Effects of remote ischemic preconditioning on astrocyte proliferation and glial scars after cerebral infarction

Shi-Min Liu,1* Xian-Min Cao,2* Xin-Hui Qu,1© Wen Cai,1 Fan Hu,1 Wen-Feng Cao,1 Lin-Feng Wu1 and Xiao-Mu Wu1

Abstract
The aim of this study was to investigate whether remote ischemic preconditioning (RIPC) can promote neurological function recovery after middle cerebral artery occlusion (MCAO) in rats and its possible mechanism. A total of 32 Sprague Dawley (SD) rats were randomly divided into RIPC group (n = 16) and MCAO group (n = 16). In the RIPC group, 1 h before induction of MCAO, the rats received bilateral femoral artery ischemic preconditioning (10 min/time), followed by 10 min of relaxation, and a total of three cycles were carried out. Then, the MCAO-2h model was established. In the MCAO group, the MCAO-2h model was established at 1 h after the separation of bilateral femoral arteries. The modified neurological severity score (mNSS) was assessed. At postmodeling day 7, triphenyltetrazolium chloride (TTC) staining and immunohistochemistry were conducted, and neurological function recovery, infarct size, and the expression levels of glial fibrillary acidic protein (GFAP), synaptophysin (SYN), and neurite outgrowth inhibitor A (Nogo-A) were observed. At postmodeling day 7, the difference in mNSS was statistically significant ($P < 0.05$). Infarct size was significantly smaller in the RIPC group than in the MCAO group ($P < 0.05$). The number of GFAP$^+$ cells was significantly lesser in the RIPC group than in the MCAO group ($P < 0.05$). The difference in thickness of the glial scar was not statistically significant ($P = 0.091$). At postmodeling day 7, the expression level of SYN integrated optical density (IOD) was significantly higher in the RIPC group than in the MCAO group ($P < 0.05$). The number of Nogo-A$^+$ cells was significantly lesser in the RIPC group than in the MCAO group ($P < 0.05$). At day 7 after MCAO, RIPC can promote neurological function recovery in rats and reduce infarct size. The mechanism may be that after 7 days, RIPC reduces GFAP expression, inhibits the trend of glial scar formation and Nogo-A expression, and increases SYN expression.

Keywords
GFAP, glial scar, middle cerebral artery occlusion, mNSS, Nogo-A, remote ischemia preconditioning, SYN, TTC

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Introduction
In recent years, the incidence of ischemic stroke has continued to increase year by year. Seeking for new therapies and approaches has become the most urgent and important research topic. Remote ischemic preconditioning (RIPC) refers to the effect of alternative sublethal and transient ischemic stimulations to nonischemic organs on subsequent ischemic organs, in order to improve its tolerance to ischemic injury and produce a strong protective effect on tissues or organs. At present, animal experiments have revealed that RIPC can also promote neurological function recovery after cerebral infarction.1 However, its

1Department of Neurology, People’s Hospital of Jiangxi Province, Nanchang, China
2Jiangxi University of Finance and Economics, Nanchang, China
*These two authors contributed equally to this paper.

Corresponding author:
Xin-Hui Qu, Department of Neurology, People’s Hospital of Jiangxi Province, No. 92 Aiguo Road, Nanchang 330006, Jiangxi, China. Email: quxh012543@163.com
mechanism remains unknown and requires further research.

**Materials and methods**

**Animal model establishment and grouping**

Sprague Dawley (SD) male rats (Shanghai Sciple & Bruel & Kjaer Experimental Animal Co. Ltd., Certification number: 2008001602051, SPF class) with a body weight of 250–300 g were used. Rats were fed at approximately 20°C–25°C under a day–night ratio of 1:1. A total of 32 SD rats were randomly divided into two groups: RIPC–middle cerebral artery occlusion (MCAO) group (n = 16) and MCAO group (n = 16). Among these rats, six rats in each group were used for the measurement of infarct size by triphenyltetrazolium chloride (TTC) staining. Before the operation, all rats were fasted without water deprivation for 12 h, anesthetized with an intraperitoneal injection of 10% chloral hydrate (0.3 mL/100 g), and the temperature of the anus was maintained at 36.5°C–37°C. In the RIPC-MCAO group, 1 h before the establishment of the MCAO model, the bilateral femoral arteries were separated from rats, clamped with arterial clamps for 10 min, and the artery clamps were relaxed for 10 min. A total of three cycles were performed. Then, the MCAO-2h reperfusion models were established according to the suture-occluded method of Zea Longa. An incision was made at the median of the neck of the rat, and the right common carotid artery, external carotid artery, and internal carotid artery were separated, the external carotid artery was ligated and cut off, and a 0.26 mm thrombus thread (Beijing Shandong Biotechnology Co. Ltd., grade AAAA) was inserted from the stump of the external carotid artery to the site at approximately 18–20 mm apart from the anterior cerebral artery, in order to embolize the middle cerebral artery. Then, the fascias and skins were sutured. After 2 h, the thrombus thread was pulled out to the external carotid artery to achieve reperfusion. In the MCAO group, the femoral artery was separated, but not clamped. After 1 h, the MCAO reperfusion models were established.

**Behavior test (modified neurological severity score)**

At days 1, 3, and 7 after MCAO reperfusion, the modified neurological severity score (mNSS) of rats was assessed. The mNSS included the following four tests: exercise, sensation, reflex, and balance beam. The neurological function score was within 0–18 points (0 point indicates normal and 18 points indicate the largest neurological deficit). If a rat cannot perform a task or lacks reflex, one point is added. Furthermore, 13–18 points indicate severe injury, 7–12 points indicate moderate injury, and 1–6 points indicate mild injury.

**Measurement of infarct size by TTC staining**

At postmodeling day 7, six rats were taken from each group, anesthetized with an intraperitoneal injection of 10% chloral hydrate at a dose of 0.3 mL/100 g, and the brains were directly obtained. Then, the brains of rats were placed in the coronal brain slice molds, placed in a refrigerator, and quick-frozen at −20°C for approximately 20 min to make it easy to slice, and then sliced into seven 2 mm sections, placed in the TTC dye, placed in a water bath at 37°C (in the dark), turned over every 5 min, and the coloration was stopped when the normal tissues were dyed into bright red and the infarcted tissues were not colored and remained white. The process took approximately 15 min. Next, the brain tissues were placed in 4% paraformaldehyde solution and fixed for 24 h, photographed with a digital camera, and percentage of infarct size was calculated by the formula \[\frac{(V_C - V_L)}{V_C} \times 100\], where \(V_C\) is the volume of control hemisphere and \(V_L\) is the volume of noninfarcted tissue in the lesioned hemisphere.

**Immunohistochemical staining**

At postmodeling day 7, 10 rats in the two groups were intraperitoneally anesthetized with chloral hydrate and underwent thoracotomy and laparotomy. The abdominal aorta was clamped, a needle with an infusion apparatus was interpolated into the left ventricle, and the right atrial appendage was cut open. Then, these were first instilled with 150 mL of saline and subsequently instilled with 4% paraformaldehyde. After the end of the instillation, the brains were collected. Then, the brain tissues were placed in a container containing 4% paraformaldehyde and were fixed for 4–6 h. The brain tissue from the center of the cerebral infarction (at approximately −1.0 to +1.0 mm around the coronal anterior fontanelle) was collected, embedded with paraffin, and sliced into sections. The thickness of the sections was 4 μm. The sections were used for
immunohistochemical staining. Microwave repair was performed. The sections were placed in 0.01 mol/L of citrate buffer solution at a pH of 6.0, placed in a microwave oven, boiled first at 100°C for three times, and the temperature of the microwave oven was adjusted to 30°C for 15 min. Then, the sections were collected and placed for natural cooling to room temperature. In two-step immunohistochemical reaction, the sections were incubated in deionized water containing 3% H₂O₂ for 5–10 min, rinsed with phosphate buffered saline (PBS) for 2 min, and repeated three times. Then, the following antibodies were first added: glial fibrillary acidic protein (GFAP, 1:1000), neurite outgrowth inhibitor A (Nogo-A, 1:1000), synaptophysin (SYN, 1:400). The sections were placed in a wet box and incubated at 4°C overnight, rinsed with PBS for 2 min, and was repeated three times. Then, these were added with goat anti-rabbit horseradish peroxidase (HRP)-labeled immunoglobulin G (IgG) antibody, placed at room temperature for 30 min, rinsed with PBS flush for 2 min, and repeated three times. Next, these were colored with diaminobenzidine (DAB), fully rinsed with distilled water, and underwent counterstain–dehydration–xylene treatment–neutral gum sealing. The sections for the negative control were treated with PBS instead of the first antibody, while the other steps were the same as mentioned earlier.

Collection and analysis of images

A medical image analysis system (Wuhan Qianping Imaging Technology Co., Ltd.) and an optical microscope (Olympus, 20×) were used to analyze the images. For each index in each rat, three slides were used, and for GFAP, SYN, and Nogo-A, six images were acquired at the periphery of the infarct (objective, 40×). For the measurement of the glial scar, four images were acquired at the periphery of the infarct (objective, 2×). The Imagepro-Plus 6.0 software was used to count the GFAP, SYN, and Nogo-A⁺ cells and measure the thickness of the glial scars.

Statistical analysis

All data were expressed as mean ± standard deviation (x ± SD). Student’s t-test was used to compare the two groups of data using the SPSS 18.0 statistical software. P < 0.05 was considered statistically significant.

| Table 1. mNSS score at days 1, 3, and 7 in the MCAO-RIPC and MCAO groups. |
|-----------------|-----------------|-----------------|---|
| Day | N | RIPC-MCAO group | MCAO group | P |
| 1 | 16 | 10.27 ± 1.03 | 10.20 ± 1.01 | 0.96 |
| 3 | 16 | 11.67 ± 0.98 | 11.47 ± 0.83 | 0.45 |
| 7 | 16 | 8.93 ± 0.88 | 9.8 ± 0.77 | 0.01 |

MCAO: middle cerebral artery occlusion; mNSS: modified neurological severity score; RIPC: remote ischemic preconditioning.
t-Test showed that there was statistical significance in the comparison of mNSS score between the two groups at day 7 (P < 0.05).

Results

Neurological function scores

The mNSS at days 1, 3, and 7 after MCAO revealed that the highest score appeared at day 3 after MCAO, while the lowest score appeared at day 7 after MCAO (Table 1). This suggests that neurological deficits were most severe at day 3 after MCAO. The comparison between the RIPC group and the MCAO group revealed that the differences in scores at days 1 and 3 after MCAO were not statistically significant, the scores at day 7 after MCAO was significantly lower in the experiment group than in the control group, and the difference was statistically significant (P < 0.05; Table 1 and Figure 1(a)).

TTC staining

At day 7 after MCAO, the right brain tissues of rats were stained with TTC, and pale infarcts could be observed (Figure 1(b) and (c)). The Imagepro-Plus software was used to calculate the infarct size. The percentage of the infarct sizes in the RIPC group and the MCAO group was 18.77% ± 0.71% and 20.72% ± 1.13%, respectively. These results reveal that the infarct size was significantly smaller in the RIPC group than in the MCAO group (P < 0.05; Table 2 and Figure 1(d)).

Immunochemistry

GFAP and glial scars. The expression of GFAP was significantly higher in the cerebral infarct side than in the contralateral side, positive cells were mainly concentrated in the periphery of the ischemic area, and GFAP⁺ glial cells surrounding the infarcts formed glial scars. The number of GFAP⁺ cells was significantly lesser in the RIPC-MCAO group than in the MCAO group (P < 0.05; Figure 2(a) and (b); Table 2). The thickness of the glial scar...
was smaller in the RIPC group than in the MCAO group, but the difference was not statistically significant ($P=0.091$; Figure 2(c) and (d); Table 2).

**SYN and Nogo-A.** SYN was found in vesicles in the presynaptic membrane, which plays an important role in the release of neurotransmitters. Its content can accurately reflect the number of synapses. Nogo-A is selectively expressed in oligodendrocytes, which can inhibit the growth of axons. Furthermore, the cumulative optical density of SYN was significantly higher in the RIPC group than in the MCAO group ($P<0.05$; Figure 2(e) and (f); Table 2), while the number of Nogo-A$^+$ cells was significantly lesser than in the MCAO group ($P<0.05$; Figure 2(g) and (h); Table 2).

**Discussion**

In 1993, Przyklenk et al. reported that the ischemic preconditioning of the circumflex branch of the coronary artery was applied in canines for the first time. Figure 1. mNSS score and TTC staining in the RIPC-MCAO and MCAO groups: (a) mNSS score in the MCAO-RIPC and MCAO groups, (b) infarct size in the RIPC-MCAO group (white part), (c) infarct size in the MCAO group (white part), and (d) TTC staining in the MCAO-RIPC and MCAO groups.

**Table 2.** Comparison of infarct size, glial scar, GFAP, SYN, and Nogo-A between the RIPC-MCAO group and the MCAO group.

| Items                        | N  | RIPC-MCAO group | MCAO group | $P$  |
|------------------------------|----|-----------------|------------|------|
| Percentage of infarct sizes (%) | 6  | 18.77 ± 0.71    | 20.72 ± 1.13 | 0.025|
| Glial scars (μm)             | 10 | 196.9 ± 15.38   | 212.2 ± 16.25 | 0.091|
| The number of GFAP$^+$ cells | 10 | 15.1 ± 2.23     | 18.7 ± 2.67  | 0.011|
| SYN (IOD)                    | 10 | 4045 ± 123      | 3743 ± 130  | 0.002|
| The number of Nogo-A$^+$ cells | 10 | 15.6 ± 2.95     | 19.7 ± 2.98  | 0.046|

GFAP: glial fibrillary acidic protein; MCAO: middle cerebral artery occlusion; Nogo-A: neurite outgrowth inhibitor A; RIPC: remote ischemic preconditioning; SYN: synaptophysin; TTC: triphenyltetrazolium chloride.

t-Test showed that there was statistical significance in the comparison of infarct size (TTC staining), GFAP$^+$, SYN, and Nogo-A$^+$ between the two groups ($P<0.05$).
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time, which could reduce myocardial infarct size and protect against myocardial damage in subsequent myocardial ischemia. Hence, the concept of ischemic preconditioning was proposed for the first time.\(^3\) In 2006, Zhao et al.\(^4\) revealed that treating cerebral infarction in rats using bilateral common carotid artery ischemic preconditioning could promote the recovery of neurological function by inhibiting apoptosis and the formation of free radicals, reducing the size of the cerebral infarct.

The results of this study revealed that RIPC can promote neurological function recovery after MCAO in rats, reduce the infarct size, and further reduce GFAP\(^+\) cells and tend to reduce the glial scar thickness. At present, the mechanism of RIPC in promoting recovery after cerebral infarction remains unknown. After cerebral infarction, astrocytes rapidly respond, many changes, such as the hypertrophy of cell bodies, the increase in intermediate filaments, the high expression of GFAP, and the atavistic expression of stem cell surface marker nidogen, begin to appear, and the proliferation of reactive astrocytes (RA) occurs, in which glial scars would form in patients with severe injury.\(^5\) RIPC can inhibit the expression of GFAP. However, the mechanism remains unclear at present. Studies have revealed that a few minutes after cerebral infarction, the damaged neurons and glial cells secrete cytokines, such as interleukin (IL)-1, and IL-6, to promote the amplification and hypertrophy of astrocytes.\(^6\) A study has revealed that RIPC could reduce the infarct size of MCAO by regulating the production and release of inflammatory factors.\(^7\) Hence, further studies are needed to determine whether it is correlated with the inhibition of the release of inflammatory factors after infarction.

SYN is widely distributed in neurons in the body and is specially distributed on the membrane of presynaptic vesicles. It is a membrane protein that is closely correlated with synaptic function. The expression of SYN can accurately reflect the occurrence and density of synapses, which is an important marker for the reconstruction of synapses.\(^8\) The central nervous system myelin-derived axonal

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**Figure 2.** Immunohistochemistry in the RIPC-MCAO and MCAO groups: (a) GFAP staining in the RIPC-MCAO group, (b) GFAP staining in the MCAO group (single-sided black arrow, ×400), (c) glial scar in the RIPC-MCAO group, (d) glial scar in the MCAO group (double-sided black arrow, ×20), (e) SYN staining in the RIPC-MCAO group, (f) SYN staining in the MCAO group (single-sided black arrow, ×400), (g) Nogo-A staining in the RIPC-MCAO group, and (h) Nogo-A staining in the MCAO group (single-sided black arrow, ×400).
growth inhibitory factor (Nogo-A) is highly expressed in cell bodies and the protuberances of oligodendrocytes and is concentrated in the innermost layer of the axon and the outermost layer of the myelin membrane. The distribution of Nogo-A in oligodendrocytes is consistent with its inhibitory effect on the growth of regenerated fibers and the remodeling of the nervous system structure.9,10 The results of this study revealed that RIPC can inhibit the expression of Nogo-A and increase the expression of SYN. This suggests that RIPC can promote synapse formation, increase neurotransmitter release, and promote the recovery of neurological function. Its mechanism may be associated with the decrease in the expression of Nogo-A and GFAP and the decrease in the occurrence of glial scars. This study revealed that at day 7 after MCAO, RIPC can promote neurological function recovery in rats and reduce infarct size. The mechanism may be that at day 7 after MCAO, RIPC reduced GFAP expression, inhibited the trend of scar formation and Nogo-A expression, and increased SYN expression. However, the mechanism that RIPC inhibits GFAP expression at day 7 after MCAO remains unclear and needs further studies.

Declaration of conflicting interests
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ORCID iD
Xin-Hui Qu https://orcid.org/0000-0001-5193-3116

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