

# Beneficial Effects of Antecedent Exercise Training on Limb Motor Function and Calpain Expression in a Rat Model of Stroke

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**Abstract.** [Purpose] In the present study, we investigated the effects of antecedent exercise on functional recovery and calpain protein expression following focal cerebral ischemia injury. [Subjects and Methods] The rat middle cerebral artery occlusion model was employed. Adult male Sprague-Dawley rats were randomly divided into 4 groups. Group I comprised untreated normal rats (n=10); Group II comprised untreated rats with focal cerebral ischemia (n=10); Group III comprised rats that performed treadmill exercise (20 m/min) training after focal cerebral ischemia (n=10); and Group IV comprised rats that performed antecedent treadmill exercise (20 m/min) training before focal cerebral ischemia (n=10). At different time points (1, 7, 14, and 21 days), limb placement test score and the levels of calpain protein in the hippocampus were examined. [Results] In the antecedent exercise group, improvements in the motor behavior index (limb placement test) were observed and hippocampal calpain protein levels were decreased. [Conclusion] These results indicated that antecedent treadmill exercise prior to focal cerebral ischemia exerted neuroprotective effects against ischemic brain injury by improving motor performance and decreasing the levels of calpain expression. Furthermore, these results suggest that antecedent treadmill exercise of an appropriate intensity is critical for post-stroke rehabilitation.

**Key words:** Antecedent exercise, Calpain, Cerebral ischemia

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## INTRODUCTION

Stroke is an acute and progressive neurodegenerative disorder that has become one of the leading causes of mortality and various disabilities<sup>1)</sup>. Ischemic stroke is the result of a transient or permanent reduction in cerebral arterial blood, and it is a leading cause of long-term motor disabilities<sup>2)</sup>. About 85% of stroke patients have had ischemic strokes, which are mainly caused by acute thromboembolic occlusion or local thrombosis of the intracranial arteries<sup>3)</sup>. The major pathological mechanisms of cerebral ischemic injury include protease activation, oxidative stress, disruption of Ca<sup>2+</sup> homeostasis disruption, inflammation, and intracellular excitotoxicity<sup>4-6)</sup>.

Exercise that is performed within a relatively short period of time results in effects on serum lipids, inflammation, oxidative stress markers and blood pressure as well as reducing the risk of stroke<sup>7)</sup>. Among several exercise paradigms, voluntary wheel running, forced treadmill running, and involuntary muscle contraction from neuromuscular electrical stimulation (NMES) are commonly adopted exercise models<sup>8, 9)</sup>. It is important to know which rehabilitation intervention is most effective in facilitating motor function recovery. In addition, upregulating the levels of proteins

in the caspase and calpain families has been shown to be a leading factor that is responsible for motor learning and neuronal plasticity following brain degenerative processes<sup>10)</sup>.

Neuroplasticity is an important mechanism in functional recovery after brain injury<sup>11)</sup>. Calpain is one of a family of natural cysteine proteases that have been implicated in cell death following brain damage<sup>12)</sup>. The main calpain isoforms are  $\mu$ -calpain and m-calpain, and their activation has been linked to synaptic plasticity, as well as to neurodegeneration, in the central nervous system<sup>13)</sup>. These proteases have been implicated in numerous cell functions, including cell survival, division, proliferation, maturation, migration, and apoptosis<sup>14, 15)</sup>. Many of the substrates of calpains have been localized to the presynaptic and postsynaptic compartments of neurons in the hippocampus and cortex<sup>16, 17)</sup>. Calpain substrates include cytoskeletal and associated proteins, kinases and phosphatases, membrane receptors and transporters, and steroid receptors<sup>18)</sup>. Calpain inhibition has been shown to be neuroprotective in most models of neurodegeneration<sup>19-21)</sup>, but it remains unclear whether the endogenous inhibition of calpain that is induced by physical exercise has neuroprotective effects after stroke. Thus, we hypothesized that antecedent exercise treadmill training would promote motor function and change the levels of expression of calpain in focal cerebral ischemic injury in rats.

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## SUBJECTS AND METHODS

Forty male 8-week-old Sprague-Dawley rats, weighing 250–260 g were used following a 1-week acclimatization period. The rats were housed at a temperature of  $25.0 \pm 1.0$  °C and a humidity level of  $50 \pm 5\%$  a 12-h light-dark cycle. They had free access to food and water. All rats were divided randomly into 4 groups. Group I comprised untreated normal rats ( $n=10$ ); Group II comprised untreated focal cerebral ischemia rats ( $n=10$ ); Group III comprised rats that performed treadmill exercise (20 m/min) training after focal cerebral ischemia ( $n=10$ ); and Group IV comprised rats that performed antecedent treadmill exercise (20 m/min, 14 days) training before focal cerebral ischemia ( $n=10$ ). All animal experimental protocols were performed in accordance with the guidelines of the institution's Animal Care and Use Committee. Focal cerebral ischemia was induced by a modified intraluminal suture, as described previously<sup>22</sup>. Briefly, the left common internal and external carotid arteries were exposed through a midline incision in the neck and then carefully dissected from the surrounding tissues under an operating microscope. After electro-coagulation of the external and common carotid arteries, a 3-0 silicon rubber-coated monofilament was inserted through the common carotid artery into the internal carotid artery to a depth of 18–20 mm beyond the carotid bifurcation at the base of the middle cerebral artery. An atraumatic aneurysm clip was placed on the internal carotid artery in order to prevent bleeding. The clip and the monofilament were removed 1 h later for transient ischemia, and they were left in place for 24 h for permanent ischemia. The incision was then sutured.

Treadmill exercise was performed according to a previously described method<sup>23</sup>. The treadmill velocity was set at 20 m/min on a 0° degree incline, and antecedent treadmill exercise was performed for 20 min/day over a 14-day period. At that same time, the rats in group III were allowed to move freely in their cages, but no additional treadmill running was employed. The rats in the groups III and IV performed treadmill exercise over a 21-day period, which began 24 h after the surgery. At the end of experiment, the animals were sacrificed by decapitation the morning following the last exercise day, and their hippocampi were removed immediately, placed on dry ice, and stored at 70 °C for protein measurements. In the limb-placement test, rats were graded from 0 to 2 in each of the 8 subtests as follows: score 0, unable to place limb; score 1, partial or delayed over 2-s placement; and score 2, immediate placement<sup>24</sup>. The hippocampus was homogenized in lysis buffer B (137 mM NaCl, 20 mM Tris, 1% NP40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 1 µg/ml leupeptin, 0.5 mM sodium vanadate, pH 8.0) for western blot analysis. The tissue was homogenized in freshly prepared lysis buffer (1:10 w/v) and centrifuged at  $12,000 \times g$  for 30 min. The supernatants were collected, aliquoted, and stored at -70 °C. The total protein concentrations of the hippocampal homogenates were determined with a MicroBCA kit with the use of bovine serum albumin as a standard. Aliquots containing an equal amount of hippo-

campal protein extracts (20 µg) were mixed with gel loading buffer and separated on 10% sodium dodecyl sulfate-polyacrylamide gels. After electrophoresis, the proteins were transferred onto polyvinylidene fluoride membranes and nonspecific binding was blocked with 5% nonfat dry milk in tris-buffered saline and Tween 20. Membranes were incubated with the following primary antibodies. After incubation with the rabbit polyclonal anti-m-calpain antibody (1:1,000 Triple Point Biologics; Forest Grove, OR, USA) and the monoclonal anti-β actin (A-5316, 1:5,000, Sigma, USA) antibody, the membranes were washed with TBST and incubated with appropriate horse radish peroxidase-conjugated secondary antibody (1:4,000 dilution). The immunocomplexes were visualized by chemiluminescence with an ECL kit according to the manufacturer's instructions. The film signals were digitally scanned and then quantified with NIH image J software.

Data analysis was performed with SPSS for Windows, version 18.0 (SPSS Inc., Chicago, IL, USA). All of the data are expressed as mean  $\pm$  standard deviation (SD) of 3 replications. The differences between 2 groups were tested by one-way ANOVA, which were followed by the Student–Newman–Keuls multiple comparison tests when differences were detected. *p*-values less than 0.05 at a 95% confidence level were considered significant.

## RESULTS

The limb placement test behavior scores were 18 for all of the rats in Group I on postoperative day 1. The scores for Group II on postoperative day 1 were  $4.5 \pm 0.8$  points, which were significantly different from those of the normal group ( $16.0 \pm 0.0$  points). On day 21, there were apparently significant differences in these scores between the ischemia group (Group II) and the exercise groups (Group III and Group IV). After antecedent treadmill exercise, the limb placement test behavior score increased from  $4.6 \pm 0.5$  points to  $8.4 \pm 0.5$  points in the treadmill exercise group, while the limb placement behavior test significantly increased from  $4.5 \pm 0.5$  point to  $11.5 \pm 0.5$  points in the exercise group ( $p < 0.05$ ) (Table 1).

We analyzed each brain protein extract for the levels of calpain protein expression. There was a trend toward lower expression levels of calpain protein in the exercise groups compared to the control group. The antecedent exercise group (Group IV) showed lower levels of expression of calpain than the treadmill exercise group (Table 2).

## DISCUSSION

Stroke patients display various symptoms and disabilities, such as motor impairment, depression, cognitive impairments, physical disabilities, sensory weakness, and limited activities of daily activities<sup>25–28</sup>. These are many of the factors that have been shown to influence the quality of life of stroke patients. Physiotherapists have tried to aid in return of upper limb function for patients, by conducting repetitive practice through several therapeutic interventions, paying special attention to strength, endurance, coordination, speed, and ways to integrate motor function into the

**Table 1.** Results of the limb-placement test before and after the treadmill exercise by mild cerebral ischemic rats (score)

Groups	1 day	7 days	14 days	21 days
Group I	16.0 ± 0.0	16.0 ± 0.0	16.0 ± 0.0	16.0 ± 0.0
Group II	4.5 ± 0.8 <sup>a</sup>	4.7 ± 0.8 <sup>a</sup>	4.9 ± 0.5 <sup>a</sup>	5.5 ± 0.7 <sup>a</sup>
Group III	4.6 ± 0.5 <sup>a</sup>	4.9 ± 0.6 <sup>a</sup>	5.6 ± 0.5 <sup>a</sup>	8.4 ± 0.5 <sup>b</sup>
Group IV	4.5 ± 0.6 <sup>a</sup>	5.0 ± 1.0 <sup>a</sup>	8.1 ± 1.1 <sup>c</sup>	11.5 ± 1.2 <sup>c</sup>

Data were presented as mean ± SD, a:  $p < 0.05$  as compared to group I, b:  $p < 0.05$  as compared to group II, c:  $p < 0.05$  as compared to group III.

**Table 2.** Effect of treadmill exercise on calpain protein expression alteration in focal brain ischemia rats (%)

Groups	Group I	Group II	Group III	Group IV
Calpain	99.85 ± 8.52	146.50 ± 8.44 <sup>a</sup>	125.50 ± 15.56 <sup>b</sup>	109.50 ± 11.80 <sup>c</sup>

Data are presented as mean ± SD, a:  $p < 0.05$  as compared to group I, b:  $p < 0.05$  as compared to group II, c:  $p < 0.05$  as compared to group III.

patient's everyday activities<sup>29, 30</sup>). A number of previous studies have investigated the role of exercise in promoting stroke rehabilitation. The pathological mechanisms underlying ischemic stroke, at least in part, converge on impaired intracellular calcium homeostasis, leading to the activation of calpain. Owing to their dependence on calcium, calpains have attracted much attention as modulators implicated in a wide variety of biological phenomena including cell migration, proliferation, cell signalling, and protein homeostasis<sup>31–34</sup>). Thus, we hypothesized that antecedent exercise treadmill training would promote motor function and change the levels of expression of calpain in focal cerebral ischemic injury in rats.

Physical exercise training has been a well-established way to protect neuronal cells against ischemia-induced brain injury<sup>35, 36</sup>). Exercise increases capillary density by inducing angiogenesis factors, and it is protective against ischemic damage<sup>37</sup>). Our results revealed significant motor function recovery, as shown-by the limb placement test score improvements in the treadmill exercise groups. Neuronal synaptic remodeling in the brain hippocampus that is induced by physical exercise has been described in a number of animal models<sup>38</sup>).

An important mechanism of neuronal cell death following brain ischemia is the disruption of  $Ca^{2+}$  homeostasis<sup>39</sup>). The activation of calpain could be just one of the various pathways that lead to neuronal injury and cell death. Calpains are abundantly present in the nervous tissue as well as in the hippocampus, cortex, and cerebellum<sup>40</sup>). The best known members of the calpain family are the  $\mu$ - and  $m$ -calpains, which are referred to occur in practically all mammalian tissues and cell types<sup>17</sup>). An increase of cytosolic  $Ca^{2+}$  in neurons that are exposed to glutamate may cause the activation of calpain, which is a cytosolic  $Ca^{2+}$ -dependent protease<sup>30, 41</sup>). The main characteristics of the different members of the calpain large family of proteases, traditionally divided into classical and non-classical calpains according to their domain structure. Cells have an intricate strategy for the tight regulation of calpain activity. This includes

the binding of calpain to its specific endogenous inhibitor calpastatin in a substrate-competitive manner, a differential intracellular localization, and the cellular control of  $Ca^{2+}$  homeostasis<sup>42</sup>). Our results indicate that physical exercise inhibited mitochondrial release of apoptotic cell death in ischemic rat brain injury.

While the deleterious effects of calpain activation in neuronal ischemia have been well documented, other factors, such as energy deficits in the cell, the activation of poly polymerase-1 and  $Ca^{2+}$  dependent phospholipase  $A_2$ , and the degradation of membrane phospholipids, are also thought to be important<sup>43–46</sup>).

However, in our study, there were significant inhibitory effects antecedent treadmill exercise groups on the levels of calpain expression, and improvement limb motor function compared to the effects in the treadmill exercise group. The changes in calpain expression might reflect neuroprotective characteristics that promote the survival of hippocampal neurons, as has been shown in animal experiments of insults, such as focal brain ischemia. Based on the findings that calpain is a critical mediator of the effects of antecedent treadmill exercise on synaptic plasticity and motor function, our results showed that changes in calpain are crucial for accomplishing this process.

Our data clearly show that antecedent treadmill exercise acted as a major homeostatic regulator of motor function and calpain expression inhibition, with important implications for neuronal plasticity. These findings suggest that the antecedent treadmill exercise is important for improving limb motor function. Thus, antecedent treadmill exercise rather than other post-stroke exercises may provide beneficial effects for stroke patients.

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