

Combination Therapy of Donepezil and Environmental Enrichment on Memory Deficits in Amyloid-Beta-Induced Alzheimer's Disease Rats

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Research Article

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Abstract

Alzheimer's disease (AD) is progressive neurodegeneration known as the most common cause of dementia, and it is the sixth leading cause of death in older people. Given the promising data on the additive effect of combination therapy with donepezil (Aricept), an acetylcholinesterase inhibitor (AChEI), and regarding the similar neuronal mechanisms through them donepezil and environmental enrichment (EE) exert their enhancing effects on cognition; we asked whether simultaneous treatment with two paradigms in amyloid-beta-induced AD rats may lead to greater cognitive improvements than either treatment individually. AD was induced by intrahippocampal injection of amyloid-beta (1-42, 6 µg), and donepezil was orally administered (4 mg/kg) for 21 days. Environmental enrichment consisted of housing animals in large cages (50× 50× 50 cm) containing a running wheel and differently shaped objects for 21 days. Spatial learning and memory were assessed in the Morris water maze (MWM) and Real-time PCR was performed to assess the expression of brain-derived neurotrophic factor (BDNF) and M1 muscarinic acetylcholine receptor (AChM1R) within the hippocampus. Spatial memory was impaired in AD animals, and while neither pretreatment with donepezil nor EE alone could significantly restore spatial memory scores in AD rats, combination therapy was effective. BDNF expression was suppressed in AD rats and pretreatment with donepezil plus EE could increase it to the saline levels. The data suggest that a cholinesterase inhibitor and cognitive stimulation can be used effectively in combination to improve cognitive loss in an AD rat model.

Introduction

Alzheimer's Disease (AD) is progressive neurodegeneration known as the most common cause of dementia and it is the sixth leading cause of death in older people. Current estimates suggest that 44 million people live with dementia worldwide at present and by 2030, the number of people with the disease is expected to rise to more than 70 million worldwide (Garre-Olmo, 2018). This profile has a lot of medical, economic, and social concerns now and in the future (Nichols et al., 2019). The accumulation of amyloid plaques and decreased levels of acetylcholine (ACh) in selectively vulnerable brain regions are the best-known pathological features of the disease (Nelson et al., 2009). Cholinergic deficiency contributes to cognitive decline and probably behavioral symptoms of AD (Terry & Buccafusco, 2003).

Donepezil (Aricept), an acetylcholinesterase inhibitor (AChEI), has for over 2 decades served as a monotherapy or in combination with NMDA-agonist memantine for either improvement or stabilization of cognitive and functional performance of the disease (J. Guo et al., 2020). It prolongs the acetylcholine's activity at the synapse by blocking its breakdown (Kasa et al., 2000). Donepezil has also been shown to decrease the amyloid plaques in the brain of humans and mice (Dong et al., 2009). None of the AChEIs has proven more than modestly effective even at the maximum tolerated doses. There are no definitive treatments so far that completely stop the progression of AD (Noetzli & Eap, 2013). Non-drug therapy is an alternative approach to preserve or even improve the cognitive abilities of AD (Klimova et al., 2016).

Recent studies have proved the clinical and epidemiological benefits of an active lifestyle in reducing the risk of incidence or slowing the progression of cognitive disorders (Arenaza-Urquijo et al., 2015). More advanced educational and occupational status has been consistently correlated with a lower risk of developing dementia in general and AD in particular (Norton et al., 2014; Sando et al., 2008). Such a situation can be imitated in experimental models. Environmental enrichment (EE) is an intervention that exposes laboratory animals to new and complex stimuli due to changes in their physical environment, leading to the amplification of their sensory, cognitive, and physical stimuli. In this paradigm, the animal is placed in larger cages where there are a variety of attractive objects such as tunnels, materials used for animal nesting, toys, and running wheels (Baroncelli et al., 2010).

The beneficial effects of EE as a potential noninvasive strategy on cognitive deficits and biochemical features of AD pathology in transgenic models have been reported (Hu et al., 2010; Petrosini et al., 2009). EE increases the expression of neurotrophic factors and other signaling molecules involved in cognitive processing and exerts neuroprotective effects in AD models (Angelucci et al., 2009; Herring et al., 2009; Hu et al., 2010). There are also some conflicting data implying that the effect of EE on AD pathology, neurogenesis, or cognitive performance is heterogeneous and variable (Arendash et al., 2004; Petrosini et al., 2009). EE could emerge as a potential non-pharmacological strategy that might affect the onset and progression of neurodegenerative diseases including AD (Bragin et al., 2005; Klimova et al., 2016).

The combination of different interventions for achieving superior cognitive enhancement has appeared as a promising therapeutic approach in health and disease. In the case of cognitive deficits in AD and other experimental models, the combination of donepezil with some treatments including piperine (Nazifi et al., 2021), resveratrol (Rao et al., 2021), estradiol (Gibbs et al., 2011), and manual acupuncture (Jiang et al., 2019) has been explored. However, to date, no study has addressed the combination of donepezil and EE for cognitive impairments in experimentally neurodegenerative status.

Given the promising data on the additive effect of combination therapy with donepezil and regarding some similar neuronal mechanisms through them donepezil and EE exert their enhancing effects on cognition, we asked whether simultaneous treatment with two paradigms in amyloid-beta-induced Alzheimer's disease rats may lead to greater cognitive improvements than either treatment individually. Moreover, we sought to address the potential contribution of molecular mechanisms involved in learning and memory by measuring the hippocampal mRNA expression of M1 muscarinic acetylcholine receptor (AchM1R) and brain-derived neurotrophic factor (BDNF) of the animals.

Materials And Method

Amyloid-beta 1-42 was a product of Sigma Aldrich (SCP0049) and donepezil was purchased from Samisaz company, Mashhad, Iran. All other chemicals used in this study were of analytical grade and high purity.

Experimental animals

All experiments and animal handling were approved by the Animal Ethics Committee of Mashhad University of Medical Sciences (IR.MUMS.MEDICAL.REC.1396.683). The experimental design is depicted in Fig.1. Male rat pups were purchased from the colony maintained by Mashhad Medical College Animal Facility.

At the age of 1 month, the animals were randomly assigned to one of the five experimental groups (n=8 each) as follow:

i) Alzheimer (Alz): Rats received bilateral intrahippocampal injections of amyloid-beta (6 μ g/4 μ l) and maintained in standard cages (50*30*25 cm).

ii) Sham-operated: Rats received the same volume of vehicles by the same route and were housed in standard cages.

iii) Alzheimer+Donepezil (Alz+Don): Rats in standard cages treated orally with donepezil (4 mg/kg) for 3 weeks and then subjected to intrahippocampal injection of amyloid-beta 1-42.

iv) Alzheimer+Enriched Environment (Alz+EE): Rats housed in larger cages (50*50*50 cm) throughout 5 weeks which were equipped with nesting materials, tunnels, ladder, shelters, houses, and toys, which were modified and rearranged weekly to increase the sense of novelty. The animals were then subjected to intrahippocampal injection of amyloid-beta 1-42.

v) Alzheimer+Don+EE: Rats subjected to 5 weeks of the enriched environment combined simultaneously with 3 weeks of donepezil treatment and then subjected to intrahippocampal injection of amyloid-beta 1-42 (depicted in fig 1).

In all groups, behavioral tests were performed at 2 months. When behavioral tests were finished, rats were euthanized for hippocampal dissection and further analysis (Fig. 1). Animals were kept in groups of four and eight in standard and EE cages, respectively, and they had free access to food and water in all conditions and were housed in a climate-controlled room (23 °C \pm 1 °C) on a 12-h light-dark cycle (lights-on 06:00–18:00 h). All cages were cleaned once a week.

Intrahippocampal microinjection of amyloid-beta 1-42

The animals were anesthetized with ketamine-xylazine (100-10 mg/kg, i.p.; Vibac Laboratories, Carros, France) and placed in a stereotaxic frame. Bilateral Burr holes were drilled in the skull over the CA1 region of hippocampi using the following coordinates: 3.6 mm posterior to bregma, 2.4 mm lateral to the sagittal suture, and 3.6 mm ventral to the skull surface. Amyloid-beta 1-42 (6 μ g in 4 μ l PBS) or vehicle was injected bilaterally (2 μ l each side) through a 27-gauge injection needle connected by a polyethylene tube to a Hamilton syringe (10 μ l). The injection was delivered slowly over 10 min. The injection needle was left for an additional 60 s to maximize diffusion away from the needle tip and minimize dorsal diffusion.

Spatial learning and memory in Morris water maze (MWM)

The MWM was a black circular metal pool (160 cm diameter and 80 cm height) filled with water (22-24°C) at a depth of 50 cm. The pool was conceptually divided into four equal quadrants, and release points were labeled in each quadrant as 1, 2, 3, and 4. A hidden circular platform (10 cm diameter) was submerged 2 cm below the water surface in the center of quadrant 3. The trials were performed in a low-light room in which a variety of fixed geometric images (e.g., squares, circles, or triangles) were attached at different locations on the walls around the maze. Animal performance was recorded automatically by a video tracking system (Borj Sanat Azma), which could be traced on a computer screen. Behavioral experiments in the MWM task were accomplished on two consecutive days (fig. 1). Each rat completed three training blocks separated by a 30-min interval during the acquisition. Each block contained a series of four successive trials of 60 s duration and three 60 s inter-trial intervals. On each trial, the animal was immersed into the water from one of the 4 quadrants of the maze and was allowed to find the hidden escape platform in 60 s (maximum time). When the animal found the platform, it was allowed to stay there for 20–30 s and returned to its home cage to wait for 20–30 s before the subsequent trial. Rats that failed to find the submerged platform within 60 s were guided to the platform. The time and distance to find the escape platform was recorded and analyzed later. A single probe trial was carried out 24 h after the last training trial to test the spatial memory in the water maze. In this trial, the animal was allowed to swim freely for 60 s without any escape platform. The percentage of the time, distance, and the number of crossing in the target quadrant (quadrant 3) was analyzed and considered the spatial memory criteria. The behavioral tasks for all groups were conducted during the same time of the lights-on phase.

Tissue dissection and Real-time PCR

After completing behavioral experiments (fig. 1), animals were decapitated, and both hippocampi were rapidly extracted and frozen in liquid nitrogen and then stored at -80 until further assessments. Quantitative real-time PCR (qRT-PCR) was performed to assess the hippocampal expression of AchM1R and BDNF mRNA. Total RNA was extracted by the RNeasy Mini kit (Parstous, Iran). A nanodrop spectrophotometer measured total RNA concentration at 260 nm absorbance (Thermo Fisher Scientific, Germany). According to the manufacturer's instructions, the first-strand cDNA was synthesized with 1000 ng of RNA using the cDNA Synthesis Kit (Yekta-Tajhiz; Cat: Yt4500). qRT-PCR amplification was performed with a CFX 96 Real-Time System (Roche Applied Science, USA) using Syber Green dye (Amplicon, Denmark). All qRT-PCR reactions were done in duplicate. Relative expression levels of the target genes were calculated using The $2^{-\Delta\Delta Cq}$ (Livak) method by normalization to the internal control (β -actin). The sequences of the primers and the annealing temperature are shown in Table 1. **Statistical analysis**

The time and distance to find the hidden platform in the MWM training blocks in the individual groups were assessed using a repeated measures analysis. These variables between the groups were analyzed using a two-way ANOVA with repeated measures (group and block as the factors). All comparisons of data collected in the probe trials, swimming speed, and gene expression were analyzed with one-way

ANOVA followed by Tukey's post hoc multiple comparison test. The values are expressed as means±SEM, and $P<0.05$ was considered statistically significant.

Results

Spatial learning and memory in MWM

In acquisition trial blocks, the repeated measures analysis revealed that animals in all individual groups successfully learned the location of the hidden platform, as revealed by the decline in escape latency and distance traveled over three subsequent blocks of training ($P=0.000$). No significant difference was found among the groups in escape latency and distance traveled by analyzing two-way repeated-measures ANOVA ($P=0.912$ for time and $P=0.759$ for distance, Fig. 2 A and B). The spatial memory parameters in the single probe trials are shown in Fig. 2C. One-way ANOVA indicated the significant differences in the percentage of time ($F(4,36)= 4.567, P=0.004$), distance ($F(4,36)= 4.635, P=0.004$), and crossing ($F(4,36)= 2.774, P=0.042$), in the target quadrant (Q3) among the groups. Tukey's post- hoc test revealed that Alzheimer's animals spent significantly less time and distance in the target quadrant than the sham and Alz+Don+EE groups ($P<0.05$). The decrease in the number of crossing in the Alz group reached a significant level only compared with sham and Alz+Don+EE ($P<0.05$). There was no significant difference in MWM swimming speed among the five experimental groups (fig 2. D).

AchM1R and BDNF mRNA expression

The results of AchM1R and BDNF mRNA expression in the hippocampus are depicted in Fig. 3A, B. The difference in AchM1R mRNA expression did not reach a significant level ($P=0.199$, A). One-way ANOVA revealed a significant difference in hippocampal BDNF between the groups ($F(4,10)= 5.838, P=0.011$, B). Tukey's post- hoc analysis showed that BDNF is decreased in Alz compared to the sham group ($P<0.05$), and donepezil plus EE could reverse it to the vehicle value ($P<0.005$).

Discussion

Based on the additive effect of combination therapy of donepezil and regarding the similar neuronal mechanisms through them donepezil and EE exert their enhancing effects on cognition, we hypothesized that an intervention paradigm combining donepezil administration as an AChEI with cognitive stimulation in AD rats would yield greater cognitive benefits than single-domain interventions. AD animals showed impaired function in spatial memory parameters as marked by decreased time, distance, and crossing in the target quadrant in MWM. Pretreatment with donepezil and EE separately could only lead to a slight enhancement in memory parameters. When animals were treated with donepezil and EE simultaneously, they exhibited significantly restored function as seen in saline-treated rats. Hippocampal BDNF expression was decreased in AD animals and combination therapy of donepezil and EE could reverse it to the control levels.

Alzheimer's disease is characterized by the progressive accumulation of senile plaques and neurofibrillary tangles in the brain, which are associated with neuronal damage, particularly in the cholinergic system. The amyloid-beta peptide is the main component of senile plaques, and it is known as a causal factor in the development and progress of AD (Nelson et al., 2009). Animal models with AD exhibit progressive and long-term deficits in memory function which are similar to the symptoms of sporadic AD (Facchinetti et al., 2018; LaFerla & Green, 2012). Our data confirmed the previous studies showing that ICV or intrahippocampal injection of amyloid-beta causes memory impairments. However, a single injection of amyloid peptide does not express all of the pathological features of AD. There is no robust animal model that reproduces all of the characteristics of the disease, and genetic mouse models, as well as ICV or intrahippocampal injection of amyloid proteins, are the most widely applied methods for experimentally induced AD (Facchinetti et al., 2018). The injection of amyloid-beta into the rat brain induces pro-inflammatory reactivity, oxidative stress, and a cascade of neurotoxicity that ultimately leads to the loss of neuronal functions involved in the behavioral symptom of AD (McLarnon, 2014). Therefore, the injection of amyloid-beta is an alternative AD animal model to transgenic animals.

Both donepezil and EE have been shown to improve cognitive ability scores in healthy experimental subjects as well as in experimental models with cognitive impairments (Berardi et al., 2007; H. B. Guo et al., 2015; T. S. Lee et al., 2013). However, these paradigms in the present study could not separately lead to a significant recovery of the impaired memory of AD animals. Among the several probable factors, the dose of donepezil used and the time course effect of EE exposure in this study may be the most important ones. By this assumption, Cavalcante et al. (Cavalcante, 2019) reported that only short-term (two weeks) but not long-term (four weeks) exposure to an EE could promote the extinction of aversive memory. The cognitive benefits of donepezil administration in AD patients are widely suggested to be dose-response related (Jelic & Darreh-Shori, 2010; J. H. Lee et al., 2015). In this study, we orally administered a 4 mg/kg dose of donepezil once a day for 3 weeks. The administered dose of donepezil in animal studies varies between 0.5 and 10 mg/kg. The approved standard doses of 5 and 10 mg/day inhibits cortical AChE activity by only 20–40%, but based on the dose-response relationship with donepezil, it was expected that increasing dosage to 23 mg/day further increases AChE inhibition (Cummings et al., 2013; Farlow et al., 2010). The dose-dependent effect of donepezil administration on the cholinergic system is also reported in animal studies (Chamoun et al., 2016; Kasa et al., 2000). Thus, the dose used in the present study may not be enough to produce separately significant improvement in the memory function of AD animals.

The primary goal of this study was to test whether co-exposure to donepezil and EE is more efficient in improving cognitive deficits in AD rats than either paradigm alone. Results showed that while neither EE nor donepezil alone could significantly restore spatial memory scores in AD rats, combination therapy was effective. It seems from the inspection of the data that the combination therapy was not synergistic but may be additive, with each paradigm alone producing some improvement which when combined reached significance. All individual groups performed the learning trials successfully; however, while the Alzheimer group showed a considerable increase in the acquisition blocks, it did not meet the significant level. Given this, and since the swimming speed was the same among the groups, it could be assumed

that the observed differences in memory function may not be related to confounding factors such as specific differences in sensorimotor integration or motivation of the animals.

Donepezil in combination with a variety of agents such as piperine (Nazifi et al., 2021), resveratrol (Rao et al., 2021), estradiol (Gibbs et al., 2011), and manual acupuncture (Jiang et al., 2019) has been examined to achieve a superior benefit for neurobehavioral deficits in AD animal models. To date, there have been no attempts to address the potentials of donepezil administration and EE in combination for the prevention of cognitive deficits in AD patients or experimentally neurodegenerative status. The partially analog studies are those that aimed to evaluate the combined benefit of cognitive rehabilitation plus donepezil for AD patients (Giordano et al., 2010; Matsuzono et al., 2016). One found that the combination of cognitive rehabilitation plus a choline esterase inhibitor donepezil resulted in a better effect on the Mini-Mental State Examination test scores in AD patients than only drug therapy (Matsuzono et al., 2016). These findings suggest that the combination pattern of donepezil with cognitive stimulation programs is more efficacious than AChEI alone for improving cognitive and behavioral deficits in the case of CNS insults and neurodegenerative status.

Next, we sought the hippocampal expression of BDNF which is a key signaling molecule in memory formation and AD neurodegeneration (Chen et al., 2017; Cunha et al., 2010). It has been reported that donepezil increases the serum levels of BDNF in patients with AD, and BDNF upregulation is involved in the protective effect of AChEI (Leyhe et al., 2008). The contribution of nerve growth factors to the enhancing effect of EE on neuronal functioning has also been well-documented (Rossi et al., 2006). Therefore, we examined the potential involvement of hippocampal BDNF in memory status by measuring its mRNA expression in animals. We found that BDNF expression was suppressed in AD rats and pretreatment with donepezil plus EE could increase it to the saline levels. These results are consistent with the previous findings showing that a suppressed hippocampal BDNF in the amyloid-beta model of AD rats (Kim et al., 2014). Thus, the observed additive outcome of donepezil and EE combination for memory performances of AD rats may be in part due to the augmented influence of this combination therapy on BDNF expression. A human study by Alvarez et al. (Alvarez et al., 2016) evaluated the effects of Cerebrolysin, donepezil, and combined therapy on BDNF serum levels and cognition at week 16 and week 28 after treatment mild-to-moderate AD patients. Cerebrolysin, but not donepezil, increased serum BDNF at week 16, while the combination therapy enhanced it at both time points. The increasing effect of combination therapy was significantly more profound than donepezil and Cerebrolysin groups at week 16 and week 28, respectively. These findings were associated with better cognitive improvements in AD patients, suggesting an advantage of combination therapy again for achieving the most benefits from medication in AD.

Results of AchM1R expression within the hippocampus revealed that while there was no significant difference among the groups, the AD group showed an increase in AchM1R levels, and don, EE, and especially don+EE diminished it toward the control levels. Intracerebral injection of amyloid-beta leads to the loss of cholinergic neurons and decreased Ach levels (Giovannini et al., 2002). Therefore, the

observed pattern of modulations in AchM1R expression can be assumed to be related to the balance between ligand concentration and receptor expression.

Conclusion

In conclusion, these data suggest that a cholinesterase inhibitor and cognitive stimulation can be used effectively in combination to improve cognitive loss in an AD rat model. Given the noninvasive nature of environmental enrichment, it can be considered as an efficient complementary strategy along with medication to protect against cognitive and neuronal deficits in AD patients.

Declarations

Author contributions: Hajali V designed, analyzed and wrote the manuscript. Sahab Negah S reviewed the final draft of the manuscript; Gholami j and Rajabian A did most of the benchwork. All authors read and approved the final manuscript.

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Data availability Data are available on request.

Ethics approval Ethical approval with number IR.MUMS.MEDICAL.REC.1396.683

was obtained from the Animal Care Committee of Mashhad University of Medical Sciences, Mashhad, Iran.

Consent for publication All authors approved the submission of the manuscript for publication.

Conflict of interest The author(s) declared no potential conflicts of interest concerning any part of this research.

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Tables

Table 1. Summary of primers used in the polymerase chain reaction

| Gene | Length (bp) | Forward: 5'→3' | Reverse: 3'→5' | TA (°C) |
|---------------|-------------|----------------------|----------------|---------|
| BDNF | 122 | CAGTGGCTGGCTCTCATACC | | 60.29 |
| | | AACAGGACGGAAACAGAACG | | 60.89 |
| AchM1R | 138 | AGTCCCTCACATCCTCCGAA | | 60.29 |
| | | TTCTTGGTGGGCCTCTTGAC | | 60.89 |
| actinβ | 98 | AAGTCCCTCACCTCCCAAAG | | 60.29 |
| | | AAGCAATGCTGTACCTTCCC | | 60.89 |

Figures

| Unweaned | | | Age (weeks) | | | | | | 10 | |
|-----------------------|---|---|---|---|---|---|---|---|-----------------|--|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | d1 | d2 |
| Sham | | | Standard housing | | | | | Stereotactic surgery, Vehicle injection, and recovery | MWM acquisition | - MWM probe test - tissue preparation |
| Alz | | | Standard housing | | | | | Stereotactic surgery, beta-amyloid injection and recovery | » | » |
| Alz + Don | | | Standard housing ----- donepezil administration | | | | | » | » | » |
| Alz + EE | | | Enriched Environment | | | | | » | » | » |
| Alz + Don + EE | | | Enriched Environment ----- donepezil administration | | | | | » | » | » |

Figure 1

Experimental design. Animals were subjected to different treatments from week 4 to week 8 of age. Following the interventions, spatial learning and memory in Morris water maze (MWM), and hippocampal extraction were performed. Alz: Alzheimer; EE: enriched environment; Don: donepezil. For further details see Sections Methods.

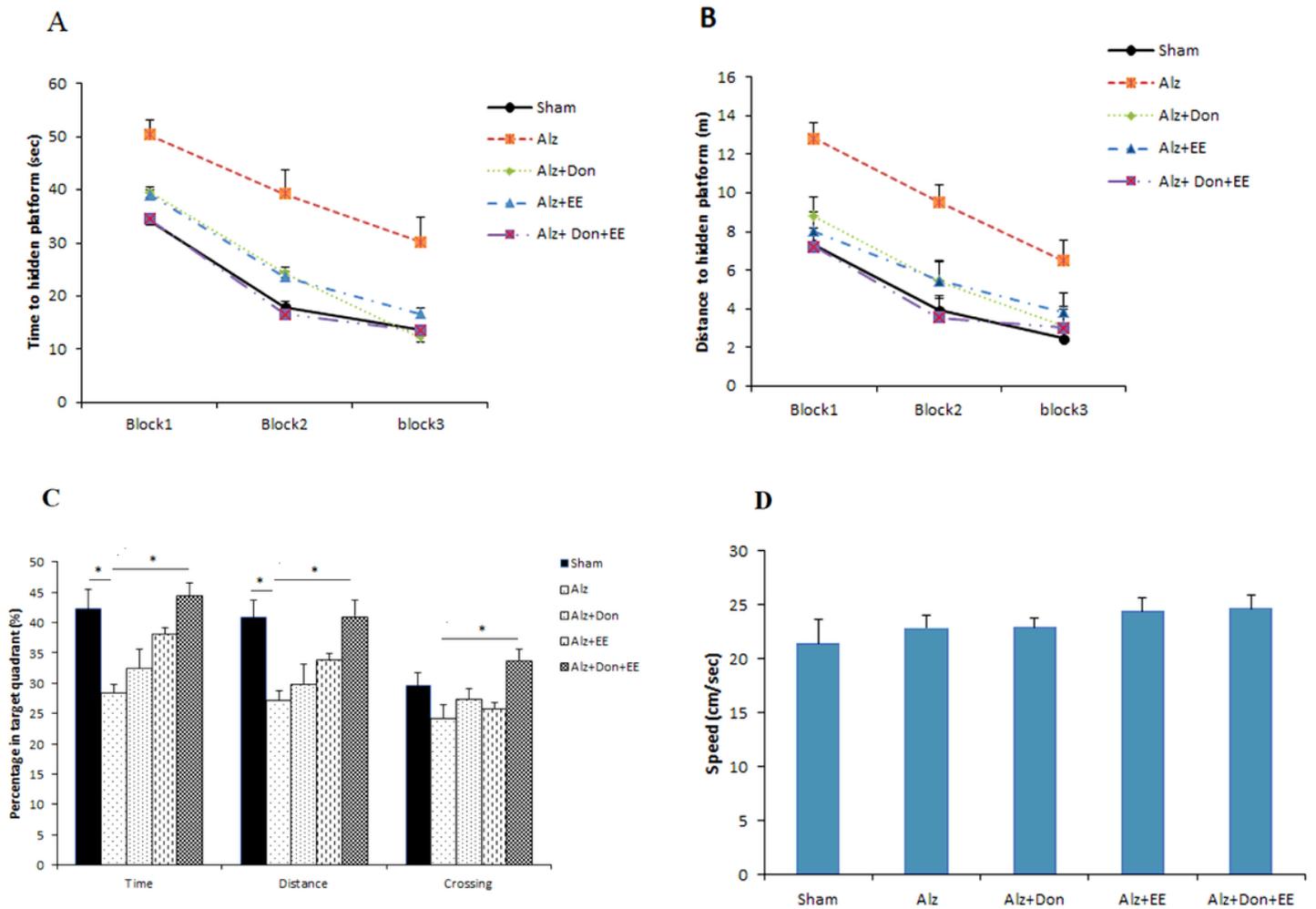


Figure 2

A, B, C, and D. (A and B) The spatial learning in the Morris water maze (MWM) test in sham, Alz, Alz+Don, Alz+EE, and Alz+Don+EE. Each block represents the mean latency (A) and distance traveled (B) of four consecutive trials to find the hidden platform. There were no significant differences in spatial learning ability among the groups. Data are shown as mean \pm S.E.M. (two-way repeated measure ANOVA). (C) The spatial memory in MWM is shown by the percentage of the time, distance, and crossing over the target quadrant. * $P < 0.05$. (D) The swimming speed in MWM. Data are shown as mean \pm S.E.M. (one-way ANOVA followed by Tukey test). Alz: Alzheimer; EE: enriched environment; Don: donepezil.

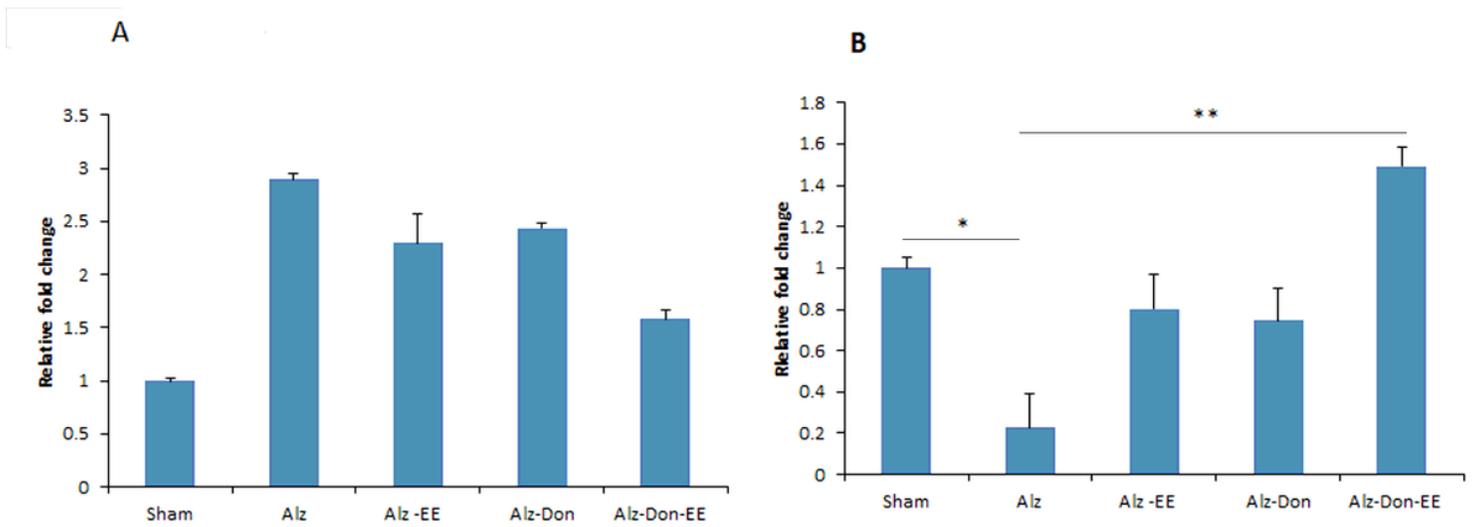


Figure 3

A and B. (A) The hippocampal gene expression of M1 muscarinic acetylcholine receptor (AchM1R) (A) and brain-derived neurotrophic factor (BDNF) (B) in sham, Alz, Alz+Don, Alz+EE, and Alz+Don+EE. The differences in AchM1R did not reach a significant level ($P=0.199$). One-way ANOVA revealed that BDNF levels were significantly decreased in the Alz group and were returned to the normal value in the Alz+Don+EE group. * $P < 0.05$ and ** $P < 0.01$. Data are shown as mean \pm S.E.M. (one-way ANOVA followed by Tukey test). Alz: Alzheimer; EE: enriched environment; Don: donepezil.