

## Research Article

# Early Electroacupuncture Extends the rtPA Time Window to 6h in a Male Rat Model of Embolic Stroke via the ERK1/2-MMP9 Pathway

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Received 26 June 2020; Revised 10 October 2020; Accepted 14 October 2020; Published 11 November 2020

Academic Editor: Lu Wang

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**Background.** Recombinant tissue plasminogen activator (rtPA) is the only recommended pharmacological treatment for acute ischemic stroke, but it has a restricted therapeutic time window. When administered at time points greater than 4.5 h after stroke onset, rtPA disrupts the blood-brain barrier (BBB), which leads to serious brain edema and hemorrhagic transformation. Electroacupuncture (EA) exerts a neuroprotective effect on cerebral ischemia; however, researchers have not clearly determined whether EA increases the safety of thrombolysis and extends the therapeutic time window of rtPA administration following ischemic stroke. **Objective.** The present study was conducted to test the hypothesis that EA extends the therapeutic time window of rtPA for ischemic stroke in a male rat model of embolic stroke. **Methods.** SD rats were randomly divided into the sham operation group, model group, rtPA group, EA+rtPA group, and rtPA+MEK1/2 inhibitor group. An injection of rtPA was administered 6 h after ischemia. Rats were treated with EA at the Shuigou (GV26) and Neiguan (PC6) acupoints at 2 h after ischemia. Neurological function, infarct volume, BBB permeability, brain edema, and hemorrhagic transformation were assessed at 24 h after ischemia. Western blotting and immunofluorescence staining were performed to detect the levels of proteins involved in the ERK1/2 signaling pathway (MEK1/2 and ERK1/2), tight junction proteins (Claudin5 and ZO-1), and MMP9 in the ischemic penumbra at 24 h after stroke. **Results.** Delayed rtPA treatment aggravated hemorrhagic transformation and brain edema. However, treatment with EA plus rtPA significantly improved neurological function and reduced the infarct volume, hemorrhagic transformation, brain edema, and EB leakage in rats compared with rtPA alone. EA increased the levels of tight junction proteins, inhibited the activation of the ERK1/2 signaling pathway, and reduced MMP9 overexpression induced by delayed rtPA thrombolysis. **Conclusions.** EA potentially represents an effective adjunct method to increase the safety of thrombolytic therapy and extend the therapeutic time window of rtPA administration following ischemic stroke. This neuroprotective effect may be mediated by the inhibition of the ERK1/2-MMP9 pathway and alleviation of the destruction of the BBB.

## 1. Introduction

Stroke is a leading cause of mortality and disability worldwide [1]; approximately 13.7 million new stroke cases, 5.5 million deaths, and 116.4 million disability-adjusted life-years due to stroke were reported in 2016 [2]. Acute ischemic

stroke, the most common subtype, accounts for 87% of all strokes [3] and primarily results from occlusion of the cerebral arteries by thrombosis or embolism [4]. Currently, intravenous thrombolysis with recombinant tissue plasminogen activator (rtPA) has been proven to be the most effective pharmacological treatment for acute ischemic stroke when

administered within 3–4.5 h after ischemia onset [5]. Unfortunately, the use of rtPA is restricted by its narrow thrombolytic time window, because it may cause thrombolytic complications, such as brain edema and hemorrhagic transformation, particularly when delayed thrombolysis is initiated after 4.5 h [6, 7]. Due to these limitations, only 3.8–8% of patients with ischemic stroke benefit from rtPA-mediated thrombolysis [8]. Therefore, any neuroprotective strategy designed to reduce complications and extend the thrombolytic time window will be very important.

Based on accumulating evidence from clinical and animal studies, the disruption of the blood-brain barrier (BBB) is the key event that leads to brain edema and hemorrhagic transformation during thrombolysis for ischemic stroke [9–11]. The BBB is composed of endothelial cells, tight junctions (TJs), pericytes, astrocytic endfeet, and extracellular matrix (ECM) [12]. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that are best known for their role in the degradation and remodeling of ECM components [13]. The level of the matrix metalloproteinase 9 (MMP9) protein has consistently been shown to increase after ischemic stroke, and it plays an important role in BBB destruction by degrading ECM and TJ proteins [14–16]. More importantly, rtPA may cross the BBB, enter the brain parenchyma, and thereby damage the neurovascular matrix by promoting MMP9 production and activation [17, 18]. Consequently, selective inhibition of MMP9 reduces brain injury, particularly the degradation of the BBB, after rtPA thrombolysis for ischemic stroke [14, 19].

Extracellular signal-regulated kinase 1/2 (ERK1/2), a critical member of mitogen-activated protein kinase (MAPK) cascades, is activated by dual phosphorylation catalyzed by MAPK kinase (MAPKK, also known as MEK1/2). The ERK1/2 signaling pathway is involved in the inflammatory response and apoptosis and plays an important role in the repair of the BBB after brain injury [20, 21]. MMP9 expression is induced by ERK1/2 signaling, and inhibition of ERK1/2 signaling reduces the hemorrhagic transformation and brain edema caused by overexpression of MMP9 following cerebral ischemia [14, 22–24].

Electroacupuncture (EA), a type of acupuncture with electronic stimulation, is a well-known complementary and alternative medical treatment for ischemic stroke in China. Based on both clinical and experimental studies, EA, a safe and effective treatment, significantly reduces the infarct volume and neurological deficit score following cerebral ischemia [25–27]. EA stimulation exerts a neuroprotective effect by increasing the expression of TJ proteins (Claudin5 and ZO-1), reducing MMP9 expression and protecting the BBB integrity in various animal models of ischemic stroke [28–30]. In addition, EA alleviates cerebral ischemia and reperfusion injury by modulating the ERK1/2 signaling pathway [31]. However, researchers have not yet clearly determined whether EA improves the safety of thrombolysis and extends the therapeutic time window during rtPA thrombolysis for ischemic stroke. Therefore, the present study was conducted to test the hypothesis that EA represents an adjunct therapy that will extend the therapeutic time window of rtPA for ischemic stroke by alleviating BBB damage and reducing

the risk of complications induced by delayed rtPA thrombolysis. Moreover, we further elucidated whether this neuroprotective effect was associated with the modulation of the ERK1/2-MMP9 signaling pathway, as well as the underlying mechanisms.

## 2. Materials and Methods

**2.1. Animals.** Adult male Sprague-Dawley (SD) rats weighing  $320 \pm 20$  g were supplied by Shanghai Xipuer-Bikai Experimental Animal Co., Ltd. (Shanghai, China; license no. SCXK (Hu): 2018-0006). All rats were housed in a temperature- and humidity-controlled room on a 12 h light/dark cycle at the Experimental Animal Centre of Nanjing University of Chinese Medicine. This study was approved by the Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine, and all procedures were strictly conducted in accordance with the guidelines of the National Institutes of Health Animal Care and Use Committee. The experiments reported here were performed in accordance with the ARRIVE guidelines.

**2.2. Establishment of the Embolic Stroke Model.** An embolic stroke model was induced by placing a blood clot into the middle cerebral artery (MCA) using the methods described by Zhang et al. [32].

- (a) *Preparation of Embolus.* The external carotid artery (ECA) of the donor rat was catheterized; blood was transferred into 20 cm long PE-50 tubing, allowed to clot for 2 h at 37°C, and then stored at 4°C for 22 h. A 5 cm segment of clot-filled PE-50 tubing was cut, and the clot was then drawn into a PE-10 tubing connected to a saline-filled syringe via a 30 G needle. The clot was drawn into and flushed out of the PE-10 tubing repeatedly to remove the red blood cells. A 4 cm segment of clot was cut and transferred to a modified PE-50 catheter (outer diameter of 0.35 mm) connected to a 100  $\mu$ l syringe and the clot was then injected into the MCA.
- (b) *Embolic Stroke Model Establishment.* Rats were anesthetized with isoflurane (5% for induction and 1.5–2% for maintenance), and then, an embolic stroke was induced. The rectal temperature ( $37 \pm 0.5^\circ\text{C}$ ) was maintained throughout surgery with an electric blanket. The right common carotid artery (CCA), internal carotid artery (ICA), and ECA were exposed via a midline cervical incision. The distal end and branches of the ECA were ligated, and the CCA and the ICA were temporarily clamped with a microvascular clip. Immediately thereafter, a partial arteriotomy on the ECA was performed, and the tip of a modified PE-50 catheter containing the clot was inserted into the ECA lumen and advanced 19–22 mm from the ECA into the lumen of the ICA until it reached the origin of the MCA. Then, the catheter was retracted 1–2 mm, and the clot was slowly injected with 5–10  $\mu$ l of saline at a rate of 10  $\mu$ l/min. The catheter was withdrawn from the arteriotomy

5 min after the injection. The cerebral blood flow (CBF) was monitored with laser Doppler flowmetry (LDF, MoorVMS-LDF1) by gently attaching an LDF probe to the dura mater. The successful obstruction of CBF by the thrombus was defined as a reduction in perfusion greater than 70% of the baseline CBF [33] (Supplementary Figure 1). The successful establishment of the model was judged based on the obstruction of CBF and neurological deficit score at 2 h after stroke. For sham-operated rats, the same surgery was performed, except that 5-10  $\mu$ l saline was injected into the MCA

**2.3. Experimental Design and Groups.** Two sets of experiments were performed, and the animals were randomly divided into various groups.

In the first experiment, rats were randomly assigned to the following groups: (1) sham, (2) model, (3) rtPA, and (4) rtPA+EA. A tail vein injection of rtPA (Boehringer Ingelheim, Germany) was administered at 6 h after stroke induction, and the doses of rtPA (10 mg/kg) were determined based on previous studies [34, 35]. An equal volume of normal saline was injected intravenously at the corresponding times in rats that did not receive rtPA. These animals were used to measure the infarct volume, brain edema, neurological deficit score, hemorrhagic transformation, BBB permeability, and expression of ZO-1, Claudin5, ERK1/2, and MMP9. In this experiment, we determined that EA attenuated delayed rtPA-induced BBB disruption and hemorrhagic transformation and improved neurological function by preventing the activation of ERK1/2 and MMP9.

We conducted a second experiment to determine whether ERK1/2 signaling affects the expression of MMP9 in rats with embolic stroke. The rats were randomly divided into the sham group, rtPA group, and rtPA+MEK1/2 inhibitor (U0126). U0126 (5  $\mu$ l, 0.2  $\mu$ g/ $\mu$ l) [36] or vehicle (0.1 M PBS containing 0.4% DMSO) was administered intracerebroventricularly (ICV) 30 min prior to embolic stroke. The ICV injection site was chosen at the following coordinates from the bregma according to the rat brain in stereotaxic coordinates: anteroposterior, 1.2 mm; lateral, 2.0 mm; and depth, 3.8 mm.

At 24 h after stroke, all animals were euthanized, and their brains were harvested for further experiments.

**2.4. Electroacupuncture Treatment.** Rats in the rtPA+EA group received EA at the Shuigou (GV26) and left Neiguan (PC6) acupoints at 2 h postembolic stroke. GV26 was located at the junction of the upper 1/3 and middle 1/3 of the upper lip. PC6 was located approximately 3 mm proximal to the palm crease above the median nerve. Stainless steel acupuncture needles (outer diameter 0.3 mm) were inserted 2-3 mm into GV26 and PC6. Then, the needles were connected to an electrical stimulator (HANS-200, Nanjing Jisheng Medical Technology Company, China) with an intensity of 1 mA and frequency of 2/15 Hz for 30 min.

**2.5. Measurement of Neurological Function.** The neurological deficit score was recorded at 2 h and 24 h after embolic stroke

and determined with a modified 6-point scoring system [37, 38] as follows: 0, no apparent deficits; 1, contralateral forelimb flexion; 2, decreased grip of the contralateral forelimb while pulled by tail; 3, spontaneous movement in all directions, contralateral circling only if pulled by tail; 4, spontaneous contralateral circling; and 5, death. The successful establishment of the embolic stroke model was confirmed by the obstruction of CBF and a neurological deficit score of no less than 2 points at 2 h after stroke.

**2.6. Measurement of the Infarct Volume and Brain Edema.** TTC staining was performed 24 h after ischemia onset to determine the infarct volume. Rats were euthanized, and the brains were rapidly removed and frozen at -30°C for 15 min. Then, brain tissues were sectioned into 2-mm-thick coronal slices and immersed in a 2% solution of TTC (Sigma) at 37°C in the dark for 30 min. Normal regions were stained red, whereas infarct regions appeared white. Finally, the stained slices were fixed with a 4% paraformaldehyde solution for 24 hours and scanned to measure the ratio of infarct area to the whole brain using an image analysis system (ImageJ software).

Brain edema was assessed at 24 h after ischemia onset by measuring the brain water content using the wet-dry weight technique. The cerebellum and olfactory bulb were removed. Then, the injured right hemisphere was weighed to obtain its wet weight and subsequently dried at 100°C for 24 hours to obtain the dry weight. The percentage of the brain water content was calculated as (wet weight - dry weight)/(wet weight)  $\times$  100%.

**2.7. Evaluation of BBB Permeability.** BBB permeability was assessed by measuring the extravasation of Evans Blue (EB, Sigma) dye in the rat brain at 24 h after embolic stroke [39, 40]. Two hours before decapitation, 2% EB was injected into rats via the tail vein at 4 mL/kg (body weight). The rats were deeply anesthetized and transcardially perfused with normal saline through the left ventricle until an outflow of clear perfusion fluid from the right atrium was observed. After decapitation, the brain tissue was removed, and the right hemisphere was weighed. The brain tissue was homogenized in a formamide solution (1 mL/100 mg) and then incubated in a water bath (60°C) for 24 h before being centrifuged at 1000  $\times$  g for 30 min. Finally, the absorbance of EB in the supernatants was measured at 620 nm using a spectrophotometer. The EB content was reported as micrograms per gram of brain tissue and was calculated from a standard curve.

**2.8. Measurement of Hemorrhagic Transformation.** Hemorrhagic transformation was determined by detecting hemoglobin levels on the ischemic side of the brain using a method reported in a previous study [41]. At 24 h after embolic stroke, rats were anesthetized and perfused transcardially with 0.1 M phosphate-buffered saline (PBS). The ischemic hemisphere was separated, homogenized in 0.1 M PBS, and then centrifuged for 30 min (13000 rpm). Thereafter, the supernatant was collected, and the hemoglobin level was measured with a hemoglobin assay kit (QuantiChrom™

Hemoglobin Assay Kit, Hayward, USA) according to the manufacturer's protocol. The optical density value of each sample was measured at 400 nm using a microplate reader.

**2.9. Western Blot Analysis.** All rats were sacrificed at 24 h poststroke, and the ischemic penumbra was separated based on a previous study [42]. Total protein was extracted using a protein extraction kit (Beyotime Biotech) according to the manufacturer's protocol. The protein content was determined using the quantitative BCA protein assay. Equal amounts of protein (30  $\mu$ g) were separated by electrophoresis on 10% SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked with 5% BSA for 2 h at room temperature, followed by an overnight incubation at 4°C with the following specific primary antibodies: ERK1/2 (1:1000, ab17942, Abcam), MEK1/2 (1:1000, ab178876, Abcam), MMP9 (1:2000, ab76003, Abcam), p-MEK1/2 (1:1000, CST9154, CST), p-ERK1/2 (1:2000, CST4370, CST), Claudin5 (1:2000, ab131259, Abcam), and ZO-1 (1:1000, 21773-1-AP, Proteintech). After washes with TBST, the membranes were incubated with HRP-labeled goat anti-rabbit IgG (1:1000, A0208, Beyotime Biotech) at room temperature for one hour. The bands were detected using an enhanced chemiluminescent substrate (ECL, Thermo Scientific) and visualized using a Bioshine ChemiQ4800 imaging system (Shanghai Bioshine Scientific Instrument Co., Ltd). Finally, the gray level ratio of target proteins was obtained using the ImageJ software, and  $\beta$ -tubulin (1:2000, 30302ES20, Yeasen) was used as the internal control.

**2.10. Immunofluorescence Staining.** The animals were perfused transcardially with a 4% paraformaldehyde solution, and the brains were dissected and fixed with paraformaldehyde for 24 h at 4°C. After dehydration with a 40% sucrose solution and embedding in OTC, the brain tissues were cut into 12- $\mu$ m frozen sections for staining using a cryostat (Leica CM1950, Germany). Next, tissue sections were washed three times with PBS, permeabilized, and blocked with PBS containing 0.1% Tween 20, 0.3% Triton X-100, and 5% BSA. Then, the sections were incubated overnight at 4°C with the following specific primary antibodies: MMP9 (1:300, ab76003, Abcam), Claudin5 (1:500, ab131259, Abcam), and ZO-1 (1:50, 21773-1-AP, Proteintech). Sections were then washed with PBS and incubated for 1 h with secondary antibodies (Alexa Fluor 594, ab150080, Abcam and Alexa Fluor 488, ab150077, Abcam) at room temperature. Nuclear counterstaining was performed using DAPI (C0065, Solarbio, China). Images of ischemic penumbral sections were randomly captured using an Olympus BX63 fluorescence microscope (Olympus, Tokyo, Japan). Fluorescence intensity was quantified using the ImageJ software, and the results are presented as average optical density (AOD) values.

**2.11. Statistical Analysis.** All data were analyzed using the SPSS 24 and GraphPad Prism 7 software, and the data are reported as mean values  $\pm$  standard deviations. Data with a normal distribution and homogeneity of variance were ana-

lyzed using analysis of variance (ANOVA). The nonparametric Mann-Whitney  $U$  test was used to analyze nonnormally distributed data. Statistical significance was defined as  $p < 0.05$ , and a high level of statistical significance was defined as  $p < 0.01$ .

### 3. Results

**3.1. EA Improved Brain Injury following Delayed rtPA Thrombolysis for Ischemic Stroke.** We evaluated neurological deficits and the cerebral infarct volume at 24 hours after stroke to determine the effects of EA on delayed rtPA thrombolysis for ischemic stroke. The neurological deficit score and infarct volume in rats with rtPA thrombolysis did not differ from the model group ( $p > 0.05$ , Figures 1(a) and 1(c)). However, the combination of EA and 6 h rtPA resulted in significant reductions in the neurological deficit score and infarct volume compared to the model group and to the group treated with 6 h rtPA alone ( $p < 0.05$ ,  $p < 0.01$ , Figures 1(a) and 1(c)). Based on the results, EA improved brain injury following delayed rtPA thrombolysis for ischemic stroke.

**3.2. EA Decreased Hemorrhagic Transformation and Brain Edema Induced by Delayed rtPA Thrombolysis for Ischemic Stroke.** We evaluated hemorrhagic transformation and brain edema at 24 hours after stroke to examine the potential effect of EA on delayed rtPA-induced complications. As depicted in Figure 2, we did not detect any significant hemorrhagic transformation in the model group compared to the sham group ( $p > 0.05$ , Figure 2(a)). The rtPA treatment at 6 h after stroke significantly increased the hemoglobin level compared with the model group; however, the combination of EA and 6 h rtPA significantly decreased the hemoglobin level compared to the 6 h rtPA alone group ( $p < 0.01$ , Figure 2(a)). In addition, the rtPA treatment at 6 h after stroke significantly increased the brain water content compared with the model group, but the combination of EA and 6 h rtPA significantly decreased the brain water content compared to the model group and to the 6 h rtPA alone group ( $p < 0.01$ , Figure 2(b)). Thus, rtPA thrombolysis beyond the time window after acute ischemic stroke aggravated the incidence and severity of hemorrhagic transformation and brain edema, but EA decreased hemorrhagic transformation and brain edema induced by delayed rtPA thrombolysis.

**3.3. EA Reduced BBB Permeability Induced by Delayed rtPA Thrombolysis for Ischemic Stroke.** We assessed BBB permeability by measuring the amount of EB leakage in the ischemic hemisphere to investigate the effect of EA on the BBB integrity in the postischemic brains subjected to delayed rtPA thrombolysis. As depicted in Figure 3, rtPA thrombolysis at 6 h after ischemia significantly increased EB leakage compared to the model group ( $p < 0.01$ , Figure 3). However, the combination of EA and 6 h rtPA significantly decreased EB leakage compared to the model group and to the 6 h rtPA alone group ( $p < 0.01$ , Figure 3). Based on these results, the administration of rtPA thrombolysis beyond the time window after acute ischemic stroke increased BBB permeability,

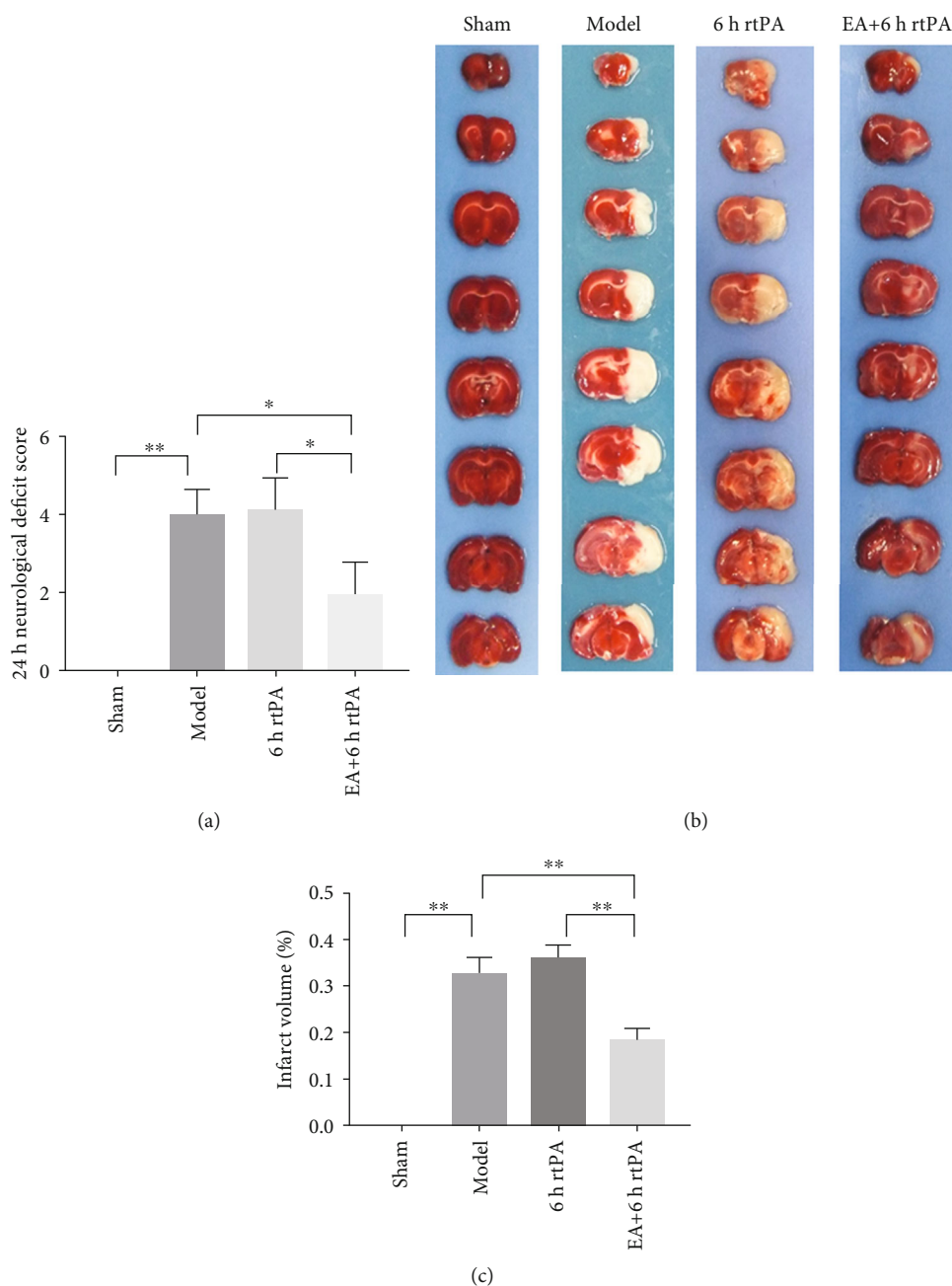


FIGURE 1: The neurological deficit score ( $n = 10$  rats) and infarct volume ( $n = 6$  rats) in each group. (a) Neurological deficit score. (b) Representative images of brain slices stained with TTC. (c) Brain infarct volume. \* indicates  $p < 0.05$ ; \*\* indicates  $p < 0.01$ .

but EA preserved the BBB integrity and reduced BBB permeability induced by delayed rtPA thrombolysis.

**3.4. EA Attenuated the Loss of Tight Junction Proteins Induced by Delayed rtPA Thrombolysis for Ischemic Stroke.** Because tight junctions play an important role in maintaining BBB integrity, we examined whether EA increased the levels of TJ proteins (Claudin5 and ZO-1) using immunofluorescence staining and Western blot analysis. As depicted in Figures 4 and 5, rtPA thrombolysis at 6 h after ischemia significantly decreased the levels of the Claudin5 and ZO-1 proteins compared to the model group. However, compared with the model group and the 6 h rtPA alone group, the com-

bination of EA and 6 h rtPA significantly increased the levels of the Claudin5 and ZO-1 proteins ( $p < 0.01$ , Figures 4(b), 4(d), 5(b), and 5(d)). Therefore, the administration of rtPA beyond the time window after acute ischemic stroke substantially disrupted the BBB, but EA increased the levels of TJ proteins and alleviated the impairment of the BBB induced by delayed rtPA thrombolysis.

**3.5. EA Attenuated MMP9 Overexpression Induced by Delayed rtPA Thrombolysis for Ischemic Stroke.** MMP9 plays a pivotal role in BBB disruption, leading to brain edema and hemorrhagic transformation [43], and rtPA enhances the activation of MMP9 [44]. Therefore, we investigated the

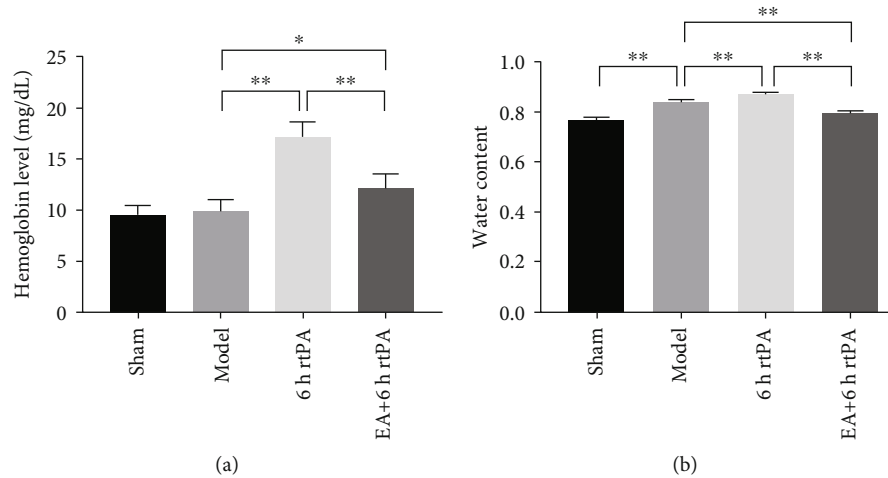


FIGURE 2: Hemorrhagic transformation and brain edema in each group ( $n = 6$  rats). (a) Hemoglobin level. (b) Brain water content. \* indicates  $p < 0.05$ ; \*\* indicates  $p < 0.01$ .

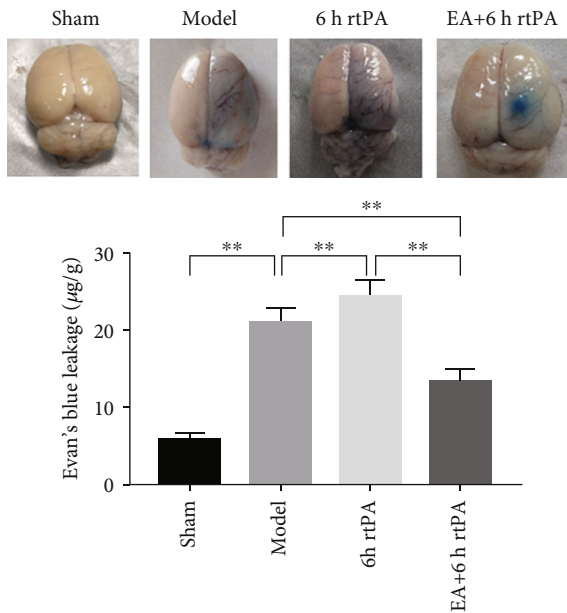


FIGURE 3: BBB permeability in each group ( $n = 6$  rats). \*\* indicates  $p < 0.01$ .

effects of EA on MMP9 activation induced by delayed rtPA thrombolysis for ischemic stroke. As depicted in Figure 6, rtPA thrombolysis at 6 h after ischemia significantly increased the levels of the MMP9 protein compared to the model group ( $p < 0.01$ , Figure 6(b)). However, compared with the model group and 6 h rtPA alone group, the combination of EA and 6 h rtPA significantly decreased the levels of the MMP9 protein ( $p < 0.01$ , Figure 6(b);  $p < 0.05$ , Figure 6(d)). Based on these results, the administration of rtPA thrombolysis beyond the time window after acute ischemic stroke caused MMP9 overexpression, but EA reduced the high levels of the MMP9 protein induced by delayed rtPA thrombolysis.

**3.6. EA Ameliorated BBB Disruption by Inhibiting the ERK1/2-MMP9 Pathway following Delayed rtPA Thrombolysis for Ischemic Stroke.** The levels of phosphorylated MER1/2 (p-MER1/2) and phosphorylated ERK1/2 (p-ERK1/2) were analyzed at 24 h after stroke using Western blot analyses to evaluate the potential relationship between the protective effects of EA on the BBB integrity and ERK1/2 signaling. As depicted in Figure 7(a), the levels of p-MER1/2 and p-ERK1/2 in the model group were significantly elevated compared with the sham group. In the 6 h rtPA group, the levels of p-MER1/2 and p-ERK1/2 were further elevated and did not differ from the levels in the model group ( $p > 0.05$ , Figures 7(b) and 7(c)). However, compared with the model group and the 6 h rtPA alone group, the combination of EA and 6 h rtPA significantly decreased the levels of p-MER1/2 and p-ERK1/2 ( $p < 0.01$ , Figures 7(b) and 7(c)). Therefore, ERK1/2 signaling was activated after acute ischemic stroke. Thrombolysis beyond the time window did not decrease the level of phosphorylated ERK1/2. However, EA inhibited the activation of the ERK1/2 signaling pathway and reduced the high level of phosphorylated ERK1/2 following delayed rtPA thrombolysis for ischemic stroke.

We randomly divided the rats into a sham group, a 6 h rtPA group and a 6 h rtPA+MEK1/2 inhibitor (U0126) to determine whether ERK1/2 signaling modulates MMP9 expression in rats with embolic stroke. Levels of p-ERK1/2 and MMP9 were analyzed at 24 h after stroke using Western blot analyses. As depicted in Figure 7(d), compared with the sham operation, levels of p-ERK1/2 and MMP9 were significantly increased in the 6 h rtPA group ( $p < 0.01$ , Figures 7(e) and 7(f)); compared with the 6 h rtPA group, levels of p-ERK1/2 and MMP9 were significantly decreased in the 6 h rtPA+U0126 group ( $p < 0.01$ , Figures 7(e) and 7(f)). Thus, MMP9 was the downstream target of the ERK1/2 signaling pathway, and inhibition of ERK1/2 further reduced the level of MMP9 in rats with embolic stroke. Based on these findings, EA ameliorated BBB disruption by inhibiting the ERK1/2-MMP9 pathway following delayed rtPA thrombolysis for ischemic stroke.

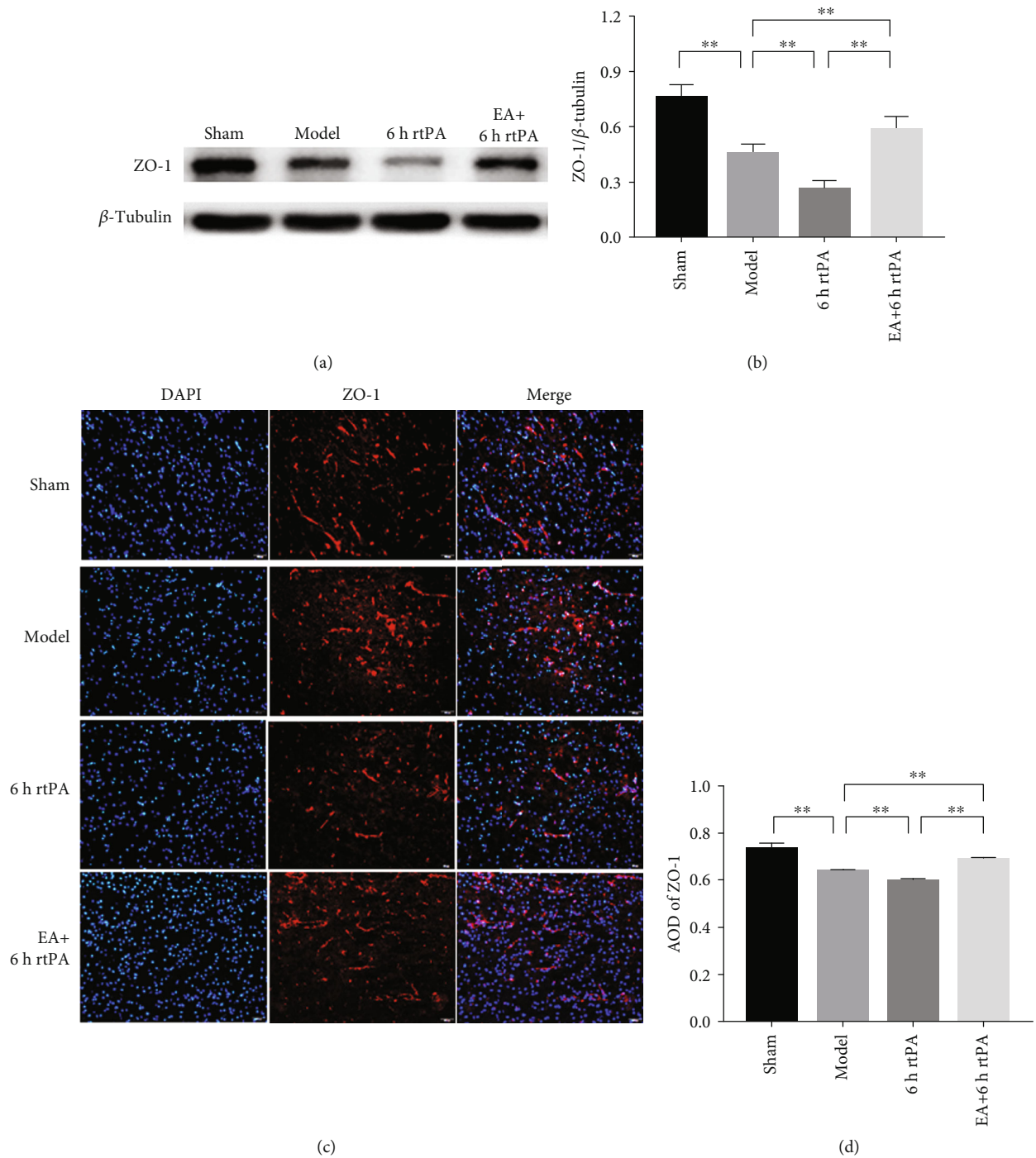


FIGURE 4: Western blot and immunofluorescence staining for ZO-1 in each group ( $n = 6$  rats). (a) Representative immunoblot showing the level of ZO-1 in the ischemic penumbra area. (b) Relative levels of the ZO-1 protein. (c) Representative images of ZO-1 staining in the ischemic penumbra area. Bar = 50  $\mu$ m. (d) Average optical density of ZO-1. \*\* indicates  $p < 0.01$ .

## 4. Discussion

**4.1. Overview of Findings.** This study was the first to investigate the important question of whether early EA extends the thrombolytic time window of rtPA by alleviating the BBB disruption and rtPA-associated complications after delayed rtPA treatment in a male rat model of embolic stroke. EA significantly improved neurological function and reduced the

infarct size, hemorrhagic transformation, brain edema, and EB leakage in rats that received EA at 2 h and rtPA at 6 h after ischemic stroke. Moreover, these protective effects were probably related to the amelioration of BBB disruption by attenuating the degradation of TJ proteins and inhibiting the ERK1/2-MMP9 pathway. Our study described a potentially effective adjunct therapy to increase the safety and thrombolytic time window of rtPA following ischemic stroke.

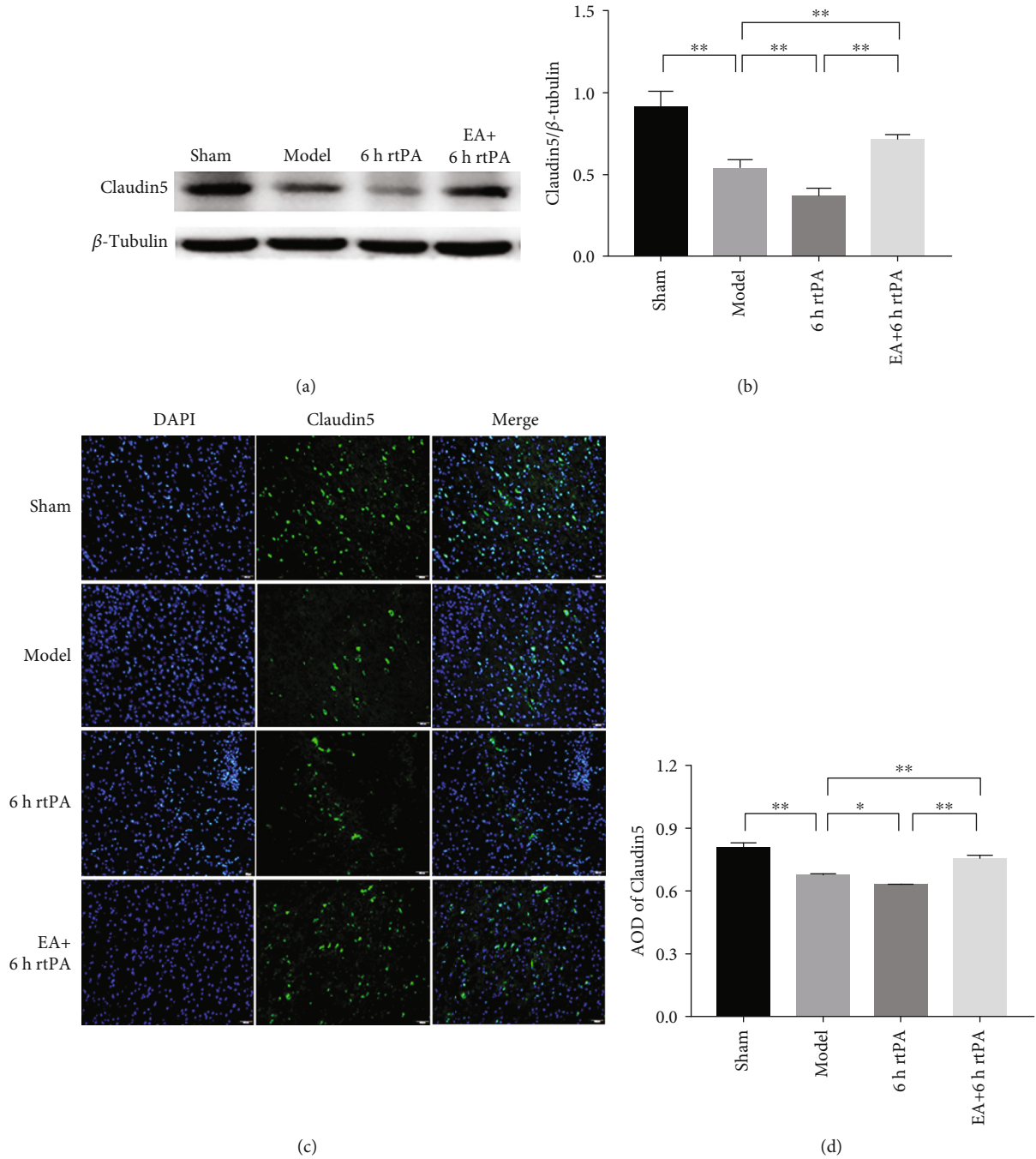


FIGURE 5: Western blot and immunofluorescence staining for Claudin5 in each group ( $n = 6$  rats). (a) Representative immunoblot showing Claudin5 levels in the ischemic penumbra area. (b) Relative levels of the Claudin5 protein. (c) Representative images of Claudin5 staining in the ischemic penumbra area. Bar = 50  $\mu$ m. (d) Average optical density of Claudin 5. \* indicates  $p < 0.05$ ; \*\* indicates  $p < 0.01$ .

**4.2. EA Application during rtPA Thrombolysis for Acute Ischemic Stroke.** Acupuncture is a traditional therapy derived from ancient China that has been used for more than 3000 years. EA is derived from the combination of traditional acupuncture and modern electrical stimulation, which has been recommended as a complementary therapy for ischemic stroke in both Asian and Western countries [26]. Additionally, EA is widely used in clinical practice and experimental studies of ischemic stroke due to its repeatability and the standardization of the frequency, intensity and duration.

According to the theory of traditional Chinese medicine, the basic mechanism of ischemic stroke is an imbalance between Ying and Yang, as well as an obstruction of Qi and blood. Shuigou (GV26) is the acupoint of the governor meridian, which collects Yang from peripheral regions and transports it to the brain. Neiguan (PC6) is the acupoint of the pericardium meridian, which exists in a close physiological and pathological relationship with the brain and promotes the circulation of Qi and blood. Consequently, the GV26 and PC6 acupoints were chosen for EA stimulation.



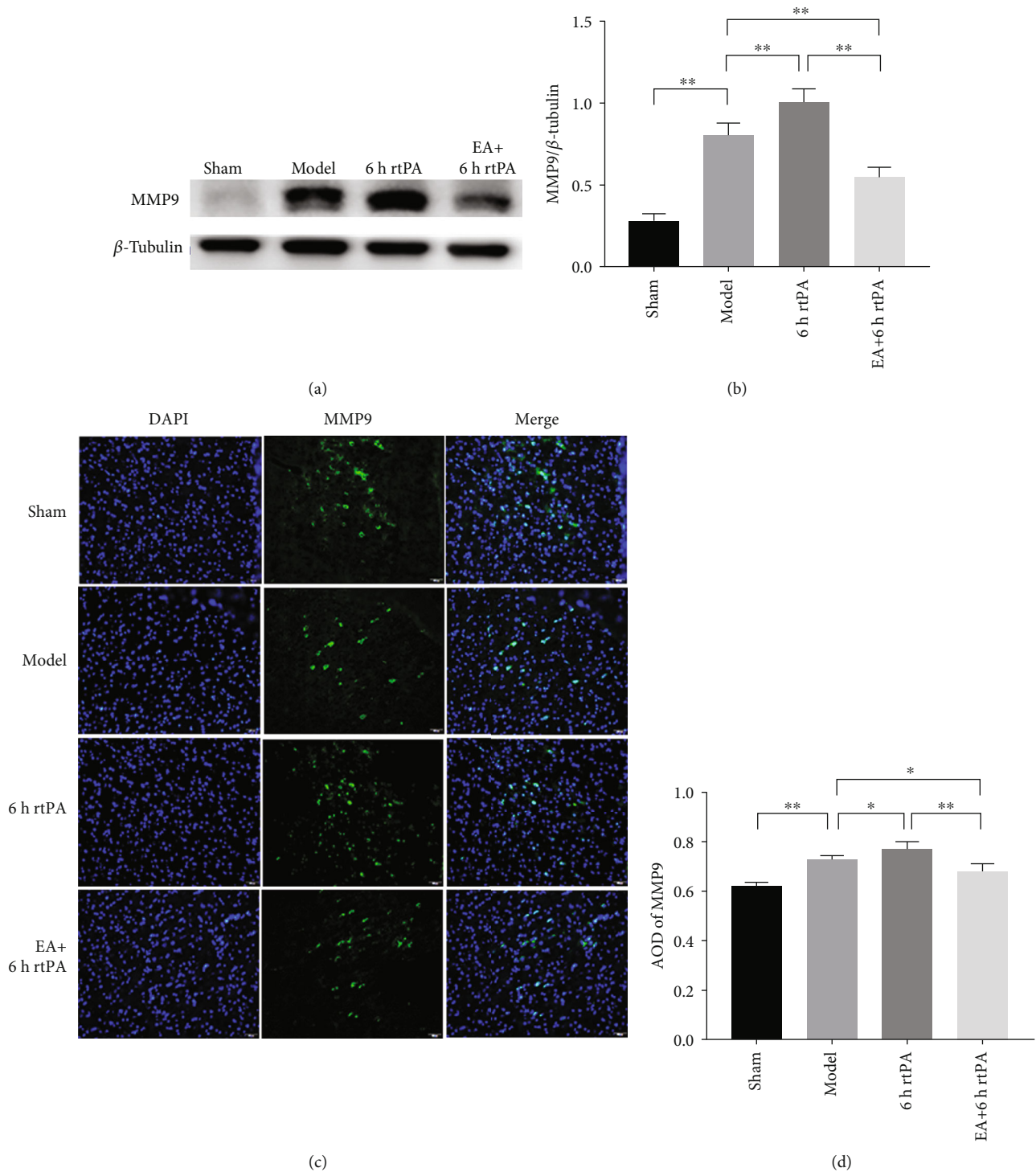


FIGURE 6: Western blot and immunofluorescence staining for MMP9 in each group ( $n = 6$  rats). (a) Representative immunoblot showing the levels of MMP9 in the ischemic penumbra area. (b) Relative levels of the MMP9 protein. (c) Representative images of MMP9 staining in the ischemic penumbra area. Bar = 50  $\mu$ m. (d) Average optical density of MMP9. \*indicates  $p < 0.05$ ; \*\*indicates  $p < 0.01$ .

In addition, many researchers have reported that EA at GV26 and PC6 facilitates the resuscitation of the brain, increases cerebral blood flow, reduces the infarct volume, and alleviates brain injury after ischemic stroke [45–47]. In our study, an embolic stroke model was used to study thrombolytic therapy, because it resembles the pathological condition of cere-

bral thrombosis in the clinic. Within 1 hour after clot injection, spontaneous clot dissolution and vascular recanalization are observed [48]. Maximum neurological deficit scores were recorded for rats as early as 2 h after the induction of ischemia [49]. Therefore, the 2-hour occlusion of the middle cerebral artery produces a large infarct volume

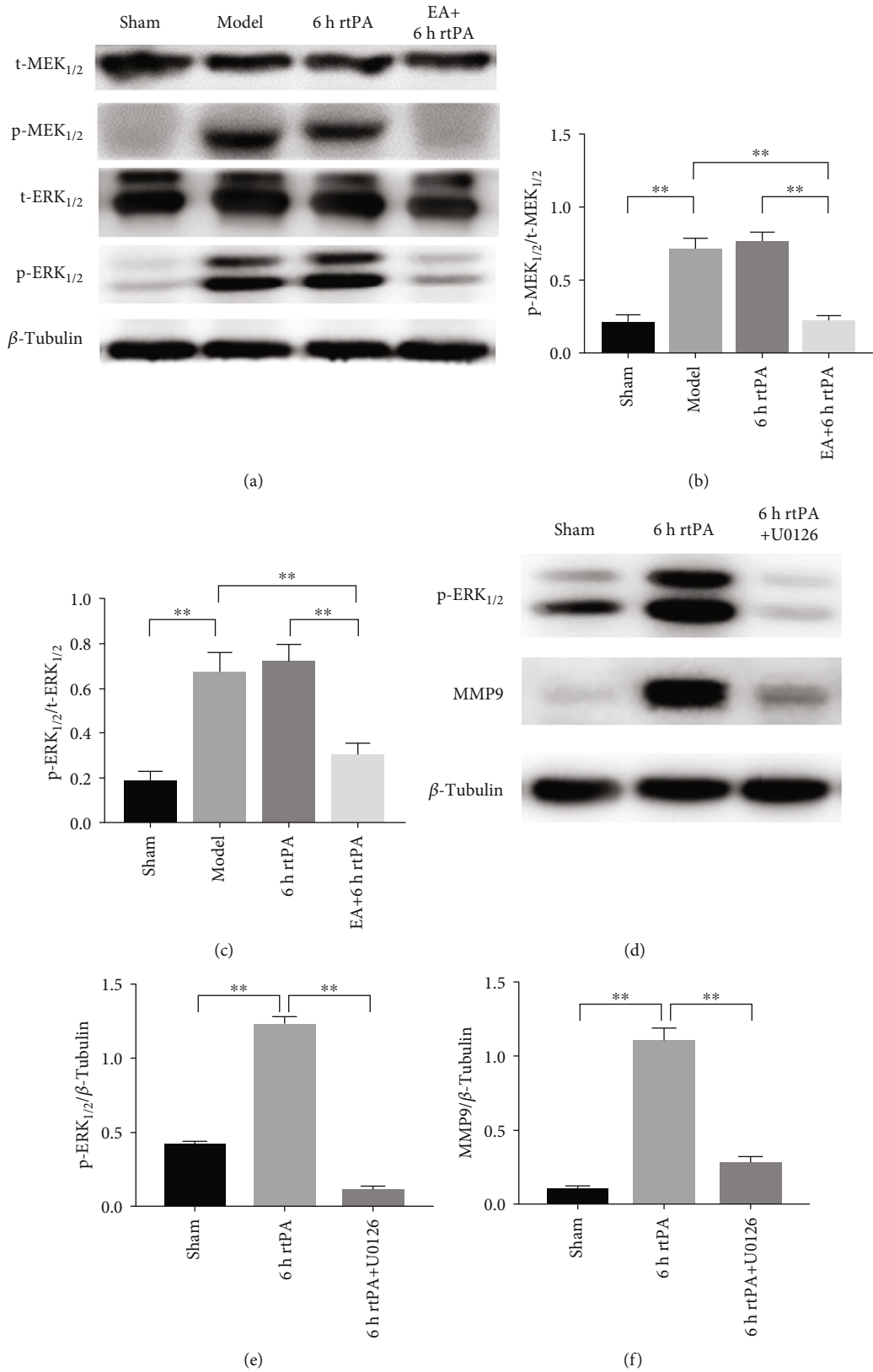


FIGURE 7: Western blots showing the effects of EA on the ERK1/2-MMP9 pathway in each group ( $n = 6$  rats). (a) Representative immunoblots showing the levels of t-MEK1/2, p-MEK1/2, t-ERK1/2, and p-ERK1/2 in the sham, model, 6 h rtPA, and EA+6 h rtPA groups. (b) Relative levels of p-MEK<sub>1/2</sub>/t-MEK<sub>1/2</sub>. (c) Relative levels of p-ERK<sub>1/2</sub>/t-ERK<sub>1/2</sub>. (d) Representative immunoblots showing the levels of p-ERK1/2 and MMP9 in the sham, 6 h rtPA, and 6 h rtPA+U0126 groups. (e) Relative levels of p-ERK1/2. (f) Relative levels of MMP9. \*\*indicates  $p < 0.01$ .

that is reliable and stable during the first 24 h after cerebral ischemia [50]. Moreover, this study aimed to observe the effect of early EA on thrombolytic complications, and another study confirmed that the application of EA at 2 h after ischemic stroke reduced the cerebral infarct size and neuronal damage in rats with cerebral ischemia-reperfusion injury [51]. Therefore, we delivered EA at 2 h after the onset of cerebral ischemia. However, no study has examined the effect of EA on thrombolysis in subjects with acute ischemic stroke, and the effect of EA on thrombolysis remains unclear. Therefore, our study investigated whether early EA improved the safety of thrombolysis and extended the thrombolytic time window during rtPA thrombolysis for acute ischemic stroke.

**4.3. MMP9 and rtPA-Associated Thrombolytic Complications.** Currently, the intravenous infusion of rtPA within 3–4.5 h is the most efficient pharmacological therapy for acute ischemic stroke [5]. However, based on accumulating data, rtPA also exerts deleterious effects on the ischemic brain that potentially compromise the overall benefit of thrombolysis during stroke [52]. In multiple animal studies, rtPA has been reported to cause neurotoxicity and brain damage after cerebral ischemia [52–54]. Moreover, delayed treatment with rtPA beyond 4.5 h generates serious hemorrhagic transformation and brain edema due to the degradation of ECM components and increased BBB permeability [6, 7]. As shown in the present study, rats treated with rtPA at 6 h after the onset of embolic stroke presented severe neurological deficits and large cerebral infarct volumes, similar to rats with embolic stroke alone. However, delayed rtPA-associated hemorrhagic transformation and brain edema were significantly increased, consistent with previous studies [55, 56]. Thus, rtPA thrombolysis for ischemic stroke at a delayed time point did not improve neurological outcomes, but increased the risk of thrombolytic complications. BBB disruption is a critical pathological process that occurs after cerebral ischemia-reperfusion and is thought to be a prerequisite for hemorrhagic transformation, brain edema, and poor treatment outcomes [10, 11, 44]. Therefore, the maintenance of BBB integrity has been one of the major targets for protecting the brain from ischemic stroke. Claudin5 and ZO-1 are important TJ proteins that are located in the tightly sealed monolayer of brain endothelial cells comprising the BBB and are sensitive markers of BBB disruption [57]. MMP9 is expressed in many cell types, including vascular endothelial cells, and plays a vital role in rtPA-mediated neurotoxicity after stroke thrombolytic therapy [58]. Based on accumulating evidence, delayed rtPA treatment activates MMP9 in ischemic brains, and the activation of MMP9 subsequently degrades the ECM and TJ proteins, leading to BBB opening, brain edema, and hemorrhagic transformation [17, 19, 56, 59]. Consistent with these studies, delayed rtPA treatment significantly increased MMP9 levels and aggravated the loss of TJ proteins (Claudin5 and ZO-1) in the ischemic penumbra of rats following cerebral ischemia compared to the model group in the present study.

**4.4. Role of ERK1/2 Signaling in rtPA Thrombolysis for Acute Ischemic Stroke.** ERK1/2 is widely expressed in the nervous system and activated in the early stage after brain injury. Activated ERK1/2 phosphorylates substrates in the cytoplasm or nucleus, thereby inducing the expression or activation of specific proteins, leading to cell proliferation, differentiation, apoptosis, and other processes [60]. ERK1/2 is immediately activated in the ischemic region after the occlusion of the middle cerebral artery, and the expression of ERK1/2 in neural stem cells of rats with ischemic stroke increases in a time-dependent manner [61]. The ERK1/2 signaling pathway regulates the inflammatory response and apoptosis and participates in the repair of BBB disruption after cerebral ischemia [20]. The role of the ERK1/2 signaling pathway in cerebral ischemic injury has become a hot topic in recent years; however, two contradictory views have been described. The ERK1/2 signaling pathway has been shown to alleviate brain injury and exert neuroprotective effects after cerebral ischemia [21, 61, 62]. Nevertheless, more studies have shown that activation of the ERK1/2 signaling pathway promotes inflammation, aggravates nerve cell death, and eventually leads to the deterioration of neurological function following cerebral ischemia [36, 63–65]. Meanwhile, a substantial loss of TJ proteins is observed in the rat brain tissue, and blocking the activation of ERK1/2 with the MEK inhibitor U0126 protects the integrity of the BBB and mitigates brain damage in rat models of ischemic stroke [66]. These dual roles of the ERK1/2 signaling pathway may be attributed to the use of different stimuli, cerebral ischemic models, and periods of ischemic stroke. Therefore, we first clarified the role of the ERK1/2 signaling pathway in ischemic brain injury during thrombolytic therapy in a male rat model of embolic stroke. ERK1/2 signaling was activated during delayed rtPA thrombolysis for acute ischemic stroke in the present study. Moreover, the decrease in ERK1/2 phosphorylation improved neurological function and reduced the infarct size following delayed rtPA thrombolysis.

**4.5. Possible Mechanisms Underlying the Effects of EA on Acute Ischemic Stroke.** EA significantly reduced the infarct size, hemorrhagic transformation, brain edema, and EB leakage and extended the therapeutic time window of rtPA in a male rat model of embolic stroke. Moreover, we further explored the potential molecular mechanism of EA treatment. The activation of ERK1/2 is accompanied by MMP9 overexpression in ischemic brain tissue, which leads to BBB destruction and brain edema in rats subjected to cerebral ischemia-reperfusion; however, U0126 reverses these changes and improves neurological function [14, 23]. U0126, a selective inhibitor of MEK1/2, was administered to inhibit the activation of ERK1/2 signaling and to determine whether ERK1/2 signaling modulates MMP9 expression in rats with embolic stroke during delayed rtPA thrombolysis. MMP9 was the downstream target of the ERK1/2 signaling pathway. In addition, EA decreased rtPA-induced MMP9 overexpression by inhibiting the ERK1/2 pathway and reduced thrombolytic complications of delayed rtPA thrombolysis for ischemic stroke in rats.

According to numerous experimental studies, the beneficial effects of EA treatment for ischemic stroke have been confirmed to be achieved by regulating a series of pathological reactions. EA administered early after cerebral ischemia protects cells against neuronal injury in cerebral ischemia-reperfusion by ameliorating nitro/oxidative stress-induced mitochondrial dysfunction and decreasing the accumulation of damaged mitochondria via Pink1/Parkin-mediated mitophagy clearance [67]. An EA pretreatment regime has also been shown to effectively reduce the cerebral infarct volume and the level of neuronal apoptosis in the hippocampal CA1 region after cerebral ischemia-reperfusion injury, and its mechanism may be related to the inhibition of the GluN2B/m-calpain/p38 MAPK proapoptotic pathway [68]. Another study found that EA reduced neurological deficit scores, impeded oxidative stress injury, inhibited inflammatory cytokine production, curbed P38 phosphorylation, and suppressed TRPV-1 expression, indicating a neuroprotective effect of the EA pretreatment on rats with cerebral ischemia-reperfusion injury [69]. Moreover, EA stimulation has been shown to reduce MMP9 expression, preserve BBB integrity, and alleviate cerebral ischemic injury by modulating the ERK1/2 signaling pathway in ischemic stroke models [28–31]. Similar to the results from the aforementioned studies, EA increased the levels of the Claudin5 and ZO-1 proteins and alleviated the impairment of the BBB induced by delayed rtPA thrombolysis in the present study. Furthermore, EA inhibited the activation of the ERK1/2 signaling pathway and reduced MMP9 overexpression induced by delayed rtPA thrombolysis.

**4.6. Limitations and Implications.** The present study has a limitation in that we only examined the neurological function, infarct size, hemorrhagic transformation, brain edema, and EB leakage at 24 h after ischemic stroke. The acute neuroprotective effect appears to be good, but the long-term effect remains unclear. For animal experiments and clinical applications, further studies are needed to determine whether the protective effect of EA on rtPA thrombolysis for ischemic stroke in the acute phase would be sustained for longer periods.

## 5. Conclusions

In conclusion, early administration of EA before the rtPA infusion improved stroke outcomes and reduced hemorrhagic transformation and brain edema associated with late thrombolysis. EA potentially represents an effective adjunct method to enhance the safety of thrombolytic therapy and extend the therapeutic time window of rtPA to 6 h in a male rat model of embolic stroke. This neuroprotective effect may be mediated by the inhibition of the ERK1/2-MMP9 pathway and alleviation of the destruction of the BBB. The findings provide a reliable theoretical basis for the clinical treatment of acute ischemic stroke with thrombolytic therapy within a broadened therapeutic window.

## Data Availability

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

## Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgments

This study was supported by the National Natural Science Foundation of China (NO. 81674067) and the Open Projects of the Discipline of Chinese Medicine of Nanjing University of Chinese Medicine Supported by the Subject of Academic priority discipline of Jiangsu Higher Education Institutions (NO. ZYX03KF013).

## Supplementary Materials

Supplementary Figure 1: cerebral blood flow was monitored during the induction of embolic stroke model. (*Supplementary Materials*)

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