**Regular Article**

**Edaravone Improves Intermittent Hypoxia-Induced Cognitive Impairment and Hippocampal Damage in Rats**

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Oxidative stress plays an essential role in obstructive sleep apnea-hypopnea syndrome-induced cognitive dysfunction in children. This study investigated the effects of edaravone, a potent free radical scavenger, on intermittent hypoxia (IH)-induced oxidative damage and cognition impairment in a young rat model of IH. IH rats were treated with edaravone for 4 weeks. Behavioral testing was performed using the Morris water maze, and hippocampal tissues were harvested for further analyses. Edaravone attenuated IH-induced cognitive impairment, reduced morphological and structural abnormalities, and increased the number of mitochondria in the IH rats. Furthermore, edaravone significantly increased the inhibition of hydroxyl free radicals; reduced expressions of superoxide anion, malondialdehyde, and 8-hydroxy-2′-deoxyguanosine; and upregulated the expression of manganese superoxide dismutase, catalase, cAMP, protein kinase A, phosphorylated-cAMP response element-binding (p-CREB), B-cell lymphoma 2, and brain-derived neurotrophic factor in the hippocampal tissue of IH rats. Our findings suggest that edaravone attenuated IH-induced cognitive impairment and hippocampal damage by upregulating p-CREB in young rats.

**Key words** edaravone; intermittent hypoxia; oxidative stress; cognitive function; obstructive sleep apnea; young rat

**INTRODUCTION**

Obstructive sleep apnea-hypopnea syndrome (OSAHS) is a common (1–4% incidence) sleep-disordered breathing disease in children that peaks at 2–8 years of age. 1) Studies have shown that long-term, repeated hypoxemia in children with OSAHS can cause neuropsychological deficits, including decline in neurocognitive functions, such as attention, memory, executive ability, and alertness. 2,3) OSAHS involves recurrent episodes of hypoxia-reoxygenation, which produce reactive oxygen species (ROS) that can be detrimental to multiple pathways in the body. The hippocampus plays an essential role in spatial memory, episodic memory, and spatial navigation. Clinical studies have shown that the patients with OSAHS have hippocampal atrophy and decreased hippocampal volume, which are positively correlated with decreased cognitive function. 4) In addition, the cAMP/protein kinase A (PKA)-cAMP response element-binding (CREB) signaling pathway is implicated in the regulation of cognitive function. 5,6) However, a few studies have investigated the role of this pathway in intermittent hypoxia-induced oxidative damage.

Edaravone is a potent free radical scavenger, which easily crosses the blood–brain barrier. 5,9) To elucidate the underlying mechanism of cognitive impairment in children with OSAHS, we established a young rat model of intermittent hypoxia to mimic OSAHS in children and explored the underlying molecular mechanisms for oxidative stress damage-induced cognitive impairment in OSAHS using the antioxidant, edaravone.

**MATERIALS AND METHODS**

**Experimental Animals** Eighty healthy 4-week-old male Wistar rats weighing 90 ± 10 g were provided by the Animal Center of Lanzhou University. The animals were fed the standard rat diet and had free access to water. Rats that could not swim and turn in a complete circle were excluded from participation in the study. After one week of adaptive feeding, they were randomly divided into the following four groups (n = 20 per group): 1) control (NC), 2) intermittent hypoxia (IH), 3) intermittent hypoxia with intraperitoneally administered 5 mg/kg edaravone (10 mg/5 mL, Nanjing Xiansheng Dongyuan Pharma, Nanjing, China) (IH + ED), and 4) intermittent hypoxia with normal saline treatment (IH + NS).

All experimental procedures were approved by the Ethics Committee of the First Hospital of Lanzhou University. All animals used in the experiment were cared for in accordance with the Guide to the Care and Use of Experimental Animals.

**Hypoxic Exposure** Rats were placed in a specially designed hypoxic chamber with a controlled delivery system that regulated the flow of compressed air, oxygen, and nitrogen from 9 a.m. to 5 p.m. every day for 4 weeks. The program length in the IH group was 120 s. During the first 40 s, pure nitrogen filled into the chamber at 10 L/min for 30 s, and the oxygen concentration gradually decreased from 21 to 6–7% (low-oxygen period). There was no gas input during the last 10 s, and the oxygen concentration was kept at 6–7% to establish a hypoxic condition. This was followed by an 80 s reoxygenation period. During this period, pure oxygen was filled at 10 L/min for 20 s. After the oxygen concentration gradually increased and was maintained at 21 ± 0.5%, compressed air
was continuously filled at 5 L/min for 60 s. Once a cycle was completed, rats in the IH group underwent another cycle of intermittent hypoxia. The program in the NC group was similar to that in the IH group, but all of the gas sources were from compressed air, and the oxygen concentration was maintained at about 21%. The oxygen concentration was monitored using a continuous oxygen analyzer (CY-12C, Zhuhai, China). After hypoxic exposure, all experimental animals were placed in the breeding boxes with free access to water and food.100

**Morris Water Maze** The Morris water maze consisted of a circular pool (180 cm diameter and 50 cm height) that was divided into four quadrants. A cylindrical platform (12 cm diameter and 30 cm height) was placed in the center of the second quadrant. External reference objects and the platform remained unchanged. The water surface was 2–3 cm higher than the platform surface, and the water temperature was kept at 25 ± 1°C. The test program included two sessions: 1) the positioning navigation (learning and memory), and 2) the space exploration (memory retention ability) sessions. For positioning navigation, the movement trajectory and time required for the rat to search and climb the platform was recorded; the time required to climb the platform was defined as escape latency. Rats trained 4 times a day with 15-min intervals of training for five days and one day of the test. The training in the first, second, third, and fourth quadrants was conducted in sequence. The rat was placed in the water facing the pool wall, and the movement trajectory of searching and climbing the platform within 120 s and the time required for the animal to climb the platform were recorded. The rat was allowed to stay on the platform for 30 s to enhance the memory effect. If the rat did not find the platform within 120 s, it was directed not to the platform and allowed to stay on the platform for 30 s. On the 6th day, the test was conducted, and the average of the 4 latencies was used as the latency time. The day after the positioning navigation experiment, the platform was removed, and the space exploration experiment was conducted. The rat was placed in the water facing the pool wall, and the movement trajectory for searching the platform within 120 s was recorded. The number of times of crossing the position corresponding to the platform within 120 s was assessed three times, and the average value was used for analysis.

**Preparation of Paraffin Sections of the Brain Tissues and Collection of the Hippocampal Tissues** After the water maze test, the brains were removed for hippocampal paraffin section analyses (n = 3, first subgroup), transmission electron microscope analyses (n = 3, second subgroup), hippocampal oxidative stress analyses (n = 7, third subgroup), and Western blotting analyses (n = 7, fourth subgroup). Rats were anesthetized with an intraperitoneal injection of 1% sodium pentobarbital (40 mg/kg) and perfused with 4% paraformaldehyde for 30 min. The brain was removed, 4 µm coronal sectioning was performed, and the tissue was stained for hematoxylin and eosin (H&E). In the remaining 3 groups, the brain skull was opened using a rongeur, and the bilateral hippocampi were removed and placed on ice. In the second subgroup, the hippocampal CA1 areas were separated under a stereomicroscope, cut into 1.0 × 1.0 × 1.0 mm tissue blocks, and placed in pre-cooled 2.5% glutaraldehyde at 4°C for 2 h. The tissues were washed 3 times with phosphate buffer, fixed in a pre-cooled 1% osmic acid solution at 4°C for 1 h, and analyzed with transmission electron microscopy. The hippocampal tissues in the third and fourth subgroups were flash-frozen in liquid nitrogen and stored at −80°C until further use.

**Determination of Oxidative Stress in the Hippocampus** After the sample was incubated with hydroxylamine, hydrochloride to form NO2, p-aminobenzenesulfonic acid and α-naphthylamine were added to convert NO2 to red azo compounds (absorption at 530 nm). The O2 levels were calculated using the absorbance values at 530 nm (A530). To determine hydroxyl radical (·OH) inhibition capacity, samples were subjected to the Fenton reaction, and the absorbance was measured at 550 nm (A550) with a UV-Vis spectrophotometer. The results were expressed in U/mg protein. The levels of malondialdehyde (MDA) were determined using the thiobarbituric acid method (i.e., absorbance at 532 nm [A532] measured with a UV-1700 spectrophotometer) and calculated according to the kit manual. The results were expressed in nmol/mg protein. The levels of catalase (CAT) were determined based on the decomposition reaction of H2O2. The remaining H2O2 reacted with ammonium molybdate to produce a pale-yellow complex. The absorbance of the complex was measured at 405 nm and used to calculate the CAT levels, and the results were expressed in U/mL. The levels of copper/zinc superoxide dismutase (Cu/Zn-SOD), manganese (Mn)-SOD, and 8-hydroxy-2’-deoxyguanosine (8-OHdG) were measured by enzyme-linked immunosorbent assays (ELISAs). The absorbance at 450 nm was measured using a microplate reader to plot a standard curve, and the levels of Cu/Zn-SOD, Mn-SOD, and 8-OHdG were calculated. The results were expressed in pg/mL, pg/mL, and ng/mL, respectively. The protein concentration of the hippocampal homogenates was determined using the bicinchoninic acid (BCA) method.

**Determination of cAMP Levels in the Hippocampus** The cAMP levels in the hippocampus were determined by ELISA (Rat cyclic Adenosine Monophosphate ELISA, Shanghai BlueGene Biotech Co., Ltd., China) using a microplate reader, and the cAMP levels were calculated according to the standard curve. The results were expressed in pmol/mL.

**Western Blotting** Hippocampal tissue (100 µg) was ground, and the protein supernatant was obtained according to the manufacturer’s protocol (Dalian Meilun Biotechnology Co., Ltd., Dalian City, China, MA0151). The protein concentration of extracted hippocampal tissue was determined using the BCA protein quantification kit (Dalian Meilun Biotechnology Co., Ltd., MA0082). Around 20 µg of the protein sample was subjected to polyacrylamide gel electrophoresis (12% separating gel and 5% concentrating gel [Dalian Meilun Biotechnology Co., Ltd., MA0159]) and transferred to a polyvinylidene fluoride or polyvinylidene difluoride (PVDF) membrane (MilliporeSigma, Burlington, MA, U.S.A.). Western blotting was performed using the following antibodies: anti-β-Actin (1:1000; MBL Beijing Biotech Co., Ltd., Beijing, China, PM053), anti-KAc (1:1000; Abcam, Cambridge, U.K., ab211265), anti-CREB (1:500; Abcam, ab32515), anti-phospho-CREB (1:5000; Abcam, ab32096), anti-brain-derived neurotrophic factor (BDNF) (1:1000; Abcam, ab108319), anti-B-cell lymphoma 2 (Bel-2) (1:500; Abcam, ab59348), and goat anti-rabbit immunoglobulin G (IgG) secondary antibody (1:5000; ImmunoWay Biotechnology Company, Tennyson, TX, USA, RS0002). The gray value ratio of a target band to internal β-actin was defined as the relative expression level of the target protein. It was calculated using Image J software.
H&E Staining  Paraffin sections were de-waxed in water, soaked in hematoxylin for 4 min, differentiated in 1% hydrochloric acid ethanol for 2–3 s, incubated in methylene blue solution for 10 s, and soaked in eosin solution for 30 s. Further, the sections were gradient dehydrated, made transparent, sealed, and imaged under a microscope.

Statistical Analysis  Data are expressed as mean ± standard deviation. One-way ANOVA and the Newman–Keuls test were used for between-group comparisons. Differences with a p-value < 0.05 were considered as statistically significant. Statistical analyses were performed using SPSS 20.0 software (IBM Corporation, Armonk, NY, U.S.A.).

RESULTS

Morris Water Maze  Escape latencies significantly increased in the IH and IH + NS groups as compared with those in the NC group (p < 0.05), but they did not differ between the NC and the IH + ED groups (p > 0.05). Significant differences in escape latencies were found between the IH + ED and the IH and IH + NS groups (p < 0.05). The traveling time in the target quadrant and the number of times of crossing the platform were decreased in the IH and IH + NS groups as compared to those in the NC group (p < 0.05); however, there were no differences in traveling time or the number of times of crossing the platform between the NC and the IH + ED groups (p > 0.05). The traveling time in the target quadrant and the number of times of crossing the platform were significantly increased in the IH + ED group as compared with those in the IH and IH + NS groups (p < 0.05). These results suggest that intermittent hypoxia may reduce learning and memory abilities in young rats and these effects may be attenuated by edaravone treatment (Table 1). During training of the positioning navigation, the changes of escape latencies for several consecutive days among the 4 groups (Fig. 1).

H&E Staining of Hippocampal CA1 and CA3 Areas In the NC group, the CA1 and CA3 areas had normal neuronal structure with densely and neatly arranged neurons. No degenerative and necrotic neurons were observed. The IH and IH + NS groups exhibited a loose intercellular matrix in the CA1 area with a widened pericellular space. Degenerative and necrotic neurons were also found. The karyon was shrunken and hyperchromatic, the nucleus was hypochromatic and partially irregular, and the nucleolus was unclear. Notably, the morphological and structural abnormalities in the CA1 area were attenuated in the IH + ED group. In addition, neurons in the CA3 area in the IH and IH + NS groups were neatly arranged. Occasionally, the karyon was shrunken and hyperchromatic, and the nucleus was hypochromatic. Irregular nuclei and unclear nucleoli were also occasionally detected. The appearance of the hippocampus in the IH + ED group was similar to the NC group. These findings suggest that intermittent hypoxia damages hippocampal neurons and that the CA1 area is more sensitive to intermittent hypoxic damage than the CA3 area.

Further, our results indicate that edaravone could alleviate this damage (Figs. 2, S1).

Oxidative Stress in the Hippocampus  The capacity to inhibit hydroxyl radicals and the expression levels of Mn-SOD and CAT were significantly decreased in the IH and IH + NS groups compared with the capacity to inhibit hydroxyl radicals and the expression levels of Mn-SOD and CAT in the NC group (p < 0.05) and the IH + ED group (p < 0.05); however, the capacity to inhibit hydroxyl radicals and the expression levels of Mn-SOD and CAT did not differ between the NC and the IH + ED groups (p > 0.05). The expression levels of Cu/Zn-SOD slightly decreased in the IH, IH + ED, and IH + NS groups as compared to those in the NC group, which indicates that intermittent hypoxia could lead to impairment in learning ability of young rats. The performance of the IH + ED group rats stabilized on the fourth day and the final escape latency was shorter than that in the IH group and IH + NS group, which suggests that edaravone could alleviate the learning ability impairment caused by intermittent hypoxia.

Table 1. Comparison of Learning and Memory Abilities of Rats in Different Groups

| Groups   | n  | Escape latency (s) | Traveling time in the target quadrant (s) | Number of times of crossing the platform |
|----------|----|--------------------|------------------------------------------|------------------------------------------|
| NC       | 20 | 20.87 ± 4.71       | 43.10 ± 5.11                             | 5.85 ± 1.35                              |
| IH       | 20 | 27.70 ± 5.43*#     | 30.11 ± 7.37*#                           | 3.30 ± 1.12**                           |
| IH + ED  | 20 | 23.15 ± 5.75       | 39.79 ± 7.96                             | 5.15 ± 1.49                              |
| IH + NS  | 20 | 27.17 ± 5.38*#     | 31.17 ± 6.28**                           | 3.50 ± 1.27**                           |

*Compared to NC group; p < 0.05; #compared to IH + ED group; p < 0.05.

NC, the control group; IH, the intermittent hypoxia group; IH + ED, the intermittent hypoxia group with edaravone treatment; IH + NS, the intermittent hypoxia group with normal saline treatment.

Altered cAMP Levels in the Hippocampus  cAMP levels were significantly decreased in the IH and IH + NS groups as compared with those in the NC group (p < 0.05) and the
IH + ED group \((p < 0.05)\), but there were no significant differences in cAMP levels between the NC and the IH + ED groups. These findings suggest that intermittent hypoxia reduces cAMP levels in the hippocampus, and edaravone treatment may attenuate this reduction (Fig. 3H).

**Effects of Intermittent Hypoxia on the Expression Levels of the CREB/PKA Signaling Pathway-Related Proteins in the Hippocampus** The expression levels of PKAc, p-CREB, Bcl-2, and BDNF in the hippocampus were significantly downregulated in the IH and IH + NS groups as compared with those in the NC and IH + ED groups \((p < 0.05)\), but the expression levels of PKAc, p-CREB, Bcl-2, and BDNF did not differ between the NC and the IH + ED groups. The expression levels of CREB did not differ among the four groups \((p > 0.05)\); however, p-CREB levels were decreased in the IH and IH + NS groups as compared with those in the NC group and the IH + ED group. There were no differences in p-CREB levels between the NC and the IH + ED group. These findings suggest that intermittent hypoxia downregulates the expression of PKAc, p-CREB, Bcl-2, and BDNF in the CREB/PKA signaling pathway, and edaravone treatment may attenuate these effects (Fig. 4).

**DISCUSSION**

Accumulating evidence indicates that OSAHS causes cognitive dysfunction in children,\(^{11-14}\) and surgery only partially improves cognitive/memory functions in children with OSAHS.\(^{15,16}\) Our animal model mimics OSAHS pathogenesis in children. During the positioning navigation session in the Morris water maze, the escape latency was increased in the IH group as compared to that in the NC group. During the space exploration experiment, the traveling time in the target quadrant and number of times of crossing the platforms were significantly reduced in the IH group as compared with those in the NC group. Notably, improved cognition function was found in the edaravone-treated group, and this suggests that edaravone may attenuate intermittent hypoxia-induced learn-
OSAHS is an oxidative stress disease, which indicates the role of intermittent hypoxia-induced oxidative stress in the pathogenesis of OSAHS. We found that intermittent hypoxia significantly increased the ROS levels in the hippocampus, significantly decreased Mn-SOD and CTA expression, and did not affect Cu/Zn-SOD expression levels. Mn-SOD is expressed in the mitochondria; therefore, oxidative stress may predominantly occur in the mitochondria. Moreover, we found that the levels of lipid peroxidation products, including MDA and DNA damage products, such as 8-OHdG, were significantly increased in the IH group as compared with those in the NC group.

After edaravone treatment, impaired cognition in young rats was significantly attenuated and ROS, MDA, and 8-OHdG levels in the hippocampus were significantly decreased. These findings suggest that edaravone alleviates intermittent hypoxia-induced neuronal damage by scavenging ROS. H&E staining revealed different degrees of neuronal damage in the hippocampal CA1 and CA3 areas in the IH group. Specifically, the CA1 area was more severely damaged than the CA3 area. Studies have also found that the expression levels of thioredoxin were lower and calcium overload, ROS levels, and the degree of activation of mitochondrial permeability transition pore were higher in the CA1 area than in the CA3 area. These findings indicate that the CA1 area may be more vulnerable to oxidative damage than the CA3 area.

The hippocampal cAMP/PKA/CREB signaling pathway plays an important role in neuronal damage, synaptic plasticity, long-term memory, and cognitive dysfunction in Alzheimer’s disease and cerebral infarction. We found that cAMP was significantly downregulated in the hippocampus region in the IH group, which might have possibly been caused by the excessive production of ROS that resulted in the reduction of ATP. At the same time, it is also possible that the decline in hormone levels caused by hypoxia activated the G protein-coupled receptor pathway and restrained the activity of adenylate cyclase. Both the abovementioned mechanisms may account for the reduction of cAMP produced by the catalysis of ATP with adenylyl cyclase. Accompanying the decrease in cAMP concentration, the concentration and expression of PKAc and p-CREB were also significantly downregulated in the IH and IH + NS groups compared with those in the NC group and the IH + ED group. However, no significant differences in the expression of CREB were found among the groups. We speculate that the concentration of cAMP directly determines the level of PKA activation and the expression of p-CREB. However, the levels of cAMP, PKAc, and p-CREB were significantly upregulated in the IH + ED group. This suggests that edaravone may upregulate cAMP/PKA/CREB signaling pathway by clearing ROS in hippocampus, thereby, modulating the downregulation of BDNF and Bcl-2 in the downstream pathway. The downregulation of BDNF and Bcl-2 may eventually impair neuronal repair and increase apoptosis and necrosis. Edaravone may upregulate p-CREB by scavenging ROS and reducing neuronal damage. Bouchez and Devin found that the activation of the cAMP/PKA signaling pathway also contributes to the reduction of ROS production. This finding suggests that edaravone may scavenge free radicals not only through single-electron transfer but also through the upregulation of the signaling pathway. We had performed terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining during this experiment (data not shown). Very few TUNEL positive cells were found in the four groups, and it is not statistically significant.

This study indicates that intermittent hypoxia impairs cognitive function in young rats via enhanced neuronal damage in the hippocampal CA1 area. Our results also suggest that oxidative stress plays an essential role in hippocampal neuronal damage through the downregulation of CREB phosphorylation in the cAMP/PKA-CREB signaling pathway. Further, edaravone may reduce intermittent hypoxia-induced cognitive impairments and hippocampal damage by upregulating CREB phosphorylation. One limitation of this study is that the impact of sleep on the present findings was not investigated. Further studies are needed to explore the role of sleep in the effects of intermittent hypoxia on cognitive function and neuronal damage in young rats.
ther studies are needed to examine the effects of other possible factors, including sleep, on intermittent hypoxia-associated cognitive impairment.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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