

# Effect of different levels of intermittent hypoxia on autophagy of hippocampal neurons

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Received: 7 September 2016 / Revised: 28 April 2017 / Accepted: 15 May 2017 / Published online: 29 May 2017  
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## Abstract

**Purpose** The current study was carried out to assess the effects of different levels of intermittent hypoxia (IH) on autophagy in hippocampal neurons, and explore the extent, frequency and duration of IH for researching on autophagy in hippocampal neurons.

**Methods** Hippocampal neurons were exposed to different levels of IH. To analyze the oxygen level of neuronal exposure environment, we detected the oxygen concentration in the chamber by O<sub>2</sub> analyzer, and monitored the oxygen partial pressure (PO<sub>2</sub>), carbon dioxide partial pressure (PCO<sub>2</sub>), and pH in the culture media by blood gas analyzer. After 4-, 8-, and 12-h IH, the morphology and quantity of neurons, as well as the expression of light chain 3 (LC3)-II positive dots were observed by immunofluorescence. The expression of apoptosis marker protein cleaved caspase-3 and autophagy marker protein LC3 were examined by western blotting.

**Results** The oxygen level in the chamber and the neuronal culture media both reached to the values set previously in three models. The level of cleaved caspase-3 and LC3 had no significant changes in IH-1 group. The morphology and quantity had no significant changes, while the levels of cleaved caspase-3 and LC3 were both increased in IH-2 group. The quantity of neurons was reduced significantly, and the chromatin condensed and nuclei fragmented in IH-3 group.

**Conclusions** The effects of varying degrees of IH on autophagy in hippocampal neurons are different. The IH model, hypoxia phase (1.5% O<sub>2</sub>, 5% CO<sub>2</sub>, and balance N<sub>2</sub>) for 5 min and reoxygenation phase (21% O<sub>2</sub>, 5% CO<sub>2</sub>, and balance N<sub>2</sub>) for 10 min, may be the best condition for researching on autophagy in hippocampal neurons.

**Keywords** Intermittent hypoxia · Autophagy · Apoptosis · Hippocampal neurons

## Introduction

Intermittent hypoxia (IH), which resulted from recurring episodes of upper airway obstruction, the typical characteristic, and the most important pathophysiologic pathway of obstructive sleep apnea-hypopnea syndrome (OSAHS), is believed to be a potential major factor causing neuronal injury which may play the key role in the development and progression of cognitive impairments [1, 2]. It is worth noting that a wide range of neurocognitive impairments have been identified in OSAHS patients, and the greater hazard of cognitive dysfunctions is associated with the severity of IH, which is demonstrated by the higher percentage of patients experiencing poor cognition in the moderate-to-severe OSAHS patients than the primary snoring and mild OSAHS patients based on the subjective symptom assess [3]. Moreover, the injury induced by severe IH was more serious than those induced by moderate IH and mild IH in vivo and vitro experiments [4]. In a word, all these above suggest that the neuronal injury caused by IH is different and that is proportional to the severity of IH.

Autophagy is a phenomenon that widely presents in eukaryotic cells, and it can provide the required starting materials for cells to the synthesis of new proteins and update of organelles to maintain cellular homeostasis [5]. Many studies

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have confirmed that autophagy is increased in numerous hypoxic-excitotoxic conditions including cerebral hypoxia-ischemia [6], ischemia-reperfusion [7], and oxygen and glucose deprivation (OGD)-reperfusion (RP) [8]. And the increased level of autophagy is connected with the severity of hypoxia-related conditions. IH, a special form of hypoxia, also causes increased expression of autophagy in pancreatic cancer cells, human neutrophils, and animal tissue [9–11]. However, in hippocampal neurons which are more susceptible to IH, the effects of varying degrees of IH on autophagy are still unclear.

In many studies about OSAHS, IH was used as the sole exposure factors to simulating the disturbances of homeostasis of OSAHS patient [12, 13]. The extent, frequency, and duration of IH are various in different studies which focus on different research purposes. The oxygen concentration of hypoxic phase is usually maintained at 5% or more in IH animal model [12, 14, 15], and is held at 0.1~5% in IH cell model [16, 17]. Furthermore, the duration of hypoxia and reoxygenation is also diverse, which ranges between tens of seconds and tens of minutes. Therefore, in present study, we have chosen 1.5% O<sub>2</sub> and 21% O<sub>2</sub> for simulating different frequencies and durations of IH, and explored the effects of IH on autophagy in hippocampal neurons, which will be meaningful for further understanding the mechanisms of hippocampal neuron injury induced by IH.

## Methods and materials

### Hippocampal neuron culture

Primary hippocampal neurons were obtained from Sprague-Dawley rat pups (<24 h), which were purchased from Chinese Academy of Military Science (Beijing, China), and cultured according to the procedure described Pont-Lezica et al. [18]. Briefly, the brain was dissected and hippocampus was removed. The meninges and blood vessels were carefully striped and the hippocampus was minced into 1 mm<sup>3</sup> fragments. Then, the hippocampal pieces were dissociated by 0.25% trypsin (Sigma, USA) and 100 ng/ml DNase (Roche, Sweden) at 37 °C for 30 min. The isolated cells were resuspended by DMEM medium (Hyclone, USA) with 10% FBS (Gibco, USA) and seeded into 6-well plates or 22 \* 22 mm<sup>2</sup> glass coverslips in 35-mm Petri dishes pre-coated with 10 µg/ml poly-D-lysine (Sigma, USA) at a density of 5 \* 10<sup>5</sup> per well. After 4 h, neurons were cultured in the neurobasal-A medium with 2% B27, 1% glutamine, and HEPES (Gibco, USA) for 7 days at 37 °C in 5% CO<sub>2</sub> and 95% O<sub>2</sub> atmosphere.

### Exposure of hippocampal neurons to IH

The culture medium was changed to fresh medium for routine culture before the cells were exposed to

intermittent hypoxia. Hippocampal neurons grown in culture plates were placed in Plexiglas exposure chamber and were exposed to IH or normoxia conditions. We developed a gas delivery system that permits the exposure of cell cultures to hypoxia/reoxygenation cycles, simulating the pattern of hypoxic episodes seen in recurrent apnea. Visual C++ computer language was used in coding a control program (Breath-Simulating system 1.0) which regulated the delivery system as previously described [19]. Briefly, a personal computer drove a single chip through serial port communicating protocol, and with this system, solid relay was adjusted to modulate a gas resource system for controlling whether the gas mixture was given into the chamber or not. The gas mixture (Tai-ya Gas Limited Company, Tianjin, China) in this study consisted of O<sub>2</sub> in particular concentration, 5% CO<sub>2</sub>, and balanced N<sub>2</sub>. The hypoxia gas mixture (1.5% O<sub>2</sub>, 5% CO<sub>2</sub>, and balanced N<sub>2</sub>, hypoxia phase) or normoxia gas mixture (21% O<sub>2</sub>, 5% CO<sub>2</sub>, and balanced N<sub>2</sub>, reoxygenation phase) was flushed alternatively into the customized small IH housing chamber to provide a designated IH or normoxia environment. The O<sub>2</sub> concentration in the chamber was rapidly decreased to about 1.5% with hypoxia gas mixture during hypoxia phase. The O<sub>2</sub> concentration was increased to a maximum of 21% by rapidly flushing the chamber with normoxia gas mixture during reoxygenation phase. In order to analyze the effect of varying degrees of IH on autophagy, cells were maintained in the cycle of 1.5% O<sub>2</sub> for 2 min and 21% O<sub>2</sub> for 5 min (IH-1 model), cycle of 1.5% O<sub>2</sub> for 5 min and 21% O<sub>2</sub> for 10 min (IH-2 model), cycle of 1.5% O<sub>2</sub> for 10 min and 21% O<sub>2</sub> for 20 min (IH-3 model), and sustained normoxia gas mixture (NC), respectively. The chamber was equipped with a humidifier, thermostat, and molecular sieve to maintain an inner temperature of 37 °C, humidity of 45%, and a relative germfree circumstance. Change of oxygen concentrations in the chamber was monitored by O<sub>2</sub> analyzer. O<sub>2</sub> levels in the culture media, 1 mm above the cell layer, were sampled and monitored by oxygen electrode (Lazar, USA) and blood gas analyzer (AVL OMNI, Swiss), with six samples per gas environment.

### Immunofluorescence staining for microtubule-associated protein 2 (MAP-2), LC3, and DAPI

The primary hippocampal neurons, grown on coverslips in 3.5-cm dishes, were observed and took photograph by optical microscope after treated with IH or normoxia. The cells were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 15 min. Then, they were permeabilized with 0.1% Triton X-100 for 10 min and blocked with BSA buffer for 30 min at room temperature. Subsequently, cells

were incubated with the anti-MAP-2 antibody (1:100, Boster Biological Technology, China) and anti-light chain 3B (LC3B) antibody (1:50, Cell Signaling Technology, USA) at 4 °C overnight. After being rinsed with PBS, the cells were incubated for 1 h at room temperature with an appropriate secondary antibody. Finally, 4,6-diamidino-2-phenylindole (DAPI) (Beyotime, C1002) was used as a counterstain. Images were taken under OLYMPUS microscope (cellSens system) and OLYMPUS confocal microscope (FV 1200). The total number of neurons was counted in individual views. LC3-II dots were counted in individual hippocampal neurons and the average of dots in at least 30 cells was presented in the figures.

### Western blotting for cleaved caspase-3 and LC3 protein expression

Hippocampal neuron protein was extracted in accordance with the instructions of the protein extraction kit extracted (Promega, USA). Protein concentrations were measured using a BCA Assay Kit (Thermo, USA). Equal amounts of the protein (10 µg) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk containing Tris buffered saline and Tween 20 (TBS-T) for 2 h on a shaker at room temperature and then incubated with appropriate dilutions of the primary specific antibody: rabbit anti-LC3B antibody (1:1000, Cell Signaling Technology, USA); rabbit anti-cleaved caspase-3 antibody (1:1000, Cell Signaling Technology, USA); and rabbit anti-GAPDH antibody (1:1000, Cell Signaling Technology, USA) in TBS-T at 4 °C overnight. The membranes were washed with TBS-T on the shaker. The secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:1000, Beyotime Biotechnology, China), incubation of which was carried out on the shaker at room temperature for 1 h. Chemiluminescence was imaged in a Molecular Imager Chemi Doc™ XRS + WB with Image Lab™ software (Bio-Rad, CA, USA), and band intensity was measured by ImageJ software.

### Statistical analysis

SPSS 16.0 (SPSS Inc., USA) software package was used for statistical analysis. The data of blood gas analysis of culture media were obtained and were displayed as a descriptive analysis. The immunostaining was analyzed with the ImageJ software. Student's *t* test was used for comparing with baseline values. Unless otherwise stated, values were reported as mean ± standard deviation (SD). The significance of variables

between groups was tested by one-way ANOVA.  $P < 0.05$  is considered statistically significant.

## Results

### Changes of oxygen concentration in the intermittent hypoxic cabin

We detected the level of oxygen concentration in the chamber. The results showed the process of hypoxia and normoxia alternately in IH models. As the Fig. 1 displayed, oxygen concentration decreased from 21 to 1.5%, and continued until 5 min. Then, it increased from 1.5 to 21%, and maintained 10 min. These results suggested that the level of oxygen in the cabin reached to the values (1.5 or 21%) set previously in three IH models.

### PO<sub>2</sub>, PCO<sub>2</sub>, and pH values in the culture media of hippocampal neurons

We collected the culture medium samples from about 1 mm above the cell layer, and analyzed the PO<sub>2</sub>, PCO<sub>2</sub>, and pH values. The results are described as Table 1, which suggested that three IH models can provide hypoxia environment at the cellular level.

### Effects of IH on morphology and quantity of hippocampal neurons

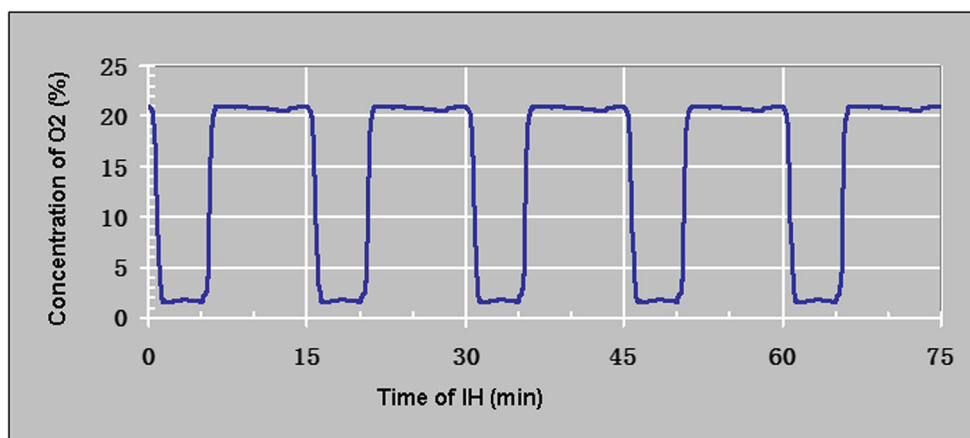
The morphology and quantity of hippocampal neurons were observed by optical microscope and fluorescence microscope after IH. The intact structure of neurons suffered severe damage, and the quantity of neurons was reduced significantly in IH-3 group compared with normoxia group ( $P < 0.05$ ) (Fig. 2a, c), while neurons of IH-1 group and IH-2 group had no significant changes compared with normoxia group both in morphology and quantity ( $P > 0.05$ ) (Fig. 2b, d).

The apoptosis induced by IH in hippocampal neurons was verified by examining nuclear morphological changes following staining of nuclei with DAPI. As indicated in Fig. 2, severe IH-treated neurons (Fig. 2e) exhibited typical late apoptotic morphological features, including condensed chromatin and fragmented nuclei. By contrast, the neurons exhibited homogenous in the normoxia group, IH-1 group, and IH-2 group (Fig. 2f).

### Effects of IH on cleaved caspase-3 and LC3 expression in hippocampal neurons

We examined the expression of cleaved caspase-3 in IH-1 group and IH-2 group, and found that the level of cleaved caspase-3 was increased in these groups. The increased level of cleaved caspase-3 in IH-1 group had no statistical significance compared with

**Fig. 1** The oxygen concentration-time curve diagram in the cabin of IH-2 model (episodic cycles of 1.5% O<sub>2</sub>, 5% CO<sub>2</sub>, and balance N<sub>2</sub> for 5 min and 21% O<sub>2</sub>, 5% CO<sub>2</sub>, and balance N<sub>2</sub> for 10 min)



normoxia group ( $P > 0.05$ ) (Fig. 3a), while it was significant higher in the IH-2 group than those in normoxia group ( $P < 0.05$ ) (Fig. 3b), especially at the time of 12 h of IH ( $P < 0.01$ ). These results suggested that the IH model which induced hippocampal neuron injury was successfully established by episodic cycles of hypoxia phase (1.5% O<sub>2</sub>, 5% CO<sub>2</sub>, and balance N<sub>2</sub>) for 5 min and reoxygenation phase (21% O<sub>2</sub>, 5% CO<sub>2</sub>, and balance N<sub>2</sub>) for 10 min.

As described above, the IH-2 model can not only provide hypoxia environment but also cause the hippocampal neuron injury. Thus, we examined the expression of LC3 in IH-2 group, and found that it was significantly increased, especially at the time of 12 h of IH ( $P < 0.01$ ) (Fig. 3d). To further explore the effect of IH on autophagy in hippocampal neurons, we also examined the expression level of LC3 in IH-1 group, and found that its increase had no statistic significant ( $P > 0.05$ ) (Fig. 3c). At the same time, compared with the normoxia group, the number of LC3-II positive dots was not statistic significant in IH-1 group (Fig. 4d) and that was significant higher at 12 h in IH-2 group ( $P < 0.01$ ) (Fig. 4b).

## Discussion

In the present study, we discussed the effects of varying degrees of IH on autophagy in hippocampal neurons, and found

that the effects of varying degrees of IH on autophagy in hippocampal neurons are different. The IH model, hypoxia phase (1.5% O<sub>2</sub>, 5% CO<sub>2</sub>, and balance N<sub>2</sub>) for 5 min and reoxygenation phase (21% O<sub>2</sub>, 5% CO<sub>2</sub>, and balance N<sub>2</sub>) for 10 min, may be the best condition for researching on autophagy in hippocampal neurons.

Firstly, we successfully established IH model in hippocampal neurons by detecting the oxygen concentration in the chamber and PO<sub>2</sub>, PCO<sub>2</sub>, and pH in the culture media of hippocampal neurons. It is widely used that the hypoxia gas mixture and normoxia gas mixture were flushed alternatively into the customized small IH housing chamber to provide a designated hypoxia or normoxia environment. In previous studies, the cycle of hypoxia gas mixture (1.5% O<sub>2</sub>, 5% CO<sub>2</sub>, and balanced N<sub>2</sub>) and normoxia gas mixture (21% or 20% O<sub>2</sub>, 5% CO<sub>2</sub>, and balanced N<sub>2</sub>) has been used in many cells, such as pheochromocytoma PC12 cell [20] and the human lung microvascular endothelial cells [16]. The PO<sub>2</sub> in the culture media of cells can more accurately detect the O<sub>2</sub> concentration. He et al. [4] also monitored the O<sub>2</sub> levels in the culture media by oxygen electrode and blood gas analyzer, and found that the PO<sub>2</sub> was associated with the pattern of IH closely.

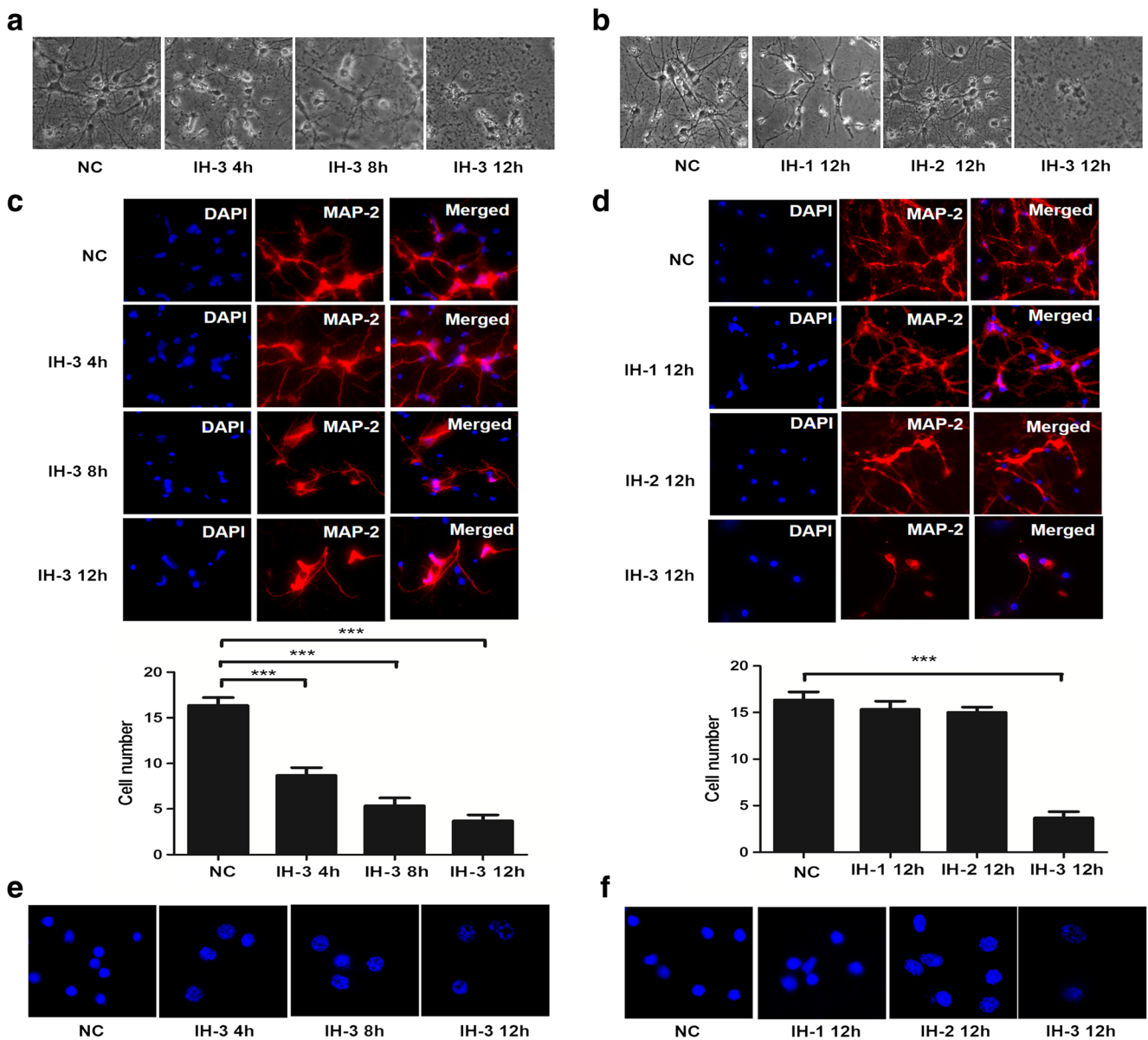
Secondly, we observed the morphology and quantity of hippocampal neurons before and after IH treatment. In this study, the morphology and quantity of neurons had no significant change in IH-1 group and IH-2 group, while the number of hippocampal

**Table 1** The exposure environment at the cellular level provided by IH model

Groups	Hypoxia phase			Reoxygenation phase		
	PO <sub>2</sub> (mmHg)	PCO <sub>2</sub> (mmHg)	pH	PO <sub>2</sub> (mmHg)	PCO <sub>2</sub> (mmHg)	pH
NC	124.67 ± 0.5773	38.33 ± 0.5773	7.28 ± 0.0100	–	–	–
IH-1	94.67 ± 1.5272	38.33 ± 1.1547	7.29 ± 0.0058	124.33 ± 2.0817	38.67 ± 1.5275	7.28 ± 0.0153
IH-2	66.33 ± 1.5275	38.33 ± 0.5773	7.28 ± 0.0115	124.67 ± 1.5275	37.67 ± 0.5773	7.28 ± 0.0153
IH-3	53.33 ± 1.5275	38.33 ± 0.5773	7.29 ± 0.0058	125.33 ± 0.5773	38.67 ± 1.5275	7.28 ± 0.0058

PO<sub>2</sub> partial oxygen pressure, PCO<sub>2</sub> partial carbon dioxide pressure





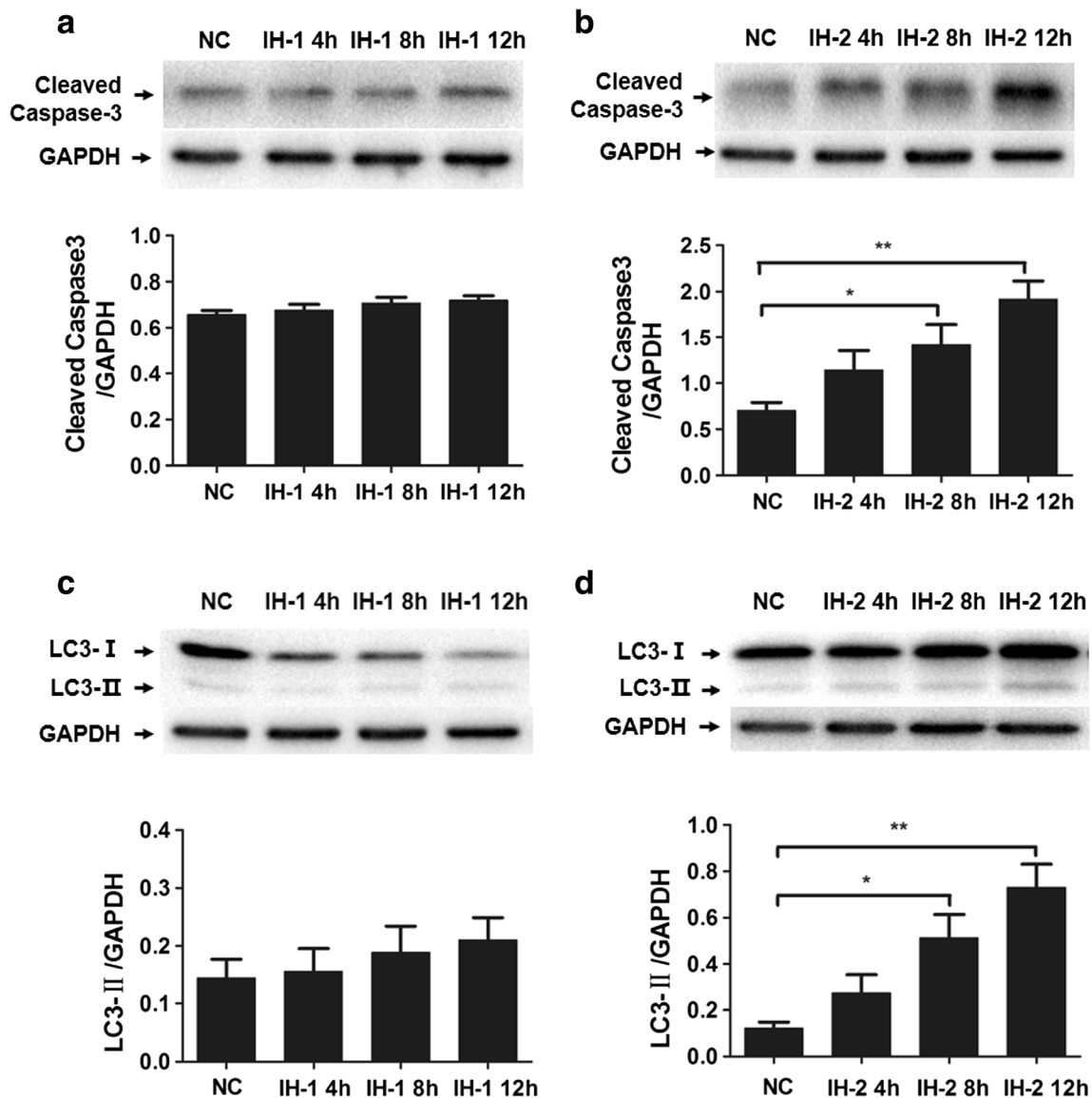
**Fig. 2** The changes of morphology and quantity of hippocampal neurons after IH. **a, b** Hippocampal neurons were observed by optical microscope. Compared to normoxia group, the damage of neuronal integrity was more serious in IH-3 group. *Scale bar:* 50  $\mu$ m. **c, d** Hippocampal neurons labeled MAP-2, DAPI, and merged. Compared to normoxia group, the

number of neuron was decreased in the IH-3 group ( $***P < 0.001$ ). *Scale bar:* 50  $\mu$ m. **e, f** Fluorescent microscopy of neurons stained with DAPI. Chromatin condensation and nuclear fragmentation were observed in IH-3 group. *Scale bar:* 20  $\mu$ m

neurons was decreased and the intact structure of neurons was rarely visible in the IH-3 group which suffered from more serious hypoxia. Then, we further investigated whether or not the apoptosis and necrosis are related to the reduction in the number of neurons. DAPI staining, a method for detecting apoptosis through observing the changes of nuclear morphology, is now widespread used [21–23]. Chromatin condensation and nuclear fragmentation are the typical morphological features in the late apoptosis [24]. In IH-3 group of our study, chromatin condensation and nuclear fragmentation were also observed. Thus, we thought that the apoptosis-induced by IH may play an important

role in the reduction of neurons, but not exclude necrosis that will require large studies.

Due to severe injury and reduction of the neurons in IH-3 group, we thought that the model of IH-3 was not suitable for further study. To investigate whether IH model can induce cell injury, we examined the expression level of apoptosis marker protein cleaved caspase-3, and found that the increased level of cleaved caspase-3 in IH-1 group was not significant and that had significant statistic significance in IH-2 group, especially at the time of 12 h of IH. These results suggested that the IH-1 model was not neurotoxic, and the frequency and

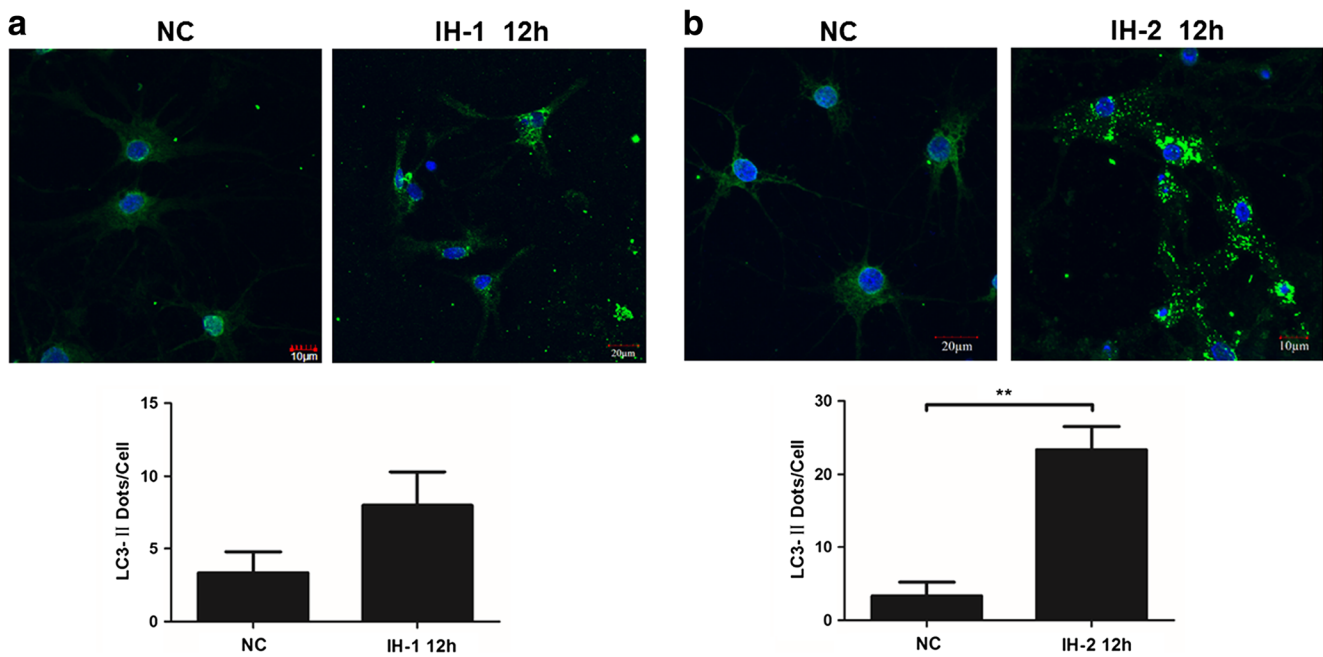


**Fig. 3** The expression level of cleaved caspase-3 and LC3 in hippocampal neurons with western blotting. **a, b** The expression level of cleaved caspase-3 in hippocampal neurons. Compared to normoxia group, the level of cleaved caspase-3 was not statistically significant in IH-1 group and that was significantly higher at 8 and 12 h in IH-2 group

(\* $P < 0.05$ , \*\* $P < 0.01$ ). **c, d** The expression level of LC3 in hippocampal neurons. Compared to normoxia group, the level of LC3 was not statistic significant in IH-1 group and that was significantly higher at 8 and 12 h in IH-2 group (\* $P < 0.05$ , \*\* $P < 0.01$ )

duration of IH-2 model could cause neuronal injury. In one study that focused on the effects of varying degrees of IH, the injury was proportional to the severity of IH, which was demonstrated through the level of systemic inflammatory markers describing as severe IH > moderate IH > mild IH [4]. The pathophysiological characteristics of IH and cerebral ischemia are similar, and the severity of cerebral ischemia is also proportional to the severity of hypoxia, which showed that the short hypoxic duration produced smaller average infarct size and more variable brain damage than the longer hypoxic duration [25]. These may be associated with the level of oxygen that affects mitochondrial function and structure [26].

Finally, we detected the expression level of autophagy. The effects of varying degrees of hypoxia-condition on autophagy are different. Shi et al. [8] established the model of cerebral hypoxia and ischemia through oxygen and glucose deprivation (OGD) and reperfusion (RP), and they found that longer OGD injury activated higher autophagic activity as determined by the greater percentage of AVO-positive neurons to total neurons in the 6-h OGD group than that of 3-h OGD. Furthermore, they also showed that the LC3 levels were increased in a time-dependent fashion according to the RP time in the 6-h OGD group. In this study, we also found that the effects of varying degrees of IH on autophagy in hippocampal neurons are different, which indicated



**Fig. 4** The expression level of LC3-II in hippocampal neurons with immunofluorescence. Compared to normoxia group, the number of LC3-II positive dots was not statistically significant in IH-1 group and that was significantly higher at 12 h in IH-2 group (\*\* $P < 0.01$ )

by these results that the increased level of LC3 in IH-1 group was not significant, while that had significant statistical significance in IH-2 group, especially at the time of 12 h of IH.

## Conclusion

In conclusion, our results highlight the fact that the effects of varying degrees of IH on autophagy in hippocampal neurons are different. The model described as hypoxia phase for 2 min and reoxygenation phase for 5 min did not cause neuron damage and autophagy changes. Neuron injury and autophagy changes both occurred in the IH model described as hypoxia phase for 5 min and reoxygenation phase for 10 min, while more serious neuron injuries were caused by the IH model described as hypoxia phase for 10 min and reoxygenation phase for 20 min. Therefore, we think that the IH model, hypoxia phase (1.5% O<sub>2</sub>, 5% CO<sub>2</sub>, and balance N<sub>2</sub>) for 5 min and reoxygenation phase (21% O<sub>2</sub>, 5% CO<sub>2</sub>, and balance N<sub>2</sub>) for 10 min, may be the best condition for researching on autophagy in hippocampal neurons. Furthermore, autophagy may be involved in the injury induced by IH in hippocampal neurons. However, the specific mechanisms are still unclear and need further study.

## Compliance with ethical standards

**Funding** This work was supported by the National Natural Science Foundation of China (Grant Nos. 81670086 and 81370183), the National Clinical Key Subject Construction Project of NHFPC Fund,

and the National Training Program of Innovation and Entrepreneurship for Undergraduates (Grant No. 201510062005).

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

**Ethical approval** All applicable international, national, and institutional guidelines for the care and use of animals were followed. The Animal Ethics Committee of Tianjin Medical University approved this study.

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