Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

Mild hypothermia therapy alleviates neuronal damage and repairs cerebral ischemia-reperfusion injury through the SIRT1/AMPK pathway

Xiaowei Li[1*], Ying Shang[2], Xiaobao Zhao[1], Ming Kong[2], Hui An[3]

[1] *Department of Anesthesiology, Baoding No.1 Central Hospital, Baoding, Hebei, 071000, China*
[2] *Department of Anesthesiology, Baoding Second Hospital, Baoding, Hebei, 071000, China*
[3] *Department of Critical Care Medicine, Baoding No.1 Central Hospital, Baoding, Hebei, 071000, China*

Article Info

Article history:

Received: March 15, 2024
Accepted: April 24, 2024
Published: August 31, 2024

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Abstract

Cerebrovascular disease, one of the high-risk diseases worldwide, is high in morbidity, disability, mortality, and recurrence rates, which brings many harms to human beings such as physical and mental harm, economic losses, and impairment of social relations. Cerebral ischemia-reperfusion injury (CIRI) is one of the most common pathological manifestations, with mild hypothermia therapy being the most commonly used treatment in clinical practice. In this study, the research team established a CIRI animal model and found that the neuronal apoptosis rate was significantly increased, accompanied by significant ferroptosis, increased inflammation and oxidative stress damage in brain tissue, and obviously inhibited SIRT1/AMPK pathway. However, after mild hypothermia treatment, the pathological changes of CIRI rats were significantly reversed, and the SIRT1/AMPK pathway was reactivated. Therefore, mild hypothermia may achieve the purpose of CIRI repair by activating the SIRT1/AMPK signaling pathway, and targeted regulation of the SIRT1/AMPK signaling pathway may be a research direction for optimizing mild hypothermia therapy or developing new treatment plans for CIRI.

Keywords: Mild hypothermia, Neuronal cells, Cerebral ischemia-reperfusion injury, SIRT1/AMPK pathway, Mitochondria, Ferroptosis.

1. Introduction

The brain is the most sensitive organ of the human body to hypoxia. Cerebral ischemia will cause damage to local brain tissue and its function, with the extent of damage associated with the length of ischemia and the amount of residual blood flow; short-term incomplete cerebral ischemia only causes reversible damage, while long-term complete ischemia or severe ischemia can induce infarction [1]. According to statistics, ischemic stroke accounts for more than 87% of all cerebrovascular accidents [2]. Among them, cerebral ischemia-reperfusion injury (CIRI), one of the most typical pathological types, is mainly manifested as brain edema and neuronal injury, with cognitive impairment, motor disorders, and sensory failure as the major clinical symptoms [3]. How to further improve the therapeutic effect of CIRI and enhance the prognosis and health of patients has been a long-standing research hotspot and difficulty in clinical practice [4]. Mild hypothermia, currently the most commonly used method in clinical treatment of CIRI, has been shown to effectively inhibit neuronal apoptosis after CIRI and reduce the release of inflammatory mediators [5, 6]. However, the specific action pathway of mild hypothermia therapy on CIRI is still unclear, and there is a lack of reliable references in clinical practice, making it difficult to further optimize the treatment effect.

The Silent mating type information regulation 2 homolog-1 (SIRT1)/Adenosine 5'monophosphate-activated protein kinase (AMPK) pathway is a classic signaling pathway in clinical research, which is considered to have a good regulatory effect on glucose, lipid homeostasis, cardiovascular function, and insulin sensitivity [7]. Besides, SIRT1/AMPK is involved in autophagy, apoptosis, differentiation, and other biological pathways of cells, which is of great clinical significance [8]. The role of SIRT1/AMPK in myocardial ischemia-reperfusion injury has been validated multiple times and is hailed as a key pathway of action for future ischemia-reperfusion injury [9, 10]. But for CIRI, its function is still unclear. At the same time, we found significant abnormal expression of the SIRT1/AMPK pathway in the mild hypothermia treatment of cardiopulmonary resuscitation [11], suggesting that SIRT1/AMPK may also be of great significance in the mild hypothermia treatment of CIRI.

Therefore, by establishing an animal model of CIRI, this study observed changes in the SIRT1/AMPK pathway

* Corresponding author.
E-mail address: 15031250138@163.com (X. Li).
Doi: http://dx.doi.org/10.14715/cmb/2024.70.8.20

during mild hypothermia treatment to help gain a better understanding of the pathway and mechanism of mild hypothermia for CIRI, providing clinical evidence for further optimization of mild hypothermia treatment regimens.

2. Materials and methods

2.1. Animal data

Forty specific-pathogen-free (SPF) healthy SD rats, all males with a weight of 250-280 g, were provided by GemPharmatech Co., Ltd. (SYXK (Su) 2023-0036). They were raised in an SPF animal room and allowed to eat and drink freely. The Animal Ethics Committee of our hospital approved this research, which was carried out strictly following the "3R (Reduction, Replacement, and Refinement)" principle.

2.2. Modeling

Twenty rats were randomly divided into a control group (n=10) and a model group (n=10), in which the control group was fed normally without treatment, and the model group was modeled as follows with reference to the study of Yang L et al. [12]: After a week of adaptive feeding, the rats were fasted but not water-deprived for 24 hours. After intraperitoneal injection anesthesia (0.3 mL/100 g) with 10% chloral hydrate and fixation in the supine position, the rat's anterior midline was sheared and disinfected, and a small incision was made to fully expose the common (CCA), external (ECA), and internal carotid artery (ICA). Then, the ECA and the communication branch between ECA and ICA were electrocoagulated, and the lower end of CCA was permanently ligated. Next, the CCA and ICA were propped up and straightened with curved forceps, and the ICA was temporarily ligated and blocked. After that, an oblique small incision was cut upwards at a distance of 3 mm from the proximal end of the bifurcation between ECA and ICA with ophthalmic scissors, and the poly-L-lysine-coated middle cerebral artery occlusion (MCAO) thread at the front end was clamped and inserted from the incision. Then, the slipknot at the ICA was untied, and the thread was pushed along the ICA direction for about 18-20 mm and stopped when it encountered resistance, thus embolizing the right middle cerebral artery. The wound was then sutured layer by layer and the part of the MCAO thread outside the body was fixed with a thin thread to ensure that the thread did not detach from the incision. After cerebral ischemia for about 120 min, the tail end of the thread was slowly pulled out for about 10 mm with curved forceps to establish a CIRI model. 120 min after modeling, when the vital signs of rats were stabilized, the Longa's neurological deficit test was performed [13]: 0 points for no neurological deficit, 1 point for no extension of the contralateral forelimb during tail lifting, 2 points for rotation to the opposite side of cerebral ischemia when crawling, 3 points for tilting to the opposite side of cerebral ischemia while crawling, and 4 points for inability to move freely and loss of consciousness. Rats with a score of 0 and 4 points were eliminated, and the number of modeled rats was supplemented according to the same modeling steps.

2.3. Detection of inflammatory cytokine levels

After the modeling was completed, rats in the control and model groups were killed by neck amputation under anesthesia. The brain tissues of rats in each group were taken out and made into a homogenate on ice, which was then centrifuged for about 15 min with a low-temperature high-speed centrifuge at 3000r/min, and the supernatant was collected to determine Superoxide dismutase (SOD), Malondialdehyde (MDA), Interleukin-1β (IL-1β), Interleukin-6 (IL-6), and Tumor necrosis factor-α (TNF-α) levels following the instructions of corresponding kits (Nanjing Saihongrui Biotechnology Co., Ltd.).

2.4. Neuronal apoptosis detection

The brain tissue was mashed and digested by trypsin, followed by PBS washing and digestion termination. The tissue was then filtered with a 200-mesh sieve and centrifuged. After controlling the cell number at 1×10^6/mL by using a cell counting plate and a cell counter, the cells were collected into a centrifuge tube and dripped with binding buffer for resuspension. Next, 10 μL of AnnexinV-FITC was added to the centrifuge tube for 20 min of staining in the dark, followed by 10 min of night-tight staining with 10 μL of PI. The apoptosis rate of neurons was finally determined by flow cytometry.

2.5. Protein detection

After thoroughly grinding and homogenizing the brain tissue, it was treated with lysis with a tissue total protein lysis buffer and low-temperature centrifugation, and the supernatant was collected to obtain the total protein. The protein concentration was detected with a bicinchoninic acid (BCA) protein assay kit. The total protein samples were then separated in the electrophoresis solution using 12% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, the samples were blotted onto a polyvinylidene fluoride (PVDF) membrane, which was sealed with 3% bovine human albumin (BSA) for 1 hour at room temperature. After blocking, it was incubated with Recombinant solute carrier family 7, member 11 (SLC7A11), Recombinant solute carrier family 3, member 2 (SLC3A2), Glutathione peroxidase 4 (GPX4), SIRT1, AMPK, and GAPDH (1:1000) and other antibodies overnight. A second antibody (1:2000) was added the next day, and finally, the membrane was developed on the computer for gray value analysis using ImageJ.

2.6. Mild hypothermia intervention treatment

The remaining 20 rats were randomized into an intervention group (n=10) and a model group (n=10), and were modeled according to the above method. After the completion of modeling, the rats in the intervention group were placed in a cryogenic metabolic cage with ice cubes, with their tympanic membrane temperature and anal temperature maintained at (31±1)℃ and (33±1)℃ for 72 hours, respectively. Rats in the model group were fed normally at room temperature. Then, inflammatory factors, oxidative stress, SIRT1/AMPK pathway expression and neuronal damage were detected according to the above methods.

2.7. Statistical methods

In this study, all experiments were run in triplicate. Data conforming to a normal distribution were represented by ($\bar{\chi}$±s), and the independent sample t-test was used for comparisons. The data that do not conform to a normal distribution were described as the median (interquartile range), and the comparison was made by the nonparametric Mann-Whitney U test. The difference was considered

statistically significant at a P-value <0.05.

3. Results

3.1. Modeling results

No rats died during the modeling process of this study. After modeling, the Longa score of the model group was (2.50±0.53), higher compared with the control group (P<0.05) (Figure 1).

3.2. Comparison of neuronal cell injury

After testing, the neuron apoptosis rate in the model group was (12.08±1.52)%, higher compared with the control group (P<0.05). In addition, SLC7A11, SLC3A2, and GPX4 protein levels were all decreased in the model group (P<0.05), suggesting obvious Ferroptosis (Figure 2).

3.3. Comparison of inflammatory response and oxidative stress damage

Compared with the control group, IL-1β, IL-6, TNF-α, and MDA in the model group were significantly higher, while SOD was lower (P<0.05), indicating the presence of obvious inflammatory response and oxidative stress damage in the model group (Figure 3).

3.4. Comparison of SIRT1/AMPK pathway expression

The model group showed elevated SIRT1 and AMPK protein expression in the brain tissue than the control group (P<0.05), indicating that CIRI can activate the SIRT1/AMPK pathway (Figure 4).

3.5. Effect of mild hypothermia on neuronal cell injury

After mild hypothermia intervention, the apoptosis rate of neurons in the intervention group was significantly reduced compared to the model group (P<0.05). In addition, SLC7A11, SLC3A2 and GPX4 protein expression levels were higher in the intervention group compared with the model group (P<0.05), indicating inhibited ferroptosis in neuronal cells (Figure 5).

3.6. Effect of mild hypothermia on inflammatory response and oxidative stress damage

Furthermore, the intervention group was found to have lower IL-1β, IL-6, TNF-α, and MDA levels than the model group, with higher SOD (P<0.05), suggesting that the inflammatory response and oxidative stress damage are alleviated in the intervention group (Figure 6).

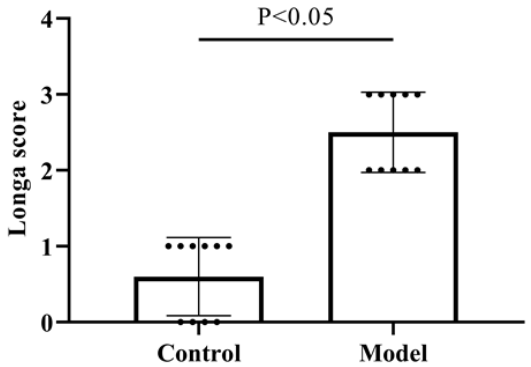

Fig. 1. Confronto del punteggio Longa.

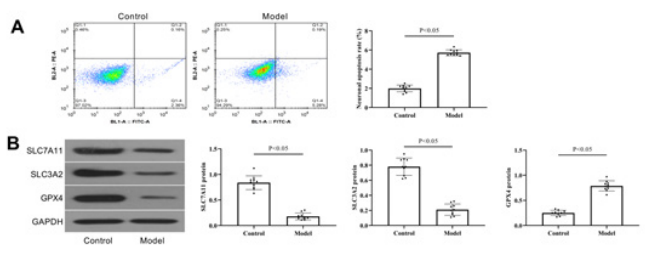

Fig. 2. Comparison of neuronal cell injury. A) comparison of neuronal apoptosis rates. B) comparison of ferroptosis proteins.

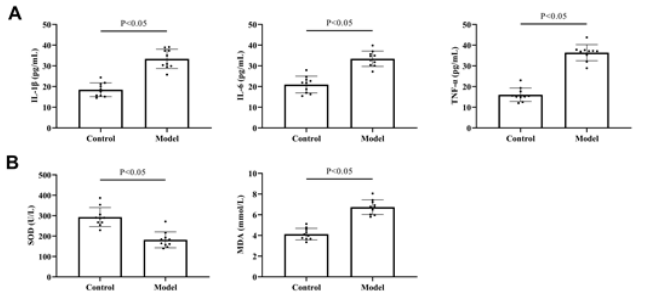

Fig. 3. Comparison of inflammatory response and oxidative stress damage. A) comparison of inflammatory factors IL-1β, IL-6, and TNF-α. B) Comparison of oxidative stress damage markers SOD and MDA.

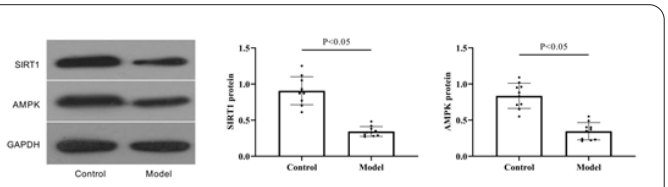

Fig. 4. Comparison of SIRT1/AMPK pathway expression.

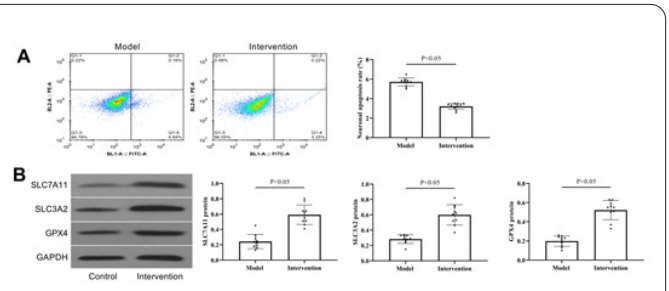

Fig. 5. Effect of mild hypothermia on neuronal cell injury. A) comparison of neuronal apoptosis rates. B) comparison of ferroptosis proteins.

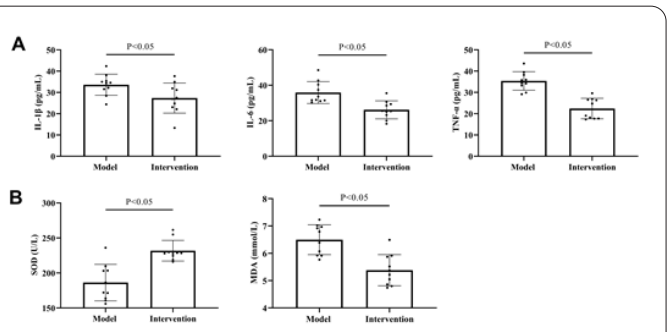

Fig. 6. Effect of mild hypothermia on inflammatory response and oxidative stress damage. A) comparison of inflammatory factors IL-1β, IL-6, and TNF-α. B) Comparison of oxidative stress damage markers SOD and MDA.

3.7. Effect of mild hypothermia on SIRT1/AMPK pathway expression

Finally, the detection results of the SIRT1/AMPK pathway showed that the SIRT1 and AMPK protein expression levels in the intervention group were (0.72±0.11) and (0.75±0.11) respectively, which were reduced compared to the model group (P<0.05) (Figure 7).

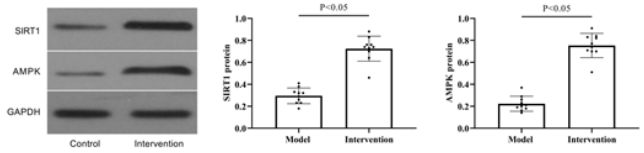

Fig. 7. Effect of mild hypothermia on SIRT1/AMPK pathway expression.

4. Discussion

CIRI can cause a series of ischemia-related cascades in brain tissue, which not only alter the morphology and function of cerebral blood vessels, but also affect the brain's material exchange ability, resulting in a decrease in blood oxygen supply and eventually damaging brain cells [14]. Mild hypothermia is one of the effective measures to alleviate CIRI, which can reduce ischemic brain injury by reducing ATP consumption, inhibiting the release of excitatory amino acids, reducing Ca+ mobilization, and relieving endoplasmic reticulum stress, in addition to blocking the cascade of multiple injury after reperfusion [15]. In this study, we further observed the influence of mild hypothermia on CIRI, which can provide more reliable clinical guidance for future applications of mild hypothermia therapy.

In this study, we found that compared with normal rats, the neuronal apoptosis rate in the CIRI model rats was significantly increased, accompanied by significant ferroptosis and increased inflammation and oxidative stress damage in brain tissue, which is consistent with previous pathological studies on CIRI [16], supporting the success and accuracy of the modeling. At present, clinical research on the pathogenesis of CIRI mainly focuses on inflammation, oxidative stress, intracellular calcium overload, excitatory amino acids, and mitochondrial dysfunction, among which post-cerebral ischemia inflammation plays an important role in ischemic reperfusion injury [17]. After reperfusion, blood flow reperfusion not only brings nutrients and oxygen to cells, but also exacerbates the inflammatory cascade, resulting in a series of biological and behavioral changes. When CIRI occurs, the cytochrome oxidase dysfunction in mitochondria and the decrease in antioxidant enzyme activity lead to an increase in free radical generation and a decrease in the ability to remove oxygen free radicals, thus exacerbating mitochondrial dysfunction, causing cell energy metabolism disturbance, and eventually leading to cell death [18]. Ferroptosis, as one of the new iron-dependent programmed cell death modalities, is an important pathological process in CIRI, with the main mechanism being the imbalance of oxidation-reduction homeostasis, manifested as mitochondrial dysfunction, weakened antioxidant function, increased oxygen free radicals, abnormal lipid metabolism in cell membranes, and excessive accumulation of lipid peroxidation products such as MDA [19]. We believe that this is also the main mechanism of accelerated neuronal cell apoptosis in the model group. However, under the intervention of mild hypothermia, all pathological changes in the intervention group were significantly ameliorated compared to the model group, which further confirms the excellent therapeutic effect of mild hypothermia on CIRI, consistent with the results of previous studies [20, 21]. In addition, mitochondria have been shown to play a relay and amplification role in various apoptotic cell signaling factors or pathways during CIRI, and changes in mitochondrial morphology and function are important factors influencing CIRI progression [22]. Therefore, the effects of mild hypothermia treatment on various signaling pathways of CIRI will be reflected in changes in mitochondrial morphology and function, such as excessive mitochondrial division and apoptosis [23]. Based on the expression changes of ferroptosis-associated proteins mentioned earlier, we speculate that mild hypothermia mainly inhibits excessive mitochondrial ferroptosis, thereby alleviating the accelerated apoptosis of neuronal cells.

On the other hand, as mentioned before the SIRT1/AMPK pathway may have significant implications in mild hypothermia treatment of CIRI. SIRT1/AMPK is also one of the clinically recognized regulatory pathways for mitochondrial activity. For example, resveratrol can increase the expression of SIRT1 and mitochondrial proteins, reduce oxygen free radicals, and ensure mitochondrial stability in myocardial cells [24]. In this study, we found significantly inhibited SIRT1/AMPK in CIRI rats, consistent with the results of Li N et al. in exploring the expression of SIRT1/AMPK in fatty liver [25]. After mild hypothermia treatment, we observed an increase in the expression of SIRT1 and AMPK in the intervention group, indicating that mild hypothermia has the effect of activating the SIRT1/AMPK pathway. This may also be due to the intervention of mild hypothermia, which can alleviate hypoxic stress damage to brain tissue, increase AMP content, and activate AMPK phosphorylation. Activated AMPK can act on various downstream substrates, inhibit ATP consumption, initiate ATP generation pathways to maintain the body's energy metabolism balance, increase intracellular dependent nicotinamide adenine dinucleotide (NAD^+) content, and subsequently activate SIRT1, thus forming a positive biological effect on CIRI.

The SIRT1/AMPK signaling pathway can regulate the metabolism and apoptosis of neuronal cells in a specific way, and may be a potential target for the treatment of CIRI. In the future, it is expected to become a research direction for optimizing mild hypothermia therapy or developing new treatments for CIRI.

However, due to limited conditions, there are still many limitations in this study. Further in vitro experiments are needed to analyze the mechanism through which mild hypothermia affects neuronal cells. Meanwhile, the SIRT1/AMPK pathway expression in CIRI needs to be validated by clinical cases. We will conduct more comprehensive experiments and analyses to address the limitations mentioned above in the future.

5. Conclusion

Mild hypothermia inhibits mitochondrial ferroptosis by activating the SIRT1/AMPK signaling pathway, alleviating excessive neuronal apoptosis and achieving the goal of repairing CIRI. In the future, targeted regulation of the SIRT1/AMPK signaling pathway may be a research direc-

tion for optimizing mild hypothermia therapy or developing new therapies for CIRI.

Conflict of interests

The author has no conflicts with any step of the article preparation.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Funding

None.

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