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Mechanism of ALDH2 improves the neuronal damage caused by hypoxia/reoxygenation

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Abstract. – OBJECTIVE: To investigate the protective effect and mechanism of ALDH2 on PC12 cells and brain nerve tissue injury under hypoxia.

MATERIALS AND METHODS: The hypoxia model of PC12 cells with low ALDH2 expression was established and screened. The eukaryotic expression vector of wild type pEGFP-N1-ALDH2 and blank plasmid pEGFP-N1 were constructed and transfected into PC12 hypoxia cells respectively. After reoxygenation culture, the morphology, quantity, ALDH2 expression level and apoptosis rate of the two groups were observed, and the role of ALDH2 in cell hypoxia injury was analyzed. Eighty SD rats were randomly divided into model group (ischemia-reperfusion injury group), Alda-1 group (intraperitoneal injection of alda-1 12 hours before and after modeling), DMSO group (intraperitoneal injection of dimethyl sulfoxide) and sham operation group, with 20 rats in each group. The neurobehavioral score, apoptosis rate of nerve cells, the content and activity of ALDH2 in active cerebral cortex and hippocampal CA1 area were compared.

RESULTS: The number of PC12 cells in hypoxia group was lower than that in control group. The expression level of ALDH2 protein in PC12 cells after 4 hours of hypoxia was lower than that in normal culture group. The number of PC12 cells transfected with wild-type recombinant plasmid was significantly more than that of blank plasmid group. Compared with the hypoxia group, the pre apoptotic and post apoptotic cells in wild type transfection group decreased after hypoxia treatment. Compared with sham operation group, nerve injury and apoptosis were increased in group M and DMSO, while ALDH2 activity and expression did not change significantly. Compared with M group and DMSO group, the nerve injury and apoptosis in Alda-1 group were improved, ALDH2 activity was increased, and ALDH2 expression was not significantly changed in Alda-1 group.

CONCLUSIONS: Increasing the expression of ALDH2 or enhancing the activity of ALDH2 can improve the injury of neurons induced by hypoxia/reoxygenation.

Key Words:

Acetaldehyde dehydrogenase 2 (ALDH2), Alda-1, Hypoxia/reoxygenation, PC12 cells, Cerebral cortex.

Introduction

Ischemic cerebrovascular disease is one of the major diseases that threaten human health and survival[1]. For the last few years, its incidence has been rising, and reperfusion of brain tissue during treatment often leads to ischemia/perfusion injury[2,3], which in turn causes oxidative stress, calcium overload[4], or massive production of inflammatory factors, resulting in irreversible cerebral ischemia/reperfusion damage. Revascularization leads to reperfusion injury, and irreversible damage will occur without sufficient reperfusion.

Mitochondria, which are important organelles involved in activities such as energy metabolism, are often involved in the early stage of ischemia and hypoxia[5], and the aldehyde dehydrogenase 2 (ALDH2) in the mitochondria is closely related to bio-oxidation and can reduce the expression of the transcription factor p53 by degrading the 4-hydroxynonenal (4-HNE) produced during ischemia and hypoxia, thus reducing ischemia-induced apoptosis. Studies have shown that ALDH2 also exhibits anti-oxidative stress damage activity and is highly transcribed in the brain[6]. If the ALDH2 activity in brain tissue can be activated, it can reduce the accumulation of aldehyde products during cerebral ischemia/reperfusion injury, which in turn may play a neuroprotective role[7].

The PC12 cell line is a rat adrenal pheochromocytoma cell line. In the presence of nerve growth factors, the cell body can grow neuronal axon-like protrusions[8] that are similar to neurons in terms of the morphology, as well as physiological and biochemical functions. This cell line has been widely used for neuronal cell research. Some scholars have used it as a cell model for studying hypoxic injury in the nervous system[9,10]. Cardiac and renal IRI research showed that preconditioning with an effective ALDH2 activator or physiological levels of ethanol provides protection for the corresponding organs, and consistently cor-

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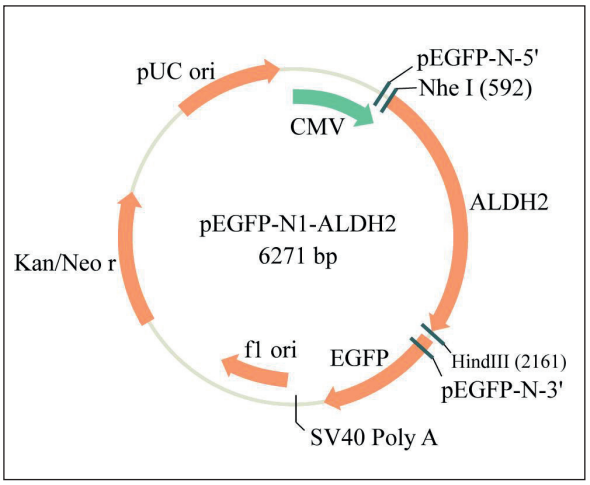

Figure 1. Wild type plasmid pEGFP-ALDH2 (primary: AT1).

relates with the phosphorylation status of ALDH2 to inhibit oxidative stress[11]. The aim of this study was to investigate the effect of ALDH2 on cell injury by observing the response of PC12 cells to hypoxia and reoxygenation after transfection with ALDH2. At the same time, animal experiments were carried out to observe the changes of neurobehavior, the content and activity of ALDH2 in cerebral cortex and hippocampus, in order to determine the protective effect of ALDH2 on nerve tissue injury.

Materials and Methods

Culture of PC12 Cells and Model Preparation

After resuscitation, PC12 cells (Kunming Institute of Animal Science) were passaged in a complete medium (DMEM/F12 basal medium, Invitrogen, Carlsbad, CA, USA), and the cells in the logarithmic growth phase were placed in a three-gas incubator (Heal Force) containing 5% CO_2 + 95% N_2 for 2 h/4 h/8 h/12 h (pre-experimental results had verified that the ALDH2 expression level was the lowest) and in a normal CO_2 incubator for 2 h (complete medium); the Western Blot method was used to detect the ALDH2 expression level in the PC12 cells (the ALDH2 antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Transfection of PC12 Cells

The PC12 cells in the logarithmic growth phase were resuspended in DMEM/F12 complete medium (Invitrogen, Carlsbad, CA, USA; 100 × 1 ml of penicillin/streptomycin double antibody + 10 ml of fetal bovine serum), seeded in 6-well plates at 1 × 10^5 cells/well, and cultured for 24 h. When the cells reached 70% to 80% confluence, the wild-type plasmid pEGFP-ALDH2 (Clontech, Figure 1) and a control plasmid were transfected into the cells with lipofectamine.

Verification of PC12 Cell Transfection

Flow cytometry (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was performed to verify the transfection of the PC12 cells; the instrument parameters were as follows: excitation wavelength Ex = 488 nm, emission wavelength Em = 530 nm. The FITC green fluorescence channel of Annexin V-FITC (FLK) was FL1, and the PI red fluorescence channel was FL2 (preferably FL3); the cells were also transfected with a non-hypoxia-treated negative plasmid to prepare the control for fluorescence compensation adjustment, to remove the spectral overlap and set the position of the cross door.

Animals and Grouping

Eighty male SD rats, weighing 240-300 g, were provided by the Experimental Animal Center of Kunming Medical University. During the study, the animal room was kept at about 22°C with a relative humidity of about 70% and an automatic illumination from 8 am to 8 pm. The animals could eat and drink freely. The use of all the experimental animals was approved by the Animal Management Agency of Kunming Medical University and complied with the relevant regulations. All the animals were treated humanely. After one week of adaptive feeding, the modeling was initiated; all the animals were prohibited food and water for 12 h before the modeling. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Kunming Medical University.

The SD rats were randomly divided into 4 groups, with 20 rats in each group: the M group, i.e., the ischemia/reperfusion injury group, in which the rats underwent cerebral ischemia-reperfusion surgery, and ether inhalation anesthesia was conducted before the surgery. After the femoral artery was separated, the distal end of the heart was ligated with silk thread, then the femoral ar-

tery was filled for 10-15 minutes. When the femoral artery is full, the proximal end of the femoral artery is clamped with a micro artery clamp, the artery is intubated with an intravenous indwelling needle, and then fixed with a silk thread. After 10 minutes of ischemia, the micro clamp was removed to remove the occlusion of bilateral common carotid arteries, and the blood was reinfused. The Sham group, in which the bilateral common carotid arteries and the femoral arteries and veins of the rats were exposed, and then sutured and observed for 24 h; the DMSO group, in which the rats underwent ischemia-reperfusion surgery with intraperitoneal injection of 1.5 ml of the Alda-1 solvent DMSO 12 h before and after the modeling; and the Alda-1 group, in which the rats underwent ischemia-reperfusion surgery with intraperitoneal injection of Alda-1 (12 mg/kg) 12 h before and after the modeling.

Preparation of Rat Cerebral Ischemia/ Reperfusion Injury Model

After intraperitoneal injection of 7.2% chloral hydrate (360 mg/kg) for anesthesia, we confirmed that the animals were not exhibited signs of peritonitis, and the animals anesthetized prior to retro-orbital blood collection. The femoral artery of each rat was isolated, and the distal end was ligated. After the femoral artery was filled, the proximal end was clamped with a micro-arterial clamp, and 150 IU/kg heparin was injected through a T tube to achieve systemic heparinization. Blood was drawn back through the T tube to achieve an average arterial pressure of 30 to 40 mmHg. A median neck incision was then performed to separate the bilateral common carotid arteries, which were clamped with micro-arterial clamps to induce ischemia. The color changes in the eyeballs and the respiratory status of the rats were observed. After 10 min of ischemia, the micro-arterial clamps were removed for blood re-supply; this was the reperfusion period. The rats were then sutured and maintained in a temperature-regulated environment (24°C) on a 12-hour light/dark cycle, housed in individual cages with soft bedding and a microisolator cover, and given free access to standard rat chow and tap water. Ketoprofen (5 mg/kg) was given for postoperative analgesia. The reperfusion period of this experiment was 24 h.

Neurobehavioral Score

According to the Neurological Severity Scores (NSS) scoring standard[12], the neurobehavioral score included aspects such as exercise, feeling, reflection, and balance. The lowest score was 0 points, and the highest was 18 points. A 0-point score was classified as normal, 1-6 points were classified as mild damage, 7-12 points were classified as moderate damage, and 13-18 points were classified as severe damage (Table I). All the neurobehavioral scores were taken 24 hours after the modeling and then animals were sacrificed.

Detection of Apoptosis

Ten rats from each group were selected and perfused with normal saline until their lungs and liver became white, and 10% formaldehyde was then perfused until their necks and forelimbs were stiff. Each rat was then decapitated, and its brain was sampled; this was immersed in 10% formaldehyde for fixation. The apoptosis was measured *via* the TUNEL assay (Nanjing Kaiji, Nanjing, China). Five fields of view were randomly chosen to take photos on both sides of the hippocampal CA1 area for calculating the apoptotic cell index (positive cell number/total cell number × 100%).

Determination of ALDH2 Protein Content

According to the instructions of the bicinchoninic acid (BCA) protein concentration determination kit (Beyotime, Shanghai, China), the total protein concentration was determined using Coomassie brilliant blue G250 method. After measuring the protein content, the volume of the solution containing 10 μg of protein was calculated and it was boiled for 5 min to denature the protein, followed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis. A 10% separation gel was prepared by referring to the kit instructions (Solarbio, Beijing, China), and then 2 μl of PageRuler (marker) and the protein samples of each group were added into different wells. The initial voltage of electrophoresis was 84 V, and when the sample line passed the boundary between the separation gel and the concentrated gel, the voltage was adjusted to 106 V until the bromophenol blue had just passed. The ALDH2 gel block (molecular weight 52.6) was then separated and the proteins were transferred onto a PVDF membrane. The ALDH2 concentration in the cerebral cortex and hippocampal CA1 region was measured using the chemiluminescence imaging method, and a gel image processing system (TS-2009) was used to analyze the net optical density value (gray value) of the target band.

Table I. Neurological scoring scale.

Item	Score
Exercise test	3
Tail rising	3
Forelimb flexion	1
Hind limb flexion	1
Vertical movement of head within 30 seconds >10 degrees	1
Walk	3
Normal walking	0
Can't go straight	1
Rotate to the temporal side	2
Falling to the temporal side	3
Feeling test	2
Placement feeling (visual, tactile)	1
Proprioception	2
Balance test	6
Stable posture	0
Can grab the balance beam edge	1
Can hold the balance beam but one limb falls	2
Can hold the balance beam but 2 limbs fall or rotate on the balance beam > 60 seconds	3
Trying to balance on the balance beam but falls, >40 seconds	4
Trying to balance on the balance beam but falls, >20 seconds	5
Falling down, doesn't want to be on the balance beam or hanging on the balance beam	6
Loss of reflection or abnormal movement	4
Wing reflection disappears (shaking head when moving ears)	1
Corneal reflection disappears (eye closing due to cotton swab touching the cornea)	1
Panic reflection disappears (sport response to sound)	1
Sudden twitching, tendon, or dystonia	1

Determination of ALDH2 Activity

Ten other rats in each group were kept under reperfusion for 24 h and then decapitated to sample the cerebral cortex and hippocampus for isolating the mitochondria using the Mitochondria Isolation Buffer. To each well of 96-well plates, 2 µl of the sample was added and supplemented with 48 µl of the assay buffer (ALDH Assay Buffer) to a total volume of 50 µl. To the background control wells were added 48 µl of Assay Buffer + 2 µl of ALDH Substrate Mix (ALDH Substrate Mix + 220 µl of dH_2O), and 50 µl of measurement solution (43 µl of ALDH Assay Buffer + 2 µl of ALDH Substrate Mix + 5 µl of acetaldehyde). The positive control was prepared by mixing 220 µl of Assay Buffer + 20% glycerol. All the wells were placed in a microplate reader, and the measurements at 450 nm were recorded at 0 min and every 2 min thereafter to plot the standard curve ($R^2 = 0.9982$).

Statistical Analysis

The experimental data were processed using the Statistical Product and Service Solutions (SPSS) 19.0 statistical software (IBM, Armonk, NY, USA), and the results were expressed as mean ± standard deviation ($\bar{x}\pm s$). The *t*-test was performed to compare the ALDH2 contents of the untransfected and transfected groups; the neurobehavioral scores, apoptosis of the hippocampal CA1 region, and ALDH2 contents and activity for different groups were compared using one-way analysis of variance (ANOVA), and the data with statistical significance underwent multiple pairwise comparisons *via* the Student-Newman-Keuls (SNK) test, with $p < 0.05$ being considered as statistically significant.

Results

Culture of PC12 Cells

Inverted microscopy revealed that the cells were in good condition, with strong light transmittance, an elliptical cell body, and long protrusions on both sides (Figure 2A); the hypoxia culturing was carried out using 5% CO_2 + 90% N_2 + 5% O_2 culture conditions (Figure 2B). Compared with that in the normal control group, the neurite outgrowth of PC12 cells in the hypoxia group was shorter, and some cells died, appeared circular, and floated (Figure 2C); the number of cells was significantly lower (155.33 ± 6.81 *vs.* 223.00 ± 8.54, $p < 0.05$). After 72 h of transfection with the recombinant plasmid pEGFP-N1-ALDH2 (wild type), the number of cells increased significantly

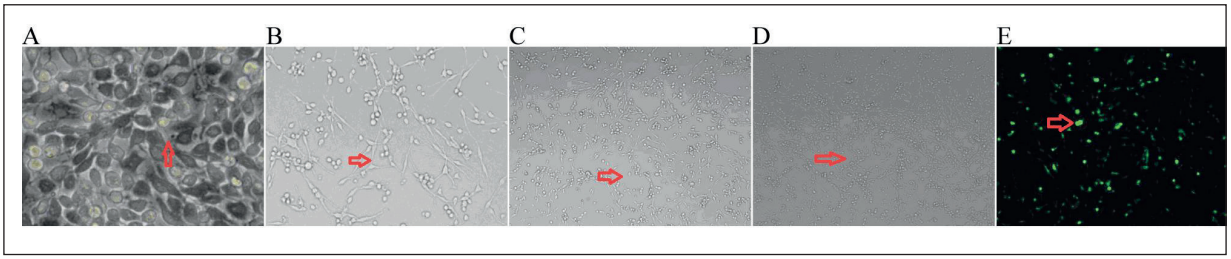

Figure 2. PC12 cells number altered in different status, the arrows showed the cells. **A**, Normal culture, 200×; **B**, 5% CO_2+90% N2+5% O_2 anoxic conditions, 100×; **C**, 4 h-hypoxia, (100×); **D**, bright field of fluorescence microscopy after wild type pEGFP-ALDH2 transfection, (100×); **E**, fluorescence field after wild type pEGFP-ALDH2 transfection, (100×).

more than that of the untransfected group (Figures 2D and 2E, $p < 0.05$).

ALDH2 Expression in Hypoxia/Reoxygenation PC12 Cell Model

The Western blot results showed that, compared with the expression in the normal group, the expression of ALDH2 protein was significantly lower in the 4-h hypoxia group, and the ratio of gray scales of ALDH2 to GAPDH decreased (Figure 3, $p < 0.05$).

Apoptosis in PC12 Cell

The proportions of cells in the pre-apoptotic phase and in the late apoptotic stage were both lower in the transfection group than in the hypoxia treatment group (Figure 4, $p < 0.05$).

Analysis of Neurobehavioral Scores

The neurobehavioral score (2.5 ± 0.8) of the Sham group was significantly lower than those of the other three groups ($p < 0.05$), followed by that of the Alda-1 group (6.8 ± 0.7), for which, although the neurobehavioral score was higher than that of the Sham group, it was still statistically lower than those of the M (9.3 ± 1.8) and DEMO groups (8.5 ± 1.2) ($p < 0.05$); there was no statistical difference between the M and DEMO groups ($p > 0.05$).

Apoptosis In Brains Of SD Rats

Unlike the Sham group, only a few apoptotic neurons were observed in the hippocampal CA1 region of the Alda-1 group; the number of neuronal apoptotic cells in the hippocampal CA1 regions of the M and DEMO groups was significantly higher (Figure 5). The apoptosis indices of the different groups were statistically significant ($p < 0.05$); the SNK method was used for further pairwise comparison, and the results revealed a significant difference between two groups (Figure 5): the Sham group had the lowest apoptosis index (9.42±0.93), which was statistically different from that of the M (25.51±2.94), DEMO (19.28±5.07), and Alda-1(13.84±1.6) groups ($p < 0.05$). There was no significant difference between the M and DEMO groups ($p > 0.05$), whereas the index of the Alda-1 group was significantly lower than those of the M and DEMO groups ($p > 0.05$).

ALDH2 Protein Expression Level in Brain of SD Rats

The results of Western blot (ALDH2/β-actin) after 24 h of reperfusion showed no significant difference between two groups (Sham: 0.81 ± 0.19, M: 1.07 ± 0.11, DMSO: 0.92 ± 0.24, and Alda-1: 0.87 ± 0.10, $F = 1.639$, $p = 0.233$).

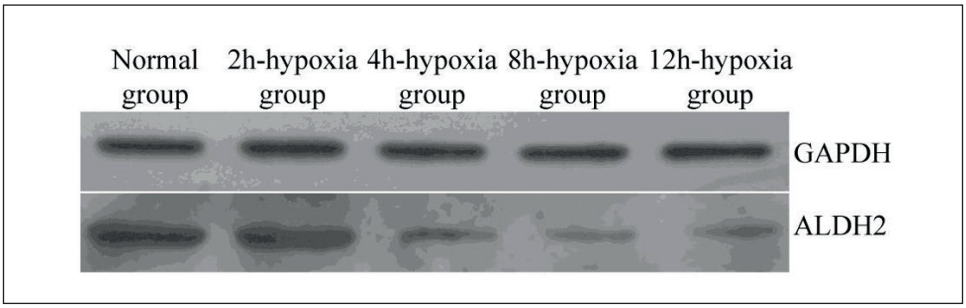

Figure 3. ALDH2 expression in hypoxia/reoxygenation PC12 cell model.

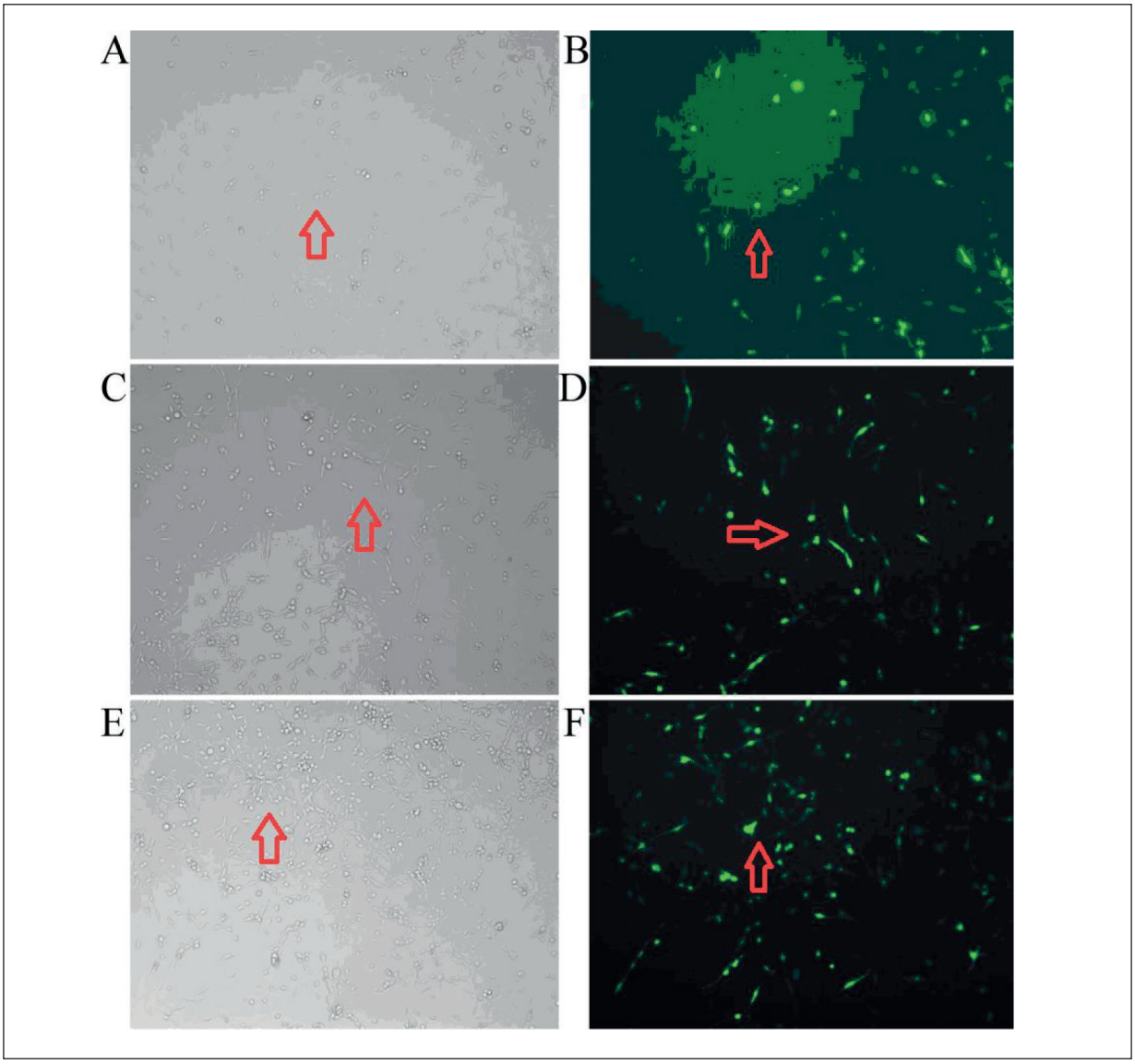

Figure 4. Analysis of the apoptosis in PC12 cells in different groups using fluorescence microscopy, the arrows showed the cells. **A**, Blank plasmid GFP (-), ALDH2(-), hypoxia, inverted microscope. **B**, Blank plasmid GFP(-), ALDH2(-), hypoxia, fluorescence microscope. **C**, Mutant type plasmid GFP (+), ALDH2(-), hypoxia, inverted microscope. **D**, Mutant type plasmid GFP (+), ALDH2(-), hypoxia, fluorescence microscope. **E**, wild type plasmid GFP (+), ALDH2 (+), hypoxia, inverted microscope. **F**, Wild type plasmid GFP(+), ALDH2(+), hypoxia, fluorescence microscope (100×).

ALDH2 Activity in Brain of SD Rats

After 24 h of reperfusion, the ALDH2 activity analysis showed that there was a statistically significant difference between the Alda-1 (7.60 ± 3.86) and Sham (0.77 ± 0.42) groups, as well as between the M (2.73 ± 2.06) and DMSO (3.38 ± 3.16) groups; however, the other groups showed no statistically significant difference ($p > 0.05$), suggesting that the level of ALDH2 activity in the brain significantly increased after the injection of Alda-1.

Discussion

In this study, we selected a differentiated PC12 cell line to simulate the changes in nerve cells in cerebral ischemia/reperfusion injury. While the number of PC12 cells decreased significantly after reoxygenated for 2 h with previous hypoxia, also the expression of ALDH2 decreased. After 72 h of transfection by pEGFP-N1-ALDH2 eukaryotic expression vector, the number of PC12 cells and the ALDH2 level were restored, and the apoptosis also reduced.

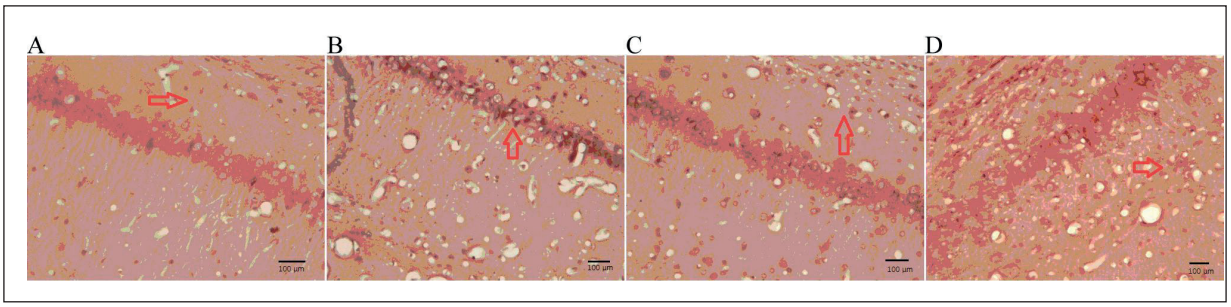

Figure 5. Detection of apoptosis in hippocampal CA1 region by TUNEL (×100). **A**, The sham operation group (Sham). Ratio of apoptosis 9.42±0.93. **B**, The DMSO group (DMSO, intraperitoneal injection of 1.5 ml of DMSO). Ratio of apoptosis 25.51±2.94*. **C**, Model group (M). Ratio of apoptosis 19.28±5.07*. **D**, The Alda-1 group (Alda-1, intraperitoneal injection of Alda-1 12 mg/kg 12 h before and after modeling). Ratio of apoptosis 13.84±1.61*$^{\#\Delta}$. Compared with group Sham, *p<0.05; compared with group M, $^{\#}p$<0.05; compared with group DMSO, $^{\Delta}p$< 0.05 (100×).

Similarly, we obtained consistent results in the animal experiments. The neurobehavioral scores of the rats showed that the neurological deficits in the M group were the most serious, the apoptosis in the hippocampal CA1 region was the most evident, and the ALDH2 activity significantly decreased. However, after the ALDH2 activator Alda-1 was administered, the neurological deficits of the rats were improved, the extent of apoptosis decreased, and the level of ALDH2 activity also significantly increased, indicating that the activity of ALDH2 was closely related to hypoxia/reperfusion and also associated with nerve damage. After the Alda-1 treatment, ALDH2 was activated, the apoptosis in the rat hippocampal CA1 region was alleviated, and the neurological function was restored to a certain extent, which further demonstrated that the ALDH2 activator (Alda-1) could pass through the blood-brain barrier and activate the enzyme ALDH2, thus acting as a neuroprotective agent. However, this process did not involve an increase in the expression level of ALDH2 protein. We think that this is the mode of action for the protective effect of ALDH2 in hypoxia/reoxygenation injury.

Ischemic cerebrovascular diseases are extremely harmful, and the main purpose of clinical treatment is to open the occluded blood vessels as soon as possible to save the penumbra. However, after revascularization of the occluded cerebral vessels, cerebral ischemia/reperfusion injury can occur in a short period of time, and can aggravate the clinical defects, resulting in poor prognosis; currently, the mechanisms of this injury are not fully understood but mainly include excessive formation of oxygen free radicals[13-15], increased intracellular free calcium[16], or a series of metabolic effects of acidosis[17].

Among them, the significance of a large number of oxygen free radicals has been recognized by most scholars. This process occurs not only in the reperfusion phase of sudden reoxygenation[18,19] but also in the ischemic phase of the brain tissue[20]. Mitochondria are extremely sensitive to ischemia and hypoxia. If there is no timely energy supply in the form of ATP or phosphocreatine, the reduction of a small amount of cerebral blood flow will cause damage and dysfunction of brain cells[21,22]. Mitochondrial ALDH2 can oxidize the intermediate of glycol metabolism (acetaldehyde) to non-toxic carboxylic acid, which plays a major role in removing aldehydes in the body. Inactivation of ALDH2 can cause the accumulation and metabolism of acetaldehyde, thus causing irreversible damage to tissues and organs. Bai et al[23] found that the overexpression of the *ALDH2* gene may attenuate 4-HNE-induced neuronal cell death, which may be achieved by regulating caspase-3 protein and reducing the production of reactive oxygen species. Eckert successfully transfected PC12 cells with plasmids and obtained stably expressing cells[24,25]. Ohta et al[26] verified the protective effect of ALDH2 on PC12 cells by transfecting the *ALDH2* gene and an inactive mutant *ALDH2* gene into these cells; their results are consistent with those of this study. ALDH2 overexpression delays the formation of 4-HNE and toxic aldehyde adducts, and thus preserves the mitochondrial function during IRI.

By studying rodent models, Chen et al[27] found that if small molecules of the ALDH2 activator Alda-1 were administered to rats before ischemic myocardial infarction events, the area of the infarction reduced by 60%, i.e., if ALDH2 is always activated in the heart, it will result in a decrease in the degree of myocardial ischemic

damage. Researchers have speculated that this may be due to the reduction in the formation of cytotoxic aldehydes.

Limitation

In our study, we just provided the protein change results, no RNA or gene results were given; the sensitivity of ALDH2 on PC12 has not been detected. More clinical studies are still needed.

Conclusions

Hypoxia-ischemia was induced by artificial hypoxia/reperfusion in rats, and Alda-1 was injected 12 h before and after modeling; the results confirmed that in the rat model of cerebral ischemia, Alda-1 could also reduce the damage caused by hypoxia-ischemia. A possible explanation for this phenomenon is that during the ischemia-reperfusion phase, the ability of the lipid peroxygen reaction to produce aldehydes far exceeds that for clearing ALDH2. If Alda-1 also has a similar effect in humans, the use of drugs to enhance the activity of ALDH2 may be a new treatment for patients who have undergone coronary artery bypass surgery or have other myocardial ischemic diseases.

Acknowledgments

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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