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Original Research

Voluntary exercise promotes neurotrophic factor and suppresses apoptosis in hippocampal ischemia

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Previous studies have demonstrated that exercise facilitates recovery from ischemia. However, the mechanisms need to be further elucidated. The current investigation was designed to study the effect of voluntary exercise on cerebral ischemia and discuss possible mechanisms using-middle cerebral artery occlusion model. Rats were randomly allocated to three groups: control, middle cerebral artery occlusion, and middle cerebral artery occlusion plus exercise. The middle cerebral artery occlusion plus exercise group was preconditioned by three weeks of voluntary wheel running prior to surgery. The accelerated rotarod test was employed to evaluate motor performance. Infarct volumes were analyzed to detect the neuroprotective effect of voluntary exercise. Brain-derived neurotrophic factor, Bax, Bcl-2, and caspase-3 protein expressions were measured by Western blot. Behavior evaluation showed the middle cerebral artery occlusion plus exercise group achieved significantly longer time on a rotarod than the unexercised group. Additionally, voluntary exercise reduced cerebral infarction and increased brain derived neurotrophic factor expression. Exercise down-regulated the apoptotic Bax/Bcl-2 ratio and caspase-3 protein expression. Results indicate that voluntary wheel running promote hippocampal brain derived neurotrophic factor and inhibit cell apoptosis in ischemia-induced impairment.

Keywords

Hippocampal ischemia; voluntary exercise; brain-derived neurotrophic factor; apoptosis; rodent

Abbreviations

BDNF	Brain-derived neurotrophic factor
CCA	Common carotid artery
ECA	External carotid artery
ICA	Internal carotid artery
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
TTC	2,3,5-Triphenyl-tetrazolium chloride solution

1. Introduction

Ischemia is the most common cerebral vascular disease and results in heavy socio-economic burden (Feigin et al., 2003; Bajaj et al., 2010; Urra and Chamorro, 2013). The mechanism of ischemia is complex. It includes an inflammation reaction, protease activation, intracellular excitatory toxicity, and calcium overload (Heo and Kim, 2013; Xing et al., 2018). Numerous drugs and therapies have been tested, however, in clinical settings, no available treatment attenuates the neural deficits observed after ischemia (Xing et al., 2018).

Brain-derived neurotrophic factor (BDNF) is a protein belonging to the neurotrophin family, which exerts a protective effect in many neurological disorders (Binder, 2004; Fumagalli et al., 2006; Zhang et al., 2012; Wu et al., 2017). The endogenous receptor of BDNF is Tropomyosin-related kinase receptor type B (TrkB). Both dendrites and axons secrete BDNF in response to neuronal activity (Lessmann and Brigadski, 2009). Several studies reported that treatment with BDNF could promote functional recovery in neural diseases. For example, BDNF promotes neurogenesis in depression (Jiang et al., 2012), anxiety (Quesseveur et al., 2013), and Alzheimer's disease (Liu et al., 2015). Treatment by lentiviral BDNF gene delivery significantly ameliorate cell loss and improve synaptophysin immunoreactivity in assembly activating protein mutant mice (Nagahara et al., 2013). In Huntington's disease, transfaction of the BDNF gene to striatal neurons could alleviate injury to both cognitive and motor functions, enlarge striatal volume and increase NeuN+ cell numbers (Connor et al., 2016). Furthermore, BDNF is also reported to increase synapse number (Sanchez et al., 2006) and regulate synaptic plasticity such as LTP (Leal et al., 2017; Kowianski et al., 2018). It has been shown that BDNF plays an important role in the pathophysiological course of stroke. For example, intracerevro-ventricular perfusion of BDNF can reduce infarct size and protect hippocampus CA1 pyramidal cells after focal ischemia (Beck et al., 1994; Schabitz et al., 1997; Yamashita et al., 1997). BDNF promotes neuroblasts to migrate to the ischemic area in the striatum ischemia mouse model (Grade et al., 2013). Clinically, serum BDNF level is closely related to the pathology of stroke. In the acute phase of stroke, BDNF concentrations are significantly lower than controls (Stanne et al., 2016). Social support is associated with increased BDNF level, which partly

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reduces the risk of stroke (Salinas et al., 2017). These observations imply that BDNF is important in the pathogenesis of ischemia.

A number of studies have documented that physical activity is effective in the treatment of stroke. Post-stroke exercise enhances stroke induced down-regulation of motor function and cognition, and modulates synaptic plasticity (Nie and Yang, 2017). Exercise improved the motor behavior index, and decreased the hippocampal calpain protein levels in focal cerebral ischemia rats (Heo and Kim, 2013). Treadmill training promoted cortical BDNF expression following ischemic stroke in a mature rat model (Quirie et al., 2012). However, to the authors knowledge, the protective mechanism of pre-stroke voluntary exercise has yet to be clarified. This paper investigates the impact of preconditioning voluntary wheel running on BDNF in intact and stroke brains. The results may further promote the application of physical exercise for stroke prevention, and provide a more reliable theoretical foundation of preventive kinesiotherapy in the clinic.

2. Materials and methods

2.1. Chemicals and Reagents

Triphenyltetrazolium chloride (TTC) solution was purchased from Sigma Aldrich (USA). Anti-cleaved Caspase-3 antibody (9664S) and anti-Bax antibody were obtained from Cell Signaling. Antibodies against BDNF, Bcl-2 and GAPDH were respectively purchased from Millipore (USA), Santa Cruz (USA) and Boster (Wuhan, China). Secondary antibodies for Western blot were bought from CWBIO (China), and ECL Prime reagent was obtained from Beyotime (China).

2.2. Animals

Thirty-two male Sprague-Dawley rats 250-280 g, aging 10 weeks, were supplied to be subjects by Tianjin Medical Laboratory Animal Center (Tianjin, China). They were housed under standard laboratory conditions (pathogen-free, 23 ± 1°C, 55% relative humidity, 12 hour light/dark cycle). Food and water (SPF-degree) were freely available. All procedures were conducted according to the Chinese Council on Animal Care Guidelines, Ethical guidelines were approved by The 940^{th} Hospital of Joint Logistics Support Force of the Chinese People's Liberation Army (Approval No. 2019KYLL035). Subjects were randomly divided into three groups by a computer-generated randomization schedule: control (n = 8), middle cerebral artery occlusion (MCAO) (n = 12), and Exercise + MCAO group (n = 12). Due to loss during the MCAO procedure, the number of subjects in these groups was kept high. After the MCAO procedure, there were eight subjects in each group.

2.3. Voluntary running

The exercise + MCAO group was equipped with an acrylic cage ($330 \times 115 \times 125$ mm) and an activity wheel. Meanwhile, the control and MCAP groups were allowed to freely explore acrylic cages without a running wheel. After 21 consecutive days of voluntary exercise, subjects received MCAO surgery.

2.4. MCAO test procedure

The MCAO procedure was performed as previously reported (Longa et al., 1989). Briefly, surgery followed an anesthetic intraperitoneal injection of 10% chloral hydrate (350 mg/kg). The left common carotid artery (CCA) was freed from its carotid sheath after a midline neck incision. The left vagus nerve, left external carotid artery (ECA), and the internal carotid artery (ICA) were next identified and carefully separated. A ligation was then performed on the arterial bifurcation of ECA and CCA and the middle cerebral artery (MCA) was blocked with a 19 mm embolus made from fishing line inserted into the ICA. The line was removed after 120 minutes to achieve reperfusion. In the control group, the CCA, ECA, and ICA were isolated, but without endovascular embolism. All procedures were performed under sterile conditions.

24 hours following reperfusion, the neurological function of subjects was assessed by the neurological deficit score: 0: no deficit, 1: failure to fully extend right forepaw, 2: circling to right, 3: falling to right, and 4: no walking and depressed consciousness. Only subjects scoring 1-3 were accepted for the study and then randomly divided into the ischemia groups (Liu et al., 2018). Three subjects died, one was motionless, four exhibited no deficit after MCAO surgery and were also excluded after neurological appraisal.

2.5. Rotarod test

The degree of hemiparesis and coordinated motor function of subjects was determined by computer aided accelerated rotating rod test as previously described (Tahta et al., 2018). Three days prior to MCAO, all subjects were trained on a rotarod cylinder (ENV-575MA, MediAssociates, Georgia, USA) for five daily sessions accelerating from four to forty rpm (revolutions per minute) over five minutes. At seven and fourteen days after surgery, subjects were placed on the rotarod cylinder for five minutes and performance was measured. This procedure was repeated three times and the average falling time recorded.

2.6. TTC staining

TTC staining was performed to verify MCAO test reliability as described previously. Following the rotarod test, animals were sacrificed. Brains were dissected and placed in a customized slicer (-20 °C, 20 minutes). Two millimeter coronal sections were cut and dark incubated in 2% TTC solution (17779, Sigma, USA) at 37 °C for a further 30 minutes. Stained sections were then immediately photographed. The white infarct area was measured by ImageJ software. The total infarct volume was calculated by the sum of the infarct area times the slice thickness (Tahta et al., 2018).

2.7. Western blotting analysis

Following decapitation, hippocampi were quickly separated on ice and mixed with ice-cold RIPA (450 mM NaCl, 50 mM Tris-HCl pH 6.8, 0.1 nM SDS, 1 mM EDTA, 1% deoxysodium cholate, 1% TritonX-100) and phenylmethylsulfonyl fluoride (PMSF). Hippocampal homogenates were centrifuged at 12000 g for 20 minutes at 4 °C and supernatants were gathered and stockpiled at -80 °C until analyzed. Protein concentration was determined using a BCA protein assay kit (Boster, China) according to the manufacturer's instructions. 50 μg protein was loaded and separated by 10% SDS-PAGE gel and transferred to polyvinyldifluoride (PVDF) membranes (Millipore, USA). After blocking by 5% non-fat milk, membranes were separately incubated at 4 °C overnight with primary antibodies of rabbit polyclonal anti-BDNF (1: 500), Bcl-2 (1: 200), Bax (1: 1000), anti-caspase-3 (1: 1000), and GAPDH (1: 100). Subsequently, after three rinses with phosphate-

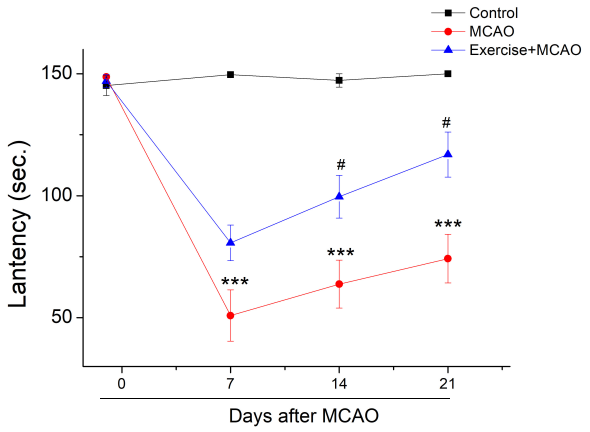

Figure 1. Rotarod test showing behavioral and motor functional analyses. The latency of falling from the rotarod was recorded at 7, 14, and 21 days post surgery (n = 8). Data presented as mean ± SEM. *** $p < 0.00$ *vs.* control group; ♯ $p < 0.05$ *vs.* MCAO group.

buffered saline (PBS), stabilized membranes were incubated with horseradish peroxide-conjugated secondary antibodies (1: 5000) for one hour at room temperature. The protein intensities was probed with ECL Prime reagent and analyzed by FluroChemTM SP software.

3. Statistical analysis

All statistical analyses were performed using IBM SPSS 25.0. All data are expressed as mean ± standard error of mean (SEM) followed by post-hoc LSD test with equal variances assumed. Differences between the groups were analyzed by one-way ANOVA and $p < 0.05$ was considered to indicate statistically significant difference.

4. Results

4.1. Exercise improved motor function recovery with rotarod test

The rotarod test was first employed to evaluate behavioral and motor function. Eight subjects were excluded by their neurological grade. Seven ($F_{(2,23)} = 42.253$, $p < 0.05$, n = 8 for each group) or 14 days ($F_{(2,23)} = 28.990$, $p < 0.05$, n = 8 for each group) after MCAO surgery, the falling latency clearly decreased (Fig. 1). The latency of the MCAO + Exercise group was also reduced, however, this group performed significantly better than the MCAO group on both days ($p < 0.05$, Fig. 1).

4.2. Exercise decreased cerebral infarct volume in MCAO rats

Following the rotarod test, subjects were sacrificed and cerebral infarct volumes measured. MCAO surgery significantly increased infarct volume ($p < 0.05$, Fig. 2), indicating successful development of the MCAO model. Additionally, clear differences were detected in the infarct volume of MCAO and MCAO + Exercise groups. Compared to the MCAO group, exercise significantly reduced infarct volume, showing exercise provides therapeutic efficacy for reduction of cerebral ischemia ($F_{(2,9)} = 101.856$, $p < 0.05$, n = 4 for each group, Fig. 2).

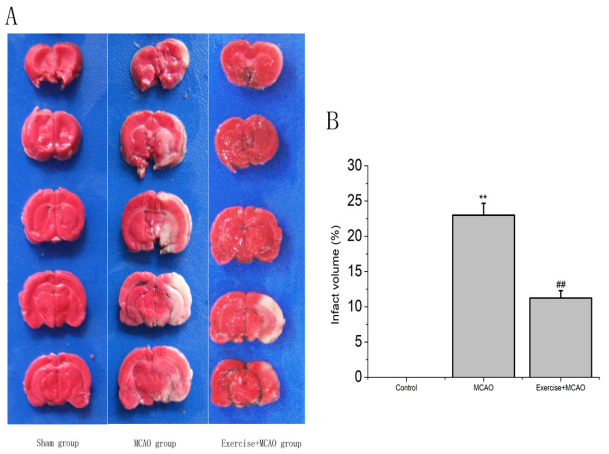

Figure 2. Effect of exercise on cerebral infarct volume in MCAO rats. Cerebral infarct volumes in the MCAO group were significantly higher, while exercise preconditioning significantly reduced cerebral infarct volumes (n = 4). Data presented as mean ± SEM. ** $p < 0.01$ *vs.* control group; ♯♯ $p < 0.01$ *vs.* MCAO group.

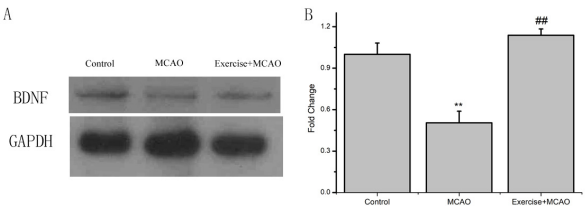

Figure 3. Protective effect of exercise on MCAO-induced down-regulation of BDNF. (A) Western blot showing BDNF expression. (B) Quantification of BDNF expression by Western blot (n = 3). Data presented as mean ± SEM. ** $p < 0.01$ *vs.* control group; ♯♯ $p < 0.01$ *vs.* MCAO group.

4.3. Exercise promoted BDNF expression in MCAO rats

To explore the mechanism of exercise as a neuroprotective effect, expression of hippocampal BDNF was tested by Western blot assay. The result demonstrated that compared with the control group, MCAO significantly decreased hippocampal BDNF expression, which increased remarkably for the MCAO + Exercise group ($F_{(2,8)} = 23.313$, $p < 0.05$, n = 3 for each group, Fig. 3).

4.4. Exercise regulates the expression of apoptosis related proteins

To evaluate the protective effects of pre-exercise on cell apoptosis, Bax, Bcl-2, and caspase-3 expression were assessed by Western blot analysis. In this analysis, MCAO surgery increased Bax protein expression, which was otherwise reduced by exercise ($F_{(2,8)} = 13.941$, $p < 0.05$, n = 3 for each group, Fig. 4A, B). Bcl-2 protein levels in the MCAO group decreased, while exercise up-regulated Bcl-2 protein levels significantly compared to the non-exercise group ($F_{(2,8)} = 12.093$, $p < 0.05$, n = 3 for each group, Fig. 4A, C). The Bax/Bcl-2 ratio profoundly increased in MCAO group but was attenuated by exercise ($F_{(2,8)} = 35.139$, $p < 0.05$,

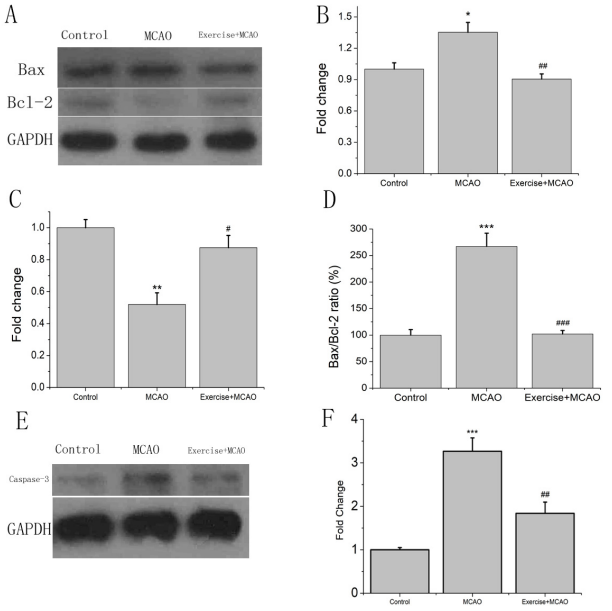

Figure 4. Effect of exercise on expression of Bax, Bcl-2, and caspase-3. (A) Effect of MCAO and exercise on Bcl-2 and Bax expression. (B) Western blot showing Bax expression (n = 3). (C) Western blot showing Bcl-2 expression (n = 3). (D) Exercise profoundly inhibited the construction-mediated up-regulation of the Bax/Bcl-2 ratio. (E) Western blot showing caspase-3 expression. (F) Quantification of caspase-3 expression by Western blot (n = 3). Data presented as mean ± SEM. * $p < 0.05$ vs. control group; ** $p < 0.01$ *vs.* control group; *** $p < 0.001$ vs. control group; ♯ $p < 0.05$ *vs.* MCAO group; ♯♯ $p < 0.01$ vs. MCAO group, ♯♯♯ $p < 0.001$ *vs.* MCAO group.

n = 3 for each group, Fig. 2D). Expression of caspase-3 protein increased after MCAO and exercise reversed this increase ($F_{(2,8)} = 23.862$, $p < 0.05$, n = 3 for each group, Fig. 4E, F).

5. Discussion

In the research described here, the preventive efficacy of exercise on ischemia-reperfusion injury was studied and the possible mechanism by which it affects BDNF and apoptosis was explored. It was found that MCAO impaired both neurological and motor function, but exercise preconditioning significantly improved neurobehavior, reduced infarct volume, and increased hippocampal BDNF expression after stroke. Furthermore, exercise suppressed the Bax/Bcl-2 ratio and up-regulated caspase-3 expression in hippocampus. These results suggested that pretreatment by voluntary wheel running showed significant neuroprotective effects in the ischemic-reperfused rat brain.

The common exercise models usually include voluntary wheel running, forced treadmill training, and involuntary muscle contraction caused by electrical stimulation (Kim, 2013). However, to the authors knowledge, many studies employed the treadmill exercise model, while only a few studies focused on the preventive effect of preconditioning by voluntary exercise on ischemia. It is reported that moderate and vigorous exercise are associated with substantial reductions in the incidence of cardiovascular events although the protective mechanism is unclear (de Waard and Duncker, 2009). Compared with treadmill exercise, the voluntary exercise model may attenuate the symptoms and inflammation response to dextran sodium sulfate (Cook et al., 2013). Hippocampal metabolic protein levels are also different for voluntary and treadmill exercise (Chen et al., 2008; Kirchner et al., 2008). In the present study, a voluntary exercise model was employed and it was found that voluntary exercise could alleviate ischemia-induced impairment.

Physically active individuals usually show lower blood pressure, a more favorable plasma lipoprotein profile, and higher insulin sensitivity, which are usually related to a decrease in the risk of developing cardiovascular disease as well as cardiovascular death (Nystoriak and Bhatnagar, 2018). Exercise also benefits the nervous system. It is reported that exercise enhances cerebral vascular integrity (Kang et al., 2011), promotes hippocampal neurogenesis (Liu et al., 2018), and modulates cognitive benefits (Lebowitz et al., 2018). Furthermore, exercise alleviates ischemia-induced memory impairment (Seo et al., 2014). It was found that voluntary wheel running before MCAO mitigated damage to neurological and motor function in the present study, which indicates the potential preventive effect of exercise preconditioning.

BDNF is beneficial in the case of nervous system disease (Binder, 2004; Fumagalli et al., 2006; Zhang et al., 2012; Castren and Rantamaki, 2010). In ischemia, BDNF could reduce infarct size and protect the hippocampus CA1 pyramidal cells (Yamashita et al., 1997). BDNF has also been reported to be anti-apoptotic *in vitro* (Kubo et al., 1995; Tong and Perez-Polo, 1998). Data reported here provides evidence that voluntary running before MCAO injury could up-regulate hippocampal BDNF protein expression. It was hypothesized that BDNF might protect neurons and glia from MCAO injury. This was the current of investigation.

Voluntary exercise may modulate cell apoptosis in chronic restraint stress (Seo et al., 2016) and the ischemia/reperfusion (Shang et al., 2018) rat model. In the present study, precondition by exercise for three weeks inhibited the ischemia-induced increase in the Bax/Bcl-2 ratio through increased Bcl-2 expression. An increase in caspase-3 protein expression was also observed in the voluntary exercise group. These findings are in accordance with numerous studies that have suggested that BDNF represses cell apoptosis in ischemia (Yao et al., 2012; Fan et al., 2015; Asadi et al., 2018) and it is surmised that the underlying mechanism of suppressed apoptosis might be related to BDNF. Furthermore, the voluntary running model is implemented in rat home cages, and this method may involve the effect of enriched environment. This will be a focus of future studies.

It was reported that stroke may induce glutamate release, which may increase hippocampal neuron injury (Wang and Harvey, 2016). However, exercise preconditioning could attenuate the overexpression of glutamate (Mourao et al., 2014) and down-regulate its receptors (Zhang et al., 2010). Furthermore, reperfusion may increase the number of free-radicals and reintroduce oxygen to neurons and glia, which is a cause of cell apoptosis (Han et al., 2019). Given that BDNF protein expression was increased after voluntary exercise (Fig. 3), it may decrease oxidative stress (Lee et al., 2011), repair DNA damage (Schmidt et al., 2016), and reduce cell apoptosis (Fig. 4).

In summary, preconditioning by voluntary exercise in MCAO rats alleviates neurological impairments of cerebral ischemia, upregulates BDNF protein level, and modulates hippocampal cell apoptosis. These protective effects may provide a therapy that might decrease cell death and ameliorate loss of function after ischemia.

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Conflict of Interest

The authors declare no competing interests.

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