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# Remote Ischemic Postconditioning Protects the Neurovascular Units in MCAO/R Rats through HIF-1$\alpha$-Mediated Pathway

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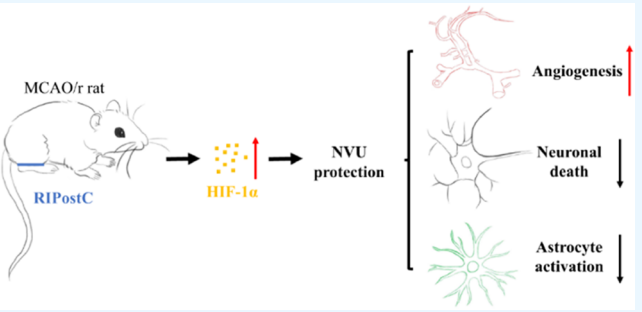
**ABSTRACT:** Remote ischemic postconditioning (RIPostC), administered after the onset of local ischemia, has been shown to have beneficial effects on neurological, vascular, and motor functions in animal models. However, the precise mechanisms and interactions underlying these functional improvements remain unclear. Our study aimed to determine whether RIPostC exerts protective effects on the neurovascular units (NVU) and to investigate whether this protection is mediated by hypoxia-inducible factor-1$\alpha$ (HIF-1$\alpha$). We used left middle cerebral artery occlusion and reperfusion (MCAO/r) to induce ischemic stroke in rats and applied RIPostC. YC-1 was used to inhibit the activity of HIF-1$\alpha$. Following the 12-day RIPostC treatment, MRI scans showed a significant reduction in infarct volume in the affected area, accompanied by an increase in HIF-1$\alpha$ protein levels and its downstream angiogenic factors in the ischemic zone. Additionally, RIPostC promoted angiogenesis in the ischemic penumbra, which, in turn, reduced neuronal loss and astrocyte activation. Behavioral assessments further indicated that RIPostC treatment partially restored the motor function in MCAO/r rats. However, the therapeutic effects of RIPostC were counteracted by the addition of YC-1, suggesting that the protective effects of RIPostC against NVU are mediated through HIF-1$\alpha$. Overall, our research demonstrates that RIPostC is an effective rehabilitative intervention for protecting NVU in MCAO/r rats through the HIF-1$\alpha$-mediated pathway.




## INTRODUCTION

Ischemic stroke continues to be a leading cause of death and disability worldwide. Cerebral infarction is the main pathological consequence of an ischemic stroke, causing severe damage to the affected brain regions, mainly affecting the health of local neurons and blood vessels. Therefore, diminishing the extent of cerebral infarction and rehabilitating the damaged neurovascular unit (NVU) are critical goals in developing effective postischemic stroke treatment strategies. The NVU, a complex system containing neurons, neuroglia cells, vascular cells, and stroma, plays a central role in the pathophysiological process of ischemic stroke and is essential for cell protection, inflammatory response, blood−brain barrier (BBB) regulation, and neurovascular repair.[1] Currently, although most ischemic stroke research focuses on alleviating neuronal death of ischemia-hypoxic neurons, the role of the other components of the NVU should not be overlooked.

The concept of remote ischemic preconditioning (RIPC) was first defined by Przyklenk et al. in 1993 as a prophylactic intervention to ameliorate subsequent prolonged ischemia by administering transient ischemia and reperfusion at distal sites such as the extremities.[2] Several researches have confirmed that RIPC plays an important role in neuroprotection against cerebral ischemia-reperfusion injury.[3−5] However, the application of RIPC has been limited by the suddenness and unpredictability of cerebral ischemia.[6] Therefore, remote ischemic postconditioning (RIPostC), as an intervention performed after the onset of localized ischemia, overcomes these limitations and becomes a potentially effective method for stroke rehabilitation. There is growing evidence that RIPostC performed in different distal regions can provide significant protection in regions such as the heart, kidney, and brain in animal models.[7−11] In the case of cerebral ischemia, a reduction in infarct volume, neuronal death, neuroinflammation, and angiogenesis is usually observed after RIPostC treatment,[12−14] suggesting that RIPostC may have an overall protective effect on the NVU.

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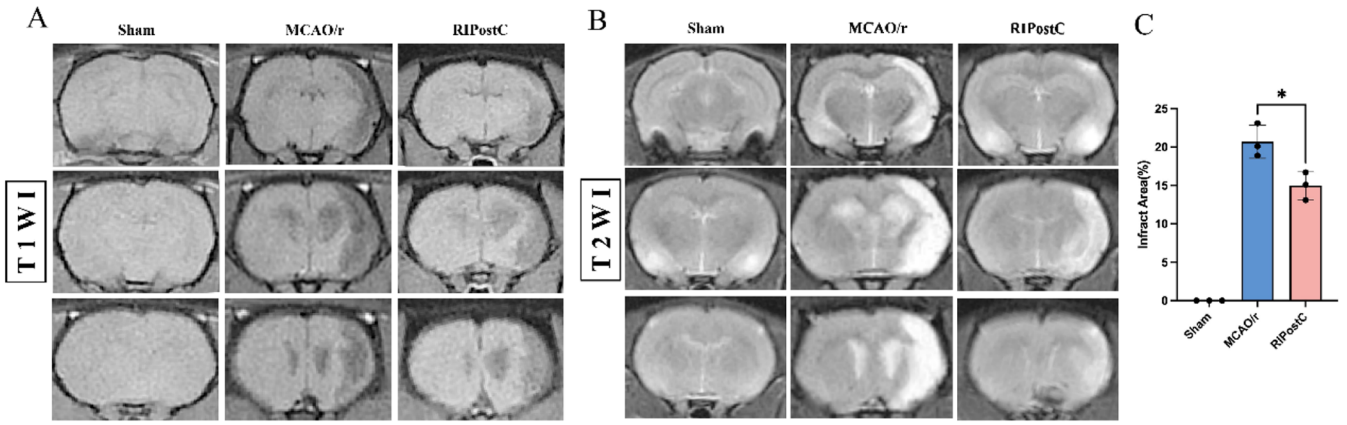


**Figure 1.** Reduction of cerebral infarct volume by RIPostC. T1WI imaging (A) versus T2WI imaging (B) of the ischemic area in the coronal plane on MRI scan at 14 days postoperatively. (C) Statistical analysis of infarct area in the three groups, $n = 3$; $^{*}p < 0.05$.

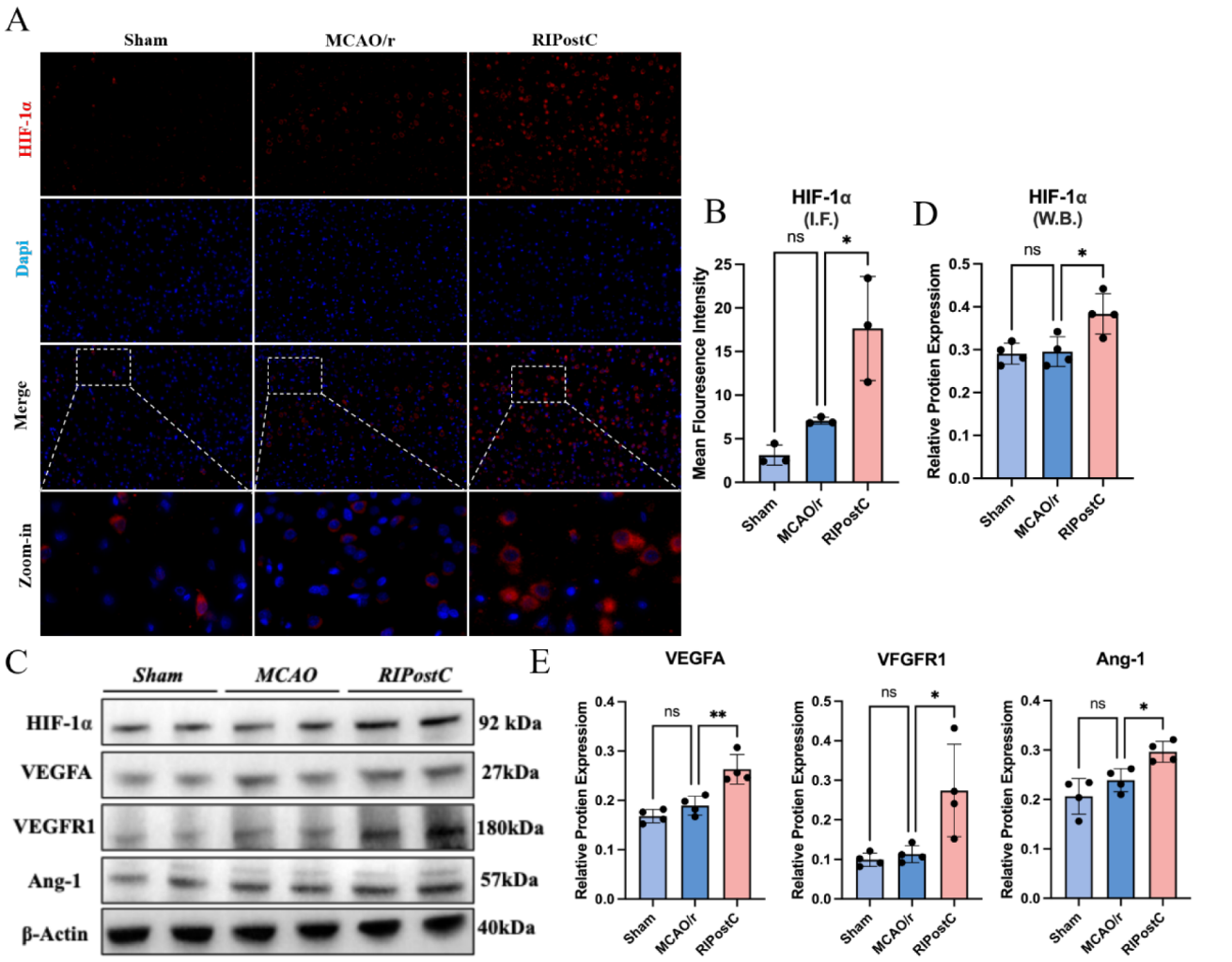


**Figure 2.** RIPostC upregulates HIF-1$\alpha$ and promotes the expression of angiogenesis-related factors. 14 days after MCAO/r, immunofluorescence, and WB were used to assess the expression of HIF-1$\alpha$ and its downstream angiogenesis-related factors in the sham, MACO/r, and RIPostC groups. (A) Representative 40× immunofluorescence images of HIF-1$\alpha$ in the three groups; white solid line squares show the enlarged panels of double-labeled HIF-1a/Dapi (Dapi, blue; HIF-1$\alpha$, red). (B) Quantitative statistics of the mean fluorescence intensity of HIF-1$\alpha$ in the three groups, $n = 3$; $^{*}p < 0.05$. (C) Representative WB images of the expression of HIF-1$\alpha$, VEGFA, VEGFR1, and Ang-1 in the three groups. (D) Statistical analysis of HIF-1$\alpha$-related protein expression in the three groups, $n = 3$; $^{*}p < 0.05$. (E) Statistical analysis of VEGFA, VEGFR1, and Ang-1 expression in the three groups, $n = 4$; $^{*}p < 0.05$; $^{**}p < 0.01$.

Hypoxia-inducible factor-1$\alpha$ (HIF-1$\alpha$) is a critical factor in regulating oxygen homeostasis and responds rapidly after hypoxia or ischemia.[15] In cerebral ischemia pathology, HIF-1$\alpha$ broadly affects neuronal survival, neuroinflammation, neo-


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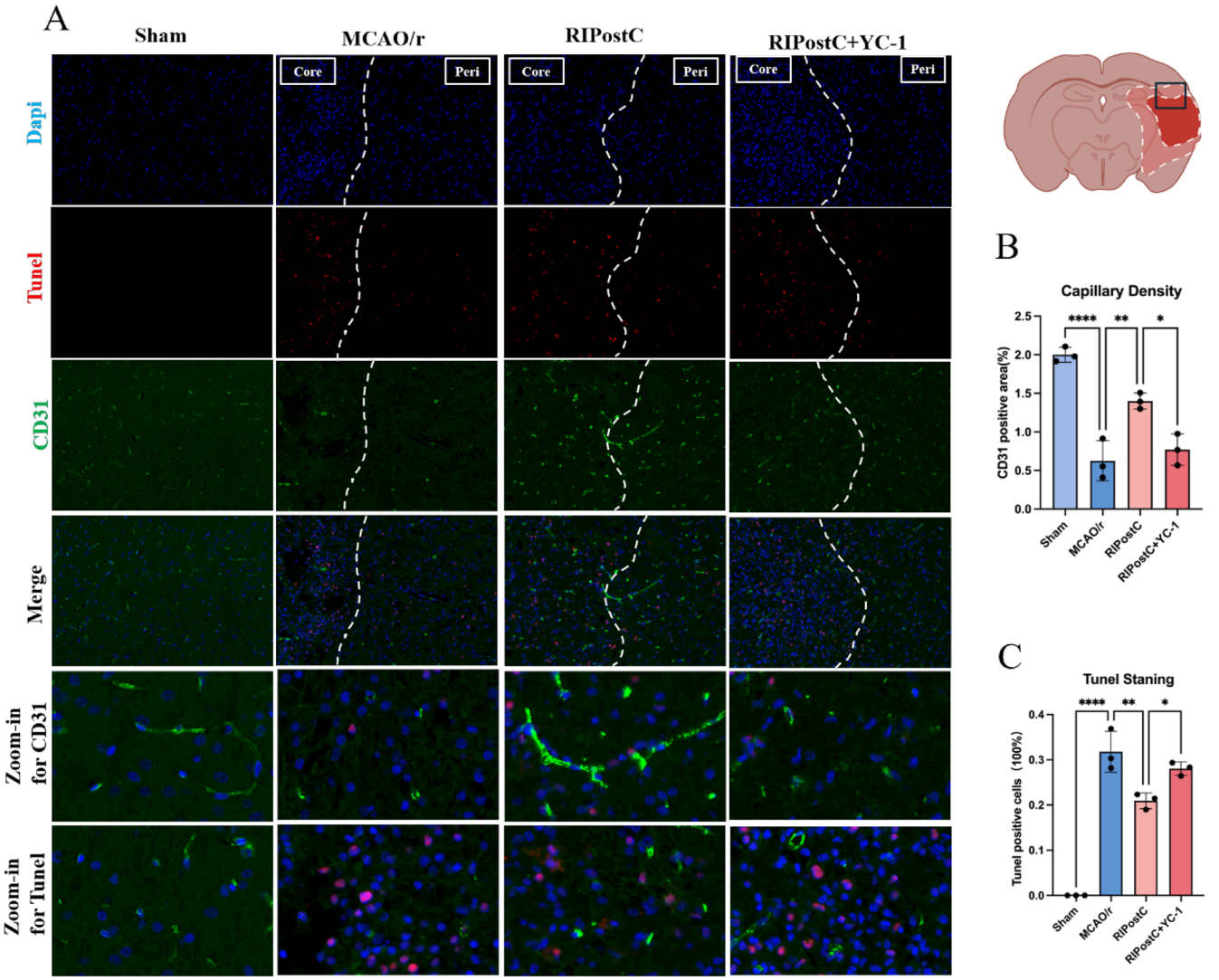


**Figure 3.** RIPostC promotes angiogenesis in the ischemic peripheral zone. At 14 days after surgery, double immunofluorescence staining was performed to study angiogenesis in the ischemic penumbra zone in the sham group, MACO/r group, RIPostC group, and RIPostC+YC-1 group. In the coronal view of the brain at the top of Figure B, the white line in the inner circle is the infarct zone, the white line in the outer circle is the peri-infarct zone, and the black box is the observation area. (A) Representative 40× immunofluorescence images of four sets of TUNEL and CD31 staining (Dapi, blue; TUNEL, red; CD31, green; white line is the demarcation line between the infarct zone and the ischemic penumbra band, with the infarct zone on the left and the ischemic penumbra on the right). (B) Capillary density was studied and statistically analyzed for four groups of CD31-positive regions, $n = 3$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$. (C) Statistical analysis of TUNEL-positive cells after TUNEL staining in the four groups, $n = 3$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$.

vascularization, glucose metabolism, and the BBB. HIF-1$\alpha$ has multiple downstream targets in different cell types of the NVU, such as astrocytes, with multifaceted impacts on cerebral ischemia.[16] As a major target of HIF-1$\alpha$, VEGF, on the one hand, acts directly on neurons as a vital regulator of neuroprotection and neurogenesis to promote the survival of new neurons and improve neurological dysfunction.[17] On the other hand, the VEGF family is known for its angiogenic capacity,[18] which stimulates angiogenesis in the ischemic penumbra, improves microcirculation, and strengthens neuroprotection.[19] Recently, it has been reported that RIPostC can significantly increase the production of HIF-1$\alpha$ in myocardial ischemia.[20] Thus, in cerebral ischemia, NVU protection may be exerted by modulating HIF-1$\alpha$ and its downstream factors.

To evaluate the effectiveness of RIPostC in poststroke neurorehabilitation, daily short-term hind limb ischemia was induced for 12 days in the MCAO/r rats. To investigate the potential role of HIF-1$\alpha$, the HIF-1$\alpha$ antagonist YC-1 was administered. The infarct volume, motor function, and key cellular components of the NVU, including blood vessels, neurons, and astrocytes, were subsequently examined.

## RESULTS

**RIPostC Reduces Cerebral Infarct Volume in Stroke Rats.** To investigate the therapeutic effect of RIPostC, we used MRI scans to assess the cerebral infarct volume. As shown by T1- and T2-weighted imaging in Figure 1, there were no significant abnormalities in the sham group 14 days after surgery. In contrast, low T1WI signals (Figure 1A) and high T2WI signals (Figure 1B) were detected in the ischemic cerebral cortex 14 days after MCAO/r, suggesting that an increase in cerebral infarct volume was severely and persistently induced due to MCAO/r injury. However, compared to the MCAO/r group, after 12 days, a significant


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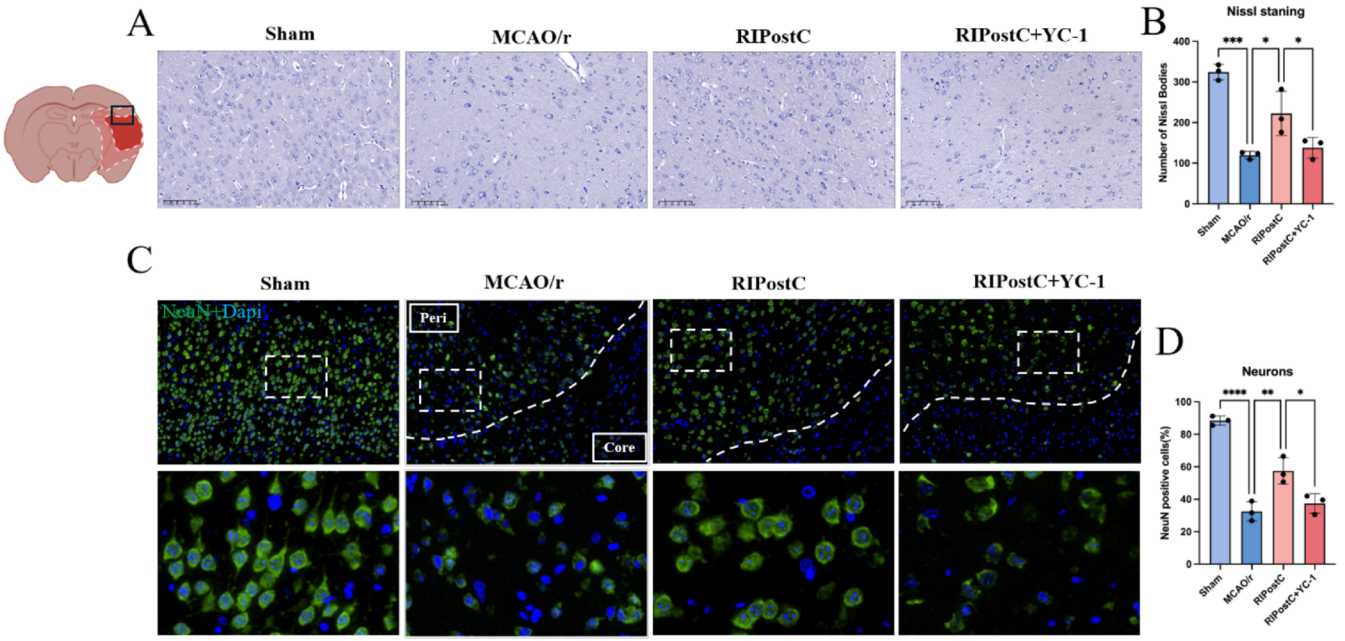


**Figure 4.** RIPostC protects neurons in the ischemic peripheral zone via HIF-1$\alpha$. Nissl staining and immunofluorescence were performed to assess neuronal integrity in the sham group, the MACO/r group, the RIPostC group, and the RIPostC+YC-1 group at 14 days after surgery. In the coronal view of the brain on the left side of Figure A, the infarcted area is shown as the white line in the inner circle, the peri-infarcted area is shown as the white line in the outer circle, and the black box is the observation area. (A) Representative images of Nissl staining of four groups of the same ischemic region (cortex). (B) Statistical analysis of the number of Nissl vesicles in four groups of Nissl staining, $n = 3$; $^{*}p < 0.05$; $^{***}p < 0.001$. (C) Representative 40× immunofluorescence images of NeuN+ neurons in the four groups (Dapi, blue; NeuN, green, white line is the demarcation line between infarcted area and ischemic penumbra zone, infarcted area on the lower right, ischemic penumbra zone on the upper left). (D) Statistical analysis of the number of NeuN + cells, $n = 3$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$.

decrease in infarct volume was observed in the RIPostC group (Figure 1C). From the MRI images, it can be primarily concluded that RIPostC was able to reduce the volume of cerebral infarction in the MCAO/r rats.

**RIPostC Upregulates HIF-1$\alpha$ and Promotes the Expression of Angiogenesis-Related Factors.** It is well known that HIF-1$\alpha$ is a pivotal factor in angiogenesis. In order to explore the potential association of RIPostC with it, we first analyzed the expression of HIF-1$\alpha$ in ischemic penumbra with immunofluorescence and WB. The immunostaining results indicated that the expression of HIF-1$\alpha$ in the RIPostC group was significantly increased compared to the MACO/r group (Figure 2A,B). Subsequently, WB was also performed to investigate the expression of HIF-1$\alpha$ and downstream factors related to angiogenesis at 14 days postoperatively. The results, as shown in Figure 2C,D, further validated the above conclusions that the RIPostC treatment promotes the expression of HIF-1$\alpha$. Additionally, we observed a significant upregulation of VEGFA, a downstream target of HIF-1$\alpha$, in the RIPostC group when compared to that in the MCAO/r group, along with an increase in VEGFA receptors, VEGFR1, and Ang-1, as illustrated in Figure 2E. Collectively, these results suggested that RIPostC initially enhances HIF-1$\alpha$ levels in the brain, which then stimulates the expression of its downstream angiogenic factors.

**RIPostC Promotes Angiogenesis in the Ischemic Peripheral Zone.** Having established that RIPostC upregulated HIF-1$\alpha$ and the expression of its downstream angiogenic factors, we proceeded to explore its impact on peri-ischemic angiogenesis with immunofluorescence and introduced a new experimental group treated with YC-1, an HIF-1$\alpha$ inhibitor. This addition allowed us to determine whether HIF-1$\alpha$ is responsible for promoting RIPostC-mediated angiogenesis. We conducted immunofluorescence staining across four distinct groups. For this analysis, we selected immunofluorescence staining markers from TUNEL, which is used to observe neuronal death[21,22] and delineate the infarct zone from the infarct margin, and CD31, a marker for vascular endothelial cells that aids in the identification and localization of these cells and the assessment of angiogenesis. Our focus of our work was on angiogenesis within the ischemic penumbra zone. As depicted in Figure 3A, the MCAO/r group exhibited significantly reduced angiogenesis and increased neuronal death compared to the sham group, while the RIPostC group demonstrated a substantial increase in angiogenesis and a concurrent decrease in neuronal death levels when contrasted with the MCAO/r group. Interestingly, after the addition of YC-1, the enhanced effect of RIPostC on angiogenesis was significantly reversed and neuronal death was exacerbated (Figure 3B,C). These findings confirm that HIF-1$\alpha$ indeed fosters angiogenesis, leading to the conclusion that RIPostC, by upregulating HIF-1$\alpha$, can effectively promote angiogenesis in the ischemic penumbra zone and ameliorate ischemia-induced neuronal death.

**RIPostC Protects Neurons in the Ischemic Peripheral Zone via HIF-1$\alpha$.** We next delved into the neurovascular unit. To assess the neuroprotective effects of RIPostC on local neurons in ischemic penumbra, we conducted a pathohistological examination of neuronal survival using Nissl staining, monitoring alterations in neuronal Nissl bodies within the ischemic penumbra. In Nissl staining, the number of surviving neurons was dramatically reduced in the MCAO/r group compared with the sham group. However, RIPostC treatment significantly mitigated the reduction, although it did not fully


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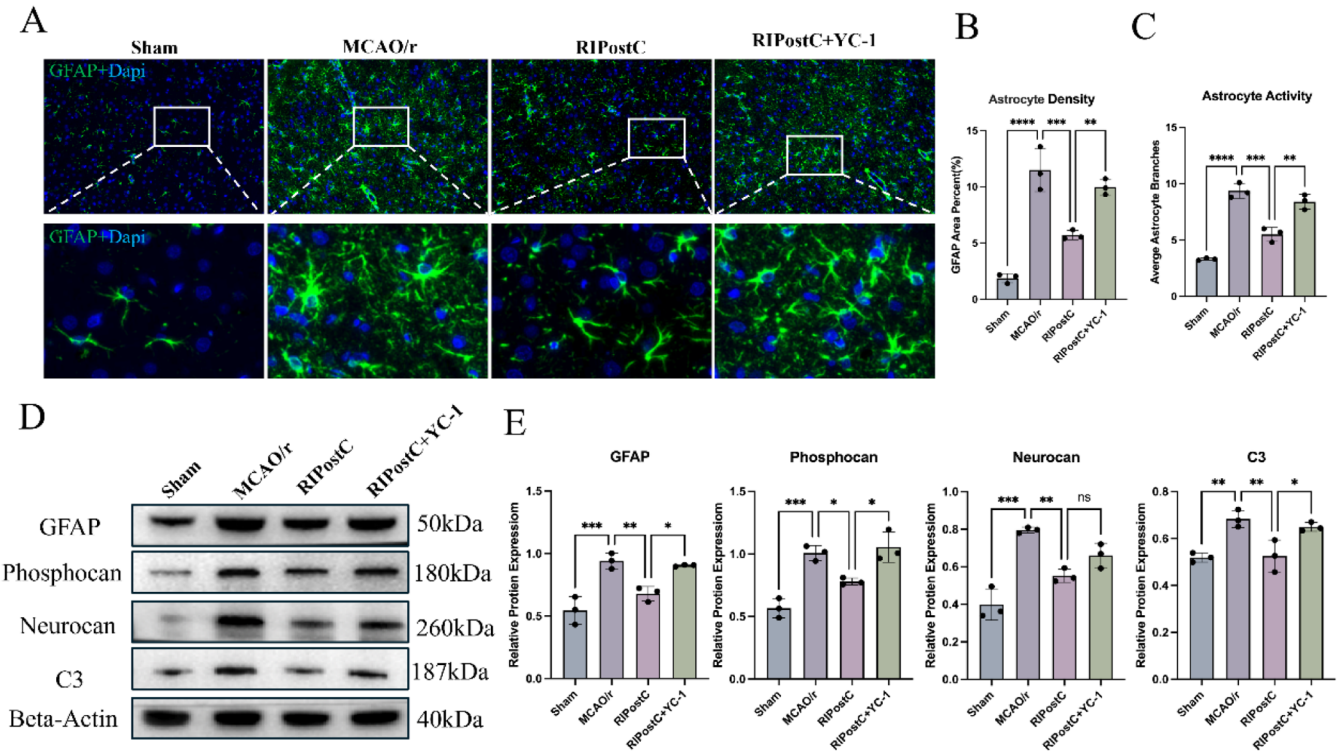


**Figure 5.** RIPostC ameliorates astrocyte activation state in the infarct zone via HIF-1$\alpha$. At 14 days postoperatively, immunofluorescence and WB were used to assess the activation status of astrocytes in the sham, MACO/r, RIPostC, and RIPostC+YC-1 groups. (A) Representative immunofluorescence staining images of the four GFAP-stained groups, white solid line squares show the number and morphology of reactive astrocytes. (B) Study of astrocyte density, statistical analysis of the percentage of GFAP areas, $n = 3$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. (C) Study of astrocyte activity, statistical analysis of mean astrocyte branching number, $n = 3$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$. (D) Representative WB images of GFAP, Phosphocan, Neurocan, and C3 expression in the four groups. (E) Statistical analysis of the expression of GFAP, Phosphocan, Neurocan, and C3 in each of the four groups, $n = 3$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, ns for no significance.

match the neuronal count observed in the sham group. Furthermore, the administration of the YC-1 partially abrogated the neuroprotective effect of RIPostC (Figure 4A,B).

Afterward, we employed immunofluorescence to scrutinize the situation surrounding the ischemic penumbra, yielding results congruent with those from Nissl staining. A comparative analysis between the RIPostC group and the MCAO/r group demonstrated that RIPostC provided support and protection for neurons following angiogenesis in the ischemic penumbra. Nevertheless, the introduction of the inhibitor YC-1 led to a reduction in angiogenesis and a subsequent inhibition of neuronal improvement (Figure 4C,D). The results above confirmed the protective influence of RIPostC on neurons within the ischemic penumbra zone through HIF-1$\alpha$.

**RIPostC Ameliorates Astrocyte Activation State in the Infarct Zone via HIF-1$\alpha$.** We subsequently investigated the impact of RIPostC on the activation status of astrocytes, given that their activation is known to impede neuronal regeneration and recovery. The activation state of the astrocytes was observed using GFAP staining. At 14 days postoperatively, the majority of astrocytes in the infarcted region of the MCAO/r group exhibited clear signs of activation, characterized by an enlarged size and dense distribution, in contrast to the sham group. Furthermore, it is particularly noteworthy that astrocytes exhibited an amoeboid morphology with enlarged cell bodies and shortened processes following ischemic insult (Figure 5A). In contrast, following RIPostC treatment, there was a marked reduction in astrocyte activation and longer astrocyte processes, with the remaining activation being significantly subdued compared with the MCAO/r group, although not completely matching the sham group. In addition, the YC-1 group counteracted the inhibitory effect of RIPostC on astrocyte activation, allowing them to return to their activated state again (Figure 5A-C).

Then, building on these findings, we conducted a WB analysis to evaluate certain proteins that are directly or indirectly linked to astrocyte activation. These included GFAP, the signature protein of astrocytes; Phosphocan and Neurocan, which are associated with glial scars; and C3, a marker for A1-type astrocytes. As shown in Figure 5D, in the RIPostC group, the expression of these proteins mentioned above decreased, approaching that of the sham group, whereas the expression of the proteins increased in the inhibitor group, aligning more with the MCAO/r group (Figure 5E).

Briefly, RIPostC can significantly reduce the activation status of astrocytes.

**RIPostC Improves Locomotor Ability through HIF-1$\alpha$ in MCAO/R Rats.** Several behavioral tests were performed to determine whether RIPostC could enhance the locomotor abilities of rats via the HIF-1$\alpha$ pathway.

The neurological functionality of the rats was evaluated through the modified neurological severity score (mNSS) on postoperative days 1, 7, and 14. As depicted in Figure 6A, aside from the sham group, all experimental groups exhibited high mNSS scores on the initial postoperative day with no significant intergroup differences. These scores progressively


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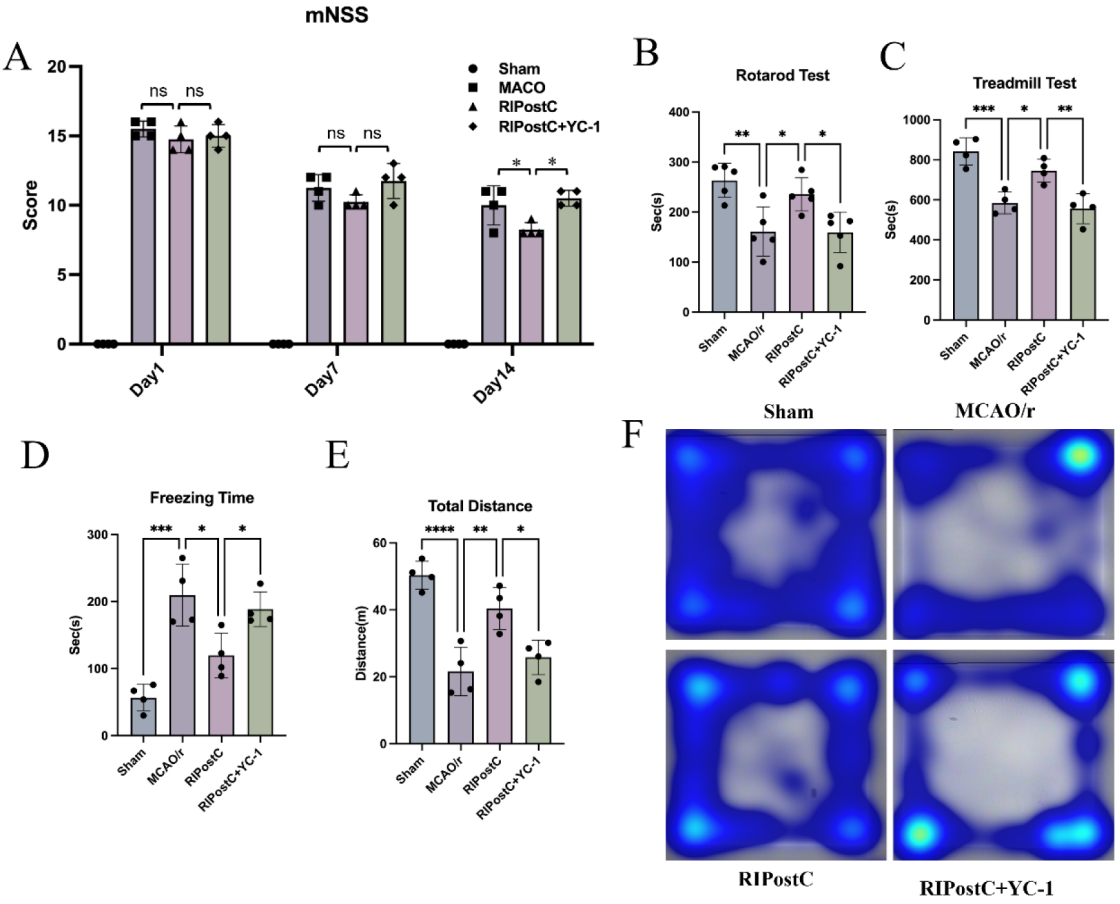


**Figure 6.** RIPostC improves locomotor ability through HIF-1$\alpha$ in MCAO/r rats. Four behavioral tests were performed to evaluate the locomotor ability of rats in the sham, MACO/r, RIPostC, and RIPostC+YC-1 groups. (A) Statistical analysis of the mNSS of the four groups at days 1, 7, and 14 postoperatively, $n = 3$; $^{*}p < 0.05$, ns is no significance. (B) Statistical analysis of the residence time on the rotating bar in the rotarod test of the four groups of rats, $n = 3$; $^{*}p < 0.05$; $^{**}p < 0.01$. (C) Statistical analysis of the time of exhaustion of the four groups of rats in the treadmill test, $n = 3$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (D) Statistical analysis of freezing time in the four groups of rats in the OFT, $n = 3$; $^{*}p < 0.05$; $^{***}p < 0.001$. (E) Statistical analysis of total distance traveled by the four groups of rats in the OFT, $n = 3$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$. (F) Heat map of the movement trajectories of the four groups of rats in the OFT.

declined over time, maintaining nonsignificant variations between groups and remained so through the seventh day. Notably, until day 14, the RIPostC group had scores lower than those of the MCAO/r group and the RIPostC+YC-1 group (Figure 6A), indicating that RIPostC indeed improves neurological function through HIF-1$\alpha$.

On postoperative day 14, the rats underwent a series of behavioral tests, including the rotarod test, treadmill test, and open field test, with ample rest periods interspersed between each test to ensure accuracy. The rotarod test, which primarily evaluates motor coordination, showed a significant reduction in the duration time on the bar in the MCAO/r group compared to the sham group. In contrast, the RIPostC group exhibited an obvious increased residence time compared to the MCAO/r group, while the RIPostC+YC-1 group showed a reduction in residence time relative to the RIPostC group (Figure 6B). Exercise fatigue was assessed in rats using the treadmill test, and the results indicated that rats in the MCAO/r group and the RIPostC+YC-1 group experienced fatigue more quickly than those in the sham group and the RIPostC group (Figure 6C). The open field test, designed to evaluate locomotor ability and exploratory behavior, demonstrated that the MCAO/r group rats displayed significantly reduced locomotion, shorter distances traveled, and increased freezing time compared to the sham group, while rats treated with RIPostC showed a notably enhanced improvement in locomotor ability. But the locomotor performance of the RIPostC+YC-1 group was weakened relative to the RIPostC group (Figure 6D–F).

Overall, the tests collectively demonstrated that RIPostC enhances the locomotor abilities of ischemic stroke rats with this improvement being mediated through the HIF-1$\alpha$ pathway.

## DISCUSSION

Ischemic stroke is caused by a sudden blockage of blood vessels, leading to the loss of blood supply to the affected brain region and causing a cascade of injuries. Following this, ischemia-reperfusion (I/R) injury, a paradoxical phenomenon that occurs upon reperfusion after ischemia, can exacerbate tissue damage. A range of pathological mechanisms contribute to I/R injury, including cellular damage, oxidative stress, inflammatory responses, disruption of the BBB, angiogenesis, myocardial hypertrophy, and fibrotic processes.[23] Despite limited treatment options for cerebral ischemia, which primarily rely on the recanalization of the occluded vessels,[24] growing evidence suggests that the effectiveness of angiogenesis in ischemic stroke prognosis is heavily dependent on


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the integrity and function of the NVU.[1,25] In this research, we explored the protective effects of RIPostC on the NVU in the MCAO/r rat model as a rehabilitative therapy. After 12 days of thrice-daily RIPostC treatment, we found that RIPostC increased the expression of HIF-1$\alpha$ in the brain, promoted angiogenesis, and exerted a protective effect on NVU within the ischemic penumbra.

The concept of the neurovascular unit (NVU) was formally proposed in 2001, pointing out the close connection between brain cells and microvasculature in terms of development, structure, and functionality, and highlighting their synchronized response mechanisms when injured.[26] Consequently, even subtle disturbances in the NVU may seriously affect brain homeostasis and health. Therefore, neurovascular signaling in microvessels composed of endothelial cells, mural cells (consisting of vascular smooth muscle cells (SMCs) and pericytes), and astrocyte end-feet has been widely emphasized. However, the role of peripheral factors and systemic influences on neurovascular function have not been as thoroughly investigated.[27] Further research is imperative to delve into these aspects, and it is with this in mind that we selected RIPostC as the focus of our research.

Remote ischemic conditioning is a promising peripheral treatment for ischemic stroke patients, aiming to reduce the infarct size and ameliorate functional prognosis. The efficacy of this approach was supported by the reduced infarct volume observed in MRI scans of rats treated with RIPostC in this research. Remote ischemic conditioning can be applied before (preconditioning; RIPreC), during (per-conditioning; RIPerC), or after (postconditioning; RIPostC) ischemia. Among these, RIPostC offers more therapeutic opportunities than preconditioning and holds greater clinical translational advantages.[28] The NVU is severely compromised after an ischemic stroke. In our previous research, we demonstrated that RIPostC, as a simple, convenient, and safe rehabilitative treatment, can reduce disability and mitigate pathological indices in the ischemic region, positioning it as a promising poststroke therapy.[13] Furthermore, numerous researches have shown that RIPostC can reduce neuronal death, alleviate neuroinflammation, and stimulate angiogenesis following ischemic stroke.[29−32] It can be inferred that RIPostC may exert a comprehensive protective effect on the NVU. While this study has explored one mechanism involving HIF-1$\alpha$, other potential mechanisms underlying its effects warrant further investigation.

As mentioned before, current researches have demonstrated that HIF-1$\alpha$ is rapidly activated under hypoxia-ischemic conditions,[33] and it, along with its numerous downstream effector molecules—particularly VEGF—exerts a broad range of regulatory effects on the NVU. VEGF, an essential downstream molecule of HIF-1$\alpha$, has the capability of directly acting on neurons to promote the survival of newly formed neurons and enhance neurological function as well as promoting angiogenesis in cerebral ischemic regions. Previous reports have also demonstrated that VEGFA can induce angiogenesis and inhibit neuronal death to promote neurological recovery after ischemic stroke.[34] In short, the HIF-1$\alpha$/VEGF axis plays a crucial role in protecting the NVU, which in turn has a significant impact on the progression and recuperation from ischemic stroke.[35,36] In our research, following a 12-day regimen of RIPostC treatment, we observed a significant increase in the expression of HIF-1$\alpha$ and VEGFA. This led us to the conclusion that RIPostC upregulated HIF-1$\alpha$ in the brain, thereby enhancing the expression of its downstream factors associated with angiogenesis. Further experiments showed that RIPostC stimulated angiogenesis in the ischemic penumbra and exerted a protective effect on neurons in the peripheral zone. These findings suggest that RIPostC activates the HIF-1$\alpha$/VEGF pathway to protect the NVU. In neurons, HIF-1$\alpha$ exerts neuroprotective and angiogenic effects through VEGF and also upregulates the expression of other factors that reduce neuronal death following cerebral ischemia-reperfusion (IR) injury.[37] Additionally, HIF-1$\alpha$ enhances glucose uptake by neurons, thereby regulating their energy metabolism.[38] Moreover, HIF-1$\alpha$ exerts beneficial effects on other cells within the neurovascular unit (NVU) after an ischemic stroke, including astrocytes, microglia, and endothelial cells. However, more in-depth mechanistic studies are needed to further elucidate the effects of RIPostC on these cellular components.

Neuroinflammation is a common feature throughout the pathological progression of an ischemic stroke. Astrocytes, the main components of NVU, are activated in response to a variety of brain injuries. Following cerebral ischemia, activated astrocytes release many factors that exacerbate the inflammatory response, disrupt the stability of the BBB, and hinder the recovery of neurological functions.[23] These effects can be alleviated to some extent by improved blood flow. When astrocyte activation is inhibited, the formation of glial scars is reduced, which in turn promotes neuronal reconnection and extension,[39] contributing to the recovery and protection of the NVU after ischemic stroke. Additionally, angiogenesis enhances the interaction between neurons and glial cells, further supporting recovery after ischemic stroke.[40] Against this backdrop, our research focused on examining the impact of RIPostC on the activation status of astrocytes. We found that RIPostC treatment facilitated angiogenesis by upregulating HIF-1$\alpha$ and then led to a significant amelioration of the activation state of astrocytes. However, our investigation was primarily concentrated on HIF-1$\alpha$ and did not extend to a deeper exploration of VEGF and the subsequent mechanisms, which need to be further explored.

In summary, while we have explored one mechanism by which RIPostC confers protection on the NVU, several aspects remain to be fully elucidated. We aim to further investigate NVU protection and broaden its potential applications to a wider range of diseases.

## CONCLUSION

In this research, we verified that RIPostC significantly reduces cerebral infarct volume, promotes angiogenesis, decreases neuronal death, alleviates astrocyte activation, protects the NVU, and enhances locomotor function in rats through an HIF-1$\alpha$-mediated pathway. This discovery offers a novel perspective and compelling evidence for the development of RIPostC-based, noninvasive therapeutic strategies aimed at combating ischemic stroke and the ensuing NVU damage.

## METHODS

**Animals.** A total of 54 adult male Sprague−Dawley rats (250−300 g) were used in this research. All rats were purchased from Shanghai Jihui Laboratory Animal Care Co., Ltd. Experiments were performed according to the Institutional Animal Care and Use Committee of Fudan University (Shanghai, China). The experimental protocol was approved



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by the Department of Laboratory Zoology, Fudan University (approval number: 2024070165). Rats were housed at room temperature under specific pathogen-free conditions using a 12 h light-dark cycle with free access to food and water. All rats were acclimatized for 1 week before the test. Rats were randomly divided into 4 groups: (1) sham group ($n$ = 12); (2) MCAO/r group ($n$ = 12); (3) RIPostC group ($n$ = 12); and (4) RIPostC+YC-1 group ($n$ = 6).

**MCAO/R Rat Model.** Rats were first anesthetized by intraperitoneal injection of 2% sodium pentobarbital (30 mg/kg). To maintain physiological body temperature, a heating pad was used to maintain the body temperature of the rats at 37 °C. Subsequently, a midline incision of approximately 2 cm was made in the neck of the rats, and the left carotid artery, external carotid artery, and internal carotid artery were finely isolated. The left middle cerebral artery was occluded using a silicone-coated monofilament inserted through the internal carotid artery with an insertion length of approximately 20 mm[13]. After the occlusion lasted for 1.5 h, the thin wire was removed to restore blood flow and achieve reperfusion. The sham-operated group had only the skin and subcutaneous tissue incised and did not suffer any additional injury.

**RIPostC.** RIPostC consisted of cyclic ischemia (10 min) and reperfusion (10 min) treatments applied to the bilateral femoral arteries of rats 48 h after MCAO/r modeling.[14] The procedure was repeated three times daily for 12 days. Ischemia was induced by temporarily occluding arterial blood flow using a thin elastic tourniquet placed on the upper third of the hind limb. Ischemia was confirmed by observing a pale hindlimb and a significant drop in subcutaneous temperature (>3 °C), while reperfusion was confirmed by the return of normal pink skin color.

**YC-1 Administration.** In the experiments, rats received injections of 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1) an HIF-1$\alpha$ inhibitor (Selleck, Houston, USA, dissolved in a solution of 1% dimethyl sulfoxide, DMSO). Its mechanism suppresses HIF-1$\alpha$ expression at the translational level through dual molecular interventions: functional inactivation of its C-terminal transactivation domain (CAD) and enhanced recruitment of the HIF-inhibitory complex (FIH).[41] The rats in the RIPostC+YC-1 group were injected intraperitoneally 2 h before MCAO/r with 5 mg/kg YC-1, and other groups received an equivalent amount of 1% DMSO every day until sacrifice day.

**Magnetic Resonance Imaging (MRI) Scanning.** All of the MRI scans were performed on a United Imaging uMR790 3.0T scanner (Shanghai, China) using a 12-channel animal coil. During the scanning process, the animals were first anesthetized and then placed firmly in a prone position on the scanning table, ensuring that their head positions were fixed. T2-weighted image (T2WI) sequences and T1-weighted image (T1WI) sequences were scanned. Lesion volumes and tissue losses were quantified. The parameters are shown in Table 1.

**Immunofluorescence.** After the rats were anesthetized, they were subjected to saline perfusion through the heart and subsequently perfused with 4% paraformaldehyde for fixation. Brain tissues were rapidly removed and fixed in 4% PFA overnight at 4 °C. The tissues were processed for dehydration and rehydration and were paraffin-embedded. The embedded brain tissue was cut into sections using a slicer, followed by passing these sections through xylene to remove the paraffin and hydration using a series of graded ethanol. Sections were subjected to antigen repair in a citrate buffer. After washing in PBS, the sections were incubated with 5% BSA and 0.5% Triton-X-100 (Solarbio, Beijing, China) for 1 h at room temperature and then incubated overnight at 4 °C with primary antibodies (1:100 dilution for GFAP [ab7260; Abcam], 1:200 for HIF-1$\alpha$ [ab51608; Abcam], CD31 [ab28364; Abcam], NeuN [ab177487; Abcam]). Next day, the sections were then washed and incubated with Alexa Fluor 488 antimouse (H+L) or 594 antimouse (H+L) secondary antibodies (1:500; Life Technologies, USA) for 1 h at room temperature. Immunoreactivity was observed and photographed by using a fluorescence microscope (ECHO Revolve, USA).

**Table 1. Parameters of MRI Used in Rats**

| | T1WI | T2WI |
|---|---|---|
| TR | 503 | 3873 |
| TE | 19 | 85 |
| FOV (mm) | 60 × 60 | 60 × 60 |
| Matrix | 272 × 272 | 272 × 272 |
| Thickness (mm) | 2 | 2 |
| Pixel size (mm) | 0.22 × 0.22 × 2 | 0.22 × 0.22 × 2 |
| Slice number | 15 | 15 |
| Scan time (min) | 4:32 | 3:44 |

**Western Blot Analysis (WB).** Rats were anesthetized and executed 14 days after MCAO/r surgery. Then, Western blot analysis was performed. Proteins were extracted from the samples and loaded onto an SDS-polyacrylamide gel for electrophoresis. The gel was then transferred to a PVDF membrane at 400 mA for 1 h. The membrane was closed with 5% skimmed milk. Incubate the membrane with primary antibodies at 4 °C for 24 h (1:1000 dilution for HIF-1$\alpha$ [ab51608; Abcam], VEGFA [ab52917; Affinity], VEGFR1 [ab32152; Abcam], Ang-1 [ab183701; Abcam], C3 [ab200999; Abcam]; 1:2000 dilution for GFAP [ab7260; Abcam], Phosphocan [ab290640; Abcam], Neurocan [ab277525; Abcam]; 1:10,000 dilution for $\beta$-actin [ab8227; Abcam]). Secondary antibodies were washed with PBS and incubated for 1 h. Protein blot images of each antibody were analyzed using an image analysis program (ImageJ 1.42, NIH, Bethesda, USA) to quantify protein expression based on relative image density.

**Histopathological Examination.** Nissl staining was used to observe the pathological changes and morphological features of the damaged neurons in the cerebral cortex.[13] On day 14 after surgery, the rats were executed, followed by cardiac perfusion, and the brains were first rinsed with saline and then fixed with 4% paraformaldehyde solution. Subsequently, the brain samples were dehydrated with incremental concentrations of alcohol and embedded in paraffin. Sections were cut from the paraffin blocks and processed for Nissl staining. The sections were observed with an Olympus microscope (Tokyo, Japan).

**Behavioral Assessments.** *mNSS.* Neurological function in the surgical group of rats was assessed by the modified Neurological severity score (mNSS), which evaluates motor, sensory, balance, and reflex responses. The score ranges from 0 to 18, with a score of 0 indicating no abnormalities and a score of 18 reflecting severe impairment.[42,43] mNSS assessments were performed on rats on days 1, 7, and 14 following MCAO/r surgery. Only rats with mNSS scores between 14 and 16 on day 1 after MCAO/r surgery were included in this study.



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*Rotarod Test.* This test utilized a rotating cylinder (Jinan Yiyan Technology Development Co., Ltd., China) with a diameter of approximately 8.5 cm to assess the motor coordination of rats. During the test, the rats were required to walk continuously to prevent falling. Two days before the MCAO/r surgery, the rats were trained twice a day for 2 days. Formal testing began 24 h after surgery, and the rats were placed on the rotating cylinder, whose speed was gradually increased from 4 to 40 rpm over 6 min. The test was repeated twice, and the average of the results from the two tests was taken for statistical analysis.

*Treadmill Test.* In our experiment, the treadmill test method was adopted to assess the physical fatigue of rats.[44] One day before the test, the rats were pretrained to adapt to the treadmill environment. The pretraining was divided into two phases: first, a 3 min exploratory period, during which the treadmill belt was kept at rest and the inclination angle was 0°; then, the treadmill was run at a speed of 10 cm/s for 5 min, followed by an increase to a speed of 20 cm/s for another 5 min. On the test day, the rats were placed on the treadmill that had been adjusted to a 5° incline, and their speed was gradually elevated to 30 cm/s over 5 min and maintained at this speed for 15 min. Rats were considered physically exhausted if they touched the electrically charged metal bar at the rear end of the treadmill more than 3 times in a row during the test.

*Open Field Test.* The open field test (OFT) is used to assess locomotor ability and exploratory behavior.[45] In OFT, rats were acclimatized to the environment for 1 h before testing. The room was kept at constant temperature and humidity, in the light and dark, and quiet. The test room was a black box divided into central and peripheral zones. The rats were placed in the central zone of the OFT and allowed to explore freely for 5 min, and their activity trajectories, time spent stationary in the open field, and total distance traveled were recorded by using video tracking software (SMART v.3.0, RWD Life Science).

**Statistical Analysis.** Statistical analysis was performed using GraphPad Prism 9.0 (La Jolla, USA) and SPSS 23.0 statistical software (Chicago, IL, USA). Significance was analyzed by Student's $t$ test, one-way ANOVA plus post hoc LSD test, and two-way ANOVA plus multiple $t$ tests. $p < 0.05$ was defined as statistically significant.

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### Notes

The authors declare no competing financial interest.


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