

## Very early-initiated physical rehabilitation protects against ischemic brain injury

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### 1. ABSTRACT

Recent clinical data suggest that very early initiated physical rehabilitation (VEIPR) within 24 hours after stroke may reduce morbidity. However, there is limited evidence to support the beneficial effects of VEIPR and the underlying mechanisms are yet unknown. The present study investigated the effect of VEIPR on brain damage, inflammation, and neurobehavioral outcomes following cerebral ischemia. Rats that underwent transient focal cerebral ischemia (tFCI) were randomly assigned to VEIPR or non-exercise (NE) groups. VEIPR was induced 24 hours after the insult by initiating treadmill training for a maximum of 14 days while the NE group remained sedentary in their cages during this period. The results indicated that VEIPR significantly improved recovery of functional behavior as measured by neurological score, foot fault test, and Morris water maze performance. We also demonstrated that VEIPR significantly reduced infarct volume, brain water content, BBB damage, and acute inflammatory response. In summary, our results provide novel evidence that VEIPR confers marked neuroprotection against experimental stroke by attenuating pro-inflammatory reactions, brain edema, BBB damage, and cognitive and behavioral deficits.

### 2. INTRODUCTION

Stroke is a major cause of mortality and chronic neurological disability worldwide (American Heart Association, 2009(1)). Most survivors from stroke suffer from motor disability, cognitive dysfunction, and problems in learning and behavior that reduce their ability to perform activities of daily living and thus reduce their quality of life (2, 3). In the past decades, there has been a rapidly growing understanding of the mechanisms underlying the pathophysiology of stroke, increasing novel therapeutic targets have been identified, and thousands of drugs have been tested in various animal models. Although those breakthroughs lead to abundant therapeutics and drugs which have been undergone clinical trials, to date, the therapeutic options for acute ischemic stroke remain very limited (4, 5). Recombinant tissue plasminogen activator (tPA) is currently the only agent shown to improve stroke outcome in clinical trials, but its use is limited by its narrow therapeutic window and risk of hemorrhage (6, 7). Consequently, the optimum treatment of acute ischemic stroke remains one of the major challenges in clinical medicine. It is therefore essential to discover therapeutic strategies that improve clinical outcomes.

Physical exercise after stroke is an effective approach of clinic rehabilitation, as it has been shown to reduce the rate of cognitive decline (8), enhance sensorimotor control (9), promote walking speed and capacity (10, 11), and improve life quality of stroke patients (12,13). In animal studies, physical exercise initiated subacutely and at delayed stage following cerebral ischemia reduced infarct size and ischemia-induced apoptosis of neuronal cells (14), improved motor function (15,16), and promoted learning and memory performance (17). The possible mechanisms involved upregulation of proteins such as BDNF and CREB (18), increased hippocampal dendritic spine density (19), and enhanced synaptogenesis (20, 21) and neurogenesis(22).

Despite massive beneficial evidences of physical exercise had been reported, the effect of very early initiated physical rehabilitation (VEIPR) remains controversial (23, 24). Some reports showed that exercise performed soon after cerebral ischemia produced detrimental effects on functional recovery and neurogenesis (25-28), while others suggested that VEIPR decreased tissue injury and improved functional outcome in experimental stroke rats (29,30). Recent clinical data show that VEIPR following stroke may offer beneficial effects in stroke patients (31). Indeed, VEIPR is recommended in plenty of stroke units (32), and has been included in the Clinical Guidelines for Stroke Management 2010 document sponsored by the National Stroke Foundation in Australia (33).

In view of the disparate information regarding the efficacy of VEIPR, we sought to evaluate the neuroprotective effect of VEIPR following experimental stroke. In the present study, we examined the effect of VEIPR on the sequelae of transient forebrain ischemia (tFCI). Our results support our hypothesis that VEIPR confers marked neuroprotection against focal ischemic brain injury in rats by attenuating infarct volume, suppressing reactive astrogliosis and production of proinflammatory cytokines, reducing brain edema, and blood brain barrier damage after ischemia and reperfusion.

### 3. MATERIALS AND METHODS

#### 3.1. Animal model of transient focal cerebral ischemia

Adult male Sprague-Dawley rats (250-270g, Shanghai SLAC Laboratory Animal Co. Ltd.) were used as subjects. All rats were housed under a 12h light/dark cycle with food and water available *ad libitum* throughout the study. After behavioral training, tFCI was induced by left middle cerebral artery occlusion (MCAO) as previously described (34). Briefly, rats were anesthetized with 1.5% isoflurane (Abbott, U.S.A) and mechanically ventilated via an endotracheal tube. After a midline cervical incision, the left common carotid artery was exposed and the external carotid artery was ligated distally. To occlude the origins of the MCA, a 4-0 nylon monofilament coated with a silicone tip was inserted into the internal carotid artery and advanced 1.9-2.0 cm from the bifurcation site. After 60 min, reperfusion was reestablished by withdrawal of the filament. Through the cannulated left femoral artery, physiologic variables (blood pressure, blood gases) were

monitored before, during, and after ischemia. To confirm the success of the model, changes in regional cerebral blood flow (rCBF) before, during, and after tFCI were recorded by laser Doppler flowmetry. Criteria used to determine successful cerebral ischemia included a drop in the rCBF of more than 80% during ischemia and ascension to more than 90% of the baseline rCBF after reperfusion. Rectal temperature was maintained at 37.0°C by a thermostat-controlled heating blanket. For the sham control group, all steps were included except for the insertion of the filament into the carotid artery. All procedures were performed according to the Animal Experimental Committee of Fudan University at Shanghai, China.

#### 3.2. Treadmill training

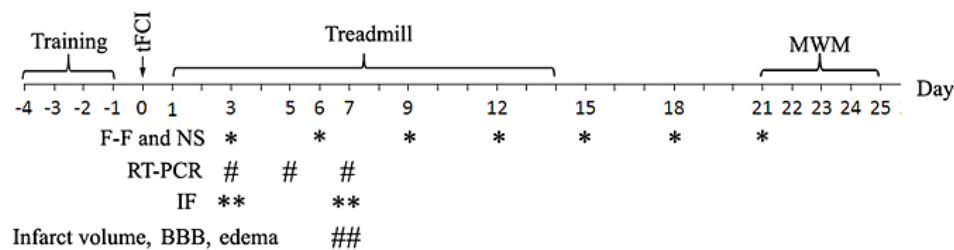
Prior to tFCI and sham surgery, all rats were habituated to the motorized treadmills at a speed of 6-9 m/min for 3 consecutive days (10 min per day). To evaluate the effect of VEIPR on behavioral recovery, rats were randomly assigned to one of the following three groups: the VEIPR group (n=10), the non-exercise (NE) group (n=10), and the sham group (n=6). Animals in the VEIPR group were induced by forced treadmill training on an electric treadmill (Litai Biotechnology Co., Ltd, China) for 14 consecutive days initiated at 24 hour post tFCI. The exercise velocity and duration was gradually increased with the following schedule: Day 1, 5 m/min for the first 10 min, 9 m/min for 10 min, and 12m/min for the last10 min; Day 2, 5 m/min for the first 5 mins, 9 m/min for 5 min, and 12 m/min for last 20 min; Day 3 (training goal) through Day 14, 12 m/min for 30 min. The slope was set at 0° for all phases of training. The rats in the NE and sham groups were placed on stationary treadmills for the same duration. All of the time points for the subsequent tests are depicted in Figure 1.

#### 3.3. Tissue section preparation

At day 3 and 7 post tFCI (Figure 1), animals were anaesthetized with chloral hydrate (360 mg/kg, i.p.) and transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Thereafter, brains were removed and transferred into a 20% sucrose solution in PBS overnight for cryoprotection. Frozen serial coronal brain sections were sliced on a cryostat (30 µm in thickness).

#### 3.4. Measurement of infarct volume

Frozen coronal brain sections (total 10 slices over a 360-µm interval with every 12th section used for analysis) from rats on day 7 post tFCI were taken for determination of infarct volume using the MAP2 staining method (35). Briefly, slices were treated with 0.3 % hydrogen peroxide to block endogenous peroxidase activity, then incubated with 10 % normal goat serum (Jackson ImmunoResearch Laboratories, U.S.A.) followed by incubated monoclonal rabbit antibodies against MAP2 (Millipore, 1:800, overnight at 4°C). The following day slices were incubated with a biotinylated goat anti-rabbit IgG secondary antibody (KPL, 1:200) for 1 hour, followed by a preformed avidin-horseradish peroxidase complex (Vectastain Elite ABC-Reagent, Vector) for 30 min. Immunostaining was developed using diaminobenzidine



**Figure 1.** Schematic illustration of the experimental design. Foot fault and treadmill training were performed prior to surgery at separate times for 4 and 3 consecutive days, respectively. On day 1 after the operation, rats in the VEIPR group were subjected to the treadmill exercise protocol described in the materials and methods section until day 14. \* Represent the foot fault test (F-F) and neurological score (NS) testing days. # represent the days when rats were sacrificed to obtain measurements of pro-inflammatory cytokines (RT-PCR). \*\* represent immunofluorescence (IF) analysis. Infarct volume, blood-brain-barrier (BBB) permeability and brain water content (edema) are indicated as ## on the figure. Evaluation of spatial learning using the Morris water maze and measuring the latency for the rat to find the submerged platform started on day 21 and continued each day until day 24 post tFCI. Spatial memory was evaluated on day 25 using the probe test where the platform was removed and the amount of time the rats spent in the correct quadrant was measured. Abbreviations: tFCI, transient focal cerebral ischemia; MWM, Morris water maze; RT-PCR, reverse transcriptase polymerase chain reaction.

(Sigma-Aldrich). All the sections were digitized with a microscope (Nikon, Japan) using the same magnification. The area of non-MAP2 staining was defined as the infarct zone. For each tissue section the area of remaining tissue (MAP2 positive staining) was traced using NIH Image software (available at: <http://rsb.info.nih.gov/nih-image/>). The percentage of infarct volume was determined according to the following equation: (MAP-2 positive area of ischemic hemisphere) / (MAP-2 positive area of nonischemic hemisphere) X 100%. The results were presented as mean  $\pm$  SE.

### 3.5. Immunofluorescence staining and image analysis

Mouse monoclonal anti-GFAP antibody (Cell Signaling Technology, U.S.A.), and rabbit anti-Iba-1 antibody (Wako, Japan) were used as the primary antibodies in this study. Brain sections processed according to section 3.3 on days 3 and 7 post tFCI were incubated with primary antibodies for 1 hour at 37°C and then at 4°C overnight, followed by incubation for 1 hour at 37°C with DyLightTM 594-conjugated goat anti-mouse and DyLightTM 488-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, U.S.A.). Sections were then counterstained with DAPI (Thermo Scientific, U.S.A.) for 2 min at room temperature followed by mounting with fluoromount-G (Southern Biotech, U.S.A.). For densitometric analysis a computerized camera based NIH Image-analysis system software (available at: <http://rsb.info.nih.gov/nih-image/>) was used as previously described (36). Briefly, areas of interest focusing in ischemic penumbra were digitized to TIFF images under the same exposure time. The images were then binarized and segmented under a consistent threshold (50%). Next, the total black pixels per image were counted. In order to minimize the differences of fluorescent intensity among immunostained sections, pixels values were calculated as ratios of injury in the ipsilateral (IL) relative to the contralateral (CL) hemisphere (IL: CL = lesion: intact hemisphere). The results used for analysis were presented as pixels of IL: CL.

### 3.6. Tissue processing and total RNA extraction

At day 3, 5, and 7 post tFCI (Figure 1), rats underwent their indicated exercise protocol were given 2 hour of rest. Thereafter they were sacrificed by decapitation under deep chloral hydrate (360 mg/kg, i.p.) anesthesia. The brain was quickly removed and the infarcted core and penumbra in hemisphere with the lesion was isolated on ice followed by immediate freezing in dry ice and stored at -80°C. Total RNA was extracted by homogenization with Trizol reagent (Applied Biosystems, USA) in accordance with the manufacturer's protocol. RNA quantity was determined by optical density measurement and prepared for cDNA synthesis.

### 3.7. Reverse transcription and semi-quantitative real-time RT-PCR

The reverse transcription was conducted with a RT reagent kit (Agilent, U.S.A.) in accordance with the manufacturer's protocol. PCR analyses were performed with gene-specific primers (Table 1), and the endogenous control was glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Real-time data were analyzed with a Mastercycler<sup>®</sup> *realplex* analysis system (Eppendorf, Hamburg, Germany). All samples were performed in triplicate. Thermal cycling condition was set according to the manufacturer's recommendations. Relative quantification of target mRNA was normalized to GAPDH expression with the comparative cycle threshold (Ct) method (37). The relative fold change of target gene expression was expressed as  $2^{-\Delta\Delta Ct}$ , where  $\Delta\Delta Ct = \Delta Ct_{\text{test animal}} - \Delta Ct_{\text{calibrator animal}}$ . Three animals in the sham group were randomly chosen as the calibrator sample. The  $\Delta Ct$  was defined as  $Ct_{\text{target}} - Ct_{\text{GAPDH}}$ .

### 3.8. Blood brain barrier permeability evaluation

Blood brain barrier permeability was determined by Evans blue (EB) extravasation at day 7 post tFCI. Briefly, EB (2% in 0.01M PBS; 5 mL/kg) was slowly administered i.v. Three hours after dye administration

**Table 1.** List of primers used in this study

Gene	Forward 5'-3'	Reverse 5'-3'
IL1-alpha	CCAAAGTTCCTGACTTGTGTTG	GAAGGTGAAGGTGGACATC
IL1-beta	CTGTCCCTGAACCTCAACTGTG	GTCCTCATCTGGAAGCTCC
IL-6	CAGGGAGATCTTGAAAATGAG	GTTGTTCTTCACAACTCC
iNOS	GGAAGTTTCTCTTCAGAGTC	CGATGGAGTCACATGCAGC
COX2	GACAGATCAGAAGCGAGGACCTG	GTAGATCATGTCTACCTGAGTG
TNF-alpha	GATCGGTCCCAACAAGGAGG	GCTGGTACCACCAGTTGGTTG
VCAM-1	GGCTCGTACACCATCCGC	CGGTTTTCGATTACACTCGT
ICAM-1	AAACGGGAGATGAATGGTACCTAC	TGCACGTCCTGGTGATACTC
GAPDH	GTGAAGGTCGGTGTGAACGG	GTTTCCCGTTGATGACCAG

intracardiac perfusion was performed with 300 ml saline to remove intravascular EB dye. The brains were quickly removed and dissected into sections of 2 mm thickness for imaging analysis. To assess the Evans blue (EB) extravasation, these sections were soaked in methanamide for 48 hours followed by centrifugation for 30 min at 14000 rpm. The absorption of the supernatant was determined at 632 nm with a spectrophotometer (Bio-Rad). The content of EB was calculated as  $\mu\text{g/g}$  of brain tissue using a standard curve.

### 3.9. Brain water content determination

At day 7 post tFCI, rats were killed by decapitation under deep chloral hydrate (360 mg/kg, i.p.) anesthesia. The brain was quickly removed and dissected along the fissure into the ischemic and non-ischemic hemispheres. Brain tissues was then weighed (wet weight), and then the brain was heated for 3 days at 100°C in a drying oven to determine the dry weight. Brain water content ipsilateral to the lesion was calculated with the following formula:  $\% \text{H}_2\text{O} = (1 - \text{dry wt/wet wt}) \times 100 \%$  (38-40).

### 3.10. Behavioral training and evaluations

#### 3.10.1. Neurological deficits

Neurologic deficits scores were performed at beginning at day 3 through day 21 post tFCI (Figure 1) as previously described (41). Each rat was scored according to a seven points behavioral rating scale: 0, no deficit; 1, failure to extend right forepaw fully; 2, decreased grip of the right forelimb when held by tail; 3, spontaneous movement in all directions, but torso turning to the right side when held by tail; 4, circling or walking to the right; 5, walks only when stimulated; 6, no spontaneous activity; and 7, dead. Animals without a deficit were excluded from the study. An observer blinded to experiment design performed neurological testing.

#### 3.10.2. Foot fault test

Measurement of coordinated locomotor movement of rat forelimb was determined using the foot fault test as previously described with some modification (42). Briefly, on a horizontal ladder with a regular arrangement of rungs (at 2 cm intervals) all rats were trained for 4 consecutive days before tFCI, and the baseline score was determined on the day before the operation. After tFCI, the foot fault test was performed every 3 days starting from day 3 until day 21 post tFCI (Figure 1). All animals were trained and tested three times per session and every session was videotaped for quantification (only consecutive steps were analyzed). The quantitative evaluation of

forelimb placement was performed using the following scoring system: Score 0, total miss, the limb completely missed the rung; Score 1, deep slip, the forelimb was placed on the rung, but slipped off and caused a fall; Score 2, slight slip, the forelimb was placed on a rung, and then slipped off but did not result in a fall and the rat continued a coordinated gait; Score 3, replacement, the forelimb was placed on a rung, but then quickly lifted and placed on another rung before it was weight bearing; Score 4, correction, the forelimb aimed for one rung, but was placed on another rung before touching the first one; Score 5, partial placement, the forelimb was placed on the rung with wrist digits; Score 6, correct placement, the forelimb was placed on the rung correctly. The scores of the three trials were averaged for analysis.

#### 3.10.3. Morris water maze task

Spatial learning and memory were assessed in a water maze task as described previously (43) with some modification. Briefly, the water maze was a black-colored circular pool filled with water and situated in a room with salient visual cues. Acquisition of spatial learning was begun at post-operative day 21 for 4 consecutive days (day 21 through day 24). Each animal performed five trials (with randomly assigned starting positions) per day to locate the platform submerged 2 cm below the water surface with an inter-trial interval of 10 s. They were allowed 60 s to locate the platform. If they failed to locate the platform within 60 s, the rat was manually guided to the platform and remained there for 10 s. The mean escape latency per day was recorded for each animal and used in the statistical analysis. One day after the final acquisition training session all rats performed a probe test with the escape platform removed. The animals were placed into the pool from the location most distal to the target quadrant (with removed platform). The percent of time spent in the target quadrant was recorded and interpreted as spatial memory (44).

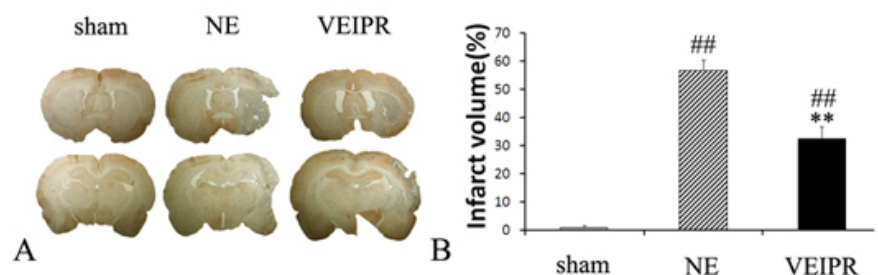
### 3.11. Statistical analysis

All data was presented as mean  $\pm$  standard error of the mean (SEM). Differences in infarction volumes, neurological deficit, foot fault, MWM, and water and Evans blue content were analyzed using ANOVA followed by post hoc Fisher's PLSD tests. Statistical significance of relative mRNA expression and immunofluorescence analysis were determined with an unpaired Student's t-test.  $P < 0.05$  was considered statistically significant.

## 4. RESULTS

### 4.1. Physiological variables

There were no significant differences between groups for all the monitored indices of physiological



**Figure 2.** VEIPR reduced infarct volume after tFCI. A, Representative images of MAP-2 stained sections on day 7 after tFCI. B, Quantitation of the infarct volume demonstrates that VEIPR treatment significantly reduced infarct volume following tFCI compared to NE-treated rats; however, the infarct volume in VEIPR-treated rats was significantly larger than sham-treated rats.  $N = 5$  sham rats and  $n = 6$  rats in both the VEIPR and NE groups). \* $p < 0.05$ , \*\* $p < 0.01$  versus the NE group; # $p < 0.05$ , ## $p < 0.01$  versus the sham group. Abbreviations: NE, non-exercise group; VEIPR, very early initiated physical rehabilitation.

variables including pH,  $paO_2$ ,  $paCO_2$ , and blood pressure before, during, or after ischemia between sham, non-exercise (NE), and VEIPR groups (data not shown). Thus, there were no physiological confounds that could contribute to the subsequent results.

#### 4.2. VEIPR reduced infarct volume

In order to provide support for our hypothesis that VEIPR is neuroprotective, we examined the infarct volume of sham, NE, and VEIPR groups at 7th day after tFCI. Compared to the NE group, VEIPR significantly reduced the infarct volume in the cortex and striatum (56.8% vs. 32.5% in NE and VEIPR groups, respectively) (Figure 2A and B). The rats in the sham group did not exhibit any ischemic damage. The data here demonstrate that VEIPR is able to mitigate brain tissue loss following tFCI.

#### 4.3. VEIPR inhibited the activation of astrocytes and microglia cells

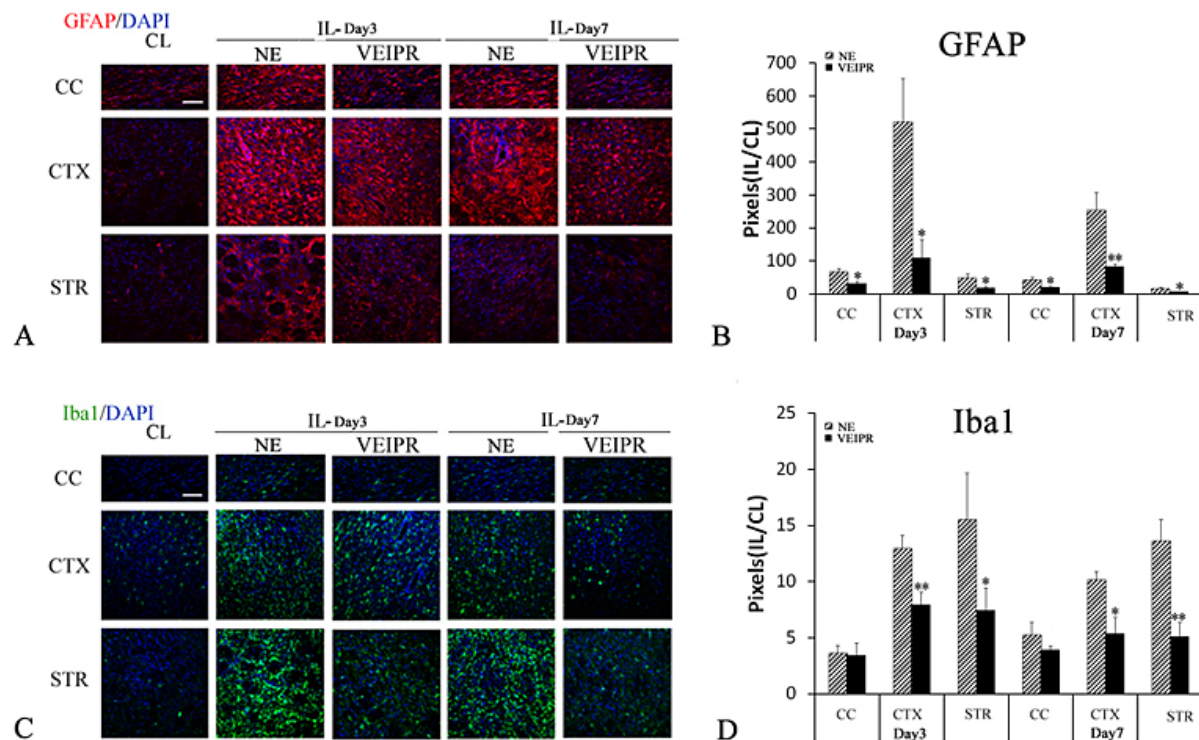
Activation of astrocytes and microglia due to tFCI have been implicated in ischemia-induced brain injury in terms of contributing to blood brain barrier (BBB) permeability, cerebral edema, cerebral perfusion, and microglia-mediated release of proinflammatory cytokines (45). To evaluate if VEIPR had an effect on astrocyte activation, immunofluorescence staining was used to probe for GFAP to assess active astrocyte and Iba1 to determine microglial activation. The results show that ramified-appearing reactive astrocytes (Figure 3A) were localized to the peri-lesional regions and infarct core, particularly in cortex and striatum in the NE-treatment group. In contrast, there is no astrocyte and microglial activation in the sham-treated rats and in the contralateral hemisphere of VEIPR- and NE- treated rats. VEIPR significantly suppressed ramified astrocytic cells in the corpus callosum, cortex, and striatum at day 3 and 7 post tFCI compared to the NE treatment group (Figure 3B). Microglia were activated and located in the infarct penumbra and exhibited amoeboid morphology after tFCI in the NE-treated group. Consistent with astrocytic cell activation, increases of activated microglia were significantly inhibited in rat brain in VEIPR-treated rats compared with the NE-treated rats in both the penumbra of cortex and striatum at day 3 and 7

post tFCI, but not in the corpus callosum (Figure 3C and D). Our results indicate that astrocyte and microglial activation induced by tFCI could be suppressed by VEIPR.

#### 4.4. VEIPR suppressed proinflammatory cytokine and cell adhesion molecule expression after tFCI

Given the finding that VEIPR suppressed microglial activation, and activated microglia is known to release proinflammatory cytokine which result in a neuroinflammatory response to ischemia (46), we next examined the effect of VEIPR on proinflammatory cytokine expression after tFCI. To determine the effect of VEIPR on tFCI-induced inflammation, we measured the expression of several inflammatory factors at day 3, 5, and 7 post exercises training using real time PCR. At all test points, the expression levels of proinflammatory cytokines IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, iNOS, COX2, and TNF- $\alpha$  were significantly increased by tFCI. On day 3 VEIPR markedly decreased the expression of all the aforementioned cytokines compared to the NE group (Figure 4A). Thereafter the expression of proinflammatory cytokines markedly declined. At day 5 post tFCI, the levels of IL-1 $\beta$ , COX2, and TNF- $\alpha$  in the VEIPR group remained significantly lower than NE group (Figure 4B); however, the differences seen at day 3 with the other proinflammatory cytokines were absent. At day 7 post tFCI, cytokine expression dropped further, but it remained higher than sham-operated rats. At this time after tFCI, only the expression of TNF- $\alpha$  was significantly reduced by VEIPR compared to the NE group (Figure 4C).

Cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), are upregulated by tFCI, mediate leukocyte migration into the CNS and contribute to ischemia-induced neuroinflammation. Notably, blocking ICAM-1 either via neutralizing antibodies or genetic knock out improves neurological outcome and is neuroprotection following stroke (for review see ref. 47). We therefore wanted to determine if the mechanism underlying the neuroprotection provided by VEIPR could also include decreasing the expression of cell adhesion molecules. Following tFCI there was an increase in the mRNA expression of ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) in the NE group compared to sham-



**Figure 3.** VEIPR inhibited the activation of astrocytes and microglia cells. A, Representative immunofluorescence photomicrographs of GFAP (red) in the infarcted hemisphere in the corpus callosum, cerebral cortex, and striatum at day 3 and 7 post tFCI. Dapi stain of nuclei is depicted in blue. B, Quantification of the densitometric analysis of GFAP immunostaining demonstrates that VEIPR significantly decreased reactive astrogliosis in all brain regions examined at both day 3 and 7 post tFCI. C, Representative immunofluorescence photomicrographs of Iba1 (green) in the infarcted hemisphere in the corpus callosum, cerebral cortex, and striatum at day 3 and 7 after tFCI, to detect activated microglia. D, Quantification of the densitometric analysis of Iba1 immunostaining shows that VEIPR decreased microglial activation in the cortex and striatum at both 3 and 7 days compared to NE-treated rats. Of note, there was no difference between groups in the corpus callosum at either time tested. The statistical results were expressed as positive pixel ratios in the ipsilateral compared to the contralateral hemisphere (IL: CL). N = 5 for each group. \* $p < 0.05$ , \*\* $p < 0.01$  versus the NE group. Scale bar = 50  $\mu$ m. Abbreviations: CC, corpus callosum, CL, contralateral hemisphere; CTX, cortex, IL, ipsilateral hemisphere; NE, non-exercise group; STR, striatum; tFCI, transient focal cerebral ischemia; VEIPR, very early initiated physical rehabilitation.

treated rats on days 3, 5, and 7 (Figure 4A-C). Similar to the reduction in proinflammatory cytokine mRNA in the VEIPR-treated rats, there was also a decrease in the expression of ICAM-1 and VCAM-1 mRNA on days 3 and 5 following tFCI (Figure 4A and B). The effect of diminished ICAM-1 in the VEIPR group compared to the NE group was absent by day 7, while VCAM-1 expression remained significantly decreased compared to the NE group (Figure 4C). Together these data suggest that animals treated with VEIPR had significantly reduced proinflammatory cytokines and leukocyte-assisting molecule mRNA expression after tFCI compared to NE animals.

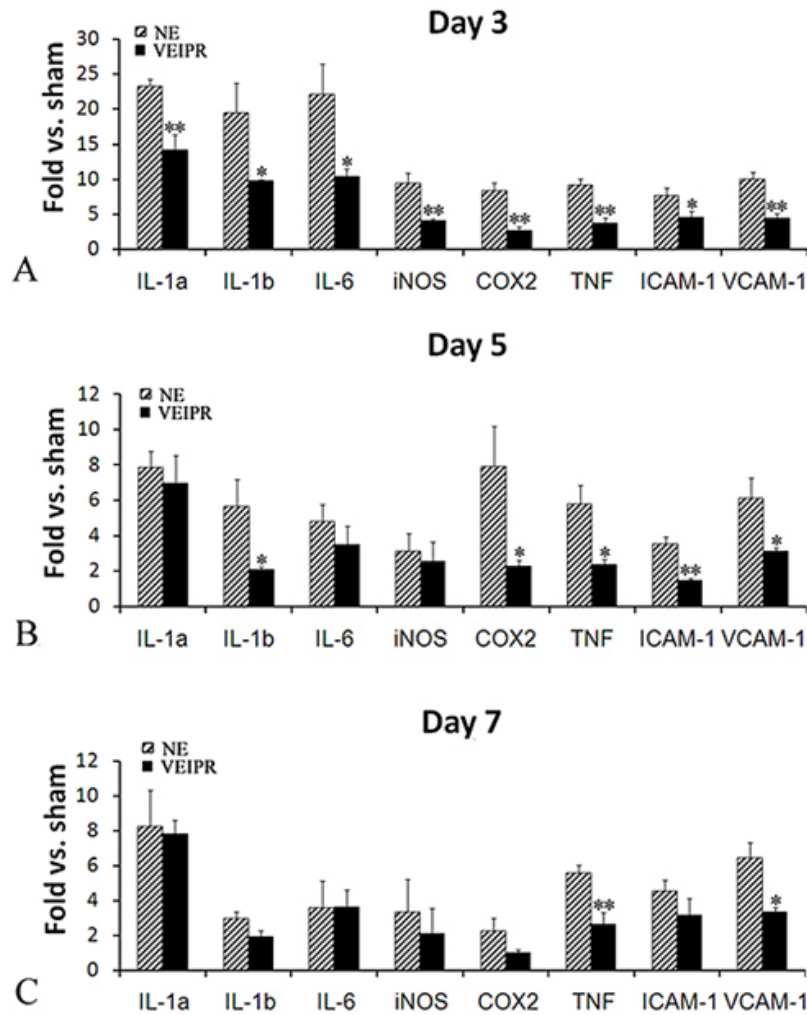
#### 4.5. VEIPR improves BBB integrity and decreases brain edema after tFCI

Impaired blood brain barrier integrity and brain edema are both induced by tFCI and have a detrimental impact following stroke. Interestingly, both BBB integrity and water ion homeostasis are intimately linked to astrocyte

function following stroke (48). Since VEIPR decreased both reactive astrogliosis and gliosis, we next attempted to determine if it also would mitigate the detrimental effects of tFCI on BBB permeability and brain edema.

Investigation of BBB integrity using Evans blue (EB) extravasations at day 7 post tFCI demonstrated an impaired BBB in the NE group compared to sham-treated controls (Figure 5A and B). Rats treated with VEIPR showed diminished BBB compromise in the ipsilateral cortex and striatum compared to the NE group; however, the amount of EB extravasation was elevated compared to sham-treated rats (Figure 5A and B).

Brain edema was evaluated by examining the water content of the brain ipsilateral to the lesion. The results show that brain water content was significantly elevated in the NE group compared to sham-treated rats at day 7 after tFCI (Figure 5C). Treatment with VEIPR significantly alleviated brain edema compared to the NE



**Figure 4.** VEIPR attenuated proinflammatory cytokine and cell adhesion molecule mRNA expression after tFCI. A, Pro-inflammatory factor (IL1-alpha, IL1-beta, IL-6, COX2, TNF-alpha) and cell adhesion molecule (ICAM-1, VCAM-1) mRNA expression at day 3 after tFCI, measured by PCR and normalized to GAPDH. Data are expressed as fold change in expression compared to sham group expression. B and C, Proinflammatory factor and cell adhesion molecule expression at day 5 and 7 after tFCI. Note that expression of all proinflammatory cytokine and cell adhesion molecule mRNA expression was attenuated by VEIPR. This effect gradually dissipated until day 7 where only TNF-alpha and VCAM-1 expression were significantly decreased in VEIPR-treated compared to NE-treated rats. N = 5 rats per group. \* $p < 0.05$ , \*\* $p < 0.01$  versus the NE group. Abbreviations: COX2, cyclooxygenase 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICAM-1, intercellular adhesion molecule-1; IL-1a, -1b, and -6, interleukin-1a, -1b, and -6; iNOS, inducible nitric oxide synthase; NE, non-exercise group; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; VCAM-1, vascular cellular adhesion molecule-1; VEIPR, very early initiated physical rehabilitation.

group, and brought the brain water content down to sham-treated levels (Figure 5C). Together these results show that VEIPR blocks brain edema and improves BBB integrity after tFCI.

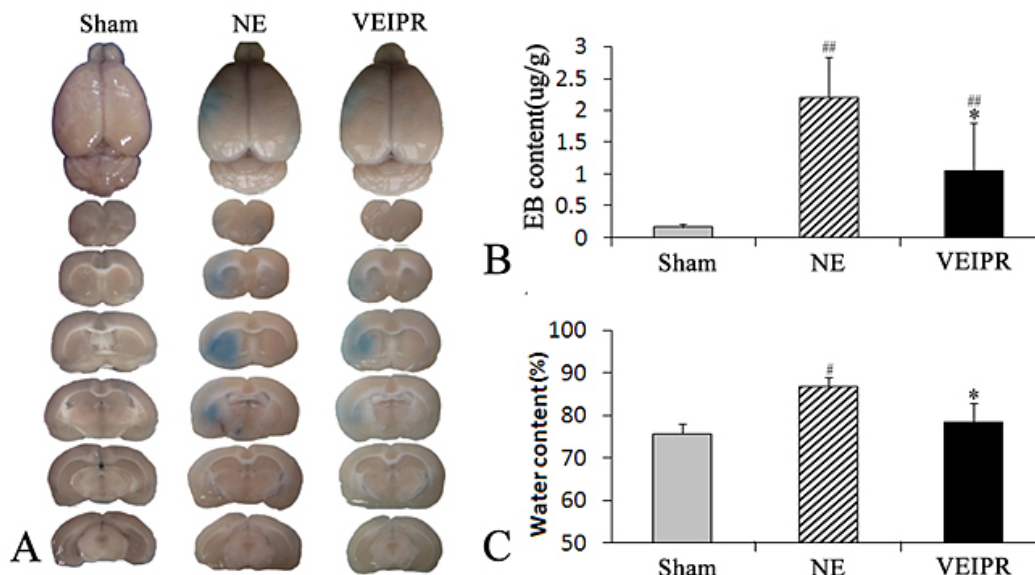
#### 4.6. VEIPR promotes functional recovery

When evaluating a potential clinical therapeutic perhaps the most important parameter to measure is its effect on overall motor and neurological deficit improvement after the ischemic insult. Here we

evaluate the effect of VEIPR on neurological function, motor function, and spatial learning and memory.

##### 4.6.1. Neurological deficits

We examined the effects of VEIPR using the 7 points neurological scale described in the materials and methods. All sham-treated rats demonstrated no neurological deficit at any of the time point measured. The NE group exhibited moderate neurological dysfunction with the first measurement at day 3 with gradual improvement until the last measured time at day 21.



**Figure 5.** VEIPR improved BBB integrity and decreased water content after tFCI. **A.** Representative images of EB-stained brain sections (2 mm thick) at day 7 day after tFCI. Note the presence of Evans blue dye in the NE-treated rats compared to the sham- and VEIPR-treated rats. **B.** Quantification of Evans blue dye content ( $\mu\text{g dye/g brain tissue}$ ). VEIPR significantly reduced the amount of EB in the ipsilateral hemisphere compared to NE-treated rats. Permeability of the BBB in VEIPR-treated rats remained compromised compared to sham-treated rats. **C.** The percentage of water content in the brains of sham-, NE- and VEIPR-treated rats was evaluated by calculating the wet weight divided by the dry weight of the ipsilateral hemisphere at day 7 post tFCI. Treatment with VEIPR significantly reduced the brain water content seen in NE-treated rats to sham-treated levels.  $N = 5$  for the sham group and  $n = 6$  for the VEIPR and NE groups.  $*p < 0.05$  versus NE group;  $^{##}p < 0.05$  versus sham group. Abbreviations: EB, Evans blue; NE, non-exercise group; VEIPR, very early initiated physical rehabilitation.

Treatment with VEIPR significantly improved neurological scores at day 6, 9, and 12 compared to NE-treated rats (Figure 6A). From day 15 to 21 the neurological score was comparable between NE-treated and VEIPR-treated rats. Of note, neither the NE- nor the VEIPR-treated rats' neurological scores returned to baseline by the last day of testing. However, the results indicated that VEIPR mitigates early neurological dysfunction as measured by this test.

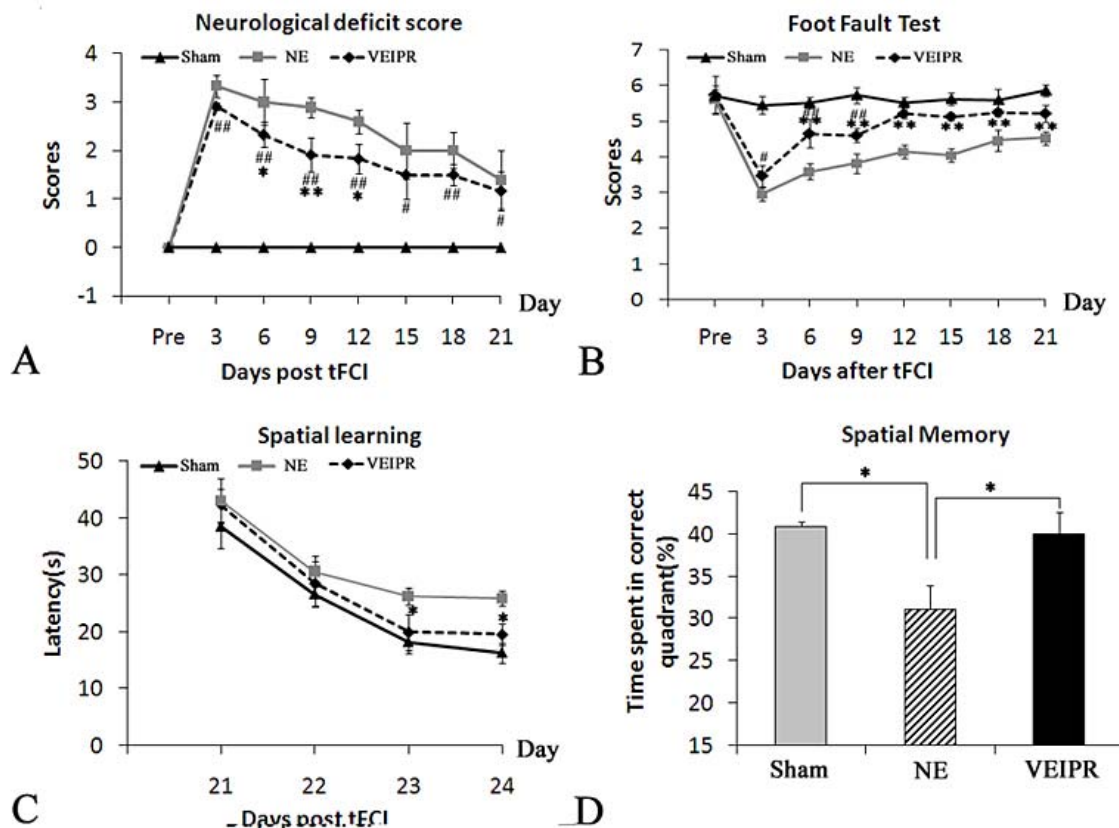
#### 4.6.2. Motor function

One of the prominent symptoms induced by occlusion of the MCA in both humans and rodents is hemiplegia of the upper extremity contralateral to the lesion (34). Motor function was therefore evaluated using the foot-fault test to determine recovery of forelimb placing, stepping, and inter-step coordination in rats subjected to tFCI. The results revealed that the NE-treated rats showed marked right forelimb impairment compared to sham-treated controls at all times tested after tFCI. Although rats that received VEIPR exhibited impaired performance on day 3 following tFCI, they demonstrated improved performance compared to NE rats at all subsequent test sessions (Figure 6B). Notably, rats treated with VEIPR recovered motor function to the level of sham-treated rats by day 12, and this recovery to baseline persisted until the last day tested, day 21. These results indicate that VEIPR robustly improves the recovery of motor function after tFCI compared to rats who did not exercise after the ischemic insult.

#### 4.6.3 Spatial learning and memory

Cerebral ischemia is known to potentially result in deficits in spatial learning and memory depending on the location and severity of the insult (44). Using the Morris water maze, we examined whether VEIPR improved learning and memory performance. The initial assessment of spatial learning using the metric of escape latency to find the submerged platform revealed that NE-treated rats showed a significant increase in latency to find the platform on day 3 and 4 of training (day 23 and 24 post tFCI, respectively) compared to both the sham-treated and VEIPR-treated group (Figure 6C). There was no statistical difference between the sham-treated and the VEIPR-treated groups in spatial learning.

To evaluate spatial memory, we performed a probe trial on the day after the last escape latency trial, day 25. During this trial, the platform was removed and the time the rat spent in the quadrant of the former platform was measured (correct quadrant). The results indicate that NE-treated rats spent significantly less time in the correct quadrant compared to both the sham-treated and VEIPR-treated groups (Figure 6D). Again, as with the previous measure of escape latency, there was no difference between sham and VEIPR rats. Collectively, the data from the Morris water maze experiments indicated that spatial learning and memory deficits caused by tFCI are abolished by VEIPR.



**Figure 6.** Improved neurobehavioral recovery with VEIPR treatment. A, Treatment with VEIPR significantly improved the neurological deficit score compared to NE-treated rats on days 6, 9, and 12 post tFCI. Note that neurological deficit scores showed significant impairment for both tFCI-treated groups compared to sham-treated rats. B, The foot fault test was evaluated on the days indicated in the figure. Treatment with VEIPR improved performance on the foot fault test from day 6 through 21 compared to NE-treated rats. Note that by day 12 the performance of the VEIPR group was at the level of the sham-treated rats. C, Evaluation of spatial learning using the Morris water maze latency test following tFCI demonstrates that NE-treated rats show impaired learning as indicated by increased latency to find the submerged platform at days 23 and 24 post tFCI. There was no significant difference between VEIPR and sham groups in terms of spatial learning. D, Determination of spatial memory using the probe test on day 25 post tFCI. The submerged platform used in the previous test was removed and the percent of time spent in the quadrant where the platform was previously located was measured. The NE-treated rats showed significant impairment in this task, while VEIPR-treated rats performed at the level of sham-treated rats. N = 10 for the VEIPR group; N = 10 for the NE group; N = 6 for the sham group. \* $p < 0.05$ , \*\* $p < 0.01$  versus the NE group; # $p < 0.05$ , ## $p < 0.01$  versus the sham group. Abbreviations: NE, non-exercise group; tFCI, transient focal ischemia; VEIPR, very early initiated physical rehabilitation.

## 5. DISCUSSION

Ample evidence suggests that rehabilitative exercise initiated after stroke improves neurological recovery from cerebral ischemia in preclinical and clinical studies (16, 29, 49-51). An important caveat is that rehabilitative exercises are necessarily delayed until the patient is medically stable enough to undergo this type of treatment. Recent clinical trials have attempted to accelerate the time until the initiation of physical rehabilitation to within 48 h after stroke, and the data suggest that very early initiated physical rehabilitation (VEIPR) may offer beneficial effects in patients (31, 32, 24,52). Currently there is limited evidence demonstrating a neuroprotective effect of VEIPR in experimental stroke,

and the underlying mechanism for VEIPR-afforded neuroprotection is unknown. In the present study, we confirm that physical rehabilitation via treadmill exercise exhibits a neuroprotective effect through reducing the infarct volume (14), and we also confirmed that treadmill exercise improves motor performance (15,16) and learning and memory (17) compared to NE controls. In addition we elucidate a possible novel mechanism underlying VEIPR-induced neuroprotection by showing that VEIPR decreases reactive astrogliosis, decreases activated microglia with subsequent decreases in proinflammatory cytokine expression and cell adhesion molecules, and it decreases BBB permeability and brain edema in a rat model of transient focal cerebral ischemia (tFCI).

The rationale to conduct rehabilitation as early as possible is seemingly evident not only because of its potential benefits on recovery after tFCI, but also because excessive bed rest in hospitalized patients results in obvious negative effects on multiple organ systems such as the nervous, cardiovascular, renal, gastrointestinal, and musculoskeletal systems (53,54). In order for VEIPR to become a well-accepted strategy, however, abundant evidence at both the basic science and clinical levels is necessary. In recent preclinical studies, the effect of intense exercise initiated soon after tFCI demonstrated that VEIPR reduced infarct volume and promoted neurological recovery (23, 30). Our findings are consistent with these results in our rat model as we also evidenced decreased infarct volume, improved motor-coordination, and improved spatial learning and memory. Thus, VEIPR was not only safe and feasible, but also promoted functional recovery.

The role of non-neuronal brain cells following ischemic injury has been increasingly delineated. They are known to increase neurotransmitter uptake, moderate calcium and water homeostasis, produce and release neurotrophic factors, contribute to neuroinflammation, and are a major component of blood brain barrier formation (45). Despite the increased knowledge of astrocyte function, their role following ischemia remains equivocal (55) as activation of astrocytes has been shown to have neuronal regenerative capacity and astrocytic activation also contributes to degenerative neuroinflammation. The discrepancy in these findings likely involves the severity and type of injury, the animal model used, and any neuroprotective therapy administered.

In the current study our intervention of VEIPR decreased reactive astrocytosis and microglial activation as measured by the expression of GFAP- and Iba-1-positive cells, and this was associated with decreased infarct volume and improved neurobehavioral recovery. Therefore, in our model, a VEIPR-induced decrease in reactive astrocytosis is beneficial. One possible mechanism underlying the reduction in reactive astrocytosis is that VEIPR reduces oxidative stress caused by ischemia. It has been shown that not only does exercise not cause oxidative stress, it may increase brain's capacity for decreasing reactive oxygen species via upregulation of antioxidant enzymes such as superoxide dismutase (56). Exercise-mediated decreases oxidative stress is an interesting hypothesis that should be explored further to delineate the role of oxidative stress and reactive astrocytosis.

There is increasing evidence that acute inflammation, which starts within hours and persist for days, contributes to neuronal death following stroke (57, 58). After ischemic brain injury, the inflammatory response is orchestrated by proinflammatory cytokines such as IL-1, IL-6, iNOS, COX2, and TNF- $\alpha$ . While the role of some cytokines is controversial (59, 60), these acute proinflammatory cytokines have been shown to markedly exacerbate neurodegenerative cascades in potentially viable tissue through production of reactive oxygen species (ROS) and inhibiting mitochondrial respiration (61-63). Inhibition

of inflammatory cytokines by antagonists, neutralizing antibodies, or gene knockouts abolished the detrimental effects and provided neuroprotection (64, 65). Recent studies indicate that pre-ischemic physical exercise may ameliorate inflammatory injury during ischemia and reperfusion (66, 67). However, the effect of physical exercise initiated after stroke on inflammatory response remains unknown. In this study, we initiated physical exercise soon after tFCI and determined the expression of proinflammatory cytokines in the acute phase. Our data show that VEIPR reduced the expression of inflammatory cytokines in the acute phase of tFCI. In addition to reducing the expression of proinflammatory cytokines directly, VEIPR significantly reduced the expression of cell adhesion molecules (ICAM and VCAM). These are two critical factors mediating the adhesion and infiltration of blood-derived leukocytes in inflammatory injury after ischemia.

Although production of pro-inflammatory cytokines has been shown to exhibit some beneficial effects in recovery from stroke (68,69), activated resident glial cells in the acute phase after ischemia also play a important role in the development of secondary brain injury through secretion of deleterious molecules, such as glutamate, ROS, and nitric oxide (70-73). Suppression of activated microglia with anti-inflammatory drugs markedly improves functional recovery after tFCI (74, 75). The results of present study show that suppression of activated glia is the possible cause for reducing the inflammatory response, and may have ultimately contributed to the neurobehavioral recovery demonstrated here.

Astrocytes are important components of the blood brain barrier, and they also moderate ion and water homeostasis (45). Therefore, changes in astrocyte integrity and function after ischemia can contribute to BBB disruption and cerebral edema. Indeed, tFCI caused increased brain water content and increased BBB permeability in the NE group. It is possible that treatment with VEIPR decreased these deleterious effects via blocking reactive astrocytosis and neuroinflammation. However, future studies will need to confirm this association.

Although our data indicated that VEIPR conferred neuroprotection against ischemic brain injury, it is important to note that different exercise protocols can lead to an entirely different outcome (76, 77). In some animal studies, excessive use of the affected limb and high intensity exercise exacerbate ischemia injury (25, 28, 78-80). Therefore, the implementation of VEIPR in clinical practice should be cautiously introduced and based on the individual patient's condition.

In conclusion, our results provide novel evidence that very early initiated physical rehabilitation confers marked neuroprotection and provides improved neurobehavioral recovery from focal ischemic brain injury in rats. The neuroprotection by VEIPR is associated with the attenuation of reactive astrocytosis, pro-inflammatory cytokines, brain edema, and blood brain barrier damage

after ischemia and reperfusion. Further work is required to elucidate the precise underlying mechanisms in order to provide effective clinical implementation.

## 6. ACKNOWLEDGEMENTS

The present study was supported by Chinese National Natural Science Foundation (30772300).

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**Abbreviations:** VEIPR: very early initiated physical rehabilitation; tFCI: transient focal cerebral ischemia; MCAO: middle cerebral artery occlusion; BBB: blood-brain-barrier; EB: evans blue; TNF: tumor necrosis factor; IL: interleukin; iNOS: inducible nitric oxide synthase; COX2: cyclooxygenase-2; ROS: reactive oxygen species; ICAM-1: intercellular adhesion molecule-1; VCAM-1: vascular cell adhesion molecule 1

**Key Words:** VEIPR, *in vivo* ischemia, Neuroinflammation, Functional recovery, Astrocytosis

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