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Original article

Mechanisms of transformation of nicotinamide mononucleotides to cerebral infarction hemorrhage based on MCAO model

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ABSTRACT

Objective: The study aims at discussing the effect of nicotinamide mononucleotides on protecting hemorrhagic transformation of cerebral infarction in the middle cerebral artery occlusion (MCAO) model.
Method: Male mice aged 4–5 weeks and weighing about 22–35 g in Shanghai Ninth People's Hospital are divided into three groups: sham group, collagenase intracerebral hemorrhage model (cICH + Vehicle) group and collagenase nicotinamide mononucleotide (cICH + NMN) group. Then, the intervention therapy research is carried out. After 24 h, the neurological function, brain edema, hematoma volume, body weight, hemorrhage volume, RNA expression level, apoptosis, inflammatory factors and reactive oxygen species (ROS) content in surrounding tissues of mice are analyzed comprehensively.
Results: Compared with the other two groups, nicotinamide mononucleotides in MCAO model have significant effects on improving neurological function, brain edema, inflammatory factors, body weight and cell apoptosis in mice, but have no significant effect on hemorrhage volume and hematoma volume in mice.
Conclusion: Nicotinamide mononucleotides can significantly improve the collagenase-induced intracerebral hemorrhage (ICH) model in mice under MCAO model, and they can protect the brain tissue of mice from RNA level to tissue cell level or mouse body weight and volume level.
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1. Introduction

Intracerebral hemorrhage (ICH), a fatal type of stroke frequently occurring and causing death, about 10–15% of stroke (Hu et al., 2017). The prevalence of ICH is ethnically related (Wang et al., 2016). For example, in Chinese, ICH accounts for about 33% of all strokes, compared with only 12% in Caucasians. It has been reported that the mortality rate of one month after the onset of ICH is about 40% (Jhelum et al., 2017; Yang et al., 2017). Improvements in medical care over the past two decades have not reduced this mortality rate, and surviving patients have to endure long-term physical and mental disorders (Li and Yang, 2016). The high recurrence rate and mortality rate of ICH is partly on account of the lack of effective treatment options (Forreider et al., 2017). Current clinical treatments are limited to primary treatment, such as controlling intracranial pressure, treating hematoma and maintaining hemodynamic stability. However, in the past decade (Khoury et al., 2018; Sarmah et al., 2019), the increased interest of researchers in ICH has improved people's understanding of the potential mechanism of cerebral injury induced by ICH. Its injury mechanism is different from that of cerebral ischemia. These findings lead to a number of clinical trials exploring the treatment of ICH. According to the cause of hemorrhage, ICH can be divided into primary type. Although many experts have carried out a lot of research on this, and found that endogenous tPA is vital in the central nervous system (CNS), neurodegeneration, and ischemic stroke, and delaying tPA can aggravate the cytotoxicity of the brain system and destroy the permeability, the mechanism of its harmful role in stroke is still not very clear.

Most cases of ICH are caused by essential hypertension, atherosclerosis and amyloid angiopathy. Secondary ICH accounts

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for 15–20% of all patients. Hypertension caused by intracranial vascular malformations, tumorigenesis, coagulopathy and the use of cerebral ischemic thrombolytics is an important risk threat for ICH, relevant with the high occurring and death rate in the elderly. Chronic hypertension induces degenerative changes in small arteries, making them more susceptible to rupture. Therefore, the treatment of hypertension can reduce the risk of ICH in patients with hypertension. Nicotinamide phosphoribosyl transferase (Nampt) is the rate-limiting enzyme of nicotinamide adenine dinucleotide (NAD) synthesis in mammalian cells, which can regulate the concentration of NAD. It is found that Nampt can control the deacetylation of SIRT1 (Sirtuin 1) by controlling the synthesis of NAD, further regulate the acetylation of upstream kinase of adenosine 5′-monophosphate (AMP) - activated protein kinase (AMPK) and the redistribution in cells, and finally regulate the activity of AMPK and play a neuroprotective role. Nicotinamide mononucleotide (NMN), a product of Nampt enzyme, can play a neuroprotective role in MCAO by intraventricular administration and improve animal neurological function. It mainly includes the reduction of cerebral infarction volume, the reduction of neurological defects, and the reduction of cell death in penumbra. In the model of MCAO reperfusion mice, NMN was injected intraperitoneally (500 mg/kg) once a day for more than one week, which can significantly reduce the mortality of stroke and promote the nerve regeneration and functional recovery after stroke.

To sum up, in this study, collagenase-induced ICH in mice model under the action of nicotinamide mononucleotide near the hematoma tissue cells and nerve function are studied. The results show that nicotinamide mononucleotides can improve the collagenase-induced ICH in mice under MCAO model, and the effect is very positive. The innovation of this study is that the mechanism and effect of nicotinamide mononucleotides on ICH in mice have been studied comprehensively, both in cell structure and RNA expression. The single administration and long-term administration in hyperacute period are used to prove that NMN plays a beneficial role in collagenase-induced ICH model in mice.

2. Materials and methods

2.1. Research subjects

Male mice, weighing 25–35 g, are purchased from Shanghai Ninth People's Hospital and bred in a clean environment. The space temperature is maintained between 20 and 25 °C, and the mice can eat and drink normally. All experimental animals were dealt with following the requirements for treating experimental animals and agreed by animal management agencies, with the consent of the relevant ethics committee, and in accordance with relevant regulations.

2.2. Experimental methods

The model of ICH is prepared as follows. Mice weighing 25–35 g are taken out and chloral hydrate (400 mg/kg) is injected into the abdomen of the mice. After the mice are completely anaesthetized, a transverse opening is made between the eyes of the mice with a scalpel to expose the skull of the mice to a clearly visible field of vision. A scalpel is used to gently scrape the bones above the mouse skull, and then they are wiped off with a cotton ball. The mouse's brain is placed on a stereotaxic device (World Precision Instruments Trading Co., Ltd., USA). The position of the mouse skull is adjusted to ensure that it is at the same level as the horizontal plate on the equipment. The input coordinates are clearly marked on the mouse skull. At the coordinate points already marked, a small hole through the skull is opened with a radius of about 0.05 mm. The skull coordinate position is adjusted so that the syringe (Shanghai Guangzheng Medical Instrument Co., Ltd., China) can pass through the skull hole smoothly and make it pass slowly at a very slow speed (2 mm/min) to reach the target position (4 mm in length). Every minute, a 0.2-l collagenase solution is slowly injected, requiring a total of 1 ul. When the injection is completed, the needle of the syringe needs to be kept in place for 10 min. Then, the needle of the syringe is slowly pulled out of the skull. Previously prepared bone wax is gently applied to the surface of the drilling mark. Then, the skin is sutured with a needle and thread, and the mouse skull is removed from the device. Then, 1 mL of saline solution is injected from the abdomen. The mice are placed at 35 °C for 1 h.

NMN administration method: In the detection of the effect of NMN administration based on brain damage caused by ICH in mice (nicotinamide mononucleotide), the NMN (400 mg/kg) is injected from the tail artery of mice 1 h after the model of ICH is successfully established. In the delayed experiment, after the construction of the collagenase cICH model, the mice are continuously injected with NMN from the abdomen. The first injection time is 40 min after the model is built. In sham group, saline is injected in a consistent manner.

Brain edema detection: The mice are completely anesthetized one day after the construction of the cICH model. The brain of the mice is divided into five parts: contralateral basal nucleus, cerebellum, diseased basal nucleus, contralateral cortex and diseased cortex. The weight of each part is weighed. Next, the tissues and organs are weighed at 100° for 48 h. Brain water content = [(wet-dry weight)/wet weight] * 100%.

Detection of hematoma volume: The mice are anesthetized completely one day after the construction of the cICH model. The mice's brains are taken out and frozen for 1 h at −30 °C. The telencephalon and cerebellum are not abandoned. The brain is made into eight equal-sized pieces. The hematoma volume ratio (%) is the sum of hemorrhage area and contralateral hemisphere area.

Blood loss test: Sample preparation: 24 h after the model of cerebral infarction is built, the mice are completely anesthetized. The PBS solution, which is 50 mL in volume, has been pre-cooled in advance. It is used for perfusion and the mouse brain is taken out. The telencephalon and cerebellum are not abandoned. The brain is divided into contralateral and affected hemispheres from the middle position. They are ground in 500 L of saline water and centrifuged (Bio-Rad, USA) at 12,000 rpm for 1 h, leaving only the supernatant liquid for later use.

Calculation of sample hemoglobin concentration:

$$MCHC = [OD_{SAMPLE} - OD_{BLANK})/(OD_{STD} - OD_{BLANKE}] * 100(mg/dl)$$

Detection procedure: A clean 98-hole plate is taken out. First, 300 ul of pure water is injected into the blank hole, and then 100 ul of samples and 300 ul of chemical reagents are injected into the standard hole. 50 ul and 300 ul chemical reagents are injected into the reaction pore of the sample. Ninety-eight plates with holes are slightly shaken (JIM-X scientific, USA) and placed at room temperature for 10 min. The absorbance of 500 mm is measured by a multifunctional enzyme label (Tacana, USA).

Total RNA extraction and RT-PCR (Reverse Transcription-Polymerase Chain Reaction): 24 h after cICH modeling, mice are anesthetized. The tissue around the hematoma is removed and frozen in liquid nitrogen and stored at −80 °C. TRIzol (Invitrogen, USA) is used to extract total RNA. Super-Script reverse transcription kits (Invitrogen, USA) and Oligo (dT) (Invitrogen, USA) are used to synthesize CDNA chains with primers. SYBRgreen (ABI Company, USA) and Beta-actin, IL-1β, IL-4, IL-6, IL-10 and TNF-α primers (Shanghai Bioengineering Company, China) are used for amplification. The Ct value is read on the fluorescence curve (ABI Company, USA), and Beta-actin is the internal reference. The rela-

tive expressions of the above-mentioned primers are calculated. The expression of the primers in the affected hemisphere of Sham is taken as the basis. The increase multiples of the primers in the cICH model group are calculated.

Immunohistochemical staining: Sample treatment and staining: One day after the completion of the cICH model, the mice are completely anesthetized. The 50 mL pre-cooled phosphate buffer solution (PBS) experimental solution is used for perfusion. Then, 300 mL of polyformaldehyde reagent with a concentration of 5% is used for cardiac perfusion. The mouse brain is taken out, and the telencephalon and cerebellum are abandoned. The rest of the unnecessary brain organs are stored in formalin (China Pharmaceutical Group Chemical Company, China) solution for 48 h at 5 °C. Formalin solution was released. The organs were then placed in 40% formalin reagent and stored at 5 °C for 48 h. When the organ reaches the bottom of the container of formalin solution, it can be used again.

TUNEL staining: For the specific operation of TUNEL staining, the treatment method is the same as that of immunohistochemical staining. Ordinary paraffin is used for encapsulation and burial, and the way of wax removal is also the same as that of immunohistochemical staining. Protease is added and placed quietly. Within 20 min, it should not be touched. The finished brine is used for repeated rinsing, at least five times. The PBS reaction solution containing 5% hydrogen peroxide solution is injected and mixed at room temperature for 10 min. PBS solution is used for full rinsing. Absorbent paper is used to absorb excess liquid. Then, 60 ul of TUNEL dye is injected and incubated for 2 h at 35 °C to avoid sunlight. The treated saline solution is used for repeated flushing, at least five times, for microscopic observation.

Weight weighing: to test the efficacy of NMN in mice's cICH model, the mice were weighed first. Firstly, the experimental animals were randomly divided into three groups: sham group, cICH model group, and cICH administration group. 30 min after the establishment of the model, the administration group was injected with 300 mg/kg NMN dissolved in normal saline through the tail, and the sham operation group and the model group were given the corresponding amount of normal saline by the same method. Record the diet of each group of mice, then place the mice on a safe and clean workbench, shake off the extra things on the mice gently to avoid errors, and then weigh and record.

TUNEL staining: it was the same as immunohistochemical staining, and paraffin embedding and dewaxing were used. Add proteinase K, stand for 15 min, wash with ultrapure water for 4 times. Add Phosphate Buffered Saline (PBS) containing 2% H_2O_2 and react at room temperature for 5 min. Then wash with PBS, remove the excess liquid with filter paper, add 50 uL TUNEL detection solution, incubate at 37 °C in dark for 1 h, wash with PBS for 3 times, make NBT coloring, and observe under microscope.

Nervous system score: there are two main methods: the first is physical sign evaluation method, and the second is Beam-walking method. The main methods of physical sign evaluation are: 0 point: no neurological injury symptoms, can act normally; 1 point: unable to make the opposite front paw fully extended; 2 points: crawling to the opposite side of the circle; 3 points: the body falls to the hemiplegic side when walking; 4 points: cannot walk themselves and lose consciousness; 5 points: died.

The Beam-walking method is mainly: place the mice on the crossbar (1.2 m long, 1.5 cm wide, and 50 cm high). If the mouse can't keep balance on the crossbar (<5 s), it will be 0 point; if the mouse can keep balance on the crossbar (>5 s), but can't move on the crossbar, it will be 1 point; if the mouse can move on the crossbar, but can't traverse the crossbar, it will be 2 points; if the affected limb stretches and doesn't touch the surface of the crossbar when the mouse traverses the crossbar, or turns around in the middle of the crossbar, it will be 3 points; if the frequency of foot slipping was 100% when the mouse traversed the crossbar, it will be 4 points; if the frequency of foot sliding is between 50% and 100% when the mouse traverses the crossbar, it will be 5 points; if the frequency of foot sliding is less than 50% when the mouse traverses the crossbar, it will be 6 points; if the frequency of foot sliding is less than 2 when the mouse traverses the crossbar, it will be 7 points. The final result is expressed as the average score of three tests. Using this method to measure the nerve function, it is necessary to train the mice to walk on the single wooden bridge one day before modeling.

2.3. Statistical methods

In addition to neurological function, the other data of Sham group, model group and drug group are analyzed by double sample equal variance hypothesis *t* test. The neurological function score data of Sham group, model group and drug group are analyzed by two independent sample rank sum tests. The data of affected hemisphere and contralateral hemisphere are analyzed by paired test.

3. Results

3.1. ICH in cICH + Vehicle model group and sham group

As Fig. 1 shows, after the cICH + Vehicle model is built, the model is tested comprehensively. In this study, compare the sham group with the model group by combining the histological sections and hemoglobin content. As can be seen from the figure, the hemoglobin content of sham group is lower than that of model group. Tissue sections also show clear bleeding in the model group (see Fig. 2).

3.2. Effects of NMN administration during acute period on neurological function of cICH + Vehicle model mice

Fig. 3 shows the neurological function score of mice 24 h after cICH modeling, with no obvious change in the recovery of nerve function in sham group ($P < 0.001$) compared with the model group, which also shows that the nerve function of mice is significantly affected by ICH model. Compared with the model group, administration group has more ideal recovery effect of neurological function ($P < 0.001$), which fully shows that NMN has a significant effect on the recovery of neurological function in mice.

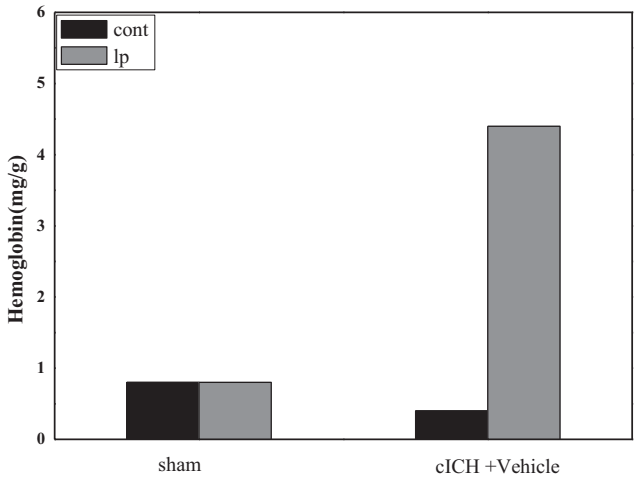

Fig. 1. ICH in cICH + Vehicle model group and Sham group.

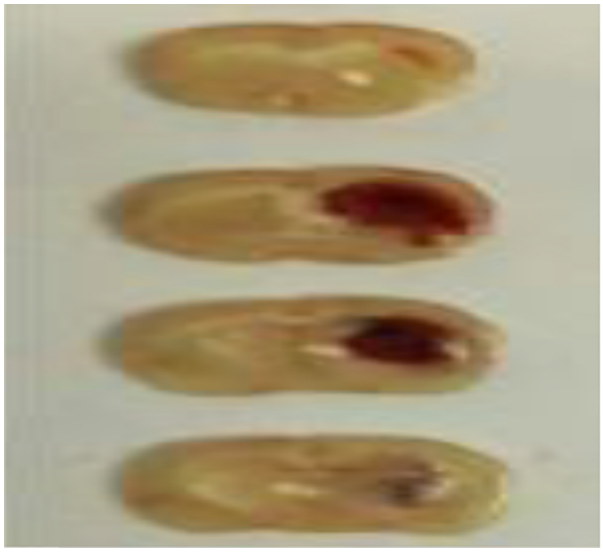

Fig. 2. Cerebral hemorrhage section.

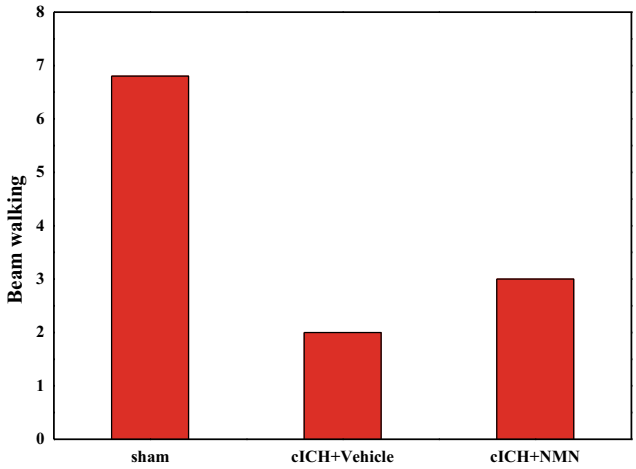

Fig. 3. Neurological score 24 h after cICH model establishment in mice.

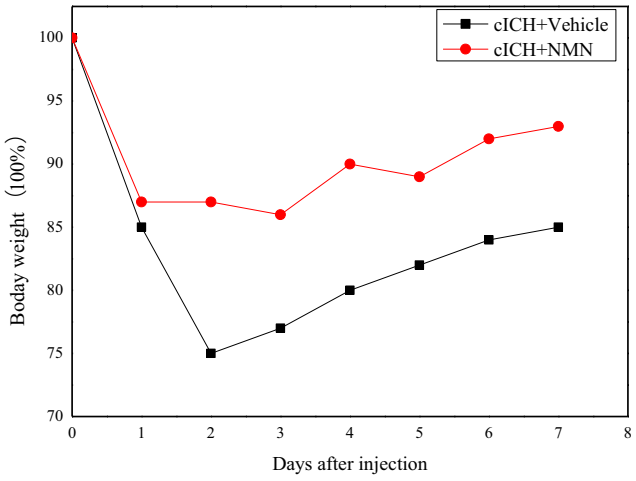

Fig. 4. Weight recovery curve of the drug and control groups in long-term drug administration experiments.

3.3. Effect of long-term administration of NMN on body weight recovery after ICH

In the long-term drug administration experiment, the weight recovery curves of the drug group and the control group are shown in Fig. 4. The data suggests that from the second day of administration, the decimal body weight begins to stabilize and then increases, indicating that the physical condition is recovering and improving. Compared with the model group, the performance of NMN administration is better, which shows that NMN administration has a very significant help for the post-operative rehabilitation of the cICH model group.

3.4. Effect of acute administration of NMN on oxidative stress in peritumoral tissue

The NOXI immunohistochemical staining of brain tissue in mouse cICH model is shown in Fig. 4. From Fig. 5, it can be seen that the expression level of NOXI in NOX1 positive cells in the model group is more obvious compared with the sham group in the detection of NOXI by immunohistochemical staining. It shows that in the collagen-guided model, the injury results in the increase of NOXI expression in the cells near the hematoma. Compared with the model group, the number of NOXI-positive cells in the administration group is lower, indicating that NMN administration can restore the NOXI situation caused by injury.

3.5. Effect of acute administration of NMN on activation of inflammatory chemokines and cell death in perihematoma tissues

TUNEL staining of brain tissue in mice cICH model is shown in Fig. 6. From Fig. 5, it can be seen that in the detection of apoptosis of mouse cells by TUNEL staining function, compared with sham group, the expression level and number of cells near the hematoma in the model group are significantly higher, indicating that hemorrhagic destruction gives rise to an increase in the number of cell death near the hematoma. Compared with the model group, the number of TUNEL positive cells near the hematoma in the administration group is significantly reduced, indicating that NMN administration can restore the cell death caused by injury. Decreased activity of glial cells and neurons can cause greater damage to the ability of nerve function. Therefore, the administration of NMN can reduce the apoptosis of nearby cells and increase the recovery of nerve function.

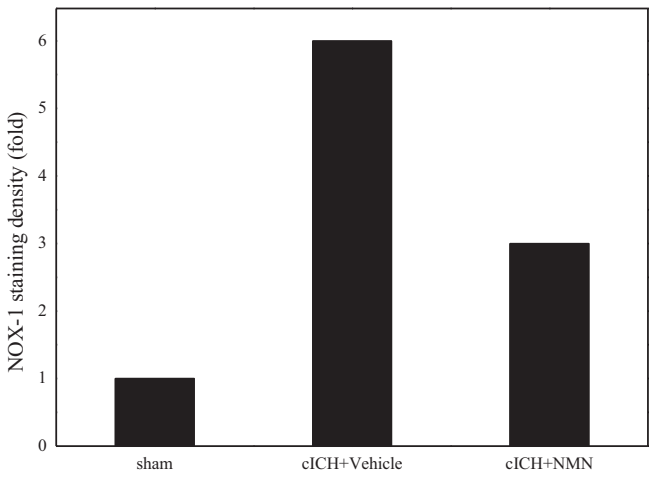

Fig. 5. NOXI immunohistochemical staining in brain tissue of mice cICH model.

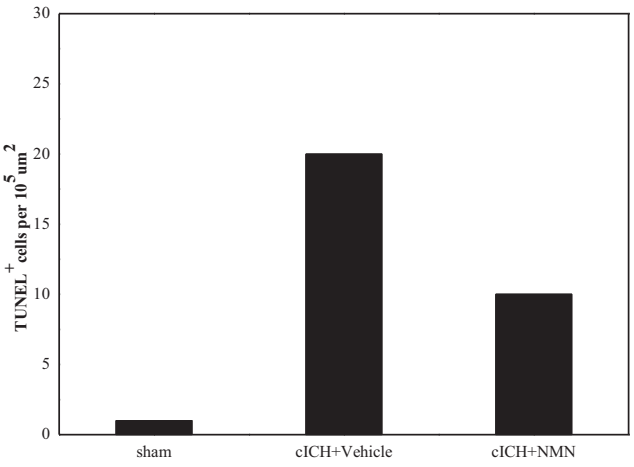

Fig. 6. TUNEL staining of brain tissue in mice cICH model.

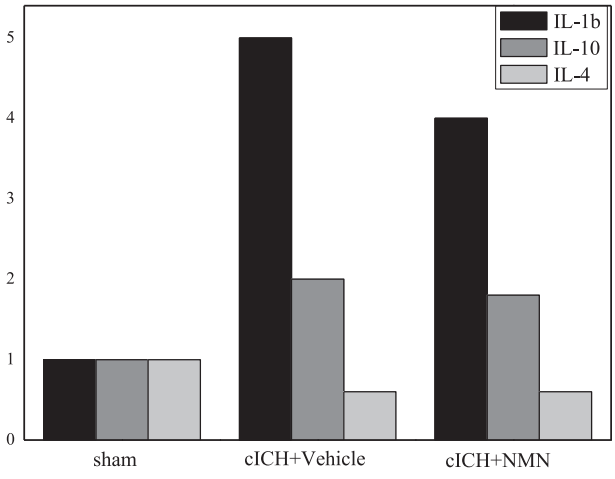

Fig. 7. IL-1b, IL-10 and IL-4 levels around CICH hematoma.

3.6. Effect of acute administration of NMN on activation of inflammatory chemokines and cell death in perihematomal tissues

The levels of IL-1b, IL-10 and IL-4 mRNA around the hematoma of cICH are shown in Fig. 7. It shows that, between the model group and the sham group, the level of L-4 near the hematoma in the former is lower when the expression of inflammatory factors is tested by RT-PCR, indicating that the hemorrhagic destruction results in the decrease of the expression of IL-4 near the hematoma. The situation of L-4 near the hematoma in the administration group is basically the same as that of the model group, indicating that NMN administration can improve the performance of IL-1b and IL-10, but the effect on IL-4 is not obvious.

4. Discussion

In this study, the tissue cells and nerve function near the hematoma in the collagenase-induced ICH mouse model under the action of nicotinamide mononucleotide are focused on. Male mice, 4–5 weeks, weighed about 22–35 g and ICR in Shanghai Ninth People's Hospital are divided into three groups: sham group, collagenase cICH + Vehicle group, and cICH + NMN group for intervention treatment. After 24 h, the neurological function, brain edema, hematoma volume, weight, hemorrhage volume, RNA expression level, apoptosis, inflammatory factors and ROS content in surrounding tissues of mice are analyzed comprehensively. The results show that the protective effect of nicotinamide mononucleotides on collagenase-induced ICH in mice is significantly better than that of the other two groups, mainly in neurological function, brain edema, inflammatory factors, body weight and cell apoptosis of mice, which indicates that nicotinamide mononucleotides are effective in treating ICH in mice. The improvement and protection of ICH in mice are significant.

Previous studies have proved that early tPA treatment can effectively improve the prognosis of patients after cerebral ischemia. However, whether tPA plays other roles in the CNS has not been clearly studied. This paper studies other roles of tPA in the CNS, which may help to reveal the mechanism of the side effects of tPA in the treatment of cerebral ischemia and prolong the time window of thrombolysis. More and more evidences suggest that endogenous tPA is involved in neurodegeneration and ischemic stroke in CNS. Some experts in the animal model of MCAO have shown that tPA gene knockout and inhibitors can improve the prognosis of stroke. The mechanism of the harmful effect of tPA in stroke is not clear, but it may be related to the aggravation of the excitatory toxicity induced by ischemia in CNS. In addition, tPA can also directly affect the permeability of blood-cerebrospinal fluid barrier. Another expert study shows that delayed administration of tPA can significantly aggravate the damage of blood-cerebrospinal fluid barrier in 24 h after stroke. This suggests that the side effects of tPA may be caused by the destruction of blood-cerebrospinal fluid barrier induced by tPA in the state of ischemia, and the increase of blood-cerebrospinal fluid barrier permeability may be the main inducing factor of hemorrhage transformation. Therefore, different grouping and administration of interventional therapy on collagenase-induced ICH in mice are studied in terms of tissue cells, gene and nerve. Nicotinamide mononucleotide NMN can effectively improve the damage of ICH to mice tissues, cells and nerves, and improve the survival of mice. Intervention therapy with nicotinamide mononucleotide NMN as the core has important research value. There are also some limitations in this study. For instance, too few samples are selected to make the experiment results convincing and unsatisfactory conditions. In the future, the experiment will be done under the satisfactory conditions with more samples and the interference will be reduced.

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