Abstract. The present study aimed to compare the expression of phosphatidylinositol 3-kinase (PI3-K), mammalian target of rapamycin (mTOR) and Beclin-1 between the hippocampi of normal rats and intermittent ischemic rats following whole brain ischemia/reperfusion (i/r), and investigate the role of the PI3K-mTOR autophagy pathway in rat nerve damage following intermittent hypoxia (iH)-aggravated whole brain ischemia. A total of 80 male Wistar rats were divided by random number table into a sham operation group (So group; 20 rats), pure cerebral ischemia/reperfusion group (i/r group; 20 rats), intermittent hypoxia for 7 days-i/r group (iH7+i/r group; 20 rats) and intermittent hypoxia for 21 days-i/r group (iH21+i/r group; 20 rats). Prior to model establishment, the rats in the iH7+i/r group and iH21+i/r group underwent intermittent hypoxia for 7 and 21 days, respectively. The optimized Pulsinelli 4-vessel occlusion method was used to prepare the i/r model. H&E staining and transmission electron microscopy were performed to observe the morphological changes of nerve cells in the hippocampus. Immunohistochemical and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses were performed to detect the expression levels of PI3-K, mTOR and Beclin-1 in the hippocampal brain tissues of the rats. A Morris water maze test was used to assess rat learning and memory. The results showed that, compared with the So group, the rats in the i/r group exhibited structural damage in neurons (shown by H&E staining), a reduced number of viable nerve cells, and decreased learning and memory ability at each time point. The results of the immunohistochemical analysis showed that the numbers of PI3-K, mTOR and Beclin-1 immunopositive cells were increased (P<0.05). The RT-qPCR analysis showed increased expression levels of PI3-K, mTOR and Beclin-1 (P<0.05). Compared with the i/r group, the rats in the IH-i/R groups exhibited aggravated structural damage in neurons, shown by H&E staining and electron microscopy. The number of viable nerve cells was decreased, and the rats exhibited decreased learning ability and memory. The immunohistochemical analysis revealed that the numbers of PI3-K, mTOR and Beclin-1 immunopositive cells were increased (P<0.05). The RT-qPCR analysis showed increased expression levels of PI3-K, mTOR and Beclin-1 (P<0.05). The above changes were more marked in the iH21+i/R group (P<0.05). Taken together, iH was shown to aggravate nerve damage following whole brain i/R. The underlying mechanism was associated with activation of the PI3K-mTOR-autophagy pathway and increased loss of nerve cells.

Introduction

Obstructive sleep apnea hypopnea syndrome (OSAHS) can aggravate the nerve damage caused by brain ischemia, which is an independent risk factor increasing the morbidity and mortality rates of ischemic stroke (1,2). It has been shown that cognitive dysfunction following brain ischemia is closely associated with cellular metabolic changes, signal transduction system disorder and nerve cell death following brain tissue ischemia and hypoxia (3,4). Autophagy, a cellular defense mechanism of eukaryotic cells, is essential in maintaining intracellular homeostasis, and determines the final outcome of cells: Recovery, repair, damage, aggravation or death (5). The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) mammalian target of rapamycin (mTOR) classic cascade signaling pathway effectively regulates multiple physiological and pathological mechanisms, is involved in various eukaryotic processes, including autophagy, proliferation and apoptosis, and is important in diseases of the central nervous system (6,7). Following OSAHS-complicated ischemic stroke, a variety of factors are aggravated, including oxygen free radicals, oxidative stress and inflammatory reactions. These factors can cause increased nerve cell death, either directly or indirectly. Therefore, the present study hypothesized that...
the PI3K-mTOR autophagy pathway is important in the pathological progress of nerve damage following intermittent hypoxia (IH)-aggravated brain ischemia. In the present study, the whole brain ischemia/reperfusion (I/R) model was prepared using rats exposed to intermittent hypoxia. Expression of the PI3K-mTOR autophagy pathway and loss of nerve cells in the hippocampus were observed. The role of the PI3K-mTOR autophagy pathway in IH-aggravated brain I/R was investigated and discussed. The results provided experimental evidence for the clinical prevention and treatment of OSAHS complicated by hypoxic brain vascular diseases.

Materials and methods

Materials. Male Wistar rats (n=80) were provided by Vital River Laboratories Co., Ltd. (Beijing, China). The certificate number for the laboratory animals was SCXK (Jing) 2012-013. Animals were kept in colony cages; under the following laboratory conditions in a ventilated room: 18-25°C, 35-50% humidity and a 12 h light dark cycle, with free access to food and water. The present study was performed with approval from the Animals Welfare Care Committee of North China University of Science and Technology (Tangshan, China). The hypoxia control program was provided by the Science and Technology Center of Tsinghua University (Beijing, China). Oxygen measurement equipment was purchased from Jiande Meicheng Electrochemical Analytical Instrument Factory (Jiande, China). The animal hypoxia chamber was purchased from China Huaibeizhenghua Science and Technology Co., Ltd. (Huaibei, China). Pure nitrogen was purchased from Tangshan General Gas High-Tech Co., Ltd. (Tangshan, China). The Morris water maze was provided by the Pharmaceutical Research Institute of China Academy of Medical Sciences (Beijing, China).

Groups of animals and model preparation. The rats were divided by random number table into a sham operation (SO) group (n=20), I/R group (n=20), IH for 7 days (IH7)+I/R group (n=20) and IH for 21 days (IH21)+I/R group (n=20). Each group was further divided into a 6 and 24 h subgroup, with 10 rats in each subgroup.

The blood vessels in the rats of the SO group were separated and exposed. Electrocoagulation of the vertebral artery and occlusion of the carotid artery were not performed. For the rats in the I/R, IH7+I/R and IH21+I/R groups, the whole brain ischemia model was prepared using the optimized Pulssinelli 4-vessel occlusion method (8). Anesthesia was induced by intraperitoneal injection of 10% chloral hydrate at a dose of 300 mg/kg, (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). An incision was made in the rear occipital and the holes on the bilateral flank were carefully exposed. The pre-heated electrocoagulation needle was inserted and electrocoagulation was performed for 2-4 sec each time to occlude the bilateral vertebral artery. The rats were fixed in a supine position on the surgical bench and an incision was made in the middle of the neck to separate the common carotid arteries on both sides. After 24 h, the bilateral common carotid arteries were occluded at the same time for 15 min to achieve complete whole brain ischemia.

The rats in the IH7+I/R and IH21+I/R groups were placed in a hypoxia chamber every day from 8:00 a.m. to 15:00 p.m. Nitrogen and air were introduced into the chamber recurrently. Each cycle lasted for 120 sec and nitrogen was added continuously for 30 sec to maintain an oxygen level of 5-21% in the chamber. The oxygen concentration was monitored using a digital oxygen analyzer for 7 and 21 days, respectively.

H&E staining. A total of five rats were randomly selected from each group at 6 and 24 h post-I/R, respectively. Cardiac perfusion was performed using 4% paraformaldehyde. The rats were sacrificed and, following decapsulation, brain tissue was removed. The tissue between the optic chiasma plane and transverse section was dehydrated, embedded in paraffin, sectioned, dewaxed, cleared in xylene, stained with H&E and observed under a light microscope (magnification, x400).

Immunohistochemical staining. The brain tissue slides were subjected to conventional dewaxing to water, blocking in 0.3% H2O2 for 10 min and heated antigen retrieval for 90 sec. Antibodies against PI3-K, mTOR and Beclin-1 were added onto the slides, respectively, and the slides were incubated in a humid box at 37°C for 30 min. The secondary antibody was added and the slides were incubated at 37°C for 40 min. Mouse monoclonal anti-PI3-K (1:200, Bioss, Product batch number: bs-0128R); Mouse monoclonal anti-mTOR (1:150, Bioss, Beijing, China, Product batch number: bs-5331R); Mouse monoclonal anti-Beclin-1 (1:200, Baomanbio, Shanghai, China, Product batch number: OA00006W). The slides were then subjected to DAB development, dehydration, clearing, neutral resin-mounting and microscope observation. Quantitative analysis of the positive rate was performed using the following method. A total of five slides were selected from each parameter, and five regions in the hippocampus were randomly selected in each slide under a light microscope (magnification, x400). The number of positive cells was counted using a FACS-like tissue cytometry analysis system (Tissue FAXS plus, TissueGnostics GmbH, Vienna, Austria).

RT-qPCR analysis. Five rats were randomly selected from each group at 6 and 24 h post-I/R, respectively. A 50-100 mg sample of tissue from the CA1 region of rat hippocampus was collected and homogenized in TRIZol solution. Total RNA was extracted. cDNA samples were blended with DEPC-treated Water and SYBR-Green Master Mix in a total volume of 20 μl. The OD260/280 was measured using a Roter-Gene 3000 Fluorescence quantitative PCR instrument. The RNA concentrations were calculated based on the OD260 and the RNA samples were stored at -80°C. The following primers were used: PI3K, forward GAAACCCAGTCACTAGGCC and reverse 5'-GGTGGGCGATGAACTCAA-3';
mTOR, forward 5'-GGTGGACGAGCTCTTTGTCA-3' and reverse 5'-AGGAGCCCTAACACTCGGAT-3'; Beclin-1, forward 5'-CTTCTGTCAGGGGTACCTTC-3' and reverse 5'-CCTTAGACCCCTCCATTCTCA-3'; GAPDH forward 5'-CTCCCCATCCRCACCTTTTG-3' and reverse 5'-CCAACCTGTGGCTGAG-3'. The RT-qPCR procedure was performed as follows: Stage 1 and 2 (RT reaction) comprising one cycle at 42°C for 50 min and 95°C for 10 sec. Stage 3 (qPCR reaction) involving repetition of 45 cycles at 95°C for 15 sec and 56°C for 20 sec. Stage 4 involved melting curve analysis following the dissociation protocol.

**Water maze test.** The laboratory animals were subjected to a water maze test at 48 h post-I/R. The escape platform was placed in the second quadrant of the water maze. The water level was 2-3 cm above the platform, and temperature was maintained at 25±1°C. The rats were placed in a fixed position in the four quadrants, respectively. The time limit for swimming was 90 sec. The length of time a rat spent locating the platform was recorded. The length of time was recorded as 90 sec if a rat failed to locate the platform within 90 sec. A water maze video tracking system (MED-SYST-VWM, MED Associates, Inc., Fairfax, VT, USA) were used to follow, image and analyze the route of a rat from the four quadrants to the platform, the latency and the number of times a rat crossed the platform.

**Statistical analysis.** A database of experimental data was established using Microsoft Excel 2003 (Microsoft Corporation, USA, Redmond, WA, USA). All data are expressed as the mean ± standard error. SPSS 17.0 statistical analysis software (SPSS, Inc., Chicago, IL, USA) was used for one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**H&E staining.** The nerve cells in the hippocampus of the SO group rats were neatly arranged and exhibited normal structures; nuclei were clear and the nucleoli were visible. Swelling of neurons was observed in majority of the nerve cells, with loose structures. The nuclei of these neurons were clearly stained and the nucleoli were visible. There was obscurity in the nerve cells, as shown in Fig. 2a-d.

**Observation using transmission electron microscopy.** In the SO group, the rat hippocampus nerve cells were in ordered arrangement with a smooth nuclear membrane. Chromatin was evenly scattered in the nuclei. There were abundant organelles in the nerve cell cytoplasm, exhibiting normal structures. In the I/R group, the nuclear membrane of the rat hippocampus nerve cells was dissolved. Chromatin had shrunk in the nuclei. The number of nerve cell cytoplasmic organelles was reduced but with integrated structure in the I/R group, nuclear shrinkage was observed and nuclear chromatin was dissolved. Cytoplasmic organelles were reduced in the nerve cells, with obscure structures, as shown in Fig. 2A-D.

**Table I. Comparison of survival rates of rat hippocampal nerve cells among treatment groups.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Animals (n)</th>
<th>6 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO</td>
<td>5</td>
<td>99.08±0.84</td>
<td>98.65±0.75</td>
</tr>
<tr>
<td>I/R</td>
<td>5</td>
<td>78.65±1.43*</td>
<td>71.71±1.39*</td>
</tr>
<tr>
<td>IH7+I/R</td>
<td>5</td>
<td>65.74±1.20*</td>
<td>60.75±1.39*</td>
</tr>
<tr>
<td>IH21+I/R</td>
<td>5</td>
<td>49.06±1.70*</td>
<td>45.12±1.51*</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± standard error. *P<0.05, compared with the SO group; **P<0.05, compared with the I/R group; ***P<0.05, compared with the IH7+I/R group. SO, sham operation; I/R, ischemia/reperfusion; IH7, intermittent hypoxia for 7 days; IH21, intermittent hypoxia for 21 days.

**Table II. Comparisons of latency and times platform crossed among rats from each treatment group in the water maze test.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Animals (n)</th>
<th>Latency period</th>
<th>Times platform crossed (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO</td>
<td>10</td>
<td>7.20±1.45</td>
<td>12.47±0.92</td>
</tr>
<tr>
<td>I/R</td>
<td>10</td>
<td>14.04±0.62</td>
<td>8.53±0.60</td>
</tr>
<tr>
<td>IH7+I/R</td>
<td>10</td>
<td>17.23±0.89</td>
<td>6.57±0.65</td>
</tr>
<tr>
<td>IH21+I/R</td>
<td>10</td>
<td>29.79±1.96</td>
<td>1.15±0.33</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± standard error. *P<0.05, compared with the SO group; **P<0.05, compared with the I/R group; ***P<0.05, compared with the IH7+I/R group. SO, sham operation; I/R, ischemia/reperfusion; IH7, intermittent hypoxia for 7 days; IH21, intermittent hypoxia for 21 days.
Immunohistochemistry

**PI3K.** The immunohistochemical staining showed that PI3K (brown) was located in the cytoplasm and predominantly expressed in neurons. Positively stained cells were occasionally observed in the SO group. At each time point (6 and 24 h), the numbers of PI3K-positive cells were increased in the I/R groups, compared with that in the SO group (P<0.05). At each time point (6 and 24 h), the numbers of PI3K-positive
cells were increased in the IH groups, compared with that in the I/r group (P<0.05). These changes were more marked in the IH21+i/r group (P<0.05), as shown in Table III and Fig. 3a1-d1.

mTOR. The immunohistochemical staining showed that mTOR (brown) was located in the cytoplasm and predominantly expressed in neurons. Positively stained cells were occasionally observed in the So group. At each time point (6 and 24 h), the numbers of mTOR-positive cells were increased in the I/r groups, compared with that in the So group (P<0.05). At each time point (6 and 24 h), the numbers of mTOR-positive cells were increased in the IH groups, compared with that in the I/r group (P<0.05). These changes were more marked in the IH21+i/r group (P<0.05), as shown in Table III and Fig. 3a2-d2.

Beclin-1. The immunohistochemical staining showed that Beclin-1 (brown) was located in the cytoplasm and predominantly expressed in neurons. Positively stained cells were occasionally observed in the So group. At each time point (6 and 24 h), the numbers of Beclin-1-positive cells were increased in the I/r groups, compared with that in the So group (P<0.05). At each time point (6 and 24 h), the numbers of Beclin-1-positive cells were increased in the IH groups, compared with that in the I/r group (P<0.05). These changes were more marked in the IH21+i/r group (P<0.05), as shown in Table III and Fig. 3a3-d3.

RT-qPCR analysis

PI3K. At each time point (6 and 24 h), the expression of PI3K was significantly increased in the I/r group, compared with that in the So group (P<0.05). At each time point (6 and 24 h), the expression of PI3K was significantly increased in the IH groups, compared with that in the I/r group (P<0.05). These changes were more marked in the IH21+i/r group, as shown in Table IV.

mTOR. At each time point (6 and 24 h), the expression of mTOR was significantly increased in the I/r group, compared with that in the So group (P<0.05). At each time point (6 and 24 h), the expression of mTOR was significantly increased in the IH groups, compared with that in the I/r group (P<0.05). These changes were more marked in the IH21+i/r group, as shown in Table IV.

Discussion

In addition to being a respiratory disease, OSAHS is able to damage and involve multiple systems, leading to damage in the brain, heart and other organs, and to the neurological system in particular (9). Studies have shown that, due to repeated hypoxia and hypercapnia, OSAHS can cause cerebral blood rheology,
cerebral metabolic change, dysfunction of neurohumoral regulation, oxidative stress and inflammation, initiating and aggravating the pathophysiological progress of cerebral I/R (10). This leads to neuron loss, and damage to the morphology and structure of nerve cells in multiple cerebral regions, thereby increasing nerve damage following cerebral I/R (11). The neurological dysfunction following brain ischemia is directly associated with the loss of nerve cells. For example, the results of a study by Sun et al (12) showed that the number of viable nerve cells was reduced and learning ability was decreased following brain I/R. Running exercise increased the survival rate of nerve cells and improved learning ability in rats. The results of the present study showed that, compared with the I/R group, rats in each OSAHS hypoxia group exhibited reduced nerve cell survival in the hippocampus, severely damaged nerve cell structure, and apparent dysfunction in learning and memory ability. This indicated that OSAHS hypoxia aggravated rat neurological damage following brain I/R, and these results were consistent with those of clinical studies (13,14).

Autophagy is a process, which is extensively present in eukaryotic organisms and functions through the lysosomal pathway, which involves the identification, degradation and reversal of functionally damaged proteins and organelles with physiological and pathological factors. It is a third mechanism of cellular death in addition to apoptosis and necrosis (15). Wang et al (16) established rat whole brain ischemia models and observed nerve cells in the CA1 hippocampal region. It was found that damage to neurons induced by whole brain ischemia was significantly reduced following the addition of autophagy inhibitor 3-MA at 1 h or 30 min prior to ischemia. This indicated that ischemia-induced autophagy aggravated neuron damage led by brain ischemia. The results of the present study showed that, compared with the I/R group, the rats in the IH groups exhibited aggravated damage in neuron structure, reduced neuron survival rates and increased expression of Beclin-1. These changes were more marked in the IH21+I/R group, indicating that autophagy in the process of I/R alone was further activated by IH to promote neuron loss following brain ischemia. IH can facilitate the activation of autophagy. For example, Liu et al (17) established mouse IH models and observed autophagy-associated protein expression in nerve cells of the mouse hippocampal CA1 region following IH. The results showed that the protein levels of LC3II/LC3 were increased and damage to the microstructure of nerve cells was aggravated, indicating that IH induced autophagy in the rat hippocampal nerve cells.

The activation of autophagy is dependent on the PI3K/Akt/mTOR signaling pathway (18). It has been shown that the activities of PI3K, Akt and mTOR are significantly increased following brain ischemia. Ishrat et al (19) prepared rat focal brain ischemia models by cerebral artery occlusion and observed significantly elevated phosphorylation levels of PI3K and Akt in addition to nerve cell apoptosis, indicating that brain ischemia activated the PI3K/Akt signaling pathway. Zhao et al (20) exposed rats with chronic brain ischemia to flavonoids from hawthorn leaves, and detected the protein expression levels of PI3K and Akt using immunohistochemistry. The results showed that flavonoids from hawthorn leaves increased the protein expression of PI3K and Akt, and reduced brain tissue damage. It has been reported that (21) under ischemia and hypoxic conditions, the cellular PI3K/Akt signaling pathway is activated and autophagy can be induced by regulating the activity of mTOR. The results from a study by Liu and Xu (22) demonstrated that sevoflurane pre-treatment reduced the autophagy of cardiac muscle cells during I/R through activating the PI3K/Akt signaling pathway and enhancing downstream mTOR activity, which had a protective effect on cardiac muscles. Gong et al (23) established focal brain ischemia models in Sprague-Dawley rats and found that the PI3K inhibitor, LY294002, inhibited the PI3K-mTOR signaling pathway and subsequently inhibited autophagy, increasing the area of infarction in rats. The results of the present study showed that the expression levels of PI3K and mTOR were elevated in all the IH groups, and this was associated with the degree of hypoxia. This indicated that IH increased autophagy and aggravated neurological dysfunction following cerebral ischemia by activating the PI3K/Akt/mTOR signaling pathway. At present, the mechanisms underlying the regulation of PI3K/Akt/mTOR signaling by IH remain to be fully elucidated. However, it has been shown that the characteristic chronic IH hypoxia in OSAHS, similar to damage from I/R, aggravates hypoxia, and induces oxygen free radical production, ATP depletion and inflammation (24), which may be one of the reasons why the PI3K/Akt signaling pathway was activated by IH following cerebral ischemia.

In conclusion, the present study demonstrated that IH promoted autophagy and aggravated neurological dysfunction following cerebral ischemia by activating the PI3K/Akt/mTOR cascade signaling pathway. These results provided experimental evidence for the prevention and treatment of OSAHS-complicated brain ischemia.

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Availability of data and materials
All data generated and/or analyzed during the present study are included in this published article.

Authors' contributions
XG conceived the present study. YNZ made substantial contributions in data analysis and wrote the manuscript. YL and RF performed the experiments. YLB, XFG, JML and CXC designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The Animal Experimentation Ethics Committee of North China University of Science and Technology (Hebei, China)
approved the experimental animal protocol of the present study.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**References**