 antibodies were obtained from Shino-Test (Kanagawa, Japan). As determined by Limulus endotoxin assays (Wako Pure Chemical Industries, Ltd., Kyoto, Japan), the content of LPS in HMGB1 solutions (5 μg/ml) was found to be 3.11 ng/ml. Edaravone was obtained from Mitsubishi Wellpharma (Tokyo, Japan). The S100 mouse monoclonal antibody, mouse anti-oligodendrocyte monoclonal antibody, and mouse neuronal marker anti-MAP-2 monoclonal antibody were purchased from Chemicon International (Temecula, CA). Anti-phospho (p) extracellular signal-regulated kinase (ERK)1/2, anti-ERK1/2, and anti-α actin antibodies were purchased from Cell Signaling Technology (Beverly, MA). The MAPK or ERK kinase (MEK)1/2 inhibitor U-0126 was obtained from Calbiochem (La Jolla, CA). The anti-cytochrome c monoclonal antibody was purchased from BD Biosciences International (Camarillo, CA). The H_2O_2 was a product of Santoku Chemical Industries Co., Ltd (Tokyo, Japan). The AnaeroPack® and its anaerobic jar were purchased from Mitsubishi Gas Chemical Co. (Tokyo, Japan).

Methods

Unilateral cerebral hypoxic-ischemia model

All animal procedures were conducted with the approval of the Animal Care Committee of the Ethics Board of the Institute of Laboratory Animal Sciences of Kagoshima University, Japan. Adult male Wistar rats (230–260 g; 8-weeks-old) were purchased from Japan SLC Inc (Tokyo, Japan) and maintained under a 12-h light/dark cycle with

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free access to food and water. Rats were anesthetized intraperitoneally with chloral hydrate (400 mg/kg body weight). Mean tail arterial blood pressure (MABP) and cerebral blood flow (CBF) were monitored by a non-preheating, non-invasive blood-pressure monitor (MK-2000, Muromachi, Tokyo, Japan). Regional cerebral blood flow (CBF) was measured over the middle cerebral artery (MCA) territory by laser Doppler flowmetry (LDF) (ALF21, Advance Co, Inc, Japan). A rectangular thin probe (7.5 × 3.5 × 1.0 mm, Type-CS, Unique Medical, Japan) for the LDF was slid through a small scalp incision into the natural pocket between the temporal muscle and the lateral side on the skull over the temporal cortex as previously reported (Harada et al., 2005). Blood gas and glucose levels were determined before and after occlusion of the middle cerebral artery (MCAO) via insertion of a PE50 tube via the left common carotid artery into the aorta (AVL OPTI critical care analyzer, AVL Scientific Corp., Roswell, GA, USA; Glucocard™ GT-1640, Aventis Pharma Ltd., Tokyo, Japan). Rectal and head temperatures were servo-controlled at 37.0°C using an electric blanket (KN-474, Natume, Tokyo, Japan). The head temperature was determined before and after reperfusion by monitoring the right temporal muscle (Model BAT-12, Physitemp Instruments Inc., Clifton, NJ). Blood gas analysis and glucose level at 10 min, body temperature and blood pressure at 45 min, and head temperature at 70 min after occlusion were determined.

Experimental left MCAO was induced for 90 min by an intraluminal filament, as reported previously (Longa et al., 1989). Mice that were administered edaravone immediately after reperfusion showed a stronger reduction of infarct volume and a more rapid functional recovery compared with mice that were administered edaravone 3, 6 and 12 after reperfusion (Zhang et al., 2005). Therefore, in the present study edaravone

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was administered immediately after reperfusion. Furthermore, we used two different doses of edaravone: 3 mg/kg and 6 mg/kg. The dose of edaravone was administered as previously described (Nishi et al., 1989; Zhang et al., 2005).

Rats either received no treatment (n=3) or cervical vein infusion (n=6) of 3, 6 mg/kg body weight edaravone immediately after reperfusion. The control sham-operated group (n=6) underwent the same protocol except for the MCAO procedure. Reperfusion was established after 90 min by withdrawal of the filament. Regional CBF (rCBF) was measured by a laser Doppler flowmeter (ALF-21, Advance Co, Inc, Tokyo, Japan) before and during MCAO. Animals without significantly reduced blood flow (more than 20% of the pre-ischemic baseline) during MCAO were excluded from further studies. At 2, 12, 24, and 48 h after reperfusion the rats were anesthetized, sacrificed by perfusion of 100 ml saline containing 10 U/ml heparin (Zhang et al., 2005), and their brains were excised. Coronal brain sections (2 mm thickness) from Bregma to 2 mm posterior were stained with 2,3,5-triphenyl tetrazolium chloride (TTC) to evaluate the infarct area, National Institutes of Health (NIH, Bethesda, MD) Image 1.63 software was used to calculate the area of the infarct. Sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for histochemical analysis. The left lateral caudoputamen, lower parietal cortex, and upper frontoparietal cortex were examined as described by Haberg et al. (Haberg et al., 2001).

A neurological grading scale was assessed according to Menzies et al. (Menzies et al., 1992): scale: 0 = no apparent deficits; 1 = right forelimb flexion, 2 = decreased grip of the right forelimb while tail pulled, 3 = spontaneous movement in all directions (right circling only if pulled by tail), 4 = spontaneous right circling.

HMGB1 ELISA

Brain tissue (120 μg/well) was gently homogenized in 10 mM *N*-2-hydroxyethlpiperazine-*N'*-ethanesulfonic acid (HEPES)/10 mM KCl buffer with 0.08% NP-40, 0.1 mM ethylenediamine tetraacetic acid (EDTA), 0.5 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and the soluble fraction derived from the cytoplasm was kept at -80°C until further testing. Serum was obtained by centrifuging (5000 rpm for 5 min) blood samples immediately after collection and storing the supernatant at -80°C until further analysis. All samples were examined for HMGB1 content using an enzyme-linked immunosorbent assay (ELISA) kit (Shino-Test).

Immunohistochemistry

Immunohistochemistry was performed by the indirect immunoperoxidase method. After deparaffinization and hydration, endogenous peroxidase was blocked with methanol containing 0.9% H_2O_2 for 10 min. After three 10-min rinses with 50 mM phosphate-buffered saline (PBS; pH 7.6), the sections were blocked with 10% non-fat milk for 20 min at room temperature, and then individually incubated in anti-HMGB1 rabbit antibody (10 μg/ml) overnight at 4°C. After three 10-min PBS rinses, sections were incubated in goat anti-rabbit immunoglobulin G (IgG) conjugated to peroxidase-labeled dextran polymer (EnVision, Dako, Carpinteria, CA) for 60 min. After rinsing with PBS, immunoreactivity was visualized with diaminobenzidine/peroxide. As negative controls, isotypic nonimmune serum (1 μg/ml) of the same species was used instead of the primary antibody. The sections were

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counterstained with Mayer's hematoxylin, mounted, and then examined with an Axioskope microscope (Carl Zeiss, Oberkochen, Germany).

Cell culture

Rat PC12 cells were obtained from the American Type Cell Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 5% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin on poly-L-lysine-coated dishes at 37°C in a humidified 5% CO_2 atmosphere. For all experiments, medium was replaced with serum-free Opti-MEM-I medium (Invitrogen Corp., Carlsbad, CA).

Oxygen-glucose deprivation

OGD on PC12 cells were performed as described previously (Hillion et al., 2005). Briefly, the original medium was removed, cells were rinsed twice with glucose-free RPMI 1640 medium (Life Technologies, CA), glucose-free RPMI 1640 medium supplemented with 2% horse serum and 1% FBS (OGD medium) was added and cells were placed in an anaerobic jar (Delaney and Onderdonk, 1997).

Immunofluorescence analysis

PC12 cells (1×10^6/ml) were cultured on Lab-Tech chamber slides (Nalge unc International, Cambridge, MA) with or without 40 μM H_2O_2 for 8 h and fixed with 4% paraformaldehyde for 5 min (Kawahara K et al., 1999). Slides were incubated with blocking buffer containing 1% BSA and PBS-0.02% Tween 20 (PBST) for 1 h, followed by an incubation of 30 min with anti-HMGB1 rabbit polyclonal antibody (1

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μg/ml) for 1 h. Subsequently, cells were washed with PBST and incubated with fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG (diluted 1:50) for 20 min. Slides were examined under an Axioskope microscope.

Sections from rat brain tissue were immunostained as described above. Slides were incubated with anti-HMGB1 rabbit polyclonal antibody (1 μg/ml), mouse anti-S100 monoclonal antibody (1:300), mouse anti-oligodendrocyte monoclonal antibody (1:500), and mouse anti-MAP-2 monoclonal antibody (1:200) overnight at 4°C. After washing with PBST, slides were incubated with FITC-labeled anti-rabbit IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and Alexa Fluor 546-labeled anti-mouse IgG diluted 1:200 in PBS for 1 h. After washing with PBST, the sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 1:500, Wako) for 5 min. After washing, the slides were examined under an Axioskop microscope (Carl Zeiss, Oberkochen, Germany).

Sample preparation for Western blot analysis

HMGB1-released samples were prepared according to our previous studies (Taniguchi et al., 2003). Briefly, PC12 cells (8×10^5/well) were exposed to OGD (2-4 h) or stimulated with 20 to 80 μM H_2O_2 for 12 h at RT. Edaravone was either added to the cultures 30 min before OGD treatment, or cells were incubated simultaneously with edaravone, H_2O_2, and HMGB1. U-0126 was pre-incubated for 1 h before H_2O_2 exposure. The supernatant (1 ml) was incubated with 50 μl heparin-sepharose 6B beads overnight at room temperature to bind HMGB1. Heparin beads bound to HMGB1 were washed three times with PBS, and sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl, 2% SDS, and 10% glycerol) was added to the beads. For MAPK

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activation, cell lysates of stimulated PC12 cells (5×10^5/dish) were obtained by adding 120 μl SDS sample buffer and lysed as described previously (Tancharoen et al., 2005). To evaluate mitochondrial cytochrome c activation, 50 μg protein from the cytoplasmic fraction of HMGB1-treated samples was subjected to MAPK activation as described above.

Western blot analysis

All samples were subjected to 12% SDS-polyacrylamide gel electrophoresis (PAGE; with the exception of cytochrome c, which was subjected to 15% SDS-PAGE), and then transferred to a nitrocellulose membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The membrane was blocked with 5% non-fat milk in Tris-buffered saline (TBS) containing 0.02% Tween 20 (TBST) for 1 h, followed by incubation with the respective primary antibodies (10 μg/ml anti-HMGB1 antibody, anti-ERK1/2 antibody diluted 1:1000, or 2 μg/ml anti-cytochrome c antibody) in TBST containing 1% non-fat milk for 3 h at room temperature. After three washes with TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG polyclonal antibody (Santa Cruz Biotechnology Inc.) or HRP-conjugated anti-mouse IgG polyclonal antibody diluted 1:3000 in TBST containing 2.5% non-fat dry milk for 1 h. The membrane was washed twice, and immunoreactive bands were visualized using the ECL detection system (GE Healthcare Bio-Sciences Corp.) and measured using National Institutes of Health (NIH) image 1.63 software.

Nuclear staining for the assessment of apoptosis

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Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with Hoechst 33258 (Sigma-Aldrich, Gillingham, UK). Briefly, cells were treated for 24 h at RT with 3 μg/ml HMGB1 in the presence or absence of edaravone (10 μM), cells were washed with PBS and fixed for 30 min with 4% paraformaldehyde. After additional washes with PBS, cells were incubated with Hoechst 33258 (final concentration 10 μg/ml) for 10 min. Nuclear morphology was examined using an Axioskope microscope. Intact blue nuclei were considered to represent viable cells and condensed/fragmented nuclei were considered to represent apoptotic cells.

MTT assay for determination of cell viability

PC12 cells were seeded at a density of 2–3 $\times$ 10^4 cells per well in 96-well dishes. Cells were treated with HMGB1 (0.5–4 μg/ml) in the presence or absence of edaravone (10 or 30 μM) for 24 h. Subsequently, 3-(4,5-dimethylthiazolyl-2)-2,5 diphenyltetrazolium bromide (MTT, Sigma-Aldrich) was added (final concentration, 0.5 mg/ml) to each well. After 3 h of additional incubation, 100 μl of a 10% SDS (pH 4.8) and 0.01 N HCl solution was added to dissolve the crystals for 16 h. Absorbance values at a test wavelength of 570 nm and a reference wavelength of 630 nm were determined with an automatic ELISA reader (Immuno Mini NJ-2300, Inter Med, Tokyo, Japan).

Statistical analysis

All experiments were performed at least three times, and representative examples are shown. Experimental values are given as the mean ± standard deviation (SD). In

HMGB1-stained sections, stained cells in three predefined areas (each 0.25 mm^2) were counted. Densitometric analysis of protein signals was assessed with the Student's t-test. One-way analysis of variance (ANOVA) followed by the *post hoc* Fisher's protected least-significant difference test was used to determine the significance of differences in the inhibition study. A P value <0.05 was considered to be statistically significant and was analyzed using StatView ver. 5.0 software or graphpad Prism ver. 5.0.

Results

Physiological variables

The physiological variables observed in the different groups of rats are shown in Table 1. No significant difference was observed between sham-operated and MCAO animals. Furthermore, administration of edaravone had no significant effect on these variables as previously described (Zhang et al., 2005). In previous reports, edaravone had no significant effect on body temperature and CBF (Ando et al., 1997; Qi et al., 2004; Zhang et al., 2005). One of the 28 rats that did not receive edaravone treatment died under anesthesia during MCAO.

Protein expression profile of HMGB1 in non-treated or edaravone-treated rats

We investigated the expression levels and cellular distribution of HMGB1 2–48 h after initiation of ischemic reperfusion in brain tissue of both edaravone-treated and non-treated rats using immunohistochemical analysis. Strong HMGB1 immunoreactivity was observed in the cytoplasm of neurons and glial cells, in the infarct area 2 h after reperfusion initiation in the non-treated group. After 12 h of reperfusion, HMGB1 immunostaining was weaker and small immunopositive cell bodies were found (Supplemental Fig.1c). Moreover, 24 h and 48 h (data not shown) after reperfusion, the HMGB1 signal was further decreased, indicating that nuclear HMGB1 had translocated to the cytoplasm, and subsequently to the extracellular space within the infarct area. The infarct area in the brains of edaravone-treated rats showed a marked decrease in HMGB1 immunoreactivity in the cytoplasm and an increased immunoreactivity in cell nuclei 2 h after reperfusion. However, edaravone treatment failed to block the HMGB1 signal at 12 h, 24 h and 48h after reperfusion. In the

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contralateral area of the cerebrum, HMGB1 was exclusively localized in the round-shaped nucleus between 2 and 48 h after reperfusion (data not shown).

Effects of edaravone on experimental cerebral ischemic injury in rats

A severe infarction was observed 24h after reperfusion by TTC staining of the cerebrum of rats that did not receive edaravone. The 24h interval was assessed as previously described (Nishi et al., 1989). A marked reduction in infarct area was observed in rats that received 3 mg/kg edaravone i.v. and a dramatic reduction of the infarct area was observed in rats that were administered 6mg/kg edaravone i.v. (Fig.1A). Next, we determined the infarct area in the lateral caudoputamen, lower parietal cortex (also named core) and upper frontoparietal cortex (also named as penumbra) of the cerebrum from edaravone treated or non-treated rats. As shown in Fig.2B, edaravone caused a significant reduction (34 ±2.2 %, $P<0.01$) of the infarct area at 6 mg/kg but not at 3 mg/kg in the lateral caudoputamen and lower parietal cortex. However, in the upper frontoparietal cortex, a dose of edaravone as low as 3mg/kg caused a significant reduction (60.6 ± 6 %, $P<0.01$) and a sharp reduction (87.9 ±4.5 %, $P<0.005$) in infarct area was observed in rats that were administered 6mg/kg edaravone compared with the non-treated group (vehicle, 0.9% NaCl). Our results are consisted with a previous report (Zhang et al., 2005). Moreover, a significant lower neurological score was observed in the edaravone (3 mg/kg) treated group compared with the non-treated group [2.3(2-3)], $P<0.05$.

We next examined HMGB1-positive cells in the lateral caudoputamen, lower parietal cortex, and in the upper frontoparietal cortex of rats that were administered edaravone (3 mg/kg i.v. or 6 mg/ kg i.v.) by double-immunofluorescent labeling using

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HMGB1 and S100 antibodies (See photographs presentation in Fig. 2B. No immunofluorescence data is shown for edaravone at 3mg/kg). It has been reported that the S100 protein is localized in the cytoplasm and/or nucleus of neuronal and non-neuronal cells (Donato, 1999; Gonzalez-Martinez et al., 2003). S100 staining was used to identify the subcellular localization of HMGB1 and to determine the cell type of HMGB1 positive cells. In sham-operated rats, we observed nuclear localization of HMGB1 (Fig. 2Ab and 2Ad) and cytoplasmic localization of S100 (Fig. 2Ac and 2Ad). Anti-MAP2 and anti-oligodendrocyte antibody staining revealed similar levels of ischemic cerebral damage in the contralateral areas of the edaravone-treated and non-treated groups compared with the control sham-operated group (data not shown). At 24 h after reperfusion, neither HMGB1 nor S100 staining was evident in the lateral caudoputamen and lower parietal cortex, regardless of edaravone treatment (Fig. 2Af–Ah and Fig. 2Aj–Al). However, in the non-treated group, HMGB1 (Fig. 2An and 2Ap) and S100 (Fig. 2Ao and 2Ap) colocalized in the cytoplasm and periphery of the nucleus throughout the upper frontoparietal cortex. Interestingly, in this region, no cytoplasmic translocation of HMGB1 was observed in the edaravone-treated group (Fig. 2Ar–2At). Furthermore, as shown in Fig. 2B, a significantly increased number of cytoplasmic-HMGB1-positive cells in non-treated groups (45.2±5.4%) compared with edaravone-treated groups (15.3±2.5% vs 45.2±5.4%, $P<0.0001$ for edaravone at 3mg/kg and 2.1±1.9% vs 15.3±2.5%, $P<0.005$ for edaravone at 6 mg/kg) indicated that more HMGB1 was shuttled into the cytoplasm and cell periphery of edaravone non-treated brain tissue 24 h after reperfusion.

We also confirmed the release of HMGB1 in the tissue and serum 24 h after reperfusion by ELISA. A significant increase in HMGB1 (25±4 μg/g of tissue vs

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10.4±1.7μg/g of tissue, $P<0.05$) was detected in the upper frontoparietal cortex area of non-treated rats compared with the sham-operated group (10.4±1.7 μg/g of tissue), whereas the HMGB1 release was significantly reduced (58.4 ± 6 %) in the upper frontoparietal cortex area (10.4±2.5 μg/g of tissue vs 25±4 μg/g of tissue, $P<0.05$) of edaravone-treated (6mg/kg) rats. No significant release of HMGB1 was observed in brain tissue collected from the lateral caudoputamen and lower parietal cortex area compared with sham-operated brain tissue.

Moreover, we found a remarkable upregulation of serum HMGB1 level in non-treated rats 24 h after reperfusion, an effect that was significantly abrogated by administration of either 3 or 6 mg/kg of edaravone (Fig.2C).

Edaravone inhibits OGD and reactive oxygen (H_2O_2)-induced HMGB1 release in PC12 cells

PC12 cells have been used previously to study neuronal injury *in vitro* (Hillion et al., 2005) and we therefore used this model system to confirm the *in vivo* results presented above. We examined whether HMGB1 was released in PC12 cells following different stresses, such as OGD and reactive oxidants (H_2O_2). Subsequently, we examined whether edaravone could block HMGB1 release. PC12 cells were treated with or without edaravone, and then exposed to OGD for 2-4 h. As shown in Fig. 3A, cells exposed to OGD for 3 h triggered a marked release of HMGB1 into the culture medium and this release was further up-regulated by 4 h. Edaravone (3-10μM) blocked this effect for 3 h strikingly.

ROS that are generated following hypoxia include superoxide ($O_2^{\bullet-}$), H_2O_2, hydroxyl radicals ($^{\bullet}OH$), and singlet oxygen ($^1O_2$) (Gechev et al., 2006). In the present

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study, cells that were exposed to H_2O_2 released significant levels of HMGB1 in a dose-dependent manner, and, as expected, this release was dose-dependently blocked by edaravone (3, 10μM) (P<0.05; Fig. 3B). However, no decrease in cell viability was observed following 24 h of exposure to up to 80 μM H_2O_2 as revealed by MTT assay (data not shown).

We next used fluorescence microscopy to examine the translocation of HMGB1 from the nucleus to the cytoplasm in PC12 cells in response to H_2O_2. Active expression of HMGB1 was observed in the periphery of the nucleus as well as in cytoplasmic regions 8 h after stimulation of cells with 40 μM H_2O_2 (Fig. 3Cd–3Cf), whereas most HMGB1 was localized in the nucleus of control cells (Fig. 3Ca–3Cc). Consistent with the *in vivo* results, 10μM edaravone treatment (Fig. 3Cg–3Ci) was found to significantly inhibit nuclear translocation of HMGB1 induced by H_2O_2 *in vitro*.

Several lines of evidence indicate that MAPKs play an important role in the active release of HMGB1, and are essential in the hypoxic signaling cascade (Haddad, 2004). Therefore, we next examined whether non-toxic doses of H_2O_2 triggered the activation of MAPKs in PC12 cells. As shown in Fig. 3D, H_2O_2 dose-dependently triggered the activation of p-ERK1/2 but not p-JNK1/2 and p-p38 MAPK following doses of up to 100 μM H_2O_2 (data not shown). We then evaluated the functional role of ERK1/2 activation on H_2O_2-induced HMGB1 release by pretreating cells with U0126, which is a potent and specific pharmacological inhibitor of MEK1/2 (an upstream signaling molecule of ERK1/2). We observed that U0126 almost completely blocked the activation of p-ERK1/2 (data not shown) and the release of HMGB1 in response to H_2O_2 (Fig. 3E).

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Edaravone abrogates HMGB1-induced apoptosis in PC12 cells

PC12 cells are a useful neuron-like model for studying programmed cell death (Mills et al., 1996). To evaluate the role of endogenously produced HMGB1 during cerebral ischemic reperfusion or under experimental hypoxic conditions, we investigated the effect of exogenous administration of HMGB1 on the viability of PC12 cells using recombinant HMGB1. For this purpose, PC12 cells were treated with various concentrations (0–4 μg/ml) of HMGB1 in the presence or absence of edaravone for 24 h. As shown in Fig. 4A, HMGB1 triggered cell death in a dose-dependent fashion. A significant decrease in cell viability was initiated at 1 μg/ml, which was further significantly decreased at 2 μg/ml. A dramatic reduction of cell viability was observed at 4 μg/ml (Fig. 4A). Furthermore, HMGB1 but not heat-treated HMGB1 significantly decreased cell viability **(**Supplemental Fig. 2). The LPS concentration in HMGB1 solutions was 0.581±0.058 ng/ml. The viability of PC12 cells did not significant decrease after treatment with LPS at 10000 ng/ml for 24 h as previously reported (Huang et al., 2002).

Next, we examined the role of edaravone (10-30 μM) on HMGB1-induced cell death. As shown in Fig. 4A (right panel), edaravone significantly rescued HMGB1-induced cell death in a dose-dependent manner. Moreover, HMGB1-induced cell death was followed by notable features of apoptosis, such as cytoplasmic and nuclear shrinkage, chromatin condensation, and nuclear fragmentation (Fig. 4B). As mitochondrial-mediated apoptotic cell death involves the release of cytochrome c into the cytosol, we investigated whether this accompanied HMGB1-induced cell death and whether it could be abrogated by edaravone. A significant release of cytochrome c was observed by Western blotting of the cytosolic protein extract 20 h after the exposure of

PC12 cells to HMGB1, which was markedly suppressed (by approximately 50%) by 10μM edaravone (Fig. 4C). These findings indicate that edaravone is a powerful regulator of the HMGB1 cascade in apoptosis.

Discussion

The present study demonstrates that HMGB1 is released from neuronal cells following ischemia, and that this release can lead to brain injury via the ROS–ERK1/2–HMGB1 signal-transduction pathway. In *in vitro* and *in vivo* experiments, the synthetic free radical scavenger edaravone significantly suppressed HMGB1 release, infarct size, neurological deficit and HMGB1-induced cell death.

In MCAO, the ipsilateral lateral caudoputamen and lower parietal cortex are rendered severely ischemic, whereas the upper frontoparietal cortex represents moderately ischemic tissue. The former is often referred to as the *ischemic core*, and the latter as the *penumbra* (Haberg et al., 2001). Necrosis occurs in the *core*, while apoptosis occurs in the *penumbra* (Benchoua et al., 2001).

We observed that HMGB1 translocated from the nucleus to the cytoplasm in the upper frontoparietal cortex (penumbra) and this translocation did not occur in neurons from healthy control rats or rats treated with edaravone. This implies that HMGB1 is a highly motile protein that shuttles to the cytosol via nuclear pores, can be released from the brain under pathological conditions (Kim et al., 2005) and that such a release is efficiently inhibited by edaravone.

HMGB1 was increased in the serum of rats 16–32 h after LPS stimulation (Wang et al., 1999) implying that HMGB1 is a “magic bullet” targeting inflammatory sites, especially in late-phase inflammation and sepsis. Recently, HMGB1 has been identified as an early mediator of hemorrhage after acute lung injury (Kim et al., 2005), and hepatic injury after liver ischemia/reperfusion (Tsung et al., 2005). Furthermore, OGD induced HMGB1 release in cultured neurons (Qiu et al., 2008). Interestingly, we found that edaravone significantly suppressed HMGB1 release in OGD-induced PC12 cells.

Our results are in agreement with a previous report describing the therapeutic efficacy of edaravone on oxidative damage early after ischemic insult (Zhang et al., 2005).

It is well established that free radicals are produced in the brain during ischemia (Fagan et al., 2005). We found that PC12 cells stimulated with non-toxic doses of H_2O_2 showed a significant increase in HMGB1 release (Fig.3B) as was shown previously for monocytes and macrophages (Tang et al., 2006). Extracellular HMGB1 induces receptor for advanced glycation end products (RAGE) signaling which induces elevated free radicals (Ding et al., 2007), followed by HMGB1 activation of NAD(P)H oxidase and increased ROS production in a toll-like receptor 4 (TLR4)-dependent manner (Fan et al., 2007). Furthermore, HMGB1 is released from necrotic cells in the infarct core after reperfusion, a result that is consistent with recent findings (Kim et al., 2006; Liu et al., 2007). These findings led us to propose a role for ROS–HMGB1 signaling in ischemic reperfusion brain injury and to suggest that a continuous HMGB1 release following cerebral ischemia induces an important amplification signal for the enhanced progression of brain damage. Moreover, extracellular HMGB1 during apoptotic cell death (Jiang et al., 2007) might also play a role in the further progression of the ROS–HMGB1 signaling, thereby inducing more-severe neuronal damage. However, further *in vivo* investigations are needed to clarify this important issue.

The MAPK signaling pathways play an important role in the development of inflammatory diseases, such as septic shock (Zhao et al., 2006) and neurodegenerative diseases (Sekine et al., 2006). We found that H_2O_2-stimulated HMGB1 release was mediated through activation of the ERK1/2 pathway, which is consistent with a previous study where an ERK1/2 antagonist inhibited ischemic neuronal cell death (Alessandrini et al., 1999). However, another recent study reported that HMGB1 release

occurred independently of ERK1/2 activation (Riuzzi et al., 2006). ERK1/2 is activated in neurons, astroglial cells, reactive microglia, and endothelial cells during human cerebral infarction (Slevin et al., 2000), while a recent study in mouse brain demonstrated that ERK1/2, p38MAPK, and SAPK/JNK were maximally activated at 120 min, 20 min, and 30 min, respectively, after focal cerebral ischemia (Wu et al., 2000). The discrepancy between these results might be due to differences in cell types and different stimulants that were used. Alternatively, these different results might reflect different MAPK cascades in cerebral infarction.

We observed that a dose of 3 and 6 mg/kg body weight edaravone effectively relieved hemispheric volume loss and suppressed HMGB1 release in rats 24 h after reperfusion. Our results are consistent with the finding that administration of 3 mg/kg body weight edaravone twice daily was effective against cerebral infarction in mice (Zhang et al., 2005), suggesting that, in the clinic, an initial edaravone injection might exert anti-oxidative effects when administered within 24 h of the insult.

Edaravone treatment was unable to rescue damage in the *ischemic core* 24 h after reperfusion because the level of cell injury was too severe. In light of our results, it appears that edaravone also targets the *penumbra*, as is the case for other drugs that are used to treat cerebral infarction (Fisher, 2006). Administration of 1.5 and 3.0 mg/kg body weight of edaravone immediately after reperfusion in a rat cerebral infarction model generated a maximum plasma concentration (Cmax) of 5.7 and 9.9 μM of the drug, respectively (Takamatsu et al., 1997). Moreover, edaravone inhibited the increase of 2, 3-ditydroxybenzoic acid (2, 3-DHBA) levels *in vitro*, with an average IC50 value of 6.7μM (Watanabe et al., 2004). Furthermore, the average Cmax for treatment of humans was 6 μM (Yokota et al., 1997). We found that edaravone (10-30 μM)

effectively protected against HMGB1-induced apoptosis in PC12 cells, a result consistent with a recent report demonstrating that concomitant addition of 10 μM of edaravone with 15-hydroperoxyeicosatetraenoic acid to cultured bovine aortic endothelial cells prevented cell death completely (Watanabe et al., 1988). Our results also support the rescue of ischemic-reperfusion-induced apoptosis by edaravone in human choriocarcinoma cells (Yung et al., 2007) and nitric oxide-induced apoptosis in cultured rat astrocytes (Kawasaki et al., 2007), indicating that the interaction between ROS and HMGB1 is important in the pathological mechanism. We propose that ROS triggers the first "bullet" in the *ischemic core*, and subsequently induces the formation of edaravone-suppressible ROS–HMGB1 signaling that causes neuronal damage, increases infarct area, increases apoptosis in the *penumbra* and continuation of these processes cause severe damage to the brain.

Our study demonstrated a strong association between HMGB1 release and antioxidant activity of edaravone. First, HMGB1 has oxidation-sensitive unpaired cysteines (Cys) at position 23, 45, and 106 (Sahu et al., 2008). The function of the Cys23-Cys45 pair in HMGB1 A-domain is essential for HMGB1 function in the nucleus and the oxidization of Cys106 in B-domain is caused by HMGB1 release by ROS such as H_2O_2 (Sahu et al., 2008). We also found that HMGB1 release is caused by oxidative stress in ischemic condition and H_2O_2-stimulated PC12 cells mediated by ERK1/2 pathway in our condition, suggesting that its release may depend on oxidized conditions. Thus, these evidences indicated that oxidized HMGB1 may be released from apoptosis and enhanced the inflammatory events, demonstrating that HMGB1 release by oxidative stress might be prevented by antioxidant activity of edaravone. On the other hand, RAGE, which is the specific receptor of HMGB1, is a multifunctional

immunoglobulin super family and its signaling cascade induces a strong ROS (Yamagishi and Takeuchi, 2004), suggesting that inhibition of HMGB1-stimulated cell death might be caused by antioxidant effects of edaravone. However, we are currently unable to elucidate the underlying mechanisms such as the direct effect of edaravone as HMGB1 and/or RAGE antagonist. Therefore, we would prefer to address this matter in our future studies.

Recently, HMGB1 short-hairpin (sh) RNA, transfected 24 h before MCAO, significantly decreased infarct volumes and suppressed microglial activation (Kim et al., 2006). Furthermore, an anti-HMGB1 monoclonal antibody ameliorated brain infarction induced by transient ischemia in rats (Liu et al., 2007). Together with our findings, these results indicate a key role for HMGB1 in both damage and delayed inflammatory processes in the post-ischemic brain (Kim et al., 2006). Our *in vitro* and *in vivo* findings showed that edaravone significantly reduces ischemic-induced cerebral infarction by abrogating the HMGB1 release, and ameliorating HMGB1-induced apoptosis in neuronal cells, suggest that edaravone could be used in the treatment of ischemia-induced cerebral infarction. However, further studies are needed in ischemic neuronal cell models to explore pharmacological strategies against expanding cerebral infarction, traumatic brain injury, and intracerebral hemorrhage in which ROS act as key mediators (Dohi et al., 2007; Nakamura et al., 2008).

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Footnotes

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Legends for Figures

Figure. 1. Effect of edaravone on experimental ischemic injury in rats. (A) Coronal sections from ischemic rat brain stained with TTC. Sections shown are 24 hours after reperfusion. Infarct area is in the right lateral caudoputamen, and to a lesser extent in the lower parietal cortex and frontoparietal cortex. (B) Infarct areas from non-treated and edaravone-treated groups (3, 6 mg/kg) are compared. Values are mean ± SD. * $P<0.01$, ** $P<0.005$.

Figure. 2. Effect of edaravone on HMGB1 in rats with cerebral infarction. (A) Double immunofluorescence staining of HMGB1 (b, f, j, n, and r) and S100 (c, g, k, o, and s) in lateral caudoputamen, lower parietal cortex and upper frontoparietal cortex. Arrow indicates the cytoplasmic shift of HMGB1 (non-treated group) in the upper frontoparietal cortex. Arrowhead indicates the nuclear localization of HMGB1 (edaravone-treated group). Bar=20 μm. (B) Quantification of cytoplasmic HMGB1-immunoreactive cells and values are presented as the percentage of HMGB1-positive cells after 24 h reperfusion. Values are mean ± SD. * $P<0.005$, ** $P<0.0001$. (C) Effect of edaravone on HMGB1 in rat serum. The serum level of HMGB1 increases 24h after reperfusion. Treatment with edaravone (3, 6 mg/kg) significantly decreases HMGB1 release into the serum. Values are mean ± SD. * $P<0.005$, ** $P<0.0001$. $n = 6$ rats per group in every experiment.

Figure. 3. Effect of edaravone on experimental ischemic injury in PC12 cells. HMGB1 release into the supernatant following (A) OGD and (B) H_2O_2 - stimulation analyzed by

Western blotting. The lower panels of (A) and (B) show a graphical representation of blots after densitometric analysis relative to the highest HMGB1 concentration, which was set to 100. Values are mean ± SD. *P<0.05, **P<0.005. (C) Nuclear translocation of HMGB1 detected by immunofluoresence analysis in response to H_2O_2 exposure for 8 h. Nuclei are labeled with DAPI (a, d, and g). The arrow in e indicates decreased HMGB1 immunoreactivity in the nucleus. The arrowhead in f indicates a shift of HMGB1 in the periphery of the nucleus. Original magnification×400. (D) H_2O_2 triggers activation of ERK1/2 in PC12 cells as determined by Western blot analysis. (E) U-0126 blocked H_2O_2-induced HMGB1 release as analyzed by Western blotting (n=3 for each group). The lower panels of (D) and (E) show a graphical representation of blots after densitometric analysis. Values are mean ± SD. *P<0.05.

Figure. 4. Edaravone abrogates HMGB1-induced apoptosis in PC12 cells. (A) Cell viability was measured by MTT assay 24 h after exposure to the indicated concentrations of HMGB1 and edaravone. Data shown are percentages of control values ± SD (*P<0.0001). (B) Hoechst 33258 staining of PC12 cells treated with HMGB1 in the presence or absence of 10 μM edaravone. The arrowhead indicates fragmented nuclei that are a characteristic feature of apoptotic cell death. Original magnification x400. (C) Edaravone suppresses HMGB1-induced cytochrome c release from mitochondria as shown by Western blot analysis. β-actin (lower blot) was used as loading control. The lower panel shows a graphical representation of the upper blot after densitometric analysis. Data shown are relative to the highest cytochrome c signal, which was set to 100. *P<0.05.

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Experimental Groupes	MCAO Edaravone (-) n=6 (24 h)	MCAO Edaravone (3mg) n=6 (24 h)	MCAO Edaravone(6mg) n=15 (6,12,48 h; n=3 24 h; n=6)
Before occlusion			
MABP(mmHg)	61±12	62±13	69±10
PaO_2 (mmHg)	62±8	64±7	79±26
$PaCO_2$ (mmHg)	53±8	46±6	47±5
pH	7.34±0.06	7.35±0.05	7.37±0.05
Ht (%)	39±1	38±8	39±4
Glucose (mg/dl)	212±9	211±52	246±60
Head temperture (℃)	36.8±0.1	36.9±0.1	36.9±0.1
Body temperture (℃)	37.0±0.1	37.0±0.1	37.0±0.1
After occlusion			
MABP(mmHg)	75±17	69±20	77±5
PaO_2 (mmHg)	94±47	76±5	87±17
$PaCO_2$ (mmHg)	50±11	47±6	47±5
pH	7.35±0.04	7.30±0.06	7.38±0.02
Ht (%)	41±0	40±4	42±1
Glucose (mg/dl)	154±46	154±99	179±26
Head temperture (℃)	36.8±0.1	36.9±0.1	36.9±0.1
Body temperture (℃)	37.0±0.0	37.0±0.1	37.0±0.1

Values are mean ±SD
MABP,mean arterial blood pressure; PaO_2,mean partial pressure of oxygen; $PaCO_2$, mean partial pressure of carbon dioxide; Ht, mean hematocrit
Blood gas analysis and glucose level at 10 min, body temperature and blood pressure at 45 min, and head temperature at 70 min after occlusion were determined.

Table 1. Comparison of physiological variables

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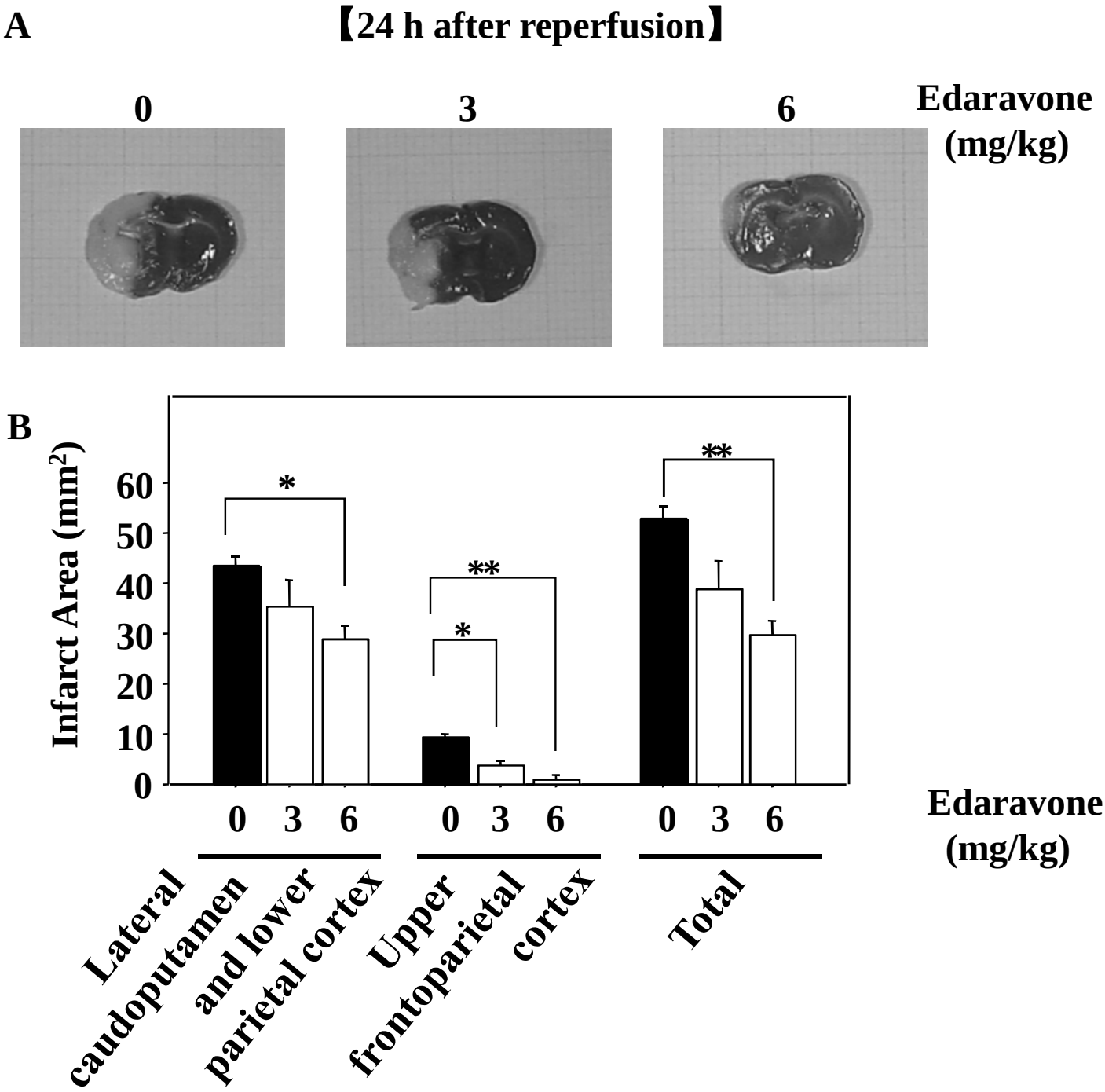

Figure 1.

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A **【24 h after reperfusion】**

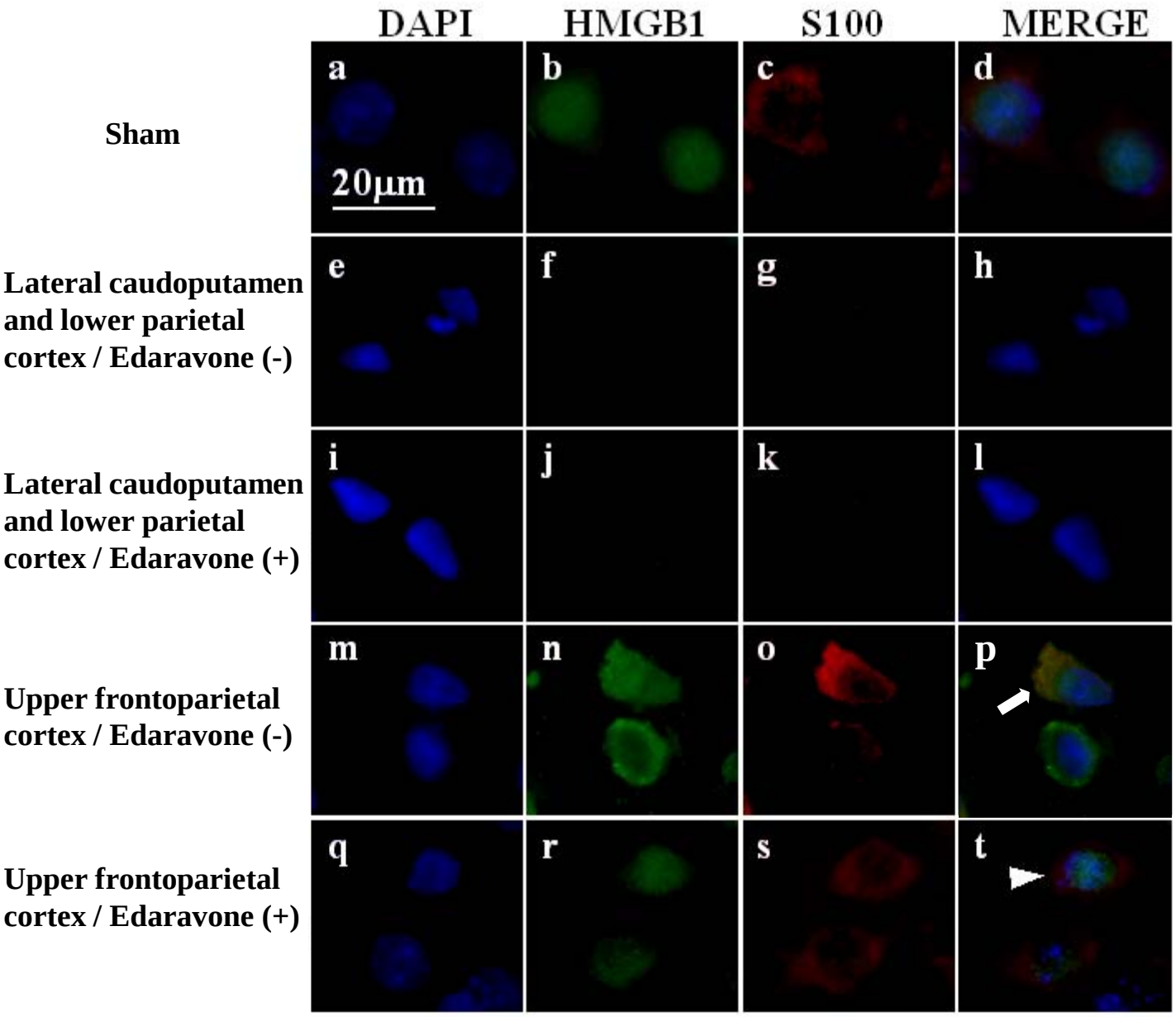

Figure 2.

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B

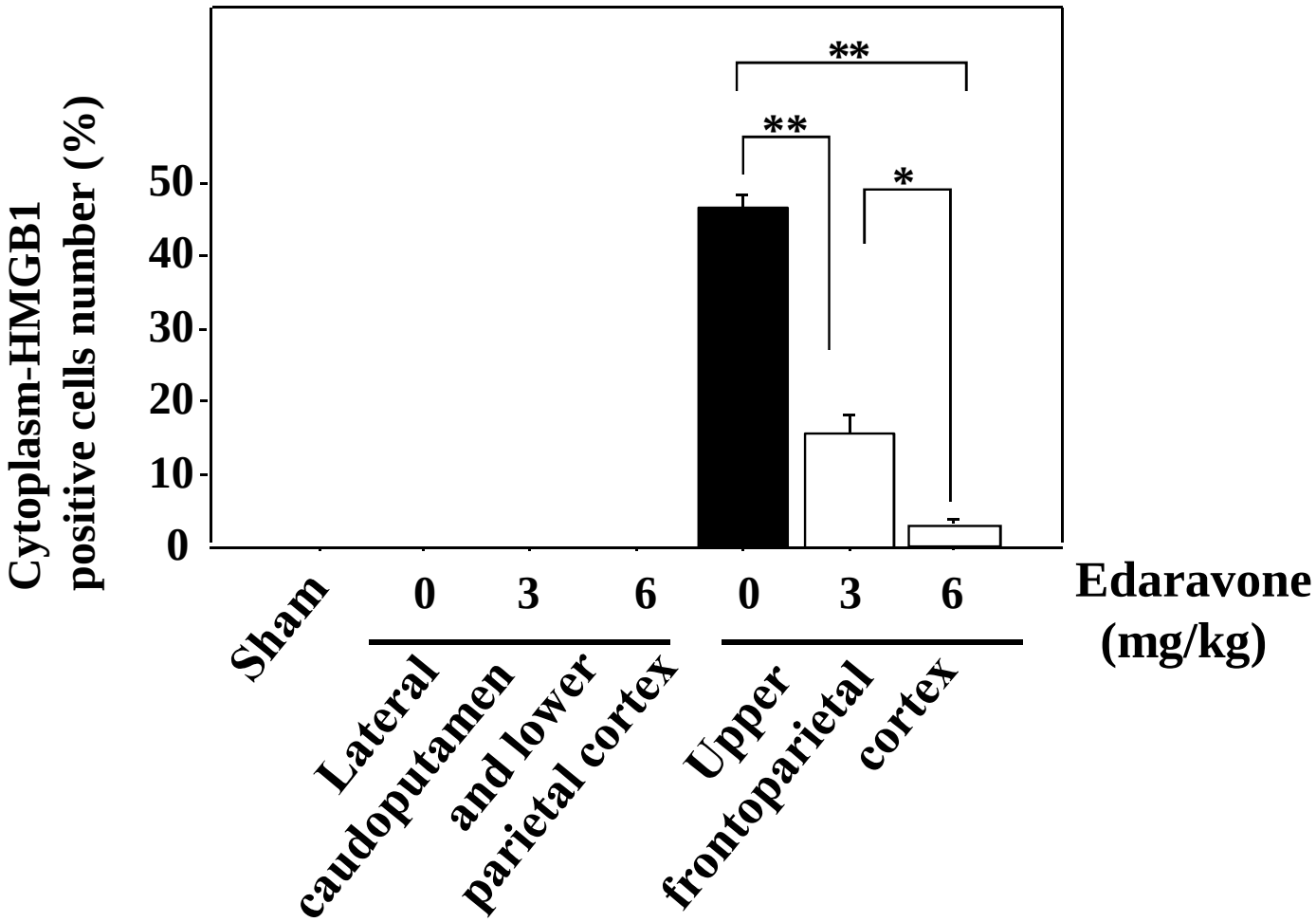

Figure 2.

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C **【24 h after reperfusion】**

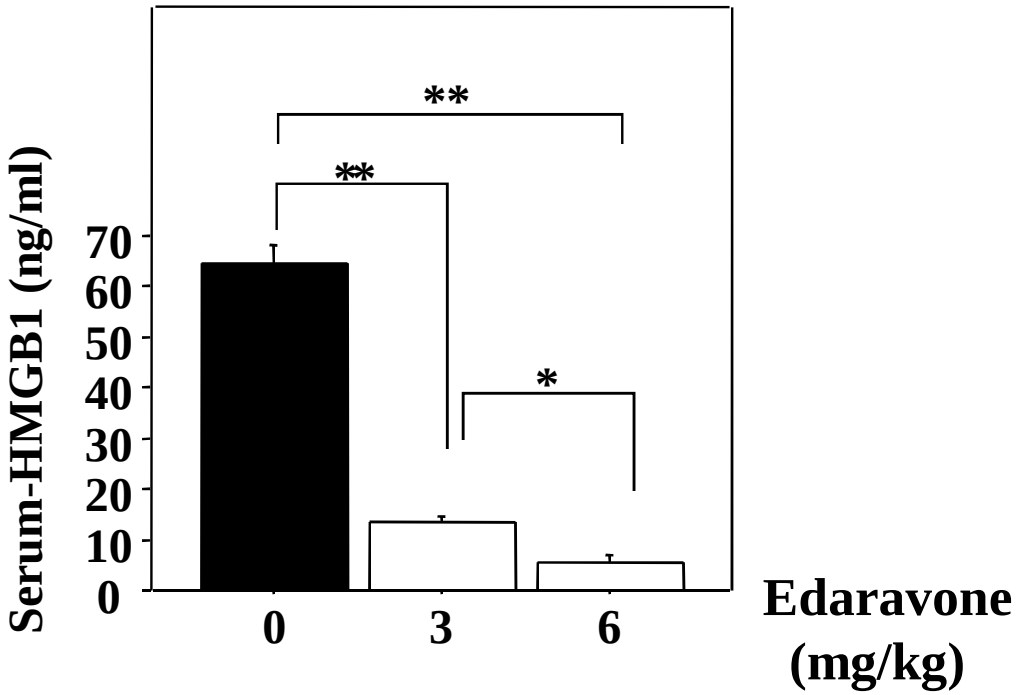

Figure 2.

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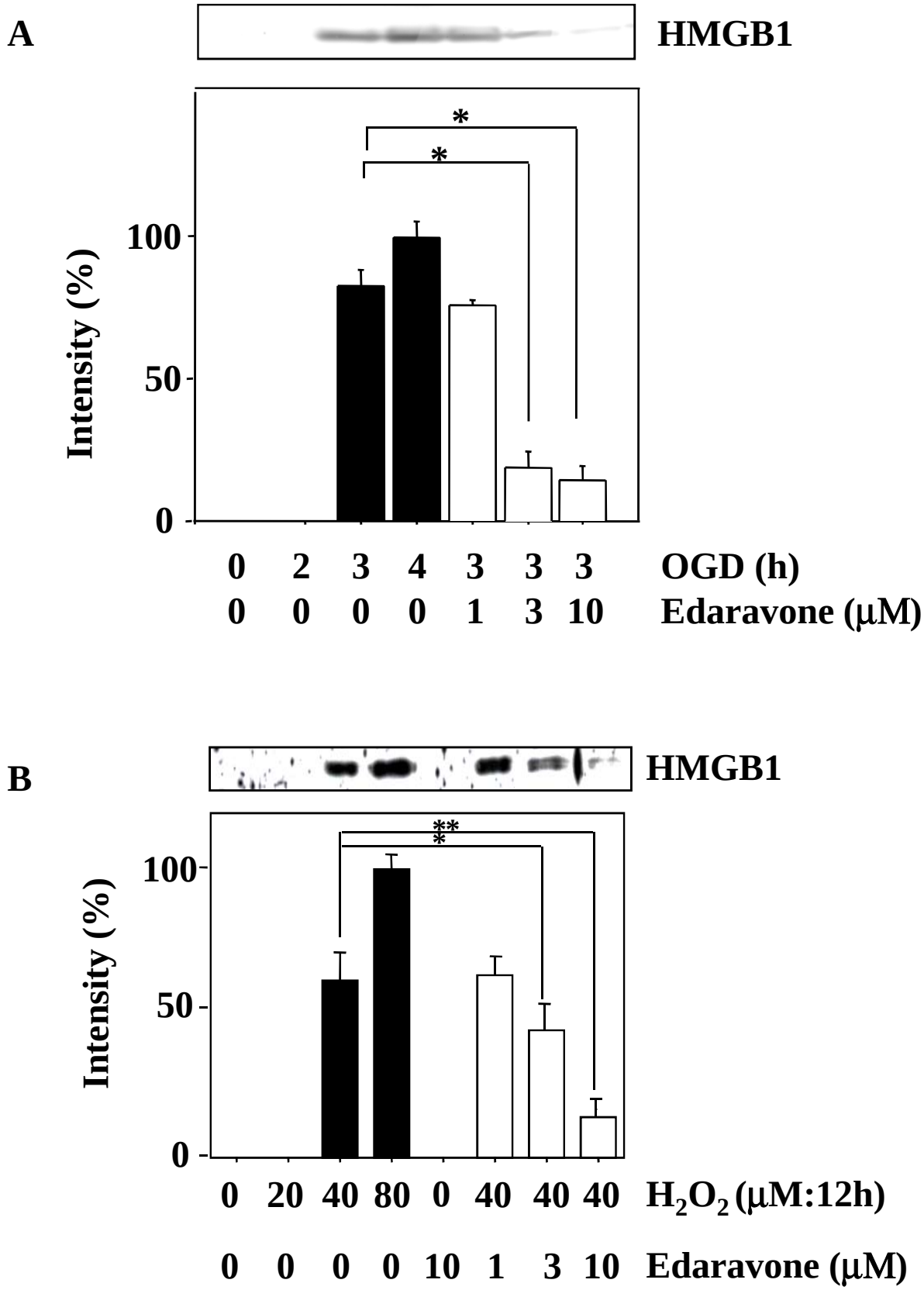

Figure 3.

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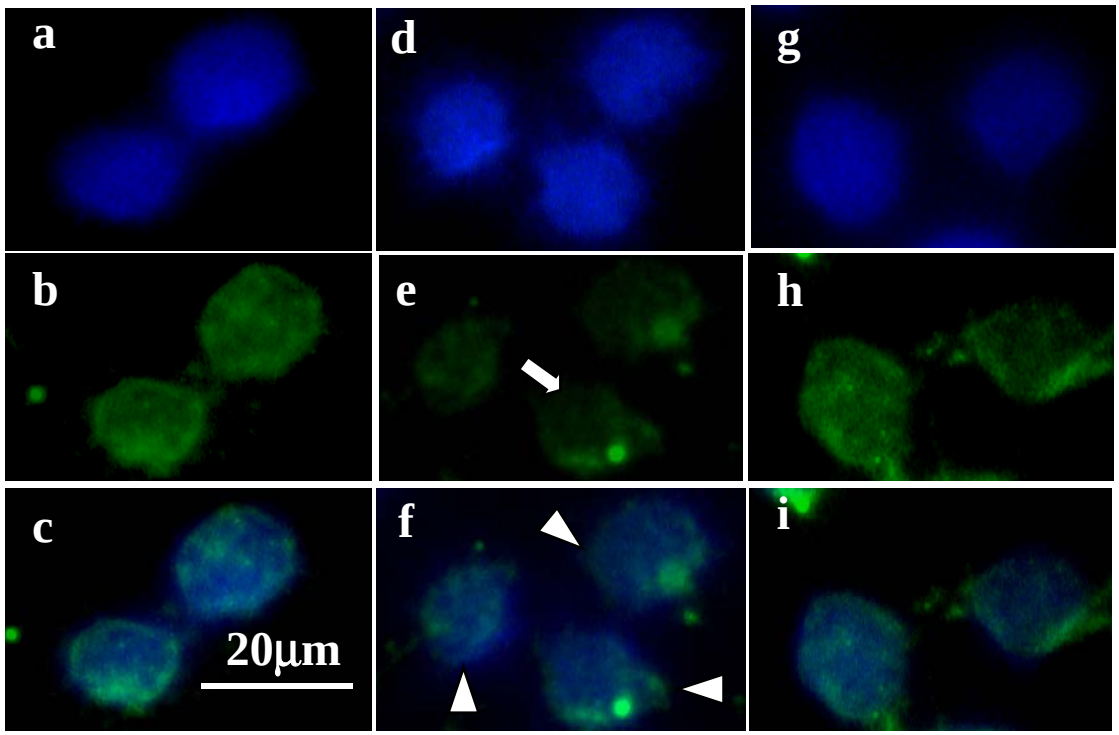
C



DAPI

HMGB1

MERGE

H_2O_2 (40μM : 8h)	(-)	(+)	(+)
Edaravone (10μM : 8h)	(-)	(-)	(+)

Figure 3.

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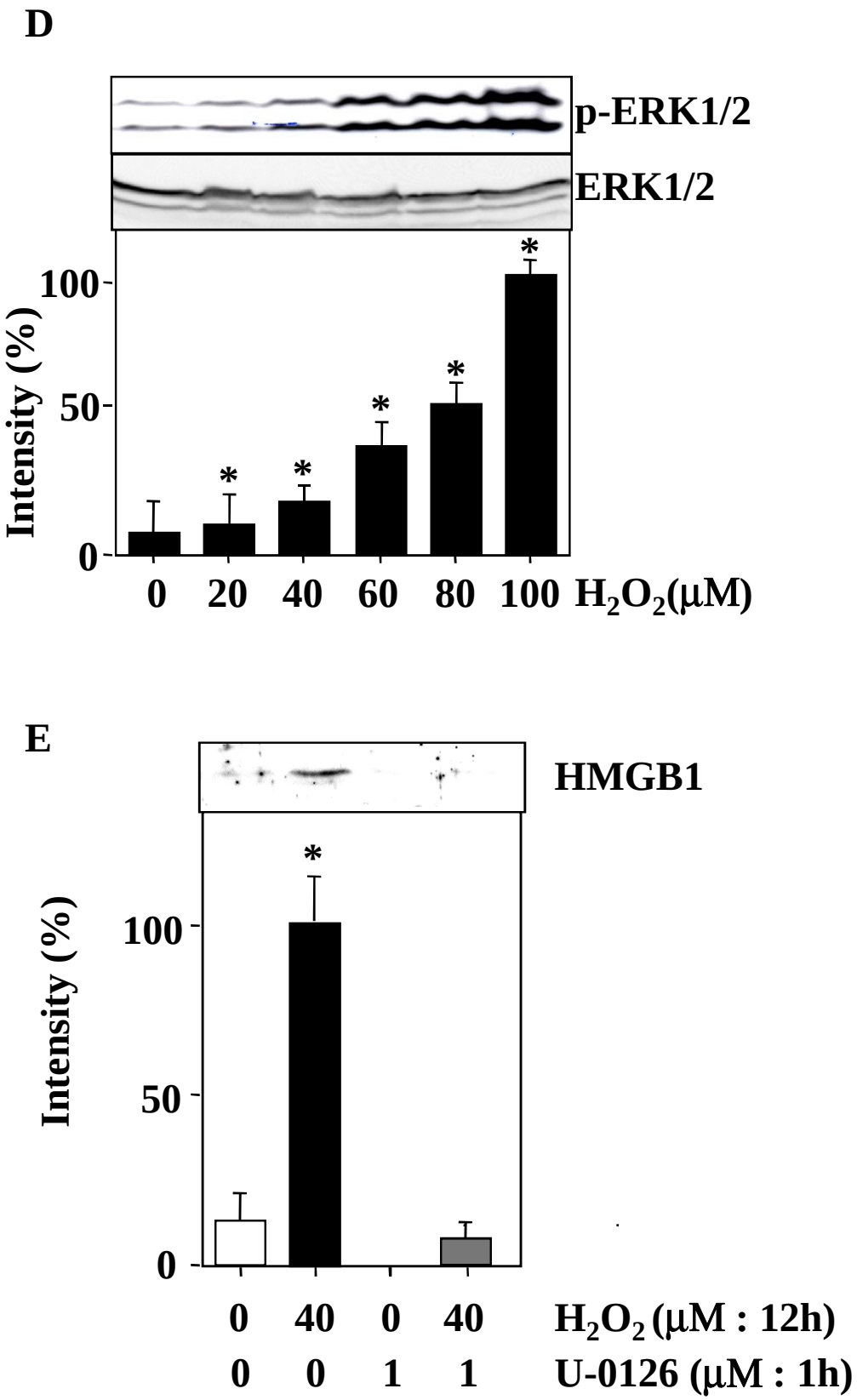

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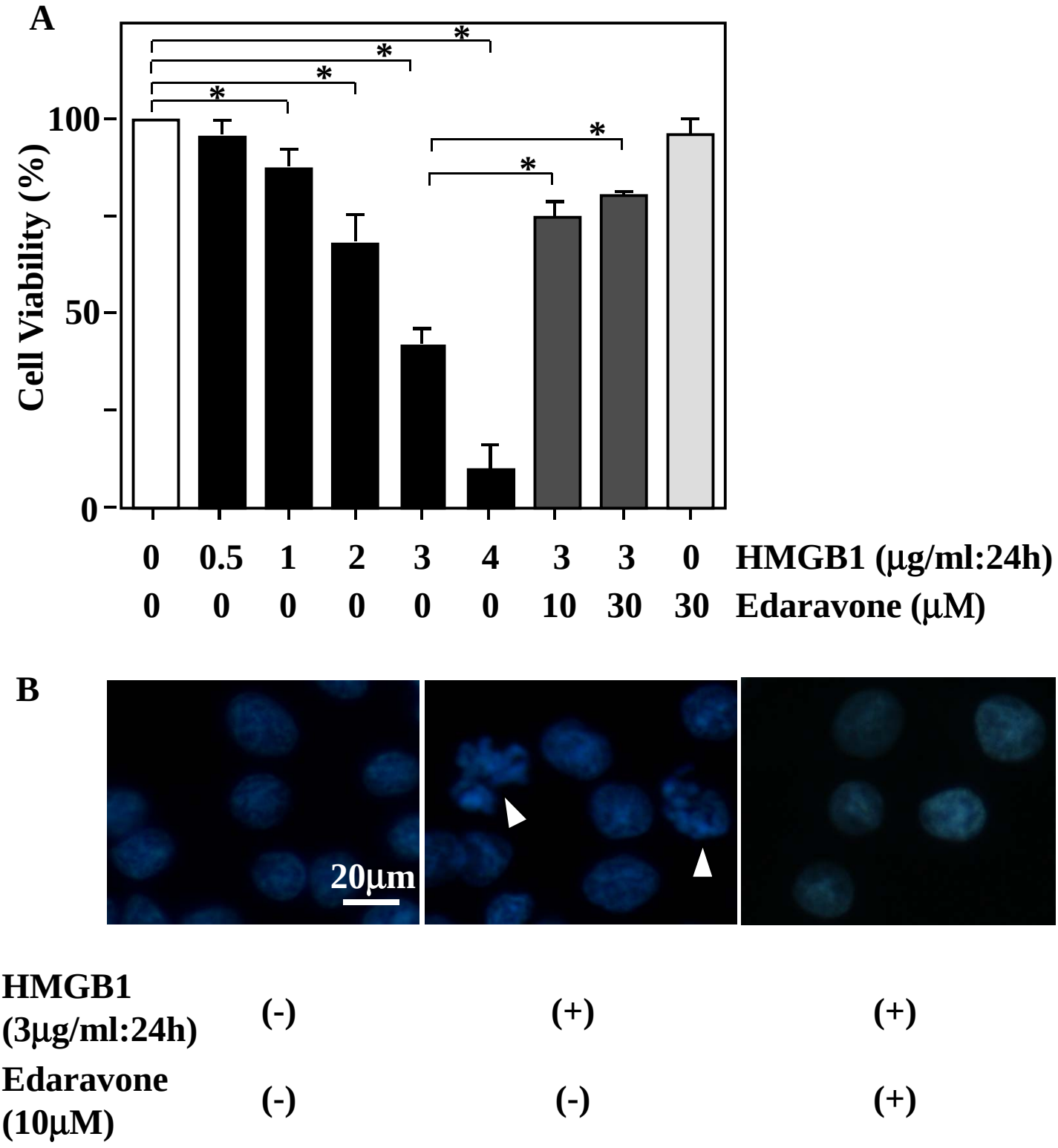

Figure 4.

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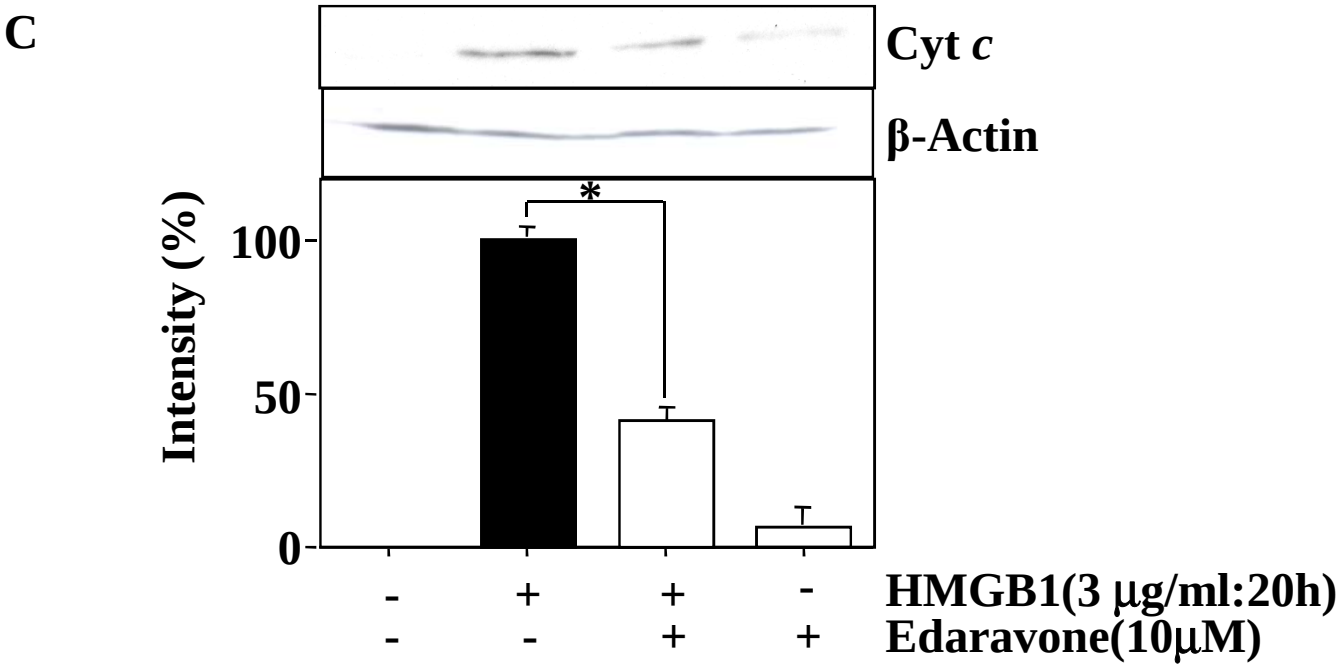

Figure 4.