

Treadmill exercise ameliorates Alzheimer disease-associated memory loss through the Wnt signaling pathway in the streptozotocin-induced diabetic rats

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Diabetes mellitus is considered as a risk factor for Alzheimer disease. The aim of the present study was to evaluate the possibility whether treadmill exercise ameliorates Alzheimer disease-associated memory loss in the diabetes mellitus. For this study, the effects of treadmill exercise on short-term memory and spatial learning ability in relation with Wnt signaling pathway were evaluated using the streptozotocin (STZ)-induced diabetic rats. Diabetes was induced by intraperitoneal injection of STZ. Step-down avoidance task and 8-arm radial maze test were performed for the memory function. Immunohistochemistry for 5-bromo-2'-deoxyridine (BrdU) and doublecortin (DCX) and Western blot for Wnt3 and glycogen synthase kinase-3 β (GSK-3 β) were conducted. The rats in the exercise groups were made to run on the treadmill for 30 min per one day, 5 times a week, during 12 weeks. In the present results, short-term memory and spatial learning ability were deteriorated by in-


duction of diabetes. Treadmill exercise improved short-term memory and spatial learning ability in the diabetic rats. The numbers of BrdU-positive and DCX-positive cells in the hippocampal dentate gyrus were decreased by induction of diabetes. Treadmill exercise increased these numbers in the diabetic rats. Wnt3 expression in the hippocampus was decreased and GSK-3 β expression in the hippocampus was increased by induction of diabetes. Treadmill exercise increased Wnt3 expression and suppressed GSK-3 β expression in the diabetic rats. The present study suggests that treadmill exercise alleviates Alzheimer disease-associated memory loss by increasing neurogenesis through activating Wnt signaling pathway in the diabetic rats.

Keywords: Diabetes mellitus, Alzheimer disease, Exercise, Wnt signaling pathway, Glycogen synthase kinase 3 beta

INTRODUCTION

Diabetes mellitus has been considered as a risk factor for inducing Alzheimer disease. Previous studies reported that glucose intolerance and impairment of insulin secretion are related with Alzheimer disease (Luchsinger et al., 2004; Rönnekaa et al., 2008). Alzheimer disease is the most common type of dementia and this disorder is characterized by loss of memory function and impairment of cognition (Querfurth and LaFerla, 2010). Because of the uncertain etiology and unclear pathology of Alzheimer disease, effective treatment for this disorder is not firmly established yet.

Dao et al. (2014) suggested that treadmill exercise exerted beneficial effects on cognitive and noncognitive functions in this Alzheimer disease rats. There are many evidences that exercise improves cognition and decreases the risk of developing to the dementia (Larson et al., 2006; Podewils et al., 2005). Intlekofer and Cotman (2013) showed that exercise improved brain function and reduced Alzheimer disease-related deterioration of hippocampal function. Physical exercise increases hippocampal neurogenesis and this increment of neurogenesis is implicated in the improvement of memory function (Clelland et al., 2009; Kim et al., 2014; Shin et al., 2013).

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Immunohistochemistry for 5-bromo-2'-deoxyridine (BrdU) has widely been used for the detection of new born neurons (Kim et al., 2010; Kim et al., 2014). Doublecortin (DCX) represents newly formed neurons in the hippocampal dentate gyrus (Friocourt et al., 2007) and DCX is associated with structural plasticity in the adult mammalian brain (Kim et al., 2013).

The effects of exercise on hippocampal function are associated with Wnt signaling pathway (Bayod et al., 2014). Wnt signaling pathway controls synaptic plasticity and regulates long-term potentiation (Chen et al., 2006). Wnt signaling pathway regulates neuronal development, differentiation, and synaptogenesis, and Wnt signaling is involved in the disease processes (Clevers and Nusse, 2012). Wnt signaling pathway inhibits glycogen synthase kinase-3 β (GSK-3 β) activity, and Wnt signaling pathway is dysregulated in the neurodegenerative diseases (Berwick and Harvey, 2012). Negative modulation of the Wnt pathway is causally related to the processes of Alzheimer disease, brain ischemia, and temporal lobe epilepsy (Caraci et al., 2008). As Wnt signaling pathway is also implicated in the cognition (Pan et al., 2016), Wnt signaling pathway is appeared as the new therapeutic target for the delay or prevention of Alzheimer disease.

The aim of the present study was to evaluate the possibility whether treadmill exercise ameliorates Alzheimer disease-associated memory loss in the diabetes mellitus. For this study, the effects of treadmill exercise on short-term memory and spatial learning ability in relation with Wnt signaling pathway were evaluated using the streptozotocin (STZ)-induced diabetic rats.

MATERIALS AND METHODS

Animals and treatments

The experimental procedures were performed in accordance with the animal care guidelines of the National Institutes of Health and the Korean Academy of Medical Sciences. Forty male Sprague-Dawley rats weighing 200 ± 10 g (7 weeks in age) were used in this experiment. Rats were housed under controlled temperature ($20^\circ\text{C} \pm 2^\circ\text{C}$) and lighting conditions (7:00 a.m. to 19:00 p.m.) with food and water made available *ad libitum*. Animals were randomly divided into four groups ($n = 10$ in each group): control group, exercise group, diabetes group, and diabetes and exercise group. All rats received 50 mg/kg BrdU (Sigma Chemical Co., St. Louis, MO, USA) intraperitoneally, once a day for 5 days, starting 3 days after STZ injection.

Induction of diabetes

To induce diabetes, a single intraperitoneal injection of STZ (50 mg/kg, dissolved in 0.01-M citrate buffer at pH 4.5; Sigma Chemical Co.) was given to each animal, as the previously described method (Kim et al., 2015). Blood glucose levels were determined 3 days after STZ injection using a blood glucose tester (Arkray, Kyoto, Japan). Only the animals with blood glucose levels of 300 mg/dL or higher were used in the diabetes groups.

Treadmill exercise protocol

The rats in the exercise groups were made to run on the treadmill for 30 min per one day, 5 times a week, during 12 weeks. The workload of the exercise consisted of running at a speed of 3 m/min for the first 5 min, 5 m/min for the next 5 min, and then 8 m/min for the last 20 min, with 0% grade of inclination.

Step-down avoidance task

In order to evaluate the short-term, the latency of the step-down avoidance task was determined on the last day of treadmill exercise, as the previously described method (Kim et al., 2014). The rats were trained in a step-down avoidance task in which they were positioned on a 7×25 -cm platform with a height of 2.5 cm, and then allowed to rest on the platform for 1 min. The platform faced a 42×25 -cm grid of parallel 0.1-cm caliber stainless steel bars, which were spaced 1 cm apart. In the training session, the animals received a 0.5-mA scramble foot shock for 2 sec immediately upon stepping on the platform. Latency was assessed at 2 hr after training session. The interval of rats stepping down and placing all 4 paws on the grid was defined as the latency in the step-down avoidance task. Any latency over 300 sec was counted as 300 sec.

8-arm radial maze test

The 8-arm radial maze apparatus was used to evaluate spatial learning ability, as the previously described method (Shin et al., 2016). The rats were water deprived for 24 hr before the training sessions and the real test. The maze consisted of an octagonal arena with eight radiating arms. The arms were 50 cm in length and 10 cm in width. The maze was placed 100 cm above the floor. Water cups (water well: 3 cm in diameter and 1 cm in depth) placed at the ends of the arms. Consecutive training was performed three times before the real test for spatial learning ability. The test was conducted on the one day before the last treadmill exercise. The test was completed when the rat found water in all eight arms or when 5 min had elapsed. Reentry into the previously visited arms was counted as an error. Correct number was de-

defined as the count of correct choice before the first error, and the error number was defined as the count of error during the test completion.

Tissue preparation

To begin the sacrificial process, the animals were fully anesthetized using Zoletil 50 (10 mg/kg intraperitoneally; Vibac Laboratories, Carros, France). After a complete lack of response was observed, the rats were transcardially perfused with 50-mM phosphate-buffered saline (PBS) and subsequently fixed with freshly prepared 500-mM phosphate buffer (pH, 7.4) containing 4% paraformaldehyde. The brains of the rats were removed and fixed in the same fixative overnight and then transferred into a 30% sucrose solution for cryoprotection. Serial coronal sections of 20- μ m thickness were obtained using a freezing microtome (Leica, Nussloch, Germany).

Immunohistochemistry for BrdU

For the detection of newly generated cells in the hippocampal dentate gyrus, BrdU immunohistochemistry was performed according to a previously described method (Kim et al., 2014). The brain sections were first permeabilized by incubation in 0.5% Triton X-100 in PBS for 20 min, then pretreated in 50% formamide-2 \times standard saline citrate at 65°C for 2 hr, denatured in 2 N HCl at 37°C for 30 min, and rinsed twice in 100-mM sodium borate (pH, 8.5). Thereafter, the sections were incubated overnight at 4°C with mouse monoclonal anti-BrdU antibody (1:600; Roche, Mannheim, Germany). Then, the sections were washed 3 times with PBS and incubated for 90 min with the biotinylated mouse secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA). Then, the sections were incubated with avidin-peroxidase complex (1:100; Vector Laboratories). For visualization, the section were incubated in 50-mM Tris-HCl (pH, 7.6) containing 0.02% 3,3-diaminobenzidine (DAB), 40-mg/mL nickel chloride and 0.03% hydrogen peroxide for 5 min. After BrdU-specific staining, counter-staining was performed on the same sections using a mouse monoclonal antineuronal nucleic antibody (1:300; Chemicon international, Temecula, CA, USA). The sections were washed three times with PBS and incubate for 1 hr with a biotinylated anti-mouse secondary antibody. For staining, the sections were incubated in a reaction mixture consisting of 0.02% DAB and 0.03% H₂O₂ for 5 min. The sections were finally mounted onto gelatin-coated slides. The slides were air dried overnight at room, and coverslips were mounted with Permount (Fisher Scientific Inc., New Jersey, NJ, USA).

Immunohistochemistry for DCX

DCX immunohistochemistry was assessed using a previously described method (Shin et al., 2013). The sections were treated with 3% H₂O₂ for 30 min at room temperature. Next, the sections were blocked with 10% normal rabbit serum in PBS with 1% bovine serum albumin and 0.2% Triton X-100 for 1 hr and incubated with goat anti-DCX antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After washing 3 times, the sections were incubated with biotinylated goat secondary antibody (1:200; Vector Laboratories) for 1 hr, followed by incubation for 1 hr with avidin-peroxidase complex (1:100; Vector Laboratories). For immunostaining, the sections were visualized in 50-mM Tris-HCl (pH, 7.6) containing 0.03% DAB and 0.03% H₂O₂. Then, the tissue samples were rinsed with PBS three times and mounted on gelatin-coated slides. The slides were dehydrated through alcohol and coverslipped with Permount (Fisher Scientific Inc.).

Western blot analysis

Western blot was conducted according to the previous method (Kim et al., 2010). Protein extracts from brain tissue were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein separation was performed using 10% polyacrylamide with 0.05% bis-acrylamide. Proteins were then transferred to nitrocellulose and the blots were probed with anti-Wnt3 rabbit polyclonal antibody (1:1,000, Cell Signaling Technology Inc., Danvers, MA, USA), anti-GSK-3 β rabbit polyclonal antibody (1:1,000, Santa Cruz Biotechnology). Peroxidase anti-rabbit IgG (1:5,000, Vector Laboratories) was used as the secondary antibody. Immunoreactivity was detected by enhanced chemiluminescence detection kit (Santa Cruz Biotechnology).

Data analysis

The area of the hippocampal dentate gyrus was measured with the Image-Pro Plus image analysis system (Media Cybernetics Inc., Silver Spring, MD, USA). The numbers of BrdU-positive and DCX-positive cells in the hippocampal dentate gyrus were counted hemilaterally under a light microscope (Olympus, Tokyo, Japan), and these numbers were expressed as the numbers of cells per mm² in the hippocampal dentate gyrus. For confirming the expressions of Wnt3 and GSK-3 β , the detected bands were calculated densitometrically using Molecular Analyst, version 1.4.1 (Bio-Rad, Hercules, CA, USA).

Differences among the groups were evaluated using IBM SPSS Statistics ver. 21.0 (IBM Co., Armonk, NY, USA). For the com-

parison among the groups, one-way analysis of variance was performed followed by Duncan *post hoc* test. All values are expressed as the mean \pm standard error of the mean. Statistically significant differences were established at $P < 0.05$.

RESULTS

Effect of treadmill exercise on the body weight and blood glucose level

Body weight changes are presented in Table 1. Body weight in the diabetes group was decreased 2 weeks after STZ injection compared to the control group. Blood glucose levels are presented in Table 2. Blood glucose level in the diabetes group was increased 2 weeks after STZ injection compared to the control group. Blood glucose level was decreased in the diabetes and exercise group at 10 weeks after STZ injection compared to the diabetes group.

Effect of treadmill exercise on short-term memory

Latency in the step-down avoidance task is presented in Fig. 1. Latency in the step-down avoidance task was decreased by induction of diabetes ($P < 0.05$) and treadmill exercise increased latency in the diabetic rats ($P < 0.05$).

Effect of treadmill exercise on spatial learning ability

Correct number and error number in the 8-arm radial maze test are presented in Fig. 2. The rats in the diabetes group showed

lower correct number and higher error number compared to the control group ($P < 0.05$). However, treadmill exercise increased the correct number and error number in the diabetic rats (Fig. 2).

Effect of treadmill exercise on the number of BrdU-positive cells in the hippocampal dentate gyrus

Photomicrographs of BrdU-positive cells in the hippocampal dentate gyrus are shown in Fig. 3. The number of BrdU-positive

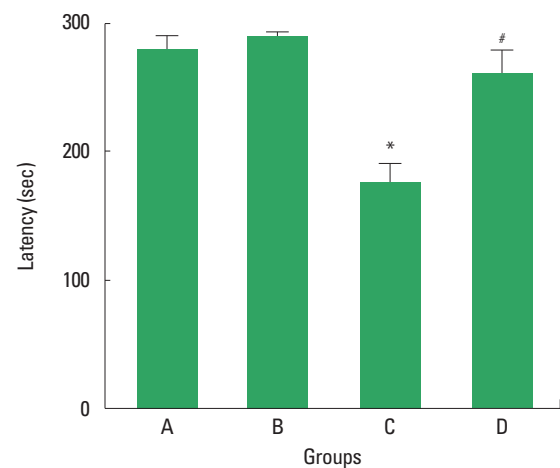


Fig. 1. Effect of treadmill exercise on the short-term memory. A, control group; B, exercise group; C, diabetes group; and D, diabetes and exercise group. Values are presented as the mean \pm standard error of the mean. * $P < 0.05$ compared to the control group. # $P < 0.05$ compared to the diabetes group.

Table 1. Effect of treadmill exercise on body weight

Group	0 Week	2 Weeks	4 Weeks	6 Weeks	8 Weeks	10 Weeks	12 Weeks
CON	264.00 \pm 2.44	322.40 \pm 7.25	395.40 \pm 7.16	400.90 \pm 8.57	411.70 \pm 11.03	415.20 \pm 11.40	453.50 \pm 7.73
EX	259.40 \pm 2.38	334.60 \pm 3.08	355.00 \pm 11.51	374.30 \pm 5.02	405.70 \pm 3.61	404.20 \pm 2.46	411.40 \pm 5.04
DM	264.60 \pm 2.51	204.70 \pm 5.61*	210.70 \pm 6.54*	205.50 \pm 7.85*	198.30 \pm 11.07*	198.70 \pm 5.53*	204.00 \pm 7.64*
DM-EX	261.20 \pm 3.18	224.90 \pm 1.72*	215.90 \pm 5.88*	213.60 \pm 3.75*	210.70 \pm 6.00*	200.90 \pm 3.41*	195.00 \pm 4.97*

Values are presented as mean \pm standard error of the mean.

CON, control group; EX, exercise group; DM, diabetes group; DM-EX, diabetes and exercise group.

* $P < 0.05$ compared to the control group.

Table 2. Effect of treadmill exercise on blood glucose level

Group	0 Week	2 Weeks	4 Weeks	6 Weeks	8 Weeks	10 Weeks	12 Weeks
CON	95.00 \pm 2.29	97.70 \pm 1.51	113.80 \pm 3.60	111.50 \pm 3.04	117.20 \pm 4.11	107.60 \pm 2.33	103.50 \pm 1.62
EX	94.60 \pm 1.85	105.80 \pm 1.88	119.50 \pm 6.40	114.70 \pm 3.46	112.40 \pm 4.99	108.00 \pm 2.39	98.90 \pm 2.20
DM	339.90 \pm 8.09	383.70 \pm 16.69*	461.20 \pm 19.80*	423.10 \pm 6.63*	406.50 \pm 15.43*	409.40 \pm 8.35*	420.80 \pm 14.95*
DM-EX	327.00 \pm 7.29	403.30 \pm 12.47*	482.50 \pm 21.13*	422.00 \pm 9.61*	406.40 \pm 8.13*	372.30 \pm 7.58*#	350.40 \pm 19.74*#

Values are presented as mean \pm standard error of the mean.

CON, control group; EX, exercise group; DM, diabetes group; DM-EX, diabetes and exercise group.

* $P < 0.05$ compared to the control group. # $P < 0.05$ compared to the diabetes group.

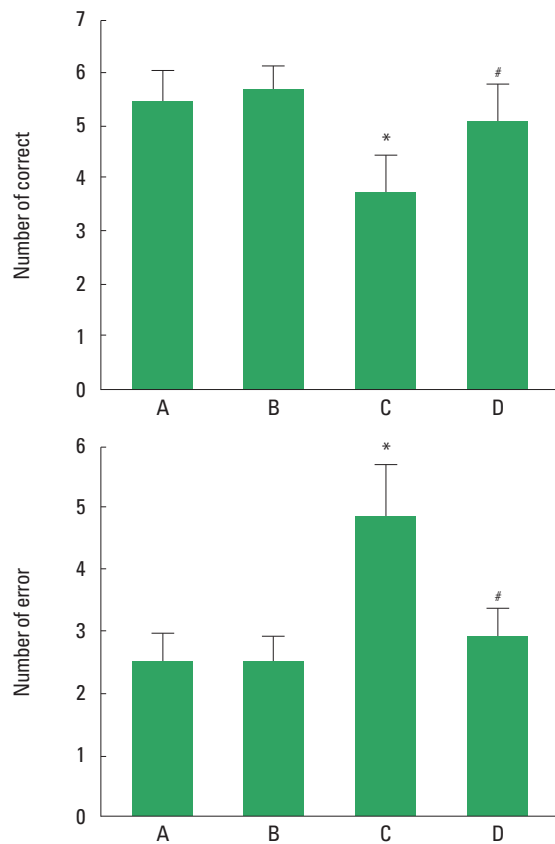


Fig. 2. Effect of regular exercise on the spatial learning ability. Upper panel: Correct number. Lower panel: Error number. A, control group; B, exercise group; C, diabetes group; and D, diabetes and exercise group. Values are presented as mean \pm standard error of the mean. * $P < 0.05$ compared to the control group. # $P < 0.05$ compared to the diabetes group.

cells in the diabetes group was lower than that in the control group ($P < 0.05$). However, treadmill exercise increased the number of BrdU-positive cells in the diabetic rats ($P < 0.05$). Treadmill exercise also increased the number of BrdU-positive cells in the normal rats ($P < 0.05$).

Effect of treadmill exercise on the number of DCX-positive cells in the hippocampal dentate gyrus

Photomicrographs of DCX-positive cells in the hippocampal dentate gyrus are shown in Fig. 4. The number of DCX-positive cells in the diabetes group was lower than that in the control group ($P < 0.05$). However, treadmill exercise increased the number of DCX-positive cells in the diabetic rats ($P < 0.05$). Treadmill exercise also increased the number of DCX-positive cells in the normal rats ($P < 0.05$).

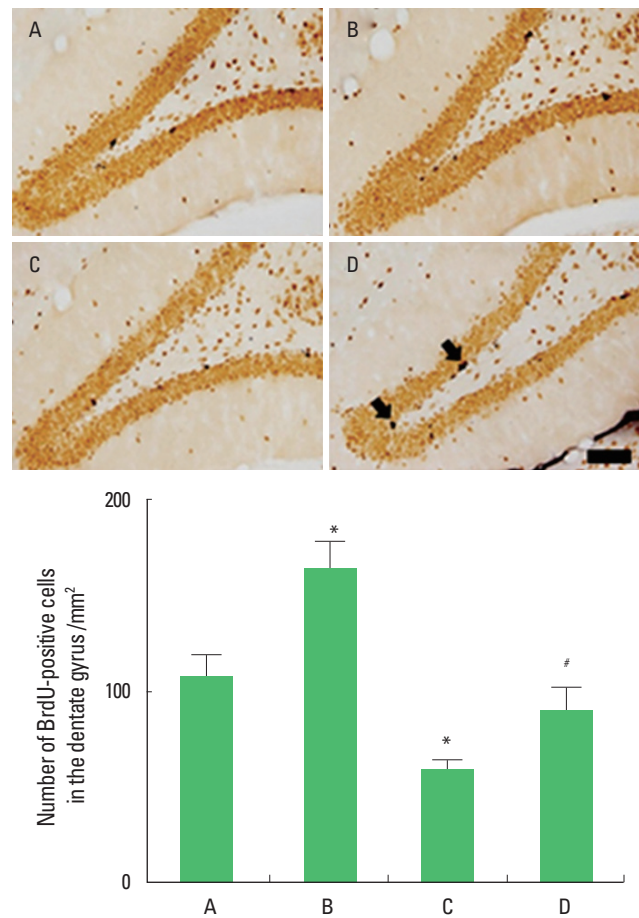


Fig. 3. Effect of regular exercise on neurogenesis in the hippocampal dentate gyrus. Upper panel: Photomicrographs of 5-bromo-2'-deoxyridine (BrdU)-positive cells in the hippocampal dentate gyrus (immunohistochemistry). Arrows indicate BrdU-positive cells. The scale bar represents 200 μ m. Lower panel: Number of BrdU-positive cells in each group. A, control group; B, exercise group; C, diabetes group; and D, diabetes and exercise group. Values are presented as mean \pm standard error of the mean. * $P < 0.05$ compared to the control group. # $P < 0.05$ compared to the diabetes group.

Effect of treadmill exercise on Wnt-3 expression in the hippocampus

Expression of Wnt-3 in the hippocampus is presented in Fig. 5. The expression of Wnt-3 was decreased in the diabetes group. However, treadmill exercise increased the Wnt-3 expression in the diabetic rats ($P < 0.05$). Treadmill exercise also increased the expression of Wnt-3 in the normal rats ($P < 0.05$).

Effect of treadmill exercise on GSK-3 β expression in the hippocampus

Expression of GSK-3 β in the hippocampus is presented in Fig. 6. The expression of GSK-3 β was increased in the diabetes group.

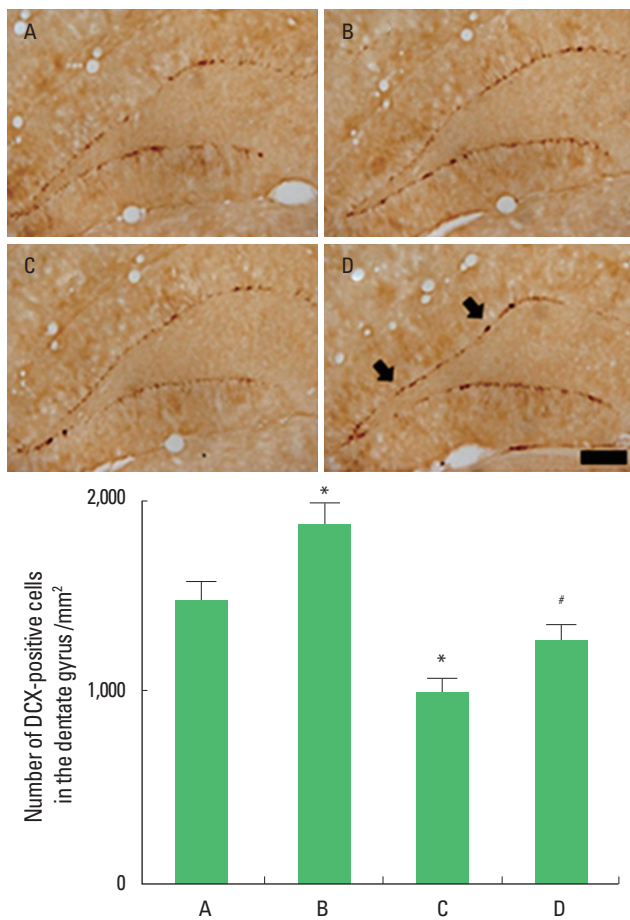


Fig. 4. Effect of regular exercise on the doublecortin (DCX) expression in the hippocampal dentate gyrus. Upper panel: Photomicrographs of DCX-positive cells in the hippocampal dentate gyrus (immunohistochemistry). Arrows indicate DCX-positive cells. The scale bar represents 200 μ m. Lower panel: Number of DCX-positive cells in each group. A, control group; B, exercise group; C, diabetes group; and D, diabetes and exercise group. Values are presented as mean \pm standard error of the mean. * $P < 0.05$ compared to the control group. # $P < 0.05$ compared to the diabetes group.

However, treadmill exercise inhibited the GSK-3 β expression in the diabetic rats ($P < 0.05$).

DISCUSSION

Many studies reported that diabetes increases susceptibility to dementia and Alzheimer disease, and then Alzheimer disease is called as the type 3 diabetes (Cole et al., 2007; Ronnema et al., 2008). In the present study, the rats in diabetes group showed impairment of short-term memory in the step-down avoidance task and deterioration of spatial learning ability in the 8-arm radial maze test. These symptoms represent Alzheimer disease-associat-

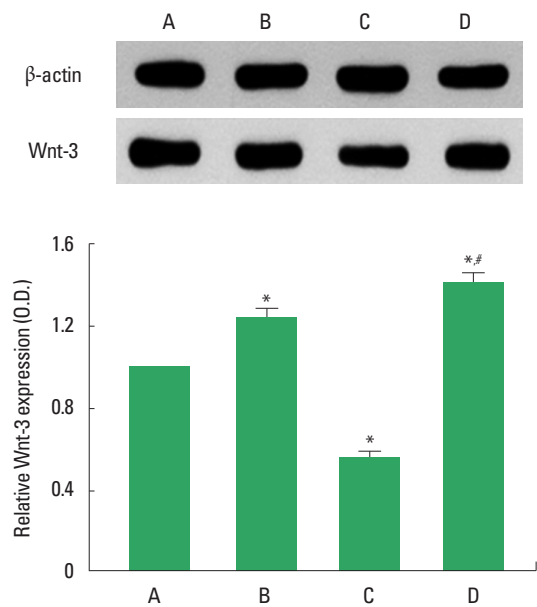


Fig. 5. Effects of treadmill exercise on Wnt-3 expression in the hippocampus. Upper panel: Representative expression of Wnt-3 and β -actin in the hippocampus. Lower panel: Relative Wnt-3 expression in each group. A, control group; B, exercise group; C, diabetes group; and D, diabetes and exercise group. Values are presented as mean \pm standard error of the mean. * $P < 0.05$ compared to the control group. # $P < 0.05$ compared to the diabetes group.

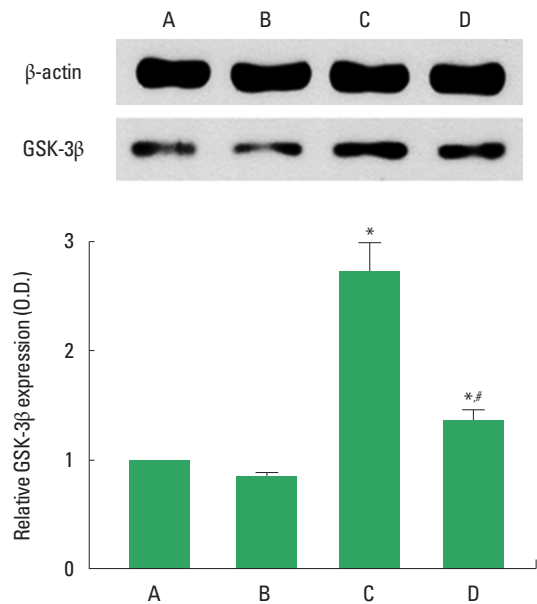


Fig. 6. Effects of treadmill exercise on glycogen synthase kinase-3 β (GSK-3 β) expression in the hippocampus. Upper panel: Representative expression of GSK-3 β and β -actin in the hippocampus. Lower panel: Relative GSK-3 β expression in each group. A, control group; B, exercise group; C, diabetes group; and D, diabetes and exercise group. Values are presented as mean \pm standard error of the mean. * $P < 0.05$ compared to the control group. # $P < 0.05$ compared to the diabetes group.

ed memory loss. Treadmill exercise ameliorated this Alzheimer disease-associated memory loss caused by diabetes. Many studies have shown that physical exercise improves memory function (Dao et al., 2014; Intlekofer and Cotman, 2013; Kim et al., 2010; Kim et al. 2014).

In the present study, the numbers of BrdU-positive and DCX-positive cells in the hippocampal dentate gyrus were decreased in the rats of the diabetes group. Treadmill exercise increased the numbers of BrdU-positive and DCX-positive cells in the diabetic rats. The present results showed that treadmill exercise alleviated defect in the neurogenesis caused by diabetes. Enhancing effect of treadmill exercise on neurogenesis is also well documented (Kim et al., 2010; Kim et al., 2014; Zhang et al., 2013). Hippocampal neurogenesis is positively correlated with enhancement of memory function (Kim et al., 2010; Kim et al., 2014; Oomen et al., 2014).

In present study, Wnt3 expression in the hippocampus was decreased and GSK-3 β in the hippocampus was increased in the rats of the diabetes group. Treadmill exercise increased Wnt3 expression and suppressed GSK-3 β expression in the diabetic rats. Our present results showed that treadmill exercise activated Wnt signaling pathway, which caused inhibition on GSK-3 β expression in the diabetic rats. Wnt3 is released hippocampal neurons in an activity-dependent manner and controls hippocampal neurogenesis (Chen et al., 2006). Stranahan et al. (2010) showed that expression of Wnt was increased by wheel running exercise. Wnt signaling pathway inhibits GSK-3 β activity, therefore, GSK-3 β negatively affects the proliferation of neural precursors and neurogenesis (Tiwari et al., 2014).

The present results showed that treadmill exercise alleviates Alzheimer disease-associated memory loss in the STZ-injection induced diabetic rats. Our study suggests that treadmill exercise may conserve memory function by increasing neurogenesis through activating Wnt signaling pathway in the neurodegenerative disorders, such as Alzheimer disease.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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