Six Weeks of Swimming Exercise Improve Memory and Brain Derived Neurotrophic Factor in CA1 Hippocampus of Diabetic Rats

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ABSTRACT

Background: Type 2 diabetes mellitus (T2DM) patients are recognized to have higher prevalence of cognitive impairment. The critical significance of brain-derived neurotrophic factor (BDNF) and irisin in cognition is well established. In animal models, exercise could increase hippocampal BDNF and irisin expression. This study aims to assess the impact of moderate-intensity swimming exercise on BDNF and irisin expression in hippocampal neurons of T2DM rats.

Method: The study involved the random division of twenty-seven male Wistar strain rats into four groups: normal control (NC, n = 6); early-stage T2DM (DM, n = 7); sedentary T2DM rats with six weeks follow-up (DMS, n = 7); and T2DM rats subjected to swimming exercise for six weeks (DME, n = 7). T2DM was induced using injections of streptozotocin with a high-fat diet. Blood glucose levels were measured from tail pricks seven days after induction. Diabetic rats are defined as those with a blood glucose level of more than 250 mg/dl. Hippocampal BDNF and irisin expression was assessed through immunohistochemistry analysis, with the number of CA1 and CA3 neurons in the pyramidal layer expressing BDNF and irisin counted using a light microscope.

Results: The BDNF expression in hippocampal CA1 region in groups DM and DMS were markedly lesser than group NC (p = 0.005 and p = 0.002, respectively). Significantly increased hippocampus BDNF expression was observed in group DME compared to group DMS after six weeks of swimming exercise training (p = 0.040). In the CA3 region, the BDNF expression in groups DM and DMS was substantially lower than that of the NC group (p = 0.035 and p = 0.024, respectively). Meanwhile, although not significant, the DME group had lower BDNF expression than the NC group (p = 0.170). The irisin expression in CA1 and CA3 hippocampus in all groups do not exhibit significant variation. In the Morris water maze test, the escape latency was substantially longer in the DM and DMS groups than in the NC group (p = 0.007 and 0.004, respectively). Likewise, the escape latency of the DM and DMS groups was notably longer compared to the DME group (p = 0.010 dan 0.005, respectively).

Conclusion: Moderate-intensity swimming exercise has the potential to enhance the BDNF expression in CA1 region of hippocampus as well as in memory performance in T2DM rats and slowed the progression of diabetes.

Keywords: BDNF, Hippocampus, Irisin, Swimming exercise, Type 2 diabetes mellitus.

INTRODUCTION

Diabetes Mellitus (DM) is a metabolic disease marked by high blood sugar levels, also called hyperglycemia, due to insulin secretion disorders. The global prevalence of DM in 2021 was estimated to reach 10.5% of the total population aged 20-79 years, approximately 536 million.[1] In Indonesia, the prevalence based on blood glucose examination results in 2013 was 6.9% and increased to 8.5% in 2018. In 2019, Indonesia ranked seventh among ten countries with the largest number of DM cases, totaling 10.7 million cases.[2] Among all cases of DM, type 2 diabetes mellitus (T2DM) is the predominant form, representing 90% of all reported cases.[1] According to the Fremantle Diabetes Study Phase I, the onset of dementia in T2DM occurs at an earlier age compared to individuals without DM.[3] Based on systematic review and meta-analysis studies, the estimated prevalence of mild cognitive impairment (MCI) in individuals with T2DM is 45%.[4] This is a serious problem considering the increasing number of T2DM cases and the increasing life expectancy.[5]

A cross-sectional study conducted on individuals with T2DM revealed a direct relationship between the cognitive function and decrease in hippocampal volume in the cornu ammonis 1 (CA1) area.[6] Meanwhile, the CA1 area is known to be an important region in the hippocampal tissue for forming long-term memories.[7] Brain-derived neurotrophic factor (BDNF) is an essential neurotrophin involved in brain function, including memory and learning processes. The hippocampal region has a high density of BDNF expression, which is well recognized for its pivotal function in the regulation of neurogenesis and synaptogenesis.[8] T2DM patients have reduced levels of BDNF in comparison to healthy control individuals.[9] Increased BDNF expression in hippocampal neurons has been associated with cognitive improvement.[10,11]

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Hippocampal neurogenesis due to increased BDNF has been correlated with cognitive improvement in experimental animal models such as rats.[12]

Inconsistent findings have been reported regarding the impact of exercise on the serum BDNF levels of T2DM patients. A systematic review of studies on T2DM indicated that out of 11 articles meeting inclusion criteria, 5 studies showed that exercise increased serum BDNF levels, while 4 studies showed a decrease, and 2 studies showed no change.[13] Studies on adolescent T2DM patients revealed no alteration in serum BDNF levels 12 weeks after engaging in aerobic activity.[14] Similarly, no increase in serum BDNF levels was observed after 16 weeks of combined aerobic and resistance exercise in T2DM patients.[15] Meanwhile, the influence of aerobic exercise on the hippocampus of animal models of T2DM has been found to enhance the levels and expression of BDNF.[16,17]

Aerobic exercise also known to improve cognitive function through muscle-brain crosstalk. With exercise, muscles release myokine mediators, which trigger the expression of hippocampal BDNF.[18] Fibronectin type III domain-containing protein 5 (FNDC5) undergoes proteolysis to produce the myokine irisin. It is known that injected irisin positively regulate hippocampal BDNF expression and serum BDNF levels in diabetic mouse models.[19]

Nevertheless, research on the impact of exercise on irisin expression in the hippocampus are still limited. Therefore, this study aims to assess the impact of moderate-intensity swimming exercise on irisin and BDNF expression in hippocampal neurons of T2DM rats, as well as on cognitive function.

MATERIALS AND METHODS

Animals

This research employed a post-test-only control group design to assess the effects of exercise on male Wistar strain rats with T2DM. Twenty-seven rats were obtained from the Animal Laboratory at the Stem Cell Research and Development Center, Universitas Airlangga, Surabaya, Indonesia. The rats weighed between 170-200 grams and were aged 11-13 weeks. The research was carried out at the Animal Laboratory, Department of Physiology and Medical Biochemistry, Universitas Airlangga. The rats were kept in a room with standard light/dark cycles and provided with a normal chow diet (Charoen Pokphand Indonesia Tbk) and unlimited access to water. They were divided randomly into four groups: Normal control group (NC, n = 6); T2DM rats without a six-week follow-up (DM, n = 7); sedentary T2DM rats with a six-week follow-up (DMS, n = 7); T2DM rats subjected to exercise (DME, n = 7). Ethical clearance for the study was granted by the Animal Care and Use Committee of Universitas Airlangga.

Induction of type 2 Diabetes

To induce T2DM in the rats, an injection of streptozotocin (STZ) and a high-fat diet were employed. For four weeks, the rats were given a high-fat diet. The composition of the diet was 60% kcal from fat, 20% kcal from carbohydrates, and 20% kcal from protein. The specific diet used was #112252 from Dyets Inc., USA. After the four-week high-fat diet regimen, rats were fasted overnight. Then, they were injected intraperitoneally with STZ at 35 mg/kg. STZ was acquired from Santa Cruz Biotechnology Inc., USA.[20] For administration, it was dissolved in a citrate buffer with a concentration of 0.01 M and a pH of 4.5. Seven days after the STZ injection, fasting blood glucose levels were measured from the tail vein using a glucometer Accu-check Instant, Roche Diagnostics, Mannheim, Germany). Diabetic rats are defined as those with a blood glucose level of more than 250 mg/dl.

Swimming exercise protocol

The exercise intervention for the designated group involved unweighted swimming sessions conducted five days per week over a duration of six weeks. The swimming intensity was set at a moderate level, corresponding to approximately 46-63% of maximum oxygen consumption (VO2 max), equivalent to around 3 metabolic equivalents (METs).[21] During the first week, rats swam for 15 minutes per session. The duration gradually increased over subsequent weeks until reaching 60 minutes per session.[22] Water tanks with a diameter of 60 cm and a height of 65 cm were utilized. The water level was maintained at a height of 50 cm. If rats were unable to keep their heads above water (sinking) for more than 3 seconds without attempting to dive, the exercise was halted. Rats failing to complete three consecutive exercise sessions were excluded from the study. Following each swimming session, rats were dried off and returned to their respective cages.

Morris water maze test

Assessments of cognitive function were performed utilizing the Morris Water Maze (MWM). In the MWM test, animals learn to swim in a circular pool with external cues to find (and climb onto) a hidden platform. Using spatial information, the animals are trained to locate the platform. The equipment used consists of a circular pool measuring 120-200 cm in diameter and 50 cm in height, filled with water to a depth of 25-30 cm. The pool is equipped with several spatial cues that are consistently positioned. A platform with a diameter of 10 cm, which is submerged 0.5-1 cm below the water surface, is the sole means of exiting the water. The platform must be situated in the center of a single quadrant, be invisible to the animals, and be positioned in the same quadrant for each trial.[23] The rats underwent two trial tests, during which the platform was placed in a pool. Next, the rats underwent a probe test, where there was no platform in the pool. The escape latency was determined by the rats' time to locate the platform.

Tissue preparation

One day following the Morris water maze test, rats were sacrificed. The process of sacrificing the rats and preparing the brain tissues for analysis involved the following steps. A combination of xylazine (10 mg/kg) and ketamine (75 mg/kg) was administered intramuscularly to anesthetize the rats. The rats were decapitated to euthanize them after achieving sufficient anesthesia. This process was conducted under anesthesia to minimize pain and distress. Following decapitation, transcardial perfusion was performed using 50 mM phosphate-buffered saline (PBS). This procedure helps to remove blood and fix the tissues. The brain tissues were carefully dissected and collected. Subsequently, they were fixed in neutral buffer formalin at 4°C overnight. This fixation process helps to preserve the tissue structure for subsequent analysis. The right hemisphere of the brain was cut sagittally (along the midline) and embedded in paraffin wax. Then, the paraffin-embedded tissue block was cut into slices with a thickness of 4 microns using a microtome (BQ-318D microtome).

Immunohistochemical analysis

Immunohistochemistry was applied to identify the BDNF and irisin expression in the hippocampus. The procedure was performed as described previously.[19] The immunohistochemistry procedure for detecting BDNF and irisin expression in the hippocampus involved the following steps. The primary antibody used was monoclonal anti-BDNF (bsm-52368R, 1:200, Bioss, USA) and anti-FNDC5 poliklonal (bs-8486R, 1:200, Bioss, USA). The primary antibody was diluted to

a concentration of 1:200 and applied to each brain tissue slice. The slices were then incubated at 4°C overnight to allow for specific binding of the antibody to the target protein. To eliminate any unbound primary antibody and other nonspecific binding substances, the brain tissue slices were cleansed with phosphate-buffered saline (PBS) following the overnight incubation time. Subsequently, a secondary antibody buffer was prepared at a dilution of 1:2000 and applied to each brain tissue slice. The secondary antibody enhances the signal for visualization by binding to the primary antibody that is already tethered to the target protein. The brain tissue slices were then incubated at room temperature for one hour to facilitate the binding of the secondary antibody to the primary antibody. Following the incubation period, the brain tissue slices were rinsed again to remove any unbound secondary antibody and other nonspecific binding substances. The brain tissue slices were stained, hydrated and examined under a light microscope (Leica DM750 equipped with Leica ICC50 HD camera, Leica Microsystems, Wetzlar, Germany) at a magnification of 400x. The counting of CA1 and CA3 neurons expressing BDNF and irisin in the pyramidal layer was conducted across five random fields for each section, and subsequently averaged. Neurons expressing BDNF and irisin are characterized by brownish cytoplasm, whereas neurons not expressing BDNF and irisin are identified by bluish cytoplasm.

Statistical Analysis

Data analysis was performed using SPSS version 21. The Shapiro-Wilk test was conducted to determine whether the data followed a normal distribution. For data with a normal distribution, such as irisin expression in CA1 and the MWM performance, ANOVA was used. Post hoc LSD tests were employed to analyze differences between groups. For data with a non-normal distribution, including BDNF expression in CA1 and CA3, as well as irisin expression in CA3, the Kruskal-Wallis test was used, followed by the Mann-Whitney test to identify differences between groups.

RESULTS

This research assessed the impact of moderate-intensity swimming exercise on the expression of BDNF and irisin in the hippocampus CA1 and CA3 region of T2DM rats using immunohistochemical analysis (Fig. 1). As shown in Fig. 2, in the hippocampal CA1 region, BDNF expression in groups DM (0.26 ± 0.07 cells/625 μ^2) and DMS (0.06 ± 0.06 cells/625 μ^2) were significantly lower than group NC (0.93 ± 0.20 cells/625 μ^2), with significant value of $p = 0.005$ and $p = 0.002$, respectively. After six weeks of swimming exercise training, the

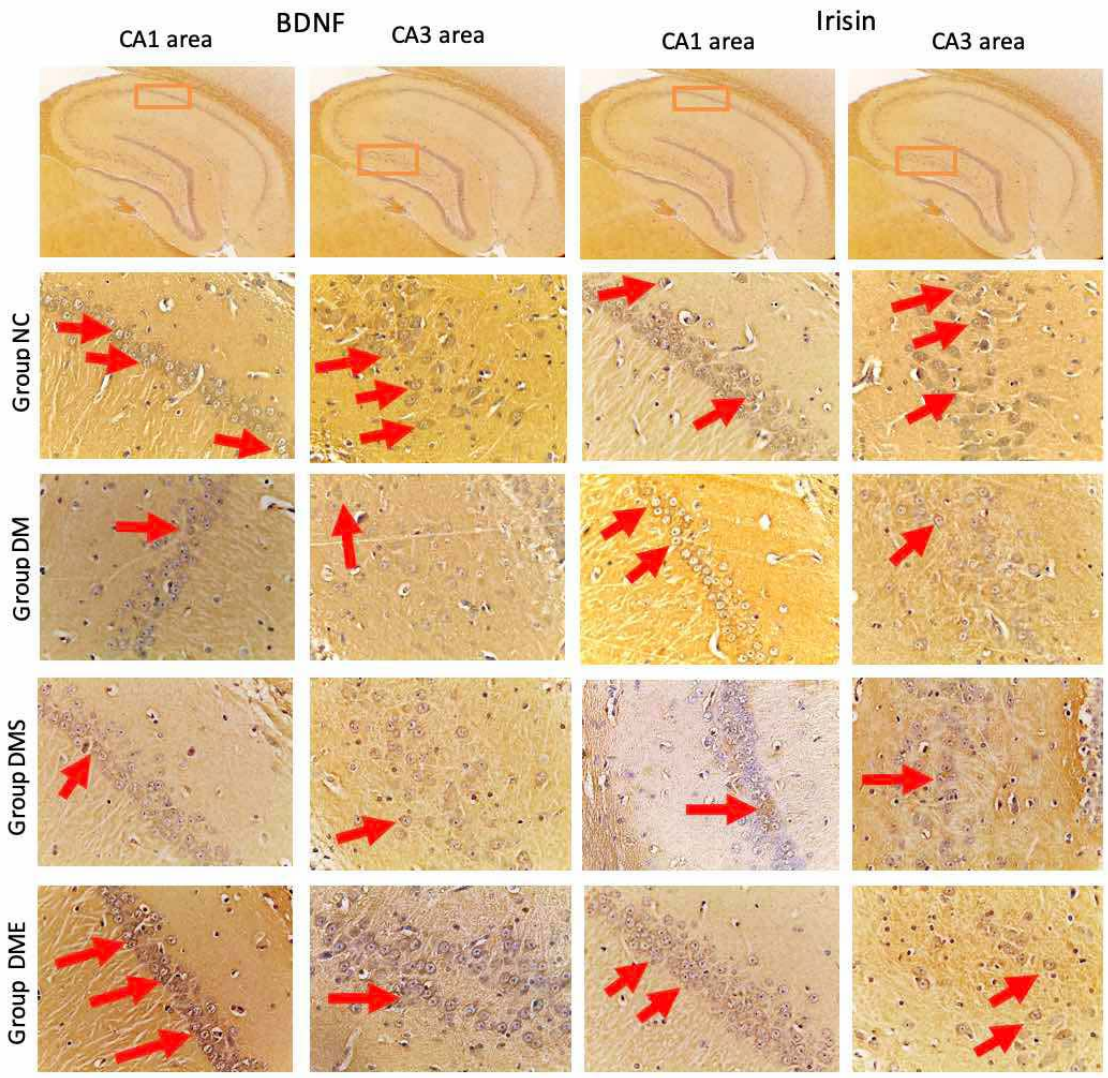

Figure 1. Expression of BDNF and irisin stained by immunohistochemistry in the pyramidal layer neuron of hippocampal tissue CA1 and CA3 region of rats in group NC, group DM, group DMS and group DME (x400). Red arrows indicated neurons with BDNF and irisin expression characterized by brownish cytoplasm, whereas neurons not expressing BDNF and irisin are identified by bluish cytoplasm.

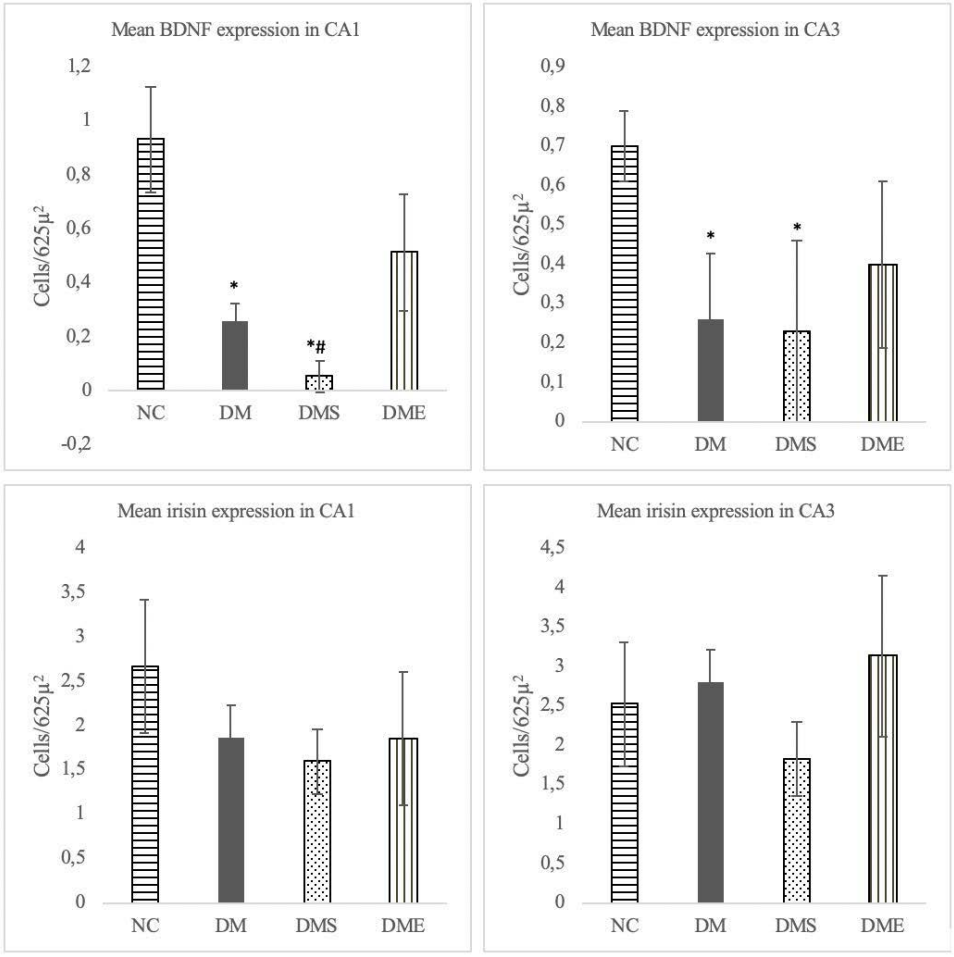

Figure 2. Quantification of BDNF expression in hippocampus CA1 and CA3 region in group NC, group DM, group DMS and group DME. *p<0.05 vs group NC; #p<0.05 vs group DME. The data are indicated as Mean ± SEM.

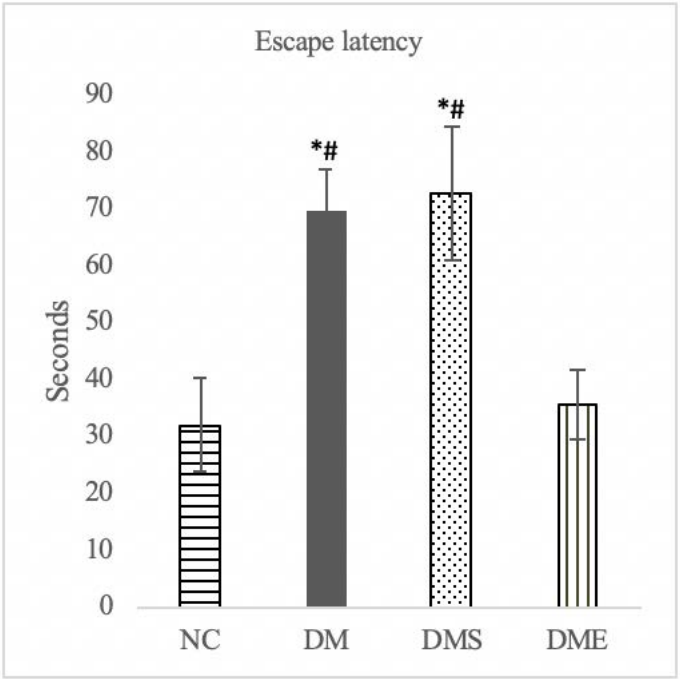

Figure 3. Escape latency of Morris Water Maze test in group group NC, group DM, group DMS and group DME. *p<0.05 vs group NC; #p<0.05 vs group DME. The data are indicated as Mean ± SEM.

hippocampal BDNF expression of group DME (0.51 ± 0.22 cells/625 μ^2) was substantially greater than group DMS (p = 0.040). However, in comparison to to the NC group, the result was not significant (p = 0.172).

The count of pyramidal neuron cells expressing BDNF in the hippocampal CA3 area of the DM group (0.26 ± 0.17 cells/625 μ^2) and DMS group (0.23 ± 0.23 cells/625 μ^2) was substantially lower than that of the NC group (0.70 ± 0.09 cells/625 μ^2) with p = 0.035 and p = 0.024, respectively. Meanwhile, the DME group (0.40 ± 0.21 cells/625 μ^2) had lower but not significant BDNF expression than the NC group (p = 0.170).

Hippocampal CA1 pyramidal neuron cells expressing irisin in the DM group (1.86 ± 0.14 cells/625 μ^2), DMS group (1.60 ± 0.38 cells/625 μ^2), and DME group (1.86 ± 0.37 cells/625 μ^2) were lower than those in the NC group (2.67 ± 0.75 cells/625 μ^2), but this difference was not significant. Likewise, in the CA3 area of the hippocampus, neurons expressing irisin in the DM group (2.80 ± 0.42 cells/625 μ2), DMS group (1.83 ± 0.47 cells/625 μ^2), and DME group (3.14 ± 1.02 cells/625 μ^2) were not significantly different compared with the NC group (2.53 ± 0.78 cells/625 μ^2).

The Morris water maze test findings (Fig. 3) revealed that the escape latency of the DM (69.71 ± 7.31 seconds) and DMS (72.86 ± 11.76 seconds) groups was substantially greater compared to the NC group (32.17 ± 8.28 seconds) (p = 0.007 and 0.004, respectively). Likewise, when compared with the DME group (35.71 ± 6.25 seconds), the escape latency of the DM and DMS groups was significantly higher (p=0.010 dan 0.005, respectively).

DISCUSSION

This study found that in the early (group DM) and late-stage (DMS) T2DM group showed significantly lower BDNF expression in both the CA1 and CA3 areas in the hippocampus compared to the NC group. This suggests progression in T2DM. However, in the rats subjected to exercise for six weeks, significantly higher CA1 hippocampal BDNF expression was observed compared to the T2DM-late group. Likewise, with BDNF expression in the CA3 area, results were similar to the healthy control group. These results indicated that moderate-intensity swimming exercise could inhibit the progression of T2DM.

This was in line with the results of the Morris water maze examination, where in the early and advanced phases of T2DM, escape latency results were found to be significantly longer compared to healthy rats. And in T2DM rats that were given 6 weeks of swimming training, the results were significantly faster compared to T2DM rats that were not given training intervention. When compared with healthy rats, the results were also similar. This condition shows that cognitive, in this case spatial memory, could improve with moderate intensity aerobic exercise intervention with swimming.

The density and structural tonicity of hippocampal neurons play a crucial role in cognitive function.[24] A cross-sectional study in patients with T2DM reported a positive correlation between the decrease in hippocampal volume in the CA1 area and cognitive function (r=0.516, p<0.001).[6] Disruptions in pyramidal cells in the hippocampus can lead to memory loss.[25] The CA1 area of the hippocampus in mice with diabetes mellitus (DM) for 6 weeks showed disorganization and changes in the arrangement of pyramidal neurons.[26] Meanwhile, neuronal density in the hippocampal CA1 area increased after diabetic animals were given exercise.[24] With exercise, hippocampal volume and function will improve.[27]

As for the types of aerobic exercise commonly used, previous study included treadmill walking and swimming. Swimming has been widely included in a variety of exercise and behavioral studies in animals. The physical movements in swimming are more uniform, do not cause injury to the legs, and are less traumatic for animals.[28] Likewise, in diabetic patients with older age, obesity, peripheral neuropathy, joint or other problems that prevent weight-bearing exercise, swimming has advantages due to the buoyancy effect of the water.[29]

Aerobic exercise has been proven to increase BDNF expression in the brain, which is associated with improving exercise capacity that supports brain growth, particularly in the hippocampus. Insulin and BDNF, along with other growth factors, channel intracellular signals in hippocampal neurons to maintain the integrity and function of the hippocampus.[30] Male C57BL/6J mice with T2DM aged 4 weeks, given treadmill exercise 5 days/week for 8 weeks, showed significantly higher levels of hippocampal BDNF protein compared to the T2DM control group.[17] Physical activity in a swimming pool for 4 weeks in diabetic mice showed significantly higher hippocampal BDNF levels in the T2DM+physical activity group compared to the non-physical activity group. The swimming method was tailored to the mice's abilities, with a minimum duration of 5 minutes and a maximum of 10-15 minutes per day, making it difficult to determine the intensity of the activity.[31]

During exercise, lactate is generated and accumulates in the bloodstream then crosses the blood-brain barrier. Once in the brain, lactate activates the Sirtuin1/peroxisome proliferator-activated receptor-gamma co-activator 1a/fibronectin type III domain-containing protein 5 (Sirt1/PGC1α/FNDC5) signaling pathway, leading to increased brain BDNF expression.[32] Another mechanism, transcription of neuronal BDNF is induced by calcium and neural activity. In neuron cultures, calcium influx through L-type voltage-gated calcium channels or N-methyl-d-aspartate-receptors (NMDARs) will increase BDNF mRNA, which can last for up to 6 hours. Increased intracellular calcium will activate the Ras/Extracellular signal-regulated kinases (ERK)1/2, cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA), and calcium/calmodulin (CaM) kinase pathways. Activation of these kinases will stimulate cAMP-responsive element-binding protein (CREB) and subsequently initiate the BDNF transcription process in neurons.[11] During exercise, the CREB and nuclear factor-kB (NF-kB) pathways are transcription factors that induce BDNF expression in the brain.[33] Post-aerobic exercise, BDNF released from brain endothelial cells may also serve as a mediator for cognitive changes. Optimal brain endothelial BDNF release is attributed to shear stress induced by cerebral blood flow and protection against oxidative stress.[34] The factor influencing endothelial BDNF transcription is endothelial nitric oxide (eNO),[35] and a positive correlation exist between BDNF and endothelial nitric oxide synthase (eNOS),[36].

Research on animal models suggests that the efficacy of memory enhancement through the BDNF pathway relies on exercise intensity, with high-intensity exercise demonstrating the most significant benefits.[37] BDNF transcription can occur at both presynaptic and postsynaptic sites. The function of BDNF release at presynaptic sites is to induce long-term potentiation (LTP), while at postsynaptic sites, it is to maintain the LTP phase.[11] In humans, serum levels of BDNF increase by 2 to 3 times following acute exercise compared to resting conditions and show a positive correlation with enhanced cognitive function.[38] The upregulation of serum BDNF levels in older adults correlates with increased hippocampal size and improvements in spatial memory performance and learning processes.[39]

Meanwhile, the results of immunohistochemical examination showed that hippocampal irisin expression in the CA1 and CA3 areas of T2DM

rats who were given swimming training was higher than those who were not given training, but not significantly. These findings are in accordance with other studies, where male C57BL/6J T2DM mice aged 4 weeks, given treadmill exercise 5 days per week for 8 weeks, showed higher hippocampal irisin protein levels than the T2DM control group, but not significantly. This study did not provide a comprehensive explanation of the time interval after the last exercise at which the mice were euthanized and their brain tissue was collected.[17]

Aerobic exercise is known to increase irisin expression in plasma and gastrocnemius muscle, but this is influenced by the time of sampling. Male C57BL/6J mice given treadmill exercise for 1 hour showed an increase in plasma irisin levels, which peaked 6 hours post-exercise and then returned to pre-exercise values within 24 hours. Meanwhile, irisin mRNA levels in the gastrocnemius muscle showed a persistent increase up to 24 hours after exercise.[40] This is one of the factors that influences the results of this study, where brain tissue samples were taken 4 days after the last exercise.

The limitation of this study was the lack of sample assessments at different time points, particularly immediately after the exercise sessions, when an increase in hippocampal irisin expression might be expected. Therefore, further studies should be conducted at multiple time points following exercise.

CONCLUSION

Moderate-intensity swimming exercise has the potential to enhance the BDNF expression in CA1 region of hippocampusas well as on memory in T2DM rats, which slowed the progression of the disease.

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