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Research article

Vitamin D3 supplementation reverses aging-related changes in cholinergic functions and improves spatial memory in aged rats

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ABSTRACT

Aim: Dietary intake and synthesis of vitamin D synthesis decline with age, increasing the risk of vitamin D deficiency. Dementia and Alzheimer's disease development are closely linked to vitamin D deficiency. In this study, we investigated whether vitamin D supplementation could attenuate age-related effects on memory and the hippocampal cholinergic system in aged rats.

Method: Thirty Wistar albino male rats (young: 4–5 months old, aged: 21–22 months old) were included in this study. Animals were divided into three groups: The Young control and the Aged control groups were administered physiological serum and the Aged + Vitamin D group was administered vitamin D (500 IU/kg/day). Spatial memory was assessed with the Morris Water Maze test. Then, ACh level and ChAT, AChE, and BChE enzyme activities in the hippocampus were examined.

Results: Vitamin D supplementation given to aged rats increased the AchE and BuChE enzyme activities and ACh levels which decreased with aging. The activity of the ChAT enzyme did not change in the aged group, and vitamin D supplementation did not affect it. Increased hippocampal cholinergic transmission improved the spatial memory of aged rats in the MWM test.

Conclusion: Vitamin D supplementation improved spatial memory in rats, probably by reversing the aging-related changes in brain cholinergic functions. Vitamin D shows promise in delaying cognitive decline associated with aging and AD.

1. Introduction

Alzheimer's disease (AD) is the leading cause of dementia and memory loss worldwide, especially after age 65. Growing evidence indicates that the brain changes caused by AD begin many years before symptoms appear. As a result, some individuals may be asymptomatic even though the disease has started, while others may show varying levels of memory loss and cognitive decline. Memory loss in AD results from the loss of cholinergic neurons in the hippocampus and basal forebrain. It is believed that normal aging does not involve the same pathological cell loss as AD, but cholinergic function gradually declines [1]. Similarly, it has been proposed that differences in memory performance among healthy older adults are also linked to the integrity of the cholinergic system [2].

Acetylcholine (ACh) is the main neurotransmitter of the cholinergic system. ACh levels in the brain are maintained by choline acetyltransferase (ChAT), which synthesizes ACh, and by two enzymes, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), which hydrolyze ACh. In AD, AChE activity decreases to 33–45 % of normal as the disease advances, while BuChE activity can increase by up to 90 % in some brain regions. This imbalance between AChE and BuChE leads to cholinergic deficits in the brain, specifically in acetylcholine. Cholinesterase (ChE) inhibitors, still commonly used in AD treatment today, work by blocking AChE and BuChE to different extents, raising acetylcholine levels [3]. However, although these treatments offer short-term symptom relief, they cannot permanently halt the progression of the disease [4].

AD has been categorized into three stages: dementia, moderate cognitive impairment (MCI), and preclinical AD, which refers to early degenerative changes in the brains of cognitively normal individuals

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[5]. Clinical research indicates that MCI, AD, and dementia are closely linked to vitamin D deficiency [6,7]. A recent study involving women over 100 years old demonstrated once again that the risk of AD decreases as serum vitamin D levels rise [8]. MCI is a clinical stage that occurs between the onset of dementia symptoms and the cognitive decline associated with normal aging. The rate at which individuals with MCI progress to dementia varies from 15 % to 38 % across different studies [9]. Vitamin D levels in MCI patients who convert to AD are significantly lower than in those who do not [10]. It has been reported that vitamin D supplementation can improve cognitive function in MCI patients [4]. A recent study examining the relationship between AD and ten different nutrients, including minerals and vitamins, also supports a negative association between 25-OH D and AD [11]. Low serum vitamin D levels in older adults may contribute to AD by significantly increasing hippocampal volume loss related to cerebral amyloid β (Aβ) and worsening neurodegeneration [12]. Experimental studies have shown that vitamin D and its analogs reduce neuroinflammation, Aβ burden, and tau protein hyperphosphorylation in the brain, which are characteristic features of AD [13,14]. Supporting this, clinical studies have found that cognitive function improved significantly and Aβ plaque accumulation decreased in elderly AD patients who received daily vitamin D supplements for a year [15].

Vitamin D can be obtained through diet or produced in the body by the effect of ultraviolet B rays from the sun. However, as we age, both vitamin D production and dietary intake decrease. Therefore, the risk of vitamin D deficiency is higher in older adults. In this age group, the prevalence of vitamin D deficiency is reported to range from 60 % to 100 % [16].

Vitamin D plays a role in many brain functions, including neurotransmitter production, neurogenesis, synaptogenesis, and defending against oxidative stress, and it offers a protective effect on the brain [17]. In humans, hippocampus-dependent cognitive abilities and adult hippocampal neurogenesis decline at the same rate with age [18]. Vitamin D supplementation has been shown to improve cognition and boost neurogenesis in both hypothyroid juvenile rats and a mouse model of Alzheimer's disease [19,20]. In numerous animal studies using experimental AD models, vitamin D significantly enhanced memory and cognitive functions. It increased brain-derived neurotrophic factor (BDNF) levels, decreased neuroinflammation and oxidative stress, and reduced the buildup of Aβ plaques in rats [21–23]. Even just two weeks of vitamin D administration increased BDNF production, decreased NOS levels, and improved learning and memory in rats [23]. Additionally, injecting vitamin D into the hippocampus of rats undergoing short-term sleep deprivation reduced astrocyte activation, limited the release of neurotoxic factors, and alleviated cognitive impairment [24].

Since there has been limited progress in treating AD through clinical and experimental studies, it is crucial to prevent AD during the "presymptomatic" or "preclinical" stages before significant cognitive decline occurs [7]. Maintaining adequate vitamin D levels in older adults may delay or prevent cognitive impairment. Although some clinical and observational human studies assessing the effects of vitamin D supplementation on memory are promising, the results remain controversial due to difficulties in standardization and varied methodologies across studies [16]. In this context, animal studies become especially important because they are easier to standardize and are not affected by placebo effects. However, the literature mainly focuses on memory impairment models such as AD, with limited research on the impact of vitamin D supplementation on normal aging. In this study, we first examined whether vitamin D could enhance memory in aged rats using the Morris water maze test. Then, we measured ACh levels and the activities of the enzymes ChAT, AChE, and BuChE in the hippocampus to understand how vitamin D influences the cholinergic system, which is vital for memory.

2. Materials and methods

2.1. Animals

For this study, thirty Wistar-albino male rats were used. Ten of these rats were young (4–5 months) and twenty were aged (21–22 months). Rats were housed in cages, 3 or 4 in each cage, with access to food and water at any time in a room maintained at a constant temperature of 21 ± 3°C. To get the animals used to human contact, they were handled every day for a week before the behavioral tests. Behavioral tests were conducted from 8:00–12:00. The care and use of animals followed the ethical principles outlined in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Appropriate permits and approvals have been obtained from the Balikesir University Animal Experiments Local Ethics Committee (2021/5–9).

2.2. Study protocol

The animals were divided into three groups of ten each. The Young control and the Aged control groups received physiological serum, while the Aged + Vitamin D group was given vitamin D. All treatments were administered via oral gavage. For three weeks, 500 IU/kg of vitamin D (D3, cholecalciferol) (Devit-3 ampoule, Deva Pharmaceutical Company, Turkey) was given daily. The dose was based on previous studies [25–27]. In the fourth week, no medication was given, and memory was tested using the Morris Water Maze (MWM) test (Fig. 1).

Following the completion of the behavioral tests, all animals were put to sleep under ketamine/xylazine (90/10 mg/kg) anesthesia. Rats' heads severed, brains removed, and their hippocampi separated on ice and stored at - 80°C.

2.3. Morris water maze (MWM) test

The MWM experiment involved filling a cylindrical tank measuring 150 cm in diameter and 50 cm in height with water that had been opacified using tempera paint. A constant water temperature of 25 ± 2°C was maintained. The tank was divided into four equal sections, labeled as the east, west, north, and south quadrants. In one quadrant, a 12 cm diameter platform was placed 1 cm below the water's surface so that the animals could not see it. Acquisition trials were conducted over the first four days of the five-day test. During these trials, rats were released into the water from predetermined points in the tank three times daily, with three swimming sessions each day. Each session lasted 60 s, during which the rats attempted to locate the platform. The mean escape time was calculated as the average time it took for a rat to find the platform. If a rat did not find the platform within 60 s, it was placed on the platform. After each swimming session, rats spent 20 s on the platform. The fifth day of the experiment was designated as the probe test. During this session, the platform was removed from the tank, and the time the rat spent in the quadrant where the platform had previously been was recorded.

2.4. Biochemical analyzes

Hippocampal samples were taken out of the freezer, thawed, and then homogenized before being centrifuged, and the supernatants were separated. All analyses were carried out in accordance with the manufacturer's instructions using commercially available kits. The colorimetric method was used to measure the activities of ChAT (BC-K125-S, Elabscience), AChE (ab138871, Abcam), and BuChE (ab241010, Abcam). The Choline/Acetylcholine Quantification Kit (MAK056, Sigma-Aldrich) was used to determine the concentration of ACh.

2.5. Statistical analysis

Prism 6.0 software (GraphPad Software, Inc., CA, USA) was used for

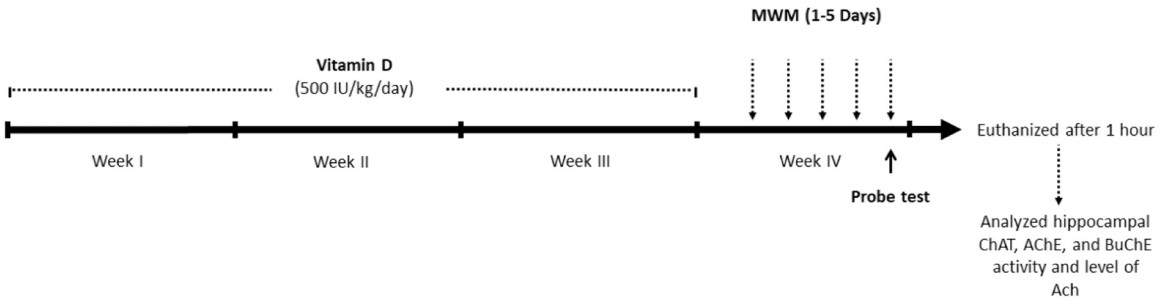

Fig. 1. Study protocol.

the statistical analysis. The data's normal distribution was evaluated with the Shapiro-Wilk test. The MWM acquisition test involved repeated measures ANOVA, followed by Bonferroni post hoc tests. All other results were analyzed using one-way ANOVA and Bonferroni post hoc tests. Relationships between parameters were assessed with the Pearson correlation test. A correlation coefficient (r) of 0.1–0.3 was considered weak, 0.3–0.5 moderate, and above 0.5 strong. Results are presented as mean ± SEM, and p values less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of vitamin D on the MWM test

In every group, the escape latency to locate the platform during the acquisition sessions decreased from day 1 to day 4 [The effect of day, repeated measures of ANOVA, $F(3.27) = 33.73$, $p < 0.05$]. The acquisition trial showed similarities among all groups (Fig. 2a).

The young and aged control groups did not differ significantly from each other. The MWM test's probe trial showed a significant difference in the time spent in the escape platform quadrant between the aged control and the aged + vitamin D groups [One-way ANOVA, $F(2.27) = 4.070$, $p < 0.05$]. Vitamin D administration increased the time spent in the quadrant of the escape platform, according to post hoc Bonferroni comparisons ($p < 0.05$, Fig. 2b).

Vitamin D increased the time spent in the quadrant of the escape platform by the aged animals. These data support that vitamin D enhances memory in the elderly.

3.2. Effect of vitamin D on ACh levels and ChAT, AChE, and BuChE activities in the hippocampus

The ACh levels of the aged control group were significantly lower than those of the young control group. However, the aging-induced decrease in ACh levels was dramatically reversed by vitamin D [$F(2.27) = 4.4$, $p < 0.05$] (Fig. 3).

ChAT activity in the hippocampus was not affected by aging or vitamin D and remained consistent across all groups [$F(2.27) = 0.47$, $p > 0.05$] (Fig. 4).

As shown in Fig. 5, there was a significant decrease in the activity of the AChE in the aged animal group compared to the control group. However, vitamin D administration significantly reversed AChE activity in the aged animals [$F(2.27) = 7.7$, $p < 0.01$] (Fig. 5).

The aged control group showed significantly lower BuChE activity compared to the young control group, as shown in Fig. 4. However, the hippocampus BuChE activity was restored after vitamin D administration [$F(2.27) = 7.1$, $p < 0.01$] (Fig. 6).

Vitamin D supplementation reversed the age-related decline in ACh levels and the activity of ChAT, AChE, and BuChE in the hippocampus. These results suggest that vitamin D administration reduced the aging effects on the cholinergic system in the hippocampus.

There was no significant correlation between the parameters in the young control group ($p > 0.05$), indicating cholinergic homeostatic balance. A strong negative correlation was observed between ChAT and BuChE in the aged control group ($r = -0.852$, $p < 0.01$). This suggests that choline esterase activity changes with aging through compensatory mechanisms. In the aged vitamin group, a strong and statistically significant positive correlation was found between ChAT and AChE ($r = 0.708$, $p < 0.05$, Table 1). These findings support the idea that vitamin D supplementation enhances the interaction between cholinergic enzymes and that vitamin D may play a role in regulating acetylcholine metabolism.

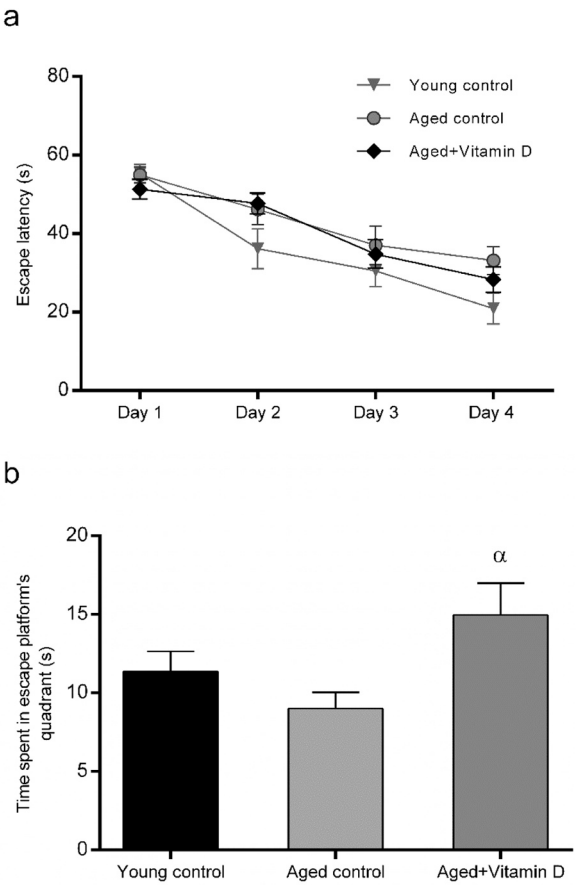

Fig. 2. (a) Impact of vitamin D on MWM test (n = 10). Each value represents the mean ± SEM. (b) Impact of vitamin D on the MWM's probe test (n = 10). Each value represents the mean ± SEM. $^{\alpha}$ $p < 0.05$ compared with the aged control group.

4. Discussion

Our study found that vitamin D (500 IU/kg/day) supplementation in

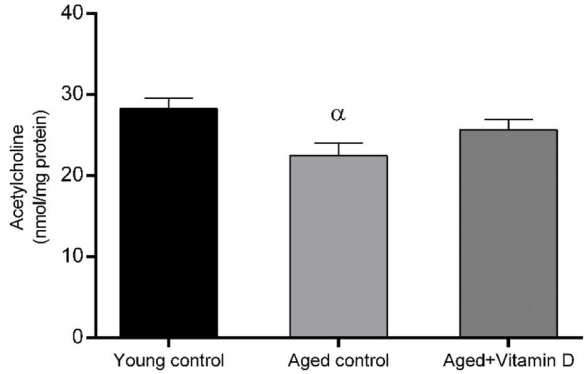

Fig. 3. The impact of vitamin D on hippocampal ACh levels (n = 10). Each value represents the mean ± SEM. $^{\alpha}$ $p < 0.05$ compared with the young control group.

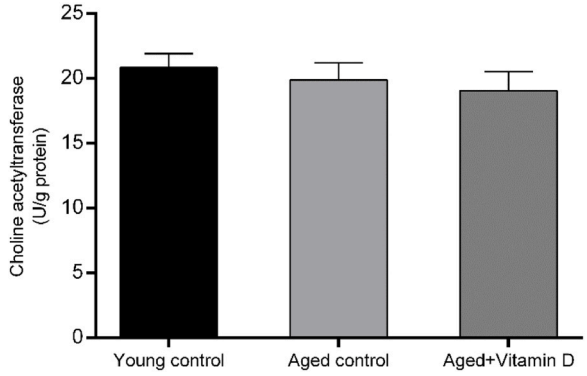

Fig. 4. The impact of vitamin D on hippocampal ChAT activity (n = 10). Each value represents the mean ± SEM.

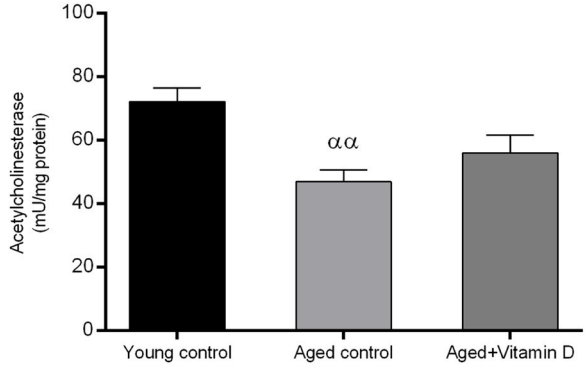

Fig. 5. The impact of vitamin D on hippocampal AChE activity (n = 10). Each value represents the mean ± SEM. $^{\alpha\ \alpha}$ $p < 0.01$ compared with the young control group.

aged rats helped reduce some age-related changes in hippocampal cholinergic system enzymes. In aged rats, AchE and BuChE enzyme activities, as well as ACh levels, declined. Vitamin D supplementation increased these enzyme activities and restored reduced ACh levels. Consequently, improved hippocampal cholinergic transmission led to better spatial memory in aged rats during the MWM test. There was no change in the activity of the ChAT enzyme in the aged group. The negative correlation between CHAT and BuChE in the aged group indicated compensatory mechanisms in the cholinergic system of the aging brain. The positive correlation between vitamin D administration and CHAT and AChE suggested that vitamin D supplementation might

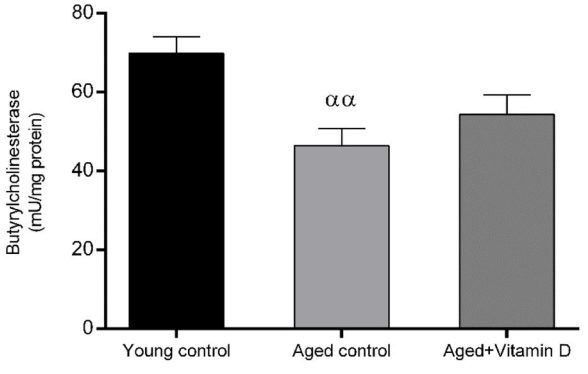

Fig. 6. The impact of vitamin D on hippocampal BuChE activity (n = 10). Each value represents the mean ± SEM. $^{\alpha\ \alpha}$ $p < 0.01$ compared with the young control group.

Table 1
Pearson correlation analysis among cholinergic system-related enzymes and acetylcholine in the young, the aged, and the aged+vitamin D group (n = 10).

Groups	Parameter 1	Parameter 2	Pearson correlation coefficient (*r*)	*p*-value
Young control group	ChAT	ACh	-0.306	0.390
	ChAT	AChE	0.365	0.299
	ChAT	BuChE	-0.197	0.586
	ACh	AChE	0.410	0.239
	ACh	BuChE	-0.081	0.824
	AChE	BuChE	-0.300	0.400
Aged control group	ChAT	ACh	0.218	0.545
	ChAT	AChE	0.202	0.576
	ChAT	BuChE	-0.852*	0.002*
	ACh	AChE	0.511	0.131
	ACh	BuChE	-0.045	0.901
	AChE	BuChE	-0.116	0.750
Aged + Vitamin D group	ChAT	ACh	0.256	0.475
	ChAT	AChE	0.708*	0.022*
	ChAT	BuChE	-0.133	0.715
	ACh	AChE	-0.042	0.908
	ACh	BuChE	0.145	0.690
	AChE	BuChE	0.004	0.991

Correlation is significant at the level *p < 0.05, and **p < 0.01 (2-tailed).

help maintain cholinergic system balance during aging.

The cholinergic system, which controls cognitive functions, is especially dense in the cortex and hippocampus, and damage to these areas impacts cognitive abilities. As cholinergic functions gradually decline with normal aging, age-related cognitive impairments start. In contrast, in pathological conditions linked to cognitive deficits, significant degeneration is seen in basal forebrain cholinergic cells. In pathological aging, such as AD, there is a severe loss of neuronal cells and cortical cholinergic innervation. However, in patients with mild cognitive impairment and early forms of AD, the issue is loss of function rather than cholinergic neurodegeneration [1].

Vitamin D plays an important role in neurogenesis, neurotransmitter synthesis, and protection against oxidative stress in the brain, and has neuroprotective effects [4]. Vitamin D deficiency has long been associated with cognitive impairment, dementia, and AD [7]. Supporting this, feeding transgenic AD mice with a vitamin D-enriched diet reduced brain pathology and prevented cognitive decline [28]. In addition, experimental studies have shown that vitamin D increases P-glycoprotein expression in the brain and reduces Aβ accumulation, a characteristic feature of AD [29]. The adverse effects of vitamin D deficiency on the brain emphasize the importance of ensuring adequate vitamin D levels to protect brain health during aging. Clinical studies have reported that vitamin D supplementation can improve cognitive impairment in patients with MCI, but its effectiveness in Alzheimer's disease is not fully understood [4].

Recent studies involving aged or Alzheimer's disease (AD) model animals have shown that vitamin D supplementation reduces various markers of AD pathology and significantly improves memory and cognitive functions. In the Scopolamine-induced Alzheimer's model, which is one of the most commonly used animal models of Alzheimer's disease, administering vitamin D to rats corrected histopathological changes in the brain, reduced amyloid peptide 1–42 levels, and improved memory [30]. In another study, Rodrigues et al. created an AD model using intracerebroventricular injection of Streptozotocin (ICV-STZ). They demonstrated that memory impairment observed in the MWM improved significantly after administering vitamin D for 21 days [22]. Yamini et al. reported that vitamin D significantly improved spatial learning and memory functions in the same AD model, and that vitamin D was more effective in reducing neuronal damage and behavioral dysfunction when given as a pretreatment [31]. Vitamin D administration has also been investigated in various models of memory impairment. It has been shown that cognitive functions impaired by Streptozotocin-induced diabetes and chronic high-fat feeding are reduced by long-term administration of 500 IU/kg/day vitamin D [26,27].

As people age, their spatial abilities decline due to physiological changes in different brain regions. In one study, vitamin D3 at doses of both 42 and 420 IU/kg improved cognitive performance in animals aged 6, 13, and 22 months. However, only 420 IU/kg of vitamin D was effective in 31-month-old rats [32]. In a study involving D-galactose (D-gal)-induced aging in mice, vitamin D enhanced learning and memory in aging mice and reduced damage to hippocampal neurons [33]. Similarly, vitamin D treatment was more effective as a preemptive measure in preventing D-gal-induced aging and memory impairment [34]. Latimer and colleagues found that when middle-aged 11- to 13-month-old F344 male rats were fed a diet with low (100 IU), medium (1000 IU), or high (10,000 IU) doses of vitamin D for 5–6 months, the group receiving the highest doses performed the MWM task much better [35]. Briones and Darwish observed that 21 days of vitamin D supplementation improved spatial memory, which was impaired in the MWM test among 20-month-old transgenic F344 male rats [36]. These studies highlight the importance of vitamin D in maintaining cognitive function in elderly rats. Consistent with previous research, the current study also suggests that vitamin D enhances spatial memory.

ChAT enzyme is the enzyme that synthesizes the neurotransmitter ACh. Research indicates that vitamin D supplementation enhances ChAT activity in specific brain regions of rats [37]. ChAT activity, which was decreased in the prefrontal cortex due to diabetes induced by intraperitoneal injection of streptozotocin in rats, was significantly increased by vitamin D (500 IU/kg/day) administration [26]. Similarly, memory impairment and decreased ChAT expression resulting from chronic high-fat feeding were alleviated by vitamin D supplementation [27]. Our study found no significant change in enzyme activity due to normal aging or vitamin D supplementation. However, the positive effect of vitamin D on cholinergic enzymes in aged animals supports that vitamin D may play a role in regulating acetylcholine metabolism in the presence of pathological changes.

Postmortem studies indicate that ChAT levels decline in the human hippocampus from middle age to old age [38]. However, autopsy brain samples from patients with MCI and early AD show no reduction in ChAT activity across several brain regions [1]. In Alzheimer's disease, enzyme activity averages 70–80 % of normal in the 60–70 age group and 30–40 % in the 80–90 age group. A similar pattern has been observed for AChE histochemical activity [38]. Supporting this, our findings demonstrate that AChE enzyme activity decreases with age. Contrary to our results, some studies have reported higher brain AChE activity in aged mice compared to young mice [39,40]. These studies used young mice aged 2–4 months and old mice aged 12–15 months, which correspond to humans aged 18–25 and 40–65 years, respectively. The rats in our study ranged from 25 to 40 years in young mice and 65–75 years in old mice [41]. Moreover, while this study focused on hippocampal content, other research analyzed overall brain tissue content. Differences between studies may stem from using rodents of different ages or examining different brain regions. Additionally, Khairy et al. observed decreased serum 25-hydroxyvitamin D levels and reduced AChE activity in older rats [25]. Our results align with this, showing that AChE activity declines in aging animals and that vitamin D supplementation can mitigate these age-related changes.

While AChE activity decreases in the brain during aging and AD, it increases in induced AD models. In two different studies, the rise in AChE in the cerebral cortex and the memory impairment seen in the AD model caused by ICV-STZ administration were prevented by 21 days of VD3 treatment [22,31]. Additionally, VD3 treatment lowered the elevated AChE enzyme activity in both the cortex and hippocampus when used as a pretreatment [31]. Similarly, the increase in AChE enzyme activity in the prefrontal cortex due to STZ-induced diabetes or chronic high-fat feeding (500 IU/kg/day) was reduced by vitamin D3 supplementation [26,27]. In another study, vitamin D treatment reduced the increased hippocampal AChE activity linked to experimentally induced hypothyroidism [19]. These findings suggest that vitamin D regulates activity by supporting decreased function in normal aging and decreasing increased activity in disease conditions.

Another enzyme that breaks down ACh is butyrylcholinesterase (BuChE). In the human brain, BuChE is found in regions such as the cerebral cortex, hippocampus, amygdala, and thalamic nuclei, which are important for cognitive functions [36]. In AD, cortical levels of BuChE are increased. Similarly, increased hippocampal BuChE activity has been observed in a scopolamine-induced AD model in rats [42]. Some studies suggest that BChE is associated with the "malignant" fibrillar Aβ plaques characteristic of AD, while others propose that BChE may inhibit Aβ fibril formation. In the brains of butyrylcholinesterase-knockout mice, fibrillar Aβ was reduced by nearly 70 % in males and 20 % in females, indicating that BChE activity could be a significant target for AD treatment [43]. We could not find any studies on changes in BuChE activity due to aging or AD with vitamin D. Consistent with the literature, hippocampal BuChE activity decreased in aging rats in this study. However, vitamin D supplementation reversed this decline.

The findings of this study demonstrate that Vitamin D supplementation improved spatial memory in aged rats, likely by reversing the aging-related changes in brain cholinergic functions. Vitamin D supplementation administered to aged rats significantly increased AchE and BuChE enzyme activities and ACh levels which had decreased with aging. This increased hippocampal cholinergic transmission resulted in the improvement of spatial memory in the MWM test. In summary, vitamin D supplementation shows promise in delaying cognitive decline associated with aging and AD.

These findings are of critical importance given the fact that dementia and AD development are closely linked to vitamin D deficiency. The risk of vitamin D deficiency increases with age due to the decline in dietary intake and synthesis of vitamin D. In this context, our study demonstrating the effectiveness of vitamin D in reversing aging-related cholinergic dysfunction suggests its potential not only as a therapeutic agent but also in the prevention of AD in "pre-symptomatic" stages before significant cognitive decline occurs. Unlike the existing literature, which predominantly focuses on models of memory impairment, this study fills a gap by detailing the effect on the cholinergic mechanism during normal aging. Future research may focus on investigating the mechanism of how vitamin D affects the cholinergic system, which is vital for memory, as well as other neurotransmitter systems, and translating vitamin D supplementation strategies into clinical applications to address their potential effects on aging and cognitive decline.

CRediT authorship contribution statement

Elif Aksoz: Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration,

Methodology, Conceptualization. **Medine Karabulut:** Writing – review & editing, Visualization, Validation, Software, Investigation, Formal analysis, Data curation. **Mustafa Hilmi Yaranoğlu:** Writing – review & editing, Visualization, Validation, Software, Investigation, Formal analysis, Data curation.

Statement of Informed Consent

Not applicable.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used Grammarly AI in order to improve language and readability]. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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Declaration of Competing Interest

The author does not declare any conflict of interest.

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