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Ipsilateral *versus* bilateral limb-training in promoting the proliferation and differentiation of endogenous neural stem cells following cerebral infarction in rats☆

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Abstract

We investigated the effects of ipsilateral *versus* bilateral limb-training on promotion of endogenous neural stem cells in the peripheral infarct zone and the corresponding cerebral region in the unaffected hemisphere of rats with cerebral infarction. Middle cerebral artery occlusion was induced in Wistar rats. The rat forelimb on the unaffected side was either wrapped up with tape to force the use of the paretic forelimb in rats or not braked to allow bilateral forelimbs to participate in training. Daily training consisted of mesh drum training, balance beam training, and stick rolling training for a total of 40 minutes, once per day. Control rats received no training. At 14 days after functional training, rats receiving bilateral limb-training exhibited milder neurological impairment than that in the ipsilateral limb-training group or the control group. The number of nestin/glial fibrillary acidic protein-positive and nestin/microtubule-associated protein 2-positive cells in the peripheral infarct zone and in the corresponding cerebral region in the unaffected hemisphere was significantly higher in rats receiving bilateral limb-training than in rats receiving ipsilateral limb-training. These data suggest that bilateral limb-training can promote the proliferation and differentiation of endogenous neural stem cells in the bilateral hemispheres after cerebral infarction and accelerate the recovery of neurologic function. In addition, bilateral limb-training produces better therapeutic effects than ipsilateral limb-training.

Key Words

bilateral rehabilitation training; affected limb; bilateral limbs; peripheral infarct zone; unaffected hemisphere; middle cerebral artery occlusion; brain; neural stem cells; proliferation; differentiation; plasticity; neural regeneration

Research Highlights

(1) According to the principle of constraint-induced movement therapy, restricting forelimb movement on the unaffected side and forcing forelimb use on the affected side of rats with cerebral infarction provides specific intensive repeated behavior training and promotes the recovery of forelimb motor function.
(2) Bilateral limb-training can promote the proliferation and differentiation of endogenous neural stem cells in the bilateral hemispheres after cerebral infarction, reduce cerebral infarct volume, and accelerate the recovery of neurologic function. Bilateral limb-training produces greater therapeutic effects than does ipsilateral limb-training.

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Abbreviations

GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2; MCAO, middle cerebral artery occlusion

INTRODUCTION

Following cerebral infarction, activation of the unaffected hemisphere is involved in the recovery of neurological functions[1-6]. Further, there is evidence that right-handed rhythmic movement can significantly activate the contralateral premotor cortex in patients with left hemispheric brain infarction, as determined by functional MRI[7]. Takeuchi *et al* [8] also reported that transcranial magnetic stimulation to the contralateral cerebral hemisphere improved the speed and accuracy of the affected handwriting after cerebral infarction in patients with right-sided hemiparesis; the efficacy lasted for about 1 week. However, it remains unclear whether bilateral limb-training can strengthen the activation of the affected hemisphere and improve the neurological function score. Compared with affected limb-training, bilateral limb functional training also leads to activation of the motor area in the ipsilateral and contralateral hemispheres of cerebral infarction patients and improves the behavioral score[9-10]. Further, Matsuda *et al* [11] reported that bilateral limb function training increased behavioral scores of rats and reduced neurological impairment and infarct volume. Bilateral functional training activates neural network in bilateral cerebral hemispheres and it also activates the connection between healthy and affected hemispheres, thereby improving the neurologic function of patients[7, 12].

Nestin, the sixth class of intermediate filament protein, is used as a marker of immature neural stem cells[13-18]. Glial fibrillary acidic protein (GFAP) is an astrocytic marker protein[19-22]. Microtubule-associated protein 2 (MAP2) is a neuronal cytoskeleton protein and an early sensitive indicator of neuronal ischemic injury, while its high expression represents neuronal structure recovery and reconstruction[23-25]. In this study, we investigated the effects of bilateral limb-training *versus* ipsilateral limb-training on neurological severity scores, brain infarct volume, and nestin/GFAP and nestin/MAP2 double immunofluorescent staining in the peripheral infarct zone and the corresponding regions in the contralateral hemisphere. We used these data to develop a new training pattern for clinical rehabilitation.

RESULTS

Quantitative analysis of animals

Forty-eight rats were initially included in the study, and were randomly divided into ipsilateral limb-training, bilateral limb-training, and untreated groups. Each group was further divided into a 3-day group and a 14-day group, with eight rats per group. All 48 rats were included in the final analysis.

Effects of ipsilateral and bilateral functional limb-training on neurological severity score of middle cerebral artery occlusion (MCAO) rats

At postoperative days 1 and 3, there was no significant difference in neurological severity score between the ipsilateral limb-training, bilateral limb-training, and untreated groups ($P > 0.05$). At postoperative day 14, the neurological severity score in the bilateral limb-training was significantly higher than that in the ipsilateral limb-training group and the untreated group ($P < 0.05$, $P < 0.01$; Table 1).

Table 1 Effects of different physical training regimes on neurological severity score in rats

Group	Days after MCAO		
	1	3	14
Untreated	9.22±1.34	9.07±0.78	12.70±0.67
Ipsilateral limb-training	10.01±0.54	10.13±1.24	13.58±1.18[a]
Bilateral limb-training	9.87±0.82	10.75±1.37	16.25±0.25[b]

MCAO: Middle cerebral artery occlusion; [a]$P < 0.05$, [b]$P < 0.01$, *vs.* untreated group. Data are expressed as mean ± SD ($n = 8$ rats per group per time point). One-way analysis of variance was used to compare differences between the groups.

Effects of ipsilateral and bilateral limb-training on cerebral infarct volume of MCAO rats

There was no significant difference in brain infarct volume between the three groups at postoperative days 3 and 14 ($P > 0.05$; Figure 1).

Effects of ipsilateral and bilateral limb-training on nestin/GFAP and nestin/MAP2 expression in bilateral hemispheres of MCAO rats

At postoperative day 3, the number of nestin/GFAP- (Figure 2) and nestin/MAP2-positive (Figure 3) cells in

the peripheral infarct region was significantly increased in the ipsilateral limb-training group and the bilateral limb-training group compared to the untreated group ($P < 0.05$); this effect lasted until 14 days after MCAO ($P < 0.05$). No significant difference was found in the number of nestin/GFAP- and nestin/MAP2-positive cells between the ipsilateral limb-training group and the bilateral limb-training group ($P > 0.05$). At postoperative day 3, the number of nestin/GFAP- (Figure 4) and nestin/MAP-positive (Figure 5) cells in the unaffected hemisphere was significantly higher in the bilateral limb-training group than in the ipsilateral limb-training group and the untreated group; this effect lasted until 14 days after MCAO ($P < 0.05$ or $P < 0.01$).

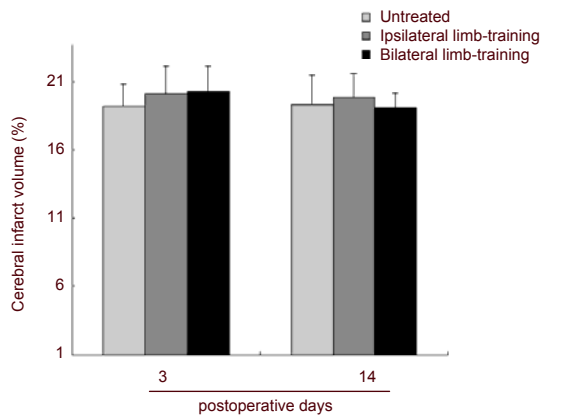

Figure 1 Effects of different physical training regimes on cerebral infarct volume in rats.

Data are expressed as mean ± SD (n = 8 rats per group per time point). One-way analysis of variance was used to compare differences between the groups. Cerebral infarct volume was calculated by cerebral infarct volume/whole-brain volume × 100%.

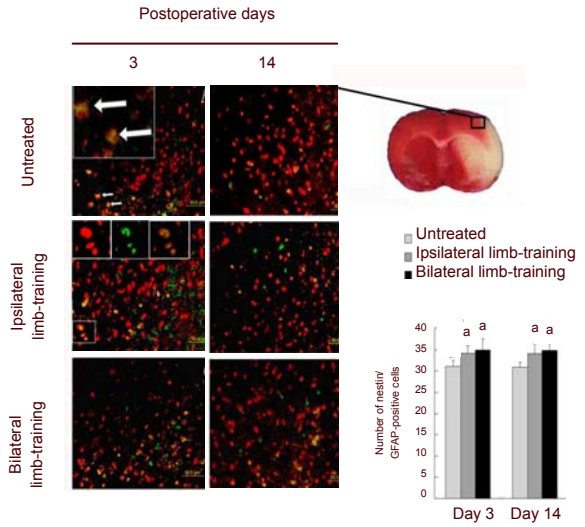

Figure 2 Nestin/glial fibrillary acidic protein (GFAP)-positive cells in the peripheral infarct zone in middle cerebral artery occlusion rats following ipsilateral or bilateral limb-training.

In the gross specimen, the white area represents the cerebral infarction area. Nestin-positive cells are stained green. GFAP-positive cells are stained red. In the cytoplasm, the nestin/GFAP-positive cells were mainly yellow.

The number of nestin/GFAP-positive cells in the ipsilateral limb-training group and bilateral limb-training group was significantly higher than that in the untreated group at postoperative day 3, and this effect lasted until postoperative day 14 ($^aP < 0.05$, *vs.* untreated group).

There was no significant difference between the ipsilateral limb-training group and the bilateral limb-training group ($P > 0.05$).

Data are expressed as mean ± SD (n = 8 rats per group per time point). One-way analysis of variance was used to compare differences between the groups.

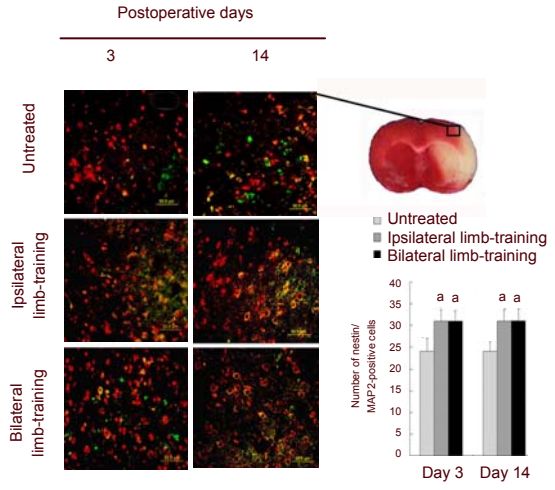

Figure 3 Nestin/microtubule-associated protein-2 (MAP2)-positive cells in the peripheral infarct zone in middle cerebral artery occlusion rats following ipsilateral or bilateral limb-training.

In the gross specimen, the white area represents the cerebral infarction area. Nestin-positive cells are stained green. MAP2-positive cells are stained red. In the cytoplasm, the nestin/MAP2-positive cells were mainly yellow.

$^aP < 0.05$, *vs.* untreated group. Data are expressed as mean ± SD (n = 8 rats per group per time point). One-way analysis of variance was used to compare differences between the groups.

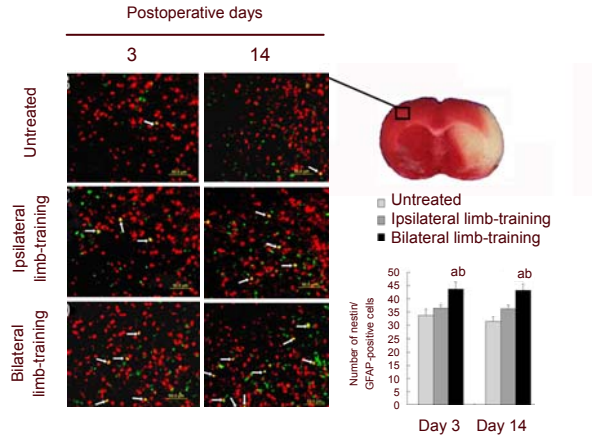

Figure 4 Nestin/glial fibrillary acidic protein (GFAP) in the unaffected hemisphere in middle cerebral artery occlusion rats following ipsilateral or bilateral limb-training.

In the gross specimen, the white area represents the cerebral infarction area. Nestin-positive cells are stained green. GFAP-positive cells are stained red. In the cytoplasm the nestin/GFAP-positive cells were mainly yellow (arrows).

[a]*P* < 0.05, *vs*. ipsilateral limb-training; [b]*P* < 0.01, *vs*. untreated group. Data are expressed as mean ± SD (*n* = 8 rats per group per time point). One-way analysis of variance was used to compare differences between the groups.

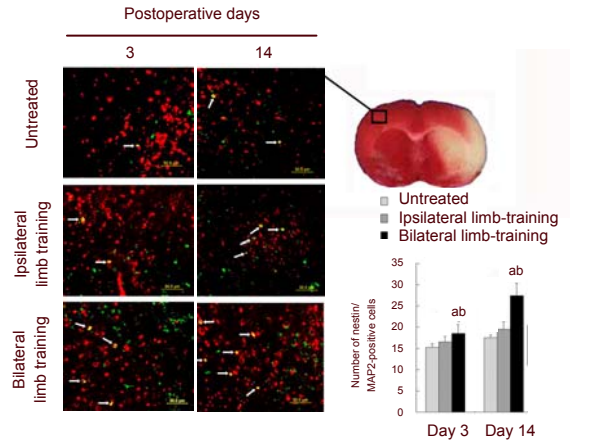

Figure 5 Nestin/microtubule-associated protein-2 (MAP2)-positive cells in the unaffected hemisphere in middle cerebral artery occlusion rats following ipsilateral or bilateral limb-training.

In the gross specimen, the white area represents the cerebral infarction area. Nestin-positive cells are stained green. MAP2-positive cells are stained red. In the cytoplasm the Nestin/MAP2-positive cells were mainly yellow (arrows).

[a]*P* < 0.05, *vs*. ipsilateral limb-training; [b]*P* < 0.01, *vs*. untreated group. Data are expressed as mean ± SD (*n* = 8 rats per group per time point). One-way analysis of variance was used to compare differences between the groups.

DISCUSSION

Symmetric bilateral movements activate similar neural network distribution in the bilateral hemispheres. The specific activated regions include the motor zone, sensorimotor cortex, motor cortex, premotor area, superior parietal lobule, and the cerebellum[26]. In the present study, we used a self-made roller, balance beam, rotating rod, and screening to train the limb grasping, balance, and creeping ability of the rats.

We found no significant difference in neurological severity score between the three groups at postoperative day 3, which may be due to cellular apoptosis and neurological impairment following acute cerebral ischemia and the relatively short period of rehabilitation training (20 days). At postoperative day 14, the neurological severity score was significantly higher in the bilateral limb-training group than in the ipsilateral limb-training group and the untreated group, suggesting that bilateral functional training is more effective at producing functional recovery.

TTC staining indicated no significant difference in cerebral infarct volume between the three groups at postoperative days 3 and 14, suggesting that brain tissue was in the edema stage after cerebral infarction. Thus, although neural cells were repaired, a longer period is required for recovery. Cerebral infarct volume also remained unchanged, suggesting that the unaffected nerve tissue was involved in the functional reorganization after cerebral infarct, possibly in the peripheral infarct zone and in the unaffected hemisphere.

In the peripheral infarct zone, the number of nestin/GFAP-positive cells was significantly increased in the ipsilateral and bilateral limb-training groups compared to the untreated group at postoperative day 3, which persisted until postoperative day 14. This recovery was associated with thickening of the glial barrier that was composed of nestin-positive astrocytes. After stroke, proliferating astrocytes are critical for restoration of infarct structure[27]. Our data suggest that functional training can further stimulate astrocyte proliferation around the infarct area, thereby accelerating lesion repair. In this study, at postoperative days 3 and 14, the

number of nestin/MAP2-positive cells in the ipsilateral and bilateral limb-training groups was significantly higher than that in the untreated group. An increase in the number of neuron-like cells is indicative of neural precursor cell differentiation. There was no significant difference in the number of nestin/GFAP- and nestin/MAP2-positive cells between the ipsilateral and bilateral limb-training groups, suggesting that bilateral limb-training had no significant impact on newborn cells around the infarct area. Thus, limb-training on the unaffected side must influence cerebral regions other than the peripheral infarct zone. In the unaffected hemisphere, the number of nestin/GFAP- and nestin/MAP2-positive cells was significantly increased in the bilateral limb-training group compared to the ipsilateral limb-training group and the untreated group at postoperative day 3. These data suggest that contralateral limb-training can promote the proliferation and differentiation of neural stem cells in the unaffected hemisphere, which lasts up to 14 days after surgery.

A large number of neural stem cells differentiated into glial cells and neurons in the unaffected hemisphere, suggesting that a series of plastic responses of the brain occurred in the corresponding cerebral region of the unaffected hemisphere, which influenced neural structure and functional reconstruction after stroke. Thus, bilateral limb-training activates cerebral plasticity and functional reconstruction in the unaffected hemisphere and improves the functional recovery after cerebral infarction in rats.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

This study was performed at the Laboratory Animal Center, General Hospital of Shenyang Military Region, Research Institute of Shenyang Brain Hospital, China between April 2010 and April 2011.

Materials

Healthy male Wistar rats of clean grade, weighing 250–300 g, were provided by the Laboratory Animal Center, China Medical University (license No. SCXK (Liao) 2003-0009). All procedures were performed according to the *Guidance Suggestions for the Care and Use of Laboratory Animals* [28] issued by the Ministry of Science and Technology of China.

Methods

Preparation of MCAO rat models

Following anesthesia by intraperitoneal injection of 1% sodium pentobarbital (30–40 mg/kg), a 1.5 cm long longitudinal incision was made in the midline of the ventral cervical skin. The right common carotid artery, right internal carotid artery, and right external carotid artery were identified and separated from the vagus nerve. A silk suture was inserted into the stump of the external carotid artery and entered into the anterior cerebral artery *via* the bifurcation of the carotid artery until some resistance was felt. The depth of insertion was approximately 17–19 mm. The common carotid artery was ligated with sutures at the bifurcation, followed by skin suture. Thereafter, rats were returned to their cages and raised normally[29]. After recovery of consciousness, MCAO induction was considered successful when rats exhibited the following signs: flexion of the left forelimb when lifting the tail, ipsilateral Hornor syndrome, left circling while climbing, and falling to the left while standing[30]. At 24 hours after MCAO, rat neurologic function was evaluated with Zea Longa's scale[31]: a score of 0, no neurologic deficit; 1, mild focal neurologic deficit, failure to extend left forepaw fully; 2, moderate focal neurologic deficits, circling to the left; 3, severe focal neurological deficits, falling to the left; 4, did not walk spontaneously and had a depressed level of consciousness; 5, died. In each group, rats were fixed by mesh equipment. Rats that scored 2–3 were included for further experimentation.

Rehabilitation training

The daily training consisted of mesh drum training, balance beam training, and stick rolling training[32], for a total of 40 minutes, once per day. The training commenced at 24 hours after surgery and was terminated when the rats died. Ipsilateral training group: the forelimb on the unaffected side was wrapped with the tape, forcing the use of the paretic forelimb. Bilateral limb-training group: the bilateral limbs were braked. Untreated group: no rehabilitation training was performed.

Assessment of neurologic function

At 1, 3, and 14 days after MCAO, neurological function in the rats was assessed including spontaneous activity, symmetry in the movement of four limbs, forepaw outstretching, climbing, body proprioception and response to vibrissae touch[33]. The examiners had no knowledge of the procedure that each rat had received. The score given to each rat at the completion of the evaluation was the summation of all six individual test scores. The minimum neurological score was 3 and the maximum was 18.

TTC staining for cerebral infarct volume

Rats were anesthetized under 4% chloral hydrate (100 mg/kg) and decapitated at 3 and 14 days after MCAO. Brains were rapidly removed and cut into 2 mm-thick coronal sections. Slices were stained with 1% TTC (Sigma, St. Louis, MO, USA) in 0.1 M PBS (PH 7.4) for 30 minutes at room temperature, and fixed in 10% buffered formalin overnight. The staining was photographed with an image acquirement system HPIAS-1000 (Beijing Kong Hai, China). Infarct volumes were blindly quantified using the formula: $V = t (A1 + \ldots An) - (A1 + An)\, t/2$, where t = slice thickness and A = infarct area.

Sampling and sections

At postoperative days 3 and 14, rats were rapidly perfused with heparinized saline, fixed with 4% paraformaldehyde (w/v), and rinsed with 0.1 M PBS (pH 7.4) for 10 minutes at 4°C. After rats were euthanized by cervical dislocation, continuous coronary blocks were cut from the frontal pole at 1.5 mm intervals, which were then fixed with 4% paraformaldehyde (w/v) and rinsed with 0.1 M PBS (pH 7.4) at 4°C for 8 hours, followed by conventional dehydration, paraffin embedding, and cutting into 4 μm thick sections.

Immunofluorescent double staining of nestin/MAP2 and nestin/GFAP

Brain slices were incubated with rabbit anti-rat nestin (1:200; Wuhan Boster, China) at 37°C for 2 hours and goat anti-rabbit IgG/Dylight 488 (1:100; Jackson ImmunoResearch Inc., West Grove, PA, USA) at 4°C overnignt, then with rabbit anti-rat GFAP or rabbit anti-rat MAP2 (1:200; Wuhan Boster) at 37°C for 2 hours followed by 4°C overnight. On the second day, slices were rinsed with PBS four times for 5 minutes each, and incubated with goat anti-rabbit IgG/DyLight 594 (1:100; Jackson ImmunoResearch Inc.) at 37°C in the dark for 1 hour, rinsed with PBS four times for 5 minutes each, and then mounted with glycerol and immediately photographed under laser confocal microscope (Leica, Wetzlar, Germany) for fluorescence observation. Negative controls for immunohistochemistry were performed by incubation with 1% bovine serum protein rather than antibodies.

Double immunofluorescent staining for nestin/GFAP double-labeled cells showed that nestin-positive cells exhibited a green cytoplasm, while the GFAP-positive cells were mainly red; in the cytoplasm, nestin/GFAP-positive cells were labeled yellow. For nestin/MAP2 double-labeled cells, the nestin-positive cells exhibited a green cytoplasm, with uniform size and irregular shape, while the MAP2-positive cells were red; in the cytoplasm, nestin/MAP2-positive cells were mainly labeled yellow. Three discontinuous sections randomly selected from each rat were used to calculate the number of positive cells in seven fields of view at high magnification (× 400).

Statistical analysis

Data were expressed as mean ± SD, and all values were tested by the homogeneity of variance and the normality test. Statistical processing was performed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Intergroup comparisons were performed using one-way analysis of variance. $P < 0.05$ was considered statistically significant.

Author contributions: Xiyao Yang and Feng Zhu were responsible for providing and integrating experimental data. Xiaomei Zhang and Zhuo Gao contributed to conception and design of the study, analysis and interpretation of the data, and writing of the manuscript. Yunpeng Cao guided and supervised the study. All authors approved the final version of the paper.
Conflicts of interest: None declared.
Ethical approval: This study received ethical permission from the Animal Ethics Committee of China Medical University, China.
Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application disputations.

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