The Effect of Combined Therapy of Exercise and Nootropic Agent on Cognitive Function in Focal Cerebral Infarction Rat Model

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Objective To investigate the effect of combined therapy of exercise and nootropic agent on cognitive function in a focal cerebral infarction rat model.

Method Forty 10-week old male Sprague-Dawley rats were subjected to photothrombotic cerebral infarction of the left parietal lobe. All rats were randomly divided into 4 groups: group A was photothrombotic cerebral infarction rats without any treatment (n=10); group B was photothrombotic cerebral infarction rats with swimming exercise (n=10); group C was photothrombotic cerebral infarction rats with oral administration of acetyl-L-carnitine (n=10); group D was photothrombotic cerebral infarction rats with swimming exercise and oral administration of acetyl-L-carnitine (n=10). Cognitive function was evaluated using the Morris water maze test on the 1st day, and the 1st, 2nd, and 4th week after the induction of cerebral infarction. The activity of superoxide dismutase (SOD) and the level of malondialdehyde (MDA) in the hippocampus were measured. The neuronal cells of the hippocampus were histopathologically evaluated.

Results The escape latency was shorter in groups B, C, and D than in group A. However, the differences were not statistically significant at the 1st, 2nd and 4th week. The activity of SOD was the highest in group D. The level of MDA was the lowest in group D. We observed more normal neuronal cells in groups B, C, and D.

Conclusion The combined therapy of exercise and nootropic agent was helpful in ameliorating oxidative stress in the focal cerebral infarction rat model. However, the effect did not translate into improvement of cognitive function.

Key Words Cerebral infarction, Cognition, Exercise, Nootropic agents

INTRODUCTION

Stroke is an important health problem because of its high incidence and various disabilities it produces. Stroke patients display various symptoms and disabilities such as cognitive impairment, motor weakness, limitation of activities of daily living (ADLs), and affective disorder. Cognitive impairment or perceptual disorders may
prevent them from participating in a neurorehabilitation program. Therefore, it is necessary to improve the abilities of cognition and perception for successful rehabilitation.\(^1\)

The blockage or decrease of blood circulation after stroke disturbs the supply of oxygen and energy sources to neuronal cells. This can cause damage to neuronal cells and produce cognitive impairment by increasing oxidative stress. Tissue damage following cerebral ischemia results from the interaction of complex pathophysiological processes such as excitotoxicity, perivascular depolarizations, inflammation as well as apoptosis.\(^2\) The search for an effective neuroprotective drug has proved to be elusive.\(^3\) One possible reason for this is the limited drug dosages that can be given because of serious side effects.\(^4\) Many investigators have paid attention to compounds that are normally present in humans and can be administered at relatively high doses without toxicity such as coenzyme Q and Acetyl-L-carnitine (ALC).\(^5\) ALC is a naturally occurring substance\(^6\) and is synthesized in the human brain, liver, and kidney by the enzyme ALC transferase.\(^6\) As ALC can cross the blood-brain barrier more efficiently than L-carnitine, it has been widely used in animal and human studies.\(^7\) ALC has been shown to be beneficial in treating Alzheimer’s disease.\(^8\) ALC is considered to be safe and without significant side effects.\(^9\)

Exercise promotes good health of the cardiovascular, pulmonary, and musculoskeletal systems. Recently, exercise has emerged as a key lifestyle factor in the prevention of dementia and age-related cognitive decline.\(^8,10\) Exercise can be a non-pharmacological and natural strategy for successful cognitive rehabilitation.\(^11\) Both ALC and exercise have positive effects on cognitive function.\(^8,11\) However, there are few reports on whether the combined therapy of exercise and nootropic agent reveals an add-on effect on cognitive function in focal cerebral infarction. Therefore, we aimed to investigate the effect of combined therapy of exercise and nootropic agent on cognitive function in a focal cerebral infarction rat model.

**MATERIALS AND METHODS**

**Animals**

All animal experiment protocols were carried out in accordance with the guidelines of the Chonnam National University Animal Care and Committee. Forty male Sprague-Dawley rats, aged 10 weeks old, weighing 300-350 grams (Samtako, Osan, Korea) were used. They were housed at a temperature of 23.0±1.0°C and a humidity of 50±5% with a 12 hours light-dark cycle and had free access to food and water *ad libitum*. All rats were pretrained on the Morris water maze (MWM) and subjected to photothermal cerebral infarction of the left parietal lobe. All rats were randomly divided into 4 groups: group A was photothermal rats without any treatment (n=10); group B was photothermal rats with swimming exercise (n=10); group C was photothermal rats with oral administration of ALC (Nicetile\(^\circledR\), Seoul, Korea) (n=10); group D was photothermal rats with swimming exercise and oral administration of ALC (n=10).

**Methods**

**Photothermal cerebral infarction rat model:** Photothermal cerebral infarction was induced based on the methods described by Watson and colleagues.\(^12\) Briefly, each rat was anesthetized with 5% isoflurane and maintained with 2% isoflurane in 70% nitrous oxide and 30% oxygen mixture during the surgical procedure. The operation was performed on a homeothermic plate (Harvard Apparatus, South Natick, USA) to maintain body temperature at 37.5±0.5°C. The rat was placed into a stereotactic frame (Stoelting, Wood Dale, USA) in the prone position. After incision and exposure of the scalp, we injected Rose Bangal dye (Sigma-Aldrich Co, St. Louis, USA) 50 mg/kg into the left femoral vein, and light exposure with KL 1500 LCD (SCHOTT, Hattenbergstrasse, Germany) was given on the left parietal lobe area about 3300 K and 150 W for 10 minutes.

**Exercise program and administration of medicine:** After the induction of photothermal cerebral infarction, ALC of 75 mg/kg was administrated to group C and group D via oral feeding tube for 5 consecutive days. The first administration was performed 12 hours after the induction of photothermal cerebral infarction.

Exercise started from the next day after the induction of photothermal cerebral infarction. Group B and D did free swimming exercise, 20 minutes per day, 5 days a week, for 4 weeks. Group D did swimming exercise after the administration of ALC for a week. Swimming exercise was performed in a round water tank, filled with 30 cm height of 21°C water.

**Morris water maze test:** Spatial learning and memory were assessed by the MWM test based on the method described by Morris et al.\(^13\) In brief, the tests were con-
ducted in a circular metal pool (diameter, 184 cm; height, 60 cm), filled with water and maintained at 20-25°C. The pool was virtually divided into four equal quadrants, and one of them was allocated as the target quadrant. Visual cues were placed at the perimeter of each quadrant. A circular escape platform (diameter, 10 cm; height, 38 cm) was placed in the center of the target quadrant. The water level in the pool was adjusted so that the platform was submerged 1 cm below the surface of the water.

The animals were placed into the water maze facing the maze wall at random entry points, which were equally distributed around the perimeter of the maze. After finding the platform, the rats were allowed to remain there for 10 seconds until the next trial. In the case that the rat could not find the hidden platform within 120 seconds, the rat was guided to the platform and allowed to stay there for 15 seconds. Then, the rat was displaced from the pool, dried and placed back in its holding bin for a period of 5 minutes, after which the second trial was conducted. All groups were acclimated to the above mentioned pre-training for 3 consecutive days right before the induction of phototothrombotic cerebral infarction. After the swimming exercise program, MWM test was performed to evaluate spatial memory for three consecutive days. In this trial, rats had to find a platform within 300 seconds. The time to reach the platform (escape latency) and the length of the swim path were recorded automatically by a video tracking system, Ethovision Color-Pro® (Nodulus, Wageningen, The Netherlands).

Biochemical analysis: The oxidant-antioxidant status of the rat brain was assessed by determining the level of malondialdehyde (MDA) and the activity of superoxide dismutase (SOD). Lipid peroxidation was determined by measuring the level of MDA, which is considered to be a standard marker for oxidative lipid damage of tissues. The activity of SOD indicates the level of anti-oxidation in tissues and organs.14,15

The left hippocampal tissues were collected and were ultrasonically homogenized in ice-cold 0.9% NaCl to produce 1:9 (g : L) homogenates. After centrifugation at 13,000 rpm for 10 min at 4.0°C, the supernatant was collected for measurements of the activity of SOD (Cayman Chemical Company, Ann Arbor, USA) and the level of MDA (OxisResearch™, Portland, USA). SOD activity was measured by the method of Sun et al.14 In brief, 500 μL aliquot of hippocampal tissue, together with 50 μmol/L xanthine and 2.5 μmol/L xanthine oxidase in 50 μmol/L potassium phosphate buffer were mixed and incubated at 37°C for 40 min, then nitro blue tetrazolium (NBT) was added. The product nitrite produced by the oxidation of oxyamine was measured by monitoring the absorbance at 460 nm with microplate readers and software (Bio-Tek, Winooski, USA). SOD activity was expressed as U/mg protein.

The MDA levels were measured by the method of Draper and Hadley.15 Briefly, 1 mL of 20% thiobarbituric acid (TBA) and 1 mL of 1% TBA reagent were added to 500 μL aliquot of hippocampal tissue, then mixed and incubated at 45°C for 60 min. After cooling on ice, samples were centrifuged at 13,000 rpm for 10 min at 4.0°C, and then the absorbance of the supernatant was read at 586 nm with microplate readers and software (Bio-Tek, Winooski, USA). The values obtained were compared with a series of standard solutions of MDA. Results were expressed as nmol/mg tissue.

Histopathological study: Each rat was transcardially perfused with 100 mL of saline and followed by 400 mL of 0.1 mol/L phosphate buffer (pH 7.4) with 4% paraformaldehyde. The brains were removed from the skull and postfixed overnight in the same fixed solution at 4.0°C. After paraffin embedding, coronal and serial sections (2 μm thick) encompassing brain tissues were taken from each brain. Light microscopic examination (Nikon, Tokyo, Japan) was carried out at a magnification of ×200 and the number of neurons at the CA3 region of the left hippocampus was determined per square millimeter by using a standardized ocular grid. The counting was made by a person blinded to the details of the experiment. The criteria used to distinguish normal from abnormal cells were as follows: (1) cells with regular-shaped soma with clear cytoplasm and intact nuclear membranes were determined as normal and (2) cells with irregular shape, with or without chromatin compaction or a nuclear membrane, DNA marginalization and darkened cytoplasm, or blackened cytoplasm with extensive vacuolarization, were considered to be abnormal.16

Statistical analysis: Statistical analyses were carried out with SPSS software program for Windows (version 13.0, Chicago, USA). Data were presented as the mean±standard deviation (SD). Repeated measures ANOVA, Kruskall-Wallis test and Wilcoxon Signed Ranks test were used for the data analyses. The differences were considered to be significant when the p-value was less than 0.05.
RESULTS

Behavioral test
On the next day after the induction of cerebral infarction, escape latencies were 47.2±20.1 sec in group A, 47.6±28.5 sec in group B, 44.8±46.6 sec in group C, and 46.1±28.5 sec in group D. At the 1st week, escape latencies were 43.6±21.8 sec in group A, 40.3±30.2 sec in group B, 40.6±45.9 sec in group C, and 38.5±29.7 sec in group D. At the 2nd week, escape latencies were 41.1±14.9 sec in group A, 39.6±26.7 sec in group B, 39.5±30.5 sec in group C, and 37.8±27.9 sec in group D. At the 4th week, escape latencies were 40.2±27.8 sec in group A, 38.3±17.6 sec in group B, 37.7±20.0 sec in group C, and 35.9±23.4 sec in group D. The escape latency was shorter in groups B, C, and D than in group A. However, the differences were not significant at the 1st, 2nd, and 4th week (p>0.05) (Fig. 1). There were no significant differences in the length of swim path among all groups at the 1st, 2nd, and 4th week (p>0.05).

Biochemical analysis
1) The activity of SOD was significantly different among all groups (p=0.000). It was more elevated in groups B, C, and D than in group A (p=0.001, p=0.001, p=0.001). Group D was more elevated than group B and groups C (p=0.018, p=0.002) (Fig. 2).
2) The level of MDA was significantly different among all groups (p=0.002). It was more decreased in groups B, C, and D than in group A (p=0.009, p=0.002, p=0.007). However, there were no significant differences among groups B, C, and D (p>0.05) (Fig. 3).

Histopathological study
Hematoxylin & Eosin staining showed severe cortical damage of the left parietal lobe in all experimental rats (Fig. 4). The number of normal neuronal cells in the CA3 region of the left hippocampus was significantly different among all groups (p=0.017) (Table 1). It was more increased in groups B, C, and D than in group A (p<0.05). However, there were no significant differences among all groups at the 1st, 2nd, and 4th week (p>0.05).
**DISCUSSION**

Excitotoxicity and oxidative stress are toxic events commonly associated with neuronal cell death in neurodegenerative diseases. Any medicine, which has antioxidant defense and mitochondrial reductive capacities, will be a valuable tool to preserve neuronal cells in toxic conditions. Many kinds of medicines were reported to be neuroprotective after ischemic brain injury in animal studies. However, their clinical usage was limited because of drug side effects or lack of effectiveness in human trials. It was reported that administration of ALC in the cerebral ischemia rat model could improve spatial memory. ALC is a naturally occurring substance without serious side effects and is a promising candidate in cognitive rehabilitation. Recent studies suggested that ALC improves spatial working memory, reduces oxidative...
tive stress, and inhibits the apoptotic cascade induced by hypoxia and hippocampal neuronal cell death in response to glutamate exposure. The acetyl component of ALC is metabolized in the brain, thereby promoting oxidative cerebral energy production and minimizing anaerobic glycolysis and lactic acidosis. ALC protects against oxidative stress indirectly through its amelioration of tissue lactic acidosis, a condition that promotes the formation of reactive oxygen species (ROS). ALC also protects against excitotoxic cell death directly through antagonism of glutamate receptors. ALC facilitates the uptake of acetyl CoA into the mitochondria during fatty acid oxidation and enhances acetylcholine production. It also increases cholinergic synaptic transmission and improves cognitive function in aging rats. In the present study, ALC administration alone resulted in slight shortening of escape latency, elevated activity of SOD, decreased level of MDA, and higher numbers of normal hippocampal neuronal cells compared to the control group. ALC may lead to elevated activity of SOD, which is involved in the direct elimination of free radicals produced by oxidative stress. Our results suggest that ALC may improve cognitive function by decreasing oxidative stress and preventing hippocampal neuronal cell death.

Exercise has positive effects on the improvement of cognitive function, especially learning and memory. There are a few studies on the effects of exercise on oxidative damage. However, the findings are conflicting. A single bout of exercise induced the formation of ROS and oxidative stress. However, regular exercise increased resistance against ROS induced lipid peroxidation, and the activity of the proteasome complex, and decreased the

Fig. 5. A greater number of normal neuronal cells are observed in groups B, C, and D (CA3 region of hippocampus, H&E stain, ×200)
accumulation of oxidative proteins and DNA damage.\textsuperscript{29} The increase in the activity of antioxidant enzymes in the brain as a response to regular physical exercise is most probably due to the excess formation of free radicals.\textsuperscript{27,30} Radak et al.\textsuperscript{31} suggested that regular exercise improved cognitive function in parallel with the decreased accumulation of oxidized proteins. In this study, the activity of SOD was increased in the three treatment groups. Especially, swimming exercise increased the activity of SOD more than ALC administration alone. The activity of SOD was most increased in the combined therapy group, which showed an add-on effect. This revealed that administration of ALC, swimming exercise, and combined therapy would protect the brain from free radicals after ischemic brain injury and suggested that the similar mechanisms of exercise and ALC would have a positive add-on effect on cognitive function.

Exercise enhances neurogenesis in the hippocampal dentate gyrus, which may contribute to cognitive improvement.\textsuperscript{32} The effects on cognitive function according to the type of exercise are controversial.\textsuperscript{33,34} Ra et al.\textsuperscript{33} demonstrated that swimming as well as treadmill running increased neurogenesis in the dentate gyrus of rats. However, van Praag et al.\textsuperscript{34} reported that swimming exercise did not affect cell proliferation. In the present study, a higher number of normal neuronal cells were seen in the three treatment groups compared with the control group. Swimming exercise would be helpful for hippocampal neurogenesis. However, the add-on effect was not evident in the combined therapy group in terms of hippocampal neurogenesis.

CONCLUSION

One of the most important findings was that both regular exercise and ALC, and moreover, the combined effects of these two natural treatments, decreased oxidative stress and increased hippocampal neurogenesis. However, these changes did not translate into improvement of cognitive function in the photothrombotic cerebral infarction rat model. Further studies using a large sample size would be needed to demonstrate the potentially important roles in natural and physiological factors, such as natural substances and regular exercise, have in cognitive function.

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