Original Article:

Improvement of Learning and Memory Deficits With Aerobic Training and Donepezil Co-therapy in Amyloid-β beta-injected Male Rats Through the CREB and BDNF Signaling Pathway

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ABSTRACT

Background: Accumulation of amyloid-β (Aß) plaques, primarily in the hippocampus, leads to neuronal death and Alzheimer disease. Exercise and medications can prevent and treat neuronal diseases. This study aimed to determine the effects of aerobic training and donepezil, a medication used in Alzheimer disease, on the improvement of learning and memory deficits in Aß-injected male rats.

Objectives: This study aimed to determine the effects of aerobic training and donepezil, a medication used in Alzheimer disease, on the improvement of learning and memory deficits in Aß-injected male rats.

Methods: Male Rats were injected with an Aβ solution into their CA1 hippocampal region. After 20 days, the rats were treated with donepezil hydrochloride at doses of 2 mg/kg/d by gavage and following treadmill exercise for 4 weeks. Then, after 24 h, they performed the Morris water maze test for five days. Additionally, we studied the molecular factors involved in neuronal plasticity, such as $Ca2^{+}$/cAMP-response element-binding protein (CREB) and brain-derived neurotrophic factor (BDNF) on day 33. The animals were also evaluated histologically to determine the deposition of Aβ in the brain tissue.

Results: Behavioral analysis showed that in the probe test, the latency to the platform zone significantly increased in the training group ($F_{1,20}=6.815$; $P<0.05$) and in the drug group ($F_{1,20}=6.369$; $P<0.05$). But there were no significant changes in the combined group compared with the control group ($F_{1,20}=3.909$; $P>0.05$). Molecular analysis showed that CREB gene expression improved in the training group ($F_{1,8}=9893.539$; $P<0.01$) and in the drug group ($F_{1,8}=631.958$; $P<0.01$). But in the combined group, there were no significant changes compared with the Aβ group ($F_{1,8}=2.556$; $P>0.05$). BDNF gene expression improved in the training group ($F_{1,8}=25.077$; $P<0.001$), and in the drug group ($F_{1,8}=45.296$; $P<0.001$). Also, in the combined group, this change was significant compared with the control group ($F_{1,8}=64.342$; $P<0.001$). Histomorphometric analysis showed that the density of survived neurons was considerably increased in the combined group ($P<0.01$), and the drug group ($P<0.05$) compared to the control group

Conclusion: In the present study, behavioral and biochemical analysis demonstrated that aerobic training and donepezil hydrochloride treatment for 4 weeks protect Aβ-injected male rats against memory impairment.

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1. Introduction

The most common neurodegenerative disease characterized by the deposition of amyloid-β (Aβ) plaques in the brain is Alzheimer disease (AD) [1]. Studies have demonstrated that soluble oligomers of Aβ are neurotoxic [2] and lead to neuronal apoptosis, as well as learning and memory deficits. $Ca2^{+}$/cAMP-response element-binding protein (CREB) is a transcription factor that has long been considered a very important factor in learning and memory [3]. CREB phosphorylation and cell proliferation in the hippocampus of transgenic mice is prevented by Aβ [4].

In AD, the first-line therapies are acetylcholinesterase inhibitors (Aches) that increase cholinergic tone in the brain and improve the mental function of patients who have mild to moderate AD [5]. To maintain the functions of neurotrophins in the pathophysiology of AD indecipherable, neurotrophin signaling is often postulated as a potential object for the development of new drugs to cure this disease [6]. Previous studies have shown that CREB and brain-derived neurotrophic factor (BDNF) gene expression is increased by exercise training [7-9]. The intensity and duration of exercise are crucial in the regulation of these genes [10-12]. As well, strength and aerobic training can produce similar or different effects on CREB or BDNF gene expression [13-15]. One of the main critical signaling pathways in the brain is the binding of BDNF to its specific receptor in various regions of the hippocampus [16]. The binding of BDNF to its receptor leads to the activation of several signaling pathways, including protein kinase A (PKC), mitogen-activated protein kinase (MAPK), and CREB [17, 18].

Studies have shown that changes in BDNF signaling are necessary to produce the effects of exercise training on the formation of the hippocampus. Furthermore, BDNF signaling can attenuate neuronal plasticity and the blockade of this signaling pathway inhibits the memory and learning improvements caused by exercise training in rodents [19]. Even a single training session can increase BDNF levels [20]. Exercise training and donepezil hydrochloride are thought to activate neurogenesis via different pathways. Thus, this study aimed to determine the effects of exercise training and donepezil hydrochloride on learning and memory deficits and CREB and BDNF gene expression in Aβ-injected male rats.

2. Materials and Methods

Animals

Male Wistar rats weighing 210-250 g were obtained from the Pasteur Institute (Tehran, Iran). Rats were housed for over one week at 23±1°C on a controlled 12:12 h light-dark cycle. Animals were housed in groups of six. Food and water were provided ad libitum. All study procedures were carried out following the National Institute of Health (NIH) guide for the care and use of laboratory animals (HHS publication 85-23, 1985). This study was conducted according to the standard and Ethics Committee of Islamic Azad University, Marvdasht Branch (Code: IR.IAU.M.REC.1398.022). Donepezil hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) (2 mg/kg/d) was administered by gavage.

Preparation of Aβ1-42

To prepare Aβ, we dissolved Aβ1-42 stock (1-42 rat, SCP0038-500UG, Sigma) powder in phosphate-buffered saline (PBS), then made aliquots and stored them at -20˚C. A solution of 200 ng/µL concentration of Aβ was prepared in PBS (0.1 M). The solution of Aβ was incubated for 5 days at 37ºC, and before injection, PBS was added to the solution to reach a final concentration of 10 ng/µL. It has been previously demonstrated that the injection of Aβ1-42 leads to brain dysfunction, which is manifested as learning and memory deficits in the Morris water maze (MWM) test [21].

Surgery and microinjection

Rats were anesthetized by intraperitoneal injection of ketamine hydrochloride (Alfasan, Netherland) (50 mg/kg) and xylazine (Alfasan, Netherland) (4 mg/kg) and secured in a stereotaxic frame. The coordinates used for injection into the dorsal hippocampus were antero-caudal: -3.2 mm from bregma; lateral: ±1.8 from bregma; and vertical: 7.2 mm from dura according to the Paxinos and Watson atlas (1986). Aβ was injected using polyethylene tubing with a 25-µg Hamilton syringe. The left and right hippocampi were microinjected with 3 µL of Aβ per side (10 ng/µL) into the CA1 bilaterally (1 µg/rat) over one minute.

Experimental design

Twenty-four adult male rats were assigned to one of the following groups:

Group 1 (Aβ): microinjection of Aβ into the CA1 bilaterally (1 µg/rat).

Group 2 (T-Aβ): microinjection of Aβ into the CA1 bilaterally (1 µg/rat) and treadmill running (30, 45, or 60 min/d) for 28 days.

Groups 3 (D-Aβ): microinjection of Aβ into the CA1 bilaterally (1 µg/rat) and treatment with donepezil hydrochloride (2 mg/kg/d by gavage, once daily) for 28 days.

Group 4 (T+D-Aβ): microinjection of Aβ into the CA1 bilaterally (1 µg/rat), treadmill running (30, 45, or 60 min/d) and treatment with donepezil hydrochloride (2 mg/kg/d by gavage, once daily) for 28 days.

Because of the neuroprotective effect of the CREB/BDNF signaling pathway, we studied the contribution of this pathway in exercise and the neuroprotective effects caused by donepezil hydrochloride against Aβ-induced disturbances. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was used to study the alteration in BDNF and CREB gene expression induced by exercise and donepezil hydrochloride treatment. Also, hematoxylin and eosin (H&E) staining was performed to analyze cell density and neurodegeneration.

Exercise protocol

Rats ran on a leveled motorized treadmill (Pishro Andishe Sanat Co, Tehran, Iran) on weekdays between 8:00 AM and 3:00 PM for 4 weeks. The rats were habituated with the treadmill while it was idle. During the first two weeks, the rats attributed to exercise ran on the treadmill for two15-min practices at a speed of 10 m/min. In the third and fourth week, rats ran for 3 and 4 practices, respectively, at a speed of 15 m/min. To prevent muscle fatigue, a 5-min rest period was given between practices. To encourage unwilling rats to continue running a mild electric tingling (intensity=0.5 mA) was constantly delivered from stainless bars placed at the start of each running lane [22].

Behavioral test: Morris Water Maze (MWM)

The maze is made of a painted black round pool 136 cm in diameter replete with water (temperature: ~ 23ºC, depth: 25 cm). The pool was located in a room with different colored visual cues on the walls. A platform 10 cm in diameter was submerged in the water (2 cm below the surface). The pool was divided into four quadrants with four points configured as starting positions (N, S, W, and E). The camera placed above the center of the pool monitored the rat's position. Rats' movements were recorded by a 3CCD camera (Panasonic Inc., Japan) located 2 m above the MWM apparatus. EthoVision software (version XT7) and a video tracking system for the automation of behavioral experiments (Noldus Information Technology, the Netherlands) evaluated locomotion tracking. Distance traveled, swimming speed, and escape latency were recorded during a 90-s window in both the training and probe trials.

Rats habituation

The rats were familiarized to the pool 24 hours before starting training, by allowing them to perform 90 s of swimming in the absence of the platform.

Study procedure

The behavioral tests started 28 days after the end of training and drug protocols. The behavioral tests included a single training session consisting of four trials over four days that each trial was started in a different quadrant of the maze. During the four training sessions, each of the four starting positions was used twice in random order. The rats were given 90 s to find the hidden platform during each trial; the animals were allowed to remain on the platform for 30 s, after finding the platform. Then, they were placed in a holding cage, until the start of the next trial. The animals were returned to their home cages when training was completed. Probe trial started 24 h later (on the test day). The hidden platform was removed and the animals were released from a fixed location (N) and allowed to swim freely for 90 s in the probe trial. All experiments were conducted between 10:00 AM and 2:00 PM.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

By using TRIzolTM reagent (Thermo Scientific Fisher, USA.) and according to the manufacturer's instructions, total RNA was extracted from 200 µg of hippocampus tissue. By using a nanodrop (ND-1000, Thermo Scientific Fisher, USA) and gel electrophoresis, we respectively assessed the quantity and quality of the extracted RNA. The RNA was treated with DNase I (Qiagen, Hilden, Germany) per the manufacturer's instructions to eliminate genomic contamination. Next, complementary DNA (cDNA) was synthesized using 1 µg of total RNA. By using a glyceraldehydes 3-phosphate dehydrogenase (GAPDH) primer as the housekeeping gene, the integrity and quality of the cDNA were examined. To evaluate the differences in the expression patterns of the BDNF

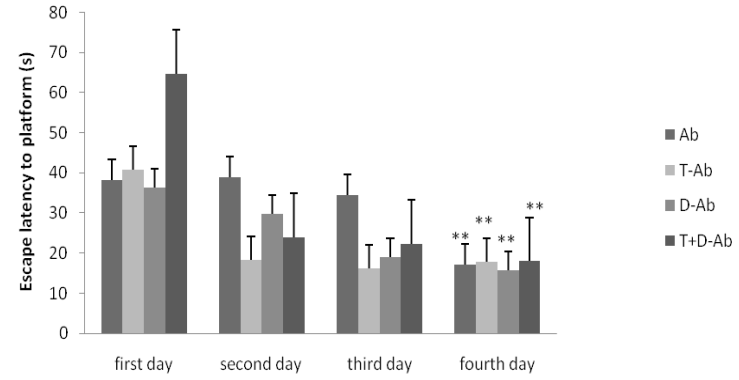

Figure 1. Morris Water Maze test results, escape latency to the platform (s) over 4 days

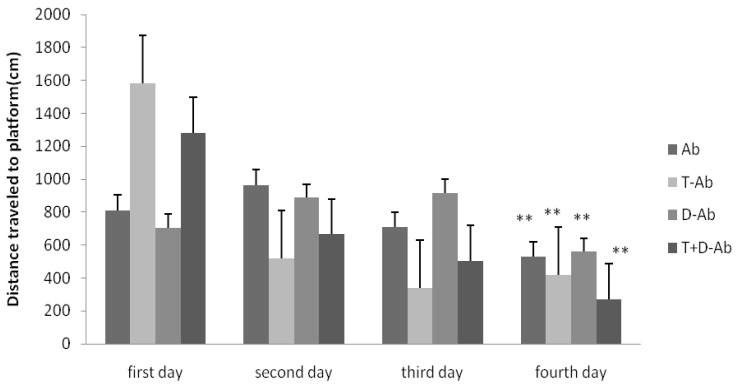

Figure 2. Morris Water Maze test results, distance traveled to the platform (cm) over 4 days

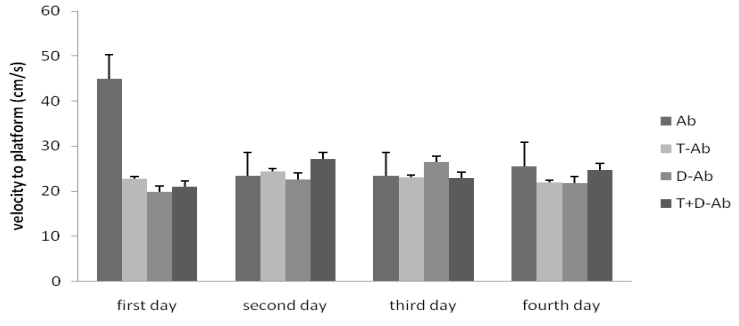

Figure 3. Morris Water Maze test results, velocity to the platform (cm/s) over 4 days

and CREB genes among the experimental groups, RT-PCR was carried out. The primers were designed using Primer 3 software version 0.4 (frodo.wi.mit.edu).

Real-time RT-PCR was performed in 20 µL reactions containing 1 µL of cDNA target, 100 nM of each the forward and reverse primers, and 1X SYBR1® Premix Ex TaqTM II (Takara, Tokyo, Japan). The experiments were carried out in triplicate using a CFX96TM real-time system (C1000TM Thermal Cycler; Bio-Rad, Hercules, CA, USA). The amplification conditions were as follows: initial denaturation at 95ºC for 10 min followed by 40 cycles (denaturation at 95ºC for 15 s, annealing and extension at 60ºC for 1 min). By comparing the cycle thresholds (CTs) of the target genes with that of the housekeeping gene (GAPDH) using the $2^{-\Delta\Delta CT}$ method and REST 2009 software [23], we calculated the relative values of the mRNA expression of the CREB and BDNF genes. For calculation of the primer set efficiencies in real-time PCR, we used serial dilutions of the cDNAs.

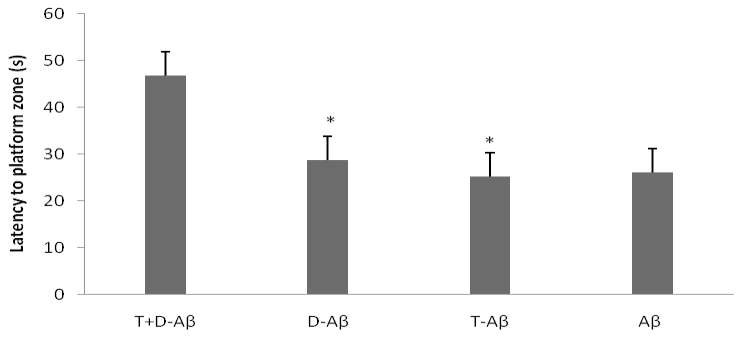

Figure 4. Morris Water Maze test results, latency to platform zone (s) in the probe test

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Histopathology

The animals were euthanized after 33 days of treatment and behavioral testing. The harvested brain tissues were fixed in 10% neutral buffered formalin (NBF, pH 7.26) for 48 h, then they were processed and embedded in paraffin. Sections of 5-µm thickness were prepared and stained with hematoxylins and eosin (H&E). An independent reviewer evaluated the slides using light microscopy (Olympus BX51; Olympus, Tokyo, Japan). The slides were assessed for histological changes, including inflammatory responses, neuronal degeneration, hemorrhage, and hyperemia.

Data analysis

Data are expressed as the Mean±SEM (standard error of the mean). Data analysis was performed in SPSS (version 17). For the behavioral testing and molecular studies, a 2-way analysis of variance (ANOVA) and 2-way repeated-measures ANOVA followed by post hoc Bonferroni test were used. A P value less than 0.05 ($P<0.05$) was considered statistically significant (*$P<0.05$, **$P<0.01$, and ***$P<0.001$).

3. Results

Behavioral results

Effects of aerobic training and donepezil hydrochloride treatment on spatial learning in Morris water maze test in Aβ-injected male rats

We used the MWM test to test spatial learning and memory because it is more useful than other test apparatuses. The data obtained in the training session showed a significant difference between the first and fourth days in escape latency and distance traveled in all experimental groups ($P<0.01$; Figure 1 and 2). The swimming speed did not show any significant change between the first and fourth days of training trials, indicating no motor disturbance in the treated animals ($P>0.05$; Figure 3).

Effects of aerobic training and donepezil hydrochloride on the Aβ-induced spatial memory impairment in Morris water maze test of Aβ-injected male rats

After training the rats, we tested spatial memory impairment on the fifth day using the MWM test. The statistical analysis revealed that in the probe test, the latency to the platform zone significantly increased in the T-A group ($F_{1,20}=6.815$; $P<0.05$) and in the D-Aβ (2 mg/kg/d) group ($F_{1,20}=6.369$; $P<0.05$). But there were no significant changes in the T+D-Aβ group compared with the Aβ group ($F_{1,20}=3.909$; $P>0.05$) (Figure 4).

Molecular results

Effects of aerobic training and donepezil hydrochloride (2 mg/kg/d) on CREB gene expression in Aβ-injected male rats

The T-Aβ group improved the CREB gene expression in the CA1 region when compared with the Aβ group ($P<0.001$) (Table 1). Furthermore, our data showed a significant difference in the CREB gene expression between the D-Aβ group and the Aβ group ($P<0.001$). T+D-Aβ group changed CREB gene expression in the CA1 region, when compared with the Aβ group, while this change was not significant in comparison to the Aβ group ($P>0.005$) (Table 1).

Effects of aerobic training and donepezil hydrochloride (2 mg/kg/d) on BDNF gene expression in Aβ-injected male rats

The T-Aβ group increased the BDNF gene expression in the CA1 region when compared with the Aβ group ($P<0.001$) (Table 1). Furthermore, our data showed a significant difference in the BDNF gene expression between the D-Aβ group and the Aβ group ($P<0.001$).

Table 1. Real-time PCR results of CREB and BDNF gene expression (relative expression compared with Aβ)

Genes	Groups	Mean	Standard Error of the Mean (SEM)	F	df	Sig.	Partial Eta Squared
CREB	Aβ	0.99769750	0.003988050	-	-	-	-
	T-Aβ	3.60505959	0.024988219	9893.539	1,8	0.001	0.999
	D-Aβ	1.62484411	0.040798915	631.958	1,8	0.001	0.987
	T+D-Aβ	4.31739547	0.078819633	2.556	1,8	0.149	0.242
BDNF	Aβ	0.01684822	0.008469295	-	-	-	-
	T-Aβ	1.10830044	0.280381134	25.077	1,8	0.001	0. 758
	D-Aβ	1.25259633	0.071361089	45.296	1,8	0.001	0.850
	T+D-Aβ	1.00014414	0.020796289	64.342	1,8	0.001	0.889

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T+D-Aβ group changed BDNF gene expression in the CA1 region when compared with the Aβ group, and this change was significant compared with the Aβ group ($P<0.001$) (Table 1).

Histopathological Results

Effects of aerobic training and donepezil hydrochloride on the density of the survived neurons in Aβ-injected male rats

Histomorphometric analysis showed that the density of survived neurons considerably increased in the T+D- Aβ ($P<0.01$), and D-Aβ ($P<0.05$) groups compared with the Aβ group (Figure 6).

4. Discussion

Acetylcholinesterase inhibitors (AChEis) are the main drugs for the treatment of Alzheimer disease. They increase cholinergic activity in the brain and improve cognitive function of the patients with AD [24]. One of the acetylcholine esterase inhibitors used for the treatment of AD is donepezil whose therapeutic effects are achieved by slowing the hydrolysis of acetylcholine at synaptic termini [25]. On the other hand, studies have shown that exercise decreases AD symptoms by increasing neuronal vitality and neurogenesis [26]. The study of exercise in rats has shown that it increases spatial learning and neuron density in the dentate gyrus of the hippocampus, which improves short-term memory [27, 28]. Studies have demonstrated that light and moderate treadmill training increases cell infiltration in juvenile rats compared with the control animals [27-29]. The amelioration of cognitive deficits has been reported for other exercise training as well [7, 27-30].

In this context, we assessed the effects of donepezil hydrochloride and aerobic training in Aβ-injected male rats. In our study, behavioral testing and biochemical analysis demonstrated that aerobic training and donepezil hydrochloride treatment for 4 weeks protects Aβ-injected male rats against memory deficits. Additionally, studies demonstrated that donepezil at doses of 0.3 and 1.0 mg/kg significantly improved ischemia-induced memory dysfunction based on the MWM test results [31]. In our study, after training the rats, we tested their spatial memory impairment on the fifth day using the MWM.

The statistical analysis revealed that in the probe test, the latency to the platform zone significantly increased in the training group and the drug group. But there were no significant changes in the combined group compared with the control group as mentioned in Figure 4. Previous studies established that behavioral alterations will appear in 20 days after injection [32, 33]. So here we chose day 20 for starting the treatment protocols. Based on the obtained results, treated rats displayed lower impaired spatial learning and memories (as assessed in MWM task), which is regarded as a good indicator of hippocampal function [33].

In AD, many cascades that have an important role in memory formation are impaired. One of the most crucial factors involved in learning and memory is the CREB transcription factor, which plays a basic role in memory process and neuronal plasticity via c-Fos that is connected to spatial and behavioral learning and memory in rats [3]. We found that donepezil hydrochloride and aerobic training increased CREB expression in Aβ-injected male rats. Donepezil increased

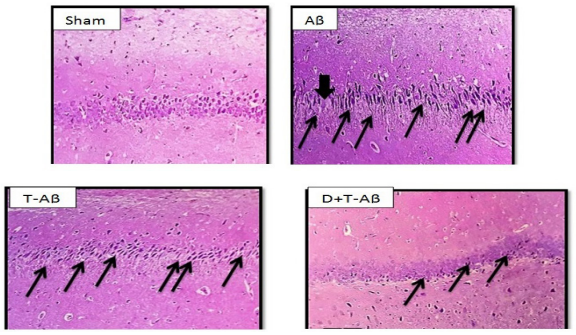

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Figure 5. Histopathologic sections of the brain in different experimental groups

The groups of: Aβ, T-Aβ, D-Aβ, D+T-Aβ

Thin arrows showing CA1 pyramidal neurons necrosis (Magnification: x400)

the phosphorylation of CREB, which is vital for TrkB-dependent hippocampal learning [34, 35].

Results demonstrated that the activation of Trk receptors by AChEis is related to the activation of many crucial intracellular signaling cascades [36]. Studies demonstrated that training attenuated the vitality of cells in the dentate gyrus by increasing CREB phosphorylation in ischemic rats, thereby improving memory [7]. In this study, training group improved the CREB gene expression in CA1 region when compared with the control group as mentioned in Figure 2. Furthermore, our data showed a significant difference in the CREB gene expression between the drug group and the control group. Combined group changed CREB gene expression in CA1 region, when compared with the control group, while this change was not significant in comparison to the control group (Figure 2).

One of the most important factors in synaptic plasticity, neurogenesis, and neuronal survival is BDNF [37]. CREB and cAMP regulate BDNF and its receptor, tropomyosin related kinase B. Besides, CREB-BDNF signaling has an important role in regulating several neural functions, including learning, memory, mood balance, and reward mechanisms [38]. Further studies demonstrated that in patients with mild to moderate AD, long-term treatment with donepezil restores BDNF serum concentrations to the levels observed in age-matched controls [5]. Most studies have shown that exercise has a positive effect on memory and learning. Two weeks of training with 7,8-dihydroxy flavones increased cellular metabolism, hippocampal activity, and synaptic plasticity in the brain of injured rats [39, 40]. Voluntary and involuntary training attenuated vascular dementia of the hippocampus and increased BDNF, PCREB, PERK1/2

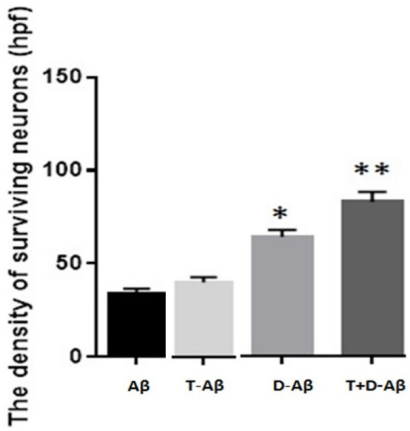

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Figure 6. The density of surviving neurons

*, **, ***: values indicates treatment groups. Aβ, T-Aβ, D-Aβ, T+D-Aβ, versus un-treatment group control; **P<0.01.

in the C1, and C2/3 dentate gyrus in rats with vascular dementi [41]. Female ovariectomized Alzheimer model rats following 3 months of training showed improvement in their memory and learning [41].

Training significantly increased BDNF and activated CREB, and APE 1 in the cerebral cortex and hippocampus of rats [42]. Therefore, exercise appears to have a feedback effect on BDNF expression and synaptic plasticity [18, 43]. In our study, the training group improved the BDNF gene expression in the CA1 region when compared with the control group (Figure 2). Furthermore, our data showed a significant difference in the BDNF gene expression between the drug group and the control group. The combined group changed BDNF gene expression in the CA1 region when compared with the control group, and this change was significant compared with the control group (Figure 2). Besides, based on our results, donepezil hydrochloride and aerobic training increased BDNF expression in Aβ-injected rats. In this study, all H&E stained sections from different experimental groups were evaluated histologically to find out the deposition of Aβ in brain tissue. The effects of Aβ administration on the survival of hippocampal neural cells were examined by histopathological evaluation. The Aβ-injected group has shown morphological signs of necrosis: cytoplasmic swelling of neurons, various degrees of vacuolization, numerous indistinct and dark cells and necrotic cells (Figure 5). In addition, the organization of this cellular layer (hippocampus CA1 neuronal layer) was generally disrupted. The histopathologic findings of training group showed lesions similar to the control group with dark stained or no visible cell boundary in the hippocampal CA1 areas (Figure 5). Micrographs of drug-treated animals showed that these treatments markedly attenuated the Aβ-induced neuronal damage as evidenced by the fewer

necrotic neuronal cells observed in this group (Figure 5). Histopathological evaluation of combined group of animals with AD showed a considerable reduction of Aβ injury in CA1 neurons at day 33 compared with the other treatments (Figure 5). Also, histomorphometric analysis showed that the density of survival neurons were considerably increased in combined and drug groups compared with the control group (Figure 6).

Studies demonstrated that donepezil hydrochloride combined with aerobic exercise training improved the learning and memory function of rats with AD. The mechanism was also related to the improved morphological structure of hippocampal neurons, reduced loss of neuronal cells, increased ChAT content and decreased AchE content [44]. Thus, drug administration and exercise training strategy may be a safe strategy. Drug and aerobic training were well tolerated by rats because we did not observe any significant weight loss over 4 weeks of treatment.

5. Conclusions

Our results in behavioral testing and biochemical analysis illustrated that the combined use of aerobic training and donepezil hydrochloride have synergistic effects on neurogenic factors. Considering the effects of donepezil hydrochloride and aerobic training, such as effectiveness, and fewer side effects make them a proper choice for AD treatment. Future studies should focus on the other mechanisms which may contribute to this issue.

Ethical Considerations

Compliance with ethical guidelines

The study protocol was approved by the Ethics Committee of Islamic Azad University, Central Tehran Branch.

Funding

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Authors' contributions

Conceptualization: Iman Mohseni; Supervisor: Maghsoud Peeri and Mohamad Ali Azarbayjani

Conflict of interest

The authors declare no conflict of interest.

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