Age-related differences in the effect of vitamin D on scopolamine-induced learning and memory impairment

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Abstract

Aim: Alzheimer’s disease is the most common type of dementia. The number of patients with Alzheimer’s is expected to reach 115 million in 2050. Due to the low effectiveness and frequent adverse effects of current treatment approaches, expected results cannot be obtained in treatment. Vitamin D supplements are reported to have protective effects against Alzheimer’s disease by increasing learning and memory performance. In this study, we aimed to evaluate the effect of vitamin D on learning and memory on cognitive deficits by scopolamine-induced memory impairment model, which is an animal model of AD, in aged rats, as well as evaluating whether the effect changes with age.

Materials and Methods: Wistar Albino male rats (Young: 4-5 months old, Aged: 21-22 months old) were used in the study. Vitamin D was given for three weeks at 500 IU/kg; scopolamine was applied 1mg/kg half an hour before behavioral experiments. Modified elevated plus maze and Morris water maze tests was performed to assess cognitive abilities during the fourth week; prefrontal cortices were then removed to assess acetylcholinesterase enzyme activity.

Results: Vitamin D administration restored memory impairment in old rats in the Morris water maze; whereas there was no effect in young group. Scopolamine significantly increased the brain prefrontal cortex AchE enzyme activity only in the young rats. Vitamin D did not create a statistically significant change in AChE activity in young rats. on the other hand, a significant increase was detected in the elderly group with vitamin D compared to the age-matched control and scopolamine groups.

Conclusion: Our results show that the effectiveness of vitamin D changes with age. Vitamin D may be a safe and effective option in preventing dementia development in the elderly group and improving cognitive dysfunction due to Alzheimer’s disease.

Keywords: Vitamin D; Alzheimer’s disease; acetylcholinesterase; scopolamine; memory

INTRODUCTION

Alzheimer’s disease (AD) is the most common type of dementia in humans (1). More than 50 million people are currently considered to have AD and this number is projected to grow by 152 million within 30 years (2). AD is defined as the deterioration of cognitive function with aging. The memory disability is attributed to the loss of cholinergic neurons in the basal forebrain and hippocampal neurons, and consequently to the impairment of cholinergic functions.

The cholinergic system has been shown to play an important role in learning and memory functions. The main neurotransmitter of cholinergic neurons is acetylcholine (Ach). It has been shown that the release of the Ach is decreased in AD patients. Additionally, when the brain tissues of AD patients were examined after death, it was found that both nicotinic and muscarinic ACh receptor binding decreased (3). Ach is broken down by the acetylcholinesterase enzyme (AChE) located in the synaptic cleft. If this enzyme is inhibited, the breakdown of acetylcholine is reduced, and cholinergic transmission increases. Acetylcholinesterase inhibitors that increase cholinergic transmission are used in the current treatment of the disease. However, using cholinergic drugs does not mean the radical treatment will occur, they only treat symptomatically. Also, side effects limit treatment success (4).
Vitamin D is important in learning and memory function in old age. Meta-analysis studies have shown that low vitamin D concentrations increase the risk of AD and cognitive decline (5). It has been found that the enzyme 1α-hydroxylase, which synthesizes the active form of vitamin D, is also produced in the brain. Also, the being of vitamin D receptors has been demonstrated in the entire brain, including memory-related areas such as the hippocampus and prefrontal cortex (6).

It was also shown that vitamin D has neurotrophic and neuroprotective effects. In addition, it plays a role in cell proliferation, differentiation, neuroplasticity, and neurotransmission in neurons. All these studies collectively show that it has a role in the development of neurological diseases and adult brain functions (7).

Today, humans generally lived in indoor areas. Therefore, sunlight providing vitamin D synthesis is not used sufficiently. Besides, vitamin D cannot be included in the diet enough (8). Last of all, vitamin D deficiency is observed in nearly half of the people (9). In this experiment, we evaluated the protective effect of vitamin D supplementation against cognitive impairment in two different age groups, young and aged. For this purpose, we applied learning and memory tests in the Scopolamine-induced memory impairment model (10). We also evaluated the activity of acetylcholinesterase enzyme, the target enzyme of the most commonly used drugs in the treatment of AD.

MATERIALS and METHODS

Subjects

Wistar Albino male rats (Young: 4-5 months old, 350-450gr; Aged: 21-22 months old, 450-550gr) were used in this study. The rats were obtained from Balikesir University Laboratory Animal Research Center. During the experiments, the rats were kept in a stable temperature (21 ± 3oC) room with feed and water intake released. One week before the behavioral experiments, the animals were accustomed to being kept and at least 24 hours before they were taken to the experiment room and adapted to the environment. All behavioral experiments were carried out between 08.00 and 12.00 in the morning. Ethical approval of the research obtained from the Balikesir University Animal Experiments Local Ethics Committee (2019/4-10).

Protocol

The animals were divided into 6 groups. There were 10 rats in each group (Table 1). Vitamin D (Devit-3 amp, Deva Pharmaceuticals, Turkey) was dissolved in sunflower oil, and scopolamine hydrobromide was dissolved in 0.9% saline. Vitamin D was given by oral gavage at 500 IU / kg for 3 weeks. The dose was determined using previous studies (11,12). Scopolamine HBr (Sigma, St Louis, USA) was applied half an hour before the first day of mEPM and the probe test of MWM by intraperitoneal (ip) route at 1 mg/kg (13) (Figure 1).

Table 1. The treatment scheme

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments</th>
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<tbody>
<tr>
<td>n</td>
<td>Three weeks (po) Before the tests (ip)</td>
</tr>
<tr>
<td>Young Control</td>
<td>10 1 ml/kg/day Saline 1 ml/kg Saline</td>
</tr>
<tr>
<td>Aged Control</td>
<td>10 1 ml/kg/day Saline 1 ml/kg Saline</td>
</tr>
<tr>
<td>Young Scop</td>
<td>10 1 ml/kg/day Saline 1 mg/kg Scop</td>
</tr>
<tr>
<td>Aged Scop</td>
<td>10 1 ml/kg/day Saline 1 mg/kg Scop</td>
</tr>
<tr>
<td>Young Vit D +Scop</td>
<td>10 500 IU/kg/day vitamin D 1 mg/kg Scop</td>
</tr>
<tr>
<td>Aged Vit D +Scop</td>
<td>10 500 IU/kg/day vitamin D 1 mg/kg Scop</td>
</tr>
</tbody>
</table>

Scop: Scopolamine

After the behavioral tests all animals were euthanized with ketamine–xylazine anesthesia. The rats were then decapitated and their brains removed, and the prefrontal cortices were separated on ice. Brain samples were weighed and after 0.9% NaCl was added, they were homogenized in an ice bath for 1 minute (1:10 w/v, 2.000 rpm/min) in PBS (pH 7.4) with a mixer (Stuart SHM 1, UK). Protein analysis of homogenate and supernatant was done (Shimadzu UV-1800, Japan) (14).

Figure 1. The experimental protocol
Morris water maze (MWM) test
A round tank was used for MWM. It was virtually divided into four equal quadrants. In one of the selected quadrants in the acquisition test of the MWM, which consisted of the first four days, a platform was placed invisibly from the surface of the water. Non-toxic black tempera paint is used to make the water opaque. Three trials were made each day of the acquisition tests. In each trial, a rat was placed in water from three specific quadrants in the tank. The time until the rat up to the platform was determined as the mean escape time. If the animal could not find the platform in 60 seconds, the maximum test time, it was placed on the platform slowly and held on the platform for 20 seconds. In this case, the delay was noted as 60 s. On the fifth day, the platform was removed from the tank during the ‘probe trial’, and the animal's swimming time on the quadrant of the platform was evaluated for 60 s (13).

Modified elevated plus maze (mEPM) test
The mEPM test was conducted by using a cross-shaped maze. The maze consists of 2 open (50 x 10 cm) and 2 closed (50 x 10 cm) arms elevated 50 cm from the ground. The time for the animal from the ending of the open arm to enter the closed arm (transfer latency, TL) with its four paws was evaluated for two consecutive days. The test time limit was 90 seconds. The rat was allowed to roam the maze for 10 seconds when it entered the closed arm. (15).

Assessment of AchE enzyme activity
Before analysis, 100 mg of the sample separated. Then it was homogenized and was centrifuged at 15000 rpm for 5 minutes + 4 OC. The resulting supernatants were pipetted into a microplate reader for analysis when it reached room temperature. AchE enzyme activity was evaluated using a commercial kit (SIGMA-ALDRICH, Acetylcholinesterase Colorimetric Assay Kit). Enzyme activity was given by adapting the protein concentration.

Statistical analysis
Data analysis was performed using Prism 6.0 software (GraphPad Software, Inc., San Diego, CA, USA). Results were expressed as mean ± SEM. When evaluating the first four days of MWM, a two-way analysis of variance (ANOVA) and then Tukey post-hoc tests were performed. One-way ANOVA test was used while evaluating other results, and then Tukey post-hoc tests were performed. Results were considered statistically significant when p values were less than 0.05.

RESULTS
The effect of vitamin D on the MWM test
Time to find the non-visible platform in animal acquisition test trials gradually decreased each day [Two-way ANOVA, the day effect, F (3.36) = 54.95 p <0.05, Figure 2]. There is no administration of scopolamine in these tests. Results of all groups were similar in the first four days trials. Only in the 4th day trial of the aged vitamin D group, the time to transition to the platform was significantly reduced compared to the aged control group [Two-way ANOVA, multiple comparisons, F (9.108) = 2.1, p <0.05, Figure 2].

![Figure 2. The effects of vitamin D on the MWM acquisition tests. Each value represents the mean ± SEM (n = 10). ^p<0.05 compared to the aged control group.](image)

Figure 3. Effects of vitamin D, scopolamine and drug combinations on the MWM probe test in young (A) and aged (B) rats. Each value represents the mean ± SEM (n = 10). ^p<0.05 compared to the control group, β p < 0.05 compared to the scopolamine group.
In the probe test, the time spent in the hidden platform quadrant significantly decreased in the scopolamine group in younger groups compared to the control group. [One-way ANOVA, $F (2.27) = 5.421$, $p<0.05$]. Vitamin D administration slightly increased the scopolamine-induced reduction in time of latency compared to the scopolamine group but did not provide a statistically significant improvement (Figure 3A).

Similar to the younger groups, the time spent in the hidden platform quadrant decreased significantly in the scopolamine group compared to the control group [One-way ANOVA, $F (2.27) = 9.233$, $p<0.05$]. Administration of vitamin D reversed to aged control group (Figure 3B).

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

**Figure 4.** Effects of vitamin D, scopolamine, and drug combination on the transfer latency in the mEPM: first-day mEPM test of young (A) and aged (C) rats, second days mEPM test of young (B) and aged (D) rats. Each value represents the mean ± SEM ($n = 10$)

**The effect of vitamin D on the mEPM test**

Figure 4A, 4B, 4C, 4D show the first and second-day latency of young and aged rats, respectively. In the mEPM test, vitamin D or its combination with scopolamine had no significant effect on the of the first and second days [$F (2.27) = 1.442$, $p>0.05$, Şekil 4.A; $F (2.27) = 0.6249$, $p>0.05$, Figure 4B], [$F (2.27) = 0.1186$, $p>0.05$, Figure 4C; $F (2.27) = 1.295$, $p>0.05$, Figure 4D].

**The effect of vitamin D on the AchE enzyme activity**

Prefrontal cortex AchE enzyme activity significantly increased in the young scopolamine group (p<0.05) compared to the young controls [$F (2.27) = 9.844$; $p<0.05$; Figure 5 A]. The administration of vitamin D slightly reduced this increase but did not produce a statistically significant change.

In aged rats, AchE enzyme activity did not change in the scopolamine group compared to the control group. However, vitamin D administration significantly increased the enzyme activity compared to the control group and scopolamine group [$F (2.27) = 9.700$; $p<0.05$; Figure 5 B].
DISCUSSION

In our study, vitamin D supplementation exerted different effects in young and aged rats in Scopolamine-induced memory impairment. Vitamin D supplementation reversed spatial learning and memory impairments induced with scopolamine in aged rats, but not young rats, in the MWM test. Scopolamine significantly increased the brain prefrontal cortex AchE enzyme activity in the young rats but did not create a significant change in the elderly. Vitamin D supplementation did not create a statistically significant change in AChE activity in young rats. However, a significant increase was observed in elderly groups with vitamin D administration compared to the control and scopolamine groups. Our results suggest that vitamin D may have the potential for preventing age-related impairment in cognitive functions associated with neurodegenerative diseases such as AD.

The cholinergic system is very important in learning and memory functions. Decreases in cholinergic markers such as AChE, choline acetyltransferase, and acetylcholine receptors have been reported in the normal aging process (16,17). ACh is the main neurotransmitter of cholinergic neurons. The release of ACh from the cerebral cortex and hippocampus and its presynaptic regulation are different in young and aged rats. Extracellular ACh levels are 42% lower in the cortex and 60% lower in the hippocampus in the elderly rats (18). ACh is destroyed by the AChE enzyme. Similarly, AChE activity is lower in the brains of the elderly. The decrease in AChE activity with increasing age indicates degeneration in cholinergic synapses due to age. (19).

The most widely used pharmacological model associated with Alzheimer's is scopolamine-induced memory impairment model. Scopolamine causes the pharmacological blockade of cholinergic neurons and induces learning and memory impairment (10). As expected, it impaired memory in both young and aged rats in our study. Vitamin D also affects cholinergic activity in the brain (20). It has been determined that vitamin D is concentrated in the nuclei of various neuron types that play a role in memory and cognitive functions (21). In recent studies, it is accepted that neurodegenerative diseases such as AD are also associated with Vitamin D deficiency (5). It has been suggested that maintaining adequate vitamin D levels in the elderly may be important in preventing neurodegeneration (7).

In a study by Taghizadeh et al., rats were administered a vitamin D supplement food diet. It has been reported that vitamin D did not improve spatial performance in the MWM test in animals (22). In another study by the same researchers, the same diet was administered to rats with AD induced by intracerebroventricular amyloid-beta, and vitamin D supplementation could not restore the deteriorating spatial performance due to AD (23). Young adult animals were used in both studies. Similar to these studies, in our study, vitamin D supplements did not affect the impaired spatial memory due to scopolamine in young adult animals.

In one study, the MWM test was performed on middle-aged male F344 rats fed a diet containing vitamin D at three different doses for 5-6 months. It has been reported that high serum level of 25 (OH) D3 prevent age-related cognitive decline (24). In another study, daily administration of 42 I.U./Kg calcitriol to aged and young male F344 rats increased cognitive performance in older rats, but not young rats (25). Consistent with these studies, vitamin D supplementation in our study also restored scopolamine-impaired memory in older rats, not young rats.

However, this memory-improving effect was observed only in the MWM test, not in the mEPM test. The elevated plus-maze test is actually a test used to assess anxiety based on the principle that rodents avoid open and high areas. It was later modified to assess spatial learning and memory in mice. On the second day of the test, it is expected that the rodents’ transition time to the closed
arms of the labyrinth will be shortened (26). In the mEPM test, different periods of memory can be evaluated. The administration of drug therapy before the first-day test affects the acquisition, and the administration immediately after the first trial affects the consolidation (27). In our experiment, the effect of vitamin D on memory acquisition was investigated by applying scopolamine before the first day test. In MWM, scopolamine was applied just before the probe test and its effect on memory consolidation was evaluated. Our findings suggest that although vitamin D improves consolidation, it does not affect the acquisition of memory.

Vitamin D also affects the AChE activity. It was found that decreased CAT activity and increased AChE activity in the prefrontal cortex and memory impairment was observed in rats with streptozocin-induced diabetes. Supplementing with 500 IU/kg of vitamin D per day for 10 weeks reduced these findings (11). In another study, vitamin D3 administered for three weeks in rats with Alzheimer’s induced by intracerebroventricular streptozocin prevented the increase of the cerebral cortex AChE activity (28). In our study, although vitamin D reduced the increased AChE activity due to scopolamine in the young group, it could not bring it to control values. But still, the results were similar to previous studies.

However, in elderly rats, vitamin D caused an increase in AChE activity with the opposite effect in the current study. Young adult rats were used in publications showing that vitamin D reduced AChE activity. Consistent with our study, in Khairy and Attia’s study, vitamin D use had different age-related effects on AChE activity. In this study, aged rats were given 500 IU/kg/day vitamin D for 5 weeks, and AChE activity significantly increased compared to aged control groups (12). This finding is consistent with the increase in AChE activity seen in aged rats in our study. The different results obtained in elderly rats in our study suggest that vitamin D may produce different results depending on age.

Memory impairment in AD is associated with cholinergic neuron loss in the basal forebrain (3). AChE reduction in cortex and hippocampus is evident especially in AD patients (29). It has been suggested that this decrease in AChE activity may be an important biomarker for dementia and AD (30). In our experiment, we found that vitamin D increased the activity of AChE and fixed the scopolamine-induced memory deficit in the aged rats. Our findings suggest that vitamin D has protective effects on cholinergic neurotransmission in the elderly. However, many different mechanisms are mentioned for the possible protective effects of vitamin D3 on cognitive functions. It has been reported that vitamin D3 supports the survival, development, and function of neural cells by regulating the expression of neurotrophins such as NGF, NT-3, and GDNF. It has also been shown to modulate inflammatory cytokines and increase the clearance of amyloid plaques through macrophage stimulation. Furthermore, it can reduce oxidative stress by increasing antioxidant levels and preventing the formation of reactive oxygen species, thus protecting cells from death (7). We could not evaluate these parameters in our study but there are many studies which have found plenty of evidence that vitamin D3 has positive effects on age-related conditions and dementia (5,7). Our findings support these studies.

CONCLUSION

In this study, we evaluated the effect of vitamin D on memory impairment as well as evaluating whether the effect changes with age. Vitamin D administration had different effects in young and aged rats in the Scopolamine-induced memory impairment model. Vitamin D restored the impaired memory in aged rats but was not effective in young rats. In addition, different effects of Vitamin D on AChE enzyme activities were observed in prefrontal cortex samples of the young and aged animals. While vitamin D administration reduced the AChE enzyme increase due to scopolamine to control levels in young rats, it increased the enzyme activity in old rats. In light of the literature and our results, we think that vitamin D supplements can be a safe and effective solution for the prevention of cognitive dysfunction.

This study was presented as an oral presentation at Izmir Democracy University International Health Sciences Congress 2020, which was held online between 20-21 June 2020. Competing interests: The authors declare that they have no conflict of interest. Financial Disclosure: This study was supported by the Scientific Research Projects Unit of Balıkesir University Rectorate with the Project Code BAP 2019/050. Ethical approval: The study protocol was approved by the Balıkesir University Animal Experiments Local Ethics Committee (2019/4-10).

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