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Roles of Isometric Contraction Training in Promoting Neuroprotection and Angiogenesis After Stroke in Adult Rats

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Summary

100 rats were randomly divided into a sham-operated group and middle cerebral artery occlusion (MCAO) modeling groups. The sham group after surgery was observed for 14 days. After MCAO, some rats received isometric contraction training (ICT) which was as follows: an atraumatic tourniquet was placed around left or right hind limb to achieve hind limb ischemia for 5 min, followed by 5 min of reperfusion, 4 cycles for one time, once a day, and five days per week. The MCAO modeling groups included the following four groups: i) a group only received MCAO, and was observed for seven days (MCAO-7d), ii) a group only received MCAO, and was observed for 14 days (MCAO-14d), iii) a group, after MCAO, received ICT for seven days (ICT-7d), and iv) a group, after MCAO, received ICT for 14 days (ICT-14d). Brain infarct area, behavioral outcomes, the number of neurons, apoptosis, cerebral edema and cerebral water content were assessed, respectively. The mRNA expression of vascular endothelial growth factor (VEGF) was assayed with RT-PCR, and protein expression of VEGF was quantified with western blot. compared with MCAO controls, cerebral infarction, neurological deficits and neuronal apoptosis were reduced significantly in the ICT groups, while the number of neurons was increased. Moreover, the mRNA expression of VEGF and protein expression of VEGF were enhanced after 1 and 2 weeks of ICT. ICT may promote angiogenesis and neuroprotection after ischemic stroke and this new remodeling method provide a novel strategy for rehabilitation of stroke patients.

Key words

Isometric contraction training • Middle cerebral artery occlusion (MCAO) • Neuroprotection • Angiogenesis • Stroke • Vascular endothelial growth factor (VEGF)

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Introduction

Ischemic stroke is one of the main causes of physical disability, which brings a heavy burden to individuals and society [1]. So far, tissue plasminogen activator (tPA) is the only drug approved by the US Food and Drug Administration to treat ischemic stroke, while the administration of tPA is limited by its narrow therapeutic time window of 3 to 4.5 h from ischemic stroke onset, and a large amount of patients do not receive timely tPA. Of the 795,000 cases with stroke that occur every year in Europe, about a quarter are recurrent cases [2]. The ways of preventing stroke recurrence include the control of risk factors, antithrombotic or antiplatelet therapies, and other interventions for atherosclerotic disease [3]. Although great progress has been made in vascular reconstruction technology and drug therapy for stroke in recent years, there is still a lack of safe, effective and non-invasive strategies for preventing stroke events and reducing the disability rate of stroke patients.

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In recent decades, with the advancement of medical technology, more and more stroke patients survived the initial injury, but most patients suffer from neurological dysfunctions such as motor, learning, memory, and cognitive dysfunction, which significantly reduce the quality of daily life [4]. The sudden decrease of focal blood flow is the main cause of the occurrence of stroke [5]. The recovery of physical functions can significantly strengthen the independent ability of stroke survivors and improve their quality of life, and many studies have shown that exercise training can improve motor function after cerebral infarction, promote functional recovery, and exert neuroprotective effects [6-9].

Clinical studies have demonstrated that patients with a history of angina pectoris have a smaller average infarct size and a lower mortality rate when myocardial infarction occurs [10]. Similarly, studies reveal that in subsequent stroke events, patients with transient ischemic attack (TIA) tend to have better recovery than non-TIA patients, suggesting that TIA might have a neuroprotective effect [11], and ischemic tolerance might play a role in TIA patients [12].

Isometric contraction is a muscle contraction in which the length remains constant but the tension changes, and due to the increased muscle tension, the resistance of blood vessels passing through the muscle increases, which can lead to different degrees of blood flow blockage, thereby increasing ventricular pressure [13,14]. We know that isometric contraction training (ICT) is an intervention based on the controlled application of ischemic tolerance [15], can create a series of noninvasive, reversible and controllable ischemic events in the normal skeletal muscles far away from the original ischemic site (i.e. the heart or brain) [16], and lead to local ischemia of normal limb contractile muscles, thus increasing ventricular pressure. It has been reported that blood flow in muscles generally includes 40-50 % of the maximum voluntary contraction (MCV) [17]. Therefore, that isometric handgrip exercises obtained more than 50 % MVC can be defined as physical ischemia training (PIT), and PIT is a reversible ischemia of normal skeletal muscle by tourniquet or isometric contractions.

Clinical studies have shown the therapeutic potential of ICT in restoring coronary blood flow through central response induced by peripheral biological effects [18-20]. Lin *et al.* [15] found that isometric grip exercise promoted the recruitment and growth of distal collateral circulation in patients with coronary heart disease. In addition, Lin *et al.* also found that isometric exercise resulted in increased coronary collateral blood flow during acute vascular occlusion [18], and PIT induced by ICT promoted the formation of collateral circulation of distal ischemic myocardium in patients with CAD [15]. Gao *et al.* investigated the patients with coronary heart disease complicated with heart failure, and found that PIT can improve the level of vascular endothelial growth factor (VEGF) in peripheral blood and quality of life [21].

ICT is a type of remote ischemic post-conditioning relying on a training effect. ICT repeatedly induces temporary ischemia in distant skeletal muscles, so that vascular endothelial growth factor (VEGF) is supposed to be released in the stimulated area [21], and VEGF circulates to the distal area to promote angiogenesis and eventually form a “biological bypass” [22]. The angiogenetic effect of ICT has been confirmed in several previous studies featuring animal models of myocardial infarction [23,24].

The angiogenetic effects of ICT may also play a role in neuroprotection after ischemic stroke. In addition, ICT may have the effect of directly enhancing the expression of VEGF, thereby further stimulating the formation of new neuronal cells [25]. There are currently limited clinical data on the roles of ICT in cerebral ischemia [24]. Hahn *et al.* [26] showed that remote per-conditioning by transient limb ischemia is simple and clinically relevant, and has a strong neuroprotective effect in the model of local cerebral ischemia reperfusion injury. Zhen *et al.* [27] found in a randomized controlled trial (RCT) of 20 patients that ICT could effectively increase the expression of VEGF and the recruitment of EPCs, and promote the formation of collateral circulation. As a cytokine, VEGF binds to and activates VEGFR on the cell membrane. The signal can be transmitted to the PI3K/AKT pathway, and the pathway and downstream factors were activated [28]. In terms of neuroprotection, the PI3K/AKT pathway can prevent nerve injury *via* nerve growth factor (NGF)/Tropomyosin receptor kinase A (TrkA) signaling [29]. PI3K/Akt pathway is an important signaling pathway that promotes neuron survival [30,31].

In the present study, we used a rat model of transient middle cerebral artery occlusion (MCAO) to test the hypothesis that ICT might have angiogenetic and neuroprotective effects after a stroke. Specifically, we hypothesized that ICT-induced angiogenesis might reduce the occurrence of cerebral edema and the infarct size, and ICT might reduce cell apoptosis and promote the formation of new nervous cells.

Materials and Methods

Statement of Ethics

All experimental procedures were approved by the Key Laboratory of Nerve Regeneration of Nantong University (Nantong, China) and were carried out in accordance with the institutional animal care guidelines of Nantong University. This study was approved by the Ethics Committee of Jiangsu Province, China, with approval No. S20141103-402.

Study design

A randomized controlled animal study was performed. The randomization was achieved with computer generated random numbers. 100 Sprague-Dawley male rats weighing 350-400 g were randomly divided into a sham-operated group and MCAO modeling groups. The sham group after surgery was observed for 14 days. The MCAO modeling groups included the following four groups: i) a group only received MCAO, and was observed for seven days (MCAO-7d), ii) a group only received MCAO, and was observed for 14 days (MCAO-14d), iii) a group, after MCAO, received ICT for seven days (ICT-7d), and iv) a group, after MCAO, received ICT for 14 days (ICT-14d). In each group, animals were randomly assigned to six different outcomes for evaluation, and each animal only was used to evaluate one of those outcomes. All procedures and measurements were performed by an investigator who was blind to the experimental groups. The concealed animal codes were revealed only after the completion of behavioral and histological analysis.

Chemicals and reagents

2,3,5-Triphenyltetrazolium chloride (TTC), propidiumiodide (PI), cresyl violet acetate, and hemoglobin assay kit were purchased from Sigma (St. Louis, MO, USA). A terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay kit was purchased from Promega (Madison, WI, USA). Mammalian protein extraction reagent (M-PER), tissue protein extraction reagent (T-PER), membrane protein extraction reagent (Mem-PER), and rabbit polyclonal antibody against vascular endothelial growth factor (VEGF) were purchased from Abcam (Cambridge, Mass, UK). Mammalian protein extraction reagent (M-PER), tissue protein extraction reagent (T-PER), membrane protein extraction reagent (Mem-PER), and Coomassie plus Bradford assay kit were purchased from Thermo Scientific Pierce (Rockford, IL).

MACA model construction and sham-operated processing

Focal cerebral ischemia was induced by the transient MCAO on the right [32,33]. Surgeries were performed by CM (physician) and SY (biologist). Both had been trained by experienced experimentalists from the Jiangsu Key Laboratory of Neuroregeneration, Nantong University, China where the surgeries were performed. Rats lay supine after anesthesia by intraperitoneal injection of sodium pentobarbital (25 mg/kg). A heating pad was used to maintain their body temperature at 37±0.5 °C during the surgical procedure. The right common carotid artery (CCA), internal carotid artery (ICA), and external carotid artery (ECA) of each rat were surgically exposed via a neck incision. The occipital artery and the superior thyroid artery were cauterized and cut. ECA was permanently ligated using 6-0 silk thread as rostrally as possible. CCA was transiently ligated as caudally as possible and ICA was transiently ligated as rostrally as possible using microvascular clips to expose enough working space. Microscissors was used to cut the ECA near the permanent ligature. 6-0 silk thread was used to ligate around the filament insertion site in the ECA, and the ligature was kept tight enough to prevent bleeding, but loose enough to allow the filament to advance. A filament (4-0 nylon suture with rounded tip) was inserted into the CCA from the insertion site, and gently entered the ICA when the microvascular clip on the ICA was removed. The origin of middle cerebral artery (MCA) lied 18-20 mm from the CCA bifurcation in rats. When the insertion was close to 18 mm (marked on the filament), please pay attention to the feeling of your hand. Once a slight resistance was felt, it meant the filament reached the MCA region. Then, we tightened the ligature around the filament and started to record the time of ischemia. After 90-min MCAO, the transient ligature around the filament was loosened, the nylon filament was withdrawn when the microvascular clips were added on the ICA and CCA again, then we permanently tighten the ligature around the insertion site to prevent bleeding, and the microvascular clips were removed again to allow reperfusion. Followingly, the area was moistened with sterile saline, lidocaine was applied as a topical analgesic, and the wound was closed with sutures. After the rat was awake, if signs of unstable standing, paralyzed left limb was and circling were observed, and the tail suspension test suggested the rat only swung to one side, it indicated that the MCAO model was successfully established. The rats in the sham group underwent the same surgical procedures except for the insertion of the filament. After the surgery, all rats were put back into their cages where they had access to food and water.

Experimental interventions

ICT started 24 h after MCAO. Rats were fixed by the conventional laboratory fixator. An atraumatic tourniquet was placed around the left or right hind-limb to achieve hind limb ischemia for 5 min, followed by 5 min of reperfusion, which were conducted a total of 4 cycles for one time, once a day, and five days per week [26]. Circulatory arrest in hind limbs was confirmed by vascular Doppler ultrasound [34].

Rats were kept under controlled environmental conditions with an ambient temperature of 22±1 °C, a relative humidity of 65 % and a light/dark cycle of 12 h (h), and they had free access to food and water during the whole experimental period. All efforts were made to reduce the number of animals used in our study and avoid unnecessary sufferings.

Outcome assessment

Animal euthanasia

The rats were anesthetized with an overdose of sodium pentobarbital (50 mg/kg), and then were sacrificed by transcardial perfusion.

Assessment of neurobehavioral deficits

Modified neurological severity score (mNSS) [35] was assessed at day 7 and 14 after MCAO. The mNSS is a comprehensive score of motor, sensory, reflex and balance tests, ranging from 0 to 18 (normal score 0; maximal deficit score 18), and can be classified into three levels: 13-18: severe impairment; 7-12; moderate impairment; and 1-6; mild impairment [35]

Edema

The examination for vasogenic edema was performed [36]. The brains of the euthanized rats were took. After removing the pons and olfactory bulb, each brain was weighed to obtain the wet weight. After the brain was dried at 60 °C for 72 h, the dry weight was obtained. Brain water content percent was calculated using the following equation: water content (%) = [(wet weight - dry weight)/wet weight]×100 %.

Infarct area

The measurement method of the infarct area has been described in many studies [37]. We took out the brains of the euthanized rats, and cut them serially into six 2-mm sections from the frontal pole using a rat brain matrix (Sunny Instruments, Beijing, China). The sections were stained with 2 % solution of 2,3,5-Triphenyl tetrazolium chloride (TTC) at 37 °C for 30 min, and then fixed with 4 % formaldehyde buffer solution for 1 h at room temperature. The area unstained by TTC was considered as the infarct area. The infarction area was calculated by the percentage of the unstained areas in the total contralateral hemisphere. A computerized image analysis system (Leica Imaging System Ltd., Cambridge, UK) was used to analyze the percentage of TTC-stained tissue with an average of more than 6 slices.

Neuron Nissl staining

Brains were taken out from the skull, post-fixed in buffered 4 % paraformaldehyde, dehydrated in a graded sucrose series, and cut into coronal sections (20-µm thick) from the anterior commissure to the hippocampus on a cryostat microtome. A total of 40-50 sections were installed and frozen at -40 °C. The frozen sections were stained with conventional Nissl staining (0.1 % cresyl violet solution) at 37 °C for 30 min, dehydrated, and installed in dibutyl phthalate in xylene. Two technicians independently calculated the number of neurons per high-power field.

Apoptosis

TUNEL assay was used for assessing apoptosis in the frozen brain sections [38]. The sections were fixed with 4 % methanol-free formaldehyde. The brain slices were stored in an equilibration buffer and then covered with DNA strand breaks labeled with fluorescein-12-dUTP. Recombinant TdT was added. Finally, the following steps were carried out in a dark room. Brain sections were incubated with 2×SSC (300 mM sodium chloride and 30 mM sodium citrate, pH 7.4) at room temperature for 15 min, and then stained with 1 µg/ml propidium iodide (PI). Apoptotic cells were positioned as bright green cells on a red background under a scanning laser confocal microscope (Leica, Germany). Data were expressed as the ratio of apoptotic cells to total cells.

VEGF mRNA expression

In order to detect VEGF gene expression, brains were taken after euthanasia. Tissue samples were collected from two cortical regions of interest (ipsilateral and contralateral to the MCAO) and stored at -80 °C until further processing.

The frozen rat brain tissue was took, and quickly placed into a centrifuge tube. The brain tissue was weighed, and Trizol was added in proportion to fully mix the brain tissue. The brain tissue homogenate was separated, chloroform was added to each tube for shaking and standing, and finally centrifugation was performed to dissolve the total RNA of brain tissue. Reverse transcription buffer and reverse transcriptase were added, respectively, and then they were placed in a 37 °C water bath for reverse transcription to generate cDNA The primers used for polymerase chain reaction (PCR) were Sense 5'-TGCACCCACGACAGAAGGGGA-3', Antisense 5'-TCACCGCCTTGGCTTGTCACAT-3' for VEGF, and Sense 5'-GAGAGGGAAATCGTGCGT-3', Antisense 5'-GGAGGAAGAGGATGCGG-3' for β-actin. Real time (RT)-PCR was performed on an ABI StepOne PCR in a 64-well plate using a final volume of 10 μl and the following cycle conditions: 95 °C for 10 min, and then 45 cycles of 15 s at 95 °C and 1 min at 60 °C. The specificity of each target amplicon was assessed by dissociation curve analysis, and the results of quantification were given according to the formula $2^{-\Delta\Delta Ct}$, using β-actin as the internal standard. RT-PCR for each sample was performed in triplicate.

VEGF protein

Western blot was used to detect the expression level of VEGF protein. The protein samples were extracted from the brain tissue of the ipsilateral/contralateral hemisphere. The protein was quantified with a Coomassie plus Bradford assay kit. For each sample, an equal amount of protein was separated in 12 % SDS-polyacrylamidegels, and subsequently blotted onto PVDF membranes. The membranes were incubated with VEGF antibody (1:1000) and β-actin (1:1000) at 4 °C overnight, washed with 0.01 % TBST three times, and then injected with purified donkey anti-rabbit IgG (1:5000) at room temperature for 2 h. The images were scanned with Odyssey infrared imaging system (LI-COR, USA), and the results were analyzed using PDQuest 7.2.0 software (Bio-Rad, USA). Integrated density was quantified by background subtraction and normalization to the β-actin signal. The results were expressed as mean standard deviation (SD) of (VEGF protein from ipsilateral/VEGF protein from contralateral hemisphere) * 100.

Statistical analysis

Data analysis was performed with SPSS 16.0 (SPSS, Chicago, IL, USA) and Stata 14 (Stata Corporation, Texas, USA). Mean differences between the groups were compared by one-way analysis of variance (ANOVA) and Bonferroni corrected pairwise *post hoc* tests in case of a significant overall F-test. Mortality across groups was examined with logistic regression followed by Bonferroni corrected pairwise comparisons in case of a significant overall Wald-test. $p<0.05$ was considered statistically significant.

Results

Isometric contraction training reducing mortality

The mortality of the sham-operated, MCAO-7d, MCAO-14d, ICT-7d and ICT-14d groups was 0, 45.0 %, 45.0 %, 30.0 %, and 20.0 %, respectively. As shown in Figure 1A, compared with that in the MCAO-14d group, the mortality in the ICT-14d group was significantly reduced (F=4.27, $p<0.05$), and however, there was no significant difference in mortality between the ICT-7d group and the ICT-14d group ($p>0.05$).

Isometric contraction training reducing edema

As shown in Figure 1B, ICT reduced stroke-induced edema. The mean water content percentage of the sham-operated, MCAO-7d, MCAO-14d, ICT-7d and ICT-14d groups was 74.3 %, 88.4 %, 87.99 %, 79.8 %, and 72.6 %, respectively. Compared with that in the sham group, brain water content in the MCAO-7d and MCAO-14d groups both significantly increased (F=5.93, $p<0.01$). Compared with that in the MCAO-7d group, brain water content in the ICT-7d group decreased significantly (F=7.29, $p<0.05$). Compared with that in the MCAO-14d group, brain water content in the ICT-14d group decreased significantly (F=10.73, $p<0.05$).

Isometric contraction training reducing neurobehavioral deficits

The modified neurological severity scores measured on the 7th and 14th days after MCAO were shown in Figure 1C. The mean mNSS of the sham-operated, MCAO-7d, MCAO-14d, ICT-7d and ICT-14d groups was 0, 4.7, 4.1, 3.1, and 1.7, respectively. Compared with that in the MCAO-7d group, the mNSS in the ICT-7d group significantly decreased (F=7.33, $p<0.05$). Compared with that in the MCAO-14d group, the mNSS in the ICT-14d group significantly decreased (F=9.94, $p<0.05$). All rats in the sham-operated group performed well without neurobehavioral deficits.

Isometric contraction training reducing brain infarct area

Figure 2A showed the cerebral infarction area evaluated by triphenyl tetrazolium chloride (TTC) staining on the 7th and 14th day after MCAO. Normal brain tissue was dark red with staining, while the infarct area was pale gray without staining. No infarction was observed in the sham-operated group. The infarct area in the ICT groups reduced significantly (Fig. 2B). The mean infarct area percentage of the sham-operated, MCAO-7d, MCAO-14d, ICT-7d and ICT-14d groups was 0, 39.64 %, 31.09 % 30.21 % and 18.39 %, respectively. Compared with that in the MCAO-7d group, the infarct area in the ICT-7d group decreased significantly (F=4.13, $p<0.05$). Compared with that in the MCAO-14d group, the infarct area in the ICT-14d group decreased significantly (F=10.53, $p<0.01$). In addition, the brain infarct area of the ICT-14d group was significantly smaller than that in the ICT-7d group (F=6.27, $p<0.05$).

The neuroprotective effect of isometric contraction training assessed by Nissl staining

As shown in the Figure 3 of the ipsilateral brain cortex, neurons in the sham-operated group had a normal shape with Nissl bodies deeply stained, and showed an integrative and granular-like configuration, while neurons in the MCAO-7d and MCAO-14d groups were apparently hypertrophic with Nissl bodies lightly stained. Neurons in the MCAO-7d and MCAO-14d groups were sparsely distributed, and the number of visible Nissl granules reduced markedly. These findings indicated that compared with the sham-operated group, transient cerebral ischemia resulted in a significant increase in the cortical necrosis in the MCAO-7d and MCAO-14d groups. Compared with that in the MCAO-7d group, the number of neurons per visual field in the ICT-7d group increased significantly (F=1.71, $p<0.05$). Compared with that in the MCAO-14d group, the number of neurons per visual field in the ICT-14d group increased significantly (F=5.48, $p<0.05$). In addition, compared with that in the sham-operated group, the number of neurons per visual field in the ICT-14d group was significantly smaller (F=0.73, $p<0.05$). These findings indicated that ICT reduced MCAO-induced neuronal injury in the cortex.

Isometric contraction training inhibiting MCAO-induced neuronal apoptosis in the rat brain

As shown in the Figure 4, in the sham-operated group, TUNEL-positive cells were hardly observed throughout the brain. The mean TUNEL-positive cells percentage of the sham-operated, MCAO-7d, MCAO-14d, ICT-7d and ICT-14d groups was 0, 43 %, 37 % 30 % and 17 %, respectively. Compared with the MCAO-7d group, the number of TUNEL-positive cells of the cortex in the ICT-7d group was significantly lower ($p<0.01$). Compared with the MCAO-14d group, the number of TUNEL-positive cells of the cortex in the ICT-14d group was significantly lower (F=2.96, $p<0.01$). In addition, compared with the ICT-7d group, the number of TUNEL-positive cells of the cortex in the ICT-14d group was significantly lower (F=5.43, $p<0.05$). These findings suggested that longer ICT could improve the recovery of brain function.

Effects of isometric contraction training on the expression of VEGF in rats with cerebral ischemia

The expression level of VEGF protein in the ipsilateral and contralateral hemisphere on the 7th and 14th days after MCAO was evaluated by western blot (Fig. 5A, 5B). Compared with that in the MCAO-7d group, VEGF protein expression in the ICT-7d group increased significantly (F=7.48, $p<0.05$). Compared with that in the MCAO-14d group, VEGF protein expression in the ICT-14d group increased significantly (F=10.79, $p<0.05$). As shown in the Figure 5C, the level of VEGF mRNA expression increased to varying degrees after ICT, and was the highest in the ICT-14d group.

Discussion

This study demonstrated that ICT reduced the infarct area, attenuated stroke-induced edema, inhibited neuronal apoptosis and improved neuroprotective recovery by activating the endogenous neuroprotective program. The possible mechanism behind these findings is that ICT promotes the expression of VEGF in the infarct area by inducing reversible ischemia in distal skeletal muscle, and VEGF in turn promotes angiogenesis and reduces nerve injury, both of which contribute to neuroprotection.

The cardioprotective effect of ICT has previously been evaluated in experimental [25,39] and clinical [40-43] studies. It was also demonstrated that the beneficial effects of ICT on the ventricular myocardium were not species specific [44]. The mechanism of ICT is

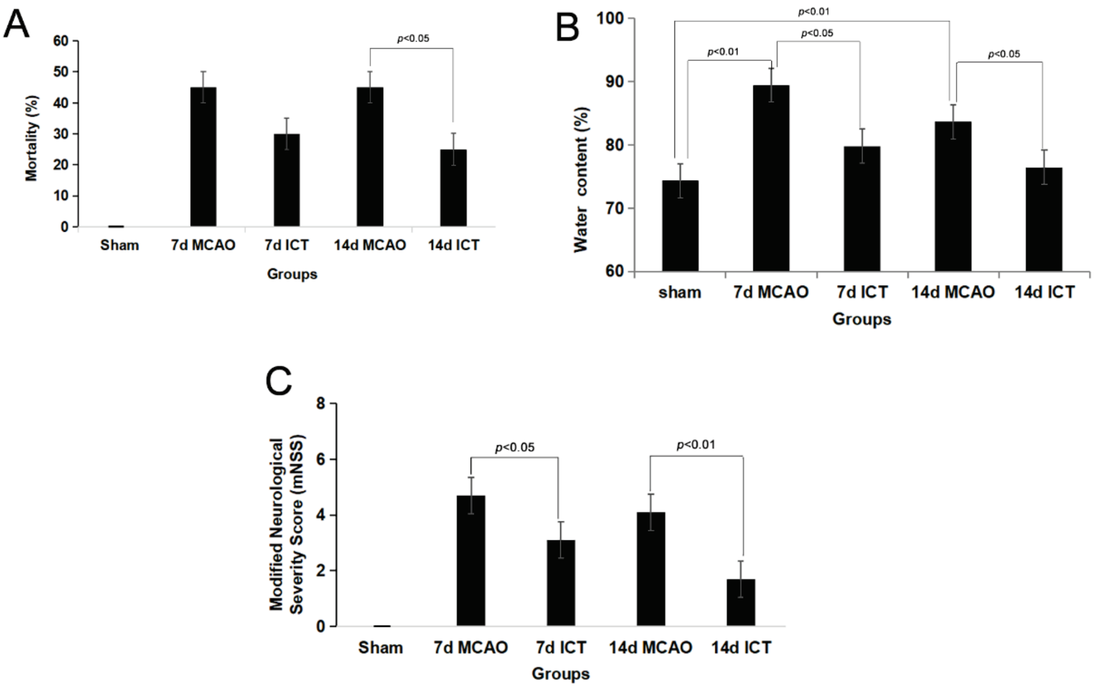

Fig. 1. Effects of isometric contraction training on mortality, brain water content, and neurobehavioral deficits. (**A**) Histogram showed the comparison of mortality between groups. (**B**) Histogram showed the comparison of brain water content between groups. (**C**) Histogram showed the comparison of the modified neurological severity score (mNSS).

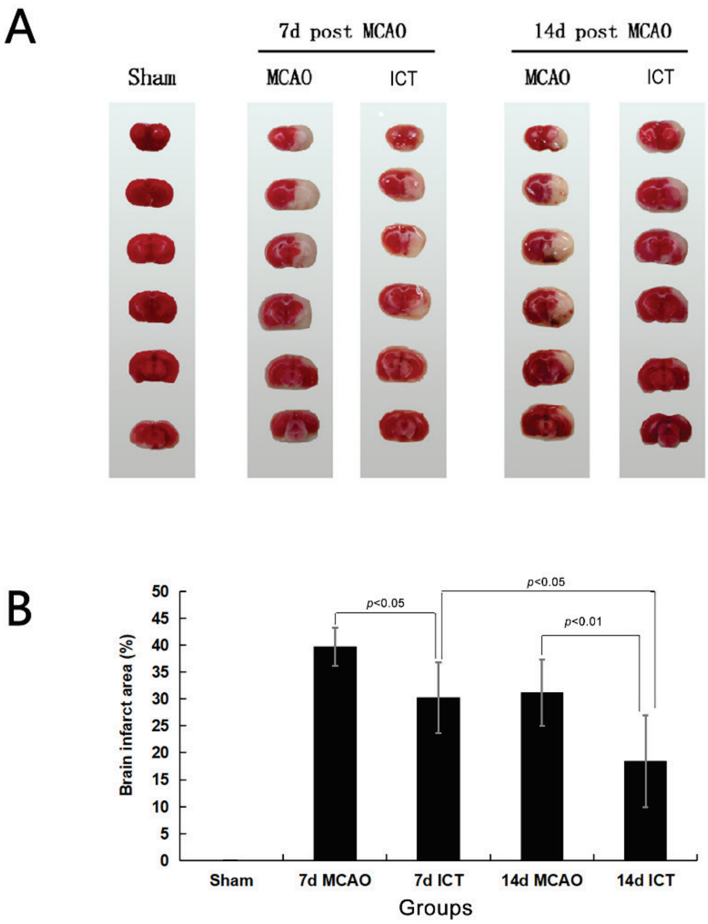

Fig. 2. Effects of isometric contraction training on the infarct area. (**A**) Triphenyl tetrazolium chloride (TTC) staining of brain slices. (**B**) Histogram showed the comparison of the infarct area between groups.

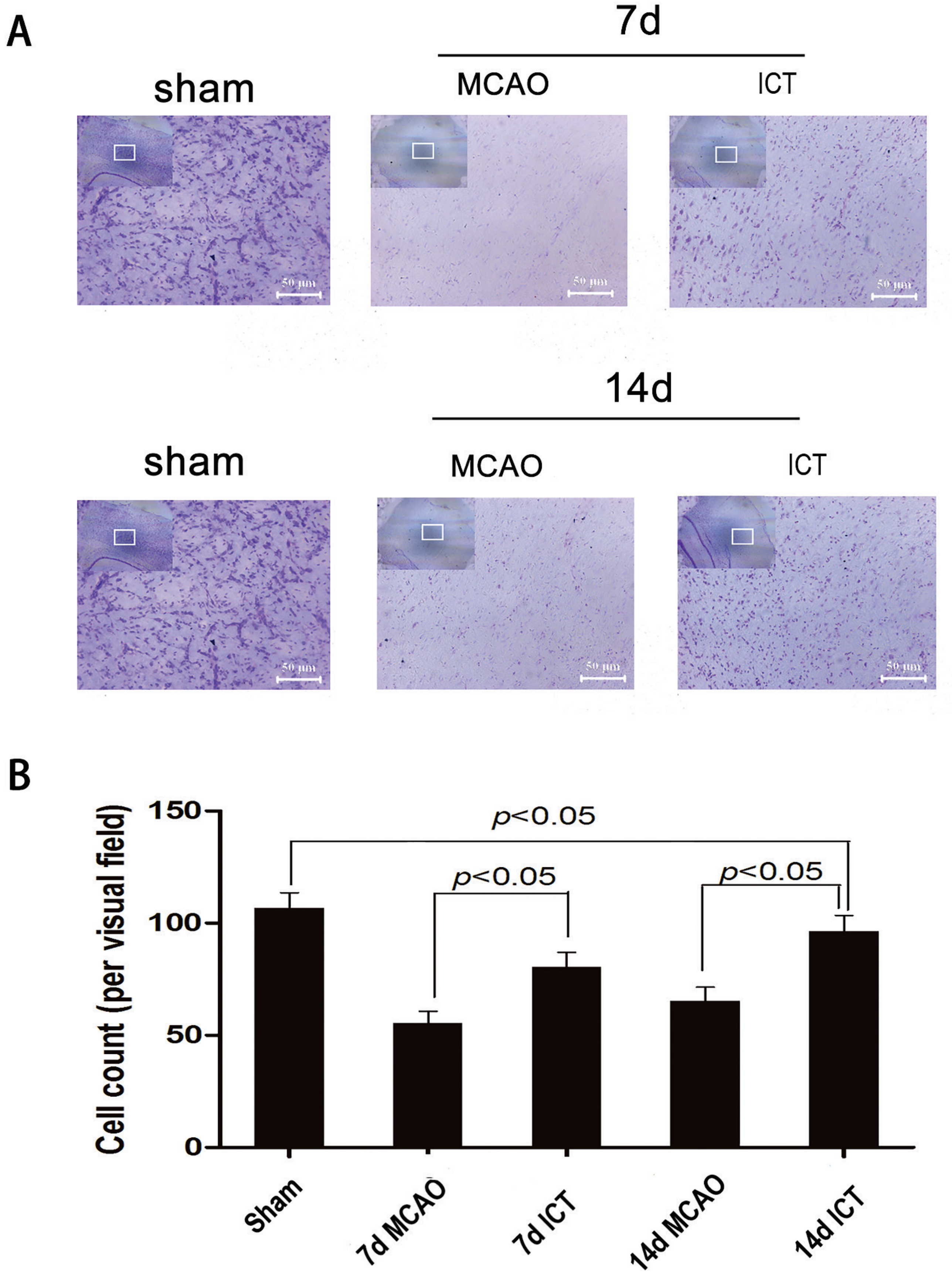

Fig. 3. Nissl staining and cell counts in the cortex region. (**A**) Nissl staining of the cortex in different groups. (**B**) Histogram showed the comparison of Nissl stained cells between groups.

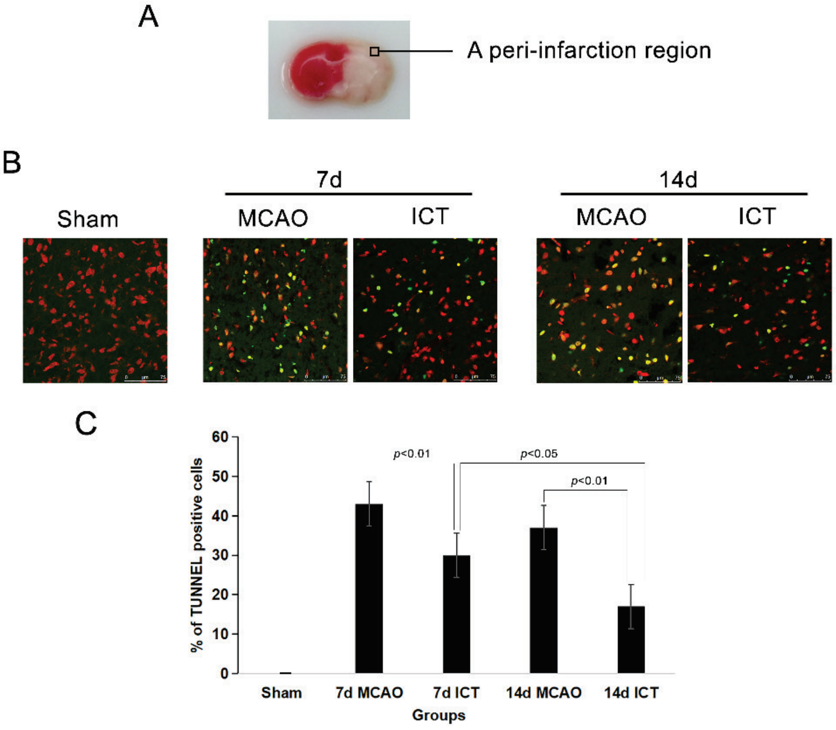

Fig. 4. Effects of isometric contraction training on neuronal apoptosis. (**A**) Rat brain tissue section. (**B**) Representative fluorescence micrographs of TUNEL staining for the cerebral cortex of different groups. (**C**) Histogram showing percentage of apoptotic (TUNEL-positive) cells in the cerebral cortex in different groups.

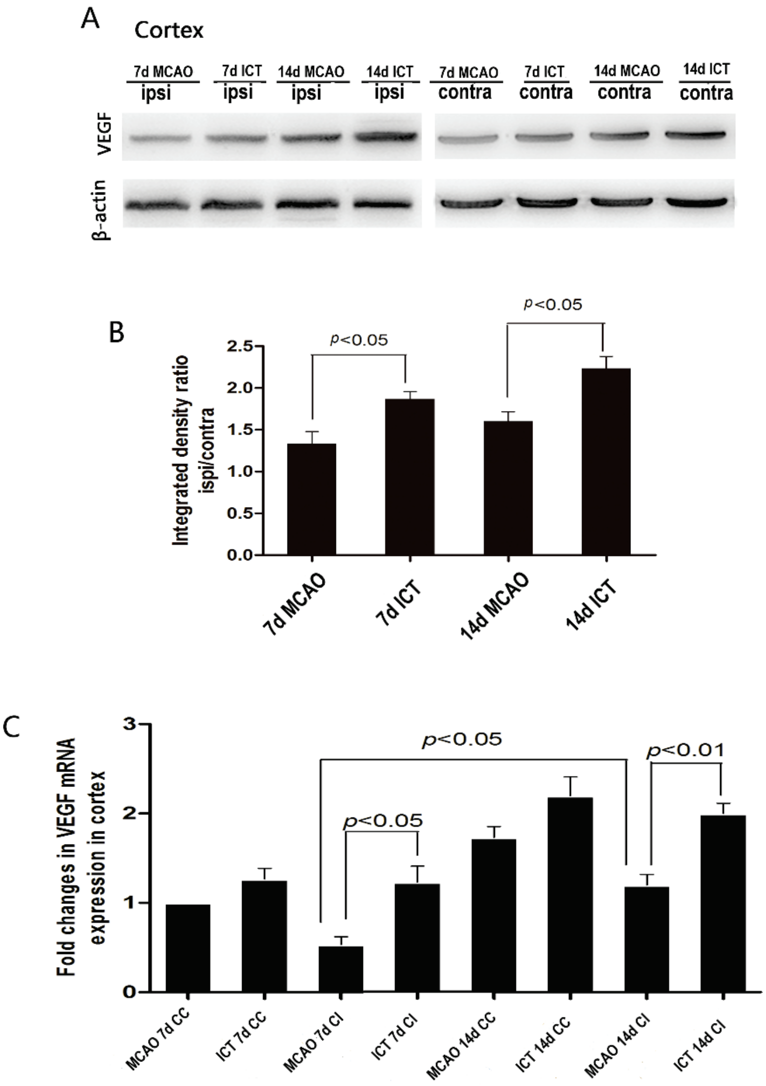

Fig. 5. Effects of isometric contraction training on the expression of vascular endothelial growth factor (VEGF). (**A**) Representative Western blots from cortical tissue samples. (**B**) Histogram showed the VEGF protein expression in the ischemic cortex of different groups. (**C**) Real-time polymerase chain reaction (RT-PCR) analysis of the effect of ICT on the level of VEGF mRNA expression. CC: cortex contra, CI: cortex ipsi.

to mobilize endothelial progenitor cells and promote vascular remodeling by up-regulating the levels of VEGF and NO [24]. Starting from the hypothesis that both cerebral ischemia and cardiac ischemia are both ischemic diseases, we demonstrated the neuroprotective effects of ICT in the brain.

The concept of ICT is different from ischemic preconditioning (IPC). IPC refers to the delay of cell death after coronary artery occlusion through short ischemic training before myocardial ischemia and infarction [45]. Unlike IPC, ICT causes transient ischemia of skeletal muscle through repeated isometric contraction training, thereby promoting the formation of arterial collateral and reducing the apoptosis of nerve cells. The cardioprotective effect of short-term skeletal muscle ischemia was evaluated in our previous experimental [24,27], and Lin *et al.* used colored microspheres to measure collateral circulation blood flow in rabbits (equation: CCBF (%) = blood flow after occlusion/blood flow before occlusion), and concluded that physiologic ischemic training of skeletal muscle may induce collateral circulation development in the myocardium [34]. Most related studies [46] have suggested that isometric contraction training could produce cardioprotection after a certain period of training (such as four weeks). Both cerebral ischemia and cardiac ischemia are ischemic disease. However, only few studies have focused on the effect of ICT on cerebral ischemia. Does ICT have neuroprotective effects? To answer this question, we designed this experiment to investigate the neuroprotective effect of ICT on the rat MCAO model.

The rat MCAO model produces obvious infarction induced by focal occlusion of the MCA. The general ischemia model is a common clinical case, and stroke mostly occurs in local perfusion after spontaneous recanalization [47]. In this study, we performed MCAO to transiently block the distal MCA, after 90-min MCA occlusion, the transient block was removed to mimic partial reperfusion after stroke that frequently occurs in patients [48]. Our ischemic model was highly reproducible and reliable. In addition, the injured region of the cortex was most likely detected in clinical stroke patients.

Using this rat model, we designed three experimental groups. The purpose of the sham group was to exclude the placebo effect or any interference from the surgical procedure itself; the MCAO group was designed to establish a model of transient MCA occlusion; and the ICT group used the same ischemic model as physiologic ischemic training, and performed daily isometric contraction training.

Our experiment found that ICT could effectively reduce the infarct area, improve behavioral recovery, promote neurofunctional recovery, and protect neurons from focal ischemia in rats. In addition, in the ICT group, the loss of Nissl granules was attenuated, the neuronal morphology was improved, and neuronal apoptosis was reduced, indicating that ICT can regulate MCAO-induced neuronal injury.

Our previous studies proved that the expressions of VEGF and VEGF mRNA increased not only in the normal skeletal muscle but also increased in remote areas [21,24]. Ren *et al.* [49] suggested that endogenous VEGF far away from the ischemic areas was also up-regulated. Our results showed that compared with the sham group, VEGF and VEGF mRNA increased significantly in both ischemic and contralateral sides in the ICT group. It was reported that VEGF not only promoted angiogenic and anti-inflammatory responses, but also regulated brain function at the neurovascular interface [50]. VEGF directly affects nerve cells and their progenitors, and indirectly affects the cerebral perfusion of the central and peripheral nervous systems [51], which is consistent with our study. This may also explain why ICT has neuroprotective effects.

We also found that ICT reduced the apoptosis of nerve cells. VEGF might be involved in the direct attenuation of cell death in the early stage of ischemic injury and the late stage of angiogenesis. Chen [52] found that TUNEL and cleaved caspase-3-positive neurons greatly reduced in AAVH9-VEGF-transduced mice, suggesting that the neuroprotective effect of VEGF was associated with the anti-apoptotic pathway. Interestingly, VEGF is also a strong survival factor in serum-deprived endothelial cells, and it also induces PI 3′-kinase activation and Akt phosphorylation. Therefore, Ang1 and VEGF have a common intracellular second messenger signaling pathway, which can prevent the apoptosis of endothelial cells under serum deprivation. Recently, Akt has been shown to promote cell survival or nitric oxide production by phosphorylating Bad and procaspase-9 or endothelial nitric oxide synthase [53]. This may be a reason for the neuroprotective effect of ICT, which needs to be investigated in future studies.

In addition, there are some limitations in our study. Firstly, we only studied the training under a single condition, and whether using different doses and time can improve the outcomes remains to be tested. Secondly, we only studied the two-week time window, and whether longer ICT can promote the functional recovery of rats also needs to be explored further. Thirdly, this study did not employ unbiased stereology method to count the number of alive neurons or TUNEL positive cells. Fourthly, regarding the potential mechanism of the neuroprotective effect of ICT, only VEGF was studied, and whether other factors and pathways can also promote brain protection and functional recovery by regulating the expression of VEGF needs to be further investigated. Finally, the mechanism of ICT's protective effect on neurofunctional recovery still needs to be confirmed in more studies.

Conclusions

In summary, our results suggested that ICT might promote neurofunctional recovery and protect neurons against focal ischemia in rats, which was manifested by the decreased animal mortality, and the reduced cerebral infarct area, brain edema and functional deficits. This phenomenon might be one of the mechanisms of neurological recovery in cerebral ischemic rats. ICT might provide a promising training approach for post-stroke rehabilitation.

Conflict of Interest

There is no conflict of interest.

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