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EXERCISE ENHANCED FUNCTIONAL RECOVERY AND EXPRESSION OF GDNF AFTER PHOTOCHEMICALLY INDUCED CEREBRAL INFARCTION IN THE RAT

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ABSTRACT

Exercise has been considered to affect the functional recovery from central nervous damage. Neurotrophic factors have various effects on brain damage. However, the effects of exercise for expression of GDNF on functional recovery with brain damage are not well known. We investigated the difference in functional recovery between non-exercise and beam-walking exercise groups, and the expression of GDNF in both groups after photochemical infarction.
Adult male Wistar rats (N = 64) were used. Animals were divided into two groups: non-exercise (N = 35), and beam-walking exercise (N = 29). All rats underwent surgical photochemical infarction. The rats of the beam-walking group were trained every day to walk on a narrow beam after a one-day recovery period and those of the non-exercise group were left to follow a natural course. Animals were evaluated for hind limb function every day using a beam-walking task with an elevated narrow beam. The number of GDNF-like immunoreactive cells in the temporal cortex surrounding the lesion was counted 1, 3, 5, and 7 days after the infarction. Functional recovery of the beam-walking exercise group was significantly earlier than that of the non-exercise group. At 3 days after infarction, the number of GDNF-positive cells in the temporal cortex surrounding the infarction was significantly increased in the beam-walking exercise group compared with that in the non-exercise group. In the exercise group, motor function was remarkably recovered with the increased expression of GDNF-like immunoreactive cells. Our results suggested that a rehabilitative approach increased the expression of GDNF and facilitated functional recovery from cerebral infarction.

Keywords: Glial cell line-derived neurotrophic factor, cerebral infarction, beam-walking exercise, functional recovery, cortical plasticity, photochemical infarction

INTRODUCTION

Exercise is an important approach in the clinical rehabilitation of hemiplegic patients. Recently, there have been numerous reports published concerning the effect of exercise on central nervous disorders in ex-

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perimental models. For instance, treadmill exercise decreased lesion size and expression of caspase-3 in an intracerebral hemorrhage model (Lee et al., 2003). Some previous studies suggested that functional recovery after stroke could be improved by complex motor training rather than simple repetitive exercise (Jones et al., 1999; Ding et al., 2002, 2004). Physical activity in an enriched environment substantially improves sensorimotor function outcome after brain injury (Johansson, 1996; Johansson and Ohlsson, 1996). In addition, previous studies suggested that brain-derived neurotrophic factor (BDNF) has a role in plasticity following cerebral infarction. Post-ischemic intravenous BDNF treatment improves functional motor recovery after photo thrombotic stroke and induces widespread neuronal remodeling (Schäbitz et al., 2004). Improvements in motor behavior index were found with an increasing level of BDNF and the level of trkB proteins in contralateral hemisphere was increased by treadmill exercise (Kim et al., 2005). However, a lot of neurotrophic factors exist besides BDNF. Glial cell line-derived neurotrophic factor (GDNF) is another potent neuroprotective factor produced chiefly by astrocytes. Intracerebroventricular and intraparenchymal administrations of GDNF reduced infarct sizes induced by transient middle cerebral artery (MCA) occlusion (Wang et al., 1997). However, few reports have described the relationship between functional recovery and GDNF after cerebral infarction. We have reported on the expression of GDNF in an accelerated phase of functional recovery in a rat model of cerebral infarction (Horinouchi et al., 2007). However, the relationships among exercise, functional recovery, and the expression of GDNF protein have to be investigated. The present study investigates the expression of GDNF protein with functional recovery upon exercise after photochemically induced cerebral infarction.

MATERIALS AND METHODS

Animals were maintained at a constant temperature under a 12:12 h light:dark cycle and allowed free access to food and water until the experiments began. This study was carried out in accordance with the Guidelines for Animal Experimentation of the Graduate School of Medical and Dental Sciences, Kagoshima University, Japan.

Initially, to investigate the effects of exercise on functional recovery after photochemically induced cerebral infarction, 22 adult male Wistar rats (231 ± 12 g) were used.

These animals were housed in plastic cages (60 x 40 x 25cm) with an environmentally controlled room with a 12/12 hour light-dark cycle, standard rat food and water were provided ad libitum. These animals were divided into two groups: non-exercise (n = 11) and beam-walking exercise (n = 11). All rats underwent photochemical infarction at the age of 7 weeks. Rats were deeply anesthetized with sodium pentobarbital (40 mg/kg). To induce a cerebral infarction, photochemical dye Rose Bengal (20 mg/kg) was injected into the tail vein and illuminated with green light (560 nm) through the intact cranium for 20 min (Watson et al., 1985; Dietrich et al., 1987; Schroeter et al., 1999). A green light beam of 10 mm in diameter was irradiated using an MHF-G150LR halogen lamp (Moritex, Japan) through optical fiber to the exposed skull localized to an area 6 mm lateral to the midline and 4 mm posterior to the bregma in order to induce infarction in the right sensorimotor area of the cerebral cortex.

The rats of the beam-walking group were trained for 20 min every day to walk on a narrow beam (122 cm long × 2.5 cm wide) from one day after infarction and those of the non-exercise group were left to follow a natural course. Animals were evaluated for hind limb function every day.

The authors chose an established seven-point performance scale. The locomotion of postoperative rats was evaluated using a beam-walking task with an elevated narrow

beam (122 cm long x 2.5 cm wide) (Feeney et al., 1982; Abo et al., 2006). The lowest score (a score of "1") was given if the animal was unable to traverse the beam and could not place the affected hind limb onto the horizontal surface. A score of "2" was given if the animal was unable to traverse the beam but placed the affected hind limb on the horizontal surface of the beam and maintained balance. A score of "3" was given if the animal traversed the beam while dragging the affected hind limb. A score of "4" was given if the animal traversed the beam and at least once placed the affected hind paw on the horizontal surface of the beam. A score of "5" was given if the animal used the affected limbs in fewer than half of its steps along the beam. The highest score (a score of "7") was given if the animal traversed the beam normally with no more than two foot slips. The day prior to the photochemical procedure, animals in all groups were given a single beam-walking test to confirm that their performance merited a score of 7. The performance of each animal was evaluated by two different observers. In the post-infarct portion of the study, rats that achieved a score of "1" postoperatively were used. The performance score was evaluated daily. Days on which a score of "7" was achieved by the non-exercise group were compared with those of beam-walking group using the unpaired-sample

Student's t test. Statistically significant differences were considered when the P value was less than 0.05.

To investigate the expression of GDNF protein, 42 adult Wistar rats (232 ± 9.5 g) were used. At various days (non-exercise group; 1, 3, 5, and 7 days; n = 6 per day, beam-walking group; 3, 5, and 7 days; 6 animals for each point) after photochemical infarction, the brains were removed under deep anesthesia.

Animals were perfused transcardially with 300 ml of 4 % paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 under deep anesthesia. The brains were removed and post-fixed in perfusion fixative overnight and then left in 20 % buffered sucrose at 4°C. Coronal sections (10 µm thick) were cut with a freezing microtome. The sections prepared as described earlier were stained using the peroxidase-antiperoxidase (PAP) technique. The sections were incubated for 15 min in 0.1 % hydrogen peroxide (Santoku Chemical Industry, Japan) in ethanol in order to inactivate the endogenous peroxidase activity, and rinsed three times with phosphate-buffered saline (PBS) at a pH of 7.4 for 5 min. The sections were then incubated for 1 h in 10 % normal swine serum (DAKO A/S, Denmark) in PBS to block the nonspecific binding of GDNF antiserum to the tissue. Sections were incubated for 24 h with the primary GDNF antibody (final antibody dilution 1:200, Santa Cruz Biotechnology, USA), rinsed for 5 min with PBS three times, and incubated for 1 h with the swine anti-rabbit secondary antibody (dilution 1:100, DAKO A/S, Denmark). The sections were then incubated for 1 h with PAP (1:100, DAKO A/S, Denmark) and rinsed three times with PBS for 5 min. Sections were washed twice for 5 min in 0.05 M Tris–HCl buffer at pH 7.6. This was followed by a dark-violet to black color reaction with 0.02 % 3,3'-diaminobenzidine (DAB: Wako Pure Chemical Industries, Osaka, Japan) and 0.6 % nickel ammonium sulfate (Nacalai Tesque, Kyoto, Japan) in 0.05 M Tris–HCl buffer at pH 7.6 containing 7 µl of H_2O_2 (nickel-enhanced DAB reaction) for 9 min at room temperature. The sections were rinsed twice for 5 min in 0.05 M Tris–HCl buffer at pH 7.6, dehydrated through a series of ethanols (90 %, 95 %, 99 %), cleared in xylene, and mounted with coverslips.

The number of GDNF-like immunoreactive cells was counted with a computer-assisted image analyzer using the public domain software Scion Image program (Scion Corp., Frederick, MD, USA). The number of immunoreactive cells in the temporal cortex surrounding the infarction was measured. One-way analysis of vari-

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ance (ANOVA) was used to compare the numbers of GDNF immunoreactive cells in non-exercise and beam-walking exercise groups over time. Post hoc analyses were performed with the Least Significant Difference (LSD) test when the F ratio for the ANOVA was significant. Statistical evaluations were performed using Dr. SPSS II for Windows 11.0.1J software package (SPSS Japan Inc., Tokyo, Japan). A *P* value < 0.05 was considered to be statistically significant.

RESULTS

Paralysis after infarction was evaluated using the beam-walking task. In this study, rats that achieved a score of "1" 24 h after the procedure were used. Functional recoveries of non-exercise and beam-walking groups evaluated with the beam-walking task are shown (Figure 1). Data are shown as mean ± standard error (S.E.). Days on which a score of "7" was achieved by the non-exercise and beam-walking groups are shown (Figure 1A). Functional recovery of the beam-walking exercise group was significantly earlier than that of the non-exercise group (P < 0.01). Recoveries of the beam-walking score in non-exercise and beam-walking exercise groups after the photochemical procedure are shown (Figure 1B). Initially, rats could not traverse the beam and struggled to maintain balance. Subsequently, in the beam-walking exercise group, recovery was remarkable, and the average score became "6.7" 6 days after infarction. In the beam-walking exercise group in particular, rats recovered remarkably at 2-4 days after the operation. However, in the non-exercise group, recovery was gradual and the average score became "6.7" 14 days after infarction.

GDNF-positive cells were located mainly in the cortical area surrounded the infarction. Localizations of GDNF-positive cells were almost equal within cortical layers II to VI, with limited expression of GDNF in layer I. In the beam-walking exercise group, the number of GDNF-like immunoreactive cells reached a maximum on day 3 and then decreased over time (Figure 2). In the non-exercise group, the number of GDNF-like immunoreactive cells reached a maximum at day 1 and then decreased over time. At 3 days after infarction, the number of GDNF-positive cells in the temporal cortex surrounding the infarction (446 ± 30 / mm²) of the beam-walking exercise group was significantly higher than that of the non-exercise group (384 ± 7.5 / mm²) (P < 0.05) (Figure 3). At 5 and 7 days after infarction, there was no statistically significant difference between the non-exercise group and the beam-walking exercise group.

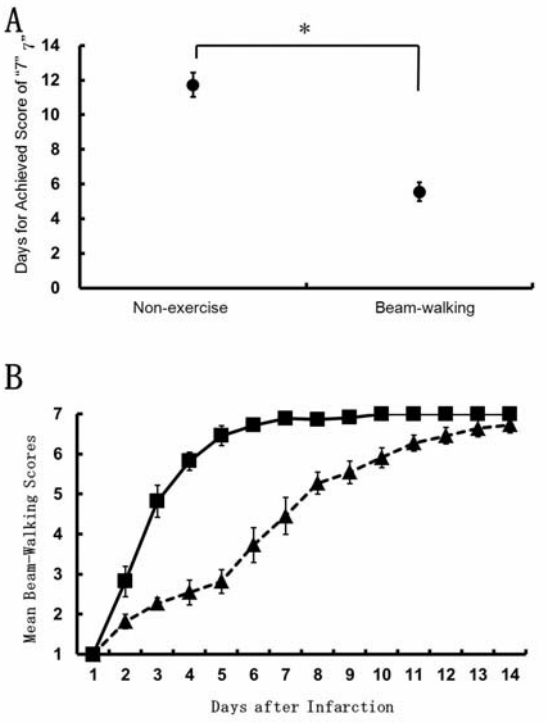

Figure 1: Mean days on which a score of "7" was achieved in non-exercise and beam-walking groups **(A)**.
*Level of significance: P < 0.01. Mean rating of beam-walking scores of rats after photochemical infarction **(B)**, triangle is non-exercise group; square is beam-walking exercise group. Values are means ± SE.

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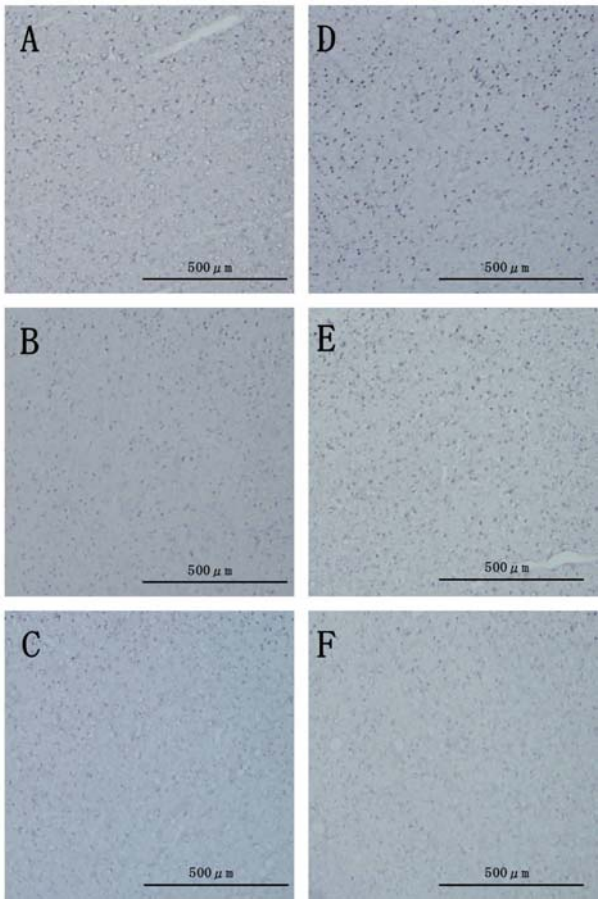

Figure 2: Representative photographs of GDNF-positive cells.
A-C represents the temporal cortex surrounding the infarction of the non-exercise group at 3 days **(A)**, 5 days **(B)**, and 7 days **(C)** after photochemical infarction. **D-F** represents the temporal cortex surrounding the infarction of the beam-walking group at 3 days **(D)**, 5 days **(E)**, and 7 days **(F)** after photochemical infarction. The scale bars are 500 μm.

DISCUSSION

In previous studies, animals exhibited significantly improved motor performance after performing various kinds of exercise.

In many studies, treadmill training was used for exercise in various contexts (Yang et al., 2003a, b; Wang et al., 2005). There have been studies comparing the quality of training with the amount of training. In other studies, experimental exercise was performed with wheel running for voluntary exercise (Kleim et al., 2002; Marin et al., 2003) and rotarod for complex motor skills training (Ding et al., 2002, 2004). An enriched environment (in a large cage with ladders, rope, a chain, and so on) promoted functional recovery after ischemic injury in rat (Biernaskie and Corbett, 2001; Risedal et al., 2002). The present results showed that the functional recovery of the beam-walking exercise group was observed significantly earlier than that of the non-exercise group. Our results indicated that the period of functional recovery was reduced by about half by the beam-walking exercise. Additionally, GDNF expression was increased in the exercise group compared with that in the control group on day 3 after infarction.

GDNF protein is a member of the transforming growth factor-β superfamily. There are few studies that have examined GDNF levels and functional recovery after cerebral infarction. However, a large number of fundamental animal studies have established its various functions. For example, focal ischemia after transient MCA occlusion induces cell proliferation, and exogenous GDNF increases the cell proliferation following focal ischemia in adult rats (Dempsey et al., 2003). Cerebral ischemia triggers an increase in gene expression of not only GDNF but also its receptors, c-Ret and GFRα-1 (Arvidsson et al., 2001). In this study, the present results showed that the number of GDNF-positive cells in the temporal cortex surrounding the infarction of the beam-walking exercise group was significantly increased compared with that in the non-exercise group. These findings suggested that the beam-walking task enhanced the expression of GDNF protein at an early stage after cerebral infarction. Interestingly, in the beam-walking exercise group, motor function was recovered remarkably and the number of GDNF-like immunoreactive cells reached a maximum at day 3. These results suggested that the expression of GDNF protein was induced by training and that functional recovery was promoted.

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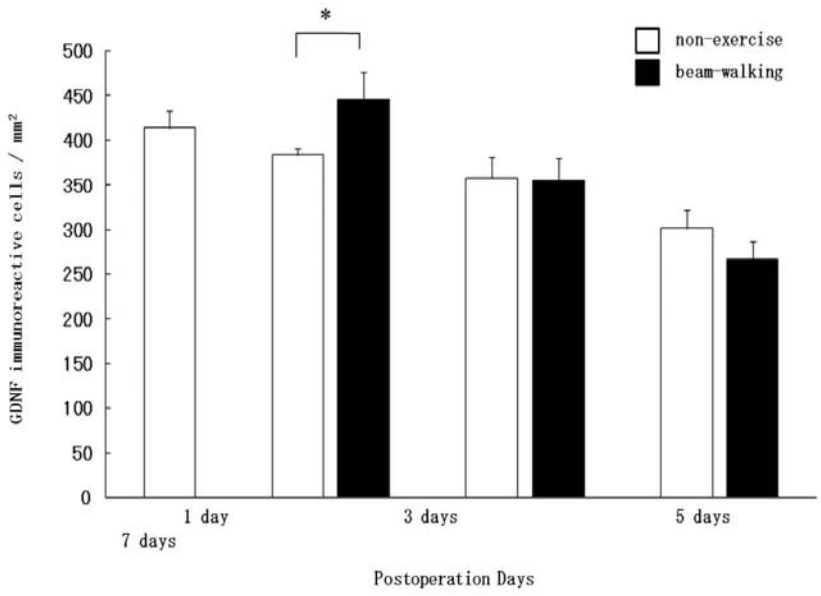

Figure 3: GDNF protein expression after photochemical infarction. Open bars represent the non-exercise group, solid bars represent the beam-walking group.
Values are means ± SE. *Level of significance: P < 0.05

Over the last decade a number of molecular mechanisms by which brain cells die, or survive, after focal cerebral ischemia have been unraveled. In the core of ischemic lesions, brain tissue undergoes necrosis; however, in penumbra, biochemical and molecular processes determine the survival of tissue. Within minutes after focal ischemia, excitotoxic mechanisms lead neurons and glia to cell death. In the following days after the onset of infarction, excitotoxic triggers contribute to the death of tissue. Such events include peri-infarct depolarization and the more delayed mechanisms of inflammation and apoptosis (Dirnagl et al., 1999, 2003). Previous studies suggested that administration of GDNF reduced infarct sizes induced by transient MCA occlusion (Wang et al., 1997). Therefore, it is possible that GDNF has a role of anti-excitotoxicity or anti-inflammation and anti-apoptosis in areas of penumbra. However, in this study, cerebral ischemia was induced by irradiation with green light. Most of the blood vessels exposed to the green light became occluded, thus abolishing all collateral circulation. Therefore, rats of photochemical infarction model have few areas of penumbra (Abo et al., 2001). In this study, the role of GDNF protein after photochemical infarction might be related to the plasticity of the brain rather than nerve protection. Recently, experimental study indicated that GDNF and its receptor GFRα-1 have a function in synapse formation in hippocampal neurons (Ledda et al., 2007).

In conclusion, this study suggested that exercise enhances functional recovery from cerebral infarction and induces increased expression of neurotorophic factor GDNF. Rehabilitation soon after cerebral infarction could enhance cortical repair and regeneration after infarction in clinical practice.

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