

Edaravone injection ameliorates cognitive deficits in rat model of Alzheimer's disease

Rui Yang¹ · Qingjun Wang² · Fang Li¹ · Jian Li¹ · Xuewen Liu¹

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Abstract Oxidative stress plays important role in the pathogenesis of Alzheimer's disease (AD). Edaravone is a potent free radical scavenger that exerts antioxidant effects. Therefore, in this study we aimed to investigate neuroprotective effects of edaravone for AD. Wistar rats were randomly divided into three groups ($n = 15$): control group, model group, and treatment group, which were injected with phosphate buffered saline, A β 1-40, and A β 1-40 together with 5 mg/kg edaravone, respectively, into the right hippocampal dentate gyrus. Spatial learning and memory of the rats were examined by Morris water maze test. 4-Hydroxynonenal (4-HNE) level in rat hippocampus was analyzed by immunohistochemistry. Acetylcholinesterase (AChE) and choline acetylase (ChAT) activities were assayed by commercial kits. We found that edaravone ameliorated spatial learning and memory deficits in the rats. 4-HNE level in the hippocampus as well as AChE and ChAT activities in the hippocampus was significantly lower in treatment group than in model group. In conclusion, edaravone may be developed as a novel agent for the treatment of AD for improving cholinergic system and protecting neurons from oxidative toxicity.

Keywords 4-Hydroxynonenal · Oxidative stress · Alzheimer's disease · Edaravone

Rui Yang and Qingjun Wang contributed equally.

✉ Rui Yang
yangruijz@163.com

¹ Department of Neurology, The First Affiliated Hospital, Liaoning Medical University, No. 2, 5th Duan, People's Street, Guta District, Jinzhou 121001, China

² Department of Oncology, The First Affiliated Hospital, Liaoning Medical University, Jinzhou 121001, China

Introduction

Up to now, Alzheimer's disease (AD) is still the most prevalent cause of dementia worldwide. Notably, AD patients present progressive decline in cognitive function [1]. The accumulation of amyloid plaques in the brain is the main pathological hallmark of AD. Amyloid plaques are primarily composed of extracellular aggregates of amyloid- β (A β) peptide, which cause damage to neurons and the development of dementia [2].

Acetylcholine (ACh) is an important cholinergic neurotransmitter and cholinergic neurons containing ACh play important role in the development and maintenance of memory and cognition [3]. A β could disrupt cholinergic system via the upregulation of calcium influx. Upon calcium influx, a cascade of events occurs such as neuron apoptosis and tau phosphorylation, leading to cognitive deficits [4]. AChE and ChAT are important markers of cholinergic neurons in the cortex and the hippocampus and play a key role in the modulation of cholinergic pathway.

Oxidative stress plays important role in the pathogenesis of AD. According to oxidative stress theory, oxidative stress could enhance the production and aggregation of A β , which then promote neuron degeneration and death, leading to the pathogenesis of AD [5, 6]. On the other hand, several lines of evidence suggest that A β induces oxidative stress. A β possesses the ability to reduce Cu²⁺ and Fe³⁺ toward Cu⁺ and Fe²⁺, which then react with oxygen to generate superoxide anion, which in turn combines with two hydrogen atoms to generate hydrogen peroxide [7]. A β could also extract protons from neighboring lipids to generate lipid peroxide [8].

However, reactive oxygen species are short-lived and it is difficult to measure them directly in the brain; therefore, oxidative stress status in the brain is frequently indicated

by the levels of oxidated proteins, lipids, or DNA. In particular, 4-hydroxynonenal (4-HNE) is an α,β -unsaturated hydroxyalkenal that is produced by lipid peroxidation in the cells and has been widely regarded as an indicator of oxidative stress. 4-HNE plays a crucial role in the pathophysiology of AD [9].

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a potent free radical scavenger that exerts antioxidant effects by inhibiting hydroxyl radical-dependent and -independent lipid peroxidation. Edaravone has shown protective effects on ischemic insults and inflammation in the heart, vessel, and brain in experimental studies [10]. In addition, edaravone has shown neuroprotective effects on amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD) in animal models [11]. Based on these results we propose that edaravone may be potent in the treatment of neurodegenerative disorders associated with oxidative stress. As described above, oxidative stress plays important role in the pathogenesis of AD. Thus, we hypothesized that edaravone may have therapeutic effects on AD. To test our hypothesis, in this study, we aimed to investigate the effects of edaravone on memory deficits, cholinergic system and oxidative stress in the hippocampus of rat model of AD.

Materials and methods

Animals

Healthy adult Wistar rats (300 ± 20 g weight, 5–7 months old) were provided by Experimental Animal Centre of Liaoning Medical University and maintained under a 12/12-h dark/light cycle at room temperature, with free access to water and ad libitum. The rats were randomly divided into three groups with 15 rats in each group: control group, model group, and treatment group. AD rat model was established as described previously [12]. Briefly, A β 1-40 (Sigma, USA) was dissolved in phosphate buffered saline (PBS) to a final concentration of 1 g/l and incubated for at least 1 week to get aggregated A β 1-40. Then the rats were anesthetized by intraperitoneal injection of phenobarbital sodium (3 g/l, 1 ml/100 g). Aggregated A β 1-40 was then microinjected into the right hippocampal dentate gyrus. Rats in control group were injected with 2 μ l PBS, rats in model group were injected with A β 1-40 (2 μ g in 2 μ l), and rats in treatment group were injected with A β 1-40 (2 μ g in 2 μ l) and edaravone (5 mg/kg dissolved in 2 μ l saline). All injections were aimed at brain areas at coordinates of 4.0 mm anterior to posterior (AP), 2.0 mm mid to lateral (ML), and 4.0 mm dorsal to ventral (DV). All animal experiment protocols were approved by Care

and Use of Laboratory Animals Committee of Liaoning Medical University.

Morris water maze behavioral test

Two weeks after the injection of A β 1-40 and administration of edaravone, the rats were examined by Morris water maze behavioral test as described previously [12]. The time of searching for platform, i.e., escape latency, was recorded within 1 min. If the rat did not find the platform, the escape latency was recorded as 1 min. For the spatial probe test, the platform was removed, and each rat was allowed to swim for 1 min. In spatial probe test, the number of times the rat passed the original platform quadrant within 1 min and the percentage of time spent in the training quadrant were measured.

Immunohistochemical analysis

At the end of the behavioral tests, five rats from each group were anesthetized with 30 mg/100 g chloral hydrate and perfused via the left cardiac ventricle with 0.9 % NaCl. The right hippocampus was fixed in ice-cold 40 g/l paraformaldehyde and cut into 8 μ m thick serial sections. The sections were treated with 3 % H₂O₂ to block endogenous peroxidase activity, and then blocked with 2 % goat serum in 0.01 M PBS for 1 h at room temperature. The sections were incubated at 4 °C overnight with rabbit polyclonal antibody against HNE (Alpha, USA), followed by incubation with secondary antibody. Finally, the sections were stained with DAB chromogen. Three sections from each rat and 5 random fields from each section were observed under the microscope. HNE contents were analyzed by comparing the intensity of HNE staining to that of an unstained reference to calculate the relative optical density (ROD) using the ImageJ software. For negative controls, the primary antibodies were replaced with PBS.

Acetylcholinesterase (AChE) and choline acetylase (ChAT) activity assay

At the end of the behavioral tests, five rats from each group were anesthetized with 30 mg/100 g chloral hydrate and were sacrificed by CO₂ euthanasia. The right hippocampus was taken from each rat, and the activities of AChE and ChAT were determined spectrophotometrically using commercial kits (Nanjing Jiancheng Biotechnology Institute, Nanjing, China). Protein concentration of the right hippocampus was determined with the Lowry method using bovine serum albumin (BSA) as the standard. Enzyme activity was expressed as unite per g protein.

Statistical analysis

Data were expressed as mean ± SD and analyzed using SPSS version 12 statistical analysis package (SPSS Inc., Chicago, IL, USA). The differences among the groups were assessed using *t* test. *P* < 0.05 was accepted as statistically significant.

Results

Edaravone improves spatial learning and memory deficits in AD rats

To evaluate the effects of edaravone on spatial learning and memory deficits in AD rats, we performed Morris water maze behavioral test on the rats in three groups. The results showed that the escape latency time became shortened with the prolongation of training in each group (Fig. 1a–f). Compared to model group, the latency time was less in control and treatment groups (Fig. 1g, *P* < 0.05). Furthermore, time spent in the training quadrant was significantly less in model group compared to control group (*P* < 0.05), but it was not significantly

different between treatment group and control group (Fig. 1h). Taken together, these results demonstrate that edaravone could improve spatial learning and memory deficits in AD rats.

Edaravone reduces HNE level in the hippocampus of AD rats

To confirm the anti-oxidative effects of edaravone in the brain of AD rats, we examined HNE, a marker of oxidative stress, in the hippocampus of AD rats. Immunohistochemical analysis showed that HNE staining was weak in the hippocampus CA1 zone of control group (Fig. 2a), but was strong in the hippocampus CA1 zone of model group (Fig. 2b). These observations showed that there was increased oxidative stress in the hippocampus of AD rats (model group). However, HNE staining was weaker in treatment group than in model group (Fig. 2c). Quantitative analysis showed that HNE content was significantly higher in model group than in control and treatment groups (*P* < 0.05), but there was no significant difference between control and treatment groups (Fig. 2d). These data suggest that edaravone reduces oxidative stress in the brain of AD rat.

Fig. 1 Cognitive function of the rats analyzed by Morris water maze test. **a, b, c** Localization trial pathway. **d, e, f** Space explore experiment trace. **a, d** Model group; **b, e** control group; **c, f** edaravone treatment group. **g** Quantitative analysis of latency period. *S* second. Data were shown as mean ± standard deviation from at least three independent experiments. **h** Time spent in the quadrant retention. *S* second. Data were shown as mean ± standard deviation from at least three independent experiments. **P* < 0.05 vs. control and treatment groups

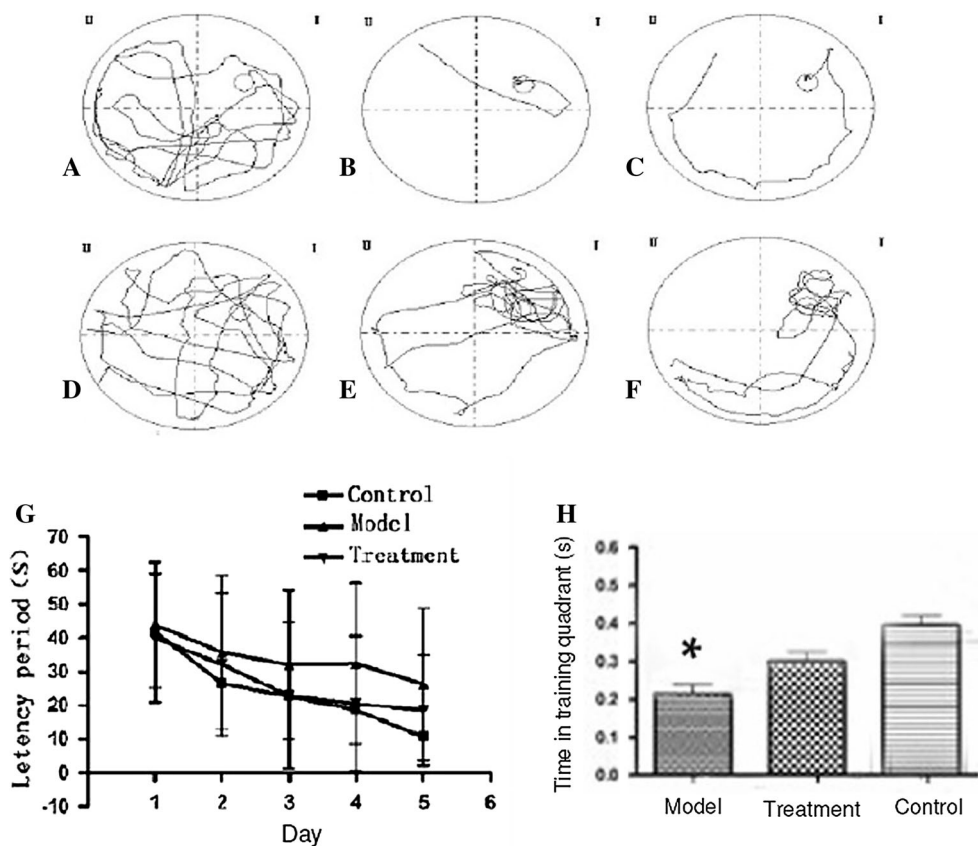
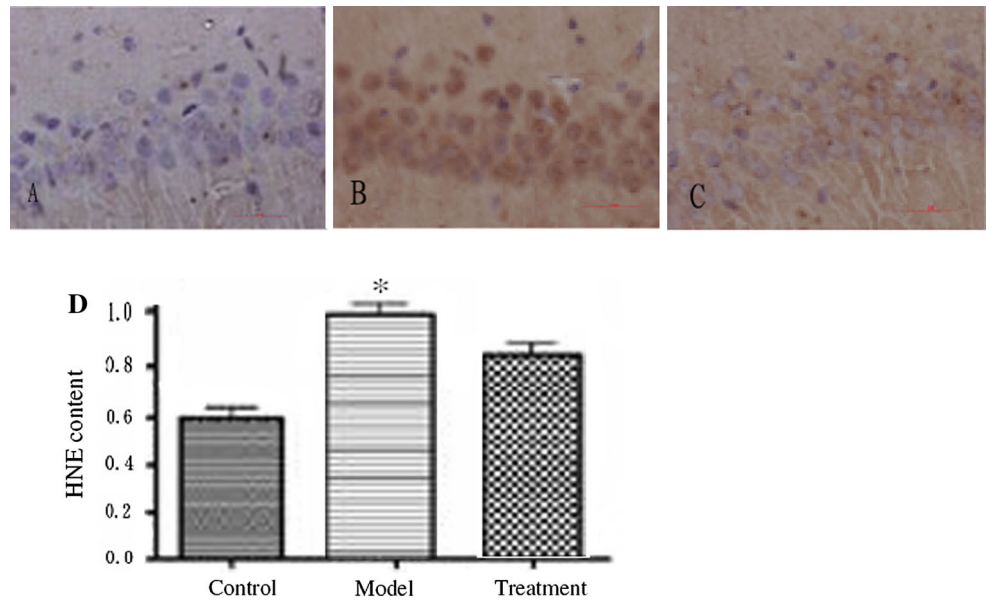


Fig. 2 Hippocampus HNE level in the rats. **a, b, c** Immunohistochemical staining of HNE in the hippocampus CA1 zone. **a** Control group; **b** model group; **c** edaravone treatment group. Bar 20 μ m. **d** Semi-quantitative analysis of HNE contents in three groups. The unit of HNE contents was set as 1 for model group. Data were shown as mean \pm standard deviation from three independent experiments. * $P < 0.05$ vs. control and treatment groups



Edaravone reverses increased AChE and ChAT activities in the hippocampus of AD rats

To understand whether edaravone could influence the activities of AChE and ChAT in the hippocampus, hippocampus samples were separated from different groups and subjected to AChE and ChAT activity assay. The results showed marked increase of AChE and ChAT activities in model group, compared to the controls ($P < 0.05$, Fig. 3). However, increased AChE and ChAT activities were reversed after edaravone treatment ($P < 0.05$ compared to model group, Fig. 3).

Discussion

In this study, we reported the main findings that edaravone attenuated cognitive deficits in rat model of Alzheimer's disease, and the neuroprotective effects of edaravone may be associated with the inhibition of oxidative stress and the improvement on cholinergic dysfunction.

AD is characterized by progressive cognitive decline due to the accumulation of A β in the brain. In this study, first we injected A β into the hippocampal dentate gyrus of the rats and observed significant spatial learning and memory deficits, demonstrating that we established rat AD model. Next, we utilized this rat AD model to evaluate the neuroprotective effects of edaravone. We found that the injection of edaravone into the hippocampus of AD rats partially relieved spatial learning and memory deficits.

Oxidative stress is crucially involved in the pathogenesis of AD [6]. Oxidative damage is considered as a very early event in the progression of AD even before the

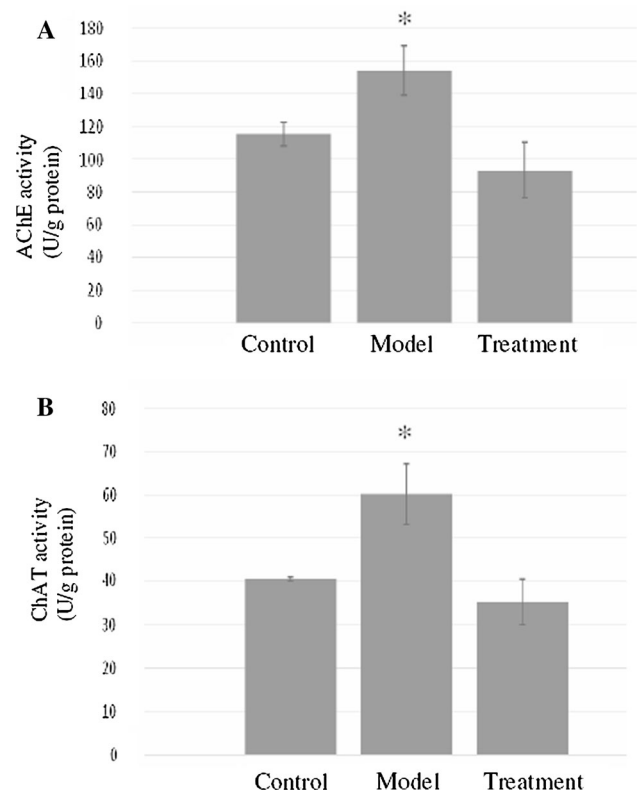


Fig. 3 AChE and ChAT activities in the hippocampus of the rats. **a** AChE activity was expressed as unit/g protein of the hippocampus. **b** ChAT activity was expressed as unit/g protein of the hippocampus. Data were shown as mean \pm standard deviation from three independent experiments. * $P < 0.05$ vs. control and treatment groups

neurons develop pathological aspects [13]. Oxidative stress induces the generation of reactive oxygen species which then interact with phospholipids in the cell

membrane to cause lipid peroxidation. 4-HNE is the main product of lipid peroxidation and its content indicates the extent of oxidative damage [14]. In the present study, we employed immunohistochemical analysis and showed that 4-HNE level was significantly higher in the hippocampus of AD rat than in control and edaravone treated rats. These data indicate that A β did induce oxidative stress in the brain but edaravone could abrogate A β induced oxidative stress.

To better understand how edaravone exerts the neuroprotective effects, we examined AChE and ChAT activities in the hippocampus of AD rats. Our results showed that AChE and ChAT activities were significantly higher in model group than in control group, but were not significantly different between control group and edaravone treatment group. These data indicate that edaravone inhibits AChE and ChAT to modulate cholinergic dysfunction and improve cognitive deficits. Consistent with our results, recent studies suggested that the improvement of cognitive deficits was associated the inhibition of AChE in the mouse and rat models [15, 16]. In addition, a recent study demonstrated that oxidative stress was involved in the cognitive impairment in AD [17]. Notably, numerous studies have shown that ChAT activity is remarkably reduced in cerebral neocortex and hippocampus of AD patients [18]. However, in this study we showed that ChAT activity was not lower but was higher in model group than in control group. Consistent with our results, DeKosky et al. could not detect reduced ChAT activity in cortical regions in patients with mild cognitive impairment (MCI) or mild AD, but instead found that ChAT activity was increased in the frontal cortex and hippocampus of MCI patients [19]. These data suggest that the selective upregulation of ChAT activity in special brain regions may represent an attempt by cholinergic neurons to compensate for functional impairments in transmitter release under stress conditions [20]. However, further studies are needed to explore the activity and role of ChAT and AChE in the pathogenesis of AD.

In summary, our data provide the evidence that edaravone ameliorates spatial learning and memory deficits in a rat model of AD, and the neuroprotective effects of edaravone are associated with the inhibition of oxidative stress and the modulation of cholinergic dysfunction in the brain. Our study suggests that edaravone may be developed as a novel agent for the treatment of AD for improving cholinergic system and protecting neurons from oxidative toxicity.

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Conflict of interest The authors declare that they have no conflict of interest.

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