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# 10- or 30-minute treadmill exercise rescues motor behaviors through inhibiting apoptosis in a rat MCAo model

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**ABSTRACT**

Ischemic stroke, one of the world's leading fatal diseases, has a high recurrence and incidence that can lead to severe mortality and disability. In this study, we investigated whether exercise can treat ischemic stroke to prevent recurrence and improve functional impairment. Experimental cerebral ischemia was induced by middle cerebral artery occlusion (MCAo) in rats, and the effect of 10- or 30-minute training for two weeks was evaluated. Following cylinder and rota-rod behavioral tests, we found that motor function was improved compared to the non-exercise group. In addition, the brain infarct volume was decreased after exercise following TTC staining. Further examination of the cell signaling mechanisms involved in the improvement showed that the immune reactivity significantly decreased the expression of the pro-apoptotic protein, Bax, and increased that of the anti-apoptotic protein, Bcl-2. Our results suggest that exercise has a beneficial effect on ischemic stroke for short- or long-term training.

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## Introduction

Stroke, one of the leading causes of morbidity and mortality, is neurological damage caused by ischemia and hemorrhage, resulting in impeded blood supply to the central nervous tissue of the brain (1-4). Worldwide, 70 million people suffer strokes each year, including ischemic stroke, which is caused by insufficient blood flow and accounts for 80% of stroke patients (1-3). Ischemic stroke, also called cerebral infarction, triggers the formation of brain tissue necrosis or death, which activates inflammatory responses and apoptotic pathways, surrounded by a peripheral reversible infarction area (3).

In the central nervous system, apoptosis, which is characterized by dense chromatin, the formation of DNA fragments, and apoptotic bodies, is crucial in ischemic injury and is the primary form of delayed neuronal death after cerebral ischemia (5-7). In the apoptotic pathway after cerebral ischemia, many apoptosis-related genes and proteins are regulated and involved (8-12). The generation of reactive oxygen species (ROS) has been shown to upregulate the pro-apoptotic proteins, including Bcl-2-associated X protein (Bax) and caspase-3 and resulted in neuronal death during ischemia/reperfusion injury (5, 13-15). Conversely, B-cell lymphoma 2 (Bcl-2), an anti-apoptotic protein, plays a critical role in cellular survival by reducing apoptosis.

After a stroke or stroke-induced cell death, mammals display functional disabilities in both motor and sensory systems, and treatment is needed for recovery, including physical therapy, rehabilitation therapy, pharmacological treatment, surgical therapy, and exercise therapy (16, 17). Exercise can effectively induce ischemia tolerance, exert neuroprotective effects, and improve organ dysfunction (12, 14, 18, 19). In addition, several studies suggested that appropriate exercise could suppress apoptosis induced by the expression of Bax and active caspase-3/8/9 and decrease the generation of ROS by the expression of Bcl-2, B-cell lymphoma-extra large (Bcl-XL), and heat-shock protein-70 (Hsp70) (8, 9, 13, 20). The intensity and duration of exercise for a patient, with stroke or brain damage, must be chosen according to the severity of the damage (21). Following several studies, light exercise is recommended to recover movement in stroke patients, who have difficulty walking on their own, rather than excessive exercise in the clinical field (14, 22-26).

Therefore, reducing apoptosis and promoting recovery following light exercise after ischemic brain injury are important therapeutic strategies for stroke patients. In this study, we hypothesized that exercise training at different durations could promote neurological recovery following reduced apoptotic cell death in ischemic stroke.

## Materials and Methods

### Animals

All experiments were approved by the Institutional Animal Care and Use Committee at Chonnam National University Medical School. Every effort to alleviate the pain of animals was made. A total of 24 male Sprague-Dawley rats (280 – 320 g, 8 weeks old) were purchased from Damul Science (Daejeon, Korea) and randomly assigned to four groups using a random table method: sham-surgery and sedentary group (sham, n = 6), ischemia and sedentary group (MCAo, n = 6), ischemia and 10-minutes exercise group (MCAo + 10Exe, n = 6), and ischemia and 30-minute exercise group (MCAo + 30Exe, n = 6). The rats were habituated under a 12-hour light/dark cycle at

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**Figure 1. The experimental procedure.** All experiments were performed following the schedule.

24°C with ad libitum access to food and water. All animals exercised on a treadmill at 6 m/min for ten minutes for two days, and behavior was tested using a rota-rod and cylinder test for two days prior to the ischemia operation. (Figure 1).

### Animal Model of Ischemic Stroke

Animals were anesthetized with an intraperitoneal (i.p.) injection of 50 mg/kg pentothal sodium (JW Pharmaceutical, Korea) and maintained under isoflurane USP (Troikaa Pharmaceuticals Ltd, India) during surgery. The rats kept breathing spontaneously during the operation. To induce MCAo, the rats were placed in the prone position on an operating table, and an incision was made in the middle of the neck with surgical scissors. The muscle layer was separated under a dissecting microscope to expose the common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA). A blunt tip filament (L.D-403556PK10, USA) was inserted into the left CCA and advanced to the ICA to occlude the origin of the MCA. After 45 minutes, the filament was removed, and the slip knot on the CCA was opened, which was reperfusion (27, 28). Rats with no evidence of acute neurological deficits or with evidence of hemorrhage were excluded from the analysis. The rats in the sham group underwent the same operation without MCAo. In sham rats, the filament was not inserted into the MCA (29).

### Treadmill Training

Prior to MCAo surgery, all rats were pre-trained to exercise for ten minutes on a treadmill (Treadmill B.S Technolab Inc. Version 10, Korea) for two days at a speed of 6 m/min, which was modified from a previous study (30). The exercise load consisted of running at a speed of 12 m/min for 10 (MCAo + 10Exe) or 30 minutes (MCAo + 30Exe) for two weeks (five days/week) (29-31). The apparatus delivered an electric shock to animals that dropped, thus stimulating them to stay on the treadmill and exercise continuously for the entire time. The sham and MCAo groups were housed in a standard cage with free access to food and water with no specific exercise.

### Behavioral tests

#### *Cylinder test*

The cylinder test is a behavioral test that can examine sensorimotor asymmetry after a stroke. Rats were placed in a transparent cylinder container (21 cm diameter x 40 cm height), and navigational behavior (touching the wall using the front paws) was recorded. A total of 20 limb movements or up to 10 minutes were recorded. Limb use asymmetry was assessed by quantifying the percentage of use of the injured and uninjured forelimb during navigation and landing (contralateral contacts/total contacts x 100%) (32, 33).

#### *Rota-rod test*

The rota-rod (Rota-Road B.S Technolab Inc. Version 1.0, Korea), which involves motor balance and coordination training, was used as the functional behavior in this study. The rod was 7 cm in diameter and 9 cm in length and is covered with smooth rubber. The animals were required to run at 4 rpm at first and gradually increased until 40 rpm for 300 seconds. The time and speed for the rat to lose balance and fall to the floor were automatically measured twice (14, 34).

### Tissue preparation

The rats were sacrificed on the 16th day of the experiment. Animals were anesthetized using 75 mg/kg sodium pentobarbital, transcardially perfused with phosphate-buffered saline (PBS), and fixed with a freshly prepared solution consisting of paraformaldehyde (PFA) in 0.1M phosphate buffer (pH 7.4). The brains were dissected and post-fixed in the same fixative overnight and transferred into a 30% sucrose solution until sinking occurred for cryoprotection. Coronal sections of 15 μm thickness were made with a freezing microtome (DE/CM1860; Leica Biosystems, Nussloch, Germany) (20, 35).

### Cerebral infarct volume assessment

The cerebral infarct volume was detected by triphenyl tetrazolium chloride (TTC; Sigma Chemical Co.) at two weeks after MCAo. Sodium pentobarbital (75 mg/kg, i.p.) was used to deeply anesthetize the rats, and then the brains were quickly removed. The brains were cut into five slices of the same thickness, immediately stained by immersion in TTC solution at 37°C for 30 minutes, and fixed in 4% PFA overnight until a clean white/red border was observed (36, 37). All brain tissue was photographed by a camera, and the normal brain tissue was shown in pink and the infarcted area in white.

### Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The prepared tissue sections were stained with the DeadEnd™ Colorimetric TUNEL System (Promega Corporation, USA) following the manufacturer's instructions. The stained images of the TUNEL results were captured by a microscope (ZEISS Axio Vert.A1, Carl Zeiss, Göttingen, Germany) at a magnification of 40x (38).

### Immunohistochemistry

For immunohistochemistry, the tissue was blocked for 20 minutes with 0.5% Triton X-100 (Sigma Chemical Co.), which included 10% normal goat serum (NGS; Vector Laboratories, Inc., Burlingame, CA, USA) in PBS. The sections were incubated with anti-Bax (1:500, sc-493, Santa Cruz) and anti-Bcl-2 (1:500, sc-492, Santa Cruz) antibodies diluted with 10% NGS and placed at room temperature for 1.5 hours. The samples were incubated with a biotinylated anti-rabbit secondary antibody (1:500, Santa Cruz) at room temperature for one hour. The secondary antibody was amplified with the Vector Elite ABC kit (1:100, Vector Laboratories). The antibody-biotin-avidin-peroxidase complexes were visualized using 0.03% DAB. Slides were mounted and then observed under a microscope (ZEISS Axio Vert.A1, Carl Zeiss, Göttingen, Germany) and images were captured (39).

### Statistical Analysis

Statistical analyses were performed with GraphPad Prism® 5.0 software (GraphPad Software Inc., San Diego, CA, USA). Differences in motor behavior between groups were assessed using two-way repeated measures ANOVA with significance levels of $p < 0.05$. If mean values between populations showed statistically significant changes, they were analyzed by post-hoc tests (Bonferroni). TTC staining, the TUNEL assay, and immunohistochemistry data were analyzed using One-way ANOVA and post-hoc tests (Tukey's) (*p < 0.05, **p < 0.01, ***p < 0.001).

## Results

### The effect of treadmill exercise on infarct volume

TTC staining was used to detect the cerebral infarction volume in each group after MCAo. Focal ischemia induced a remarkable tissue loss with necrosis and apoptosis in the cortex and striatum and resulted in increased infarct volume after stroke. The results showed that the volume of MCAo, MCAo + 10Exe, and MCAo + 30Exe were 21.54 ± 1.95, 11.24  1.42, and 10.60 ± 1.322 (mean ± standard error), respectively. The volume of cerebral infarction gradually decreased in the treadmill exercise group (Figure 2). These results suggest that exercise remarkably reduced the infarct volume and brain damage compared to the MCAo group. In contrast, the rats in the sham group did not show any infarct area.

### The effect of treadmill exercise on sensorimotor function

Cylinder tests were performed at 3, 5, 7, 10, 12, and 14 days after surgery. Following the behavioral tests, right forelimb utilization in both MCAo + 10Exe or MCAo + 30Exe groups was greater than in the MCAo group during the period. The right forelimb utilization in the MCAo + 30Exe group was significantly higher than in the MCAo group at day 12 (*$p < 0.05$ compared with MCAo).

Those results indicated that MCAo with exercise training-induced improvement of sensorimotor function compared to MCAo without exercise. In addition, the overall sensorimotor function in the MCAo + 30Exe group was slightly greater than in the MCAo + 10Exe group, indicating that exercise for 30 minutes could be more efficient (Figure 3).

### The effect of treadmill exercise on locomotor function

A duration and a speed of Rota-rod tests were performed at 3, 5, 7, 10, 12, and 14 days after surgery. The animals in the exercise group more remained than the ones without exercise. Moreover, the rota-rod test showed that the locomotor function in the MCAo + 10Exe group was significantly greater than in the MCAo group on day 12. There was no significant change in the speed of the rota-road. However, locomotor function in the MCAo + 10Exe group was generally higher than in the MCAo + 30Exe group.

Those results imply that MCAo with exercise training could increase locomotor function compared with MCAo without exercise. Moreover, the overall locomotor function in the MCAo + 10Exe was slightly greater than MCAo + 30Exe group, suggesting that exercise for 10 minutes could be more effective in improving locomotor function (*$p$<0.05 compared with MCAo, Figure 4).

### The effect of treadmill exercise on apoptotic cell death

To analyze cell death after the stroke, apoptotic cells were evaluated via a TUNEL assay. The number of apoptotic cells was calculated as 100 ± 7.67, 180 ± 5.14, 116 ± 5.95, and 116 ± 5.47 (Mean ± Std. Error) in the sham, MCAo, MCAo + 10Exe, and MCAo + 30Exe groups, respectively (***$p < 0.001$ compared with MCAo, Figure 5).

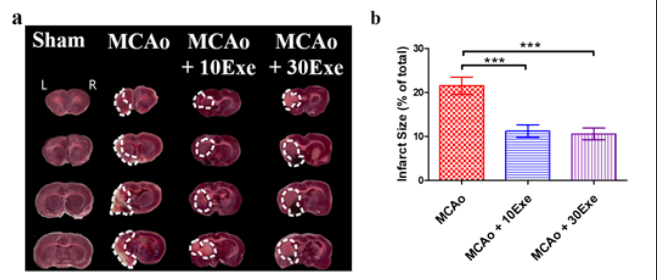


**Figure 2. Cerebral infarct volume assessment.** (a) TTC staining results in brain damage. A white line indicated infarct size. (b) Infarct size was measured and calculated using Image J (***$p < 0.001$ compared with MCAo).

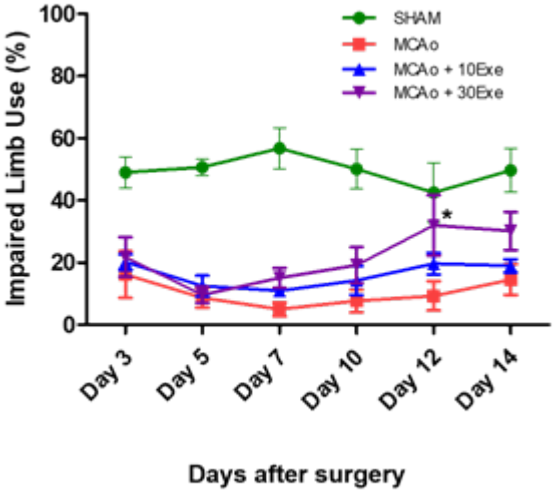


**Figure 3. Cylinder test results for impaired limb use.** The cylinder tests were performed with a total of 20 limb movements or up to 10 minutes and the percentage of use of the injured and uninjured forelimb was quantified (contralateral contacts/total contacts x 100%) (*$p < 0.05$ compared with MCAo).

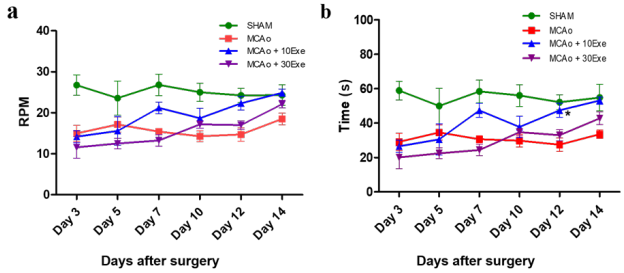


**Figure 4. Rota-rod test for balance and motor function.** All animals ran at 4-40 rpm, which gradually increased, for 300 seconds on the road. The time and speed for the rat to lose balance and fall to the floor were automatically measured twice. (a) The time of the rota-rod test. (b) The speed of the rota-rod test. (*p < 0.05 compared with MCAo).

The apoptotic cells were increased by the induction of ischemia and decreased after treadmill exercise in the ischemic rats. These results indicate that exercise could reduce apoptotic cell death after ischemic injury.

### The effect of treadmill exercise on apoptotic signaling

Apoptotic signaling was evaluated by Bax/Bcl-2 immunohistochemistry (Figure. 6). The number of Bax-positive cells was 22.45 ± 4.61, 56.66 ± 3.45, 22.08 ± 2.94, and 33.38 ± 4.16 (mean ± standard error) in the sham, MCAo, MCAo + 10Exe, and MCAo + 30Exe groups, respectively. The number of Bax-positive cells was increased by the induction of ischemia, while the treadmill exercise decreased the number of Bax-positive cells in the ischemic rats.

The number of Bcl-2-positive cells was 7.18 ± 0.60, 16.72 ± 2.28, and 15.99 ± 2.08 ((mean ± standard error) in the MCAo, MCAo + 10Exe, and MCAo + 30Exe groups, respectively (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with MCAo respectively). The number of Bcl-2-positive cells was decreased in the MCAo group, while exercise increased the number of Bcl-2-positive cells. These results indicated that both of the exercise groups reduced apoptosis after ischemic injury.

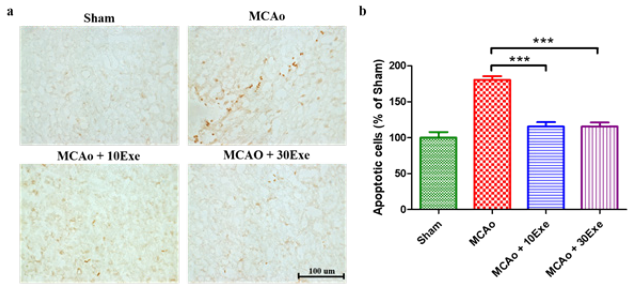


**Figure 5. Determination of the number of apoptotic cells.** To confirm the reduced apoptotic cells with exercise, TUNEL staining was investigated in the brain. Apoptotic cells were stained (a) and quantified (b). The scale bar indicated 100 μm (***$p < 0.001$ compared with MCAo).

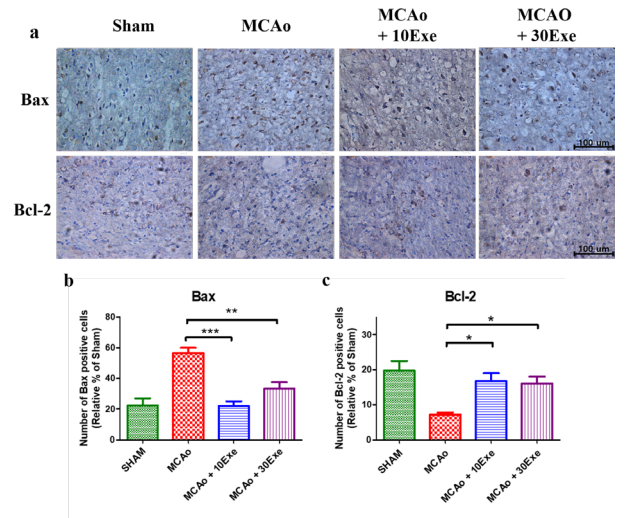


**Figure 6.** Immunohistochemical assay for Bax and Bcl-2. Apoptotic signaling was detected by DAB staining (a) and the number of cells positive for Bax (b) and Bcl-2 (c) was quantified. The scale bar indicated 100 μm (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with MCAo, respectively).

## Discussion

In ischemic stroke-induced rats, we demonstrated the improvement of locomotor function after 10- or 30-minute exercise following a reduction in the brain infarct size and decreased apoptotic cell death. Both 10- and 30-minute durations were effective behavioral improvements. Our results suggest that exercise has a beneficial effect on ischemic stroke for short- or long-term training.

Stroke is currently a major cause of disability, and there is no effective drug for clinical treatment. It is known that the pathological injury mechanisms of stroke are complex. The neurological and motor functions of patients are damaged, leading to an inability to return to normal social life or permanent disability (28). Therefore, exploring strategies, such as drugs and exercise, and discovering the mechanisms of neurological recovery after a stroke is crucial to improving the quality of life of patients.

Zhang's group demonstrated that early exercise had a protective effect by inhibiting neuronal apoptosis in cerebral ischemic injury (9, 40). One of the mechanisms of protection is reducing cardio-cerebrovascular complications (40, 41). Furthermore, combining pharmacological treatment with exercise therapy reduces the risk of secondary stroke by 80% (42, 43). Several exercise programs for stroke utilized sling, resistance, aerobic, and high-intensity aerobic exercises (43-47).

Recently, aerobic exercise has been confirmed to reduce cerebral infarct volume, improve behavioral function, and decrease neuronal death, in stoke (31, 32, 48). In addition, several groups indicated that treadmill exercise within 14 days reduces the region of brain hemorrhage, increases synaptic plasticity, and influences enhancing neuroprotection (31, 37, 48-51). After a stroke, the intensity and duration of exercise must be chosen according to the severity of the stroke (14, 21, 22, 25, 26). This study confirmed the reduction in cerebral infarction volume, the improvement of behavioral function, and the decrease of cell death through aerobic exercise training with a different duration, 10 or 30 minutes, after stroke.

Bcl-2 and Bax, two prototypic proteins of the Bcl family to suppress and promote apoptosis, are important makers regulating neuronal cell death in an experimental model of ischemic brain injury (52-54). Bax is upregulated during periods of reduced oxygen transfer resulting in neurodegeneration during ischemia-reperfusion injury (14, 55, 56). Bax promotes cell suicide by interfering with the pro-survival protein, Bcl-2 (56). Bcl-2 is an anti-apoptotic protein that acts as an apoptosis inhibitor and plays an important role in cell survival (14, 56, 57). In the clinical field, patients with a low level of apoptotic cells survived longer after stroke, suggesting that suppressing apoptosis has a positive impact on rapid recovery after stroke (58). During periods of reduced oxygen delivery, the pro-apoptotic factor Bax is upregulated and interferes with the pro-survival protein Bcl-2 to promote apoptosis (14, 55, 56). These apoptotic factors were found to be reduced by exercise training (50, 59, 60).

In this study, we demonstrated decreased apoptosis and regulated apoptotic signaling with Bax and Bcl-2 levels following exercise. As a result, it was confirmed that Bax-positive cells were decreased in the exercise group, especially in the 10–minutes group. In contrast, Bcl-2-positive cells were increased after exercise, especially after 10 mi-

nutes, and a large amount of Bcl-2 was expressed. In this study, treadmill exercise reduced apoptotic cell death in brain injury. However, the apoptotic mechanism induced by treadmill training needs more research.

However, this study has several limitations. First, stroke is a disease that often occurs in the elderly, but the rats used in this experiment were young-aged s that it may recover faster. Second, only the effects of two different durations of exercise (10- and 30- minutes) were studied, and the effects of various exercise durations should also be studied. However, short durations of exercise may be more appropriate for the elderly population which has physical weakness or difficulty walking, because longer durations of exercise may cause extra physical stress. Third, it is difficult to ascertain the exact apoptotic pathway through in vitro experiments, as this study was focused on exercise, a strictly *in vivo* approach.

## Conclusions

This study positively implicates both 10- and 30-minute exercises in post-stroke recovery. We determined that exercise reduced apoptotic factors and promoted improvement in behavioral function. In summary, different duration of exercise may promote neurological recovery by reducing apoptotic cell death and increasing motor function in ischemic stroke. Even a short 10-minute exercise was effective, suggesting that for patients who have difficulty walking or who are older, short periods of exercise may be beneficial for post-stroke recovery.

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## Interest conflict

The authors declare no conflict of interest.

## Consent for publications

Not applicable.

## Availability of data and material

Not applicable.

## Authors' Contribution

Conceptualization, J.Y., and D.K., investigation, J.Y., data curation, J.Y., writing-original draft preparation, J.Y., writing-review and editing, D.K., supervision, D.K. All authors have read and agreed to the published version of the manuscript.

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## Ethics approval and consent to participate

Not applicable.

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