Treadmill exercise ameliorates symptoms of Alzheimer disease through suppressing microglial activation-induced apoptosis in rats

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Alzheimer disease (AD) is a most common form of dementia and eventually causes impairments of learning ability and memory function. In the present study, we investigated the effects of treadmill exercise on the symptoms of AD focusing on the microglial activation-induced apoptosis. AD was made by bilateral intracerebroventricular injection of streptozotocin. The rats in the exercise groups were made to run on a treadmill once a day for 30 min during 4 weeks. The distance and latency in the Morris water maze task and the latency in the step-down avoidance task were increased in the AD rats, in contrast, treadmill exercise shortened these parameters. The numbers of terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling-positive and caspase-3-positive cells in the hippocampal dentate gyrus were decreased in the AD rats, in contrast, treadmill exercise suppressed these numbers. Expressions of glial fibrillary acidic protein (GFAP) and cluster of differentiation molecule 11B (CD11b) in the hippocampal dentate gyrus were increased in the AD rats, in contrast, treadmill exercise suppressed GFAP and CD11b expressions. Bax expression was increased and Bcl-2 expression was decreased in the hippocampus of AD rats, in contrast, treadmill exercise decreased Bax expression and increased Bcl-2 expression. The present results demonstrated that treadmill exercise ameliorated AD-induced impairments of spatial learning ability and short-term memory through suppressing apoptosis. The antiapoptotic effect of treadmill exercise might be ascribed to the inhibitory effect of treadmill exercise on microglial activation.

Keywords: Alzheimer disease, Apoptosis, Microglial activation, Rats

INTRODUCTION

Alzheimer disease (AD) gradually impairs learning ability and memory function (Sim, 2014), and the incidence of AD is 50%–80% of dementia cases. The exact pathogenesis of AD is still not known. However, cerebral accumulation of amyloid-β peptide, the primary component of senile plaques, has been suggested as the principal cause of AD pathogenesis (Selkoe, 2001). Intracerebroventricular (ICV) injection of streptozotocin (STZ) has been used for the animal model of AD (Jee et al., 2008).

Apoptosis in the hippocampus affects memory functions. Apoptosis has two main pathways (the extrinsic and intrinsic pathways). A class of cysteine proteases, such as caspase-8, caspase-9, and caspase-3, is commonly involved in these pathways (Aggarwal, 2000) and caspase-3 is one of the most widely studied members of the caspase families and it is one of the key initiators of apoptosis (Fan et al., 2005). Furthermore, terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining is an assay that detects DNA fragmentation, which is the characteristic of apoptotic cell death (Kim et al., 2010). Caspase-3 expressions and the number of TUNEL-positive cells were enhanced in the hippocampus of AD and intracerebral hemorrhage rats (Lee et al., 2005; Song et al., 2014).

Besides caspases, the Bcl-2 family proteins also play an important role in the regulation of apoptosis. The Bcl-2 family proteins are classified into antiapoptotic proteins, including Bcl-2 and Bcl-2XL, and pro-apoptotic proteins, such as Bax and Bid. The balance between pro-apoptotic and antiapoptotic Bcl-2 family mem-
bers determines the mitochondrial response to apoptotic stimuli (Upadhyay et al., 2003). The ratio of Bax to Bcl-2 was increased in the hippocampus of AD rats (Kitamura et al., 1998; Song et al., 2014).

Microglia are a type of glial cells that are the resident macrophages in the brain and spinal cord, thus microglia act as the first and main form of active immune defense mechanism in the central nervous system (Ransohoff and Perry, 2009). Activated microglial cells exert neurotoxic effect (Block et al., 2007) and increased microglial reactivity induces cellular apoptosis (Hooper and Pocock, 2007). Gliarial fibrillary acidic protein (GFAP) is an intermediate filament protein that is expressed in the numerous types of glial cells. Cluster of differentiation molecule 11B (CD11b) is the surface marker located on the plasma membrane of microglia. Expressions of GFAP and CD11b are used to identify glia cells and microglia, respectively (Singh et al., 2013; Sung et al., 2012).

Exercise is known to inhibit apoptosis and improves memory functions (Kim et al., 2014a; Kim et al., 2014b; Sim, 2014). However, the effects of treadmill exercise on AD in relation with microglial activation are not evaluated. In the present study, we investigated the effects of treadmill exercise on the microglial activation-induced apoptosis using streptozotocin-induced AD rats. For this study, Morris water maze task, step-down avoidance task, TUNEL assay, immunohistochemistry for caspase-3, GFAP, CD11b, and western blot for Bax and Bcl-2 were performed.

MATERIALS AND METHODS

Animals and treatments

Adult male Sprague-Dawley rats, weighing 400±20 g (40 weeks old), were used for the experiments. 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. The rats were housed under controlled temperature (23°C±2°C) and lighting (08:00 a.m. to 20:00 p.m.) conditions with food and water available ad libitum. The animals were randomly divided into four groups (n=10 in each group): sham-operation group, sham-operation and treadmill exercise group, AD-induced group, and AD-induced and treadmill exercise group.

Induction of AD

AD was induced according to the previously described method (Jee et al., 2008; Sim, 2014). The rats were anesthetized with Zoletil 50 (10 mg/kg, intraperitoneally; Vibac Laboratories, Carros, France) and placed in a stereotaxic frame. Burr holes were drilled in the skull on both sides over the lateral ventricles using the following coordinates: 0.8 mm posterior to bregma, 1.5 mm lateral to sagittal suture, and 3.6 mm beneath the surface of brain. Through a hole drilled in the skull, a 26-gauge needle was lowered manually into each lateral ventricle. The rats in the AD-induced groups received bilateral ICV injection of STZ (1.5 mg/kg, 5 μL in saline). The rats in the sham-operation group underwent the same surgical procedures, but same volume of saline was injected instead of STZ.

Exercise protocol

The rats in the exercise groups were forced to run on a motorized treadmill. The rats in the exercise groups were made to run on a treadmill once a day for 30 min during 4 weeks, starting 3 days after STZ injection. The exercise load consisted of running at the speed of 8 m/min with a 0° inclination.

Morris water maze task

Spatial learning ability was determined using Morris water maze task, according to the previously described method (Heo et al., 2014). The water maze was a black circular pool (diameter, 140 cm; height, 45 cm) filled with water (room temperature) and positioned in a room with many visual cues. On the 21 days after starting exercise, the rats were trained on a water maze, with two trials per day for 5 days. Each trial lasted either until the rats had found the platform or for a maximum of 3 min. At the end of each trial, the rats were allowed to rest on the platform (diameter, 15 cm; height, 35 cm) submerged 2 cm below the water surface for 15 sec. If the rats were unable to locate the platform within 3 min, they were guided to the platform and left there for 15 sec before being removed from the pool. The length of the swim path (distance) and the time to reach the platform (latency) were recorded by a video racking system (Smart version 2.5, Panlab, Barcelona, Spain).

Step-down avoidance task

The latency in the step-down avoidance task was determined to evaluate the short-term memory, according to the previously described method (Jin et al., 2014). The rats were trained in a step-down avoidance task on the 28 days after starting the treadmill exercise. The rats were placed on a 7×25-cm platform which was 2.5 cm in height. The platform faced a 42×25-cm grid of parallel stainless steel bars, 0.1 cm in caliber, spaced 1 cm apart. In the
training sessions, the animals received a 0.5-mA scramble foot shock for 2 sec immediately upon stepping down. Two hours after training, the latency (sec) in each group was measured. The interval of time which elapsed between the rats stepping down and placing all four paws on the grid was defined as the latency. Latency over 300 sec was counted as 300 sec.

**Tissue preparation**

The animals were sacrificed immediately after determining the latency in the step-down avoidance task. The rats were anesthetized using Zoletil 50 (10 mg/kg, intraperitoneally; Vibac Laboratories), transcardially perfused with 50-mM phosphate-buffered saline (PBS), and fixed with a freshly prepared solution consisting of 4% paraformaldehyde in 100-mM phosphate buffer (pH, 7.4). Brains were dissected, and storage overnight same fixative, then it was transferred to 30% sucrose for cryoprotection. The hippocampal tissues were collected from each rat.

**Tissue preparation**

To detect apoptosis and microglial activation, immunohistochemistry for caspase-3, GFAP, and CD11b was performed, according to the previously described method (Kim et al., 2014b; Sung et al., 2012). The sections were then washed three times with PBS and incubated for 1 hr with biotinylated anti-mouse caspase, anti-rabbit GFAP and, anti-mouse CD11b secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA). Bound secondary antibody was then amplified with Vector Elite ABC kit (1:100; Vector Laboratories). The antibody-biotin-avidin-peroxidase complexes were visualized using 0.03% DAB and the sections were finally mounted onto gelatin-coated slides. The slides were air dried overnight at room temperature, and coverslips were mounted using Permount (Fisher Scientific).

**Western blotting for Bax and Bcl-2**

Bax and Bcl-2 expressions were determined by Western blot analysis, according to the previously described method (Kim et al., 2010). The hippocampal tissues were dissected and collected, and then were immediately frozen at -70°C. The hippocampal tissues were homogenized on ice, and lysed in a lysis buffer containing 50-mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) (pH, 7.5), 150-mM NaCl, 10% glycerol, 1% Triton X-100, 1-mM PMSF (phenylmethylsulfonyl fluoride), 1-mM EGTA (ethyleneglycol-bis-(b-aminoethylether)-N,N,N’,N’-tetraacetic acid), 1.5-mM MgCl₂·6H₂O, 1-mM sodium orthovanadate, and 100-mM sodium fluoride. Protein content was measured using a Bio-Rad colorimetric protein assay kit (Hercules, CA, USA). Protein samples (30 μg) were separated on sodium dodecyl sulfate-polyacrylamide gel and transferred onto a nitrocellulose membrane. The membranes were incubated with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 and then incubated overnight at 4°C with the following primary antibodies: mouse anti-β-actin, mouse anti-Bcl-2, and mouse anti-Bax (1:1,000; Santa Cruz Biotechnology). Subsequently, membranes were incubated for 1 hr with attempt secondary antibodies (1:2,000; Vector Laboratories), and band detection was performed using the enhanced chemiluminescence detection kit (Santa Cruz Biotechnology).

**Statistical analysis**

For the confirmation of the expression of apoptotic proteins, the detected bands were calculated densitometrically using Molecular Analyst ver. 1.4.1 (Bio-Rad). The numbers of TUNEL-positive and caspase-3-positive cells in the hippocampal dentate gyrus were counted hemilaterally under a light microscope (Olympus, Tokyo, Japan), and they were expressed as the numbers of cells/mm² of the dentate gyrus area. The area of the dentate gyrus region was measured by Image-Pro Plus image analysis system (Me-
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via Cyberbetics Inc., Silver Spring, MD, USA). The optical densities of GFAP and CD11b immunoreactive fibers were measured in 100 μm × 100 μm square images in the hippocampal dentate gyrus using an image analyzer (Multiscan, Fullerton, CA, USA).

To estimate the GFAP and CD11b staining densities, the optical densities were corrected for the nonspecific background density, which was measured in the completely denervated parts of the hippocampal dentate gyrus. The GFAP-positive and CD11b-positive fiber densities were calculated as follows: the optical density in the lesion side/the optical density in the intact side.

Statistical analysis was performed using one-way analysis of variance followed by Duncan post hoc test, and the results are expressed as the mean ± standard error of the mean. Significance was set as $P < 0.05$.

**RESULTS**

**Effect of treadmill exercise on the spatial learning ability**

AD-induced rats showed increase of distance and latency for finding target than those in the sham-operation group. In other hand, treadmill exercise reduced the distance and latency in the AD rats (Fig. 1).

**Effect of treadmill exercise on the short-term memory**

AD-induced rats showed decrease of latency time than those in the sham-operation group. In other hand, treadmill exercise increased the latency time in the AD rats (Fig. 2).

**Effect of treadmill exercise on the DNA fragmentation in the hippocampal dentate gyrus**

AD-induced rats showed enhanced DNA fragmentation in the hippocampal dentate gyrus than those in the sham-operation group. In other hand, treadmill exercise suppressed the DNA fragmentation in the AD rats (Fig. 3).

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**Fig. 1.** Spatial learning ability in the Morris water maze task. Upper panel: Swimming path; Lower panel: Distance and latency. The data represent mean standard error of the mean. Sham, Sham-operation group; Sham-EX, sham-operation and treadmill exercise group; AD, Alzheimer disease-induced group; AD-EX, Alzheimer disease-induced and treadmill exercise group; NS, nonsignificant. *$P<0.05$ compared to the sham-operation group. †$P<0.05$ compared to the Alzheimer disease-induced group.

**Fig. 2.** Short-term memory in the step-down avoidance task. The data represent mean standard error of the mean. Sham, Sham-operation group; Sham-EX, sham-operation and treadmill exercise group; AD, Alzheimer disease-induced group; AD-EX, Alzheimer disease-induced and treadmill exercise group; NS, nonsignificant. *$P<0.05$ compared to the sham-operation group. †$P<0.05$ compared to the Alzheimer disease-induced group.
Effect of treadmill exercise on the caspase-3 expression in the hippocampal dentate gyrus

AD-induced rats showed enhanced caspase-3 expression in the hippocampal dentate gyrus than those in the sham-operation group. In other hand, treadmill exercise suppressed the caspase-3 expression in the AD rats (Fig. 4).

Effect of treadmill exercise on the GFAP expression in the hippocampal dentate gyrus

AD-induced rats showed enhanced GFAP expression in the hippocampal dentate gyrus than those in the sham-operation group. In other hand, treadmill exercise suppressed the GFAP expression in the AD rats (Fig. 5).
Effect of treadmill exercise on the CD11b expression in the hippocampal dentate gyrus

AD-induced rats showed enhanced CD11b expression in the hippocampal dentate gyrus than those in the sham-operation group. In other hand, treadmill exercise suppressed the CD11b expression in the AD rats (Fig. 6).

Effect of treadmill exercise on the Bax and Bcl-2 expressions in the hippocampus

AD-induced rats showed enhanced Bax expression and decreased Bcl-2 expression, therefore, induction of AD enhanced Bax to Bcl-2 ratio. In other hand, treadmill exercise suppressed Bax expression and increased Bcl-2 expression, resulting in decrease of Bax to Bcl-2 ratio (Fig. 7).
DISCUSSION

Injection of STZ into the ventricles caused structural alterations and physiological impairments of the hippocampus, and such changes contribute to memory impairments (Jee et al., 2008; Muller et al., 2012). Injection of STZ in rats induced desensitization of insulin receptors and biochemical changes, such as glucose and energy metabolism (Muller et al., 2012). In the present study, the distance and latency in the Morris water maze task were shortened and the latency in the step-down avoidance task was also shortened by injection of STZ in rats. Treadmill exercise increased the distance and latency of the Morris water maze task and also increased the latency of the step-down avoidance task in the AD rats. The present results represent that treadmill exercise ameliorated AD-induced deterioration of spatial learning ability and short-term memory.

Caspase-3 is one of the most widely studies members of the caspase family and it is involved in apoptosis as the principal executor (Jin et al., 2014; Ko et al., 2009; Martin et al., 2002). Furthermore, caspase-3 is up-regulated and activated in the early-stage of apoptosis (Benchoua et al., 2001; Kang et al., 2013). TUNEL assay is a common method for detecting DNA fragmentation that is the hallmark of apoptosis (Choi et al., 2013; Ji et al., 2013; Jin et al., 2014). In the present study, expression of caspase-3 and the number of TUNEL-positive cells in the hippocampal dentate gyrus were enhanced in the AD rats. Treadmill exercise suppressed caspase-3 expression and the number of TUNEL-positive cells in the AD rats, showing that treadmill exercise inhibited AD-induced apoptotic cell death in the hippocampus.

The Bcl-2 family includes anti-apoptotic molecules, Bcl-2, and pro-apoptotic molecules, Bax, Bid, and Bad. The Bcl-2 family is critical factor in determining whether neurons survive or die (Cory et al., 2003). Increased Bax expression and decreased Bcl-2 expression were observed in the AD (Kitamura et al., 1998; Song et al., 2014; Um et al., 2011). In the present study, the expression of Bax was increased and the expression of Bcl-2 was decreased, as a result, the ratio of Bax to Bcl-2 was enhanced in the AD rats. Treadmill exercise suppressed Bax expression and enhanced Bcl-2 expression, thus the ratio of Bax to Bcl-2 was decreased in the AD rats. These results also showed that treadmill exercise inhibited AD-induced apoptotic cell death in the hippocampus.

Inflammation and microglial activation are implicated in the pathogenesis of AD (Bamberger and Ladreth, 2002; Cameron and Landreth, 2010). Enhanced GFAP and CD11b expressions represent microglial activation in AD (Ruan et al., 2009; Sung et al., 2012). In the present study, GFAP and CD11b expressions were increased in the AD rats. Treadmill exercise decreased GFAP and CD11b expressions in the AD rats, representing that treadmill exercise suppressed AD-induced microglial activation in the hippocampus.

The present results showed that treadmill exercise ameliorated AD-induced impairments of spatial learning ability and short-
term memory through suppressing apoptosis. The antiapoptotic effect of treadmill exercise might be ascribed to the inhibitory effect of treadmill exercise on microglial activation. According to these effects of treadmill exercise, treadmill exercise may be suggested as a useful strategy for symptom improvement of several neurodegenerative diseases, including AD.

CONFLICT OF INTEREST

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

ACKNOWLEDGMENTS

This work was supported by the 2015 Research Grant of Sungmyung University.

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