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Chronic intermittent hypoxia exposure induces memory impairment in growing rats

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The study was aimed to examine the effect of chronic intermittent hypoxia (CIH) on spatial memory of growing rats and to explore the possible underlying mechanisms. Sixty two rats were trained to perform the 8-Arm radial maze task and were divided into four groups: 2-week-CIH (2IH), 2-week-control (2C), 4-week-CIH (4IH) and 4-week-control (4C). There were more reference memory errors, working memory errors and total memory errors in 2IH and 4IH groups compared to the controls. The levels of 8-iso-Prostaglandin $F_{2\alpha}$ (8-ISO-$PGF_{2\alpha}$), an in vivo marker for oxidative stress, in serum, hippocampus and prefrontal cortex were higher in CIH groups than the control groups. There were significant correlations between the levels of 8-ISO-$PGF_{2\alpha}$ and numbers of memory errors. The ultrastructural changes were evident in the hippocampal and prefrontal cortical tissues from the CIH groups. These results indicate that CIH can induce oxidative stress in brain tissues involved in spatial memory function.

Key words: intermittent hypoxia, isoprostane, memory impairment, ultrastructure

INTRODUCTION

Obstructive sleep apnea hypopnea syndrome (OSAHS), a condition characterized by repeated episodes of upper airway obstruction during sleep, affects about 2% of children (Brunetti et al. 2001, Schlaud et al. 2004, Anuntaseree et al. 2005). OSAHS can occur in children from newborn to adolescent, mostly in preschool children. While controversy still exists in terms of the association between OSAHS and neurocognitive dysfunction, increasing evidence from the last few decades suggests that cognitive performance and behavior may be affected in children with OSAHS (Halbower et al. 2006). It has been demonstrated that children with OSAHS have increased attention deficit (Guilleminault et al. 1982, Ali et al. 1996, Blunden et al. 2000, Owens et al. 2000), impaired learning and school performance (Gozal. 1998), and increased problematic behavior (Stradling et al. 1990, Ali et al. 1993, Owens et al. 2000), while at the more severe end of the spectrum, impaired memory and reduced intelligence (Rhodes et al. 1995). On the other hand, Beebe and coworkers (2004) reported that they compared the neuropsychological functioning of school-aged children with OSA to that of healthy children, minimal effects were observed on measures of intelligence, verbal memory, or processing speed. Exploratory analyses failed to indicate any clear relationship between neuropsychological functioning and objective indexes of hypoxia or sleep disruption (Beebe et al. 2004). Also, Calhoun and coauthors (2009) recently reported that no significant relationship was found between children with a mild apnea-hypopnea index and any measures of neuropsychological functioning (intelligence, verbal and nonverbal reasoning ability, attention, executive functioning, memory, processing speed, and visual-motor skill) (Calhoun et al. 2009). Therefore the studies describing OSAHS-related cognitive and behavioral impairment are controversial and the underlying mechanisms remain largely undefined.

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Previous animal studies have demonstrated that exposure to intermittent hypoxia (IH) during sleep cycle is associated with significant spatial learning memory deficits as well as with increased neuronal apoptosis within susceptible brain regions (Row et al. 2002, Kheirandish et al. 2005). The oscillation of O_2 concentrations during chronic intermittent hypoxia (CIH) mimics the processes of hypopnea/re-oxygenation. It has been demonstrated that repetitive episodes of hypoxia and re-oxygenation lead to an increased production of reactive oxygen species (ROS) (Prabhakar 2001) and increased levels of ROS have been detected in OSAHS patients (Schulz et al. 2000, Dyugovskaya et al. 2002). F2-isoprostanes (F2-IsoP), a series of prostaglandin F2α (PGF2α) epimers, are produced from arachidonic acid-containing lipids mainly through non-enzymatic free radical catalysed oxidation and have been proposed as in vivo markers for oxidative stress (Morrow et al. 1990).

Normally, a delicate balance exists between systems that generate ROS like superoxide ($O_2^{\cdot-}$), or hydrogen peroxide (H_2O_2) and endogenous antioxidant defenses. The increased production of ROS following CIH may tip this balance. The aim of this study was to test the hypothesis that oxidation stress induced-damages to the hippocampus and prefrontal cortex play important roles in the pathogenesis of CIH associated neurocognitive dysfunction in growing rats.

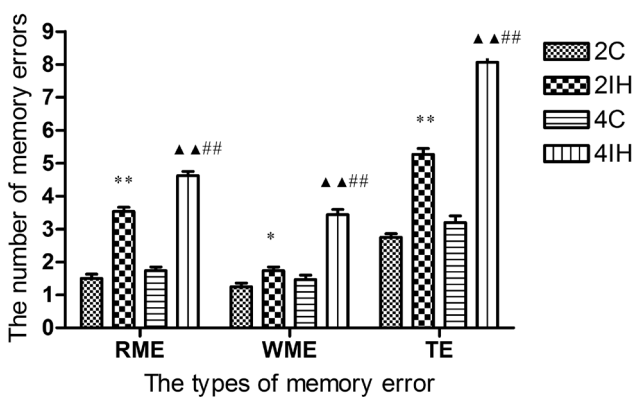

Fig. 1. The effect of chronic intermittent hypoxia on the spatial memory of rats as determined by 8-Arm radial maze test. There were significantly more RME, WME and TE in 2IH (n=15) and 4IH (n=16) groups as compared to the 2C (n=16) and 4C (n=15) control groups. The 4IH group had more errors than that of 2IH group. There were no significant differences in RME, WME and TE between 2C group and 4C group. *p<0.05 vs 2C group; **p<0.01 vs 2C group; ▲▲p<0.01 vs 4C group; ## p<0.01 vs 2IH group.

METHODS

Animals

This study was approved by the Ethics Committee of Wenzhou Medical College. Four weeks old male Sprague-Dawley rats, weighing 70-85 grams, were housed in the animal care facility with 12h light/dark cycle, and with free access to chow and water. A total of 73 rats were trained to perform the 8-Arm (4-arm baited) radial maze task. Among them, 62 rats were successfully trained and therefore were included in the study. After training, the rats were randomly divided into four groups by the method of random number table: 2-week-CIH group (2IH, n=15), 2-week-control group (2C, n=16), 4-week-CIH group (4IH, n=16) and 4-week-control group (4C, n=15).

8-Arm (4-arm baited) radial maze test

Similar to the method described in detail by others previously (Zou et al. 1998), a radial eight-arm maze with four arms-baited was used to test the spatial memory. In the 8-armed radial maze, animals can be trained to memorize the comparative distance between the bait and themselves by observing the environment, and it can be discerned and analyzed for working memory and reference memory. Thus, within a given session the animal must remember which arm is never baited (reference memory) and those arms which are rewarded but have been previously entered during that session (working memory) (Zou et al. 1998).

The maze was constructed with plastic and the floor was painted black. The central area was 30 cm in diameter and 8 arms (50×12×10 cm) extended radially. Food cups were located near the distal end of each arm. It was 2 cm in diameter and 1 cm in depth. The maze was elevated 40 cm from the floor and was positioned in a testing room with many visual signs. Prior to the experiments, animals were restricted from chow for 7 days until their body weights were reduced to 85% of the baseline weight. This weight level was maintained throughout the experiment. Next, an acclimation trial was conducted: day 1: food pellets were scattered throughout the arms and platform, and rats were allowed to eat food in the maze freely; day 2: food pellets were placed in the arms and the food cups only. During the trial periods, 45 mg food pellets were located only in the food cups of arms 3, 5, 6 and 8

while the remaining 4 arms were empty. After adaptation, all rats were trained with two trials per day. The rat was placed in an opaque box (20×20×20 cm) in the central platform. After 15 sec, the box was lifted and the rat was allowed to freely walk through the maze.

The performance of the rat in each session was assessed by the number of error choices. A correct choice occurred if the rat entered the baited arm and ate the food pellets. Entry into the never-baited arm was regarded as a reference memory error (RME), while re-entry into the arms where the pellet had already been eaten was regarded as a working memory error (WME). RME plus WME was called as the total error (TE). The rats that fulfilled the criterion (less than one error in a training trial and less than two errors in total over 3 consecutive training trials) were used in the experiment. The test took place between 08:00 AM and 05:00 PM daily. After 10 days of training, 11 rats could not perform the 8-Arm radial task and were excluded for the following CIH experiments, therefore a total of 62 rats were included in the experiments. After CIH for 2 weeks or 4 weeks as detailed below, the 8-Arm radial maze test was performed once again to test the performance of spatial memory in the rats.

Intermittent hypoxia exposure

The CIH model was performed according to McGuire and others with modifications (Gozal et al. 2001, McGuire et al. 2002, Polotsky et al. 2006). Briefly, a steel cabin for generating intermittent hypoxia and air control was created. The cabin is 125×48×24 cm in size. There are 4 air input valves and 4 air output valves in the lateral part. The pressure remained atmospheric inside the cabin. A single-chip microcomputer was used to control a series of silent solenoid valves, triggered by an electronic switch, to maintain desired gas flow into the chamber. O_2 concentration in the chamber could be reduced to a nadir of 9.0%±1.5% in 30 sec by infusion of 99.99% nitrogen with the pressure kept at 0.3KPa, stabilized at that level for 30 sec, and then gradually increased to 21.0± 0.5% over the next 12 sec by infusion of 99.50% oxygen (25L/min) into the chamber by the computer controlled timed solenoid valves. This cycle was repeated every 90 sec over 7.5 hours during the animals' diurnal sleep period for certain days according to the experimental design. Ambient temperature was kept at 22–24°C. The 2IH group rats were exposed to CIH for 2 weeks and the 4IH group rats were exposed to CIH for 4 weeks. Two control groups were placed in cabin filled with compressed air instead of hypoxia gas for 2 weeks as 2C group or for 4 weeks as the 4C group respectively. The O_2 concentration was kept at 21.0±0.5% in the control cabin.

Collection of the specimens

After 8-Arm radial maze test was completed, two rats from each group were randomly selected for electron microscopy specimen preparation (see below). The remaining 54 rats were anesthetized with 35mg×kg^{-1} sodium pentobarbital (Sigma, USA) by intraperitoneal injection. Up to 4 ml of arterial blood was obtained from the abdominal aorta. Blood was centrifuged with 3 500 rpm for 10 minutes at 4ºC and the serum was collected and stored at -80ºC for ELISA analysis. The hippocampus and prefrontal cortex tissues were collected rapidly and stored at -80ºC for ELISA analysis.

8-ISO-PGF2α measurement

We obtained serum and cerebral tissue samples of 8 animals per group selected by the method of random

Table I

The levels of 8-ISO-$PGF_{2\alpha}$ in serum, hippocampus and prefrontal cortex (mean ± SD, n=8 for each group)

group	serum (×10^{-6}g/l)	hippocampus (×10^{-6}g/g)	prefrontal cortex (×10^{-6}g/g)
2IH	17.17±3.08$^{\#\#}$	6.21±1.34$^{\#\#}$	6.24±1.09$^{\#\#}$
2C	1.88±0.69	4.26±0.79	3.95±0.53
4IH	27.10±7.03**$^{\blacktriangle}$	6.11±1.46**	5.94±0.99**
4C	1.71±0.78	3.17±0.72	3.07±0.62
F	82.30	13.76	26.62
P	<0.01	<0.01	<0.01

$^{\#\#}p$<0.01 vs 2C group; **p<0.01 vs 4C group; $^{\blacktriangle}p$<0.05 vs 2IH group;

number table. The expression of 8-ISO-$PGF_{2\alpha}$ was detected by ELISA assay using a commercially available kit (8-Isoprostane EIA Kit, Assay designs, USA). Serum samples were processed as described as follows. One part 10N NaOH was added to 4 parts serum sample and then heated at 45ºC for 2 hours. After cooling, 100 μL of concentrated (12.1N) HCl was added into 500 μL of hydrolyzed sample and then centrifuged for 5 minutes at 14 000 rpm in a microcentrifuge. The supernatant was collected for the assay. For tissue samples, hippocampus or prefrontal cortex tissue was homogenized in the 2N NaOH solution (1 mg/ml). Samples in 2N NaOH were covered and heated at 45ºC for 2 hours to ensure hydrolysis. After hydrolysis, the samples were cooled and treated with an equal volume of 2N HCl. The neutralized samples were centrifuged at 3 000 rpm in a microcentrifuge. The pH of the neutralized samples was adjusted to keep at the range of 6-8. The clear supernatant was used for the assay.

The levels of 8-ISO-PGF2α were determined by an enzyme immunoassay (EIA) in one 96-well ELISA plate and the optical density was read at 405 nm as per manufacturer's manual. The data were analyzed by an immunoassay software package utilizing a 4 parameter logistic curve fitting program.

Ultrastructure observation of hippocampus and prefrontal cortex with electron microscopy

After 8-Arm radial maze test, two rats were randomly selected from each group for electron microscopy. The rats were anesthetized with 35mg×kg^{-1} sodium pentobarbital by intraperitioneal injection and were perfused transcardially with 100 ml ice cold normal saline followed by 200 ml ice cold 4% glutaraldehyde (Sigma, USA). The hippocampus and prefrontal cortex were removed rapidly. The electron microscopy specimens were processed in conventional way, fixed in 2.5% glutaraldehyde and post-fixed in 1% osmium tetroxide (Sigma, USA) at room temperature. The specimens were embedded by Epon812. Ultra-thin sections were cut using a LKB2088 vibratome and placed on the single-hole grids. The ultra-thin sections (80 nm) were then stained with 2% lead citrate (Merck, Germany) and 1% uranyl acetate (Merck), and examined using a transmission electron microscope (Hitachi H-7500 electron, Japan).

Table II

The correlation coefficients of spearman correlation analysis between the levels of 8-ISO-PGF2α and number of memory errors (r_s)

	8-ISO-PGF2α		
Memory error	serum	hippocampus	prefrontal cortex
RME	0.796**	0.605**	0.731**
WME	0.663**	0.532**	0.572**
TE	0.798**	0.627**	0.723**

** $p<0.01$

Statistical analyses

All data were analyzed by the SPSS (Version 12.0) software. The data were expressed as the means ± SEM. All normally distributed data were analyzed by ANOVA followed by the LSD or Tamhane's T2 test for detecting the significance of differences among the groups. The Spearman correlation analysis was used to test the relationships between the levels of 8-ISO-PGF2α and the numbers of memory errors in the radial maze test. A $p<0.05$ was considered to be statistical significant.

RESULTS

The effect of intermittent hypoxia on the spatial memory of rats as determined by 8-Arm radial maze test

CIH exposure caused substantial spatial memory impairment as demonstrated by the results obtained from the 8-Arm radial maze test (Fig.1). There were significantly more RME , WME and TE in the rats that had been exposed to IH for 2 or 4 weeks as compared to the corresponding control group ($p<0.01$). The detrimental effect of CIH on the spatial memory is dependent upon the duration of intermittent hypoxia since the 4IH group had more errors than that of the 2IH group in the 8-Arm radial maze test. There were not significantly different memory errors between 2C group and 4C group ($p>0.05$).

The serum and brain tissue 8-ISO-$PGF_{2\alpha}$ levels were increased after CIH

To determine if oxidative stress was induced in rats that had exposed to CIH, we measured the levels of 8-ISO-$PGF_{2\alpha}$ in the serum and specific cerebral tissues, including hippocampus and prefrontal cortex. As shown in the Table I, the levels of 8-ISO-$PGF_{2\alpha}$ in serum, hippocampus and prefrontal cortex were significantly higher in CIH groups than the corresponding control groups ($p<0.01$, respectively). The levels of 8-ISO-$PGF_{2\alpha}$ were not significantly different between the two control groups ($p>0.05$). Theses results indicate that an intermittent hypoxia can induce oxidative stress in brain tissues which are commonly thought to be involved in brain functions related to spatial memory. As shown in the Table II, the Spearman correlation analysis showed that there were significant relationships between the levels of 8-ISO-$PGF_{2\alpha}$ in the serum, hippocampus and prefrontal cortex and the numbers of memory errors in the radial maze test ($p<0.01$, respectively).

CIH induced ultrastructural changes in hippocampus and prefrontal cortex

To further determine if CIH can induce neuronal injury in the specific brain regions which are involved in brain functions related to spatial memory, we evaluated the ultrastructural changes in hippocampus and prefrontal cortex with electron microscope. As shown in Fig. 2, the signs of neuronal damage including the destruction of the nuclear membrane and the swollen mitochondria vacuoles in the hippocampus were evident in the CIH exposed groups, especially in the 4IH group. The neuron and mitochondria were normal in two control groups as

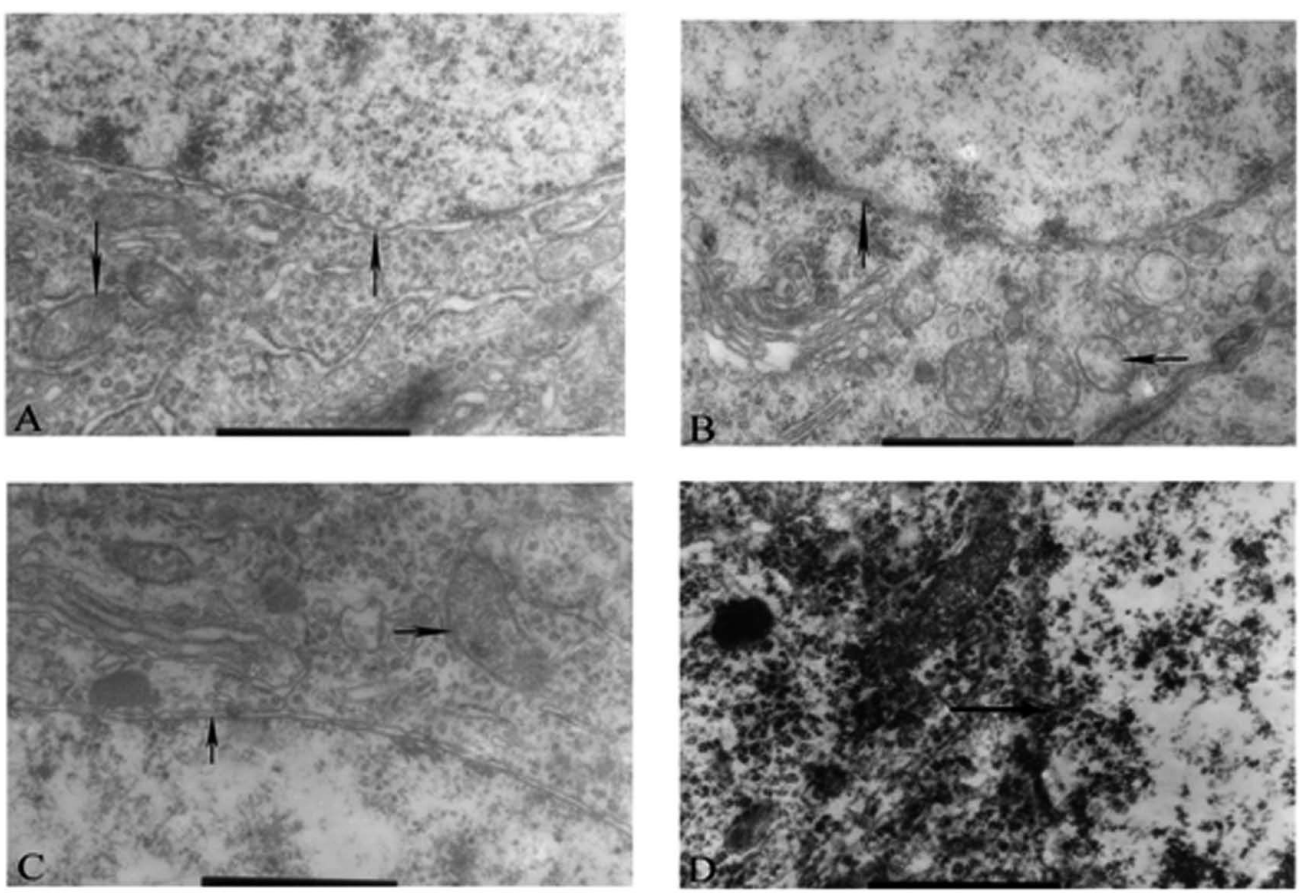

Fig. 2. The ultrastructural changes of the hippocampal tissues from the rats exposed to chronic intermittent hypoxia and the control rats. Normal nuclear membrane and mitochondria are seen in 2C (panel A) and 4C (panel C) groups. Mild fuzzy nuclear membrane and swollen mitochondria vacuoles are evident in 2IH group (panel B, up arrow and left arrow). Severe fuzzy nuclear membrane, cell swelling and lysis are seen in 4IH group (panel D, arrow). Magnification × 25, 000. Bar represents 384nm.

showed in the Fig. 2. Mild fuzzy nuclear membrane was seen in the 2IH group, and severe nuclear membrane damage, cell swelling and lysis were seen in the 4IH group. In prefrontal cortex, normal neuron and mitochondria was found in control groups and mild fuzzy nuclear membrane was observed in IH groups (Fig. 3).

DISCUSSION

Increasing evidence over the last few decades suggests that CIH plays a major role in the pathophysiology of neurocognitive dysfunction of OSAHS patients. In this study we examined the effect of CIH on the changes of spatial memory in growing rats by using an 8-Arm radial maze test and a modified computer controlled CIH model. We demonstrated that CIH exposure during a critical period of neuronal development can lead to substantial deficits in spatial memory. There was ultrastructural evidence of neuronal injury in both hippocampus and prefrontal cortex and the levels of 8-ISO-$PGF_{2\alpha}$ in the serum and in the hippocampal and prefrontal cortical tissues were higher in CIH animals, suggesting that an intermittent hypoxia-induced oxidative stress injury may be one of the mechanisms underlying the memory impairment in CIH rats.

An optimal animal model to reproduce the clinical and pathological features of OSAHS has not been well established. Researchers (Gozal et al. 2001) reported a model of sleep-disordered breathing in rats in which two episodic hypoxia profiles were used and consisted of alternating room air and 10% oxygen either every 90 sec or every 30 min during sleep. Considering the degree and frequency of hypoxia and actual sleep time in OSAHS children, we modified Gozal's model. In

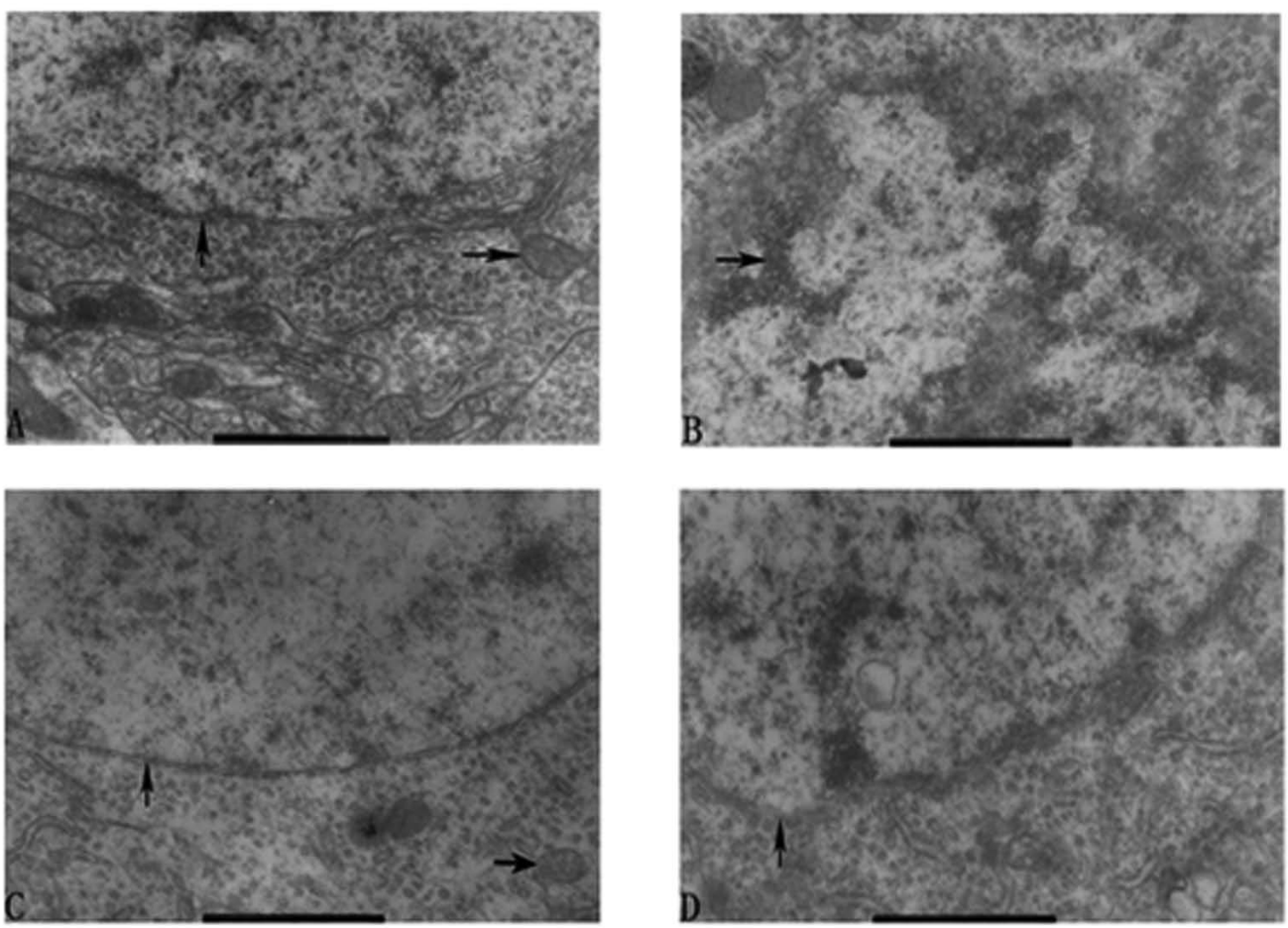

Fig. 3. The ultrastructural changes of the prefrontal cortical tissues from the rats exposed to chronic intermittent hypoxia and the control rats. Normal nuclear membrane and mitochondria are seen in 2C (panel A) and 4C (panel C) groups. Mild fuzzy nuclear membrane is evident in 2IH (panel B, arrow) and 4IH (panel D, arrow) groups. Magnification × 25, 000. Bar represents 384nm.

our study, the O_2 concentration was changed from 9.0±1.5% to 21.0±0.5% every 90s over 7.5 h during the animals' diurnal sleep period for 2-4 weeks to mimic the typical intermittent hypoxia seen in OSAHS patients. This rat model of CIH enabled us to further investigate the possible mechanisms underlying the possible CIH-associated neurobehavioral deficits.

Maze procedures offer a variety of ways of assessing spatial memory in animals. The two classic types are the Morris water maze and the radial-arm maze. Compared with the Morris water maze, the radial-arm maze measures relatively stable asymptotic reference-memory performance, and/or working memory, and is suitable for repeated measures (Hodges 1996). So, in this study, we choose the radial-arm maze to test the effects of CIH on spatial memory in growing rats. Furthermore, we examined the brain hippocampus and prefrontal cortex tissues using a transmission electron microscope to determine the effect of CIH on neuronal cell damage. The hippocampus is the most important region associated with acquired new information and stores long-term memory in cortex (Debiec et al. 2002) and these cell populations are more vulnerable to hypoxia/intermittent hypoxia than neurons from other regions (Gozal et al. 2002)**.** Our result that a CIH exposure during a critical period of neuronal development can lead to substantial deficits in spatial memory is similar to the previous reports (Row et al. 2002, 2003, Xu et al. 2004). The ultrastructural changes such as destruction of the nuclear membrane and swollen mitochondria vacuoles were more evident in the hippocampus than the prefrontal cortex tissues from the CIH groups, indicating that CIH-induced hippocampal and prefrontal cortical neuronal injury might contribute to the memory deficit.

Oxidative stress is a well established mechanism of cellular injury in mammals. Brain tissue contains a large amount of polyunsaturated fatty acids, which are highly susceptible to oxidative reactions (Halliwell and Chirico 1993). Oxidative stress and increased gliosis have been associated with aging related behavioral impairments on spatial learning tasks in the rodent (Nicolle et al. 2001), and similar increases in gliosis in the cortex and hippocampal CA1 region have been observed in the rat after exposure to CIH (Gozal et al. 2001). The repetitive episodes of hypoxia and re-oxygenation during CIH can lead to an increased production of ROS (Prabhakar 2001). In fact, increased levels of ROS have been detected in OSAHS patients (Schulz et al. 2000, Dyugovskaya et al. 2002). One study found that exposure to CIH increased the levels of protein oxidation, lipid peroxidation and nucleic acid oxidation in mouse brain cortex (Xu et al. 2004). Furthermore, exposure of rats to CIH induced caspase-3 activation and increased cortical neuronal cell apoptosis which suggests that the increased ROS production and oxidative stress propagation contribute, at least partially, to CIH-mediated cortical neuronal apoptosis and neurocognitive dysfunction (Xu et al. 2004). These findings provide initial support for the concept that oxidative stress contributes to the cellular damage and consequent behavioral impairments associated with severe forms of OSAHS.

It has been proposed that the levels of F2-isoprostanes (F2-IsoP), a series of PGF2α epimers, in urine, plasma, and tissue could be used as a quantitative index for the generation of free radicals and lipid peroxidation in vivo (Morrow and Roberts. 1999, 2000). Our study found that the levels of 8-ISO-$PGF_{2\alpha}$ in serum, hippocampus and prefrontal cortex were significantly higher in CIH groups than the corresponding control groups. Moreover, there were significant relationships between the levels of 8-iso-prostaglandin F2α and number of memory errors. All these results strongly suggest that hypoxia-induced oxidative stress injury may be part of the mechanisms underlying the hippocampals and prefrontal cortical neuronal injury in this model.

CONCLUSIONS

In summary, our study in growing rats demonstrated that CIH exposure during a critical period of neuronal development can lead to substantial deficits in spatial memory. The ultrastructural changes such as destruction of the nuclear membrane and swollen mitochondria vacuoles were evident in the hippocampus and, in lesser degree, the prefrontal cortical tissues from the CIH groups. Hypoxia-induced oxidative stress injury may be one of the mechanisms underlying the hippocampal and prefrontal cortical neuronal injury in this model.

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