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Edaravone, a free radical scavenger, protects components of the neurovascular unit against oxidative stress in vitro

Brian J. Lee, **Yasuhiro Egi**, **Klaus van Leyen**, **Eng H. Lo**, and **Ken Arai**
Neuroprotection Research Laboratory. Departments of Radiology and Neurology, Massachusetts General Hospital and Harvard Medical School, USA

Abstract

The concept of the neurovascular unit suggests that to be successful, stroke therapies must protect all neuronal, glial and endothelial components in brain. In this study, we tested the efficacy of the free radical scavenger edaravone in three cellular models of oxidative stress. HT22 neuronal cells were subjected to oxidative stress using the standard glutamate-induced glutathione depletion model. Primary rat astrocytes were exposed to H2O2. Oxidative stress was induced in human brain endothelial cells with sodium nitroprusside (SNP). Edaravone significantly reduced oxidative cell death in both HT22 neuronal cells and primary rat astrocytes in a dose-dependent manner. SNP did not kill brain endothelial cells but instead reduced their production of brain-derived neurotrophic factor (BDNF). Edaravone significantly ameliorated this response. These data suggest that free radical scavengers are effective in all cell types of the neurovascular unit, and should still be considered as a potential therapeutic approach for stroke.

Keywords

oxidative stress; neuron; astrocyte; endothelial cell; neurovascular unit; stroke

1. Introduction

Stroke is the third leading cause of death and a leading cause of adult disability in the United States. Thrombolytic therapy with tissue-plasminogen activator remains the only FDA-approved method to reperfuse and salvage ischemic brain tissue (Hacke et al., 1999). However, relatively narrow time windows for thrombolysis means that only a limited number of patients can be feasibly treated (Lo et al., 2003).

Over the past decade, impressive advances have been made in understanding the basic molecular mechanisms underlying neuronal death. However, clinically effective neuroprotectants have not yet been discovered. The Phase III trial of the radical spin-trap NXY-059 was unsuccessful (Shuaib et al., 2007). Post-hoc analyses have suggested that this might have been due to difficult drug delivery and efficacy issues related to the compound itself (Ginsberg, 2007; Proctor and Tamborello, 2007; Savitz and Fisher, 2007). But the body

Corresponding author: Ken Arai, Neuroprotection Research Laboratory, MGH East 149-2401, Charlestown, MA 02129, USA, Tel: 617.724.9503, karai@partners.org.

of experimental data supports the thesis that oxidative stress should still be a potentially viable target for stroke therapy (Niizuma et al., 2009).

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a free radical scavenger that is widely used in Japan for acute ischemic stroke (2003). Some studies suggest that the neuroprotective effects of edaravone may take place in penumbral-like regions where oxygen radicals are typically generated (Amemiya et al., 2005;Kawai et al., 1997). Furthermore, by quenching hydroxyl radicals and inhibiting lipid peroxidation and the lipoxygenase pathway, edaravone may also reduce brain edema (Abe et al., 1988;Nishi et al., 1989;Toyoda et al., 2004;Watanabe et al., 1994;Yamamoto et al., 1997).

The neurovascular unit is an emerging concept in stroke research that emphasizes the need to protect not just neurons, but all cells in the brain (del Zoppo, 2006; Iadecola, 2004; Lo et al., 2003; Lok et al., 2007). Hence, to be successful, stroke therapy should be broadly effective in neuronal, glial and endothelial cell types. In this study we tested the effects of edaravone in three models of oxidative stress: glutamate-induced glutathione depletion in HT22 neuronal cells, H2O2 in primary rat astrocytes, and sodium nitroprusside (SNP) injury in human brain endothelial cells.

2. Results

To demonstrate the efficacy and safety of edaravone on the components of neurovascular unit, we used three brain cell types: HT22 neuronal cells, primary astrocytes, and cerebral endothelial cells. First, we tested the safety of edaravone in those cells. Because most papers examining the effects of edaravone in cell cultures used around 100 uM edaravone, we subjected the cells to edaravone at 1, 10, 100, 300 uM for 24 hours. The cell lysis LDH assay showed that those concentrations of edaravone did not induce cell death in the all three cell types (Table 1). This point was important because efficacy and safety of edaravone might be different depending on cell types (Yoshida et al., 2008;Yoshida et al., 2005).

HT22 cells are a mouse hippocampal neuronal cell line. They do not express N-methyl-D-aspartate receptors. But HT22 cells express a glutamate/cystine antiporter that takes in cystine to produce the endogenous antioxidant glutathione. Hence, exposure to excess glutamate inhibits the glutamate/cystine antiporter and leads to glutathione depletion and oxidative cell death in HT22 neuronal cells (Li et al., 1997; van Leyen et al., 2008). For our experiment, we confirmed that glutamate induced HT22 cell death using both cell viability (WST) assay and cell lysis (LDH) assays (Figs. 1A-B). Co-treatment with edaravone significantly reduced HT22 cell death in a dose-dependent manner (Fig. 2A-C).

Next, we tested the efficacy of edaravone in primary rat astrocytes. Oxidative damage with hydrogen peroxide (H_2O_2) clearly caused cell death in both viability (WST) and lysis (LDH) assays (Figs. 3A-B). Once again, co-treatment with edaravone significantly protected astrocytes in a dose-dependent manner (Figs. 4A-C).

Finally, we examined effects of edaravone on cerebral endothelial cells. We have previously shown that consistent with the known literature, cerebral endothelial cells can be relatively resistant to oxidative cell death. But oxidative stress can downregulate the ability of endothelial cells to produce neurotrophic factors (Dugas et al., 2008; Guo et al., 2008; Guo and Lo, 2009). Here, sodium nitroprusside (100 uM, SNP a NO donor) did not induce overt cell death in our human brain endothelial cells (Figs 5A-B). But SNP clearly suppressed endothelial BDNF levels (Figs. 5C). Co-treatment with edaravone significantly ameliorated the SNP-induced decrease of BDNF secretion (Fig. 5C).

3. Discussion

We have shown that edaravone, a free radical scavenger, protected neuronal, glial and brain endothelial cells against oxidative stress in vitro. For HT22 cells and primary rat astrocytes, edaravone blocked oxidative stress-induced cell death. For human brain endothelial cells, edaravone ameliorated the decrease of BDNF secretion. These data suggest that oxidative stress may still be a viable target for the development of stroke therapies.

Oxidative stress is a major mechanism implicated in a variety of neurodegenerative diseases including stroke (Lin and Beal, 2006). During ischemia, reactive oxygen and nitrogen species can be generated in the ischemic penumbra (Broughton et al., 2009; Shi and Liu, 2007). And during thrombolysis, reactive species can also be produced during reperfusion injury (Broughton et al., 2009; Chan, 2001). In addition, two recent studies using rat stroke models have suggested that edaravone reduced t-PA-induced hemorrhage (Yagi et al., 2009; Yamashita et al., 2009). Hence, drugs that can reduce oxidative stress-induced insults may be useful for not only for providing neuroprotection and improving outcome after stroke but also for combination therapy with t-PA.

The molecular mechanisms of oxidative stress have been extensively dissected using in vitro cell culture systems. By scavenging free radicals and reducing the intracellular Ca^{2+} concentration, edaravone inhibits the mitochondrial apoptotic pathways (Yoshida et al., 2006). Others have shown that edaravone protected cultured neuronal cells against 6-hydroxydopamine (Yuan et al., 2008) and oxygen-glucose deprivation (Wu et al., 2006). In astrocytes, edaravone protected against MPP+ (Chen et al., 2008) and nitric oxide (Kawasaki et al., 2007). Our findings are consistent with these other reports.

Our current study suggests that edaravone may protect all three major cell types of the neurovascular unit. But an important caveat is that we did not examine the type of cell death involved. After stroke, it is likely that mixed forms of cell death occur, including necrosis, apoptosis, necroptosis and autophagy. For HT22 cells, a mixture of necrosis and apoptosis has been reported. At relatively early time points following glutamate exposure (8-12 hours), HT22 cells showed features of necrosis, but at late time points (16-24 hours), morphologies resembling apoptotic cell death also emerges (Fukui et al., 2009). As for H2O2-induced astrocyte death, the mode of cell death might depend on experimental conditions such as exposure time and concentration of H2O2. With severe injury from very high concentrations of H2O2, outright necrosis should take place. But with more moderate degrees of H2O2, apoptosis can also occur. Mazlan et al used the same cell culture model as ours to show that H2O2 (100 uM, 24 hours) induced apoptotic cell death in cultured rat astrocytes (Mazlan et al., 2006). In addition, Kitamura et al used cultured mouse astrocytes to report that apoptotic cells were observed after H2O2 treatment in time- (0-24 h) and concentration-(0-1 mM) dependent manner (Kitamura et al., 1999). Finally, edaravone has been reported to protect against apoptosis in rat stroke models (Amemiya et al., 2005). Hence, our cell culture data here may be consistent with in vivo studies as well. In the end, it is likely that oxidative stress is a common trigger for mixed forms of cell death in the brain.

In contrast to the known vulnerability of neurons and astrocytes, it is thought that endothelial cells tend to be more resistant to ischemic or oxidative injury (Lu et al., 1993). We have previously shown that low to moderate levels of oxidative stress do not kill endothelial cells but instead makes them dysfunctional enough so that they downregulate their baseline production of neurotrophic factors (Arai and Lo, 2009; Guo et al., 2008). Our present study shows that edaravone was able to ameliorate these deleterious effects of oxidative stress in brain endothelial cells. Thus, free radical scavenging can protect not only neuronal and glial

cells, but also rescue the trophic coupling that exists between vascular and parenchymal components in the neurovascular unit.

Taken together, this study suggests that edaravone was broadly effective in protecting all major cell types of the neurovascular unit. However, there are still several important limitations in our experimental design and data analysis. First, our study only used cell culture models. To be rigorous, the neuro-, astro- and endo-protection found here must also be rigorously documented in vivo. Second, our experiments cannot prove the mechanisms involved. While the chemical scavenging properties of edaravone are well known, it would be important to demonstrate that these mechanisms (reduced radical levels, healthier ionic homeostasis, preserved mitochondrial integrity, reduced apoptosis etc) do indeed occur in vivo. Third, we used a variety of models including primary cultures as well as cell lines. While these were all standard models of oxidative stress, we cannot unequivocally exclude the caveat that differences between primary cultures and cell lines may exist. Finally, our experiments fundamentally represent a proof-of-concept study. The translation of molecular and cell mechanisms and therapies into in vivo models and clinical applications is extremely challenging (Fisher and Henninger, 2007). Nevertheless, despite the failure of the NXY-059 spin trap clinical trial, it is likely that oxidative stress is still a relevant mechanism and target for stroke therapy.

In conclusion, we have demonstrated that free radical scavenging can be effective in all components of the neurovascular unit (neuron, astrocyte, and cerebral endothelium). These findings suggest that oxidative stress may still be a potentially viable target for stroke therapy. Furthermore, the use of multiple cell-type assays such as the one used our study may provide a useful screening approach for the development of future compounds for stroke and other CNS disorders.

4. Experimental Procedure

Cell Culture

A mouse hippocampal neuronal cell line HT22 cell was cultured in Dulbecco's Modified Eagle's medium containing 10% fetal bovine serum and penicillin/streptomycin (van Leyen et al., 2008). Primary astrocytes were prepared from cerebral cortices of 2-day-old neonatal Sprague-Dawley rats and cultured in Dulbecco's Modified Eagle's medium containing 10% fetal bovine serum and penicillin/streptomycin as previously described (Arai et al., 2003). Human brain microendothelial cell (hBMEC, purchased from CSC Systems, Kirkland, WA, USA) was cultured in EBM-2MV containing EGM-2MV SingleQuots kit (Arai and Lo, 2009).

Preparation of Edaravone

Edaravone was dissolved in DMSO at 100 mM and the stock solution was kept at -80C until use.

Determination of cell death/survival

Cytotoxicity was quantified by a standard measurement of lactate dehydrogenase (Roche LDH kit) according to the manufacture's instructions (Arai et al., 2003). Cell survival was assessed by WST reduction assay (Dojindo) according to the manufacturer's instructions (Arai and Lo, 2009). For those experiments, HT22 cells, cultured rat astrocytes, hBME cells were plated on non-coated 24-well plates, collagen-coated 48-well plates, and collagen-coated 6-well plates, respectively. Both LDH and WST assays were conducted 24-hours after treatment with oxidative stress inducers.

BDNF ELISA

BDNF levels in endothelial-conditioned media were measured by using the BDNF Emax ImmunoAssay System (Promega) according to the manufacture's instructions. Endothelial conditioned media were collected 24-hours after SNP with or without edaravone treatment. Before the assay, the culture medium was concentrated from 1 mL to 100 uL using VIVASPIN (10,000 MWCO PES, Sartorius) (Arai and Lo, 2009).

Statistical analysis

Statistical significance was evaluated using the unpaired t-test to compare differences between two groups and ANOVA followed by Bonferroni tests for multiple comparisons. Data are expressed as mean ± SD. A value of $p < 0.05$ was considered significant. Experiments were repeated at least three times.

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Abbreviations

BDNF	brain-derived neurotrophic factor
SNP	Sodium nitroprusside
t-PA	tissue plasminogen activator

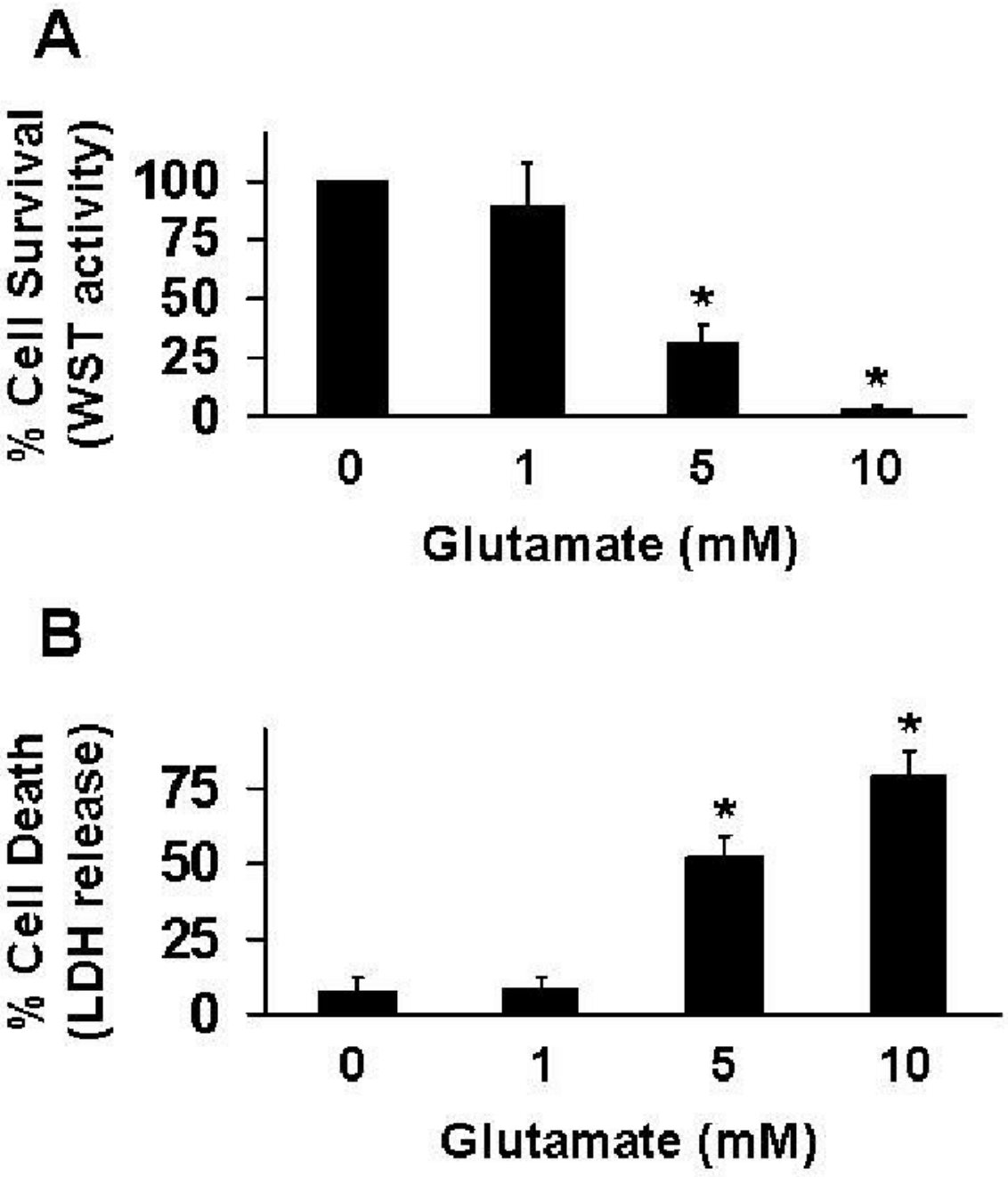

Figure 1. Glutamate-induced oxidative damage in the HT22 neuronal cell line

A WST assay **(A)** and a LDH assay **(B)** show that glutamate treatment induced HT22 cell death in a concentration-dependent manner. Both WST assay and LDH assay were conducted 24-hours after glutamate treatment (see Experimental Procedure). *P<0.05 vs 0 mM glutamate.

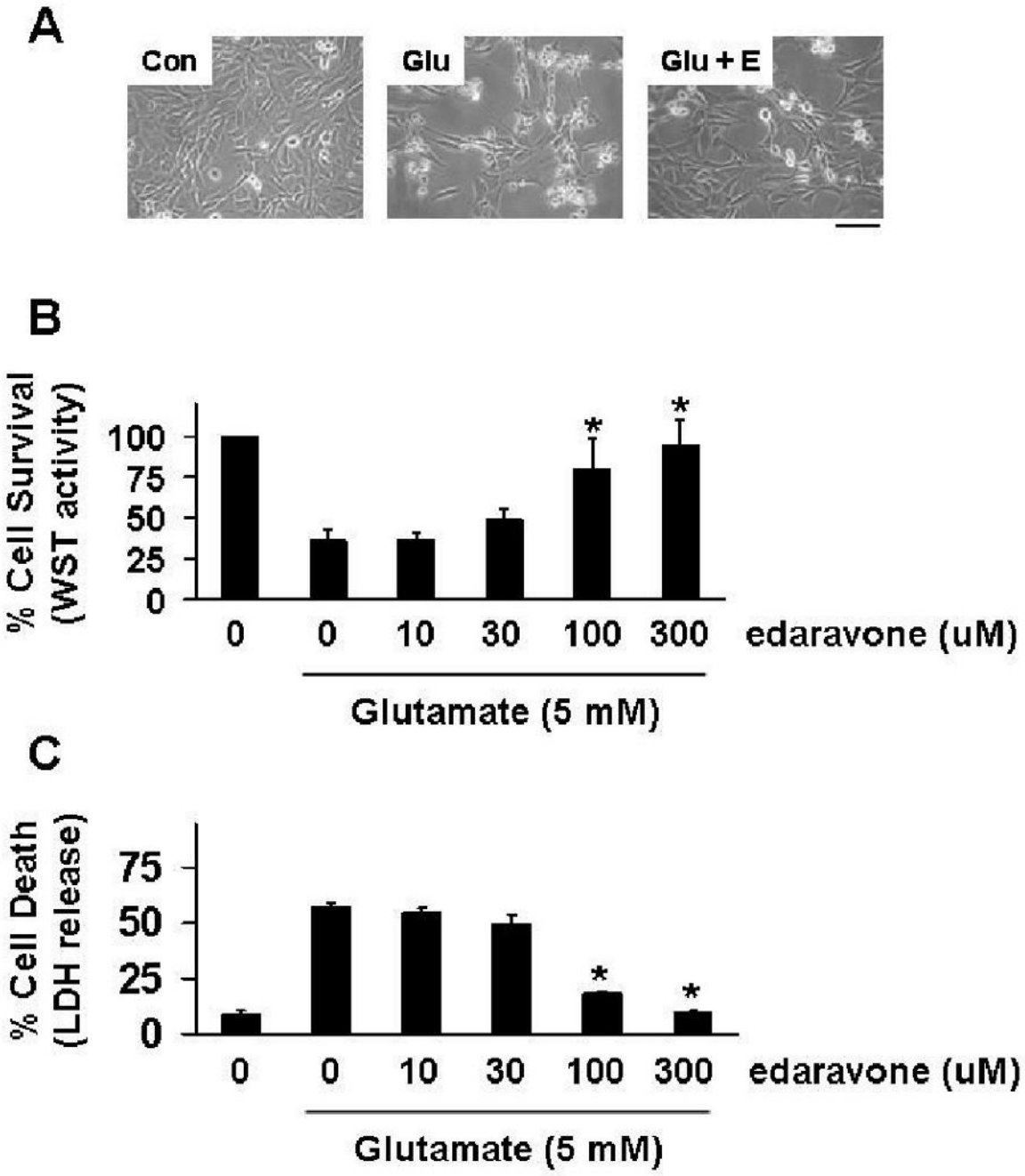

Figure 2. Edaravone protected HT22 cells against glutamate-induced oxidative stress

(A) Representative photomicrographs showing that glutamate treatment (5 mM for 24-h) induced cell death in HT22 cells, and co-treatment with edaravone (100 uM) reduced cell death. Scale bar = 100 um. A WST assay **(B)** and a LDH assay **(C)** show that protective effects of edaravone against glutamate-induced HT22 cell death were concentration-dependent. Both WST assay and LDH assay were conducted 24-hours after glutamate treatment (see Experimental Procedure). *P<0.05 vs 5 mM glutamate.

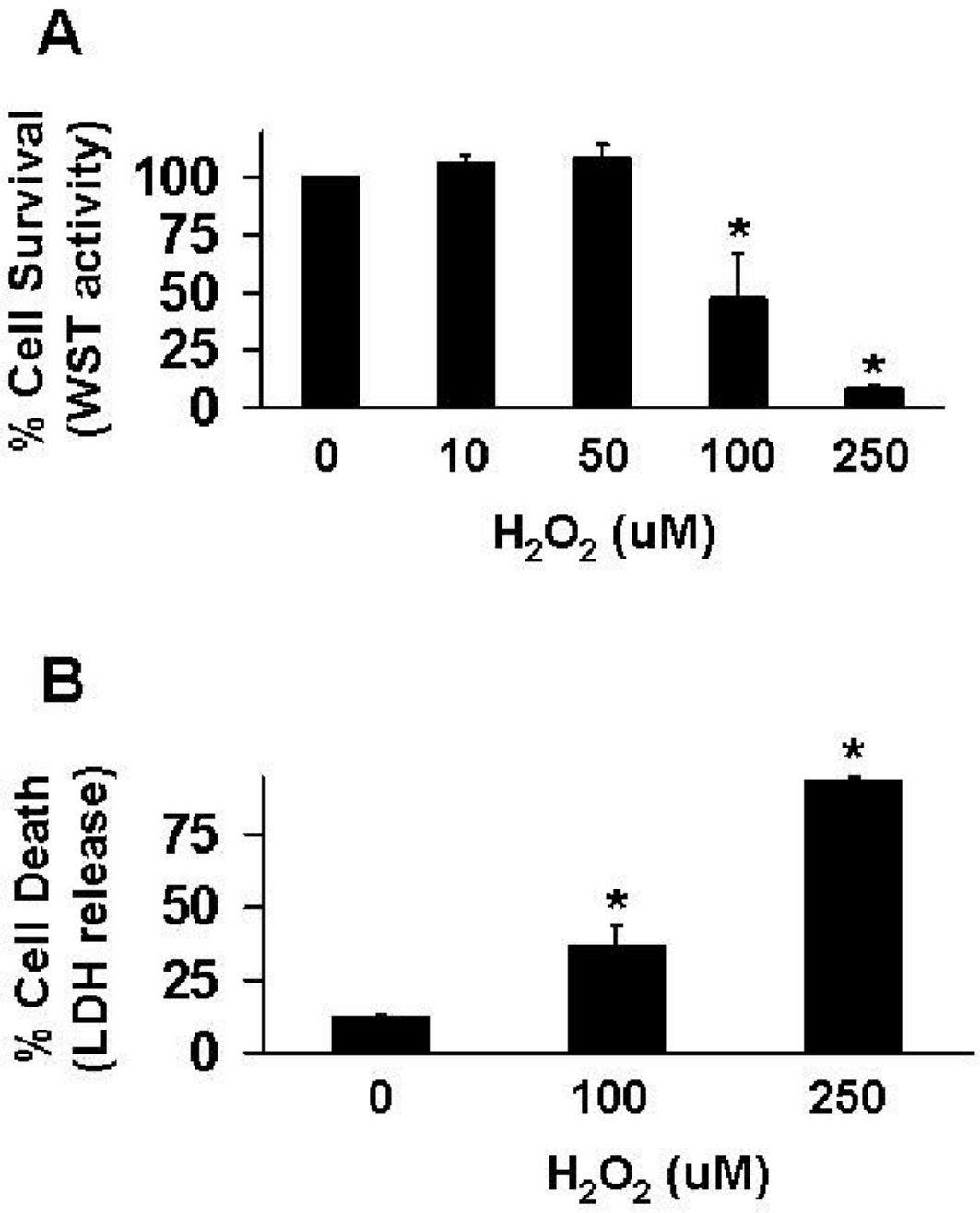

Figure 3. Hydrogen peroxide (H_2O_2)-induced cell damage in cultured rat astrocytes

A WST assay **(A)** and a LDH assay **(B)** show that H_2O_2 treatment induced astrocyte death in a concentration-dependent manner. Both WST assay and LDH assay were conducted 24-hours after H_2O_2 treatment as shown in Materials and Methods. *P<0.05 vs 0 uM H_2O_2.

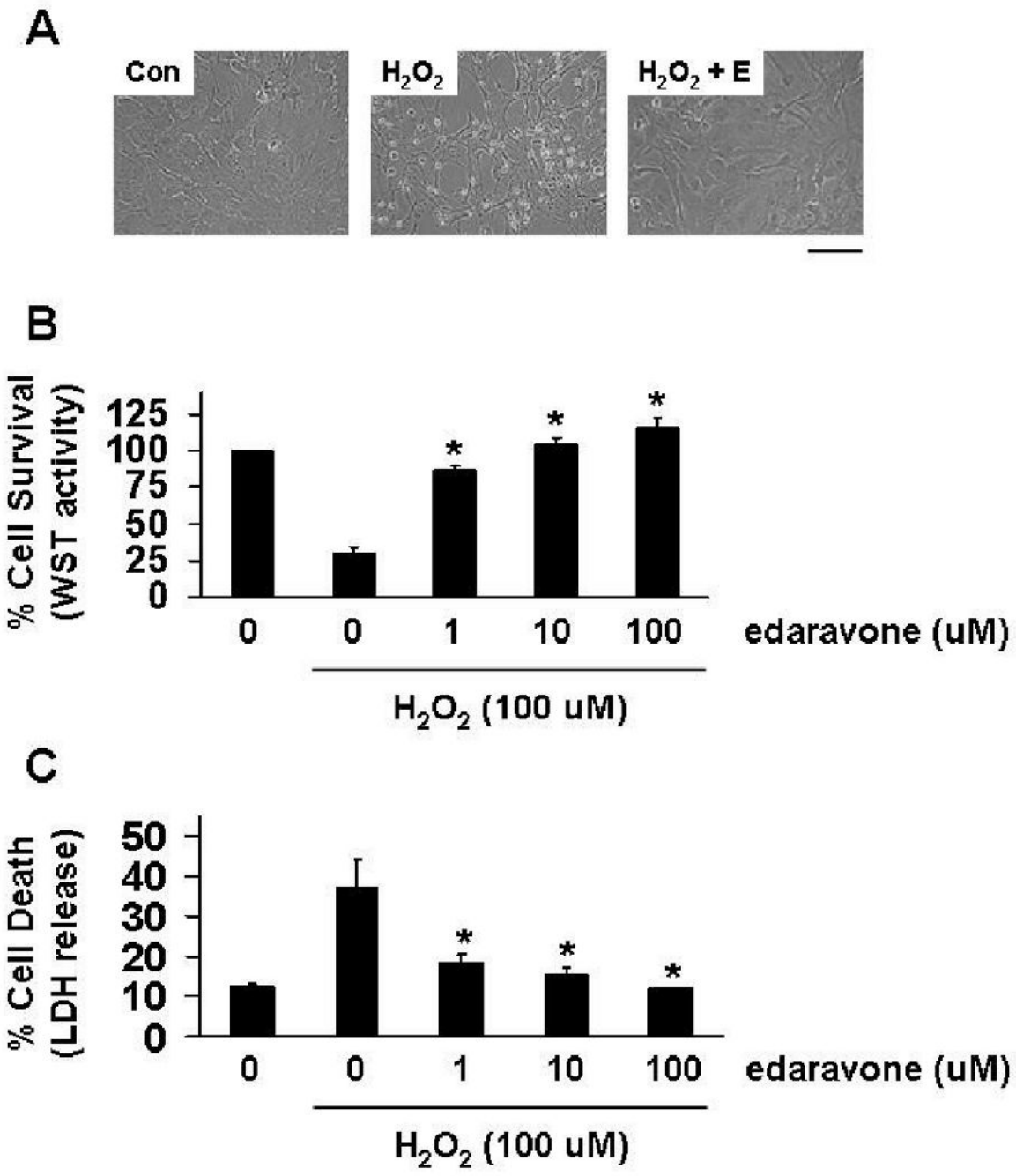

Figure 4. Edaravone protected cultured rat astrocytes against hydrogen peroxide (H_2O_2)-induced oxidative stress

(A) Representative photomicrographs showing that H_2O_2 treatment (100 uM for 24-h) induced cell death in cultured rat astrocytes, and co-treatment with edaravone (100 uM) reduced cell death. Scale bar = 100 um. A WST assay **(B)** and a LDH assay **(C)** demonstrated the concentration-dependent protective effects of edaravone against H_2O_2-induced astrocyte death. Both WST assay and LDH assay were conducted 24-hours after H_2O_2 treatment (see Experimental Procedure). *$P<0.05$ vs 100 uM H_2O_2.

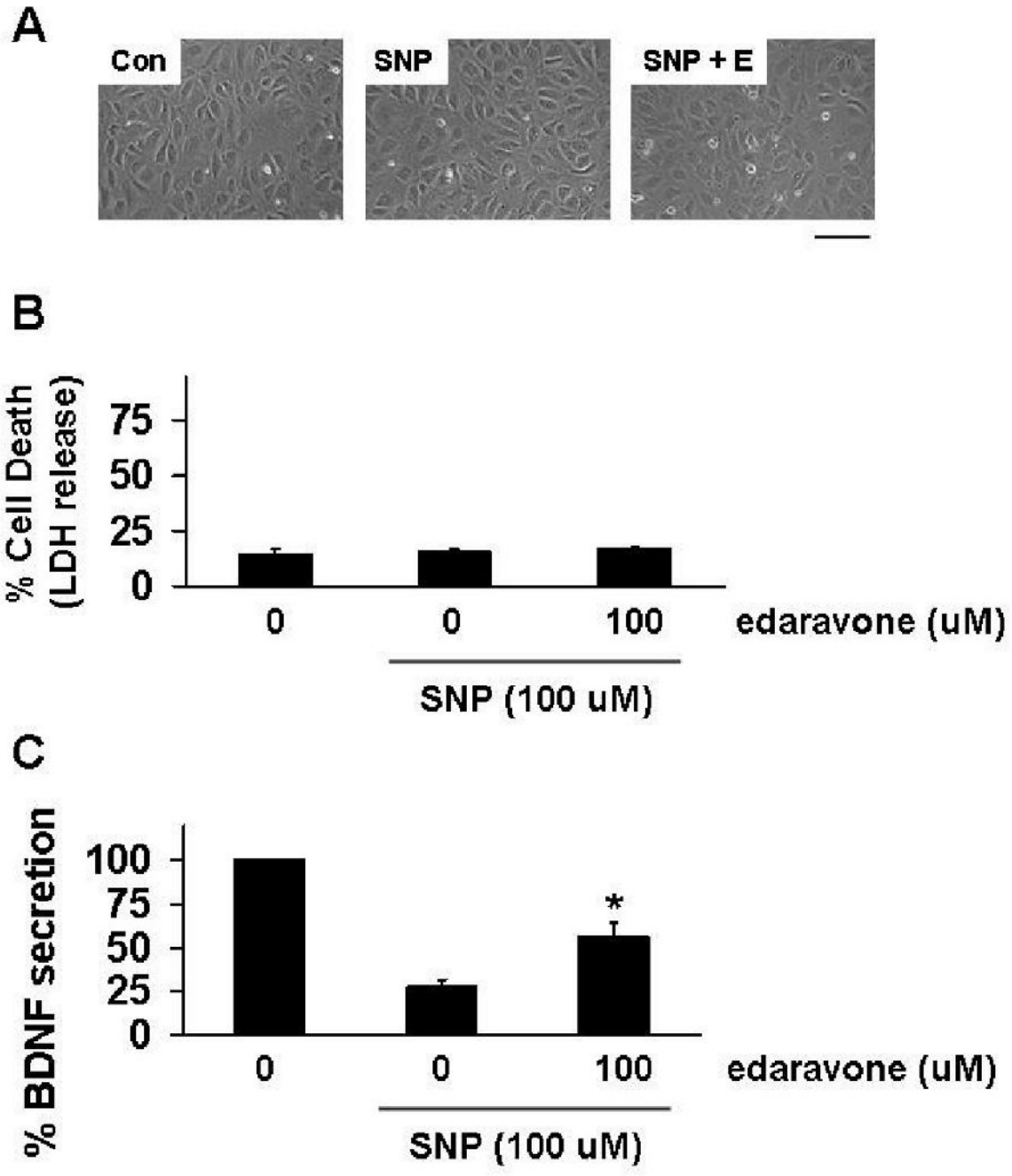

Figure 5. SNP-induced downregulation of BDNF secretion from human cerebral endothelial cells

(A) Representative photomicrographs showing that SNP treatment (100 uM for 24-h) did not induce cell death in human brain microendothelial cells. Scale bar = 100 um. **(B)** A LDH assay confirmed that 100 uM SNP was not cytotoxic to endothelial cells (see Experimental Procedure). **(C)** Nonlethal SNP reduced BDNF secretion from the endothelial cell cultures. Co-treatment with edaravone inhibited the decrease of BDNF secretion (quantified via ELISA). Conditioned media were collected 24-hours after SNP with or without edaravone treatment. *P<0.05 vs SNP.

Table 1

Effects of edaravone on cell viability in HT22, astrocyte, and cerebral endothelial cell

	Edaravone (uM)				
	0	**1**	**10**	**100**	**300**
HT22	7.0 ± 0.1	6.4 ± 0.5	6.5 ± 0.2	6.7 ± 0.2	7.7 ± 0.4
Astrocyte	9.4 ± 0.4	8.8 ± 0.9	8.2 ± 0.6	9.4 ± 0.6	9.7 ± 0.2
Cerebral endothelium	13.5 ± 3.4	13.3 ± 6.5	12.4 ± 1.8	12.0 ± 1.4	15.9 ± 1.6

A LDH assay shows that 24-h treatment of edaravone did not induce significant cell death in all three cell types. Data represents mean percent LDH release with SD for each concentration of edaravone.