

## Mild hypothermia alleviates excessive autophagy and mitophagy in a rat model of asphyxial cardiac arrest

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**Abstract** Mild hypothermia is an effective therapeutic strategy to improve poor neurological outcomes in patients following cardiac arrest (CA). However, the underlying mechanism remains unclear. The aim of the study was to evaluate the effect of mild hypothermia on intracellular autophagy and mitophagy in hippocampal neurons in a rat model of CA. CA was induced in Sprague–Dawley (SD) rats by asphyxia for 5 min. After successful resuscitation, the surviving rats were randomly divided into two groups, the normothermia (NT) group and the hypothermia (HT) group. Mild hypothermia (32 °C) was induced following CA for 4 h, and animals were rewarmed at a rate of 0.5 °C/h. Neurologic deficit scores (NDS) were used to determine the status of neurological function. Cytoplasmic and mitochondrial protein from the hippocampus was extracted, and

the expression of LC3B-II/I and Parkin were measured as markers of intracellular autophagy and mitophagy, respectively. Of the 60 rats that underwent CA, 44 were successfully resuscitated (73 %), and 33 survived until the end of the experiment (55 %). Mild hypothermia maintained eumorphism of nuclear and mitochondrial structures and significantly improved NDS ( $p < 0.05$ ). Expression of LC3B-II/I and Parkin in hippocampal nerve cells were significantly increased ( $p < 0.05$ ) in the NT group relative to the control. Meanwhile, mild hypothermia reduced the level of LC3B-II/I and Parkin ( $p < 0.05$ ) relative to the NT group. Mild hypothermia protected mitochondria and improved neurological function following CA and resuscitation after ischemia/reperfusion (I/R) injury, likely by reducing excessive autophagy and mitophagy in neurons.

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## Introduction

Despite recent improvements in resuscitation techniques, the survival rate of patients with cardiac arrest (CA) is unchanged [1–3]. Following return of spontaneous circulation (ROSC), manifestation of poor neurological outcomes is closely linked to high post-resuscitative mortality and poor quality of life [4, 5]. A primary reason for these poor outcomes is the absence of available medications that ameliorate ischemia/reperfusion (I/R) injury and promote neuroprotection during the post-resuscitation period. Interestingly, mild hypothermia represents a new treatment paradigm that has been confirmed clinically to improve neurological outcomes during ROSC [6–8].

The maximum therapeutic efficacy and the neuroprotective mechanism of action of mild hypothermia during ROSC remain unclear. The underlying pathophysiological processes, including oxidative stress, excitotoxicity, disrupted calcium homeostasis, pathological protease cascades, and activation of cell death signaling pathways, are activated within minutes to hours after global brain I/R injury following CA and resuscitation [9]. Zhang et al. [10] indicated that autophagy may play different roles in cerebral ischemia and subsequent reperfusion. In fact, during reperfusion, a protective role may be attributed to autophagy through mitophagy-related mitochondrial clearance and inhibition of downstream apoptosis.

Following a loss of mitochondrial membrane potential ( $\Delta\Psi_m$ ) or in the presence of low oxygen stress, a selective mitochondrial autophagy pathway called mitophagy is activated [11]. Mitophagy specifically degrades damaged mitochondria and removes excess radical oxygen species (ROS) to maintain cell viability. Although mitophagy is an active process that enhances the adaptive ability of cells in a low oxygen environment, excessive autophagy promotes a bioenergetic failure and cellular necrosis through the degradation of essential proteins and organelles and may ultimately result in cell death.

The level of microtubule-associated light chain 3B-II (LC3B-II) is a reliable marker for the level of intracellular autophagy. Expression of Parkin, which was first described in patients with Parkinson's disease, precedes mitophagy and is a suitable marker of mitophagy.

In the present study, we utilized a rat model of asphyxial CA to test the hypothesis that whole-body mild hypothermia attenuates excessive autophagy and mitophagy in the hippocampus following ROSC.

## Materials and methods

### Animal preparation

This study was carried out in strict accordance with the guidelines for animal care and use established by our University Animal Care and Use Committee. The protocol was approved by the Committee on the Ethics of Animal Experiments of our university (Permit Number: 120410). All surgeries were performed under anesthesia and analgesia, and all efforts were made to minimize suffering of the animals.

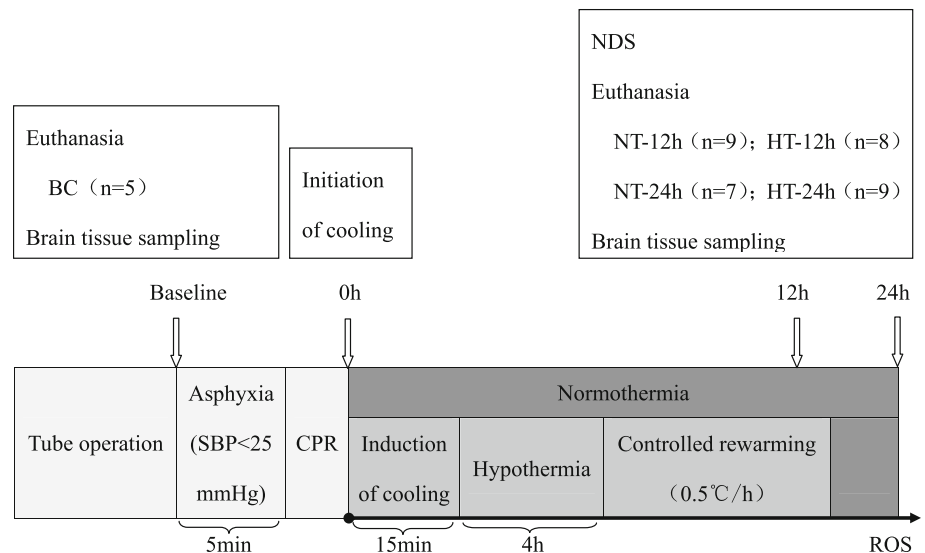
In this study, 65 male Sprague–Dawley rats (Shanghai Slac Laboratory Animal Co. Ltd, Shanghai, China), between 16 and 18 weeks old and weighing  $450 \pm 50$  g, were given free access to rat chow and water. Animals were anesthetized with an intraperitoneal injection of 3.6 % chloral hydrate (10 ml/kg) and then restrained in the supine position on the operating table. After endotracheal intubation, two trocars were placed into the rat's left femoral artery and right femoral vein, respectively. The animals were ventilated with a volume-controlled ventilator (Institute of Cardiopulmonary Cerebral Resuscitation, Guangdong, China), with a tidal volume of 6 ml/kg, a fraction of inspiration oxygen ( $FiO_2$ ) of 0.21, and a ventilation rate of 100 breaths/min.

During the procedure, rats were placed on a temperature-controlled carpet (RWD Life Science Co., Ltd, Shenzhen, China), and a temperature sensor (Philips Medical Systems, Andover, MA, USA) was placed rectally to monitor core temperature. Standard lead II electrocardiogram surface electrodes were placed to monitor heart rhythm and respiration, and a heparin sodium-filled catheter was connected to the left femoral artery to monitor arterial blood pressure. All vital signs were acquired by the Philips monitoring system (Philips Medical Systems, Amsterdam, Netherlands) and the 8-Channels Biological Signal Acquisition System (ZhengHua Biological Instrument Equipment Co., LTD, Huaibei, Anhui, China).

### Experimental protocol

For 10 min during hemodynamic stabilization, rectal temperature was adjusted to  $37.0 \pm 0.5$  °C using a temperature-controlled carpet and ice bags. Arterial blood gas analysis was performed to determine baseline breathing and acid–base parameters. Asphyxia was induced by reducing the ventilator and clipping the endotracheal catheter. Onset of CA was denoted as the time where systolic arterial blood pressure was  $\leq 25$  mmHg (Fig. 1). After  $\sim 5$  min of untreated CA, animal cardiopulmonary resuscitation (CPR) was initiated using endotracheal ventilation with 100 % oxygen at 50 breaths/min. Mechanical

**Fig. 1** Experimental procedure



**Table 1** CA time and associated data of resuscitation ( $\bar{x} \pm s$ )

Group	Total (num)	The time from asphyxia to SBP $\leq 25$ mmHg (s)	The time of SBP $\leq 25$ mmHg (s)	The time of resuscitation (s)	The rate of successful resuscitation (num, %)	The rate of survival from ROSC to observational time (num, %)
NT-12	15	212.43 $\pm$ 33.07	241.43 $\pm$ 32.04	81.14 $\pm$ 30.57	11 (73.3)	9 (60.0)
NT-24	15	244.13 $\pm$ 33.56	248.25 $\pm$ 31.18	84.13 $\pm$ 23.16	12 (80)	7 (46.7)
HT-12	15	229.83 $\pm$ 47.14	249.33 $\pm$ 35.72	70.17 $\pm$ 8.30	10 (66.7)	8 (53.3)
HT-24	15	219.33 $\pm$ 28.88	247.17 $\pm$ 21.25	79.83 $\pm$ 15.99	11 (73.3)	9 (60)

Values are mean  $\pm$  SD

SBP systolic blood pressure, ROSC recovery of spontaneous circulation, num number of rats, s second, HT-12 or 24 post-ROSC 12 or 24 h of mild hypothermia group, NT-12 or 24 post-ROSC 12 or 24 h of normothermia group

chest compressions at 200 times/min at a depth of compression one-third of the rat’s anteroposterior diameter of the chest were performed with an animal cardiopulmonary resuscitator (Institute of Cardiopulmonary Cerebral Resuscitation, China). After 10 s of CPR, epinephrine (0.1 mg/kg) and 5 % sodium bicarbonate (1 ml/kg) were injected via the right femoral vein. When ROSC was achieved, defined by the presence of an autonomic cardiac rhythm and a mean arterial blood pressure  $>60$  mmHg, chest compressions were stopped and the ventilation rate was adjusted to 100 breaths/min. A  $FiO_2$  of 100 % was maintained for 15 min after ROSC, then decreased to 45 % for 15 min, and then decreased to 21 % until the animal had spontaneous respirations. Resuscitation procedures were terminated if animals were unresponsive to CPR for 10 min.

Of the 60 rats that underwent asphyxiation and resuscitation, 33 rats survived until the completion of the study (Table 1). After successful ROSC, rats were randomly assigned to either the normothermia group (NT,  $n = 16$ ) or the mild hypothermia group (HT,  $n = 17$ ). Rats in these groups were further divided into two subgroups based on

the time of euthanasia, either 12 or 24 h after ROSC (NT-12,  $n = 9$ ; HT-12,  $n = 8$ ; NT-24,  $n = 7$ ; HT-24,  $n = 9$ ; Fig. 1). Rats in the blank control group (BC,  $n = 5$ ) underwent the same anesthesia and surgical procedures as experimental rats with the exception of CA and mild hypothermia.

Immediately after ROSC, mechanical ventilation settings were resumed to those prior to CA, and rats received standardized post-resuscitative intensive care until the end of the rewarming phase. 30 min after ROSC, arterial blood gas was measured to adjust ventilator settings. 4 h after ROSC, arterial blood gas was measured again to observe acid–base parameters. Mild hypothermia was induced as previously described in Bernard et al. [7]. Animals were actively cooled to a target body temperature of 32 °C within 15 min, maintained at this temperature for 4 h, then slowly rewarmed to 36.5 °C at 0.5 °C/h, and returned to the home cage (ambient room temperature at 25 °C. In the NT group, rectal temperature was maintained at  $37 \pm 0.5$  °C for 4 h after ROSC before being returned to the cage.

After evaluation for neurologic deficit scores (NDS) at 12 or 24 h after ROSC, animals were anesthetized with an

intraperitoneal injection of 3.6 % chloral hydrate (10 ml/kg) and euthanized with an intracardial injection of 2-ml potassium chloride (10 mol/l).

#### Extraction of cytoplasmic and mitochondria protein

Rat brains were removed at either 12 or 24 h after ROSC by craniotomy and hippocampi were isolated and snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Hippocampal cytoplasmic and mitochondrial protein was extracted for subsequent LC3B-II and Parkin analysis with a mitochondria isolation kit for tissue (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's instructions. Before extraction, all solutions were cooled below  $0^{\circ}\text{C}$  until the slight appearance of ice. Hippocampal tissues were rinsed in ice-cold phosphate-buffered saline (PBS, pH = 7.4) to remove excess blood. The tissues were weighed and decuple volumes ( $1\ \mu\text{g} = 1\ \mu\text{l}$ ) of mitochondrial separation reagent with phenylmethylsulfonyl fluoride (PMSF) were added. Samples were gently homogenized on ice with a glass homogenizer. The homogenate was centrifuged at  $1,000\times g$  at  $4^{\circ}\text{C}$  for 5 min, and the supernatant was collected. The supernatant was centrifuged at  $3,500\times g$  at  $4^{\circ}\text{C}$  for 10 min, and the sediment was collected as mitochondria. The sediment was dissociated with the mitochondrial cracking liquid to yield the mitochondrial protein. The supernatant was centrifuged again at  $12,000\times g$  at  $4^{\circ}\text{C}$  for 10 min, and the collected supernatant contained the cytoplasmic protein.

#### Western blot analysis of LC3B and Parkin

Cytoplasmic protein was used to determine the level of LC3B, and mitochondrial protein was used to determine the level of Parkin. When autophagosomes are formed, the cytoplasmic form of LC3B (LC3B-I, 18 kDa) is hydrolyzed to yield a short peptide and the autophagosome type LC3B (LC3B-II, 16 kDa); the LC3B-II/I ratio was used to estimate the level of intracellular autophagy. Protein concentration in hippocampal extracts was determined using the bicinchoninic acid (BCA) assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). Proteins (50 g) were resolved on a 12 % SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. After membranes were blocked with 5 % nonfat milk in Tris-buffered saline with Tween-20 (TBST), they were incubated at  $4^{\circ}\text{C}$  overnight with primary antibody, including rabbit polyclonal anti-LC3B (1:1,000; Abcam, Cambridge, MA, USA), rabbit polyclonal anti-Parkin (1:1,000; Abcam), and mouse polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:800; Sigma-Aldrich, St. Louis, MO,

USA). Membranes were rinsed with TBST and incubated at room temperature for 1 h with secondary antibody, including goat anti-rabbit IgG (1:3,000; Beyotime Institute of Biotechnology) and rabbit anti-mouse IgG (1:4,000; Sigma-Aldrich). ECL reagent (Biological Industries Israel Beit Haemek Ltd., Beit HaEmek, Israel) was used for protein detection. Optical density of the immunoreactive bands was calculated by Quantity One 1-D analysis software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). LC3B-II, LC3B-I, and Parkin levels were normalized to GAPDH, and the level of intracellular autophagy was defined as the ratio of LC3B-II/I.

#### Electron microscopy (EM)

Approximately 1-mm thick sections of hippocampus were sliced on ice and fixed overnight at  $4^{\circ}\text{C}$  with 2.5 % (v/v) glutaraldehyde, postfixed with 1 % (v/v) osmic acid for 1 h, and dehydrated and embedded with acetone and embedding medium. Ultra-thin sections ( $\sim 40\text{--}50\ \text{nm}$ ) were cut, stained with 2.0 % (w/v) lead citrate, and blindly evaluated with regard to treatment condition with a HITACHI H-600 transmission EM (Hitachi Scientific Instruments, Mountain View, CA, USA). To detect minor changes in cellular structure, at least four different electron microscopic micrographs representing independent areas in each section were selected for analysis.

#### Neurologic deficit scores (NDS)

NDS is a hierarchical scoring system for the evaluation of neurological dysfunction. NDS criteria include the level of consciousness, motor and sensory function, respiratory pattern, and behavior. Each animal was rated at either 12 or 24 following ROSC in each category and the scores were combined. A maximum score of 80 indicated that animals were normal, whereas a minimum score of zero represented brain death [12]. Investigators, blind to treatment condition and who were specially trained on NDS, evaluated the rats.

#### Statistical analysis

All data are expressed as the mean  $\pm$  standard deviation (SD) except for the NDSs, which are expressed as median and range. A one-way ANOVA was used to assess overall differences among groups for each variable, followed by Bonferroni test for multiple comparisons. Levene's test for equality of variances was used to confirm the multiple-comparison procedure used. Differences were considered statistically significant when  $p < 0.05$ . Analysis was performed using the software package IBM SPSS Statistics 19 (IBM Inc., Armonk, New York, USA) and Microsoft Office Excel 2007 (Microsoft Inc., Chevy Chase, MD, USA).

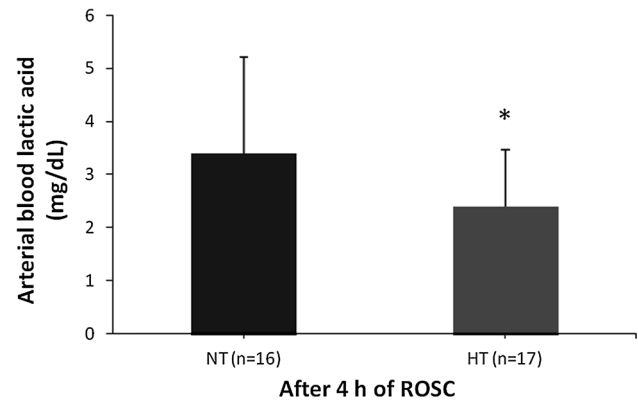
**Table 2** Animals' physiologic, hemodynamic and resuscitation data

Group	Tc (rectal, °C)	HR (bpm)	RR (bpm)	MAP (mmHg)	pH	PaO <sub>2</sub> (mmHg)	PaCO <sub>2</sub> (mmHg)	SO <sub>2</sub> c (%)	Lac (mg/dl)
<b>NT (n = 16)</b>									
Baseline	36.63 ± 0.68	254.87 ± 41.89	77.87 ± 3.93	94.27 ± 21.48	7.36 ± 0.06	91.11 ± 28.59	41.53 ± 6.19	93.67 ± 7.83	2.2 ± 0.89
PR-0.5 h	36.47 ± 0.47	232.40 ± 57.90	77.73 ± 0.80	66.27 ± 9.44	7.23 ± 0.11	83.92 ± 24.19	56.40 ± 16.18	92.40 ± 8.52	6.9 ± 2.78
PR-4 h	36.55 ± 0.53	248.33 ± 61.36	88.6 ± 11.42	90.80 ± 28.35	7.29 ± 0.07	87.38 ± 27.24	49.13 ± 9.67	90.40 ± 9.26	3.4 ± 1.81*
<b>HT (n = 17)</b>									
Baseline	36.35 ± 0.49	238.33 ± 37.37	77.42 ± 1.31	99.50 ± 21.38	7.39 ± 0.03	85.00 ± 9.43	40.75 ± 6.15	96.42 ± 1.68	2.1 ± 0.97
PR-0.5 h	32.38 ± 0.61	259.58 ± 61.85	77.42 ± 0.79	78.00 ± 14.78	7.35 ± 0.11	81.17 ± 12.72	50.25 ± 11.47	93.50 ± 6.29	7.2 ± 2.92
PR-4 h	32.12 ± 0.20	256.67 ± 56.63	74.92 ± 9.24	95.83 ± 17.64	7.33 ± 0.08	70.67 ± 10.41	43.75 ± 6.21	91.58 ± 4.38	2.3 ± 1.06*

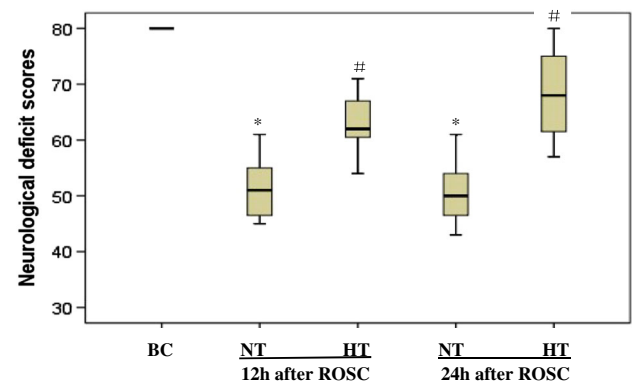
Values are mean ± SD

HT mild hypothermia group, NT normothermia group, PR post ROSC, bpm beats per min, Tc central temperature, HR heart rate, R respiratory rate, MAP mean aortic pressure, PaO<sub>2</sub> pressure of oxygen, PaCO<sub>2</sub> pressure of carbon dioxide, SO<sub>2</sub>c oxygen saturation, Lac lactic acid

\* p < 0.05 vs. NT group



**Fig. 2** Arterial blood lactic acid level after 4 h of ROSC



**Fig. 3** Neurologic deficit scores

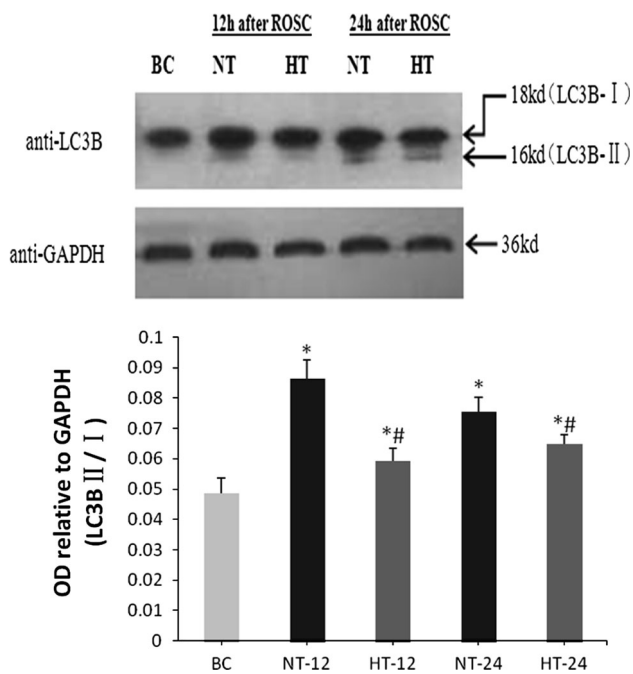
**Results**

**Animal physiologic, hemodynamic, and resuscitation data**

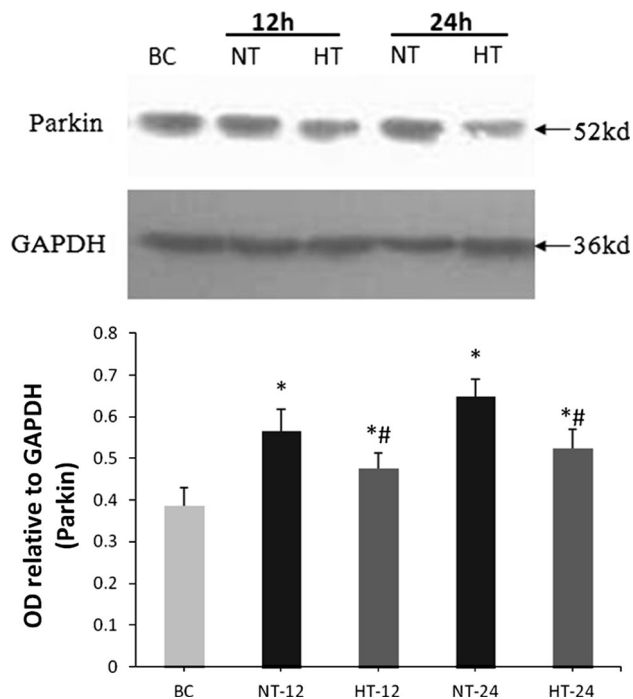
The time of CA and associated resuscitation data were documented and were not statistically different among the experimental groups (Table 1). Central temperature, heart rate, respiratory rate, mean artery pressure, pH, pressure of oxygen, pressure of carbon dioxide, and oxygen saturation during baseline at 0.5 and 4 h after ROSC were not statistically different among the groups (Table 2). Arterial blood lactate 4 h after ROSC in the NT group was significantly decreased relative to the HT group (\*p < 0.05; Fig. 2).

**Neurological outcome**

NDSs were determined immediately before animals were euthanized at 12 and 24 h after ROSC. The median NDS of rats in the NT group was significantly lower than the HT group at 12 h (NT: 9, 49.8 ± 8.8, HT: 8, 63.0 ± 5.3) and 24 h (NT: 7, 50.6 ± 5.8, HT: 9, 68.3 ± 8.3) after ROSC (#p < 0.05; Fig. 3). These findings indicated that mild



**Fig. 4** Effects of mild hypothermia on LC3B-II/I expressions in the rat hippocampus. LC3B-I, cytoplasm type LC3B, 18kd; LC3B-II, autophagosome type LC3B, 16kd



**Fig. 5** Effects of mild hypothermia on Parkin expressions in the rat hippocampus

hypothermia for 4 h after CA improved neurological outcome relative to untreated animals, likely by reducing ischemia/reperfusion (I/R) injury of nerve cells.

#### Effects of mild hypothermia on intracellular autophagy in the hippocampus

The ratio of the expression of LC3B-II and I was increased in the both NT and HT groups at 12 h (NT:  $0.087 \pm 0.006$ , HT:  $0.059 \pm 0.004$ ) and 24 h (NT:  $0.075 \pm 0.005$ , HT:  $0.065 \pm 0.003$ ) relative to the BC group ( $0.049 \pm 0.005$ ) ( $*p < 0.05$ ; Fig. 4). However, the LC3B-II/I expression was significantly decreased in the HT group relative to the NT group ( $\#p < 0.05$ ; Fig. 4).

#### Effects of mild hypothermia on mitophagy in the hippocampus

Parkin expression levels were significantly increased in the NT and HT groups at 12 h (NT:  $0.566 \pm 0.0526$ , HT:  $0.475 \pm 0.038$ ) and 24 h (NT:  $0.647 \pm 0.042$ , HT:  $0.524 \pm 0.047$ ) relative to the BC group ( $0.386 \pm 0.044$ ) ( $*p < 0.05$ ; Fig. 5). Similar to the other autophagy marker LC3B-II/I, Parkin levels were significantly decreased in the HT group relative to the NT group ( $\#p < 0.05$ ; Fig. 5).

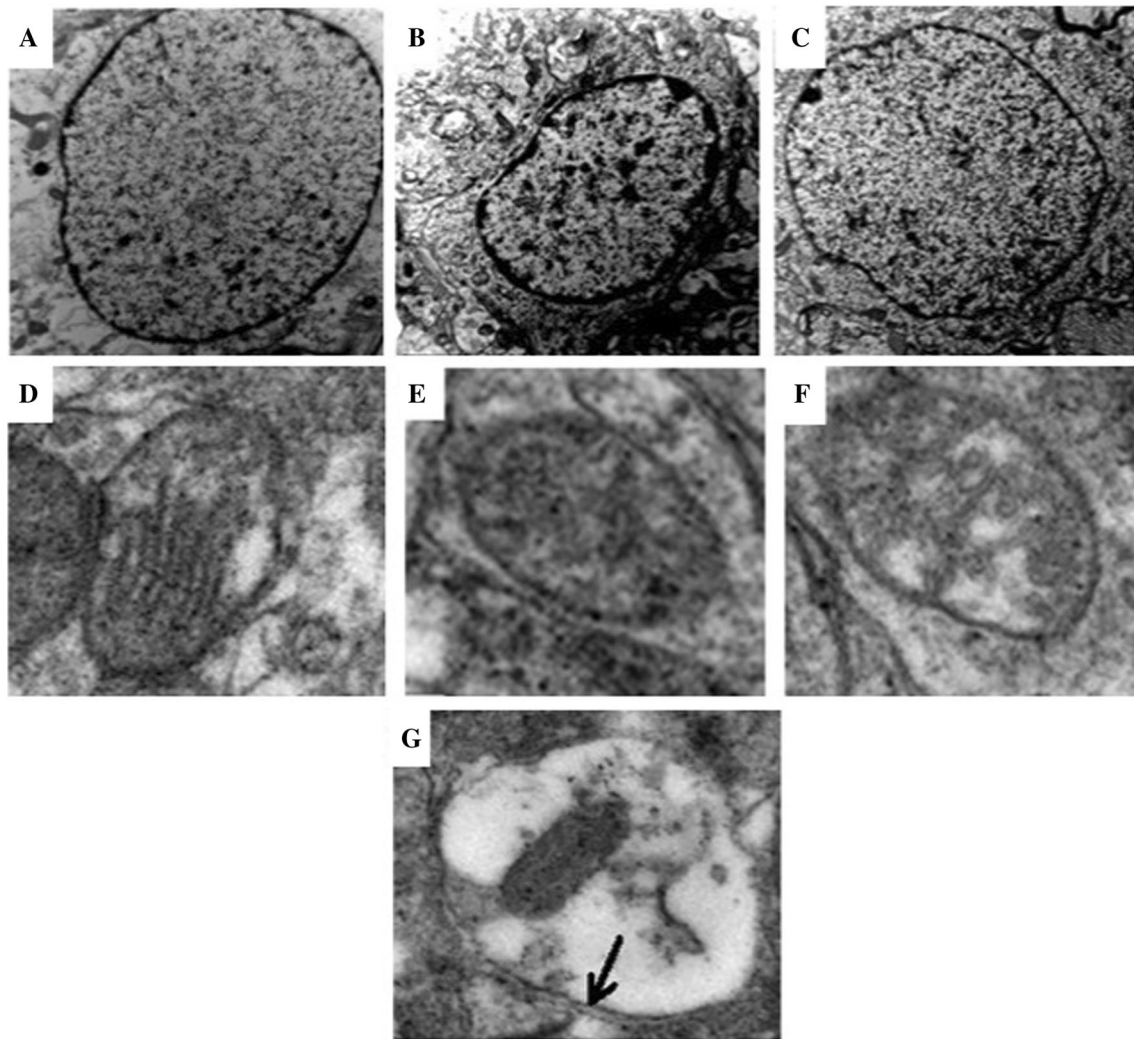
#### Effect of mild hypothermia on nuclear and mitochondrial morphology as determined by EM

Analysis of intracellular structures by EM at 24 h after ROSC showed that the extent of ultrastructural damage of nuclei in the hippocampus was reduced in the HT group relative to the NT group. In the BC group, the nucleus appeared normal with intact nuclear membrane and smooth chromatin (Fig. 6a). In contrast, the nucleus in the NT group was markedly damaged with chromatin margination and condensed nucleoplasm (Fig. 6b). The nucleus appeared slightly damaged in the HT group (Fig. 6c). In the BC group, the mitochondrion was maintained with intact membrane cristae and a smooth matrix (Fig. 6d). In contrast, the mitochondrion was markedly damaged with disrupted cristae and a damaged matrix in the NT group (Fig. 6e). The mitochondrion was slightly damaged in the HT group (Fig. 6f). Autophagolysosomes were also observed in the NT group (Fig. 6g).

#### Discussion

Mitochondrial dysfunction and oxidative stress are key factors determining the extent of cellular injury during cerebral ischemia [13]. Impairment of mitochondrial function leads to reduced ATP production, impaired calcium buffering, and the overproduction of ROS. Under normal physiological conditions, ROS do not cause injury because they are quickly scavenged by the intramitochondrial antioxidant system. However, during pathologic





**Fig. 6** Representative electron micrographs of nucleus ( $\times 8,000$ ) and mitochondria ( $\times 40,000$ ). Tissue was isolated from the rat hippocampus at 24 h following ROSC. **a** Normal nucleus with intact nuclear membrane and a smooth chromatin in the blank control group. **b** Markedly damaged nucleus with chromatin margination and condensed nucleoplasm in the normothermia group. **c** Slightly damaged nucleus with basically normal nuclear membrane and a slightly damaged chromatin in the hypothermia group. **d** Normal

mitochondria with intact membrane cristae and a smooth matrix in the blank control group. **e** Markedly damaged mitochondrion with disrupted cristae and a damaged matrix in the normothermia group. **f** Slightly damaged mitochondrion with basically normal cristae and a slightly damaged matrix in the hypothermia group. **g** damaged mitochondria engulfed by autophagolysosome in the normothermia group (*black arrow* double membrane structure of autophagosome)

conditions, such as prolonged cerebral I/R, ROS and its products overload the scavenging capacity of the endogenous antioxidant system of mitochondria, and oxidative stress occurs. As a result, the excess production of mitochondrial ROS can damage mitochondria by initiating peroxidation of intramitochondrial lipids and proteins, inhibiting the activity of mitochondrial respiratory enzymes, and breaking mitochondrial DNA [14], leading to induction of cellular apoptosis or necrosis [15].

Autophagy is a cellular process that protects against neurotoxicity by eliminating, degrading, and digesting damaged, degenerative, aged, dysfunctional organelles, denatured protein, nucleic acid, and other biological

macromolecules via the lysosome [16, 17]. Autophagy occurs after I/R injury [18], likely stimulating a cascade of gene transcription, where appropriate autophagy can protect cells and excessive autophagy can be detrimental to cell health [19]. Neurons in the rat hippocampus suffered I/R and intracellular calcium overload, which led to increased ROS and decreased  $\Delta\Psi_m$  in mitochondria [20]. These changes induced excessive autophagy and resulted in degradation of essential proteins and organelles causing bioenergetic failure and cellular necrosis [21].

Mitophagy is a subtype of autophagy where impaired mitochondria are scavenged to maintain cellular survival. Parkin, first identified in an autosomal recessive form of

Parkinson' disease, is a cytosolic protein under basal conditions, but rapidly translocates to mitochondria upon loss of  $\Delta\Psi_m$ . Parkin then ubiquitinates mitochondrial proteins, which serve as a signal for mitophagy. It has been shown that Parkin adheres to the surface of impaired mitochondria following  $\Delta\Psi_m$  induced by I/R [22].

Previously, both basic and clinical studies indicated that mild hypothermia may be neuroprotective because of its associated reduction in brain metabolism, inhibition of excitatory amino acid release, attenuation of the immune response, and/or modification of cell death signaling pathways [4, 8, 23–26]. However, the effect of mild hypothermia on mitochondrial oxidative stress and details regarding the potential mechanisms involved in CA patients were unknown.

In this study, we demonstrated that: (1) neurological outcome of the rat is improved after treatment with whole-body mild hypothermia following CA, (2) expression of proteins implicated in intracellular autophagy was significantly upregulated in the hippocampus after prolonged ischemia and anoxia, (3) this increase in intracellular autophagy in the hippocampus was attenuated with mild hypothermia treatment, and (4) expression of a protein involved in autophagy of mitochondria (mitophagy) was reduced with mild hypothermia treatment.

Previous evidence from both animals and humans reported that mild hypothermia improved the neurological outcome. We observed similar findings with a significantly higher NDS in the HT group relative to the NT group at 12 or 24 h after ROSC [4, 6–8]. Morphologically, we observed that mild hypothermia alleviated microscopic and ultrastructural changes in the hippocampus caused by global I/R at 24 h after ROSC.

We found that hypothermia can protect the structural integrity of both the nucleus and mitochondria following CA. Hypothermia may protect some temperature-dependent respiratory enzyme which scavenges ROS [9]. In addition, arterial blood lactate level 4 h after ROSC was decreased in the HT group, suggesting that upregulation of lactate dehydrogenase may be neuroprotective by eliminating excessive blood lactate.

Here, NDS and ultrastructural changes of the nucleus and mitochondria were measured to evaluate prognosis based on the level of autophagy. In this experiment, expression of LC3B-II/I was significantly increased in the NT group relative to controls, and hypothermia reduced this expression and improved the neurological function in the HT group. Therefore, we can assume that the autophagic level exceeded the normal range and caused programmed cell death in the NT group, and mild hypothermia restored the normal level of intracellular autophagy. Taken together, these data suggest that autophagy in hippocampal nerve cells after I/R injury was excessive and caused cellular dysfunction, whereas mild hypothermia can reduce

excessive autophagy to normal levels and improve neurological outcome.

In this experiment, Parkin expression was significantly elevated in the NT group relative to controls, and hypothermia appeared to reduce this increase. The pattern of LC3B-II/I expression, a marker for autophagy, was similar. Therefore, mitophagy likely participated in intracellular autophagy, leading to excessive impairment of mitochondria. Mild hypothermia decreased the level of Parkin, and we hypothesize that mild hypothermia decreased excessive mitophagy and promoted cell survival. Studies have confirmed that the main pathophysiological process in ROSC after CA is neural apoptosis, and recent studies have demonstrated that the reduction of autophagy via hypothermia can attenuate I/R-induced cell death [27, 28]. In this study, I/R injury activated LC3B-II/I and Parkin expression, and hypothermia reduced this expression while simultaneously increasing neurological function. Therefore, excessive activation of intracellular autophagy, including mitophagy, after I/R contributed to neuronal injury, and hypothermia decreased this injury by reducing excessive autophagy, including mitophagy.

## Conclusions

Long-lasting cerebral I/R can cause excessive autophagy including mitophagy in neurons, resulting in damage to neurologic function. Mild hypothermia can reduce excessive autophagy by inhibiting LC3B-II/I and Parkin expression and preventing cell death and associated poor neurological outcomes. Our findings have implications for the prevention of poor neurological outcomes by identifying abnormally regulated pathways following ROSC. Future studies will focus on determining the apoptosis induced by I/R and the proportion of mitophagy in excessive autophagy. The development of therapeutic strategies targeting autophagy has important clinical ramifications and will aid in the reduction of secondary neurologic dysfunction.

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

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