Neurology Asia 2022; 27(4) : 1017 – 1024

Explore the molecular mechanism of controlled hypothermic surgery to promote autophagy and inhibit oxidative damage to reduce the damage of neurovascular unit barrier

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Abstract

Background: Controlled hypothermia surgery is a method to lower the body temperature to a certain level in order to reduce the body's metabolism and maintain or delay cell activity. Autophagy can antagonize the apoptotic effect of cells by promoting cell survival. *Methods:* Normal and hypothermic cerebral ischemia models in mice were established. Nissl staining and HE staining were used to analyze the pathological damage characteristics of mouse brain tissue. Microplate reader was used to measure the activity of SOD, and the fluorescent probe DCFH-DA was used to detect the content of ROS. The brain tissue sections were stained with MDC to observe the characteristics of autophagy. The characteristics of autophagosomes were observed by TEM. Western blot analysis was used to determine the expression of related proteins in brain tissue. RT-PCR analysis was used to determine the mRNA expression of genes Bcl-2 and Bax in brain tissue. *Results:* HE staining and Nissl staining indicated that controlled hypothermia effectively reduced pathological damage. The activity of SOD and the content of ROS indicated that LI group has the lowest degree of oxidative damage. MDC staining, TEM imaging and Western blot analysis showed that controlled hypothermia surgery also increased the content of autophagosomes, and then promoted autophagy and apoptosis. RT-PCR also showed that LI group has the highest Bax/Bcl-2 ratio.
Conclusion: Controlled hypothermia can protect brain tissue and reduces damage caused by cerebral ischemia by promoting cell autophagy.

Keywords: Controlled hypothermia surgery, autophagy, cerebral ischemia, neurovascular unit.

INTRODUCTION

Controlled hypothermia surgery is a method that reduces the body temperature to a certain level in order to reduce the body's metabolism and maintain or delay cell activity. It aims to improve the body's tolerance to hypoxia by reducing tissue metabolism and oxygen consumption.[1,2] The implementation of controlled hypothermia surgery can reduce the pathophysiological state of neurons in cellular oxygen-glucose deprivation (OGD) to preserve the activation of neurons, microglia, astrocytes and other cells, and reduce hypoxic brain damage mediated inflammatory damage.[3]

Neurovascular unit (NVU) is a physiological unit with specific structure and function composed of neurons, glial cells, intracerebral vascular endothelial cells, smooth muscle cells, pericytes and extracellular matrix, forming a fully functional blood brain barrier (BBB).[4] NVU constitutes a huge network of many components, and the interaction between the components precisely regulates the balance of oxygen demand and oxygen supply in brain tissue, so that brain function can reach the optimal state. Among them, the tight connections between vascular endothelial cells is to maintain the nerve center and blood material exchange, and it is also a key structure to maintain the balance of NVU. The various components of NVU are related to each other.[5,6] After hypoxia and ischemia, each component is damaged to different degrees, causing the relative balance between the components to be broken, causing a series of cascades of inflammatory cascades, and finally causing brain injury.[7,8]

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Date of Submission: 13 January 2022; Date of Acceptance: 11 July 2022

https://doi.org/10.54029/2022yac

Autophagy can antagonize the apoptotic effect of cells by promoting cell survival. For example, it is possible to remove the organelles damaged by oxidative stress, or to degrade and denature macromolecular substances, to provide the hungry cells with the nutrients and energy needed for survival, or to inhibit endoplasmic reticulum stress by degrading unfolded proteins. These functions of autophagy will inhibit the generation of pro-apoptotic signals, thereby antagonizing cell apoptosis.[9,10]

This project explores the molecular mechanisms that reduce the body's production of some free radical molecules and reactive oxygen species (ROS) after controlled hypothermic surgery, inhibit oxidative damage of cerebrovascular nerve units, promote cell autophagy, and reduce brain barrier damage. A mouse model of controlled hypothermia cerebral ischemia was established, and one group of mice was injected with the autophagy inhibitor Baf A1 through the tail vein. Collect mouse brain tissue, Nissl staining and HE staining to analyze the pathological damage characteristics of mouse brain tissue. Microplate reader was used to detect superoxide dismutase (SOD) activity. Fluorescent probe DCFH-DA method was applied to detect intracellular activity oxygen free radicals (reactive oxygen species, ROS) content. Stain brain tissue slices with acid-tropic fluorescent dye MDC and transmission electron microscopy was utilized to observe the characteristics of autophagy in brain tissue. Western blot analyzed the expression levels of middle protein LC3I, LC3II, Beclin-1, Caspase-3, cleaved Caspase-3 and RT-PCR analyzed the mRNA expression levels of genes Bcl-2 and Bax in brain tissue.

METHODS

Experiment design

Purchase 80 healthy, clean-grade C57BL/6 male mice of 3 weeks old and divide them into four groups at random: normal control group (NC group), normal temperature cerebral ischemia group (NI group), controlled hypothermia cerebral ischemia group (LI group), and controlled hypothermia cerebral ischemia Group + autophagy inhibitor Baf A1 (LI+ Baf A1 group). Establish a mouse model of controlled hypothermia cerebral ischemia according to the following steps: Inject chlorpromazine into the abdominal cavity of the mouse, then place them in an incubator to cool them to a mild hypothermia state of 18.5±0.5°C, and then clamp the bilateral common carotid artery of the mice for 120 minutes, and the establishment of the DHCA model was completed. Besides, a group of mice were injected with the autophagy inhibitor Baf A1 through the tail vein. After the mouse model was established, the mice were anesthetized with isopentane and their necks severed. Collect mouse brain tissue, Nissl staining and HE staining were used to analyze the pathological damage characteristics of mouse brain tissue. For the collected brain tissue samples, a microplate reader was used to measure the activity of superoxide dismutase (SOD), and the fluorescent probe DCFH-DA method was used to detect the content of reactive oxygen species (ROS). The brain tissue sections were stained with acid-tropic fluorescent dye MDC to observe the characteristics of autophagy. The characteristics of autophagosomes in brain tissue were observed by TEM. Western blot analysis was used to determine the expression of the proteins LC3I, LC3II, Beclin-1, Caspase-3, and cleaved Caspase-3 in brain tissue. RT-PCR analysis was used to determine the mRNA expression of genes Bcl-2 and Bax in brain tissue.

HE staining and Nissl staining

The histopathological characteristics of the brain tissue was observed with hematoxylin-eosin staining (HE staining) under light microscope. The detailed steps of HE staining are as followed. After fixing with 4% paraformaldehyde (PFA) at 4°C overnight, the retina tissues were dehydrated by gradient ethanol, permeabilized by xylene, and embed with paraffin. Following this, tissues were cut into 4 μm slices and stained with hematoxylin-eosin solution (Nanjing Jiancheng, Nanjing, Jiangsu, China) according to the manufacturers' protocols. Finally, slices were mounted with neutral resins and analyzed by a light microscope (Nikon Eclipse 1000, Tokyo, Japan).

The procedure of Nissl staining was as follows. The brain tissue was fixed with 10% formalin for 48 hours, and dehydrated with gradient ethanol. The tissue was transparentized with xylene and then embedded in paraffin. Use a microtome to cut the composition into slices of 4-7 μm. The sections were deparaffinized and hydrated, and then placed in Cresyl violet stain for staining. After rinsing with deionized water, use Nissol Differentiation for 1-3min until the background was close to colorless under the microscope. Finally, it was made transparent with xylene and sealed with neutral gum.

The detection of ROS content and SOD activity

Dilute the 5 mM DCFH-DA reagent stock solution (prepared above) in HBSS/Ca/Mg or suitable buffer to make a 5 μM DCFH-DA reagent working solution. Apply 1.0–2.0 mL of 5 μM DCFH-DA reagent working solution to cover cells adhering to coverslips. Incubate cells for 10 minutes at 37˚C, protected from light. Then wash cells gently three times with warm buffer. Stain cells with counterstains as desired and mount in warm buffer for imaging. Then the fluorescence intensity of ROS was measured.

The SOD activity was measured according to the procedure of the kit. Mix the reagents in the kit with a vortex mixer according to the proportions, and place them in a constant temperature water bath at 37°C for 40 minutes. Then add 2ml of color developer to both the measuring tube and the control tube and mix them evenly, and place them in a room temperature environment for 10 minutes. Finally, deionized water was used as a zero adjustment control, a 1cm optical path cuvette was used, and a microplate reader was used for color comparison at a wavelength of 550nm.

Acid-tropic fluorescent dye MDC staining

Dilute 10×Wash buffer to 1× with deionized water. Centrifuge and collect the cells, wash the cells once with 1×Wash buffer, and discard the supernatant. Add an appropriate amount of 1×Wash buffer to resuspend the cells, count and adjust the cell concentration to 10^6/ml. Take an appropriate amount of cell suspension into a new EP tube, add MDC Stain, and mix gently. Avoid light staining at room temperature. The cells were collected by centrifugation, washed twice with 1×Wash buffer, and the supernatant was discarded. Add Collection buffer to resuspend the cells, drop them onto the glass slide and add a cover glass. Observe under a fluorescence microscope (excitation filter wavelength 355nm, blocking filter wavelength 512nm), count and take pictures.

TEM observation

Place the brain tissue section in TEM, zoom in to observe the inside of each cell, and determine the characteristics of autophagosomes in the cell.

Western blot analysis

Collect cells from each group, and add 200 μl of cell lysate to each six-well plate. After sonication, the cells were lysed on ice for 1 hour. The lysed cell sample was centrifuged at 12,500 rpm for 15 minutes at 4°C. Then, transfer the supernatant in the centrifuge tube to a clean centrifuge tube. β-actin protein quantification kit was used to quantify protein concentration. The measured protein samples were stored at -80°C. In Western blot electrophoresis, the protein loading concentration was 50 μg per well. After SDS-PAGE electrophoresis, the membrane was transferred and blocked. Proteins LC3I, LC3II, Beclin-1, Caspase-3 and cleaved Caspase-3 primary antibody (1: 500, anti-human, Thermo-Fisher, USA) were diluted to use concentration. The samples were incubated overnight on a shaker at 4°C. After washing with PBS, the samples were incubated with the secondary antibody (1: 1000, anti-human, Thermo-Fisher, USA) for 30 minutes at room temperature in the dark. Finally, the developer was used for development and photography.

RT-PCR analysis

The brain tissue cells were treated with TRIzol reagent to extract the total RNA in the cells. Revert Aid TW first Strand cDNA Synthesis Kit was used to synthesize the first chain of DNA. QuantiNova SyBr Green PCR Kit was used to perform PCR analysis. Reaction conditions: pre-denaturation at 95°C for 1 minute, denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 30 seconds, running 40 cycles.

Statistical analysis

The experimental results are expressed as mean ± standard deviation. Statistical analysis was performed using SPSS 22.0 software. The figures were produced with Origin 2022 and Adobe Illustrator 2020 software.

RESULTS

Controlled hypothermia effectively reduced pathological damage

The results of HE staining and Nissl staining are shown in Figure 1. It can be seen from the figure that the pathological damage in the NI group was very serious, and the pathological damage was effectively suppressed by performing controlled hypothermia surgery. After adding Baf A1, the pathological damage has increased, indicating that the mechanism of controlled hypothermic surgery to inhibit damage is closely related to the autophagy process.

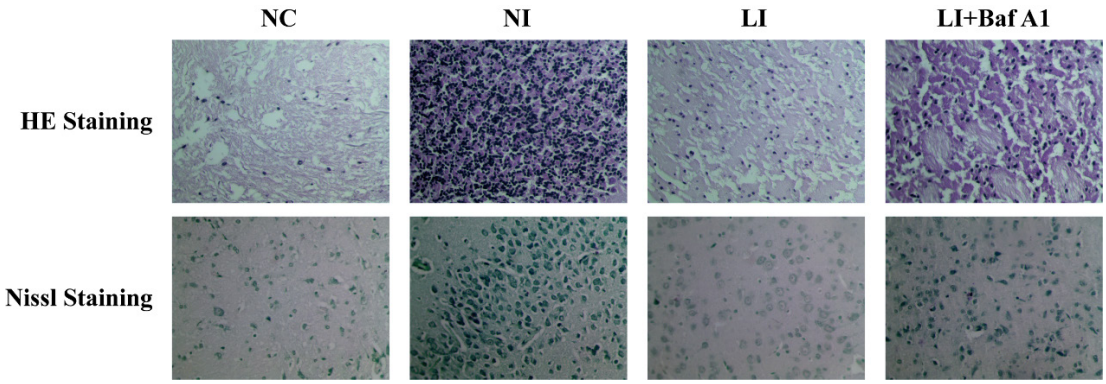

Figure 1. The results of HE staining and Nissl staining to analyze the characteristics of pathological damage in mouse brain tissue. The staining results of the NC group accorded with the physiological characteristics of normal mice.

Activity of SOD and content of ROS indicated the degree of oxidative damage

The detection results of SOD activity and ROS content are shown in Figure 2. SOD is an important component of the antioxidant enzyme system in the biological system, and plays a vital role in the balance of oxidation and antioxidant in the body. The sharp increase in ROS levels can cause serious damage to the cell structure. Therefore, the higher the activity of SOD and the lower the content of ROS, the less oxidative stress damage. The results in the figure indicate that controlled hypothermia surgery effectively reduces the degree of oxidative stress damage through autophagy.

Controlled hypothermia surgery increased the content of autophagosomes

The results of acid-tropic fluorescent dye MDC staining are shown in Figure 3. The results of TEM imaging are shown in Figure 4. It can be seen from the two figures that both the MDC staining and the TEM imaging results indicated that the controlled cryogenic surgery effectively increased the number of autophagosomes, thereby promoting the occurrence of autophagy. Autophagy can inhibit oxidative stress damage, thereby reducing the damage to the body caused by cerebral ischemia.

Controlled hypothermia surgery promoted autophagy and apoptosis

The results of Western blot analysis are shown in Figure 5. LC3 is protein light chain 3, which is the most critical marker of the autophagy process, and its function is mainly to participate in the formation of autophagosomes. In the process of autophagy,

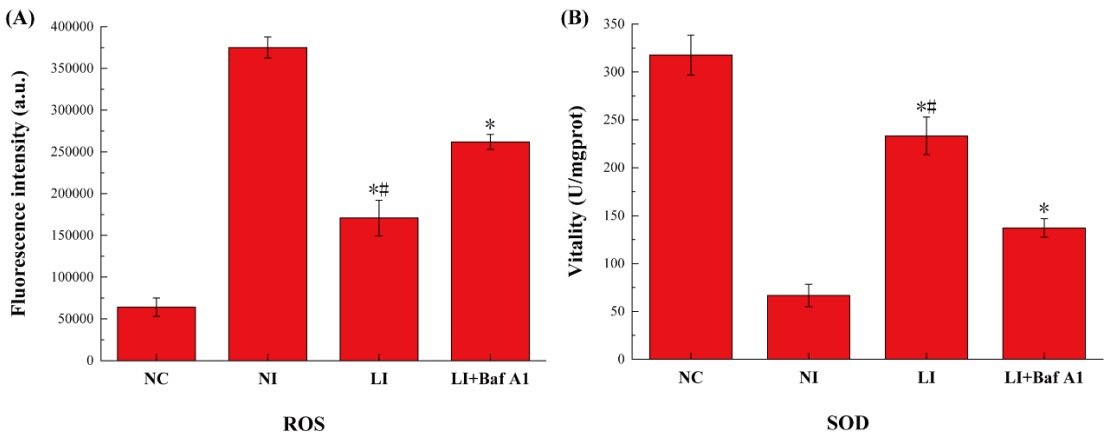

Figure 2. The results of SOD activity measured by microplate reader and the intracellular ROS content detected by the fluorescent probe DCFH-DA method. The data of NC group are consistent with the normal value. Symbol * means $p < 0.05$ compared to NI group. Symbol # means $p < 0.05$ compared to LI+Baf A1 group.

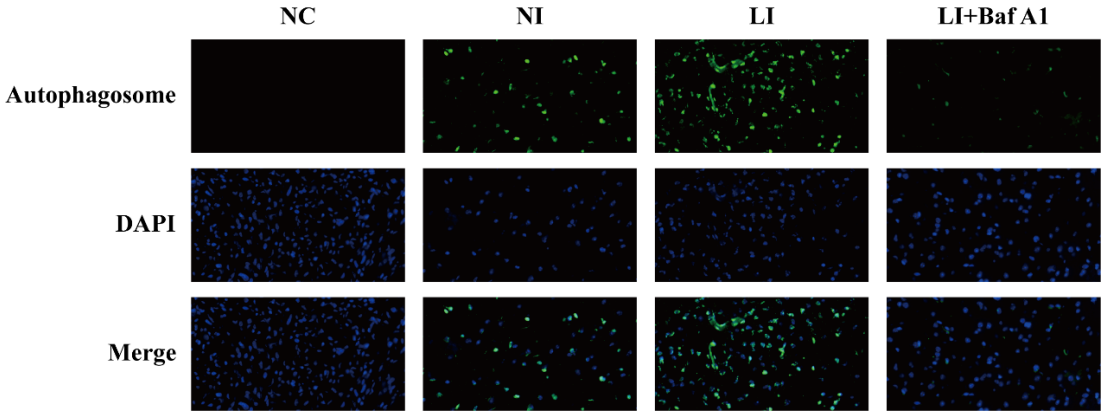

Figure 3. The results of staining brain tissue sections with acid-tropic fluorescent dye MDC. The staining results of the NC group accorded with the physiological characteristics of normal mice.

LC3-I gradually transforms into lipidated LC3-II. When the degree of autophagy is relatively high, the amount of LC3-I is lower, while the amount of LC3-II will increase. When the total amount of LC3-I and LC3-II is constant, LC3-II/LC3-I is used to indicate the level of autophagy. Beclin1 is a key protein in autophagy and is responsible for the assembly of autophagosomes in the early stage of autophagy. Caspase-3 is a key element in the process of cell apoptosis. When a cell receives an apoptotic stimulus, Caspase-3 will be cleaved into cleaved-caspase 3 to be activated, thereby inducing cell apoptosis. Both autophagy and controlled apoptosis can inhibit tissue damage

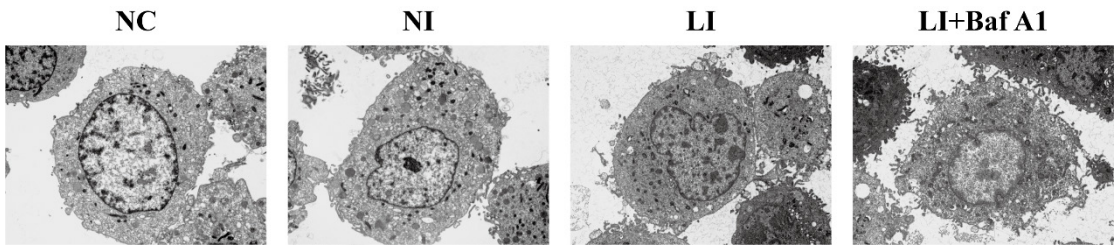

Figure 4. The results of TEM for the characteristics of autophagosomes in brain tissue. The number of autophagosomes in the LI group was significantly more than that of the other three groups.

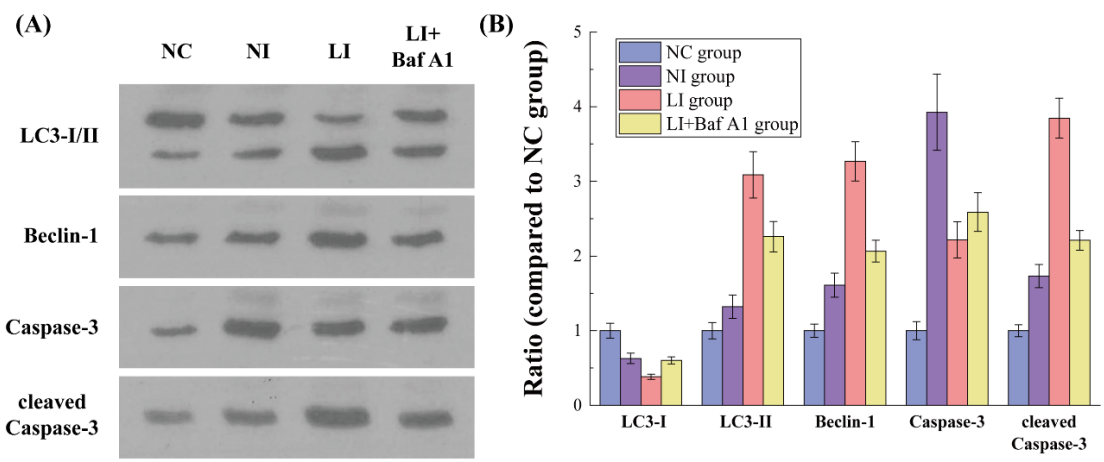

Figure 5. The results of Western blot analysis of the expression of the proteins LC3I, LC3II, Beclin-1, Caspase-3, and cleaved Caspase-3 in brain tissue. All the data of NC group are consistent with the normal value.

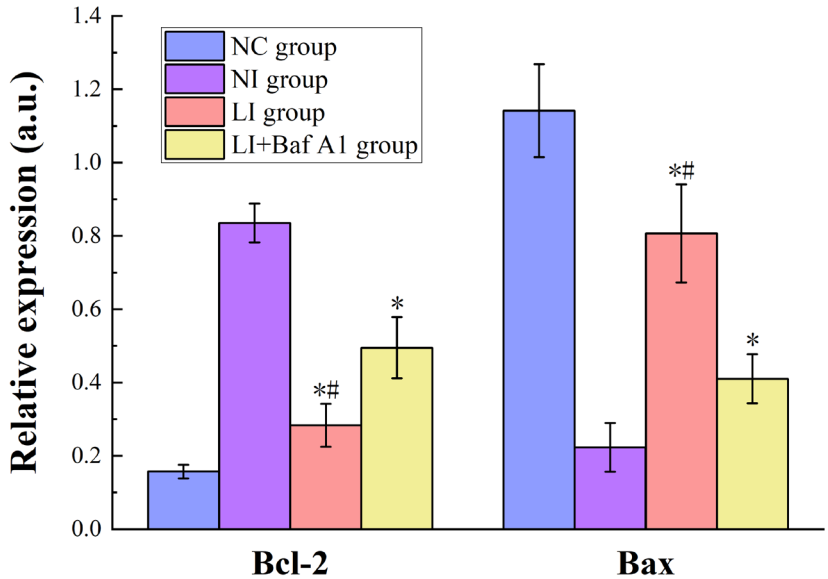

Figure 6. The results of RT-PCR analysis of mRNA expression of genes Bcl-2 and Bax in brain tissue. The data of NC group are consistent with the normal value. Symbol * means $p < 0.05$ compared to NI group. Symbol # means $p < 0.05$ compared to LI+Baf A1 group.

from oxidative stress. Therefore, the five proteins shown in the results all indicated that controlled cryogenic surgery effectively promoted autophagy and apoptosis, thereby protecting the body.

The ratio of Bax/Bcl-2 is closely related to apoptosis

The results of RT-PCR are shown in Figure 6. Bax and Bcl-2 genes are both important genes that regulate cell apoptosis, Bax is a pro-apoptotic gene, and Bcl-2 is an anti-apoptotic gene. When the expression ratio of Bax/Bcl-2 increases, the degree of apoptosis increases. It can be seen from the figure that the degree of apoptosis in the LI group was the highest, which was consistent with the results of Western blot analysis and once again confirmed the previous conclusions.

DISCUSSION

Controlled hypothermia is indicated for neuroprotection after cardiac arrest, neonatal asphyxia, and neonatal encephalopathy, with improved outcome in the intensive care unit setting. It has been shown that decreasing core temperature is protective when there is a risk of ischemia and hypoxia.[11,12] The brain has high metabolic demands, requiring a constant glucose and oxygen supply, which make it highly vulnerable to injury. Hypothermia is applied intraoperatively in neurosurgical and cardiac surgeries, both of which are associated with high risk of tissue hypoxia and ischemia.[13] Cardiac surgery requiring cardiopulmonary bypass (CPB) exposes multiple organ systems, including the brain, to the risk of hypoxia and ischemia. Hypothermia during CPB reduces whole body oxygen consumption.[14,15] However, routine application of induced hypothermia continues to be controversial.

Autophagy is an evolutionary conservative process that involves the transport of damaged organelles, misfolded proteins and other macromolecular substances to the lysosome for degradation and reuse through the lysosomal mechanism of the cell's own structure.[16,17] Autophagy is a phenomenon widely present in eukaryotic cells, and can be divided into three categories: macroautophagy, microautophagy and molecular chaperone-mediated autophagy.[18] This is a tightly regulated step. This step is a routine step in cell growth, development and homeostasis, helping cell products to maintain a balanced state during synthesis, degradation, and subsequent cycles.[19,20] Autophagy has played a vital role in this project. Controlled hypothermia surgery protects cells from oxidative stress by promoting autophagy, thereby reducing the severity of cerebral ischemia.

The effect of controlled hypothermia on the

promotion of autophagy has been verified in many ways. The setting of LI+Baf A1 group is just for comparison with LI group. In all the experiments carried out in this subject, the results of the LI group and the LI+Baf A1 group have significant statistical differences. MDC staining and TEM imaging experiments directly showed the quantitative characteristics of autophagosomes. The number of autophagosomes in the LI group was significantly higher than the other three groups. In Western blot analysis experiments, the ratio of LC3-II/LC3-I is the main detection index of the molecular level of autophagy. The extremely high ratio of LC3-II/LC3-I in the LI group also proves the large-scale occurrence of autophagy. Autophagy helps protect brain tissue from oxidative stress, making controlled hypothermic surgery an effective treatment for cerebral ischemia.

In summary, this project has established an animal model to study the inhibition of oxidative stress damage caused by cerebral ischemia by controlled hypothermia. Through the verification of various macroscopic and microscopic experiments, it is finally proved that controlled hypothermia can protect brain tissue and reduces damage caused by cerebral ischemia by promoting cell autophagy. This provides a new potential strategy for the treatment of cerebral ischemia. Nevertheless, more in-depth research is still needed to improve the conclusions of this topic in order to have a deeper understanding of cerebral ischemia and controlled hypothermia surgery.

ACKNOWLEDGEMENT

We would like to acknowledge everyone for their helpful contributions in this paper.

DISCLOSURE

Ethics approval: The research protocol has been reviewed and approved by the Ethical Committee and Institutional Review Board of the RuiJin Hospital, Shanghai Jiao Tong University School of Medicine.

Conflict of interest: None

Availability of data and materials: The data are free access to available upon request.

REFERENCES

1. Sun XF, Yang H, Li XY, *et al*. Randomized controlled trial of moderate hypothermia versus deep hypothermia anesthesia on brain injury during Stanford a aortic dissection surgery. *Heart Vessels* 2018; 33(1):66-71. doi: 10.1007/s00380-017-1037-9.
2. He ZY, Liu P, Zhang SZ, et al. A freezing-induced turn-on imaging modality for real-time monitoring of cancer cells in cryosurgery. *Angew Chem Int Ed Engl* 2019; 58(12):3834-7. doi: 10.1002/anie.201813239.
3. He XM, Bischof JC. Quantification of temperature and injury response in thermal therapy and cryosurgery. *Crit Rev Biomed Eng* 2003; 31(5-6):355-422. doi: 10.1615/critrevbiomedeng.v31.i56.10.
4. Maoz BM, Herland A, FitzGerald EA, *et al*. (2018). A linked organ-on-chip model of the human neurovascular unit reveals the metabolic coupling of endothelial and neuronal cells. *Nat Biotechnol* 2018; 36(9):865-74. doi: 10.1038/nbt.4226.
5. Zagrean AM, Hermann DM, Opris I, Zagrean L, Popa-Wagner A. Multicellular crosstalk between exosomes and the neurovascular unit after cerebral ischemia. Therapeutic implications. *Front Neurosci* 2018; 12:811. doi: 10.3389/fnins.2018.00811.
6. Thurgur H, Pinteaux E. Microglia in the neurovascular unit: Blood-brain barrier-microglia interactions after central nervous system disorders. *Neuroscience* 2019; 405:55-67. doi: 10.1016/j.neuroscience.2018.06.046.
7. Li PY, Stetler RA, Leak RK, *et al*. Oxidative stress and DNA damage after cerebral ischemia: Potential therapeutic targets to repair the genome and improve stroke recovery. *Neuropharmacology* 2018; 134: 208-17. doi: 10.1016/j.neuropharm.2017.11.011.
8. Zhang XN, Yan HJ, Yuan Y, *et al*. Cerebral ischemia-reperfusion-induced autophagy protects against neuronal injury by mitochondrial clearance. *Autophagy* 2013; 9(9):1321-33. doi: 10.4161/auto.25132.
9. Levine B, Kroemer G. Biological functions of autophagy genes: A disease perspective. *Cell* 2019; 176(1-2);11-42. doi: 10.1016/j.cell.2018.09.048.
10. Scherz-Shouval R, Elazar Z. Regulation of autophagy by ROS: physiology and pathology. *Trends Biochem Sci* 2011; 36(1):30-8. doi: 10.1016/j.tibs.2010.07.007.
11. Khademi R, Mohebbi-Kalhori D, Razminia A. (2019). Thermal analysis of a tumorous vascular tissue during pulsed-cryosurgery and nano-hyperthermia therapy: Finite element approach. *Int J Heat Mass Transfer* 2019; 137: 1001-13. doi: 10.1016/j.ijheatmasstransfer.2019.03.123.
12. Hoffmann NE, Bischof JC. Cryosurgery of normal and tumor tissue in the dorsal skin flap chamber: Part II - Injury response. *J Biomech Eng* 2001; 123(4):310-6. doi: 10.1115/1.1385839.
13. Zhang Y, Lei YB, Yao XX, Yi JP, Feng GH. Pinoresinol diglucoside alleviates ischemia/reperfusion-induced brain injury by modulating neuroinflammation and oxidative stress. *Chem Biol Drug Des* 2021; 98:986-96. doi:10.1111/cbdd.13956.
14. Liu GL, Liang Y, Xu M, *et al*. Protective mechanism of Erigeron breviscapus injection on blood-brain barrier injury induced by cerebral ischemia in rats. *Sci Rep* 2021; 11(1):18451. doi:10.1038/s41598-021-97908-x.

15. Meng JN, Ma HX, Zhu YF, Zhao QP. Dehydrocostuslactone attenuated oxygen and glucose deprivation/reperfusion-induced PC12 cell injury through inhibition of apoptosis and autophagy by activating the PI3K/AKT/mTOR pathway. *Eur J Pharmacol* 2021; 174554. doi:10.1016/j.ejphar.2021.174554.
16. Luo J, Chen JL, Yang CH, *et al*. 6-Gingerol protects against cerebral ischemia/reperfusion injury by inhibiting NLRP3 inflammasome and apoptosis via TRPV1 / FAF1 complex dissociation-mediated autophagy. *Int Immunopharmacol* 2021; 100:108146. doi:10.1016/j.intimp.2021.108146.
17. Hou K, Xu D, Li FY, Chen SJ, Li YM. The progress of neuronal autophagy in cerebral ischemia stroke: Mechanisms, roles and research methods. *J Neurol Sci* 2019; 400:72-82. doi: 10.1016/j.jns.2019.03.015.
18. Park E, Chung SW. ROS-mediated autophagy increases intracellular iron levels and ferroptosis by ferritin and transferrin receptor regulation. *Cell Death Dis* 2019; 10:822. doi: 10.1038/s41419-019-2064-5.
19. Kong LM, Deng J, Zhou X, *et al*. Sitagliptin activates the p62-Keap1-Nrf2 signalling pathway to alleviate oxidative stress and excessive autophagy in severe acute pancreatitis-related acute lung injury. *Cell Death Dis* 2021; 12(10):928. doi:10.1038/s41419-021-04227-0.
20. Yu L, Chen Y, Tooze SA. Autophagy pathway: Cellular and molecular mechanisms. AUTOPHAGY 2018; 14(2):207-15. doi: 10.1080/15548627.2017.1378838.