Remote Limb Ischemic Postconditioning Ameliorated Microglia-Mediated Inflammation Against Cerebral Ischemia/Reperfusion through Modulating Microglial Activation and Polarization via TLR4 Pathway

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Research Article

Keywords: cerebral ischemia/reperfusion, remote ischemic postconditioning, microglia, neuroinflammation, TLR4

Posted Date: November 12th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1057666/v1>

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Remote limb ischemic postconditioning ameliorated microglia-mediated inflammation against cerebral ischemia/reperfusion through modulating microglial activation and polarization via TLR4 pathway

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Author Contributions

DH, ZW took part in conception and design;

DH, LZ, ZZ, YZ, GY took part in acquisition, analysis or interpretation of data;

DH, LZ contributed to drafting the article;

ZW critically reviewed the article for intellectual content.

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Abstract

Accumulating researches have indicated that remote limb ischemic postconditioning (RIPC) mediates neuroprotection by inhibition inflammatory response against cerebral ischemia/reperfusion (I/R), which is proved to correlated with microglial activation and polarization. However, the underlying mechanism remains unclear. In this study, middle cerebral artery occlusion /reperfusion model in Sprague-Dawley rats were conducted and treated with vehicle or RIPC immediately after reperfusion. Infarct size, and neurological scores were performed to assess stroke outcomes for 7 days. Brain damage and neuronal survival were detected using HE and Nissl staining. ELISA, western blotting and immunohistochemistry staining were utilized to evaluate inflammatory response, neuronal apoptosis, and microglial activation and polarization to M1- or M2-subtypes respectively. Results showed that RIPC significantly attenuated infarct size at 3d and alleviated the neurological deficits of rats at 3d and 7d post-ischemia. Furthermore, RIPC decreased expression of inflammatory cytokines IL- β , TNF- α and neuronal loss, and increased expression of cytokines IL-10 and Bcl-2. In addition, RIPC suppressed microglial activation and promoted microglial polarization to M2 type along with downregulation of TLR4 expression. These results suggested that RIPC was neuroprotective against ischemic stroke by modulating the activation of microglia/macrophages and encouraging polarization to M2 phenotype possibly through TLR4 signaling pathway.

Keywords cerebral ischemia/reperfusion; remote ischemic postconditioning; microglia; neuroinflammation; TLR4

Introduction

Stroke is prevalent globally and disabled more than 80 million survivors in 2016 (Collaborators., 2019). With significant progress and advances in medical and surgical therapies, age-standardized deaths induced by stroke have been decreased from 1990 to

2016 (Collaborators., 2019). However, the morbidity and overall burden of stroke is still increasing due to population growth and aging, among which ischemic stroke accounted for 84.4% (Collaborators., 2019). In recent dozen years, ischemic postconditioning (IPC) or remote IPC (RIPC) has been investigated as a novel therapeutic approach in ischemic diseases, including myocardial infarction and cerebral ischemia (Guo et al., 2019;SinghandSharma, 2020;Wei et al., 2011). Moreover, accumulating experimental studies suggest that IPC or RIPC could decrease neuronal death, alleviate oxidative stress injury and reduce infarct size during the acute phase after stroke (Esposito et al., 2018;Han et al., 2021;Li et al., 2015), and improve neurological outcomes, promote neurogenesis and angiogenic remodeling during the recovery phase as well (Esposito et al., 2015;Huang et al., 2017;Liang et al., 2018). Despite the fact that neuroprotection of RIPC has been further validated in clinical studies (England et al., 2019;Li et al., 2020;Song et al., 2021), the underlying mechanisms remain poorly understood.

Microglia/macrophages, the resident neuroimmune cells of the central nervous system, play a pivotal role in modulating immune and inflammatory responses (ColonnaandButovsky, 2017), as well as neuronal apoptosis and synaptic plasticity (Sandvig et al., 2018). After cerebral ischemia, a mass of microglia are immediately activated and dynamically polarized into neurotoxic (M1)- or neuroprotective (M2)-phenotype (Xiong et al., 2016), which play biphasic effects in the pathological process. For example, the M2 phenotype ameliorates neuronal survival and tissue repair, while the M1 phenotype hastens neuronal necrosis and aggravates inflammation (Hu et al., 2015;Ma et al., 2017). Thus, regulation the activation and polarization of microglia may represent a potential target for cerebral ischemia. Recently, Han and colleagues (Han et al., 2021) declared that RIPC promoted microglia/macrophages transferring from M1 to M2 phenotype after focal cerebral I/R in mice, thus providing therapeutic effects.

TLRs (Toll-like receptors) are the main signal transduction molecules for regulating endogenous and exogenous inflammation (Wang et al., 2013), and TLR4 was proved to play a critical role in modulating neuroinflammation induced by cerebral I/R injury (Zhao et al., 2018). Under ischemic stimuli, TLR4 would be activated and highly

expressed on microglia, which would be an important signal to further trigger microglial activation and regulate the function (Ye et al., 2019), thus inhibition of TLR4 signaling would alleviate brain damage (Zhao et al., 2018). Therefore, whether the neuroprotective effects of RIPC on regulation of microglial activation and polarization against cerebral ischemia through TLR4 signaling remains further explored.

In this study, we explored the therapeutic efficacy of RIPC by detecting the inflammatory cytokines, neuronal apoptosis and microglial activation and polarization markers in the brain cortex of rats after cerebral I/R.

Materials and methods

Animals

Male Sprague-Dawley rats weighting 260–280g were purchased from the Centre of Laboratory Animals of Chongqing Medical University in Chongqing, China. Rats were maintained in a room with controlled temperature (22 ± 1 °C) on a 12-h light/dark cycle and allowed free access to standard rodent chow and tap water for 3–7 days before surgery. A total of 96 rats were included for experiments and were randomly allocated to 3 groups: sham group (n = 18), MCAO group (n = 48), and RIPC group (n = 48). All experiments were performed following protocols approved by the Institutional Animal Ethics Committee of Chongqing Medical University and conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals provided by the National Institutes of Health (NIH publication no. 80–82).

Transient focal cerebral ischemic stroke and intervention

Transient cerebral ischemia was performed as previously described with some modifications (Ling et al., 2021). Briefly, animals were initially anesthetized with 5% isoflurane, and then maintained narcotism with 2% isoflurane (30% oxygen, 70% nitrous oxide) and fixed supinely. After middle neck incision, the right common, external and internal carotid arteries were isolated and exposed. After the ligation and transaction of the external carotid artery, a nylon suture with a silicone-coated tip (diameter of 0.28 mm, Beijing Cinontech Co. Ltd., China) was inserted through

common carotid artery, and advanced for approximately 19 ± 1 mm until mild resistance was felt. Reperfusion was achieved after 90 min of ischemia by withdrawing the suture. Animals' body temperature was maintained at 36.8 – 37.2 °C with an infrared heat lamp and a heating pad until they recovered from anesthesia. Rats in the sham group underwent the same surgical procedures without the MCA occlusion. RIPC was performed as previously described (Han et al., 2021), as it was conducted immediately after cerebral ischemia with 10 min ischemia of bilateral hind limbs and 10 min reperfusion for three continuous cycles.

Laser Doppler Flowmetry (PeriFlux System 5000, Stockholm, Sweden) was performed for continuous monitoring of regional cerebral blood flow (rCBF) at the following coordinates: 1 mm posterior and 5 mm right lateral to the bregma (Taninishi et al., 2015) to confirm whether the occlusion of MCA was succeeded. Ischemia animals with a reduction of $>70\%$ rCBF after the occlusion of MCA and $>70\%$ rCBF reservation after reperfusion were eligible for subsequent experiment.

Neurobehavioral evaluation

Neurological deficits (motor, sensation, balance and reflexes) were evaluated by the 18-point modified Neurological Severity Score (mNSS) scale on days 1, 3 and 7 post-ischemia by a blinded observer according to Garcia et al. (Garcia et al., 1995). All animals were trained three times over 3 consecutive days before the surgery and the last datasets were regarded as the preoperative value. At the end of each evaluation, total scores were calculated and recorded ($n = 5$ – 10 per time point).

Coordination function was evaluated by Rota-rod test system (ENV-575A, Med Associates, USA) as previously described (Yang et al., 2019). Briefly, rats were repeatedly trained on accelerating treadmills for 3 days prior to surgeries. The speed was gradually accelerated from 4 to 40 rpm in 5 minutes. Prior to or after I/R, each rat was evaluated for triplicates. The mean value of latency to fall from the rotarod was calculated.

Infarct size assessment

After 3 days reperfusion, the rats were deeply anesthetized and transcardial perfused with 0.9% NaCl (n=6 per subgroup). The brains were harvested immediately and sliced into consecutive 2mm coronal sections. They were incubated in 2% (w/v) 2, 3, 5-triphenyltetrazolium chloride (TTC, Sigma Aldrich, USA) solution (0.2mol/ml, pH8.7) at 37°C in the dark for 20 min and then fixed in 4% paraformaldehyde at 4 °C for 24h. TTC-stained slices were photographed with a high-resolution digital camera (Canon EOS 500D, Japan) and analyzed using Image-Pro Plus 6.0 software. The ratio of infarct size to normal volume was calculated from the 6 slices according to this equation (Zhao et al., 2014): Ratio of infarct size= [(contralateral area -ipsilateral noninfarct area)/contralateral area] ×100%.

Tissue preparation for pathological evaluation and western blotting

For pathological evaluation, animals in three groups were sacrificed on day 1, 3 and 7 post-reperfusion (rats in sham group were sacrificed on day 3; n=6 per subgroup). The animals were transcardial perfused with 0.9% NaCl rapidly, and then slowly prefixed with 4% paraformaldehyde, and the brains were removed quickly. Coronal sections containing cortical tissue supplied by MCA from -2.0 to 2.0 mm posterior to the bregma were retained and then postfixed in 4% paraformaldehyde for at least 24h. Brain sections were then embedded in paraffin and cut into 6 µm slices on a microtome (Leica RM 2245, Germany) for subsequent histological staining.

For western blotting, fresh tissues were obtained from the ipsilateral brain cortex after deep anesthesia on day 1 and 7 post-reperfusion (tissues of sham group were gained on day 1; n=6 per time point) and homogenized in RIPA lysis buffer. Tissue homogenates were centrifuged and supernatants were collected for western blotting analysis.

Hematoxylin-eosin staining

Briefly, slices were deparaffinized, hydrated in water, then stained with hematoxylin for 15 minutes, and washed in running tap water for 20 minutes. Next, slices were counterstained with eosin for 2 minutes, dehydrated in 95% absolute alcohol until

excess eosin was removed, permeabilized in xylene, and mounted. Pathological changes were observed under high magnification with a light microscope.

Toluidine blue (Nissl) staining

The slices were washed three times and were dyed at 37°C with 1% toluidine blue solution for 30 min. The tissue was decolorized and dehydrated with ethanol and sealed with neutral resin. Under light microscopy (Olympus Corporation), the Nissl bodies of neurons were blue and purple.

Immunohistochemistry staining

The coronal brain slices were permeabilized with 3% Triton X-100 for 10 min, blocked with 10% normal donkey serum in phosphate buffer saline (PBS) for 60 min and incubated with the following diluted primary antibodies at 4° C overnight: Iba1 (ab5076, Abcam, 1:1000), Cox2 (ab16191, Abcam, 1:2000), Arg1(cst#93668, Cell Signaling Technology, 1:100) and Caspase-3 (ab184787, Abcam, 1:1000). Then, slices were incubated with secondary antibodies (Jackson ImmunoResearch, 1:250) at 37° C for 1 h. Sections omitting the primary antibodies were used as negative controls. Thorough washes with double-distilled water were applied between each step specified above. After all the procedures, slices were mounted for microscopic observation and images were captured by a light microscope. The numbers of positive cells and expression intensity of protein in the target areas were analyzed using Image Pro-Plus 6.0 software (Media Cybernetics, Inc., USA). Six samples were used for each group at different time points.

Western blotting

The process of western blotting was conducted as previously described (Dong et al., 2021). Diluted primary antibodies: CD206 (ab64693, Abcam, 1:500), Cox2 (ab16191, Abcam, 1:500), Iba1 (ab5076, Abcam, 1:200), Caspase-3 (ab184787, Abcam, 1:2000), Bcl-2 (sc-7382, Santa Cruz Biotechnology, 1:1000), Bax (cst#2772, Cell Signaling Technology, 1:1000), TLR4 (sc-293072, Santa Cruz Biotechnology, 1:500) and β -

tubulin (cst#2146, Cell Signaling Technology, 1:1000) were applied at 4°C overnight on a rocker. Secondary antibodies (anti-rabbit, cst#7074, anti-mouse cst#7076, and anti-goat, Cell Signaling Technology) conjugated with horse-radish peroxidase-(HRP) were incubated at room temperature for 1h on the rocker at 80 rpm. Positive bands were visualized using ECL kit (Millipore, USA) on a chemiluminescence imaging system (Bio-Rad, Hercules, CA, USA). Protein levels were measured with Image Pro-Plus 6.0, and β -tubulin was used as a loading control for densitometry analyses.

ELISA assay

To investigate the levels of inflammatory cytokines, rats were sacrificed at 1d, 3d, 7d after reperfusion and the brain tissue homogenates were obtained. The concentrations of IL-1 β , TNF- α and IL-10 were measured using specific ELISA kits according to the manufacturers' instructions (Boster Biological Technology, Wuhan, China).

Statistical analysis

Data are represented as the means $\pm$ SD. All statistical analyzes were performed using the IBM SPSS 19.0 software. Student t-test was used to compare infarct size between the ischemic groups. Other data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons (multiple groups). Prism 8 for windows (GraphPad Software Inc., USA) was used for figure generation. A value of $p < 0.05$ was considered statistically significant.

Results

1. RIPC decreased infarct size and attenuated neurological outcome after cerebral I/R.

According to TTC staining, the infarct area was unstained or white, while the normal tissue was pink or red. As showed in Fig. 1A), no white infarct area was observed in the sham group, while the white area in the I/R group was approximately 44.23%. The

infarct size of the RIPC group ($(22.97 \pm 4.28) \%$) was significantly decreased in comparison to the I/R group at day 3 ($P < 0.001$, Fig. 1B).

The neurological function of rats in all experimental groups were assessed by mNSS and rotarod test, and rats in the I/R 1-day group and RIPC 1-day group all exhibited significant deficits after the establishment of cerebral I/R, with high mNSS scores and short latency to fall. In the subacute phase, the deficits of the I/R and RIPC groups were mitigated gradually until day 7, and rats in the RIPC group gained lower mNSS scores and longer latency compared to those in the I/R group both at day 3 and 7 ($P < 0.05$).

2. RIPC mitigated neuronal damage after cerebral I/R.

Hematoxylin-eosin staining was used to evaluate the morphological changes of cells in the cortex. As shown in Fig.2A, the sham group showed no cerebral ischemia, with well-arranged and intact-structured neurons, containing consistent staining of nucleolus in the middle. In the I/R 1-day group, the brain tissue of the infarct area exhibited distinct edema, obvious enlarged perivascular space, swollen neurons with atrophic structures and pyknotic nuclei. The edema in the brain tissue was gradually alleviated and newly formed blood vessels appeared with prolonged reperfusion time, accompanied by increasing number of infiltrated glial cells at the infarction border zone. Under RIPC treatment, the extent of brain edema, disordered array of neurons, neuronal loss and nuclei pyknosis were mitigated at all time points, and there were more proliferous glial cells along the ischemic border zone.

3. RIPC ameliorated Nissl body loss in neurons after cerebral I/R.

Nissl staining showed well-arranged dark blue nucleoli of neurons in the sham group (Fig. 2B). After cerebral I/R, significant morphological changes were detected, including large area of neurons loss with disappeared or scattered Nissl bodies in the infarct area, nuclei shrinkage or dark staining of neurons in the peri-infarct zone. After 3 days of reperfusion, numerous neurons with irregular and light stained Nissl bodies were observed in the peri-infarct zone and minority in the infarct area as well. On the 7th day of reperfusion, evidently increased Nissl bodies were detected in the infarct

area with almost uniform distribution. In addition, the changes of neurons loss, nuclei shrinkage and morphology were significantly reversed under RIPC treatment compared to the I/R injury, and larger number of glial cells with hyper-chromatic appeared along the peri-infarct zone at the same time in the RIPC group. The proportions of survival neurons in the RIPC groups were significantly higher than that in the I/R groups at all time points (Fig. 2b).

4. RIPC treatment ameliorated apoptosis after cerebral I/R.

To observe the distribution of apoptosis after cerebral I/R, immunohistochemistry staining of Caspase-3 was carried out. As shown in Fig. 3A, few Caspase-3 positive cells were detected in the sham group. After 24h cerebral I/R, some positive cells were observed in the peri-infarct zone, while less or even null in the infarct area, and no statistical difference was found in the expression level of Caspase-3 between I/R 1-day group and sham group ($P>0.05$) (Fig. 3B). As the reperfusion time increased, significantly increased positive cells were detected in the cortex, especially in the peri-infarct zone. Under RIPC treatment, the expression levels of Caspase-3 were inhibited at the same time points, and the mean density of Caspase-3 expression was significantly decreased at 3d and 7d compared to the I/R group ($P<0.001$).

As shown in Fig. 3C-E, the expression of Bax and Caspase-3 in 1-day group significantly increased following cerebral I/R, which were significantly higher than the sham group ($P<0.01$). And the expression levels of Bax and Caspase-3 were further elevated in 7-day group, and greater augmentations were noted in the Caspase-3 expression. RIPC treatment significantly down-regulated the expression of Bax and Caspase-3 both in 1-day and 7-day groups in comparison with that of the I/R group ($P<0.05$), and there were no significant differences between the sham group and RIPC 1-day group ($P>0.05$).

On the contrary, the expression of Bcl-2 was significantly decreased after cerebral I/R, and further decreased by day 7 (Fig. 4 C, F). Under RIPC treatment, the expression of Bcl-2 proteins was up-regulated both in the 1-day and 7-day groups ($P<0.001$), but still lower than the level of the sham group ($P<0.001$).

5. Effect of RIPC treatment on the activation and morphology of microglia/macrophages after cerebral I/R.

Immunohistochemical staining of Iba1 was performed to detect the activation and distribution of microglia/macrophages in cortex (Fig. 4). In the sham group, scattered and ramified microglia/macrophages with small cell bodies and long processes were observed (Fig. 4A). After 1 day cerebral I/R, microglia/macrophages were immediately activated in the infarct area, and were stimulated to morph from a ramified to an intermediate or amoeboid state, with enlarged cell bodies and shortened and thickened processes, while they were still in a resting ramified state in the penumbra (Fig. 4A, B, C). After 3 days of I/R, a large number of microglia/macrophages in an amoeboid shape with very short or no processes were observed both in the infarct area and in the penumbra. With prolonged reperfusion time to 7 days, significantly increased Iba1 positive cells were observed in cortex. Round shape microglia/macrophages with disappeared processes accumulated at the infarct area, while bipolar rod-shape sub-round microglia/macrophages remaining short bulges maintained scattered in the penumbra. In the RIPC group, the morphological and quantitative changes of microglia/macrophages were comparable to those in the I/R group after cerebral I/R (Fig. 4A, B). However, there were considerable number of microglia/macrophages remaining short and thicken processes in the infarct area and slender branches in the penumbra at the 7th day after cerebral I/R. Furthermore, the number of Iba1 positive cells were significantly decreased in the RIPC group in comparison with that of I/R group at day 3 and 7 ($P < 0.01$) (Fig. 4B).

6. RIPC regulated the polarization of microglia/macrophages induced by cerebral I/R.

To detect the polarization of microglia/macrophages, immunolabeling of Arg1 and Cox2 was performed to detect the M1-type or M2-type microglia/macrophages respectively. As shown in Fig. 5A, only a small amount of Arg1 positive cells without bulges were observed in the outermost layer of brain cortex. Under the stimulation of cerebral I/R, increased Arg1⁺ cells firstly appeared at the periphery of the infarct tissue

after 24h (Fig. 5A, B), and then profusely proliferated with short and thick bulges in the infarct area at the 3rd day, particularly at the infarction border zone. With prolonged reperfusion to 7 days, more evident changes in the quantity and morphology of Arg1⁺ cells were detected, which exhibited with longer and thicken bulges in morphologically. Moreover, RIPC treatment further augmented the proliferation of Arg1⁺ cells and promoted the extension and thicken of bulges in comparison with that of I/R group at the same time points. No noticeable changes were found in the penumbra and intact tissue after cerebral I/R (Fig. 5C).

Similarly, few Cox2 positive cells were seen in the sham group, while the cell numbers increased dramatically after the stimulation of cerebral I/R (Fig. 6A), and the expression level of Cox2 was significantly higher than the sham group ($P<0.001$) (Fig. 6B). With the extension of reperfusion time, Cox2⁺ cells displayed a gradual reduction in number and expression level. Under RIPC treatment, the cell numbers and expression level of Cox2 were further reduced at the same time points compared with that of I/R group ($P<0.05$).

7. RIPC regulated the protein expression of microglia/macrophages after cerebral I/R.

To further examine the effect of RIPC on the activation and polarization of microglia/macrophages in the cortex, protein expression levels of Iba1, CD206, and Cox2 were tested.

Consistent with the immunohistochemistry staining results, the expression level of Iba1 protein was low in the sham group (Fig. 6C, D, F). Whereas cerebral I/R stimulated the expression of Iba1, and further enhanced the expression level till day 7. Under RIPC treatment, the expression level of Iba1 protein were significantly inhibited at day 1 and day 7 compared to the I/R group ($P<0.01$). In comparison to the sham group, expression of CD206 and Cox2 protein were substantially increased after 1 day cerebral I/R ($P<0.001$). After prolonged reperfusion, the protein expression levels of CD206 and Cox2 dropped at day 7, but the Cox2 expression level remained higher than that of sham group ($P<0.001$). Following with RIPC intervention, the expression level of

CD206 and Cox2 was significantly elevated and inhibited respectively after both at day 1 and day 7 compared to the I/R group ($P<0.05$, $P<0.001$ respectively).

8. NILP regulated TNF- α , IL-1 β , and IL-10 cytokines expression in rats after cerebral I/R.

The expression level of pro-inflammatory cytokines (TNF- α , IL-1 β) and anti-inflammatory (IL-10) cytokines were detected by ELISA on day 1, 3 and 7 after cerebral ischemia to evaluate the inflammation response and the outcome of RIPC on microglia/macrophage polarization. As shown in Fig. 7 A-C, IL-1 β , TNF- α , and IL-10 increased significantly in the I/R group compared to the sham group at 24h postinjury ($P<0.001$). Then, the expression level of IL-1 β continued to increase in the following days. On the contrary, the expression level of TNF- α showed continuous downtrend post-reperfusion, and they approached to the level of sham group at day 7 ($P>0.05$). Under RIPC treatment, the expression of IL-1 β and TNF- α , although showing similar tendencies with the I/R group, were significantly inhibited compared to the I/R group at each time point ($P<0.05$). The expression level of anti-inflammatory cytokines IL-10 in the I/R group significantly increased after cerebral ischemia and peaked at day 3 and then decline at day7, which was much higher than the sham group ($P<0.001$). As was expected, the expression level of IL-10 in the RIPC group exhibited similar variation tendency with the I/R group, and RIPC intervention significantly enhanced the expression level of IL-10 compared to the I/R group at all time points ($P<0.001$).

9. RIPC treatment down-regulated the protein expression of TLR4.

In comparison to the sham group, cerebral I/R induced the expression of TLR4, and further enhanced it with the prolongation of reperfusion ($P<0.001$, Fig. 7D, E). RIPC treatment reversed the amplification of protein expression of TLR4 both at day 1 ($P<0.05$) and day 7 ($P<0.001$).

Discussion

RIPC is a novel postconditioning treatment with great potential for neuroprotection following ischemic stroke in recent years. In this study, we replicated the therapeutic effect of RIPC applied on the bilateral hind limbs for 3 cycles of 10min-ischemia in cerebral I/R rats, resulting in better neurological outcomes, reduced infarct size, ameliorated neuronal survival, and alleviated inflammatory responses for 7 days, as reported in other researches (Cheng et al., 2014;Esposito et al., 2018). Simultaneously, RIPC suppressed the activation of microglia/macrophage and promoted M2-type polarization. Lower expression level of TLR4 in the cortex under RIPC treatment suggested that the observed effect of RIPC on brain damage and microglial activation and polarization might partially through TLR4 signaling pathway.

As previously reported, the Bcl-2 family plays an important role in apoptosis after ischemic stroke (Qi et al., 2015;Zhao et al., 2003), as the balance between Bax and Bcl-2 proteins determines whether cells survive or undergo apoptosis. Therefore, the increase in Bax and Caspase-3 expression after cerebral I/R indicated activation of the apoptotic cascade (Chelluboina et al., 2014), while decreased expression of Bax and Caspase-3 combined with increased expression of Bcl-2 by RIPC reinforced the point that RIPC ameliorated neuronal apoptosis and promoted neuronal survival (Cheng et al., 2014), which was also demonstrated by the result of Nissl staining.

Microglia/macrophages is prone to be activated by certain stimuli or injury, and plays an important role in the subsequent process of neuroinflammation and recovery (ColonnaandButovsky, 2017). And it was reported that there is a positive correlation between microglial activation and impaired motor function in transient cerebral ischemic mice (Lartey et al., 2014), indicating that inhibition of microglial activation is beneficial to alleviating brain damage and rescuing neurological function. However, blockade of microglial activation and proliferation inversely exacerbated brain damage in adult MCAO mice using transgenic technology (Lalancette-Hébert et al., 2007). These results revealed that proliferated and activated microglia may serve as a potential target for endogenous therapeutic approaches of stroke, and modulation rather than suppression may be important. After cerebral ischemia, microglia/macrophages progresses through four morphological states from ramified to round shape correlated

to increasing activation (Thored et al., 2009), and plays a double-edged sword dynamically depending on their phenotypes (Ma et al., 2017). In this present study, all four states of microglial/macrophages were observed in the cortex following the reperfusion, and greater number of and higher activated microglia were discovered closer to the infarct center, as displayed in other researches (Hu et al., 2012; Thored et al., 2009), which is considered to be associated with various factors, such as ischemic severity, inflammatory response and proteins released from damaged cells and etc. (Anttila et al., 2017). In addition, the morph of microglia/macrophages from amoeboid to round was delayed under RIPC treatment in the subacute phase after cerebral I/R, as well as the proliferation of microglia/macrophages, which were consistent with the results in HE, Nissl and Caspase-3 staining, indicating that appropriate inhibition of microglial over-activation leads to better outcomes.

After cerebral ischemia, microglia/macrophages were activated and polarized dynamically into M1- or M2-phenotype (Xiong et al., 2016), which can be detected by M1 markers CD16, CD86, Cox2, iNOS or M2 markers CD206 and Arg1. It has been reported that the number of M2-type microglia/macrophages in the cortex was significantly increased at 1 day, persisted for up to 5 days and declined to 14 days after focal cerebral I/R (Hu et al., 2012; Perego et al., 2011), which is consistent with the results in our study by detecting Arg1 positive cells (Fig.5). Contrary to M2-type microglia/macrophages, the number of M1-type microglia/macrophages (Cox2) sharply increased at 1 day and then gradually declined in the subacute phase (Fig. 6A, B), the changing tendency of which was also confirmed by the detection of CD16-labeled M1-type with an increase at 24h and then a decrease at 72h after 60min cerebral I/R in mice in another study (Han et al., 2021). Given the dynamic changes in the phenotypes and direct correlation between the phenotypes and function (Lartey et al., 2014), inhibiting M1 phenotype or promoting M2 phenotype leads to neuroprotective effects for ischemic stroke (Hu et al., 2012). Immunohistochemistry staining data indicated that RIPC brought an increase of Arg1⁺ cells and a reduction of Cox2⁺ cells from day 1 to day 7, indicating that RIPC promoted the polarization towards M2-type, and inhibited the polarization towards M1-type after cerebral I/R. Consistent with

immunohistochemistry results, RIPC also increased M2 marker expression (CD206) and decreased of M1 marker (Cox2) expression post-I/R. These findings suggested that RIPC was protective towards cerebral I/R by promoting microglia/macrophages polarization to M2 phenotype in rats, as well as in mice (Han et al., 2021).

Changes in morphology may signal a change in functional state of microglia, but in fact morphology alone cannot be used to predict a functional outcome (Colton and Wilcock, 2010). Under ischemic stimuli, M1 -type secretes pro-inflammatory cytokines and neurotoxic mediators, such as TNF- α , IL-1 β , IL-6, and etc., participating in the pathogenesis process and aggravating neuronal injury (Xiong et al., 2016), while M2-type secretes anti-inflammatory cytokines, such as IL-10, IL-1 β , TGF- β , IGF-1, and etc., promoting angiogenesis and tissue repair (Hu et al., 2015). Thus, we further investigated the inflammatory response by detecting the expression of inflammatory cytokines. Results indicated that RIPC increased IL-10 level and decreased IL-1 β and TNF- α level from 1d to 7d post-I/R. These results revealed that RIPC not only modulated the phenotypes of microglia/macrophages, but also regulated its function on inflammation after cerebral I/R in rats.

TLRs are the main molecules in mammals that transduce extracellular information into cells and trigger an inflammation response (Lester and Li, 2014), among which TLR4 is highly expressed on microglia and plays a vital role in the activation of microglia (Ye et al., 2019) and regulation of inflammatory response (Lu et al., 2008). Inhibition of TLR4 signaling by antagonist TAK-242 could alleviate inflammatory response against acute cerebral I/R (Ling et al., 2021), and IPC and RIPC were also reported to show similar inhibiting effects resulting in better neurological outcomes (Qi et al., 2016; Wang et al., 2014). In this study, RIPC suppressed the expression of TLR4 induced by cerebral I/R from 1d to 7d post-I/R. Thus, overall, we conclude that the neuroprotective effect of RIPC is strongly relevant to the ameliorated neuronal apoptosis and alleviated inflammation by modulation of microglial activation and polarization through TLR4 signaling pathway.

There are some limitations in the present study. Firstly, a single marker such as Cox2 or Arg1 is unlikely to provide enough information to characterize the specified

phenotypes of microglia. Thus, double-immunostaining of biomarkers or detection of specific genes may provide a more precise analysis of microglia/macrophages activation and polarization state. Moreover, detection of downstream proteins and phosphorylated protein following the TLR4 signaling would be more helpful to clarify the mechanism.

Conclusions

Overall, the present study showed that RIPC was protective towards ischemic stroke by modulating the activation of microglia/macrophages and encouraging polarization to M2 phenotype in cerebral I/R rats possibly through TLR4 signaling pathway. The mechanism underlying the therapeutic effect of RIPC still needs to be further investigated.

Funding This work was supported by National Natural Science Foundation of China grants to Dan Huang (NO.81802242) and to Zhenyu Wang (NO.81674066), and a grant from Yongchuan Hospital of Chongqing Medical University to Dan Huang (YJJC201903).

Data availability All data generated or analyzed during this study are included in this published article (and its supplementary information).

Declarations

Ethical approval All animal procedures were performed with the approval of the Ethics Committee of Cairo University Institutional Animal Care and Use Committee (IACUC) and according to its standards.

Conflict of interest The authors declare no conflict of interest with respect the research, authorship, and/ or publication of this article.

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Figures

Figure 1

Remote limb ischemic postconditioning (RIPC) reduced acute infarct size and improved neurological outcomes after cerebral I/R: (A) Representative images of TTC-stained coronal slices in sham, I/R and RIPC groups after 3 days reperfusion; (B) brain infarct size; (C) modified neurological severity scores (mNSS); (D) latency to fall in the rota-rod test. Data are mean $\pm$ SD ($n = 6$). *** $P < 0.001$ vs. sham; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. I/R (student t-test for (B), two-way ANOVA with Tukey post testing for (C, D)).

Figure 2

RIPC mitigated neuronal damage and neuronal loss after cerebral I/R: (A) Representative images of HE stained coronal slices in sham, I/R and RIPC groups at different time points after cerebral I/R. (A(a)) Sketch picture demonstrates the infarct area and the peri-infarct area defined by HE staining 3 days after cerebral I/R. Scale bar: 2000 μ m. (B) Representative images of coronal brain sections showing the distribution of Nissl bodies in the infarct area and peri-infarct area at indicated time points after cerebral I/R. Scale bar: 100 μ m. (C) Quantification of the proportion of survival neurons numbers in the I/R and RIPC groups relative to the sham group in the infarct area and the peri-infarct area at indicated time points. Data are mean $\pm$ SD (n = 6). ###P < 0.001 vs. I/R (student t-test).

Figure 3

RIPC ameliorated neuronal apoptosis after cerebral I/R. (A) Representative images of coronal brain sections of Caspase-3+ cells in the infarct area and peri-infarct zone at different time points after cerebral I/R. Scale bar=100 μ m. (A(a)) Sketch picture demonstrates the distribution of Caspase-3+ cells in the brain tissue after cerebral I/R. Scale bar =2000 μ m. (B) Quantification of mean expression density of Caspase-3 in the ischemic regions exhibited in A at different time points after cerebral I/R. (C) Western blot analysis of Bax, Caspase-3, and Bcl-2 in the peri-infarct area of all experimental groups. Quantitative analysis of (D) Bax, (E) Caspase-3, and (F) Bcl-2 in the peri-infarct area of the experimental groups at day 1 and 7 after cerebral I/R. RIPC inhibited the protein expression of Caspase-3 and Bax, while enhanced the expression level of Bcl-2 both at day 1 and day 7 after cerebral I/R. β -tubulin was used as a loading control; data are mean $\pm$ SD (n = 6). ***P < 0.001 vs. Sham; ###P < 0.001 vs. I/R (two-way ANOVA with Tukey post testing).

Figure 4

RIPC inhibited the activation of microglia/macrophages after cerebral I/R. (A) Representative immunohistochemistry images of coronal brain sections of Iba1+ cells in the infarct and peri-infarct cortex at different time points after cerebral I/R. Scale bar=100 μ m. The inset images on the bottom-left were further magnified images of the cells in box, which are selected from the peri-infarct zone (the left one) and the infarct area (the right one). (B) Quantitative analysis of the number of Iba1 positive cells in the ischemic regions exhibited in A at indicated time points. Data are mean $\pm$ SD (n = 6). ***P < 0.001 vs. Sham; ##P<0.01, ###P < 0.001 vs. I/R (two-way ANOVA with Tukey post testing). (C) Representative images of Iba1 staining in coronal brain sections 3 days after cerebral I/R at different magnifications: (a) scale bar=2000 μ m, (b) scale bar=200 μ m, (C) scale bar=100 μ m, (d) scale bar=50 μ m, demonstrate the activation, distribution and morphological changes of microglia/macrophages in the infarct area and peri-infarct area induced by ischemia/reperfusion stimulation.

Figure 5

RIPC promoted the M2-type microglia/macrophages polarization after cerebral I/R. (A) Representative immunohistochemistry images of coronal brain sections of Arg1+ cells in the ischemic cortex at different time points after cerebral I/R. Scale bar=100μm. The inset images on the bottom-left were further magnified images of the cells in box, which are selected from the border of infarct area (the left one) and the infarct area (the right one). (B) Quantitative analysis of the number of Arg1 positive cells in the ischemic regions exhibited in A at indicated time points. Data are mean ± SD (n = 6). ***P < 0.001 vs. Sham; ###P < 0.001 vs. I/R (two-way ANOVA with Tukey post testing). (C) Representative images of Arg1 staining in coronal brain sections 3 days after cerebral I/R at different magnifications: (a) scale bar=2000μm, (b) scale bar=200μm, (c) scale bar=100μm, (d) scale bar=50μm, demonstrate the distribution of M2-type microglia/macrophages in the coronal brain section.

Figure 6

RIPC regulated the polarization of microglia/macrophages and related protein expression after cerebral I/R. (A) Representative immunohistochemistry images of coronal brain sections of Cox2+ cells in the ischemic cortex at different time points after cerebral I/R. Scale bar=100μm. (B) Quantitative analysis of the number of Cox2 positive cells in the ischemic regions exhibited in A at indicated time points. Data are mean ± SD (n = 6). ***P < 0.001 vs. Sham; #P<0.05, ###P < 0.001 vs. I/R (two-way ANOVA with Tukey post testing). (C) Western blot analysis of Iba1, CD206 and Cox2 in the peri-infarct area of all experimental groups. Quantitative analysis of (D) Iba1 (E) CD206 and (F) Cox2 in the peri-infarct area of the experimental groups at day 1 and 7 after cerebral I/R. β-tubulin was used as a loading control; data are mean ± SD (n = 6). ***P < 0.001 vs. Sham; #P<0.05, ##P<0.01, ###P < 0.001 vs. I/R (two-way ANOVA with Tukey post testing).

Figure 7

RIPC regulated neuroinflammation in brain cortex after cerebral I/R, and RIPC treatment exerted neuroprotection possibly through TLR4 signaling pathway. ELISA shows that RIPC decreased the levels of IL-1 β (A) and TNF- α (B), but increased the level of IL-10 (C) after cerebral I/R in rats. (D) Western blot analysis of TLR4 in the peri-infarct area after cerebral I/R. (E) Quantification shows that RIPC decreased the expression of TLR4 after cerebral I/R. β -tubulin was used as a loading control; data are mean $\pm$ SD ($n = 6$). *** $P < 0.001$ vs. Sham; # $P < 0.05$, ### $P < 0.001$ vs. I/R (two-way ANOVA with Tukey post testing).