Neuroprotective effects of edaravone on cognitive deficit, oxidative stress and tau hyperphosphorylation induced by intracerebroventricular streptozotocin in rats

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ABSTRACT

Oxidative stress is implicated as an important factor in the development of Alzheimer's disease (AD). In the present study, we have investigated the effects of edaravone (9 mg/kg, 3-methyl-1-phenyl-2-pyrazolin-5-one), a free radical scavenger, in a streptozotocin (STZ-3 mg/kg) induced rat model of sporadic AD (sAD). Treatment with edaravone significantly improved STZ-induced cognitive damage as evaluated in Morris water maze and step-down tests and markedly restored changes in malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE) adducts, hydroxyl radical ($^\bullet$OH), hydrogen peroxide (H_2O_2), total superoxide dismutase (T-SOD), reduced glutathione (GSH), glutathione peroxidase (GPx) and protein carbonyl (PC) levels. In addition, histomorphological observations confirmed the protective effect of edaravone on neuronal degeneration. Moreover, hyperphosphorylation of tau resulting from intracerebroventricular streptozotocin (ICV-STZ) injection was decreased by the administration of edaravone. These results provide experimental evidence demonstrating preventive effects of edaravone on cognitive dysfunction, oxidative stress and hyperphosphorylation of tau in ICV-STZ rats. Since edaravone has been used for treatment of patients with stroke, it represents a safe and established therapeutic intervention that has the potential for a novel application in the treatment of age-related neurodegenerative disorders associated with cognitive decline, such as AD.

1. Introduction

Alzheimer's disease (AD) is an age-related, progressive neurodegenerative disorder. This condition is characterized by deterioration of cognitive function including behavioral impairments and memory deficits and is associated with pathological aggregations of the amyloid β (Aβ) peptide and neurofibrillary tangles of aggregated hyperphosphorylated tau protein in the brain. Although the exact etiology of AD is unknown, there exists considerable evidence implying that abnormal generation of free radicals may underlie the development and accompanying neuronal deterioration of AD (Markesbery, 1999; Su et al., 2008). Excesses in reactive oxygen species (ROS) and free radical generation, as induced by oxidative metabolism imbalance, lead to a range of changes in cellular structure and function (Smith et al., 2010). These changes including protein and DNA injury, energy deficiency, inflammation, mitochondrial dysfunction, tau hyperphosphorylation and Aβ overexpression, all of which play important roles in the acceleration of aging and age-related neurodegenerative disorders (Pratico and Delanty, 2000; Melov et al., 2007; Zawia et al., 2009; Coppieters and Dragunow, 2011). Therefore, free radical scavengers and antioxidants, which have the capacity of neutralizing free radicals, have been proposed as therapeutic agents for delaying, inhibiting or reversing the pathological process of neurodegenerative disorders (Uttara et al., 2009).

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a potent free radical scavenger and it has been demonstrated that edaravone exerts beneficial effects against oxidative stress in acute ischemic stroke patients (Yoshida et al., 2006). In addition to

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its use in cerebral infarction, edaravone has been found to have neuroprotective effects upon Amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD), and in animal models of these conditions as demonstrated in vivo and in vitro (Kikuchi et al., 2011). Taken together, these findings suggest that edaravone may be effective in the treatment of neurodegenerative disorders associated with oxidative stress, such as AD.

Streptozotocin (STZ), a glucosamine compound which, when metabolized, generates a cytotoxic product that induces preferential damage in pancreatic β cells. Interestingly, intracerebroventricular (ICV) administration of a sub-diabetogenic dose of STZ in rats has been shown to induce cognitive dysfunction, impaired intracerebral glucose and energy metabolism, increased oxidative stress, hyperphosphorylation of tau protein and other neuropathological and biochemical changes which are similar to those observed in sporadic Alzheimer's disease (Sharma and Gupta, 2001; Grunblatt et al., 2007; Salkovic-Petrisic and Hoyer, 2007). Therefore, ICV-STZ was applied to establish a valid experimental model of the early pathophysiological alterations in sAD (Salkovic-Petrisic and Hoyer, 2007; Salkovic-Petrisic et al., 2011).

The present study was designed to investigate the effects of edaravone, a potent free radical scavenger, in the intracerebroventricular streptozotocin (ICV-STZ) rat model. Specifically, we focused upon the capacity for edaravone to modulate learning and memory impairments, oxidative stress and tau protein phosphorylation in this rat model of sAD.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats weighing 320–350 g (5–7 months) were obtained from the Beijing Vital River Laboratory Animal Technology Co. Ltd in the People's Republic of China. The animals were placed in a quiet room with a 12 h light/dark cycle, an ambient temperature of 23 ± 2 °C and a relative humidity of $56 \pm 4\%$. All surgeries were performed under anesthesia using a 10% chloral hydrate solution. All efforts were made to minimize animal suffering. All animal procedures performed in this work followed guidelines in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals, and approved by the animal care and welfare committee of Harbin Medical University.

2.2. Drugs and chemicals

Streptozotocin (STZ) from Sigma Chemical Co., St. Louis, USA was dissolved in artificial cerebrospinal fluid (a-CSF – 2.9 mM KCl, 147 mM NaCl, 1.7 mM CaCl$_2$, 1.6 mM MgCl$_2$, 2.2 mM D-glucose) in a 25 mg/ml solution. This solution was prepared immediately prior to injection. Edaravone (MCI-186, 3-methyl-1-phenyl-2-pyrazolin-5-one) purchased from Calbiochem, Germany was dissolved in 1 N NaOH, diluted with distilled water, and pH-adjusted to 7.4 with 1 N HCl according to the manufacturer's instructions. All bioluminescent assay kits were purchased from Nanjing Jiancheng Bioengineering Institute, Jiangsu, China. The nissl staining kit was purchased from the Beyotime Institute of Biotechnology, Shanghai, China. Total tau, ser396-phosphorylated tau, thr181-phosphorylated tau and β-actin-specific antibodies were purchased from Santa Cruz Biotechnology Inc., USA and the polyclonal 4-HNE antibody was purchased from Abcam Biotechnology Inc., USA. The secondary antibodies were purchased from the Zhongshan Company of Beijing, China. All other chemicals were of analytical reagent grade.

2.3. Experimental design

After a week of adaption to colony room conditions the rats were randomly divided into six groups: Group 1-Sham (S): sham operated rats were infused with a-CSF in each ventricle on days 1 and 3, and the animals were administered with distilled water (0.5 ml b.i.d. intraperitoneally) as a vehicle of edaravone for two weeks following the first day after surgery in each group. Group 2-Edaravone + S: sham operated rats were administered with 9 mg/kg b.i.d. i.p. of edaravone. Group 3-Lesion (L): rats were performed with bilaterally ICV infusion of STZ on days 1 and 3, and the rats were treated with the same vehicle as S group. Group 4-Edaravone 1 + L: ICV-STZ rats were immediately administered with edaravone 1 mg/kg b.i.d. i.p. after first STZ infusion for two weeks. Similar as group 4, animals in groups 5 (Edaravone 3 + L) and 6 (Edaravone 9 + L) were infused with ICV-STZ on days 1 and 3, and treated with edaravone at doses of 3 mg/kg and 9 mg/kg respectively for two weeks following the first day after surgery.

All behavioral tests were carried out to evaluate the capacity of learning and memory for each group between day 15 and day 24. On day 25, the rats were sacrificed for assay of T-SOD, MDA, GSH, GPx as a dose dependent study. Based on the above results, the dose of 9 mg/kg was choosed for further study, including the content of content of PC, H$_2$O$_2$ and hydroxy radical, the content of 4-HNE adducts and phosphorylation levels of tau protein. Fig. 1 shows the treatment schedule design and time intervals for estimations of the various parameters.

2.4. Surgical procedure

Animals were anesthetized using a 10% chloral hydrate solution of 3 ml/kg administered intraperitoneally. The head was positioned in a stereotaxic apparatus and skull exposed. Two holes were drilled through the skull for bilateral placement of a micro-injector into the lateral cerebral ventricles using the following coordinates: 0.8 mm posterior to bregma; 1.6 mm lateral to the sagittal suture; and 4.0 mm ventral from the surface of the brain (Ishrat et al., 2009b). The STZ solution was infused slowly (1 μl/min) into each ventricle on days 1 and 3. The sham group received the same surgery but infused with an equal volume of a-CSF only.

2.5. Memory and learning test

2.5.1. Morris water maze test

Spatial learning and memory were tested with use of a Morris water maze (MWM) (Morris, 1984). The water maze was a circular black tank (150 cm diameter, 60 cm height) with an automatic
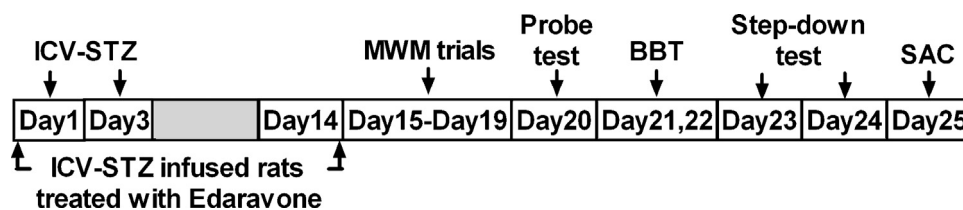
Fig. 1. The experimental design treatment schedule and intervals for estimation of various parameters. ICV-STZ = intracerebroventricular streptozotocin, MWM = Morris water maze, BBT = Beam balance test, and SAC = sacrificed.

heater to maintain a water temperature of $23 \pm 1\,^{\circ}C$. The water maze was filled with water to a depth of 40 cm. Four equally spaced locations at the periphery of the pool (North, South, East and West) divided the pool into 4 quadrants, and were used as start points. An escape platform of 10 cm diameter was placed 2 cm below the surface of the water at a constant location in the center of one of the quadrants. The animals were subjected to a daily session of four training trials with an inter-trial interval of 30 min for five consecutive days. Prior to training, rats were allowed to swim freely in the pool for 60 s without the platform. The rats were permitted a maximal time of 60 s to find the hidden platform and allowed to remain on the platform for 30 s before commencement of the next trial. If the rat failed to locate the hidden platform within 60 s, it was gently placed on the platform and allowed to remain there for a 30 s period. Latencies to locate the platform were recorded in training trials. One day after the training phase, a probe test was conducted by removing the platform. Rats were allowed to swim freely in the pool for 60 s. The time spent in the target quadrant in which the platform had previously been located during training phase was recorded. This parameter provided an index of the degree of memory consolidation.

2.5.2. Balance beam test

The balance beam is a test of motor coordination. After completion of the MWM test, the balance beam test was administered. Since balance can influence performance on the step-down test (described below), it was considered important to include an assessment of balance. Animals were trained to traverse a cylindrical beam with a length of 200 cm and diameter of 2.5 cm. A black platform (7 cm × 4 cm) was positioned at one end of the beam as a start, and a black plastic box (15 cm × 15 cm × 8 cm) was placed at the other end as a type of reinforcement for rewarding the animal to traverse the beam. The apparatus was suspended 90 cm above a cushion, which protected animals from injury due to falls, and was positioned 50 cm from a wall. Rats were moved into the testing room one hour before the test to adapt to the environment. The time required to traverse the beam during two trials was recorded. The maximal transversal time allowed was 60 s.

2.5.3. Step-down test

The step-down test was selected as a method to evaluate potential deficits in memory retention. This test was administered after all rats had completed the MWM test. The step-down passive avoidance apparatus consisted of a box (25 cm × 25 cm × 75 cm) with a stainless-steel grid floor and a round safe platform (8 cm diameter, 5 cm height) positioned in one corner on the grids. In the acquisition trial period, each rat was placed gently on the safe platform. The initial latency (IL) to step down from the platform was recorded, and an electric foot shock (3 s 0.4 mA) was immediately delivered through the floor grids. After a series of repeated trials, the rats eventually remained on the platform. This training session lasted 300 s. Twenty-four hours later, the retention latency (RL) time was measured and recorded in the same manner as described for the acquisition trial. If the rat did not step down from the platform within 300 s, the trial was terminated and the retention latency was recorded as a maximum value of 300 s. Whenever the rat stepped down from the platform this was counted as an error and the number of errors was recorded. Each rat received only one training session and only one test session. Deficits in acquisition and retention of memory were indicated by decreased latencies and increased number of errors in the task.

2.6. Measurement of biochemical parameters

2.6.1. Tissue preparation

After completion of all behavioral tests on day 25, the rats were sacrificed by decapitation. Whole brains were removed quickly and prepared for assay. The brains were rinsed with chilled saline and cerebral cortex and hippocampus were dissected on ice according to coordinates indicated in the rat brain atlas. The two dissected brain structures were weighed and homogenized in ice-cold normal saline to achieve a 10% homogenate solution (1/9, tissue/saline, w/v). The homogenate was centrifuged at 4000 rpm for 10 min at $4\,^{\circ}C$ to separate nuclear debris and an aliquot of the supernatant removed for estimation of SOD, MDA, GSH, GPx, H_2O_2 and $^{\bullet}OH$. The remaining supernatant was further centrifuged at $10,000 \times g$ for 20 min at $4\,^{\circ}C$ to generate the post-mitochondrial supernatant (PMS), which was used for PC assays.

2.6.2. Biochemical analysis

The analyses of T-SOD, MDA, GSH, GSH-Px, H_2O_2 and $^{\bullet}OH$, and PC levels were performed according to the protocols provided by the Nanjing Jiancheng Bioengineering Institute (Jiangsu, China) with a UV1700 spectrophotometer (Shimadzu, Japan) and the method described in the references.

SOD activity was analyzed according to the directions described in the kit. A competitive inhibition assay was administered using xanthine–xanthine oxidase-generated O_2 to reduce nitroblue tetrazolium (NBT) to blue formazan. One unit (U) of SOD activity was defined as the amount of enzyme required to reduce NBT to 50% of maximum. The maximum absorbance was read at 550 nm and enzymatic activity was presented as U/mg protein/min.

MDA content was determined using thiobarbituric acid reactive substances, and the adduct was measured at 532 nm. MDA content was expressed as nmol/mg protein. Blanks for each sample were prepared and assessed identically to correct for the contribution of A532 to the sample.

A thiolspecific reagent, dithionitrobenzoic acid (DTNB), was used to assay the GSH content, and the yellow mixture was measured at 420 nm. GSH content was expressed as mg GSH/g protein.

GSH-Px activity was estimated by the analysis of reduced GSH in the enzymatic reaction (Sedlak and Lindsay, 1968). One unit of GSH-Px activity was defined as the amount of decrease in 1 μmol/mg wet tissue per minute GSH concentration after subtraction of the non-enzymatic mode at $37\,^{\circ}C$ as suggested in the protocol of the commercial kit. GSH-Px activity was expressed as U/mg protein/min.

Hydrogen peroxide can form a complex with ammonium molybdate and the mixture was measured at 405 nm. Hydrogen peroxide content was expressed as mmol/g protein.

Hydroxy radical level was measured as previously described (Steiner and Babbs, 1990) and the concentration of hydroxyl radical was expressed as nmol/mg protein.

The PC level was measured as previously described (Ishrat et al., 2009a). Absorbance was recorded at 360 nm in a spectrophotometer. The PC level was then expressed as nmol carbonyl/mg protein.

2.6.3. Total protein assay

Protein concentrations were determined by the Coomassie brilliant blue protein binding method (Bradford, 1976) using bovine serum albumin (BSA) as a standard. The sample was diluted to a 1% concentration and absorbance was measured at 595 nm.

2.7. Nissl staining

Rats were deeply anesthetized with 10% chloral hydrate and subjected to transaortic perfusion with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde to fix the brain. The entire brain was then carefully extracted and maintained in 4%

paraformaldehyde overnight. After fixation the tissue was dehydrated and embedded in paraffin. Coronal sections were evaluated at 1.8–2.0 mm rostral to the optic chiasm. Sections (4 μm) were prepared and subjected to nissl staining as described in the protocol of the instructions.

2.8. Western blot

Cerebral cortex and hippocampus were dissected, snap-frozen in liquid nitrogen and stored at −80 °C. Western blot was used to assay the levels of 4-HNE protein adducts, tau, ser396-phosphorylated tau and thr181-phosphorylated tau. In brief, tissues were homogenized in a RIPA buffer (Applygen Technologies Inc.) with protease inhibitors. The total amount of protein was determined by the bicinchoninic acid protein assay (Applygen Technologies Inc.). Samples were separated by SDS–PAGE and transferred to nitrocellulose membranes, which were blocked by incubation in 3% (w/v) BSA dissolved in TBS-T. The membranes were incubated at room temperature with 4-HNE, total tau, ser396-phosphorylated tau, thr181-phosphorylated tau or β-actin-specific antibodies. Protein bands were then detected as resulting from incubation with conjugates of secondary antibodies.

2.9. Statistical analysis

The data were expressed as the mean ± SD and were evaluated using one-way ANOVA followed by LSD (equal variances or homogeneity of variance assumed after the variable transformation) or Dunnett's T3 (equal variances not assumed after the variable justification) tests for post hoc comparisons between groups. Statistical significance was accepted when $P < 0.05$.

3. Results

3.1. Behavioral observations

3.1.1. Edaravone treatment decreased escape latency in training trials and increased time in target quadrant in Morris water maze probe test in ICV-STZ rats

Latencies to locate the hidden platform below the water surface gradually diminished in all six groups over the five days of MWM training. Average latency to locate the hidden platform on the fifth day of training trials was significantly ($P < 0.05$) prolonged in the L group as compared with the S group. Edaravone (9 mg/kg) treatment significantly ($P < 0.05$) reversed the STZ induced learning deficits as observed in the Edaravone 9 + L group (Fig. 2A). The amount of time in the target quadrant was significantly less in the L group as compared with the S group. Edaravone (9 mg/kg) treatment significantly ($P < 0.05$) attenuated STZ induced memory deficits in the L group as indicated by increased amounts of time in the target quadrant in the Edaravone 9 + L group (Fig. 2B). No statistically significant differences in latencies and the amount of time in the target quadrant were obtained between low doses (1 mg/kg and 3 mg/kg) Edaravone treatment group and L group. Taken together, these results revealed an improved acquisition and retention of memory with edaravone treatment in ICV-STZ rats.

3.1.2. Edaravone does not alter balance in ICV-STZ rats as assessed in the balance beam test

In the balance beam test, rats were placed on the beam to assess their balance and coordination by measuring the time required to traverse the beam. No statistically significant differences in transversal times were obtained among the six groups (Fig. 3, $P > 0.05$).
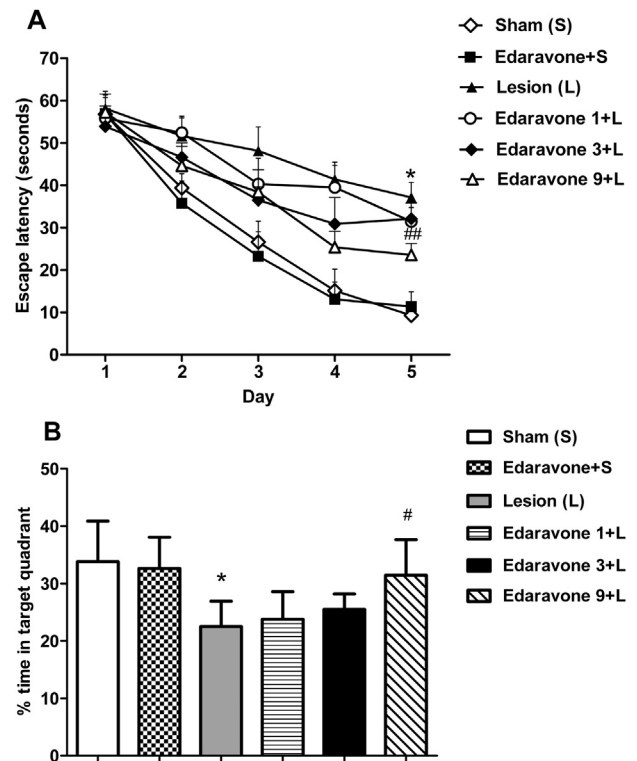

Fig. 2. Effect of edaravone treatment on the performance of training (A) and probe (B) trials in a MWM test. Values are expressed as mean ± SD in seconds. The average escape latency on the fifth day was significantly prolonged in the L group as compared with the S group, and the average time in the target quadrant was significantly reduced in the L group as compared with the S group (*$P < 0.05$, S vs. L group), indicating a learning performance deficit in the ICV-STZ lesioned rats. The prolonged escape latency and reduced time in the target quadrant observed in the L group were significantly attenuated by Edaravone treatment in Edaravone + L group (#$P < 0.05$, ##$P < 0.01$, L vs. Edaravone 9 + L group).

3.1.3. Edaravone treatment increased retention latency and reduced the number of errors in the step-down test in ICV-STZ rats

In the step-down test, latency was defined as the time for the first step-down onto the electric grid. In addition, the number of errors as recorded whenever the rat stepped down from the platform, was counted. As shown in Fig. 4A and B, latencies decreased while error-number significantly ($P < 0.05$) increased in the L group as compared to the S group. Latencies were prolonged while error-number significantly ($P < 0.05$) reduced in Edaravone 9 + L group as compared with the L group, suggesting that edaravone (9 mg/kg) improved acquisition and retention of memory in ICV-STZ rats.
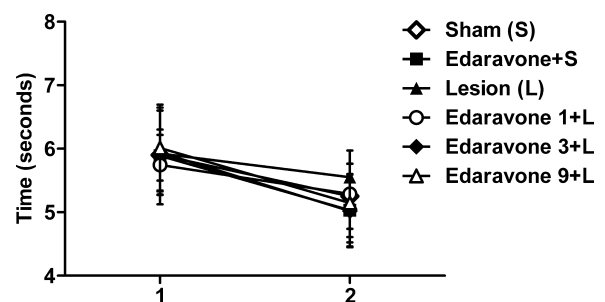

Fig. 3. Balance beam test scores of the four groups. Values are expressed as mean ± SD in seconds. No overall statistically significant differences were obtained among the four groups. Administration of edaravone does not alter balance as indicated in the latency times to traverse the elevated beam in this test using a 2.5 diameter cylindrical beam ($P > 0.05$).
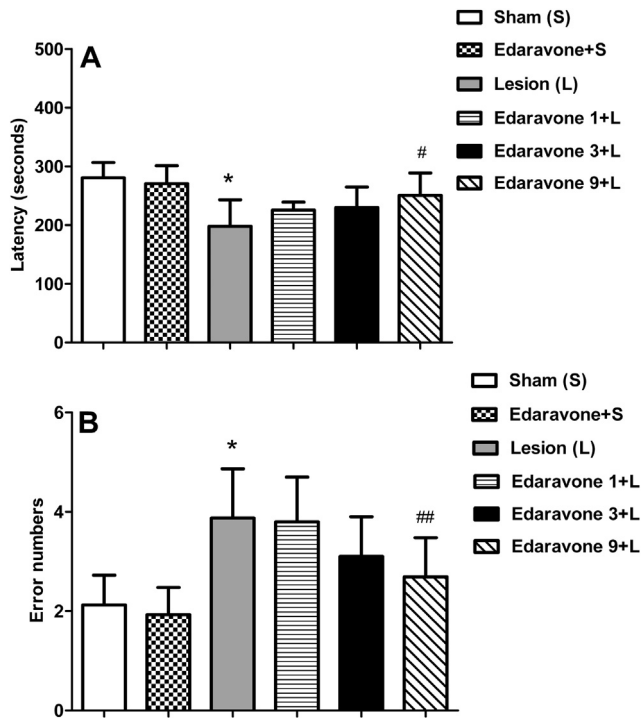
Fig. 4. Effect of edaravone treatment on latency (A) and error number (B) in the step-down test. Values are expressed as mean ± SD in seconds (A) and number (B). The latency of the L group was significantly reduced and error number significantly increased as compared with that of the S group (*$P < 0.05$, S vs. L group). The latency increased and error number decreased significantly in Edaravone 9 + L group as compared with L group (#$P < 0.05$, ##$P < 0.01$ Edaravone 9 + L group vs. L).

3.2. Biochemical results

3.2.1. Edaravone treatment restored T-SOD and GPx activities in the cerebral cortex and hippocampus of ICV-STZ rats

The effects of edaravone treatment on the activities of T-SOD and GPx in the cerebral cortex and hippocampus of ICV-STZ rats are shown in Table 1. Activities of T-SOD and GPx were significantly ($P < 0.05$) decreased in the L group as compared with the S group in cerebral cortex and hippocampus. Edaravone supplement significantly ($P < 0.01$) increased T-SOD and GPx activities in the Edaravone 9 + L group as compared with the L group. Edaravone treatment (3 mg/kg) also significantly increased T-SOD activities in the cortex in the Edarvone 3 + L group as compared with the L group. There were no significant ($P > 0.05$) differences between the Edaravone + S and S groups.

3.2.2. Edaravone treatment reduced MDA levels in the cerebral cortex and hippocampus of ICV-STZ rats

The effect of edaravone treatment on MDA levels in the cerebral cortex and hippocampus of ICV-STZ rats is shown in Table 1. MDA levels were significantly ($P < 0.05$) increased in the L group as compared with the S group. The augmentation of MDA in the L group was significantly decreased by edaravone 3 mg/kg ($P < 0.05$) and 9 mg/kg ($P < 0.01$) treatment in the cortex. However, the augmentation of MDA in the L group was significantly ($P < 0.01$) decrease by edaravone 9 mg/kg treatment only in the hippocampus. There were no significant ($P > 0.05$) differences between the Edaravone + S group and the S group.

3.2.3. Edaravone treatment restored GSH levels in the cerebral cortex and hippocampus of ICV-STZ rats

The effect of edaravone treatment on GSH levels in the cerebral cortex and hippocampus of ICV-STZ rats is shown in Table 1. Levels of GSH were significantly ($P < 0.05$) depleted in the L group as compared with the S group. GSH reductions by ICV-STZ were significantly ameliorated by edaravone (9 mg/kg) supplementation in the hippocampus ($P < 0.05$) and cortex ($P < 0.01$). The levels of GSH were not significantly ($P > 0.05$) changed by edaravone alone as revealed by the absence of significant differences between the Edaravone + S group and the S group.

3.2.4. Edarvone treatment restored hydroxyl radical, H_2O_2 and PC levels in the cerebral cortex and hippocampus of ICV-STZ rats

The effect of edaravone treatment on hydroxyl radical, H_2O_2 and PC levels in the cerebral cortex and hippocampus of ICV-STZ rats is shown in Table 2. Levels of hydroxyl radical, H_2O_2 and PC were significantly ($P < 0.05$) increased in the L group as compared with S group. The augmentations of hydroxyl radical and H_2O_2 levels by ICV-STZ were significantly ($P < 0.01$) ameliorated by edaravone (9 mg/kg) administration in both hippocampus and cortex. The increasing of PC level by ICV-STZ was significantly ($P < 0.01$) restored by edaravone (9 mg/kg) in the cortex. The levels of hydroxyl radical, H_2O_2 and PC were not significantly ($P > 0.05$) changed between the Edaravone + S group and the S group.

3.3. Histomorphological observation

Nissl stain was used to detect histomorphological changes in cerebral cortex and hippocampal neurons following ICV-STZ injection as shown in Fig. 5. Photomicrographs of coronal sections were taken at 200× magnification from the S (A, D), L (B, E) and Edaravone 9 + L (C, F) groups. Nissl staining revealed cell loss and injured neurons in the cerebral cortex and hippocampus of the L group. Compared to the L group, quantitative cell count revealed an

Table 1

Values are presented as mean ± SD and were analyzed by one-way ANOVA. ICV-STZ caused a significant decrease in the activities of antioxidant enzymes (T-SOD and GPx) and the level of an antioxidant agent (GSH) in the L group as compared with the S group (*$P < 0.05$ L vs. S group). Administration of edaravone significantly restored the activities of these enzymes in the Edaravone treatment groups (#$P < 0.05$ Edaravone 3 + L vs. L group, ##$P < 0.01$ Edaravone 9 + L vs. L group). Administration of edaravone significantly restored the content of GSH in the Edaravone 9 + L group (#$P < 0.05$, ##$P < 0.01$ Edaravone 9 + L vs. L group). ICV-STZ caused a significant increase in the levels of MDA in the L group as compared with the S group (*$P < 0.05$ L vs. S group). Administration of edaravone significantly restored the levels of MDA in the Edaravone treatment groups (#$P < 0.05$ Edaravone 3 + L vs. L group, ##$P < 0.01$ Edaravone 9 + L vs. L group). Measuring unit of each parameter: T-SOD U/mg protein/min, MDA nmol/mg protein, GPx U/mg protein/min, GSH mg/g protein.

Groups	SOD		MDA		GPx		GSH	
	Cortex	Hippocampus	Cortex	Hippocampus	Cortex	Hippocampus	Cortex	Hippocampus
Sham (S)	51.7 ± 4.1	52.4 ± 3.0	5.4 ± 0.7	3.5 ± 0.72	29.3 ± 2.6	42.4 ± 5.6	17.8 ± 3.2	19.0 ± 2.3
Edaravone + S	50.4 ± 3.1	53.3 ± 4.2	5.4 ± 0.8	3.9 ± 0.5	27.5 ± 2.9	45.6 ± 6.4	18.1 ± 4.4	18.7 ± 3.5
Lesion (L)	37.7 ± 3.3*	34.8 ± 3.2*	10.9 ± 1.9*	5.3 ± 0.8*	17.8 ± 1.5*	34.4 ± 5.3*	10.9 ± 1.9*	12.3 ± 2.5*
Edaravone 1 + L	39.1 ± 3.0	36.0 ± 3.6	9.1 ± 0.8	5.3 ± 0.8	17.5 ± 2.4	35.6 ± 3.1	10.7 ± 2.0	13.3 ± 3.5
Edaravone 3 + L	43.6 ± 6.2#	38.1 ± 4.9	7.8 ± 0.6#	4.8 ± 0.6	18.8 ± 4.0	37.2 ± 3.6	12.0 ± 3.1	16.0 ± 1.4
Edaravone 9 + L	48.6 ± 4.7##	44.9 ± 4.1##	6.0 ± 0.5##	4.0 ± 0.5##	23.3 ± 4.1##	41.0 ± 6.4##	15.5 ± 2.6##	20.0 ± 3.7#

Table 2

Values are presented as mean $\pm$ SD and were analyzed by one-way ANOVA. ICV-STZ caused a significant increase in the levels of H_2O_2, hydroxy radical and PC in the L group as compared with the S group (*$P < 0.05$ L vs. S group). Administration of edaravone significantly restored the levels of H_2O_2, hydroxy radical and PC in the Edaravone 9 + L group (##$P < 0.01$ Edaravone 9 + L vs. L group). Measuring unit of each parameter: H_2O_2 mmol/g protein, hydroxy radical nmol/mg protein, PC nmol/mg protein.

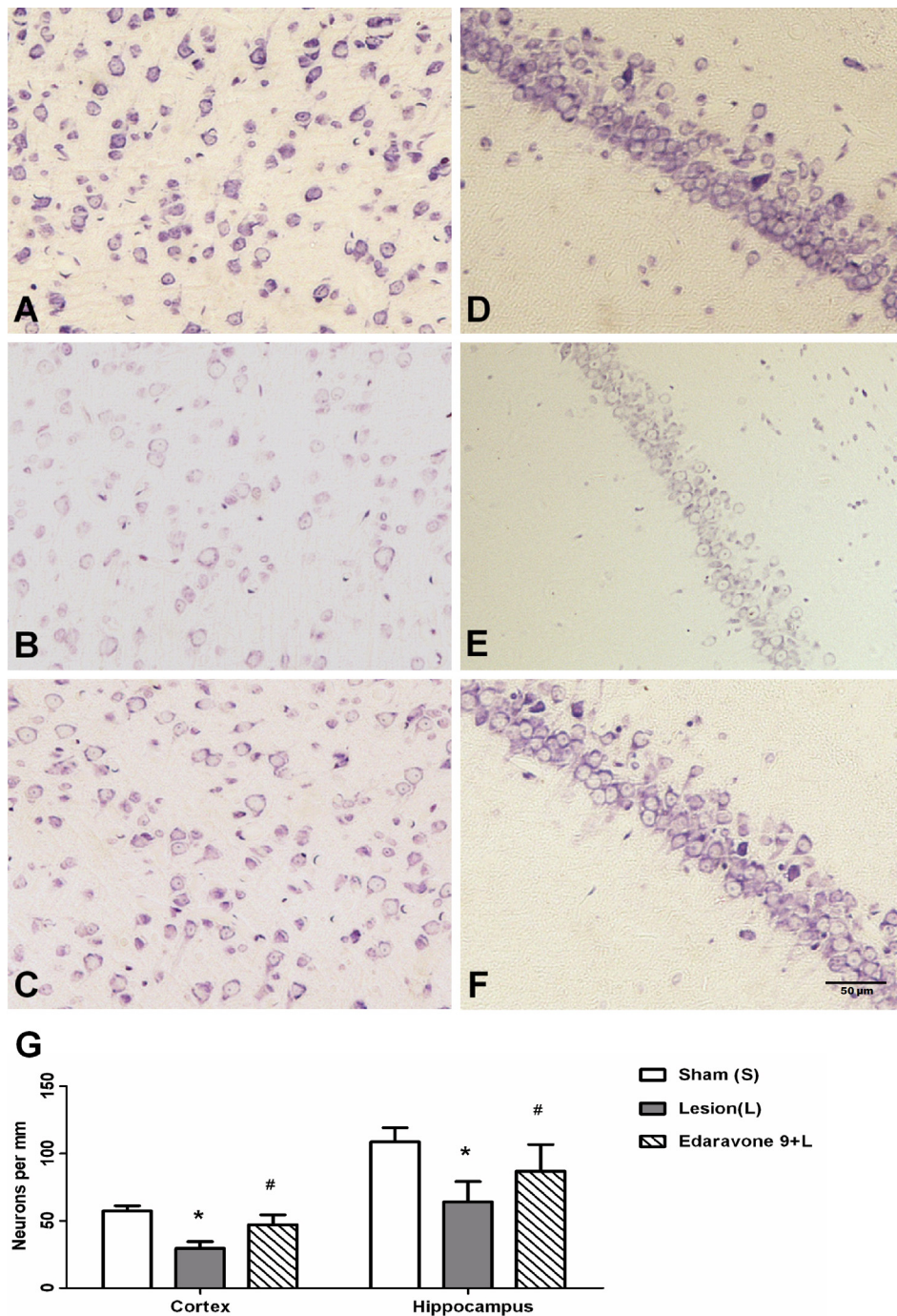| Groups	H_2O_2		Hydroxy radical		PC	
	Cortex	Hippocampus	Cortex	Hippocampus	Cortex	Hippocampus
Sham (S)	36.8 ± 1.6	40.2 ± 3.3	1.4 ± 0.1	1.9 ± 0.2	26.0 ± 2.6	24.0 ± 2.5
Edaravone + S	35.6 ± 2.7	37.8 ± 2.8	1.4 ± 0.1	1.8 ± 0.2	26.2 ± 2.0	23.3 ± 2.5
Lesion (L)	76.0 ± 9.1*	80.8 ± 5.7*	2.6 ± 0.2*	3.0 ± 0.2*	35.6 ± 3.1*	31.0 ± 3.9*
Edaravone 9 + L	54.8 ± 6.9##	55.8 ± 10.8##	2.0 ± 0.2##	2.6 ± 0.3##	31.2 ± 3.4##	28.4 ± 2.9

Fig. 5. Nissl stain in the cerebral cortex and the CA1 region of hippocampus. S group (A, D) indicate the normal neurons in cerebral cortex and hippocampus (A, D). L group (B, E) revealed cell loss in these zones after ICV-STZ in rats. Edaravone markedly decreases the loss of the cells in cerebral cortex and hippocampus (C, F). Quantification of neuron numbers was shown in G. (*$P < 0.05$ L group vs. S group, #$P < 0.05$ Edaravone 9 + L group vs. L group).
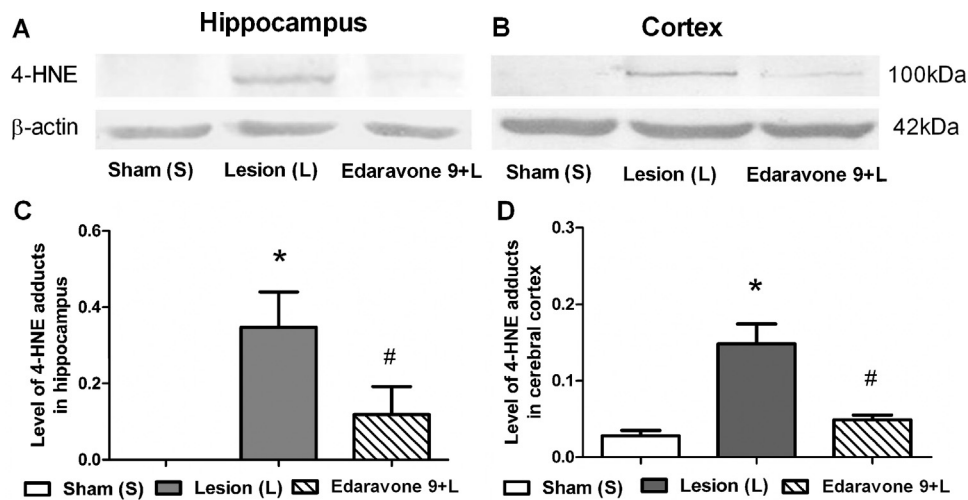
Fig. 6. Effect of edaravone treatment on 4-HNE adducts. 4-HNE adducts in the hippocampal (A) and cerebral cortical (B) proteins were determined by Western blots. Quantification of adducts (C, D) was normalized against β-actin. The relative values from each group were presented as mean ± SD. The level of 4-HNE adducts was significantly increased in the L group as compared to the S group. Treatment with edaravone significantly restored these enhanced levels of 4-HNE adducts as induced by ICV-STZ (*$P < 0.05$ L group vs. S group, #$P < 0.05$ Edaravone 9 + L group vs. L group, $n = 6$).

increase in the number of surviving neurons in the Edaravone 9 + L group (Fig. 5G).

3.4. Effect of edaravone on 4-HNE adducts levels

4-HNE is an α,β unsaturated aldehyde produced during oxidation in membranes. 4-HNE can react with proteins to form 4-HNE adducts, and the levels of 4-HNE adducts provide an indication of lipid peroxidation (Cutler et al., 2004). Levels of 4-HNE adducts were measured in the hippocampus and cerebral cortex of ICV-STZ rats by Western-blot. Western blots of 4-HNE protein adducts in hippocampal and cortex from S, L and Edaravone 9 + L rats are shown in Fig. 6A and B and quantified results of 4-HNE protein adducts levels from the above three groups of rats are shown in Fig. 6C and D. ICV-STZ infused rats had significantly increased 4-HNE adducts as compared to the S group in both hippocampus and cerebral cortex. In contrast, ICV-STZ infused rats with edaravone treatment showed a significant reduction in 4-HNE protein adducts.

3.5. Effect of edaravone on phosphorylated tau proteins

Total tau-protein expression was not significantly changed among the groups ($P > 0.05$) as shown in Fig. 7A and D. Phosphorylated tau was evaluated in the hippocampus and the cerebral cortex using the ser396-p-tau and thr181-p-tau antibodies. Hyperphosphorylation of tau at ser396 and thr181 as induced by STZ was significantly ($P < 0.05$) reduced in both hippocampus and cerebral cortex after administration of edaravone, Fig. 7A–C.

4. Discussion

The present results demonstrate that ICV-STZ induced learning and memory impairments in rats while edaravone treatment of these rats significantly improved their performance on behavioral tests of learning and memory as well as on parameters of oxidative stress, neuronal histomorphology and tau protein phosphorylation. Edaravone, a potent free radical scavenger, has been used safely for more than 10 years. In addition to functioning as a scavenger of free radicals, edaravone has shown anti-oxidative, anti-apoptotic, anti-necrotic and anti-cytokine effects in animal

models of various diseases (Kikuchi et al., 2012). However, effects of edaravone in sAD-related cognitive impairment and neuropathological changes have not been reported. Accordingly, we considered it is worthwhile to investigate the potential for edaravone to function as a neuroprotectant in an animal model of experimental sporadic dementia induced by STZ. In this study we investigated the effects of edaravone in modulating ICV-STZ induced cognitive dysfunction in rats, as well as the major biochemical components of neurodegeneration in this sAD model.

In our initial studies, we selected the doses of edaravone (1 mg/kg, 3 mg/kg and 9 mg/kg) to study. In previous studies, these doses exerted no toxic effects in rats (Tabrizchi, 2000). Our results showed that the dose of 9 mg/kg edaravone exerted the maximal effectiveness in the treatment of ICV-STZ rats. Previous studies have provided data on the concentrations of edaravone in the brain of rats. These investigators have found that the relative ratio of plasma to cerebral spinal fluid levels of edaravone was estimated to be between 50 and 65%. During administration of edaravone at 1 mg/kg/h for 3 h, concentrations of edaravone in plasma and cerebral spinal fluid were 375 ng/ml and 244 ng/ml, respectively (Tabrizchi, 2000). Moreover, another study found that edaravone has preventive effects on the degeneration of spinal motor neurons in the ALS model mice. In addition, the results of serum concentration of edaravone after a single intraperitoneal injection into the mice showed that a dose dependent elevation of serum concentration of edaravone was observed and the max serum concentration was 600–1100 ng/ml at 5 min after 10 mg/kg administration (Ito et al., 2008).

The Morris water maze test was employed to assess spatial learning and memory based on a reward mechanism. Rats readily attempt to escape from the water bath by locating an escape platform in a target quadrant. Therefore, escape latencies, which are the times required to locate the hidden platform, gradually decrease in training trials and more time is spent in the target quadrant in probe trials. Improvement of spatial memory by edaravone treatment in these ICV-STZ rats was indicated by shorter escape latencies and longer durations in the target quadrant.

The step-down passive avoidance test was used to evaluate non-spatial long-term memory based on the learned inhibition of a behavior to avoid punishment (Zhao et al., 2011). In our study, this aspect of memory showed an improvement in ICV-STZ rats
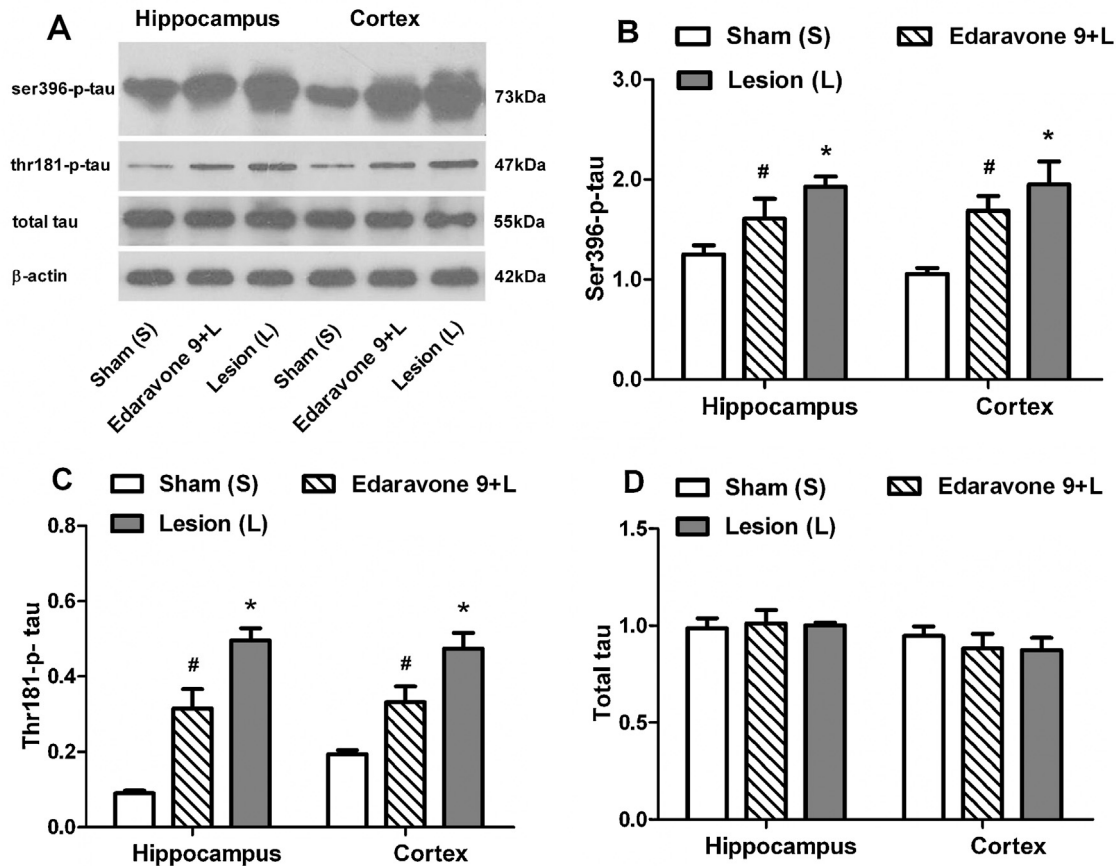
Fig. 7. Effects of edaravone on ICV-STZ-induced phosphorylated tau. The antibodies specific for ser396-phosphorylated tau, thr181-phosphorylated tau, total tau and β-actin were used to detect levels of intracerebral phosphorylated tau proteins by Western immunoblotting in each group ($^*P < 0.05$ L group vs. S group, $^#P < 0.05$ Edaravone 9 + L group vs. L group, $n = 6$).

receiving edaravone as revealed by longer latencies and fewer errors in step-down performance. In addition to the amygdala and insula, recognition of fear and the detection of aversion rely on the hippocampus and cortex (Arendt, 2001; Adolphs, 2002). Interestingly, all brain structures mentioned above play vital roles in learning and memory processing and seem to be more susceptible to oxidative damage.

The balance beam test was included to exclude the influence of balance upon performance in the step-down task. No significant differences were obtained among the groups, in particular between the ICV-STZ and edaravone treated ICV-STZ rats. Such findings suggest that the capacity for edaravone to prevent passive avoidance memory deficits in ICV-STZ rats was not due to difference in their balance.

Oxidative stress which is characterized by an imbalance in reactive oxygen species (ROS) and antioxidative defense has been demonstrated to play a critical role in several neurodegenerative diseases (Lin and Beal, 2006). Increasing evidence suggests that oxidative stress is a prominent and early feature of Alzheimer's disease (AD) and plays an important role in its pathogenesis (Christen et al., 2000; Barnham et al., 2004). Impaired antioxidative systems including antioxidant enzymes (e.g. SOD and GPx) and antioxidants (e.g. GSH) induce increased free radical generation. The unpaired electron in the outer obit of free radicals makes them unstable and capable of reacting with macromolecules such as proteins, sugars, lipids and nucleic acids, leading to changes in structure and function of neural cells in the brain (Tuppo and Forman, 2001). Consequently, these changes induce cellular damage, dysfunction, and cell death, eventually leading to pathological changes as observed in conditions such as AD, aging

and other neurodegenerative diseases (Fang et al., 2002). The most abundant ROS superoxide radical ($O_2^{\bullet-}$) may induce formation of the highly toxic hydroxyl radical, $^\bullet OH$, which represents one of the most reactive oxidants of proteins and nucleic acid. The SODs are a group of metalloenzymes that catalyze the transformation of superoxide radical into hydrogen peroxide, H_2O_2. Under physiological conditions, increased SOD activity usually accompanies increasing GPx levels and this increase of GPx detoxifies the high levels of H_2O_2 produced by SOD (Dringen et al., 2005). During the detoxification process, oxy-radicals are reduced by GPx at the expense of GSH, the most abundant non-protein thiol that buffers free radicals in brain tissue (Dringen et al., 2000).

Increased levels of free radicals initiate the oxidation of polyunsaturated fatty acids leading to lipid peroxidation (Dringen et al., 2000) and results in protein carbonylation (Smith et al., 1992). Lipid peroxides further lead to production of more stable compounds like malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE) and acrolein, which initiates oxidative stress. Oxidative damage to lipids (lipid peroxidation) and proteins (PC formation) initially results in an alteration of cell structure, inactivation of enzymes, followed by loss of normal physiological cell function and eventually neuronal cell death (Mattson, 2004). Mubeen and Stephen have shown that elevated oxidative stress resulting from PC and 4-HNE can lead to delayed neuronal death in the frontal cortex and is associated with mild cognitive impairment (MCI) and AD (Ansari and Scheff, 2010). In the present study, levels of MDA, 4HNE and PC increased and level of GSH decreased with down-regulated biochemical activity of SOD and GPx in the brain of ICV-STZ rats. Edaravone is effective in reducing oxidative stress and is a promising candidate for neuroprotection in

reducing brain damage when applied in this model of sAD (Kaur and Ling, 2008). Several reports have related the modulatory effects of edaravone on lipid peroxidation, PC formation and antioxidant enzymes as observed in hypoxia/ischemia and ischemic–reperfusion injury experiments (Takahashi et al., 2003; Zhang et al., 2005). In agreement with these findings, we report here that edaravone treatment markedly restored the biochemical changes as well as cognitive deficits induced by ICV-STZ infusion. It seems likely that this effect of edaravone could, in part, be attributed to its potent antioxidant property.

Consistent with previous reports (Chen et al., 2012; Correia et al., 2012), we also observed tau hyperphosphorylation induced by ICV-STZ. Findings from several sources have indicated that increased oxidative stress induces tau phosphorylation (Melov et al., 2007; Clausen et al., 2012). 4-HNE and PC are the two main products of oxidative stress. 4-HNE is a major aldehyde generated during lipid peroxidation of unsaturated lipids in the membrane, and it can conjugate to specific amino acid residues (e.g. lysine, cysteine, arginine) of proteins to impair their function. For example, HNE causes dysfunction of ion-motive ATP-ases, glucose and glutamate transporters, enzymes involved in amyloid metabolism, and cytoskeletal proteins (Mattson, 2009). Ikuroh Ohsawa et al. established a transgenic mice model in which the ability to scavenge HNE was decreased. As a result, an accumulation of HNE was present, ultimately, producing hyperphosphorylated tau proteins within the brain of these mice. Furthermore, under conditions of modified HNE, normal tau proteins are altered to phosphorylated tau, and epitopes of these modified tau represent the major conformational changes contributing to the formation of neurofibrillary tangles (NFTs), which is a prominent pathological marker of AD (Liu et al., 2005). In addition, increased protein carbonyl levels were found in the brains of tau transgenic mice and this change preceded tau pathology (Dumont et al., 2011). Our findings suggest that 4-HNE and PC may represent two of the main toxic products in ICV-STZ induced brain injury, in part, through their capacity to facilitate the occurrence of tau protein hyperphosphorylation. In support of this postulate, the current findings showed that edaravone protected the brain against ICV-STZ lesion by detoxifying 4-HNE and PC.

NFTs exist as intracellular abnormal protein fibers made up of paired helical filaments (PHF), which are mainly composed of aggregations of hyperphosphorylated tau. Physiologically, the main function of tau is to maintain intracellular microtubule stabilization. Hyperphosphorylation of tau decreases this capacity for binding and stabilizing microtubules (Hanger et al., 2009). Aggregation of hyperphosphorylated tau in neurons leads to axonal transport dysfunction (Yang et al., 2007), proteasome activity inhibition (Ren et al., 2007), consequent impairment of the structure and function of neurons and eventually neurodegeneration. A growing body of evidence is accumulating which indicates that the relationship between cognitive impairment and tau was more complex than that which can be attributable to that of Aβ alone (Arriagada et al., 1992; Roberson et al., 2007). These findings inspired us to study the effect of edaravone administration upon tau hyperphosphorylation as induced by STZ. In our study, we found that edaravone could reduce hyperphosphorylation of tau protein at ser396 and thr181 in the cerebral cortex and hippocampus of ICV-STZ rats.

In conclusion, our results demonstrate that edaravone ameliorated behavioral impairments of memory deficit, attenuated oxidative stress and blocked tau hyperphosphorylation in rat brains as produced by administration of ICV-STZ. Taken together, the present study provides further support for the potential use of edaravone in the treatment of aging and age-related neurodegenerative disorders.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgment

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