

# Tirofiban combined with rt-PA intraarterial thrombolysis improves the recanalization rate of acute middle cerebral artery occlusion in rabbits

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**Abstract.** – **OBJECTIVE:** To investigate the curative effect of tirofiban combined with recombinant tissue-plasminogen activator (rt-PA) selective intra-arterial thrombolysis on acute middle cerebral artery occlusion (MCAO).

**MATERIALS AND METHODS:** A total of 60 adult male Japanese white rabbits weighing 2.5-3.0 kg were selected, and the acute cerebral infarction model was established *via* autologous thromboembolism of middle cerebral artery. Rabbits were randomly divided into 4 groups: tirofiban group (Ti group, 5 µg/kg, n=15), rt-PA group (rt-PA group, 2 mg/kg, n=15), tirofiban + rt-PA group (Ti + rt-PA group, 3 µg/kg Ti + 1 mg/kg rt-PA, n=15), and control group (Co group, n=15). The vascular recanalization rate of intra-arterial thrombolysis was observed via digital subtraction angiography (DSA), relative apparent diffusion coefficient (rADC) was observed via diffusion-weighted imaging (DWI), and neurologic impairment was observed via modified Bederson's scoring method. Rabbits were executed after 24 h, then the volume of cerebral infarction was measured via triphenyl tetrazolium chloride (TTC) staining, pathological examinations were performed using the optical microscope and electron microscope, and immunohistochemical examination was performed for brain-derived neurotrophic factor (BDNF).

**RESULTS:** In Ti + rt-PA group, the vascular recanalization rate was 91.7%, and there was no significant bleeding in pathological examination. The rADC value, neurologic impairment score and cerebral infarction area in Ti + rt-PA group were superior to those in Co group, Ti group and rt-PA group. Immunohistochemical results of BDNF showed that the expression of BDNF in Ti + rt-PA group was increased compared with those in Co group, Ti group and rt-PA group. In Ti group and rt-PA group, there were neuronal degeneration, moderate organelle swelling, moderate mitochondrial swelling, enlarged volume and decreased number of cristae, and rupture and disappearance of some mitochondrial cristae. In Co group, neuronal karyopyknosis, nuclear chromatolysis and disappearance of cellular structure could be seen. Results of electron mi-

croscopy showed that the shape of neuronal nuclei in Ti + rt-PA group was basically normal, and there were mild mitochondrial swelling and enlarged volume of cristae.

**CONCLUSIONS:** Early application of tirofiban combined with rt-PA in intra-arterial thrombolysis for ultra-early cerebral ischemia can improve the recanalization rate of cerebral artery. The time of cerebral ischemia and hypoxia is short, and the neuronal ischemia-reperfusion injury is mild, whose thrombolysis effect is better than the single application of tirofiban or rt-PA.

*Key Words:*

Tirofiban, rt-PA, Cerebral thrombosis, Recanalization rate.

## Introduction

In recent years, the incidence rate of acute cerebral thrombosis has been increased significantly, bringing heavy burden to the society and family<sup>1</sup>. Acute cerebral thrombosis is characterized by high incidence, mortality and disability rates. About 80% stroke is focal ischemia caused by arterial occlusions, most of which are middle cerebral artery thrombosis. The fatality rate of such cerebral thrombosis is 53-92%<sup>2</sup>. After acute cerebral ischemia, it is essential to quickly recanalize the vascular thrombosis to recover the reperfusion in ischemic brain tissues as soon as possible. The recovery of reperfusion saved ischemic dying brain tissues and alleviated cerebral ischemia and hypoxia injury, thus improving the quality of life. Currently, thrombolytic therapy is a preferred method to save patients with stroke. Over the past 2 decades, thrombolytic therapy has been rapidly developed in the treatment of acute ischemic stroke<sup>3,4</sup>. However, the vast majority of patients come to the hospital for treatment over 6 h after onset, so only 2% patients with acute ischemic stroke meeting the indications are able to receive thrombolytic thera-

py according to statistics<sup>5,6</sup>. At the same time, the clinical efficacy of thrombolytic therapy is limited due to the inherent risk of cerebral hemorrhage in thrombolytic therapy, insufficient reperfusion or delayed perfusion with the activation of coagulation system, microcirculation disturbance and occlusion of recanalized vessels<sup>7-9</sup>. Therefore, a new drug that is relatively safe, effective and economical for the treatment of acute ischemic stroke is urgently needed.

The mechanism of acute cerebral infarction caused by acute middle cerebral artery occlusion (MCAO) is related to energy metabolism disorders, calcium overload, neurotoxicity of excitatory amino acids, free radical damage, degradation of phosphor lipid membrane and toxic effect of lipid mediators<sup>10</sup>. Tirofiban is the first strong and extensive non-peptide platelet IIb/IIIa receptor antagonist, which effectively blocks the platelet activation and aggregation induced by various pathways through inhibiting the specific binding of fibrinogen (Fg) to the platelet glycoprotein GPIIb/IIIa receptor. Tirofiban, which has short half-life, no antigenicity and few adverse reactions, has a high degree of specificity and selectivity for its receptor in a reversible manner. Recombinant tissue-plasminogen activator (rt-PA) is a preferred thrombolytic drug at present, but there are few reports on the curative effect of tirofiban combined with rt-PA thrombolytic therapy on ultra-early acute MCAO. In this investigation, the difference in curative effect between combined medication and single medication was compared, providing evidence for the clinical treatment of acute MCAO.

## Materials and Methods

### Experimental Animals

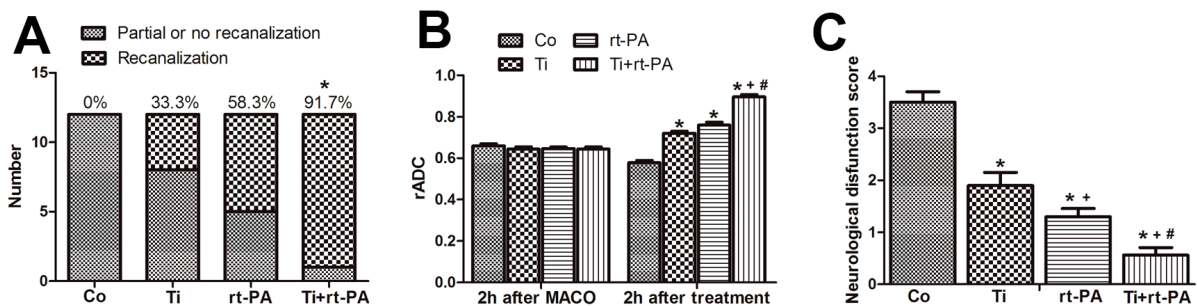
A total of 60 male Japanese white rabbits weighing 2.5-3.0 kg were provided by Weifang Medical University Animal Center. This study was approved by the Animal Ethics Committee of Affiliated Hospital of Weifang Medical University Animal Center.

### Thrombosis Preparation

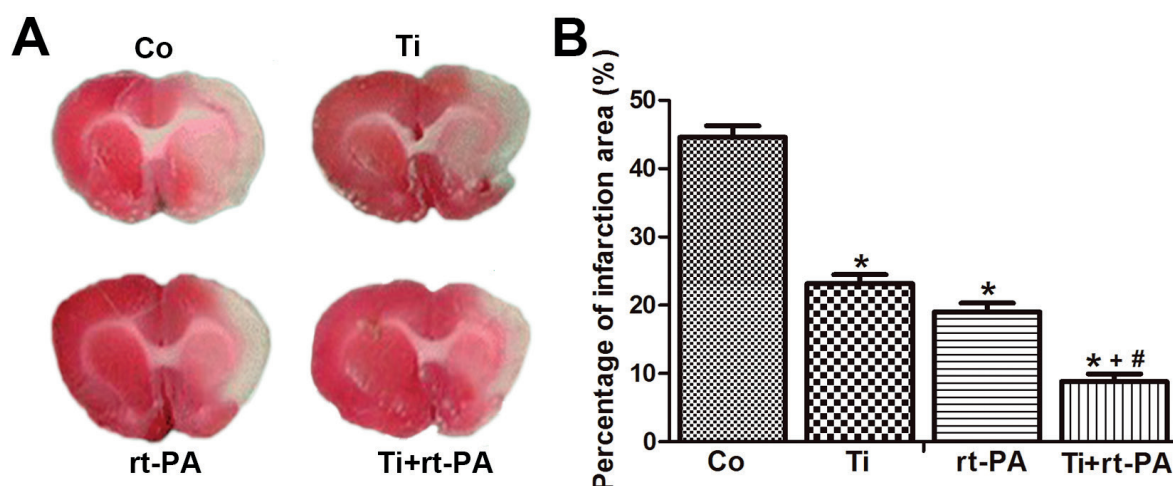
After anesthesia of rabbits via intravenous injection of 3% pentobarbital, a modified lumbar spinal needle was used to puncture and scratch about 2 cm-long endarterium in rabbit ears. The blood vessel was ligated using the suture line in the proximal part of artery in rabbit ears to reduce blood flow, thus increasing the chance of embolus formation. After 24 h, rabbits were anesthetized, and the scratched artery in rabbit ears was cut off. The intravascular thrombus was removed under a magnifying glass and placed in sterile saline for later use.

### Establishment of MCAO Model<sup>11</sup>

After experimental rabbits with arterial thrombus in ear removed were anesthetized and fixed on the operating table, the right femoral artery was exposed and soaked with papaverine locally to prevent femoral arterial spasm. The femoral artery was punctured with an 18 G trocar, and a 4F arterial sheath was inserted through the right femoral artery. 200 U/kg heparin (Chemical Book, Dalian, Liaoning, China) were given *via* arterial sheath to heparinize the artery. Echelon-10 microcatheter (eV3, Plymouth, MN, USA) combined with Silver-Speed-10 micro-guide wire (MTI, New York, NY,



**Figure 1.** The effect of tirofiban combined with rt-PA on acute middle cerebral artery occlusion in rabbits. **A**, Tirofiban combined with rt-PA improved the recanalization rate. **B**, Tirofiban combined with rt-PA increased rADC 2 h after treatment. **C**, Tirofiban combined with rt-PA improved neurologic impairment. \* $p < 0.05$  vs. Co group, + $p < 0.05$  vs. Ti group, # $p < 0.05$  vs. rt-PA group.



**Figure 2.** Tirofiban combined with rt-PA reduced the cerebral infarct area on acute middle cerebral artery occlusion in rabbits. **A**, The representative images of infarct area in different group by TTC staining. **B**, Analysis of the percentage of infarct area. \* $p < 0.05$  vs. Co group, + $p < 0.05$  vs. Ti group, # $p < 0.05$  vs. rt-PA group.

USA) was inserted into the right or left common carotid artery, and the head end of catheter was parallel to the inferior margin of the second cervical vertebra for selective angiography (300 g/L ultravist, diluted to 150 g/L, Bayer, Leverkusen, Germany). The catheter was inserted into the proximal part of internal carotid artery across the opening of occipital artery for lateral angiography to determine the direction of blood vessels. After 3 strips of thrombus were injected into the internal carotid artery using a syringe, angiography was performed again to confirm MCAO, and the catheter was extracted. The femoral arterial sheath was retained and fixed. Animals were fed in an incubator at about 37°C after operation, and angiography was performed at 2.5 h after embolism to review the vascular recanalization. The catheter was washed with heparin saline throughout the procedure.

#### Grouping and Treatment

After the successful modeling was confirmed via digital subtraction angiography (DSA), rabbits were randomly divided into tirofiban group (Ti group, 5 µg/kg, n=15) (Chemical Book, Dalian, Liaoning, China), rt-PA group (rt-PA group, 2 mg/kg, n=15) (Boehringer Ingelheim, Shenzhen, Guangdong, China), tirofiban + rt-PA group (Ti + rt-PA group, 3 µg/kg tirofiban + 1 mg/kg rt-PA, n=15) and control group (Co group, n=15).

#### DSA

At 1 h after treatment, digital subtraction angiography (DSA) (Philips, Amsterdam, The Neth-

erlands) was performed to observe the vascular recanalization. Vascular recanalization rate = number of rabbits with recanalization of intra-arterial thrombolysis/total number of experimental rabbits × 100%.

#### Magnetic Resonance Imaging (MRI) Examination

At 2 h after successful modeling and at 2 h after thrombolytic therapy, rabbits were immediately sent to the MRI (Philips, Amsterdam, The Netherlands) room for  $T_1$  weighted image ( $T_1$ WI),  $T_2$ WI and diffusion-weighted imaging (DWI) examinations. The apparent diffusion coefficient (ADC) was measured, and the contralateral cerebral hemisphere without embolism in the symmetric position was used as control. The ratio of them indicated the relative apparent diffusion coefficient (rADC).

$$\text{rADC} = \frac{\text{ADC}_{\text{infarct region}}}{\text{ADC}_{\text{corresponding contralateral normal region}}}$$

#### Assessment of Neurologic Impairment

After thrombolytic therapy, rabbits were fed for 24 h, and the neurological function was scored using Bederson's 5-point method: 0 points (no symptoms of nerve injury), 1 point (fail to fully stretch the contralateral forepaws), 2 points (circle to the contralateral side), 3 points (incline to the contralateral side), and 4 points (fail to walk spon-

taneously and loss of consciousness). The higher the score is, the more serious the neurologic impairment will be.

**Triphenyl Tetrazolium Chloride (TTC) Staining**

At 24 h after thrombolytic therapy, animals were executed under anesthesia. The brain was removed, cut into 5 mm-thick brain slices, placed into TTC solution, incubated at 37°C for 30 min and fixed in formalin solution. The ischemia range in brain tissues was observed.

**Immunohistochemistry**

At 24 h after thrombolytic therapy, animals were executed under anesthesia. The parietal cortex tissues at the embolism side were taken, fixed via 4% paraformaldehyde and dehydrated via ethanol, followed by transparency via xylene and paraffin embedding. Then, it was cut into 5 mm-thick slices. Immunohistochemical staining was performed using avidin-biotin complex (ABC) method; sections were sealed via neutral gum and observed under a microscope.

**Pathological Examination**

Brain tissues after immunohistochemical staining in each group were retained, and tissues of 4 rabbits were selected randomly. The parietal cortex tissues at the embolism side were taken and fixed in fixing solution, followed by routine paraffin embedding, sectioning and hematoxylin-eosin (HE) staining, and observation under an optical microscope.

**Electron Microscopy**

Fresh brain tissues in parietal cortex at the embolism side were selected, and fixed with 3% glutaraldehyde solution, followed by dehydration step by step, re-fixation via osmium tetroxide, embedding in epoxy resin, position of semi-thin sections, ultrathin sectioning, uranium-lead double staining and observation under a transmission electron microscope.

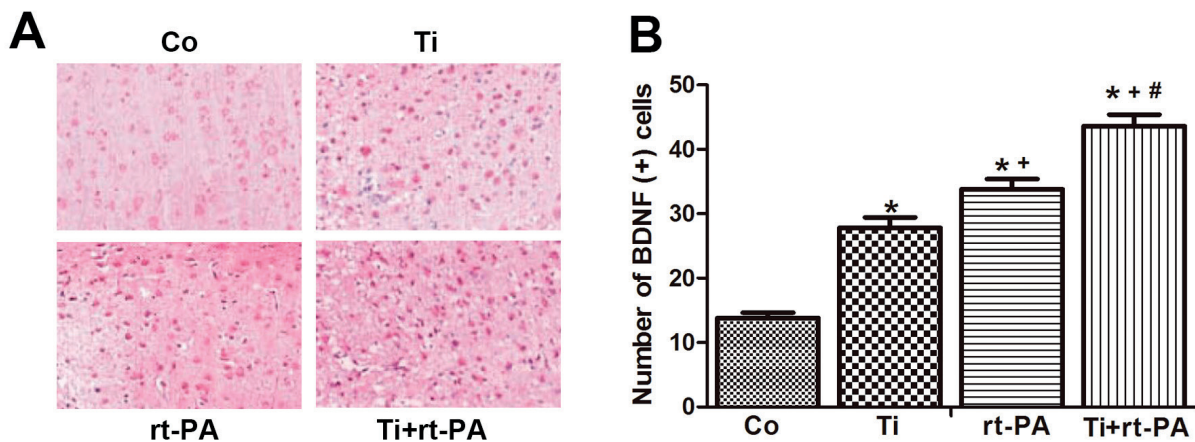
**Statistical Analysis**

Statistical product and service solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) was used for data processing. Measurement data were presented as  $(\bar{x} \pm s)$ , and one-way analysis of variance was used for the comparison among groups. Least significant difference (LSD) test was used for multiple comparisons of means in line with the homogeneity of variance, while Welch method and Brown-Forsythe method were used for comparisons of means in line with the heterogeneity of variance. Enumeration data were presented as case (%),  $\chi^2$ -test and exact probability method were used for the intergroup comparison, and Bonferoni method was used for the multiple comparison of rates among groups.  $p < 0.05$  suggested that the difference was statistically significant.

**Results**

**Comparison of Vascular Recanalization Rate in Each Group**

The vascular recanalization rate had statistically significant differences among the four groups.



**Figure 3.** Tirofiban combined with rt-PA increased the level of BDNF on acute middle cerebral artery occlusion in rabbits. **A**, The representative images of the expression of BDNF by immunohistochemistry (400×). **B**, Analysis of the number of BDNF positive cell. \* $p < 0.05$  vs. Co group, + $p < 0.05$  vs. Ti group, # $p < 0.05$  vs. rt-PA group.

According to the average rank, it was inferred that the vascular recanalization rate was the highest in Ti + rt-PA group and the lowest in Co group. There were no significant differences in the vascular recanalization rate between Co group, Ti group, and rt-PA group. There were no significant differences in the vascular recanalization rate between Ti group and rt-PA group and Ti + rt-PA group. Besides, there was no significant difference in the vascular recanalization rate between rt-PA group and Ti + rt-PA group.

#### ***Comparison of rADC in Each Group***

There were no significant differences in rADC among groups at 2 h after embolism, but there were significant differences in rADC among groups at 2 h after treatment. There were significant differences between Co group and Ti group, rt-PA group and Ti + rt-PA group. There was no significant difference between Ti group and rt-PA group. There were significant differences between Ti + rt-PA group and Ti group and rt-PA group. rADC in Ti group, rt-PA group and Ti + rt-PA group, except Co group, was increased compared with that before treatment, and it was the largest in Ti + rt-PA group after treatment.

#### ***Neurologic Impairment Score***

The neurologic impairment score was the lowest in Ti + rt-PA group, and the highest in Co group. There were significant differences in the neurologic impairment score among groups. There were significant differences in the neurologic impairment score between Co group and Ti group, rt-PA group and Ti + rt-PA group. Besides, there was a significant difference between Ti group and rt-PA group. There were significant differences between Ti + rt-PA group and Ti group and rt-PA group.

#### ***Comparison of Cerebral Infarct Area***

The cerebral infarct area was the smallest in Ti + rt-PA group and the largest in Co group. There were significant differences in the cerebral infarct area among groups. There were significant differences between Co group and Ti group, rt-PA group and Ti + rt-PA group. There was no significant difference between Ti group and rt-PA group. Besides, there were significant differences between Ti + rt-PA group and Ti group and rt-PA group.

#### ***Immunohistochemical Examination of Brain-Derived Neurotrophic Factor (BDNF)***

BDNF protein positive signal was mainly located around the cytoplasm and cell membrane,

and it was observed under the light microscope that neuronal cytoplasm and neurites were stained brown yellow, the main dendrites and cytoplasm near the membrane were stained deeply, while the area around the nucleus was stained lightly. There were significant differences in the number of BDNF positive cells among groups. There were significant differences between Co group and Ti group, rt-PA group and Ti + rt-PA group. There was also a significant difference between Ti group and rt-PA group. Besides, there were significant differences between Ti + rt-PA group and Ti group and rt-PA group.

#### ***Observation Under the Optical Microscope After HE Staining***

In Co group, neuronal karyopyknosis and nuclear chromolysis could be seen, cytoplasm was strongly stained by eosin, and cell structure disappeared. Significant edema was observed in the brain tissues, and there were a large number of vacuole-like neuronal cells and reticular cells showing severe changes. In Ti + rt-PA group, the histological morphology was basically normal, and there were occasionally vacuole-like neuronal cells showing slight changes. In Ti group, there was brain tissue edema, and a large number of vacuole-like neuronal cells showing moderate changes. In rt-PA group, there was degeneration of some neurons, and rare necrosis.

#### ***Ultrastructure Observation under the Electron Microscope***

In Co group, there was a wide range of neuronal necrosis: chromatin margination, karyopyknosis, karyorrhexis, and nuclear membrane dissolution and disappearance. The mitochondria showed significant swelling and vacuolization, the mitochondrial cristae were reduced, broken and disappeared, the endoplasmic reticulum was highly expanded, and the ribosomes shed. In rt-PA group, there were moderate swelling in organelle, vacuolization in the feet of astrocytes, and edema in astrocytes. In Ti group, there were significant swelling in organelle, vacuolization in some mitochondria, and vacuolization in the feet of astrocytes. In Ti + rt-PA group, there was mild neuronal degeneration.

## **Discussion**

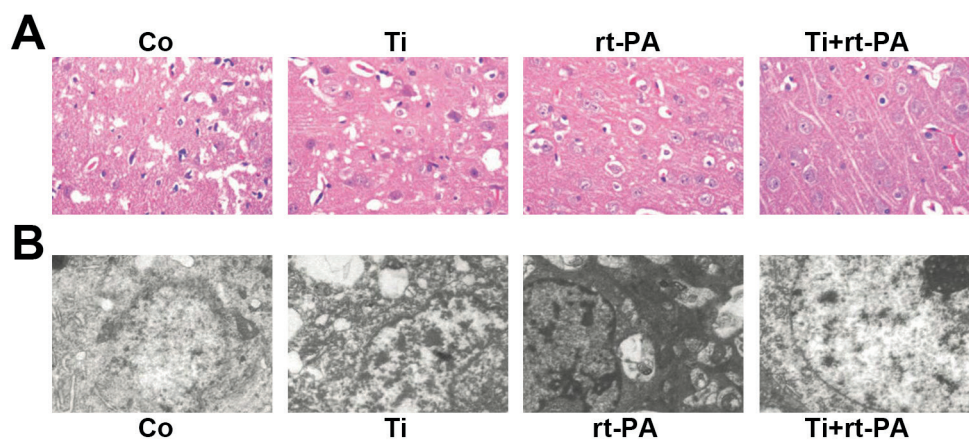
Cerebral embolism is caused by the cerebrovascular occlusion due to emboli produced by a

variety of diseases into the blood. Middle cerebral artery is a common pathogenic site of acute cerebral ischemia<sup>12</sup>. In this experiment, the interventional technique was used to selectively embolize the middle cerebral artery, and the internal carotid artery was maintained unobstructed after operation, which was more in line with the pathophysiological changes of clinical acute cerebral ischemia. After acute cerebral arterial thrombosis, necrosis will occur in brain tissues in the central ischemic area surrounded by a larger area of ischemic penumbra. Thrombolytic therapy is based on the theory of ischemic penumbra. It is expected to save the ischemic brain tissues and reduce mortality and morbidity rates if early vascular recanalization can be realized to recover local blood flow perfusion. With the further understanding of pathophysiological process and the development of pharmacology, microcatheter technique and interventional radiology technique, local intra-arterial thrombolytic therapy has attracted increasingly attention in the treatment of acute cerebral thrombosis<sup>13-15</sup>.

rt-PA is the only thrombolytic drug approved by the Food and Drug Administration (FDA) for the treatment of acute ischemic stroke. According to the experimental analysis of the National Institute of Neurological Disorders and Stroke, the earlier the patients receive rt-PA after stroke occurs, the greater the benefit will be<sup>16,17</sup>. Blood platelet plays an important role in acute ischemic stroke. The combined application of antiplatelet agents is a necessary measure to enhance the thrombolytic effect. Tirofiban, an effective drug for platelet aggregation induced by various stimulating factors,

has been used alone or as an ancillary drug in intra-arterial superselective thrombolysis and mechanical thrombectomy in the treatment of acute cerebral ischemic disease. The application of the drug increased the vascular recanalization rate, improved neurological function and reduced the incidence of thrombolytic complications. Results of this experiment showed that the vascular recanalization rate was 91.7% when tirofiban + rt-PA were given for intra-arterial thrombolysis at 2 h after preparation of acute MCAO model, that was 58.3% in rt-PA group, and that was 33.3% when tirofiban was given alone. There was no vascular recanalization in Co group. These results suggest that the vascular recanalization rate of tirofiban combined with rt-PA in the treatment of acute cerebral infarction is much higher than that of the single application of tirofiban and rt-PA.

DWI, based on the sensitivity to the free movement or dispersion of water molecules, can quickly detect high-signal brain lesions within a few minutes after cerebral ischemia. Previous researches<sup>18-20</sup> have shown that the ischemic high signal displayed by early DWI can be at least partially recovered after good reperfusion. This work revealed that rADC in Ti + rt-PA group after thrombolytic therapy was significantly increased compared with that before treatment, which was significantly higher than those in Co group, Ti group and rt-PA group, indicating that the effects of tirofiban + rt-PA thrombolytic therapy on improving the ischemic state of ischemic penumbra and saving dying neuronal cells were superior to those in Ti group, rt-PA group and Co group. There were significant differences in the cerebral in-



**Figure 4.** The effect of tirofiban combined with rt-PA on pathology under optical microscope and electron microscope. *A*, The representative images of pathological changes by HE staining under optical microscope (100 $\times$ ). *B*, The representative images of pathological changes under electron microscope (20000 $\times$ ).

infarct area between Co group and Ti group, rt-PA group, and Ti + rt-PA group, indicating that there was vascular recanalization in a certain degree in Ti group, rt-PA group and Ti + rt-PA group. There was no significant difference in the cerebral infarct area between Ti group and rt-PA group, which might be related to the poor thrombolytic effect of tirofiban or small sample size. The cerebral infarct area in Ti + rt-PA group was significantly smaller than those in Ti group and rt-PA group, indicating that tirofiban has a synergistic effect with rt-PA, enhancing the thrombolytic effect.

It was found *via* observation under the light microscope and electron microscope that there was no significant hypoxic-ischemic injury in the brain tissues in Ti + rt-PA group, and the neuronal damage was mild. In rt-PA group, there was brain tissue ischemia, but no necrosis of brain tissues. In Ti group, brain tissues were still in the state of ischemia and hypoxia, there was neuronal damage but no necrosis yet, and it would develop into irreversible necrosis if the state of ischemia and hypoxia was not improved. In Co group, brain tissues had been in an irreversible state of necrosis. These results indicate that intra-arterial medication of tirofiban combined with rt-PA has a good therapeutic value for the recovery of brain tissues after embolism, and there is no new intracerebral hemorrhagic focus.

In this work, there were still varying degrees of neurologic impairment despite of the vascular recanalization and blood flow recovery in some rabbits after thrombolytic therapy due to the neuronal cell injury in central infarct region was irreversible. Neurological function index is a good index reflecting the recovery of neurological function, and the lower its value is, the better the functional recovery will be. The neurologic impairment score in Ti + rt-PA group was significantly lower than those in Co group, rt-PA group and Ti group, indicating that tirofiban combined with rt-PA thrombolytic therapy can not only effectively recanalize the blocked blood vessels, but also effectively restore the microcirculation reperfusion, reduce the neuronal hypoxic ischemic injury, maintain the neuronal survival and improve the neurological function.

After brain injury, in addition to an active programmed cell apoptosis process, there is a parallel active neuronal survival process in tolerant cells, in which BDNF is involved<sup>21,22</sup>. When cerebral ischemia occurs, endogenous BDNF can improve the resistance capacity of neurons to ischemia through its own compensatory regulation. BDNF

can promote the repair and regeneration of damaged neurons, regulate the reconstruction of nerve structure and promote the cognitive function recovery after brain injury<sup>23,24</sup>. In this experiment, the number of BDNF positive cells was significantly different among groups at 24 h after treatment. The number of BDNF positive cells in Ti group, rt-PA group and Ti + rt-PA group was significantly larger than that in Co group, and it was significantly increased in Ti + rt-PA group compared with those in Ti group and rt-PA group, indicating that there are excessive neuronal cell death and apoptosis with the prolongation of cerebral ischemia time, leading to decreased secretion of endogenous BDNF. Moreover, the decreased BDNF expression limits its effects on neuronal repair and regeneration in turn, resulting in a vicious cycle. In view of focal ischemic injury in brain tissues, taking positive and effective measures early to improve blood circulation in ischemic brain tissues and prevent further neuronal degeneration and necrosis in ischemic region of brain tissues, is of great significance in neuronal protection. In addition, it is important to increase the expression level of endogenous BDNF in neuronal protection.

## Conclusions

We showed that the early application of tirofiban combined with rt-PA in intra-arterial thrombolysis for ultra-early cerebral ischemia can improve the recanalization rate of cerebral artery. The time of cerebral ischemia and hypoxia is short, and the neuronal ischemia-reperfusion injury is mild, whose thrombolysis effect is better than the single application of tirofiban or rt-PA.

## Conflict of Interest

The Authors declare that they have no conflict of interest.

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