

Effect of treadmill exercise on spatial navigation impairment associated with cerebellar Purkinje cell loss following chronic cerebral hypoperfusion

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Abstract. In addition to roles in motor coordination, the cerebellum is also associated with cognitive function. The aim of the present study was to investigate the effect of treadmill exercise on spatial navigation deficit induced by chronic cerebral hypoperfusion (CCH). Furthermore, whether decreased loss of Purkinje cells, which contain the calcium-binding protein in the posterior lobe of the cerebellum, attenuates the spatial navigation deficit induced by CCH was also investigated. Wistar rats were randomly divided into three groups: Sham group, bilateral common carotid arteries occlusion (BCCAO) group and a BCCAO + exercise (Ex) group. The rats in the BCCAO + Ex group ran on a treadmill for 30 min once a day for 8 weeks, starting at 4 weeks post-birth. CCH was induced by performing BCCAO at 12 weeks post-birth. The Morris water maze test was performed to determine the spatial navigation function of the rats. To investigate the histological features of the cerebellum in all of the experimental groups post-treatment, terminal deoxynucleotidyl transferase dUTP nick end labeling staining, as well as immunohistochemical analysis revealing the expression of calbindin, parvalbumin, glial fibrillary acidic protein, ionized calcium-binding adaptor molecule 1 and caspase-3, was performed. The results of the present study revealed that treadmill exercise improved spatial navigation, decreased the expression of reactive astrocytes and microglial cells, and decreased apoptotic rates in the cerebellar vermis post-CCH. Treadmill exercise also attenuated the loss of Purkinje cells following CCH. The number of Purkinje cells was revealed to be negatively correlated with spatial navigation performance.

These results indicate that treadmill exercise may attenuate spatial navigation impairment via inhibition of Purkinje cell loss in the posterior lobe of the cerebellum following CCH. Therefore, treadmill exercise may represent a therapeutic strategy for the treatment of patients with spatial navigation impairment following CCH.

Introduction

The role of the cerebellum was previously considered to be confined to movement, along with the cortex and basal ganglia; however, it has recently been redefined to represent a broader area of functions, including cognitive functions (1,2). The cerebellum receives and sends information from various brain regions, including the hypothalamus, hippocampus and cortex (3). The function of the cerebellum is strongly associated with the hippocampus, and the cerebellum has been revealed to affect hippocampal activity, including spatial navigation and working memory (3-5). Although it has not been fully determined, the cerebellum may affect the hippocampus in two ways (1). The cerebellar brain region is primarily associated with cognitive functioning, particularly spatial navigation and executive tasks, and is located in the posterior lobe of the cerebellum. A previous neuroimaging study has demonstrated that cognitive functions are associated with the posterior lobe of the cerebellum (6). Damage to the posterior lobe of the cerebellum has been revealed to be associated with impairment of spatial working memory, and transgenic mice with cerebellar dysfunction have been demonstrated to exhibit spatial navigation deficit (7,8).

Rats with Purkinje cells removed in the cerebellum exhibited impaired spatial navigation performance following analysis using the rotor-rod and Morris water maze tests (9). Purkinje cells are located the Purkinje layer of the cerebellum, which exhibits the greatest abundance of calcium-binding proteins in the cerebellum, such as calbindin D28k and parvalbumin. Such proteins have important roles in physiological processes, including cell cycle regulation, muscle contraction and the regulation of intracellular Ca²⁺ concentration associated with apoptosis (10). Purkinje cells, which have important roles in cerebellar function, are particularly vulnerable to damage in ischemia (11). Purkinje cell death induced by ischemia in the

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cerebellum has been reported to be induced by excitotoxicity due to glutamate release and intracellular calcium (12).

Chronic cerebral hypoperfusion (CCH) is a major pathological feature of vascular dementia that results in progressive cognitive impairment due to interference in the circulatory system (13-15). CCH has been demonstrated to induce hypoxia and oxidative stress, as well as cause inflammation of the cerebral cortex, white matter, hippocampus and striatum, resulting in impaired cognitive function (16-19). However, the pathological mechanism of CCH has not been fully determined. Research concerning the effects of CCH on the cerebellum and determining novel therapeutic strategies to enhance spatial navigation of patients suffering from CCH has been limited.

Treadmill exercise has been demonstrated to ameliorate neurological impairments following various brain disorders, including ischemia and Alzheimer's disease (20,21). Treadmill exercise has also been revealed to improve cognitive function by reducing the apoptosis rate of neurons and the reactivity of astrocytes in the cerebellum following transient global ischemia (22), and also protect against age-associated Purkinje cell loss (23). The aim of the present study was to investigate whether CCH induces a loss of Purkinje cells, and to investigate the effect of treadmill exercise on spatial navigation impairment associated with CCH.

Materials and methods

Experimental animals. Male Wistar rats ($n=30$; weight 80 ± 10 g, 3-weeks old, Orient Bio, Inc., Seongnam, Korea) were used in the present study. The rats were housed under controlled temperature ($20\pm 2^\circ\text{C}$), humidity ($60\pm 5\%$), and lighting conditions (7:00 a.m. to 7:00 p.m.) with food and water available *ad libitum*. All animal experimental procedures conformed to the regulations stipulated by the National Institutes of Health (Bethesda, MD, USA) (24) and the guidelines of the Korean Academy of Medical Science (Seoul, Korea). The present study was approved by the Kyung Hee University Institutional Animal Care and Use Committee [Seoul, Korea; KHUASP (SE)-16-149]. The rats were randomly divided into three groups: Sham group ($n=10$), bilateral common carotid arteries occlusion (BCCAO) group ($n=10$) and a BCCAO + treadmill exercise (BCCAO + Ex) group ($n=10$).

Treadmill exercise protocol. The rats in the BCCAO + Ex group were made to run on a treadmill for 30 min once a day for 8 weeks starting at 4 weeks post-birth, according to a previously described method (21). The treadmill exercise load consisted of running at 2 m/min for the first 5 min, 3 m/min for the following 5 min and 5 m/min for the last 20 min, at 0 degrees of inclination. The rats in the non-exercise groups were placed on the treadmill without being made to run for the same time period as the exercise group.

BCCAO. Rats were anesthetized via subcutaneous injection of 250 mg/kg tribromoethanol (Avertin[®]; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). BCCAO was performed to induce CCH and was performed carefully to avoid damage to the vagus nerve and surrounding tissues. Both carotid arteries were exposed by a ventral midline incision and double ligated with 3-0 silk (Ailee, Seoul, Korea) immediately below the

carotid bifurcation. The rats in the sham group underwent the same operation procedure without vessel ligation.

Open field test. Activity was determined using the open field test. As a previously described method (25), the rats were randomly assigned to an order of testing and placed in a white square open-field arena (100x100 cm) made of wood, enclosed by 40-cm-high walls and exposed to strong illumination (200 lux). The arena was divided into 25 squares (20x20 cm), consisting of 9 central and 16 peripheral squares. The rats were placed in the center of the arena and were allowed to explore the environment for 1 min. Following this time, the number of squares crossed was recorded for 5 min.

Morris water maze test. In order to investigate the spatial navigation in rats, the latency time in the Morris water maze test was determined according to a previously described method (1). The Morris water maze test consisted of a circular pool (painted white, 200 cm diameter, 60 cm high) filled with water ($22\pm 2^\circ\text{C}$, 37 cm deep) that was made opaque by the addition 1 kg skim milk powder. A platform (15 cm diameter, 35 cm high) was submersed 2 cm below the water surface in one of four quadrants in the pool. A video recorder was hung from the ceiling and was connected to a tracking device (SMART; Panlab, Barcelona, Spain). The animals were subjected to three trials per session. In each session, the rats were permitted to search for the platform for 60 sec. If the rat located the platform, the animals were permitted to remain on the platform for a further 10 sec. If the rat did not locate the platform within 60 sec, the rat was guided to the platform and permitted to remain for a further 10 sec. The latency times taken to locate the submerged platform were recorded. The animals were tested in this way for a total of 4 days with 3 trials per day.

Immunohistochemistry. A total of 6 animals per group were used for immunohistochemistry staining. Serial sagittal sections (40 μm) were obtained using a freezing microtome (-20 to -25°C ; Leica Microsystems GmbH, Wetzlar, Germany). Immunohistochemistry was performed to investigate the expression of glial fibrillary acidic protein (GFAP), ionized calcium-binding adaptor molecule 1 (Iba-1), calbindin D28k and parvalbumin in each cerebellar vermis. Free-floating sections were initially incubated in 3% H_2O_2 for 30 min at room temperature. Following this, the sections were incubated in blocking solution (1% bovine serum albumin and 0.2% Triton X-100; Vector Laboratories, Inc., Burlingame, CA, USA) for 2 h at room temperature (27°C). The sections were subsequently incubated overnight with the following primary antibodies: GFAP (1:500; AB5804; Chemicon; Merck KGaA), Iba-1 (1:500; ab178846; Abcam, Cambridge, UK), calbindin D28k (1:500; ab11426; Abcam), parvalbumin (1:1,000; ab11427; Abcam), and caspase-3 (1:500; sc-56053; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4°C . Following this, sections were incubated with biotinylated mouse (BA-2000), goat (BA-9500) and rabbit (BA-1100) secondary antibodies (1:200; Vector Laboratories, Inc., Burlingame, CA, USA) for 1 h at room temperature (27°C). The sections were then incubated with an avidin-biotin-peroxidase complex (1:1,000; Vector Laboratories, Inc.) for 1 h at room temperature

(27°C). For staining, the sections were incubated in a solution consisting of 0.02% DAB and 0.03% H₂O₂ in 50 mM Tris-HCl (pH 7.6) for approximately 5 min, following which they were washed with PBS and mounted onto gelatin-coated slides. Cover slips were mounted using Permount® (Fisher Scientific). Sections were assessed in a quantitative fashion, according to a micro-densitometrical method based on optical density using Image Pro® Plus software version 6.0 (Media Cybernetics, Bethesda, MD, USA). The number of positive cells per section was counted in 5 random fields from every specimen with a Nikon Eclipse 80i microscope (magnification, x40; Nikon Corporation, Tokyo, Japan).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. To visualize DNA fragmentation, which is a marker of apoptosis, TUNEL staining was performed using an *in Situ* Cell Death Detection kit with fluorescein (Roche Diagnostics GmbH, Mannheim, Germany). Serial sagittal brain sections (40 μm) were fixed in 4% paraformaldehyde at room temperature for 15 min, post-fixed in ethanol-acetic acid (2:1) for 5 min at -20°C and then rinsed with PBS. Following this, sections were incubated with proteinase K (100 μg/ml), rinsed with PBS and incubated in 3% H₂O₂ for 30 min at room temperature (27°C). Sections were subsequently permeabilized using 0.5% Triton X-100 for 1 h at room temperature (27°C), rinsed again and incubated in the TUNEL reaction mixture for 1 h at 37°C. The sections were rinsed and visualized using a Converter-POD for 1 h in a humidified 37°C chamber. The sections were incubated in a solution containing in 50 mM Tris-HCl (pH 7.6) containing 0.03% DAB, 40 mg/ml nickel chloride, and 0.03% hydrogen peroxide. The slides were counterstained with Nissl staining and mounted onto a gelatin coated slide. The slides were coverslipped with Permount® (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Statistical analysis. Data are presented as the mean ± standard error of the mean. SPSS software version 23.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Statistical analysis was performed using one-way analysis followed by Tukey's post-hoc test or Pearson's correlation analysis. P<0.05 was considered to indicate a statistically significant with 3-6 independent experiments.

Results

Treadmill exercise alleviates spatial navigation impairment induced by CCH. The Morris water maze test was performed to investigate the effect of treadmill exercise on spatial navigation impairment induced by CCH. The results indicated that the BCCAO group demonstrated an increased escape latency time compared with the sham group (P<0.05; Fig. 1). However, the BCCAO + Ex group demonstrated a significantly shorter escape latency time compared with the BCCAO group on days 3 and 4 of testing (P<0.05; Fig. 1).

Treadmill exercise reduces levels of reactive astrocytes and microglial activation in the cerebellum. To investigate the effect of treadmill exercise on glial cell activation, immunohistochemistry was performed. Photomicrographs of GFAP- and Iba-1-positive cells in the posterior regions of the

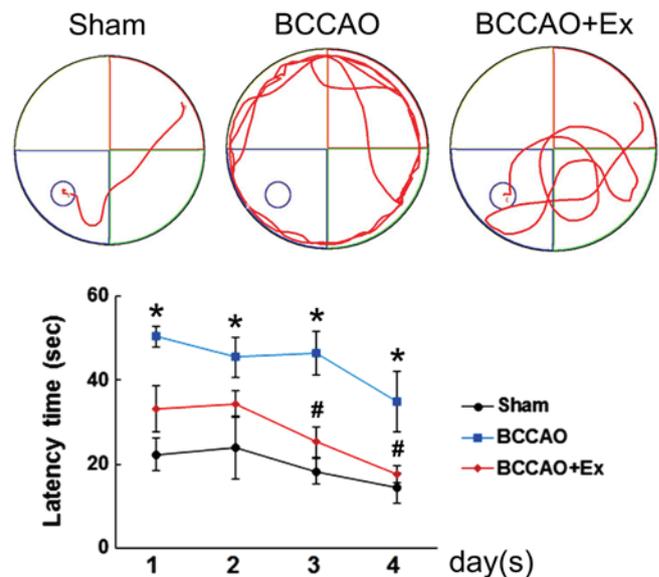


Figure 1. Effect of treadmill exercise on spatial navigation impairment. Spatial navigation impairment in rats was examined by Morris water maze testing. Representative paths and latency time taken to locate the hidden platform for each group are presented. Data are presented as the mean ± standard error of the mean. *P<0.05 vs. sham group; #P<0.05 vs. BCCAO group. BCCAO, bilateral common carotid arteries occlusion; Ex, exercise.

cerebellar vermis are presented in Fig. 2. GFAP, a marker of reactive astrocytes, was revealed to be significantly increased following cerebral ischemia (26); however, this effect was significantly attenuated following treadmill exercise (P<0.001; Fig. 2A). Iba-1 is a specific marker of microglia. Following transient focal cerebral ischemia induced by BCCAO, Iba-1 expression was demonstrated to be significantly increased in the BCCAO group compared with the sham group. However, this effect was significantly attenuated following treadmill exercise (P<0.001; Fig. 2B).

Treadmill exercise reduces apoptotic cell death in the cerebellum. Apoptotic neuronal cell death was determined via TUNEL analysis, which detects DNA fragmentation (27). The results demonstrated that the BCCAO + Ex group exhibited a significantly decreased number of TUNEL-positive cells in the posterior region compared with the BCCAO group (P<0.001; Fig. 3).

Caspase-3, a member of the caspase family, has an important function in apoptotic cell death (28). The BCCAO + Ex group demonstrated a significantly decreased number of caspase-3-positive cells in the posterior region compared with the BCCAO group (P<0.001; Fig. 3). Furthermore, the BCCAO group demonstrated increased TUNEL and caspase-3-positive cells in the granular and molecular layers of the cerebellar vermis compared with the sham group (Fig. 3).

Treadmill exercise attenuates the loss of Purkinje cells in the cerebellum. Calbindin D28k has regulatory roles in motor coordination, sensory integration and important functions of Purkinje cells (29). The BCCAO + Ex group demonstrated a significantly increased number of calbindin-positive Purkinje cells in the posterior regions compared with the BCCAO group (P<0.001; Fig. 4A). However, calbindin D28k-positive

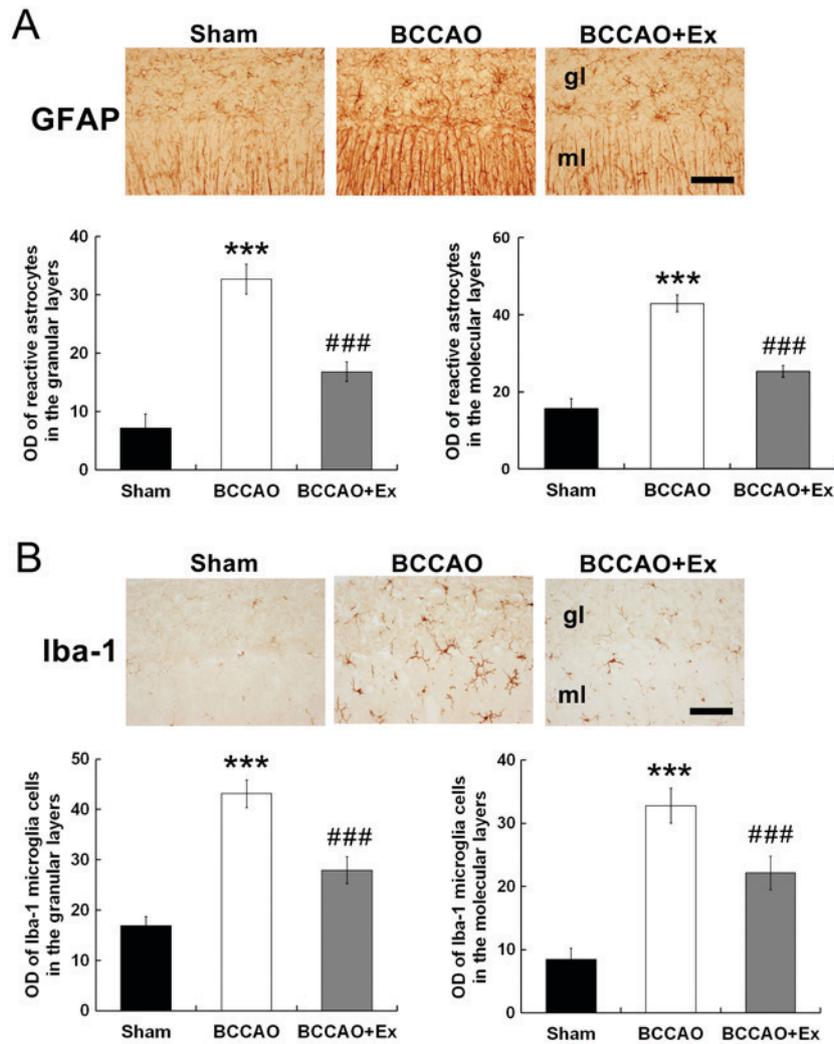


Figure 2. Effect of treadmill exercise on reactive astrocytes and microglial activation in the cerebellum. (A) Representative photomicrographs and OD of GFAP immunostaining in the granular and molecular layers of the cerebellum. Scale bar, 50 μ m. (B) Representative photomicrographs and OD of Iba-1 immunostaining in the granular and molecular layers of the cerebellum. Scale bar, 50 μ m. Data are presented as the mean \pm standard error of the mean. *** P <0.001 vs. sham group; ### P <0.001 vs. BCCAO group. OD, optical density; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adaptor molecule 1; BCCAO, bilateral common carotid arteries occlusion; Ex, exercise; gl, granular layer; ml, molecular layer.

Purkinje cells did not demonstrate a correlation with the latency time in the Morris water maze test ($r=-0.278$, $P=0.316$; Fig. 4B).

Parvalbumin, a small protein that is involved in Ca^{2+} signaling, protects neurons from cell death via suppression of intracellular Ca^{2+} concentrations (29). The BCCAO + Ex group demonstrated a significantly higher number of parvalbumin-positive Purkinje cells in the posterior region compared with the BCCAO group ($P<0.001$; Fig. 4A). Furthermore, the number of parvalbumin-positive Purkinje cells was revealed to be negatively correlated with the latency time in the Morris water maze test ($r=-0.561$, $P=0.030$; Fig. 4B).

Discussion

Physical exercise has numerous positive effects on brain functions, and running exercises have been revealed to improve various neurodegenerative disorders, such as ischemia, Alzheimer's and Parkinson's disease (20,30,31). In the present study, a low-intensity form of treadmill exercise was employed.

The low intensity exercise used in the present study represents ~50-60% of maximum oxygen uptake (32). Previous studies have demonstrated that low-intensity treadmill exercise induced positive effects on neurogenesis in the hippocampal dentate gyrus as well as the recovery of cognitive function in stroke models (33,34). The present study was performed to investigate the effect of treadmill exercise on spatial navigation impairment associated with cerebellar Purkinje cell loss following CCH. The results revealed that treadmill exercise significantly ameliorated the spatial navigation performance of rats in the Morris water maze following CCH. This result is consistent with previous studies, which demonstrated that treadmill exercise alleviated spatial navigation impairment in the radial 8 arm maze and Morris water maze (31,35). Thus, the results of the present study indicate that treadmill exercise may attenuate spatial navigation performance following CCH.

Animal activity by open field test was investigated in the present study (data not shown). The results demonstrated that the activity score in the open field test was not significantly different among the different experimental groups. The present

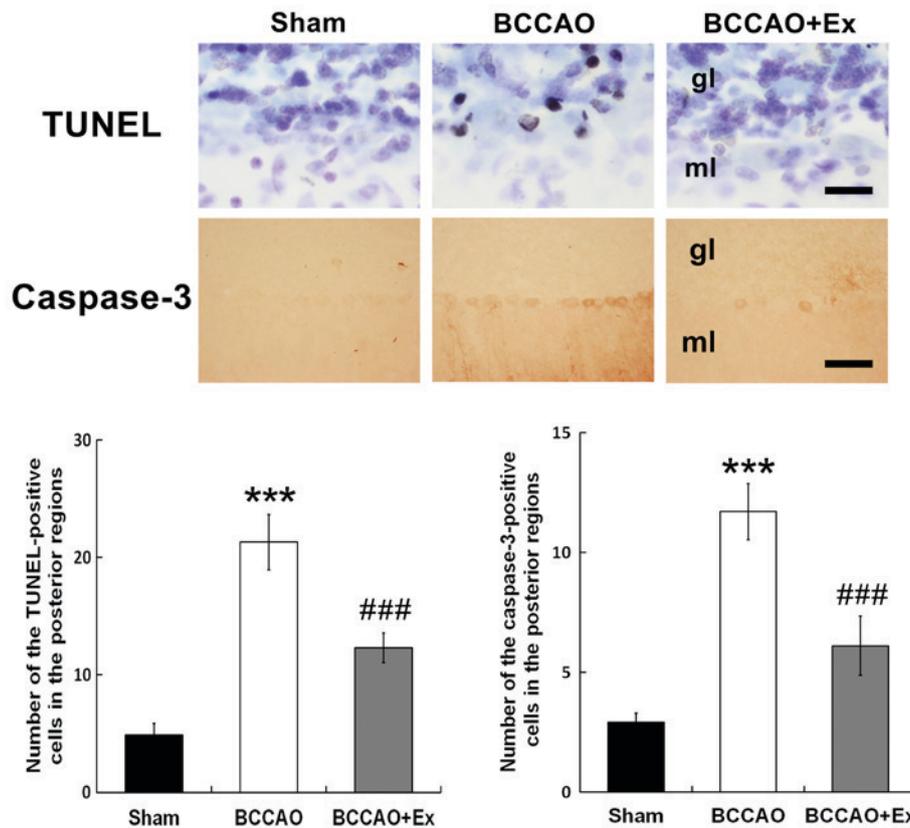


Figure 3. Effect of treadmill exercise on apoptotic cell death in the cerebellum. Representative photomicrographs of immunostaining and the numbers of TUNEL- and caspase-3-positive cells in the cerebellar vermis are presented. The scale bars represent 10 and 50 μm for TUNEL and caspase-3 staining images, respectively (blue staining: Nissl substance stained by Cresyl violet; black staining: TUNEL staining). Data are presented as the mean \pm standard error of the mean. *** $P < 0.001$ vs. sham group; ### $P < 0.001$ vs. BCCAO group. TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; BCCAO, bilateral common carotid arteries occlusion; Ex, exercise; gl, granular layer; ml, molecular layer.

study did not include a sham + Ex group, so it is difficult to expect the effect of exercise in normal group. Heo *et al* (21) previously revealed that treadmill exercise in normal mice did not exhibit a significant effect on short-term memory. Furthermore, an additional study demonstrated that the number of incorrect choices made in a radial 8-arm maze test by rats in a sham + Ex group was not decreased compared with the sham group (36). The present study investigated whether involuntary exercise attenuates spatial navigation impairment via inhibition of Purkinje cell loss in the cerebellum following CCH. Therefore, a sham + Ex group was not deemed necessary for inclusion in the study design.

Reactive astrocytes and microglia activation following brain injury enhance neuronal death (37,38). Reactive astrocytes result in neuroinflammation, neurotoxic function and the formation of a glial scar (39). Activation of microglial cells, the resident immune cells of the central nervous system, is an important factor involved in central nervous system injury responses and neuronal cell death (38). The results of the present study revealed that treadmill exercise decreased the levels of reactive astrocytes, and activation of microglial cells in the posterior lobe of the cerebellum are consistent with previous studies (40,41). Reactive astrocytes following brain damage have previously been demonstrated to result in increased GFAP expression in neurodegenerative diseases, such as Alzheimer's disease and ischemia (41). Activated microglial cells have been implicated in neuronal apoptosis following ischemic stroke, and enhanced

expression of Iba-1 has previously been revealed to be upregulated following transient middle cerebral artery occlusion (27). Therefore, the suppression of reactive astrocytes and microglia activation may represent the underlying neuroprotective effect of treadmill exercise against neuronal damage in the posterior lobe of the cerebellum.

Several studies have demonstrated that cerebral ischemia animal models exhibit increased apoptotic cell death in the brain (28,42). TUNEL and caspase-3-positive cells have also been revealed to be increased in ischemic brains (38). In the present study, treadmill exercise significantly decreased the levels of TUNEL- and caspase-3-positive cells in the posterior region of the cerebellum. Marques-Aleixo *et al* (43) revealed that a rat treadmill endurance training group demonstrated significantly increased expression levels of the anti-apoptotic protein Bcl-2 in the cerebellum compared with the expression levels exhibited by the sedentary group. A further study revealed that treadmill exercise reduced the rate of apoptosis in the cerebellums of autistic rats (44). The results of the present study indicated that treadmill exercise significantly decreased apoptosis in the cerebellum of rats suffering from CCH and may have a role in attenuating the loss of Purkinje cells.

Intracellular Ca^{2+} in neurons is important for neural excitability, as calcium enters Purkinje cells during action potentials following activation of voltage-gated Ca^{2+} channels (45). Cerebral ischemia is reported to induce an increase in intracellular Ca^{2+} levels, which subsequently results in a

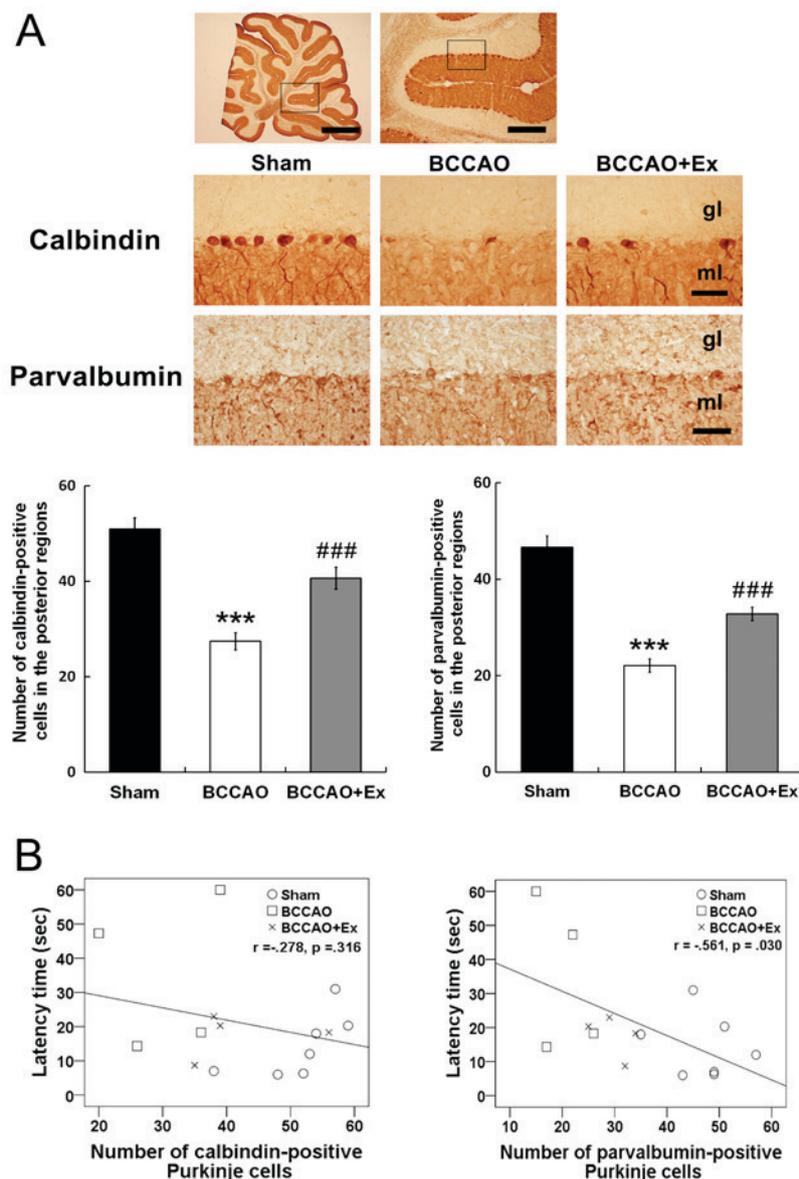


Figure 4. Treadmill exercise attenuates chronic cerebral hypoperfusion-induced Purkinje cell loss in the posterior regions of the cerebellum. (A) Representative photomicrographs of immunostaining and the number of calbindin- and parvalbumin-positive cells in the posterior lobe of the cerebellar vermis are presented. The scale bars represent 1,000 μm for the upper left image, 200 μm for the upper right image and 50 μm for the lower calbindin and parvalbumin staining images. (B) Pearson's correlation analysis was performed to determine the correlation between the number of calcium binding protein-positive Purkinje cells and latency time in Morris water maze tests. Data are presented as the mean \pm standard error of the mean. *** $P < 0.001$ vs. sham group; ### $P < 0.001$ vs. BCCAO group. BCCAO, bilateral common carotid arteries occlusion; Ex, exercise; gl, granular layer; ml, molecular layer.

marked increase in the activation of proteases, thus resulting in apoptotic cell death (46). Calbindin D28k and parvalbumin regulate motor coordination and sensory integration, which represent important functions of Purkinje cells (47). Thus, the regulation of intracellular Ca^{2+} levels by calcium-binding proteins is important for the neuronal viability in the brain (10). The results of the present study demonstrated that treadmill exercise significantly attenuated the loss of calbindin D28k- and parvalbumin-positive Purkinje cells following CCH. These results are consistent with a previous study that revealed that treadmill exercise attenuated the loss of calbindin D28k-positive Purkinje cells in the cerebellar vermis of rats suffering from amyloid β_{25-35} -induced Alzheimer's disease (41). Investigation into whether there was a correlation between loss of Purkinje cells and cognitive impairment revealed that there

was a significant negative correlation between the number of parvalbumin-positive Purkinje cells and spatial navigation ability. Furthermore, a marginal negative correlation was demonstrated between the number of calbindin D28k-positive Purkinje cells and spatial navigation ability, though this correlation was not significant. Consistent with these observations, a previous study investigated loss of Purkinje cells in the cerebellum by administration of OX7-saporin, and the results revealed that rats exhibited an impaired spatial navigation performance in the Morris water maze test compared with the control group (48). Thus, the results of the present study indicated that treadmill exercise decreased the loss of the Purkinje cells in the cerebellum following CCH in rats and that a negative correlation may exist between loss of Purkinje cells and spatial navigation performance.

In conclusion, the results of the present study indicate that treadmill exercise may exert a neuroprotective effect against the loss of Purkinje cells via the suppression of glial cells and apoptosis. Therefore, treadmill exercise may have potential as a therapeutic intervention strategy for the attenuation of memory impairment in patients with CCH.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

JL, JP and YK designed the experiments and the study. JL and MS looked after the animals and performed the experiments. CK participated in designing and discussing the study. JL, JP and YK wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Kyung Hee University Institutional Animal Care and Use Committee [Seoul, Korea; KHUASP (SE)-16-149].

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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