STUDY ON THE MECHANISM OF INHIBITING AUTOPHAGY BY ISCHEMIC PRECONDITIONING ON THE NEUROPROTECTIVE EFFECT OF ISCHEMIA REPERFUSION RATS

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ABSTRACT

Objective*: This study explores the mechanism of inhibiting autophagy during ischemia on the neuroprotective effect in ischemia-reperfusion rats.*

Methods*: Forty-five healthy male Sprague Dawley rats of clean grade were selected and randomly divided into five groups with nine rats in each group: the sham operation group, model group, and 24 h, 72 h and 120 h ischemic preconditioning groups. An animal experimental model was established. In the sham operation group, only the blood vessels were isolated and no sutures were inserted. In the model group, the rats were directly reperfused after 2 h of ischemia. The rats in the 24 h, 72 h and 120 h ischemic preconditioning groups underwent ischemia for 10 min and reperfusion for 10 min, with three cycles at intervals of 24 h, 72 h and 120 h, respectively, and then reperfused directly after 2 h of ischemia again. The neurological function of rats in each group was evaluated using the Zea-Longa neurological dysfunction score. The volume of cerebral infarction in each group was determined by the triphenyl tetrazolium chloride method. The HE staining method was used to detect the changes in the hippocampal nerve cells in each group of rats. The expression of autophagy-associated protein Beclin-1 and the microtubule-associated protein light chain 3-II (LC3-II) in rat hippocampal neurons in each group was detected using the immunohistochemistry method.*

Results*: Compared with the sham operation group, the neurological dysfunction score, cerebral infarction volume, hippocampal nerve cell injury and the expression level of Beclin-1 and LC3-II were significantly increased in the model group ($P<0.05$). Compared with the model group, the neurological dysfunction score, cerebral infarction volume, hippocampal nerve cell injury and the expression level of Beclin-1 and LC3-II in the ischemic preconditioning group were remarkably lower ($P<0.05$). In the sham operation group, the hippocampal neurons were arranged closely, the morphology was normal, and the nucleolus was obvious. In the model group, the arrangement of hippocampal nerve cells was disordered, the number was lower, and karyopyknotic, intercellular oedema and vacuole-like changes were observed. The number of cells, intercellular oedema and vacuole-like changes in the ischemic preconditioning group were significantly improved compared with the model group. The improvement was the most obvious in the 72 h ischemic preconditioning group.*

Conclusion*: Ischemic preconditioning can play a neuroprotective role by inhibiting the expression of Beclin-1 and LC3-II and by suppressing autophagy, and was found to have the best effect at 72 h.*

Keywords: *autophagy, ischemia-reperfusion, neuroprotective effect, mechanism.*

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Introduction

Cerebral apoplexy is the death of brain tissue caused by decreased cerebral blood flow or insufficient cerebral oxygen supply, which ranks second among the common causes of human death. It is also the main cause of disability in human beings, including ischemic stroke and haemorrhagic stroke, among which ischemic stroke is the most common[1].

At present, the timely recovery of blood perfusion in the ischemic area is the main method for the treatment of ischemic stroke, but in the process of treating an ischemic stroke, an ischemia-reperfusion injury inevitably will occur. It has been found that an ischemia-reperfusion injury can cause a delayed neuronal injury and severely affect prognosis and neurological recovery[2]. Therefore, it is of great significance to find an effective protective mechanism for ischemia-reperfusion injuries and reduce the damage to nerve cells. Autophagy is a highly conserved cellular process that degrades and digests unwanted or damaged organelles and macromolecular proteins and can cause cell death. It has been reported that when cerebral ischemia-reperfusion occurs,

protecting damaged nerve cells by inhibiting apoptosis is one of the most important measures[3]. With the further study of cerebral ischemia-reperfusion injuries, ischemic preconditioning gradually moved from basic experiments to clinical study. Cerebral ischemia preconditioning refers to the blocking of cerebral vessels by mechanical means. This leads to one or more transient cerebral ischemia of brain tissue, and the subsequent recovery of the blood supply leads to cerebral tissue reperfusion, after which brain cells spontaneously produce protective effects[4]. This study investigates the mechanism of inhibiting autophagy by ischemic preconditioning on the neuroprotective effect in ischemia-reperfusion rats.

Data and methods

Experimental animal

Forty-five healthy male Sprague Dawley (SD) rats of clean grade were randomly selected (purchased from Nanjing Junke Bioengineering Co., Ltd., production license SCXK [Ning] 2018-0001), with a body weight of 254±46 g. All the rats were adaptively fed for 7 days at 25±2 oC, with a humidity of 55±15% for 12 h each day and night.

Main instruments and reagents

The following were used in the experiment: inverted microscope (Shenzhen Chensheng Optical Instruments Co., Ltd., model: NIB-900); low temperature and high-speed centrifuge (Beijing Times Beili Centrifuge Co., Ltd., model: GT16-3); ultra-low temperature refrigerator (Shanghai Tianfeng Industrial Co., Ltd., model: TF-86-200-WA); two electronic balances (Shanghai Hengping Scientific Instrument Co., Ltd., model: AE323, and Shanghai Yixin Scientific Instruments Co., Ltd., model: FA-A); paraffin slicer (Shenyang Hengsong Technology Co., Ltd., model: HS-S7220); phosphate buffer solution (Shanghai Thermo Fisher Scientific Co., Ltd.); rabbit anti-mouse Beclin-1 polyclonal antibody (Shenyang Wanlei Biotechnology Co., Ltd.); and rabbit anti-mouse LC3-II polyclonal antibody (Beijing Huaxia Yuanyang Technology Co., Ltd.).

Establishment and experimental grouping of ischemia-reperfusion rats

The rats were randomly divided into five groups: the sham operation group, model group, and 24 h, 72 h and 120 h ischemic preconditioning groups, with nine rats in each group.

The rats were anesthetized after fasting for 8 h and fixed on the test bench. A modified Zea-Longa suture-occluded method was used to establish a model of ischemia-reperfusion injury to the right middle cerebral artery in rats. An incision of about 3 cm was cut in the middle of the neck of the rat, and the subcutaneous glands and tissues of the neck were separated bluntly. The right common carotid artery, internal carotid artery and external carotid artery were isolated, and the right common carotid artery and external carotid artery near the heart were ligated. A small incision was cut at the stump of the common carotid artery, and a suture was slowly inserted at about 18 mm to 20 mm to block the blood flow of the middle cerebral artery. Under anaesthesia, the rats' blood supply was recovered by gently pulling the suture outward. After the operation, the rat incision was sutured, during which the rat body was maintained at room temperature. After waking up, the rats were put back into their cage to eat freely. The model was successfully constructed according to the movement or walking disorder of the contralateral limb, such as the adduction and buckling of the left forelimb or coma in severe cases.

In the sham operation group, only the blood vessels were isolated and no suture was inserted. In the model group, the rats were directly reperfused after 2 h of ischemia. The rats in the 24 h, 72 h and 120 h ischemic preconditioning groups underwent ischemia for 10 min and reperfusion for 10 min, with three cycles at intervals of 24 h, 72 h and 120 h, respectively, and then reperfused directly after 2 h of ischemia again.

Experimental methods and observation indexes

The neurological function of rats in each group was evaluated with the Zea-Longa neurological dysfunction score. The score ranges from 0 to 5 points, and the higher the score, the more obvious the neurological dysfunction, with 5 points indicating death (rat should be excluded).

The triphenyl tetranitrogen chloride (TTC) method was used for staining. Three rats in each group were selected and the volume of cerebral infarction was measured. Ischemic tissue sites were white and non-ischemic tissue sites were red. The volume of cerebral infarction is the product of the area of each cerebral infarction and its thickness (all 2 mm).

The HE staining method was used to detect the changes in the hippocampal nerve cells in each group of rats. The expression of autophagy-

associated protein Beclin-1 and microtubule-associated protein light chain 3-II (LC3-II) in rat hippocampal neurons in each group was detected using the immunohistochemistry method.

At the end of the experiment, all the rats were euthanized.

Statistical method

All the statistical data were analysed using the SPSS22.0 software package. The measurement data were compared with a one-way ANOVA analysis and Fisher's LSD test. P<0.05 indicates that the statistical results were statistically significant.

Results

Comparison of neurological dysfunction score in each group of rats

Compared with the sham operation group, the neurological dysfunction score significantly increased in the model group (P<0.05). Compared with the model group, the neurological dysfunction score in the ischemic preconditioning groups was markedly reduced (P<0.05), with the most reduction in the 72 h ischemic preconditioning group. These results are shown in Table 1.

Groups	n	Neurological dysfunction score (points)
Sham operation group	9	0
Model group	9	2.91±0.57[a]
24h ischemic preconditioning group	9	2.21±0.35[abc]
72h ischemic preconditioning group	9	1.71±0.48[ab]
120h ischemic preconditioning group	9	2.42±0.48[abc]
F		9.81
P		<0.001

Table 1: Comparison of neurological dysfunction score in each group of rats ($\bar{x}$±s).

Notes: The superscript a represents P<0.05 compared with the sham operation group, b represents P<0.05 compared with the model group, and c indicates P<0.05 compared with the 72 h ischemic preconditioning group.

Volume change of cerebral infarction in each group of rats

Compared with the sham operation group, the cerebral infarction volume of the model group significantly increased (P<0.05). Compared with the model group, the cerebral infarction volume in the ischemic preconditioning groups was remarkably reduced (P<0.05), with the most reduction in the 72 h ischemic preconditioning group. The results are shown in Figure 1 and Table 2.

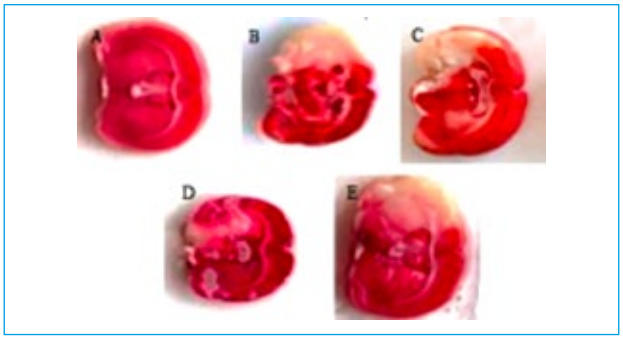

Figure 1: Volume change of cerebral infarction in each group of rats.

A: sham operation group; **B**: model group; **C**: 24 h ischemic preconditioning group; **D**: 72 h ischemic preconditioning group; **E**: 120 h ischemic preconditioning group.

Groups	n	Cerebral infarction volume (%)
Sham operation group	3	0
Model group	3	41.45±1.48[a]
24h ischemic preconditioning group	3	36.23±1.62[abc]
72h ischemic preconditioning group	3	31.39±1.47[ab]
120h ischemic preconditioning group	3	38.82±1.65[abc]
F		22.03
P		<0.001

Table 3:Volume change of cerebral infarction in each group of rats ($\bar{x}$±s).

Notes: The superscript a represents P<0.05 compared with the sham operation group, b represents P<0.05 compared with the model group, and c indicates P<0.05 compared with the 72 h ischemic preconditioning group.

Changes in hippocampal nerve cells in each group of rats

In the sham operation group, the hippocampal neurons were arranged closely, the morphology was normal, and the nucleolus was obvious.

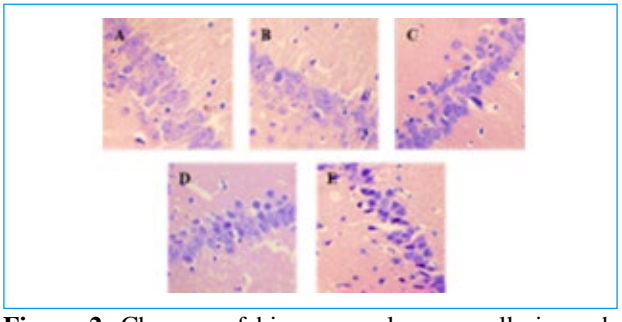

Figure 2: Changes of hippocampal nerve cells in each group of rats

A: sham operation group; **B**: model group; **C**: 24 h ischemic preconditioning group; **D**: 72 h ischemic preconditioning group; **E**: 120 h ischemic preconditioning group.

In the model group, the arrangement of hippocampal nerve cells was disordered, the number was decreased, and karyopyknotic, intercellular oedema and vacuole-like changes were observed. The number of cells, intercellular oedema and vacuole-like changes in the ischemic preconditioning groups were significantly improved compared with the model group, particularly in the 72 h ischemic preconditioning group. The results are shown in Figure 2.

Expression of autophagy-related proteins Beclin-1 and LC3-II in hippocampal nerve cells in each group of rats

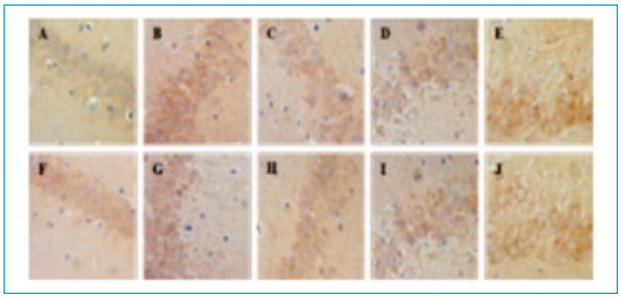

Figure 3: Expression of autophagy-related proteins Beclin-1 and LC3-II in hippocampal nerve cells in each group of rats
A: Beclin-1 expression in sham operation group; **B**: Beclin-1 expression in the model group; **C**: Beclin-1 expression in 24 h ischemic preconditioning group; **D**: Beclin-1 expression in 72 h ischemic preconditioning group; **E**: Beclin-1 expression in 120 h ischemic preconditioning group; **F**: LC3-II expression in sham operation group; **G**: LC3-II expression in the model group; **H**: LC3-II expression in 24 h ischemic preconditioning group; I: **L**C3-II expression in 72 h ischemic preconditioning group; **J**: LC3-II expression in 120 h ischemic preconditioning group.

Groups	n	Beclin-1	LC3-II
Sham operation group	3	2.51±0.98	2.71±0.83
Model group	3	35.21±0.92 [a]	47.61±1.58 [a]
24h ischemic preconditioning group	3	31.28±0.77 [abc]	38.21±0.93 [abc]
72h ischemic preconditioning group	3	22.81±1.95 [ab]	29.31±1.71 [ab]
120h ischemic preconditioning group	3	32.21±0.93 [abc]	39.45±0.81 [abc]
F		374.66	590.44
P		<0.001	<0.001

Table 3: Expression of autophagy-related proteins Beclin-1 and LC3-II in hippocampal nerve cells in each group of rats ($\bar{x}\pm s$).
Notes: The superscript a represents P<0.05 compared with the sham operation group, b represents P<0.05 compared with the model group, and c indicates P<0.05 compared with the 72 h ischemic preconditioning group.

Compared with the sham operation group, the expression of Beclin-1 and LC3-II in the model group increased significantly (P<0.05). Compared with the model group, the expression levels of Beclin-1 and LC3-II were markedly lower in the ischemic preconditioning groups (P<0.05), with the highest reduction in the 72 h ischemic preconditioning group. The results are shown in Figure 3 and Table 3.

Discussion

Cerebral stroke is a disease caused by a cerebral blood circulation disorder, hypoperfusion of brain tissue or a blood vessel rupture and bleeding between tissues. Some studies have found that when cerebral infarction occurs, the blood vessels are blocked, and the blood flow in the corresponding area drops or disappears completely, resulting in deformation, necrosis and even dysfunction of nerve cells[5]. At present, the most effective way to alleviate an ischemic brain injury and reduce the nerve function defect is to restore the blood supply of the brain tissue as soon as possible. However, if the blood supply is restored beyond the time window of brain tissue tolerance to ischemia and hypoxia, the structure and function of nerve cells will be seriously damaged, thus resulting in an ischemia-reperfusion injury[6]. Therefore, finding effective ways to reduce ischemia-reperfusion injury to nerve cells has become the focus of clinical attention.

Ischemic preconditioning was first proposed by Murry et al., who suggested that it could alleviate a myocardial ischemia-reperfusion injury. Some studies have found that ischemic preconditioning has important endogenous neuroprotective effects. Ischemic preconditioning can induce adenosine to release endogenous neurotransmitters, which then combine with the endogenous neurotransmitters of the corresponding receptors to activate the corresponding signalling pathways to make the relevant genes express and produce cellular protective substances, thus preventing or slowing down their apoptosis or necrosis[7, 8].

With the development of medical technology and the deepening knowledge of modern immunology and molecular biology, it has been found that the pathogenesis of ischemia-reperfusion injury mainly includes an inflammatory reaction, excitatory amino acid toxicity, oxidative stress injury, energy metabolism disorder, apoptosis and intracellular calcium overload[9].

It has been reported that apoptosis is closely related to the occurrence and development of a cerebral ischemia-reperfusion injury[10]. Some scholars have found that autophagy may also be involved in the regulation of neuronal apoptosis[11]. Autophagy refers to the process of degradation or recycling damaged organelles or macromolecular proteins by the lysosome. When the cells are short of nutrients, autophagy can maintain the energy balance of the cells by removing their damaged or aging organelles and can have a protective effect on the survival of the cells. However, excessive autophagy not only leads to autophagic cell death but also induces apoptosis[12].

At present, there are 31 autophagy-related genes, and the formation of autophagy is closely related to them. Beclin-1 is a specific gene of autophagy in mammal cells and is a marker gene for autophagy initiation. The over-expression of Beclin-1 can accelerate the occurrence of autophagy[13]. Zhang et al.[14] prove that increased Beclin-1 expression after the ischemic and hypoxic injury of nerve cells may be its stress response to changes in the living environment. LC3, a mammalian analogue of Atg8, is the only credible autophagy marker currently identified and includes LC3-I and LC3-II subtypes. LC3-II can bind to the autophagy membrane, and its level is closely related to the number of autophagosomes[15].

The results of this study indicate that the neurological dysfunction score, cerebral infarction volume, hippocampal nerve cell injury and the expression levels of Beclin-1 and LC3-II significantly increased in the model group compared with the sham operation group ($P<0.05$). Compared with the model group, the neurological dysfunction score, cerebral infarction volume, hippocampal nerve cell injury and the expression levels of Beclin-1 and LC3-II in the ischemic preconditioning groups were remarkably lower ($P<0.05$), with the most obvious improvement in the 72 h ischemic preconditioning group. It is suggested that ischemic preconditioning can significantly improve the neurological function after cerebral ischemia, improve the volume of cerebral infarction and the injury of hippocampal nerve cells, decrease the expression of Beclin-1 and LC3-II and inhibit autophagy.

In conclusion, ischemic preconditioning can play a neuroprotective role by inhibiting the expression of Beclin-1 and LC3- II and by suppressing autophagy and was found to have the most significant effect at 72 h.

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