

Effects of rosuvastatin on neuronal apoptosis in cerebral ischemic stroke rats *via* Sirt1/NF-kappa B signaling pathway

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Abstract. – OBJECTIVE: To investigate the effects of rosuvastatin on nerve cell apoptosis in rats with cerebral ischemic stroke through Sirt1/NF- κ B pathway.

MATERIALS AND METHODS: 30 model rats were divided into three groups: normal group, stroke group (rats with cerebral ischemic stroke) and stroke+RVT group (cerebral ischemic stroke rats treated with rosuvastatin). The expression of Sirt1/NF- κ B, areas of stroke infarction, cell cycles, as well as apoptosis situation in different groups were detected by Western Blot, immunohistochemistry, histomorphological observation, triphenyl tetrazolium chloride (TTC) staining and flow cytometry as well as immunofluorescent staining.

RESULTS: Optical microscope observation showed cells in normal group presented complete and clear cellular hierarchical structure, regular cell arrangement, bluish violet cell nucleus and pink cytoplasm. No damage or necrosis was observed under normal condition. In stroke group, the boundary line between cytoplasm and nucleus was blurry and some apoptosis bodies were also observed. However, after rosuvastatin treatment, necrosis disappeared in stroke+RVT group. Western Blot analysis showed that the expression of SIRT1 decreased and NF- κ B elevated in stroke group compared with those in normal group ($p < 0.05$). However, rosuvastatin could reverse the effects of stroke on SIRT1 and NF- κ B ($p < 0.05$). The results of immunohistochemistry and immunofluorescent staining also confirmed our findings in SIRT1 and NF- κ B expression after stroke. The areas of cerebral infarction increased significantly in stroke group and this effect could also be reversed by rosuvastatin treatment ($p < 0.05$). Besides, cell cycle detection also showed that rosuvastatin treatment could inhibit the shortening of G1, S as well as G2 periods in cell cycles after stroke ($p < 0.05$).

CONCLUSIONS: Rosuvastatin may have great effects on improving cerebral infarction condition in rats with cerebral ischemic stroke. The mechanisms may be through Sirt1/NF- κ B pathway, thereby reducing the apoptosis rate and improving cell cycle of brain cells.

Key Words:

Sirt1, NF- κ B, Ischemia, Cerebral stroke, Apoptosis.

Introduction

Cerebral ischemic stroke is a symptom from cerebral ischemia that is caused by disordered brain blood supply due to cerebrovascular stenosis or obstruction. It may develop into cerebral ischemia and anoxia, leading to cerebral necrosis, malacia, or even neural deletion¹. Considering its very high incidence rate, mortality and disability rate, cerebral ischemic stroke has become the second killer threatening human health worldwide. Approximately 2 million people in the world (900 thousand in China) die from cerebral stroke each year. Patients having cerebral ischemic stroke shared only low survival rate and even though survived one may suffer from a lot of sequelae. Cerebral anoxia, necrosis and other illnesses are all complications to cerebral ischemic stroke. Hence, to investigate the pathogenesis for a better prevention and early treatment has been clinically a priority among priorities. The pathological basis of cerebral stroke is atherosclerosis. Formation of atheromatous plaque is a key factor to the attack and recurrence of cerebral ischemic stroke. As blood lipid and glucose become abnormal and blood viscosity increases, the artery blood vessel may be blocked, causing an ischemic stroke. Thus, reducing blood lipid is the foremost mean for treatment². Rosuvastatin is capable of lowering lipid, regulating body immunity as well as diminishing inflammation as widely accepted in clinical treatment. But an increased dose of rosuvastatin causes hepatitis, rhabdomyolysis and other adverse reactions³. Sirt1, dependent on enzymes, serves as a regulatory factor in cells. It also plays a role in the negative regulation of inflammation. NF- κ B was found mainly in the cytoplasm. As an inflammatory factor NF- κ B will be activated where there is cerebral injury

and then involved in the inflammatory responses after brain injury⁴. Generally, an inhibitor will be activated only when there is cell stimulation, and then degraded to help the activation of other factors relevant to it⁵. We aimed at investigating the effects of rosuvastatin on brain cell apoptosis in rats with cerebral ischemic stroke through Sirt1/NF- κ B pathway.

Materials and Methods

Primary Reagents and Instruments

Doxorubicin hydrochloride for injection (HISUN, Shanghai, China); Rosuvastatin calcium (AstraZeneca, London, UK); Rabbit anti-mouse Sirt1 monoclonal antibody and nuclear factor- κ B (NF- κ B) (Proteintech, Rosemont, IL, USA); Normal saline (Beijing University of Chinese Medicine, Beijing, China); kit (BioTek, Winooski, VT, USA); superoxide dismutase (SOD) (Eppendorf, Germany); TTC (Beyotime Biotechnology, Shanghai, China); alanine aminotransferase (Shanghai Supeng Chemical Reagent, Shanghai, China); Color Development Kit (Nanjing Jiancheng Technology, Nanjing, China); Dehydrator and embedding machine (Universal, Germany); Microtome and optical microscope (Olympus, Tokyo, Japan); Centrifugal machine (Proteintech, Rosemont, IL, USA); Electrophoresis apparatus (Universal, Germany).

Experimental Methods

Animals Grouping and Model Building

30 Wistar Specific-Pathogen-Free (SPF) rats (about 240 g) provided by Animal Lab, Southeast University, were fed 7 days as required in the shielded environment: 25°C + 38-75% RH. They were randomly divided into normal group, stroke group (rats with cerebral ischemic stroke) and stroke+RVT group (cerebral ischemic stroke rats treated with rosuvastatin). After 24 h abrosia, rats in the stroke group and stroke+RVT group were anesthetized with 2 mL/kg pentobarbital. An incision was made at the front-left of the neck. The left common carotid artery was dissociated to ligature external carotid artery and the monofilament was inserted into the left internal carotid artery. This incision was then sutured. 2 h later, the monofilament was pulled out and the anesthesia recovery waited until the rat model was successfully established. Rats in the stroke+RVT group were then injected with rosuvastatin calcium suspension of 10 mg/(kg·d) for 2 weeks. Those in the

normal group and stroke group were given the same amount of normal saline for 2 weeks.

Specimen Collection and Histomorphological Observation

24 h after cerebral ischemia-reperfusion, rats being anesthetized were executed immediately to collect the cerebral parietal cortex into 4% poly formaldehyde phosphate buffer for fixation. Through successive processes including dehydration, hyalinization, paraffin embedding and hematoxylin and eosin (HE) staining, the obtained brain tissues were observed with a microscope.

Expressions of SIRT1 and NF- κ B by Western Blot

Separated parietal tissues were dissociated with 2 ml RIPA Lysis Buffer. A crusher was used to make tissue homogenate in the centrifuge tube followed by centrifugation at 14000 r for 25 min. The collected protein was extracted, and 10 ml of separation gel solution were prepared and added into a centrifuge tube used for gel extraction, followed by an electrophoresis and membrane transfer. 5% non-fat powder buffer was used for blocking within 1 h. After that, 5 ML Western primary antibody diluent was utilized for incubation within 90 min and the second antibody was used for another incubation lasting 30 min. After that, the membrane was washed 5 times with Tris-Buffered Saline and Tween-20 (TBST) for the purpose of development. The obtained grey levels were subjected to quantitative analyses.

Expressions of SIRT1 by Immunohistochemistry

The obtained parietal cortex was dried in an oven followed by dewaxing and hydration. It was then rinsed with water and soaked into 2 ml H₂O₂ lasting 10 min, followed by another 3 times rinse with PBS for the sake of antigen retrieval. Tissues without the supernatant were dripped into 0.01 μ l phosphate buffer. Once the Antigen binding sites were found, it was rinsed 3 times with PBS, blocked, and incubated for 20 min. With the addition of primary and secondary antibodies, it was incubated again for 60 min. SP was also dripped and then transferred into a centrifuge tube. In the mixed DAB and H₂O, the color-developing agent should be added to observe the dyeing. When yellowish-brown SIRT1 appeared, hematoxylin dye liquor was added for redyeing and dehydration as well as mounting. Under the microscope, the number of positive cells was observed and recorded.

Areas of Cerebral Infarction by TTC Staining

Brain tissue slices in the thickness of 1 mm were made and properly placed into TTC staining fluid container for the following 1 h-incubation at normal temperature, during which the color of these samples was observed. Next, the original TTC stain was drawn out. Slices were stored at low temperature in another container for 1 h to observe the imaging and calculate cerebral infarction rate.

Expressions of SIRT1 and NF- κ B Detected by Immunofluorescence Staining

Brain tissue slices were prepared and fixed with 4% paraformaldehyde (PFA). Sheep serum was used to block, followed by 30 min incubation at room temperature. Afterwards, with the addition of rabbit anti-mouse Sirt1 monoclonal antibody and NF- κ B, brain tissue slices were incubated overnight at low temperature. FITC was prepared to mark goat anti-rabbit II antibody. Marked tissues were incubated again for 60 min. DAPI was selected to redyeing cell nucleus within 30 min with mounting in glycerinum. It was washed 3 times using PBS and observed under a fluorescence microscope.

Detection of Cell Cycles by Flow Cytometry

Brain cells were digested with trypsin and then centrifuged for 3 min, 2 000 r/min. Cells of group C were injected with rosuvastatin calcium. After the cultivation, it was rinsed 3 times using PBS and digested with the said trypsin and centrifuged in a centrifugal machine, 3 000 r/min, 6 min. Later, it was rinsed twice with phosphate-buffered saline (PBS). Suspension cells and FITC dye liquor were then mixed with the solution for the

purpose of reacting within 15 min in dark place. Flow cytometry was utilized for quantitative analysis. Cell cycles distribution and proliferation index were recorded.

Statistical Analysis

The experimental data were expressed as mean + standard deviation (SD). Chi-square test and One-Way ANOVA were used for statistical significance analysis. Tukey's HSD (honestly significant difference) test is used in conjunction with an ANOVA to find means that are significantly different from each other. In terms of the morphological observation, expressions of SIRT1 and NF- κ B, areas of infarction, cell cycles and apoptosis, the three groups were all analyzed using SPSS19.0 software (IBM, Armonk, NY, USA). $p < 0.05$ represents a significant difference.

Results

Effects of Rosuvastatin on Brain Morphology After Stroke in Rats

Based on optical microscope examination, it was observed that brain tissues from normal group have a completely hierarchical structure, regular arrangement, bluish violet nucleus as well as pink cytoplasm in good conditions. No damage or necrosis was observed. In stroke group, the brain tissues were partly denatured and necrotic, and the boundary lines between cytoplasm and nucleus were blurry. Besides, some apoptosis bodies were also observed in stroke group. After rosuvastatin treatment, the boundary lines between cytoplasm and nucleus became clear and those apoptosis bodies shrank in stroke+RVT group (Figure 1).

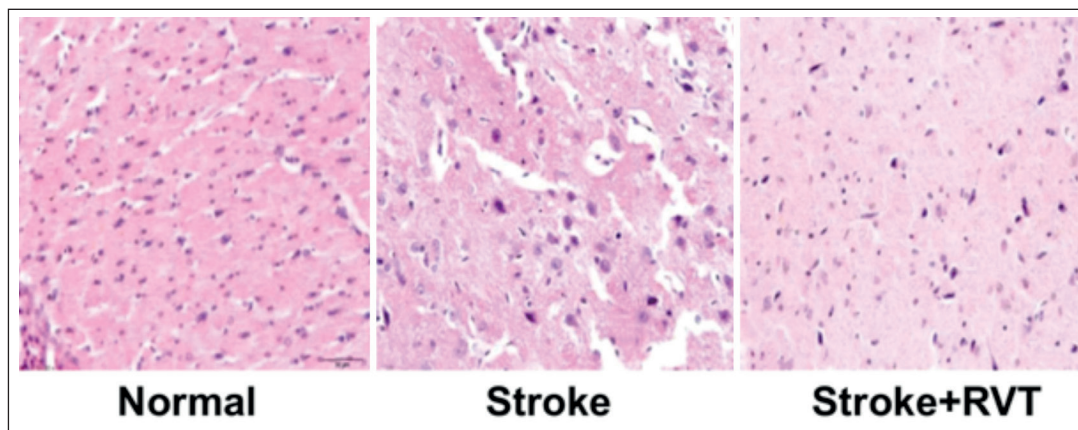


Figure 1. Morphological observation of rat brain tissues in each group (Magnification 400*).

Rosuvastatin Could Reverse the Sirt1 Down-Regulation and NF- κ B Up-Regulation in Rat Brain Tissues after Stroke

The expression of SIRT1 and NF- κ B proteins in brain tissues were detected by Western blot. Compared with normal group, the relative expression of Sirt1 was decreased while the relative expression of NF- κ B was increased significantly in stroke group ($p < 0.05$) (Figure 2A). After treatment with rosuvastatin in stroke+RVT group, the expression of Sirt1 enhanced and NF- κ B reduced compared to those in stroke group

($p < 0.05$) (Figure 2A-B). We also performed the immunohistochemistry of Sirt1 in brain tissues and the yellowish-brown expression in nucleus and microexpression in cytoplasm means positive Sirt1 expression in brain tissues (Figure 2C). The results showed that positive expression of Sirt1 significantly decreased after stroke (1.10 ± 0.32) compared with that in the normal condition (3.10 ± 0.69). And the relative positive expression of Sirt1 in stroke+RVT group reversed to 2.18 ± 0.82 , indicating the effects of Rosuvastatin on Sirt1 expression in brain tissues after cerebral ischemic

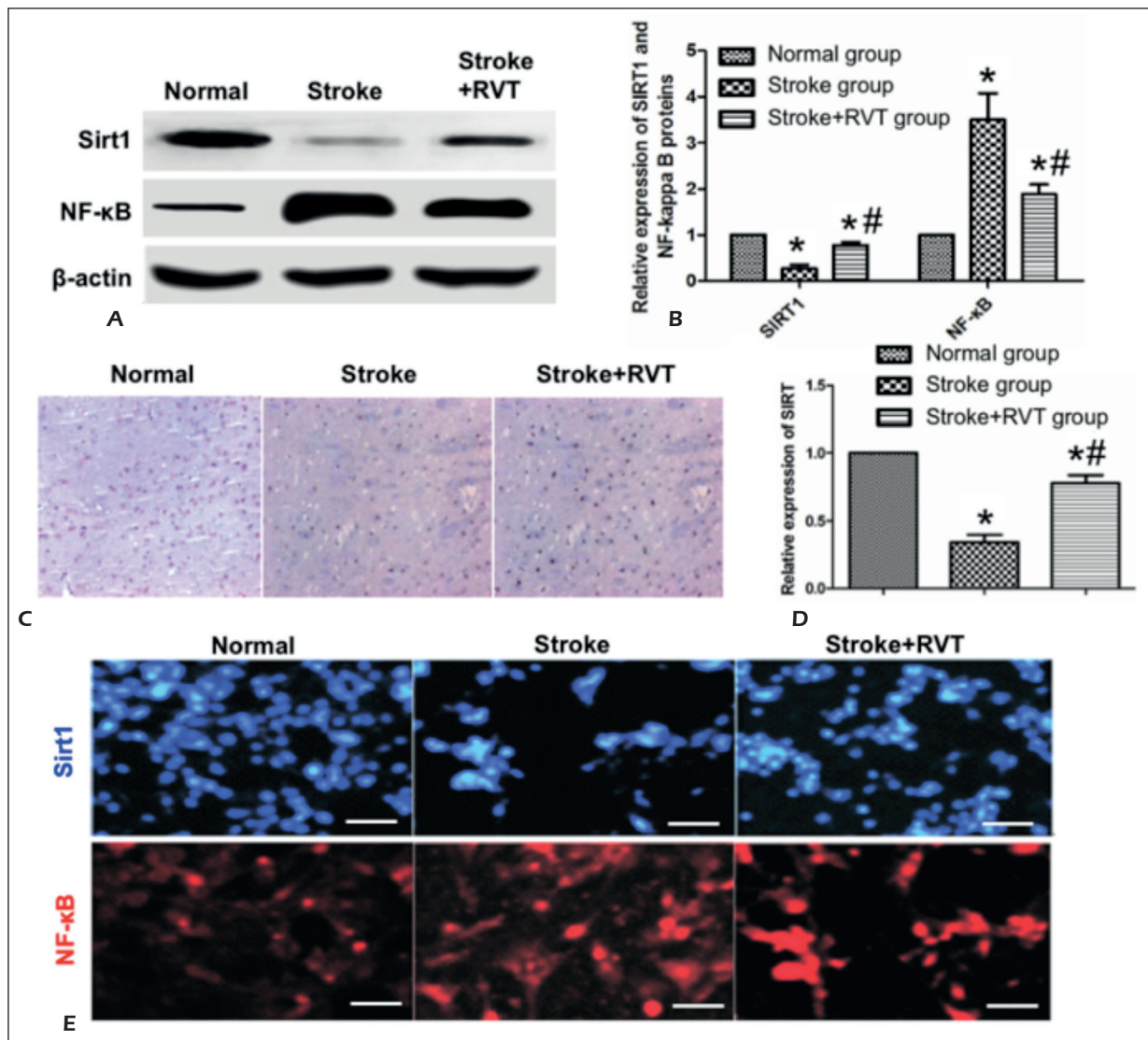


Figure 2. The expression of Sirt1 and NF- κ B in brain tissues of rats in each group (Magnification 400*). **A**, The protein expression of Sirt1 and NF- κ B in each group detected by Western Blot. **B**, The statistical analysis of Sirt1 and NF- κ B relative expression. **C**, The protein expression of Sirt1 and NF- κ B in each group detected by immunohistochemistry. **D**, The statistical analysis of Sirt1 relative expression in immunohistochemistry results. **E**) The immunofluorescence images to show Sirt1 and NF- κ B expression in cells isolated from brain tissues of each group (bar, 50 μ m). (*: $p < 0.05$ compared with normal group. #: $p < 0.05$ compared with stroke group).

stroke (Figure 2C-D). We then isolated cells from brain tissues of each group and detected the expression of Sirt1 and NF- κ B by immunofluorescence (Figure 2E). Consistently, the results also confirmed the effects of Rosuvastatin on SIRT1/NF- κ B pathway under stroke condition.

Rosuvastatin May Improve Cerebral Infarction and Cell Cycles in Rats after Stroke

The brain tissues of each group were subjected to TTC staining and the areas of cerebral infarction were calculated by Image J software (Figure 3A). The results showed that infarction area in

stroke group (infarction rate: $57.56 \pm 6.78\%$) was significantly larger than that in normal group ($p < 0.05$). Rosuvastatin may have greatly improved the conditions of cerebral infarction in rats with cerebral ischemia (infarction rate: $42.13 \pm 8.70\%$) (Figure 3A-B). Besides, we also measured cell cycles with flow cytometry. The results showed that cell cycle periods of G1, S and G2 were significantly shortened in stroke group compared with that in normal group (Figure 3C). This process could also be improved by Rosuvastatin, suggesting that protective effects of Rosuvastatin on cerebral infarction after stroke may be related to the variation in the cell cycle in brain tissues.

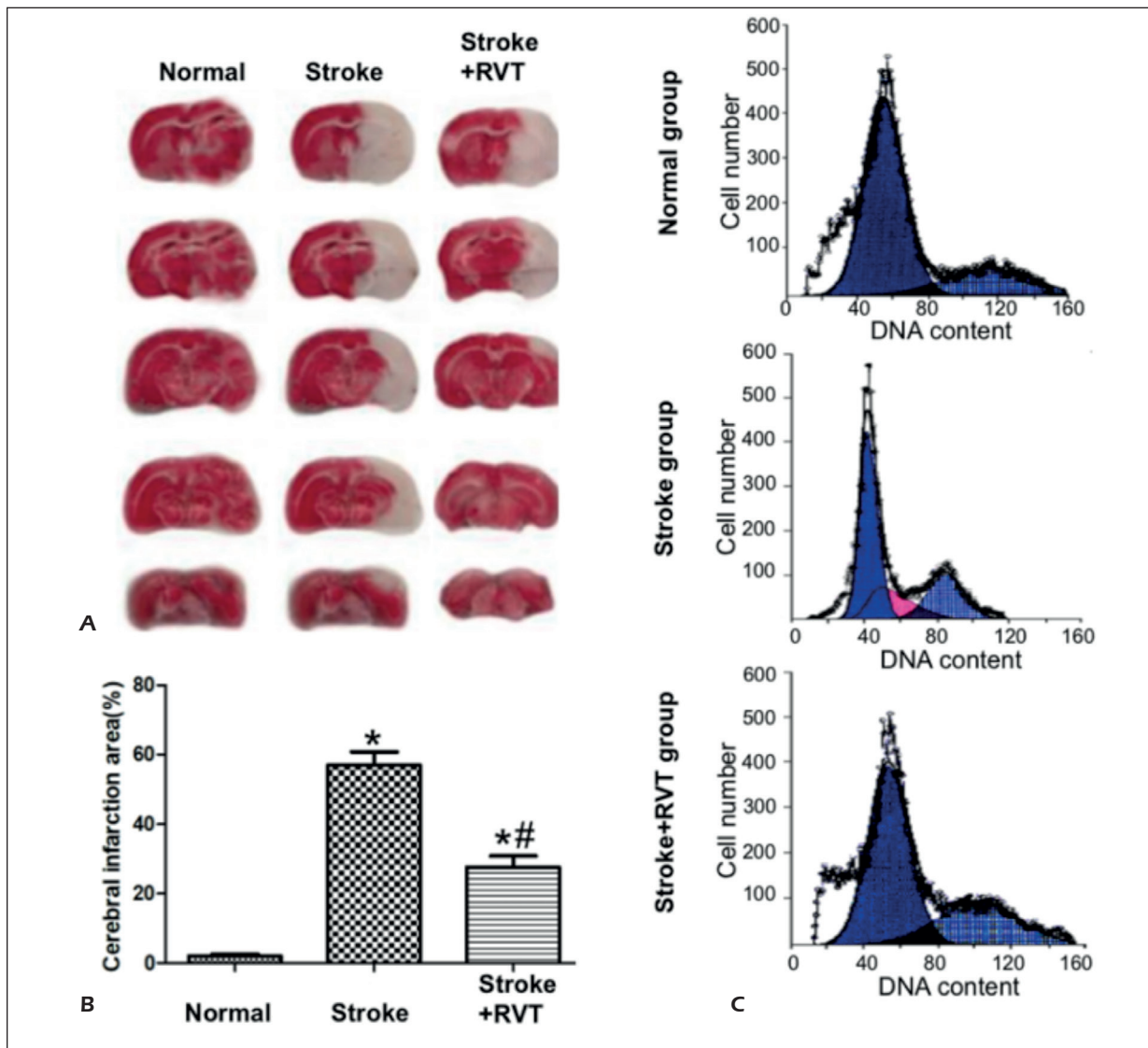


Figure 3. Cerebral infarction area and cell cycle measurements in stroke rats after treated with Rosuvastatin. **A**, TTC staining results for brain infarction area in each group. **B**, The statistical analysis of cerebral infarction area in each group. **C**, Cell cycle of rat brain tissues in each group (*: $p < 0.05$ compared with normal group. #: $p < 0.05$ compared with stroke group).

Discussion

Cerebral ischemic stroke is a kind of cranial nerve disease mainly due to the toxicity of excitatory amino acid nerves that may alter the flow of ions, causing cerebral edema. A long period of ischemia leads even to death. More than 2 million people worldwide develop cerebral ischemic stroke every year. Surveys indicated that 900 thousand people in China used to be attacked by cerebral ischemic stroke every year, and its mortality rate reaches 80%⁶. Atherosclerosis is the pathological basis of cerebral ischemic stroke. Scholars⁷ dissected 7 dead bodies that, when alive, had cerebral ischemic stroke. They found that some thrombus fragments and solid microembolus existed in carotid artery and middle cerebral artery. Currently many studies have shown that positive microembolus indicates the instability of atherosclerotic plaques. Researchers⁸ pointed out that positive microembolus suggests active embolus. Once being attacked by cerebral ischemic stroke, blood flow would have changed and promoted cerebral thrombosis. Duan et al⁹ have confirmed cerebral tissue ischemia and anoxia not only lead to neuronal necrosis but also cause nerve cell apoptosis. Therefore, finding effective treatment programs through clinical researches is of great significance. Statins are the most commonly used drugs for cerebral stroke treatment. Rosuvastatin intervention in patients with cerebral ischemic stroke reduced the encephalic microembolus, showing a great effect on patient prognosis¹⁰⁻¹². Sirt1 protects nerves, reducing damages to cerebral neuron and thrombosis through deacetylation. This work aims to investigate the effects of Rosuvastatin on nerve cell apoptosis in rats with cerebral ischemic stroke through Sirt1/NF- κ B signaling pathway. In grouping experiments, optical microscope observed in group A complete and clear cellular hierarchical structure, regular cell arrangement, bluish violet cell nucleus and pink cytoplasm. No damage or necrosis was observed. In group B, the boundary line between cytoplasm and nucleus was blurry. Also, there were some apoptosis bodies. In group C those necroses disappeared since rosuvastatin acting. De Las Heras et al¹³ have confirmed that rosuvastatin reduces AMI by regulation of Akt, leading to myocardial apoptosis which may further develop into myocardial necrosis. In this paper, Western blot and immunofluorescence method were used to determine the expressions of Sirt1 and NF- κ B. The results showed that the levels of SIRT1 in group B decreased compared with that

in group A, and those in group C increased (both $p < 0.05$). Further, the expressions of NF- κ B in group B increased compared with that in group A, and those in group C decreased (both $p < 0.05$). Immunofluorescence suggested the expressions of Sirt1 proteins in group A was 3.10 ± 0.69 , in group B 3.10 ± 0.69 and in group C 2.18 ± 0.82 . The levels in group B were significantly lowered in comparison with group A ($p < 0.05$); those in group C were significantly elevated ($p < 0.05$), indicating rosuvastatin may up-regulate the Sirt1 and down-regulate the NF- κ B. TTC staining gave the areas of cerebral infarction as follows: areas of group B were larger than that of group A ($p < 0.05$); areas of group C were significantly reduced ($p < 0.05$). Rosuvastatin may have greatly improved the conditions of cerebral infarction and remitted the occurrence of cerebral ischemic stroke. Hutcheson et al¹⁴ observed that rosuvastatin enhanced the stability of carotid atherosclerotic plaques and delayed the development of conditions by inhibiting inflammatory reaction through Sirt1/ NF- κ B pathway. Srivastava et al¹⁵ found statins up-regulate the Sirt1, improve oxidation resistance and inhibit NF- κ B, to protect the body from cerebral injury. Corsetti et al¹⁶ reported rosuvastatin may reduce cerebral injury by inhibiting NF- κ B and block the activation of inflammatory factor. Hukkanen et al¹⁷ evidenced that rosuvastatin protects blood vessel endothelium as well as inhibits atherosclerosis and reduces inflammation. Also, it enhances the elasticity of small blood vessels, maintains self-regulating ability, increases blood flow velocity, and improves areas of infarction. Cell cycles were measured with flow cytometry. The periods of G1, S and G2 in brain cells in group A were the longest and the shortest in group B. All these periods in group A and group C, compared to that in group B, significantly increased (both $p < 0.05$). Zhang et al¹⁸ found that rosuvastatin has important neuroprotective effects on the brain. Its mechanism was confirmed as the down-regulation of inflammatory expressions to fight against apoptosis. Lin et al¹⁹ showed rosuvastatin imposes deacetylation effects on proteins by acting on Sirt1. It also participates in metabolism, cell apoptosis and other processes, and regulates many genes. Guo et al²⁰ prompted that infarction cells having been treated with rosuvastatin showed reduced cell proliferation and cell survival rate, but increased apoptosis rate. The survival rate had a downtrend with the extension of processing time, while the apoptosis was on the rise. Rosuvastatin, therefore, may suppress the proliferation of infarction cells caused by cerebral stroke.

Conclusions

We found that rosuvastatin may have great effects on improving cerebral infarction condition in rats with cerebral ischemic stroke. The mechanisms may be through Sirt1/ NF- κ B pathway and thereby reduce the apoptosis rate and improve the cell cycle of brain cells.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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