Effect of Vitamin D Supplementation on the Progression of Alzheimer's Disease in Rats: A Mechanistic Approach

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Research Article

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Abstract

Purpose: A multifaceted treatment approach can be effective for Alzheimer's disease (AD). However, currently, it involves only symptomatic treatment with cholinergic drugs. Beneficial effects of high vitamin D levels or its intake in the prevention and treatment of cognitive disorders have been reported. Thus, the present study examined the preventive effect of vitamin D supplementation on AD progression and evaluated its impact on the accumulation or degradation of Aβ plaques.

Methods: A single intraperitoneal injection of scopolamine was used to induce AD in rats. Treatment of vitamin D was provided for 21 days after the injection. Various behavioral parameters like learning, spatial memory and exploratory behavior, biochemical alterations in the brain homogenate and histology of the hippocampus were investigated.

Results: Our results indicated that scopolamine-induced rats depicted cognitive deficits with high Aβ levels and hyperphosphorylated tau proteins in the brain tissue, while vitamin D supplementation could significantly improve the cognitive status and lower these protein levels. These results were supported by the histopathological and immunohistochemical staining of the hippocampal brain region. Furthermore, mechanistic analysis depicted that vitamin D supplementation improved the Aβ protein clearance by increasing the neprilysin levels. It also reduced the accumulation of Aβ plaques by lowering neuroinflammation as well as oxidative stress.

Conclusion: The present findings indicate that vitamin D supplementation can delay AD progression by an increase in Aβ plaques degradation or reducing inflammation and oxidative stress.

Introduction

The reach of concern for Alzheimer's disease (AD) spans the globe as the most prevalent form of senile dementia. Currently, 50 million people live with dementia, of which 60-70% are suffering from AD. In 2050, it is predicted to increase to 152 million (WHO 2020). The pathological hallmarks of AD comprise amyloid (Aβ) plaque deposition, hyperphosphorylated Tau (p-Tau), and an abundance of inflammatory markers in the cerebral neurons. All these lead to neuronal injury. Besides, cholinergic deficits, synaptic loss, and oxidative stress are also seen in the neuronal tissue of AD patients leading to neurodegeneration (Grimm et al. 2017; Chai et al. 2019). The key player, Aβ plaques, are formed initially from the degradation of amyloid precursor protein (APP). The enzymes responsible for it includes β-secretase 1 (BACE1) and γ-secretase (Durk et al. 2014; Grimm et al. 2017). The degradation products include Aβ1-42 or Aβ1-40, of which Aβ1-42 is more prone to plaques' formation (Durk et al. 2014). These plaques are further cleared via neprilysin (NEP) and insulin-degrading enzyme (IDE) (Grimm et al. 2014; Grimm et al. 2017).

A multimodal treatment approach for AD is effective; however, it currently includes only drugs affecting the pathways of cholinergic systems. Also, none of the current therapies can delay or prevent the progress of AD. Interestingly, preventive strategies (dietary and lifestyle habits) can effectively curb the
in brain. It usually exerts its neuroprotective actions through the vitamin D receptors present widely in the hippocampus and cortex (Annweiler et al. 2014; Jayedi et al. 2019). High vitamin D levels or its intake have depicted favorable effects in the prevention and treatment of cognitive disorders, shown in some clinical and preclinical studies (Jorde et al. 2015; Moon et al. 2015; Feart et al. 2017; Beydoun et al. 2018; Jayedi et al. 2019). Further, vitamin D deficiency is proposed as a risk factor for AD progression (Annweiler et al. 2014; Grimm et al. 2017; Chai et al. 2019), while some studies have shown its involvement in the pathology also (Grimm et al. 2017). Thus, the linkage between vitamin D and AD has been developed; however, mechanisms underlying the benefits of vitamin D supplementation in AD patients are yet unresolved (Grimm et al. 2014; Chai et al. 2019; Jayedi et al. 2019).

Vitamin D supplementation may show beneficial effects on the formation or clearance of Aβ plaques through BACE1, IDE, or NEP enzymes (Chai et al. 2019; Jia et al. 2019). It may affect APP processing, such as decreased BACE1 protein levels leading to reduced Aβ formation or upregulating the gene expression of degrading enzymes (NEP or IDE) leading to Aβ degradation (Grimm et al. 2017). Further, it also reduces senile p-Tau deposition and thus intercepts neuronal death (Grimm et al. 2014). Vitamin D also promotes hippocampal neurodevelopment by upregulating the gene expression of proteins involved in new synaptic genesis (Latimer et al. 2014). Thus, this study examined the effect of vitamin D supplementation on the accumulation or degradation of Aβ plaques in Alzheimer's disease using a scopolamine-induced AD rat model. Further, the impact of long-term administration of vitamin D on the cognitive function of the AD rats was also evaluated.

### Material And Methods

#### Animals

Healthy male albino Wistar rats weighing 250-300 gm were used for the experimental study. They were housed in the Institute vivarium (22°C ± 1°C and relative humidity 55%-75%) under 12-h:12-h dark/light cycle and were provided with rodent chow and water *ad libitum*. The behavioral studies were carried out in quiet lab and the animals were transported everyday 30 min prior to the commencement of the evaluation to provide time for acclimatization. All the animals were handled with gentle care to avoid any stressful impact on their behavior. The experimental protocol was approved by the Institutional Ethics Committee (IP/PCOL/PHD/23/2018/024) as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of fisheries, Animal husbandry, and dairying, Govt. of India, New Delhi and National Institute of Health Guide for the Care and Use of Laboratory Animals.

#### Drugs and chemicals

(-)-Scopolamine hydrobromide trihydrate (scopolamine) was obtained from Sigma Aldrich, Steinheim, Germany and was dissolved in physiological saline. Donepezil hydrochloride was a kind gift from Emcure
Pharmaceuticals Pvt. Ltd., India and was freshly prepared in physiological saline before administration. Calcitriol (vitamin D₃) was a kind gift from Sun Pharmaceuticals Industries Ltd., India.

**Experimental protocol and treatment groups**

The rats were randomized into 5 groups (6 animals/group) as follows: Group I (NC) received normal saline solution and served as normal control, while Group II (DC) received scopolamine and served as disease control. Both the groups received normal saline orally for 21 days (8th – 28th day). Groups III (CAL 2.5) and IV (CAL 5) received vitamin D (2.5 and 5 µg/kg/day, respectively) while Group V (DPZ) received donepezil (5 mg/kg/day) for 21 days (8th–28th days), orally. Groups II-V received scopolamine (2 mg/kg) as a single dose, intraperitoneally (ip), on the 8th day. The doses of scopolamine (Deb et al. 2015; Mahdi et al. 2019), calcitriol (Verma et al. 2016), and donepezil (Barai et al. 2019) were selected based on published literature.

**Neurobehavioral evaluation**

The animals were tested for neurobehavioral tests such as Morris water maze (MWM), Modified Y-maze, and fear conditioned avoidance tests. The animals were subjected to these behavioral paradigms during the first week for learning and memory. The tests were repeated post 30 min of the scopolamine injection on the 15th day and 28th day (end of the study period) (Fig 1).

**Morris water maze test:**

The MWM tests assessed spatial memory and learning. It mainly assesses the ability of the animals to navigate a submerged, hidden platform from a start location in an open circular tank. The tailor-made circular tank (110 x 60 cm) was designed for the rats and divided into 4 equal-sized quadrants (N, E, W, and S). The water was filled up to a depth of 35 cm and made opaque using apple green food grade color. A hidden platform (30 cm in height) was submerged 2 cm below the water level in the SW quadrant. Briefly, animals were initially trained for four days (3 trials each day) to navigate a hidden submerged platform in a circular tank using a random set of start locations. The experiment was performed in a dark and silent room. The time taken to locate the platform was termed escape latency time (ELT). The mean ELT was recorded for each animal in all the groups. They were allocated 60 sec to locate the platform in the target quadrant. If the animal failed, it was returned onto the start platform and made to repeat the test after 20 sec. Finally, without any escape platform, the probe trial was conducted on the 5th day, and provided 120 sec to locate the platform. The time spent in the target quadrant was measured and was reported in seconds (Morris 1981, Vorhees and Williams 2006, Bromley-Brits et al. 2011).

**Modified Y-maze test**

This test evaluated short-term spatial memory and exploratory behavior. The test mainly depends on the natural ability of the rats (exploring the novel environment). The modified Y-maze consisted of 3 arms (A, B, and C) 40 cm long, 2 cm wide, and 18 cm high. The surface of all the arms was covered with corn husk
to prevent any anxious stimuli to rats. During the training session, rats explored the two arms (A and B) for 15 min while the C arm (novel arm) was blocked. After an interval of 1-2 hr, another session was conducted. All the arms were kept open during this session, and the rats were given 5 min to explore them. The number of entries in the novel arm C and the time spent in the novel arm C were recorded (Conrad et al. 1997).

**Fear Conditioned Avoidance Test:**

This test evaluated associative learning behavior in rats. It was conducted using a transparent box (30 x 30 x 30 cm) with an electric floor framework made of stainless steel. A sound stimulus was provided to deliver foot shocks. Briefly, the experiment was conducted in three phases: familiarization, learning, and retention. The animals were initially placed, individually, on the electric grid and allowed to explore for 4 min. Later, a sound stimulus (conditioned stimulus, 2.9 kHz, 20 sec, 80 dB) followed by a foot shock (0.8 mA, 2 sec) was given. This stimulus was repeated in 5 trials conducted at a time interval of 1 min. The retention test was conducted after 24 hours. The animals were placed on the grid again, and the freezing behavior was recorded for 3 min in the absence of sound and shock (contextual recording). After 3 min, the conditioned stimulus (sound stimulus) was provided, and freezing behavior was again recorded for 2 min (Curzon et al. 2009). The absence of any movements during the observation period except respiration was defined as freezing behavior. The total freezing time of each animal during the contextual recording and under the fear conditioning was recorded in sec.

**Biochemical parameters**

The animals were euthanized by cervical dislocation after the completion of the study period. Brain samples were collected followed by decapitation and washed with ice-cold saline. The samples were divided into two halves for biochemical analysis and histopathological as well as immunohistochemical analysis. For biochemical analysis, the weight of isolated brain tissues was measured, followed by homogenization with ice-cold phosphate buffer saline (pH 7.4, 0.1 M) and centrifugation at 10,000 rpm for 15 min. The supernatant was used for biochemical estimations. The samples for histopathological and immunohistochemical analysis were preserved in 4% formaldehyde at 4°C.

**Protein Estimation**

The protein content of the brain tissue was measured by Lowry's method using bovine serum albumin as standard (1 mg/mL) (Lowry et al. 1951). The results were stated as tissue concentration/mg protein.

**Estimation of enzyme activity**

The Acetylcholine-esterase (AChE) inhibitory activity was analyzed in the supernatant using the Ellman method (Ellman et al. 1961) and was expressed as enzyme unit/mg protein. The BACE-1 activity from brain homogenate was analyzed using a β-secretase fluorometric assay kit (Biovision, California, USA) according to the instructions provided by the manufacturer. It was expressed as relative fluorescence...
Estimation of inflammatory and AD biomarkers

Inflammatory biomarkers in brain homogenate [interleukin-6 (IL-6), IL-1β, tumor necrosis factor-α (TNF-α), interferon-γ, nuclear factor (NF)-κβ] and AD markers (Aβ1-40, Aβ1-42, p-Tau, IDE, and NEP) were measured using rat ELISA assay kits procured from Krishgen Biosystems, India. The assays were performed as per the instructions provided by the manufacturer. The results were expressed as pg/mg protein.

Estimation of oxidative stress parameters

The brain lipid peroxidation was determined by measuring malondialdehyde (MDA) levels (expressed as nmol/mg protein) using thiobarbituric acid reacting substances (Ohkawa et al. 1979). Reduced glutathione (GSH, expressed as nmol/mg protein) (Jollow et al. 1974) and superoxide dismutase (SOD, expressed as IU/mg protein) activity (Sun and Zigman 1978) were measured in the brain homogenate.

Histopathological and immunohistochemical analysis

The standard procedure was followed for H&E staining of the tissue. The brain tissue was fixed, dehydrated, impregnated, and embedded in paraffin for sectioning. Further, the sections were stained with hematoxylin and eosin. For immunohistochemical detection, the hippocampus was observed after fixation in 4% formaldehyde. Later, the paraffin-embedded tissue was sectioned (5 µM) and immunohistochemical staining was performed as per manufacturer’s instructions, using antibodies against synaptophysin (Krishgen Biosystems, India). The stained sections of both the analysis were studied and captured with a camera that was connected to an inverted light microscope (Olympus CKX41, Japan, 400x).

Statistical analysis

Results are expressed as mean ± standard error of mean (SEM). Statistical difference between the groups was analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni’s posthoc test using GraphPad Prism® software (version 5.01, California, USA). The data were considered statistically significant at a p-value of <0.05.

Results

Effect of vitamin D supplementation on the neurobehavioral performance of rats in MWM The mean ELT was significantly increased (p<0.001) in the DC rats post 21 days of scopolamine injection compared to normal controls. The same was significantly reduced (p<0.01) by the treatment with vitamin D (5 µg/kg/day) as compared to DC rats. Insignificant differences were found for vitamin D (2.5 µg/kg/day) and donepezil (5 mg/kg/day) (Fig 2a). A similar beneficial effect was observed for the mean time spent in the target quadrant by the scopolamine-induced AD rats treated with vitamin D (5 µg/kg/day, p<0.01) in the MWM test as compared to DC rats, while insignificant effect was observed with the low dose (2.5
µg/kg/day) (Fig 2b). This result showed an improved retrieval index in the rats supplemented with vitamin D (Fig 2).

**Effect of vitamin D supplementation on the spontaneous alternation behavior of rats in modified Y-maze test**

In the modified Y-maze test, scopolamine injection in the DC rats significantly decreased the number of entries (p<0.001) as well as the time spent in the novel arm C (p<0.01) as compared to the NC rats. Treatment with vitamin D (5 µg/kg/day) was able to significantly increase the number of entries (p<0.001) (Fig 3a); while the time spent in the novel arm was improved (p<0.05) by vitamin D (5 µg/kg/day) supplementation as well as donepezil (5 mg/kg/day) (Fig 3b) compared to the DC rats. However, vitamin D supplementation at the dose of 2.5 µg/kg/day could not show the beneficial effects in the Y-maze test (Fig 3).

**Effect of vitamin D supplementation on the associative learning behavior of rats in fear-conditioned avoidance test**

The present findings indicated a significant reduction (p<0.001) of the freezing time in the contextual and conditioned avoidance test after scopolamine administration on the 21st day in the DC rats compared to the NC rats. The calcitriol supplementation at both doses (2.5 and 5 µg/kg/day, p<0.001) as well as donepezil (5 mg/kg/day, p<0.001) led to significant improvement of the freezing time in the conditioned avoidance tests (Fig 4a). Additionally, a significant improvement of the freezing time in the contextual test was observed with only 5 µg/kg/day calcitriol (p<0.001) and donepezil (p<0.001) (Fig 4b).

**Effect of vitamin D on biochemical parameters**

After scopolamine injection, the AChE activity in the brain homogenate was significantly elevated (p<0.001) due to AD induction in DC rats compared to NC rats. Vitamin D (2.5 µg/kg/day, p<0.01), vitamin D (5 µg/kg/day, p<0.001) and donepezil (5 mg/kg/day) supplementation were able to significantly reduce the elevated AChE activity as compared to DC rats (Table 1).

**Effect of vitamin D on AD markers**

Since Aβ proteins are majorly responsible for the formation of plaques in AD, we evaluated both the Aβ proteins formed by the activity of BACE1 and γ-secretase, i.e., Aβ1-42 and Aβ1-40, respectively. The results in Fig 5a and 5b depicted that Aβ1-42 and Aβ1-40 were significantly elevated (p<0.001) due to AD induction by scopolamine compared to the normal rats. Vitamin D (5 µg/kg/day) and donepezil (5 mg/kg/day) treatment were able to reduce significantly (p<0.001) the elevated Aβ1-42 (Fig 5a) and Aβ1-40 (Fig 5b) in AD rats. In contrast, supplementation of vitamin D in low dose (2.5 µg/kg/day) was able to significantly lower only Aβ1-42 (Fig 5a; p<0.001) and not Aβ1-40 (Fig 5b).
In the same context, the present study indicated that the mean values of p-Tau proteins in the brain homogenate significantly increased \((p<0.001)\) in the DC rats compared to the NC rats. Administration of calcitriol (2.5 and 5 µg/kg/day) and donepezil (5 mg/kg/day) in the AD-induced rats significantly reduced \((p<0.001)\) the elevated levels of p-Tau proteins compared to the DC rats (Fig 5c).

In line with the above parameters, the enzymes such as BACE1, NEP, and IDE, were also evaluated. A surge in BACE1 activity \((p<0.05)\) in the brain homogenate was found in the DC rats compared to NC rats. Significant improvement was observed in BACE1 activity via treatment with vitamin D at doses 2.5 µg/kg/day \((p<0.05)\) and 5 µg/kg/day \((p<0.001)\) as well as donepezil (5 mg/kg/day, \(p<0.01)\) (Fig 5d). The IDE levels were significantly reduced \((p<0.001)\) in DC rats compared to the normal rats. However, insignificant differences were found between vitamin D (both doses), or donepezil treated rats and DC rats (Fig 5e). In contrast, the NEP levels were significantly improved via treatment with vitamin D (2.5 µg/kg/day, \(p<0.01)\), vitamin D (5 µg/kg/day, \(p<0.001)\), and donepezil (5 mg/kg/day, \(p<0.001)\) compared to the DC rats (Fig 5f). Thus, a shift in the proteolytic processing of the APP in the amyloidogenic pathway was observed by vitamin D supplementation at a high dose after collating the results of all AD markers.

**Effect of vitamin D on inflammatory markers**

The inflammatory markers (IL-1β, IL-6, TNF-α, IFN-g and NF-κβ) play a fundamental role in the pathophysiology of AD. We studied the levels of these cytokines in the AD-induced rats. Induction of AD with scopolamine significantly increased \((p<0.001)\) the levels of IL-1β (Fig 6a), IL-6 (Fig 6b), TNF-α (Fig 6c), IFN-g (Fig 6e) and NF-κβ (Fig 6e) in the brain homogenate compared to the normal rats. However, treatment with vitamin D (5 µg/kg/day) was able to significantly reduce the elevated levels of IL-1β \((p<0.001),\) IL-6 \((p<0.01),\) TNF-α \((p<0.001),\) IFN-g \((p<0.001)\) and NF-κβ \((p<0.001)\) compared to the DC rats. A dose-dependent effect was observed for vitamin D supplementation as the low dose (2.5 µg/kg/day) could also reduce the IL-1β \((p<0.001),\) IL-6 \((p<0.001),\) TNF-α \((p<0.05),\) IFN-g \((p<0.001)\) and NF-κβ \((p<0.05)\) compared to the DC rats (Fig 6).

**Effect of vitamin D on oxidative stress markers**

The levels of MDA depict the extent of lipid peroxidation in the brain, which was significantly elevated \((p<0.001)\) in the scopolamine-induced DC rats compared to the normal rats. Additionally, GSH was found to be significantly elevated \((p<0.001)\). At the same time, SOD was reduced significantly \((p<0.001)\) in the brain homogenates of DC rats compared to the normal rats. Treatment with vitamin D (both doses) significantly improved the lipid peroxidation \((p<0.001)\) and reduced glutathione levels \((p<0.001)\) in the brain tissue compared to the DC rats. The levels of SOD were found to be significantly reduced by administration of vitamin D only at a higher dose (5 µg/kg/day) as compared to the DC rats (Table 1).

**Effect of vitamin D on histology**
The qualitative histopathological observations of the hippocampal sections of DC rats depicted the loss of cell density and pyramidal cell structure compared to the NC rats. The intercellular spaces were found to be increased due to cell loss, which led to the loss of the dense neuronal network in the hippocampus of AD-induced rats following scopolamine injection post 21 days. Further, loss of cognitive function was derived from the collapse of the three primary functional layers of dentate gyrus correlated with progressive memory decline. Supplementation of vitamin D for 21 days prevented the degradation in the cellular density and the functional layers of the dentate gyrus, thus preventing cognitive loss. However, the higher dose was more effective qualitatively than the lower dose of vitamin D (2.5 µg/kg/day). The standard drug, donepezil, restored the hippocampal cellular density, but the beneficial effect on the dentate gyrus function was not prominent (Fig 7).

**Effect of vitamin D on immunohistochemical analysis**

The immunohistochemical staining of the rat hippocampal sections for synaptophysin indicated that the hippocampal cells in the normal rats appeared with light staining and lean processes. In contrast, the DC rats, treated with scopolamine, showed thick processes and densely stained bodies. This indicated loss of synaptic plasticity in the DC rats. The rats treated with vitamin D (both doses) and donepezil (5 mg/kg/day) for 21 days showed a reduction in the stain density compared to DC rats. This reduction could be inferred as restoring functional neuronal cells indicating neurogenesis in the hippocampus (Fig 8).

**Discussion**

The present study demonstrates a potential beneficial role of vitamin D supplementation in delaying AD progression in rats due to improvements in the formation or clearance of amyloid plaques, inflammation, and oxidative stress. The scopolamine-induced amnesia model for induction of AD induction in rats was employed in the study. Scopolamine gains access to the brain via blood-brain barrier and causes an increase in AChE levels. It also increases oxidative stress and neuroinflammation in the rat brain, finally leading to cognitive decline and developing AD-like symptoms (Zaki et al. 2014; Bhuvanendran et al. 2018; El-Marasy et al. 2018; Barai et al. 2019).

Decrease in hippocampal function like short-term memory, learning, and spatial recognition are early characteristic signs in AD patients (Jahn 2013). The altered exploratory behavior in AD involves the frontal cortex, hippocampus, and basal ganglia (Zufferey et al. 2013). In the present study, significant impairment in the spatial memory, learning, and exploratory behavior of rats was observed in the neurobehavioral tests. These observations sync with previous studies (Zaki et al. 2014; Hafez et al. 2017; Rajashri et al. 2020), indicating hippocampal damage in rats. The MWM and modified Y-maze tests are prominent tools to detect hippocampal dependent memory and synaptic plasticity (Vorhees and Williams 2006, Ngatanko Abaissou et al. 2020). Improvement in MWM and modified Y-maze performance by treatment with vitamin D (5 µg/kg/day) evidenced its beneficial effect in restoring the rats' spatial learning, memory, and exploratory behavior. This behavioral improvement may indicate positive effects of
vitamin D in delaying neurodegeneration, as observed in other studies (Rodrigues et al. 2019; Sultan et al. 2020).

Further, the fear-conditioned avoidance test was conducted to evaluate emotional memory and associative learning. Fear conditioning response is a hippocampus and amygdala dependent memory function. Loss of functional connectivity between the hippocampus and amygdala may be responsible for memory loss in the early stages of AD (Hamann et al. 2002; Varinthra et al. 2021). The rats injected with scopolamine indicated a reduction in fear memory retrieval (decrease in freezing time), while treatment with vitamin D or donepezil significantly improved the contextual as well as conditioned fear memory retrieval. This improvement may indicate the ability of vitamin D to restore the synaptic plasticity between the hippocampus and amygdala, as observed earlier in an experimental model of AD (Durk et al. 2014). Moreover, the loss of fear conditioning memory is associated with the burden of Aβ plaque deposition (Hanna et al. 2012), and improvement in this memory retrieval may indicate a decrease in Aβ plaque deposition.

Further, the results of the neurobehavioral test corroborate with the loss of synaptophysin staining in the hippocampal cells during immunohistochemistry analysis. Synaptophysin is a presynaptic vesicle protein in the hippocampus, basal ganglia, and cortex, used as a biomarker for synaptic plasticity. A decrease in this protein is associated with the cognitive deficit in AD patients (Hajjar et al. 2013; Zufferey et al. 2013). Vitamin D supplementation depicted a dose-dependent protective effect observed as more viable neuronal cells than DC rats (Fig 8). In a nutshell, these results provide proof for improving cognitive behavior and effective memory consolidation in scopolamine-induced AD rats by supplementing vitamin D.

The pathological hallmarks of AD, i.e., extracellular Aβ plaque formation and intracellular p-Tau resulting in neurofibrillary tangles, are held responsible for the cognitive decline, including memory loss (spatial, episodic, emotional, recognition) as well as significant inflammatory response (Wang et al. 2015). The balance between the formation (BACE1 and g-secretase) and degradation (IDE and NEP) of Aβ proteins maintains the Aβ levels in the brain (Hafez et al. 2017). In the current study, the beneficial effects of vitamin D supplementation (5 µg/kg/day) and donepezil (5 mg/kg/day) were observed in reducing the disease burden. Previous studies reporting the lowering of Aβ proteins due to vitamin D supplementation corroborate our results (Yu et al. 2011; Grimm et al. 2014; Grimm et al. 2017; Jia et al. 2019; Bivona et al. 2021). The decreased Aβ protein levels may be attributed to the ability of vitamin D to stimulate the phagocytosis of Aβ plaques or even increase the permeability of Aβ proteins through blood-brain barrier (Banerjee et al. 2015; Bivona et al. 2021). The vitamin D induced reduction in BACE1 enzyme activity compared to DC rats was in sync with Grimm et al. (2017). The significant improvement in the APP degrading enzymes like NEP and a minor effect on IDE was observed via vitamin D supplementation. These results are consistent with a study on mice with hypovitaminosis, indicating improvement in the NEP enzyme while no statistically significant effect on IDE by vitamin D supplementation was recorded (Grimm et al. 2014). Thus, vitamin D induced Aβ clearance may be via the NEP pathway and less by the...
Additional anti-inflammatory mechanisms of vitamin D may be responsible for the decrease in Aβ protein accumulation and improvement in cognitive functions in AD rats. Accumulation of Aβ plaques and p-tau proteins can activate a cascade of neuroinflammation including proinflammatory mediators like IL-1β, IL-6, TNF-α, or IFN-g from microglial cells. These proinflammatory cytokines can mediate neurotoxicity leading to AD (Wang et al. 2015). These mediators can also reduce the endocytosis and phagocytosis of Aβ proteins by the microglial cells (Varinthra et al. 2021). Further, NF-κβ is reported to be important in maintaining learning, memory as well as synaptic plasticity. Activation of NF-κβ signaling can stimulate the BACE1 activity and thus increase the Aβ protein accumulation (Jha et al. 2019). In addition, IFN-g is reported to be significantly high in AD patients (Belkhelfa et al. 2014) that can result in increased hyperphosphorylation of soluble tau proteins (Zheng et al. 2016). In the current study, supplementation of vitamin D in AD-induced rats demonstrated significant anti-inflammatory activity in a dose-dependent manner. Our results are consistent with another study showing a decrease in inflammatory mediators (IL-1β, IL-6, NF-κβ) in the hippocampal regions of the brain due to vitamin D supplementation (Farhangi et al. 2017). The decrease in the inflammatory mediators may also inhibit the over-activation of hippocampal cells and thus can reduce the accumulation of Aβ plaques (Varinthra et al. 2021).

Oxidative stress is a characteristic feature of AD brains along with Aβ plaques and neurofibrillary tangles (Zhao and Zhao 2013). Aβ peptide is associated with the generation of reactive oxygen species that can further lead to aggregation and plaque formation (Ahmad et al. 2017). Lipid peroxidation is significantly high in several brain regions in AD. Additionally, glutathione and antioxidant enzymes like SOD are also reduced in AD brains. Together, the raised oxidative status may result in neuronal damage and thus, neuronal death as well as synaptic loss (Zhao and Zhao 2013; Ahmad et al. 2017). Scopolamine can increase glutathione and SOD activity and increase lipid peroxidation in AD-induced rats (Rahimzadegan and Soodi 2018). This increased oxidative stress in the AD-induced rats was significantly reduced by vitamin D as well as donepezil supplementation. Thus, it can be proposed that vitamin D may delay the formation of Aβ plaques. Moreover, in the present study, significantly elevated AChE activity in AD-induced rats is in tune with the previous reports (Zaki et al. 2014; El-Marasy et al. 2018; Barai et al. 2019; Rajashri et al. 2020), causing increased degradation of acetylcholine and impairment in learning and memory. These AChE levels were reinstated by vitamin D. Thus, vitamin D supplementation could prevent cholinergic neuronal loss and the development of cognitive deficit.

The histological changes of the hippocampal regions were also investigated in the current study. Loss of cell density and hippocampal architecture (pyramidal cells) was observed due to scopolamine administration compared to normal rats indicating neuroinflammation (El-Marasy et al. 2018; Rajashri et al. 2020). Restoration of the hippocampal architecture by treatment with vitamin D depicted neuronal protection. This observation is consistent with the brain homogenate's biochemical investigations (inflammatory and AD markers), indicating reduced inflammatory status and neuronal damage found in the current study.

Thus, based on the aforementioned findings, the neuroprotective effects of vitamin D supplementation
improvement in learning and memory.

**Abbreviations**

AD: Alzheimer's disease, Aβ: amyloid plaques, p-Tau: hyperphosphorylated Tau proteins, APP: amyloid precursor protein, BACE1: β-secretase 1, NEP: neprilysin, IDE: insulin-degrading enzyme, MWM: Morris water maze, ELT: escape latency time, AChE: Acetylcholine esterase, RFU: relative fluorescence units, IL: interleukin, TNF-α: tumor necrosis factor-α, IFN-g: Interferon-g, MDA: malondialdehyde, GSH: reduced glutathione, SOD: superoxide dismutase, SEM: standard error of mean.

**Declarations**

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**Conflicts of interest/Competing interest:** The authors have no conflicts of interest to declare that are relevant to the content of this article.

**Availability of data and material:** The data is available with the authors and can be produced on request.

**Code availability:** Not applicable

**Authors' contributions:** Ms. Parmi Patel contributed to design the experiments, data acquisition and analysis, and manuscript writing. This work is done as a part of her Ph.D. work. Dr. Jigna Shah supervised the Ph.D. work of Ms. Parmi Patel. She contributed to the conceptualization, critical review of the draft, and final approval of the version to be published. All authors read and approved the manuscript.

**Ethics approval:** The Institutional Ethics Committee of Nirma University approved the experimental protocol (protocol approval number IP/PCOL/PHD/23/2018/024 dated 3/8/2018). The study was conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Fisheries, Animal Husbandry, and Dairying, Govt. of India, New Delhi and National Institute of Health Guide for the Care and Use of Laboratory Animals.

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Tables

Table 1: Effect of vitamin D supplementation on the anticholinesterase levels and antioxidant status of brain after 21 days of treatment in scopolamine induced Alzheimer’s disease in rats.

| Sr. No. | Parameter                                      | NC     | DC    | CAL 2.5 | CAL 5.0 | DPZ    | p-value |
|---------|-----------------------------------------------|--------|-------|---------|---------|--------|---------|
| 1       | AChE levels (nmoles/mg protein)               | 0.06 ± 0.004 | 0.10 ± 0.003$$$$ | 0.07 ± 0.003** | 0.04 ± 0.006*** | 0.05 ± 0.004*** | <0.0001 |
| 2       | Lipid peroxidation (MDA, nmoles/mg protein)   | 2.53 ± 0.04 | 14.37 ± 0.03$$$$ | 2.56 ± 0.08*** | 1.80 ± 0.07*** | 2.36 ± 0.08*** | <0.0001 |
| 3       | Reduced Glutathione (nmoles/mg protein)       | 0.06 ± 0.005 | 0.05 ± 0.008 | 0.10 ± 0.006 | 0.37 ± 0.025*** | 0.02 ± 0.01 | <0.0001 |
| 4       | Superoxide dismutase (IU/mg protein)          | 1.01 ± 0.066 | 0.72 ± 0.032$$ | 0.80 ± 0.449 | 1.00 ± 0.064** | 1.08 ± 0.038*** | <0.0001 |

NC: Normal control rats receiving normal saline solution; DC: Disease control rats treated with scopolamine (2 mg/kg, single dose, *ip*); CAL 2.5: Alzheimer’s induced rats treated with vitamin D (2.5 µg/kg/day, *po*); CAL 5: Alzheimer’s induced rats treated with vitamin D (5 µg/kg/day, *po*); DPZ: Alzheimer’s induced rats treated with donepezil (5 mg/kg/day, *po*). AChE: Anti-cholinesterase, MDA: malondialdehyde.

Data expressed as means ± SEM (n=6 animals per group). *- p<0.05, **- p<0.01, ***- p<0.001 compared to DC rats; $ - p<0.05,

\[- p < 0.01,

$ - p<0.001$ vs. NC rats based on one-way ANOVA followed by Bonferroni’s post-hoc test.

Figures
Figure 1

Experimental design, dosage schedule and behavioral assessment schedule for the induction of scopolamine induced Alzheimer's disease in rats

Figure 2

(a) Effect of vitamin D supplementation on the mean escape latency in Morris water maze test after 21 days of treatment. (b) Effect of vitamin D supplementation on the mean % time spent in the target quadrant in Morris water maze test after 21 days of treatment NC: Normal control rats receiving normal saline solution; DC: Disease control rats treated with scopolamine (2 mg/kg, single dose, ip); CAL 2.5: Alzheimer's disease induced rats treated with vitamin D (2.5 µg/kg/day, po); CAL 5: Alzheimer's disease induced rats treated with vitamin D (5 µg/kg/day, po); DPZ: Alzheimer's disease induced rats treated with donepezil (5 mg/kg/day, po). Data expressed as means ± SEM (n=6 animals per group). *- p<0.05, **-
$ - p < 0.001$ vs. NC rats.

(a) Effect of vitamin D supplementation on the number of entries in the novel arm during Y-maze test after 21 days of treatment. (b) Effect of vitamin D supplementation on the % time spent in the novel arm during Y-maze test after 21 days of treatment. NC: Normal control rats receiving normal saline solution; DC: Disease control rats treated with scopolamine (2 mg/kg, single dose, ip); CAL 2.5: Alzheimer’s disease induced rats treated with vitamin D (2.5 µg/kg/day, po); CAL 5: Alzheimer’s disease induced rats treated with vitamin D (5 µg/kg/day, po); DPZ: Alzheimer’s disease induced rats treated with donepezil (5 mg/kg/day, po). Data expressed as means ± SEM (n=6 animals per group). *- p<0.05, **-p<0.01, ***-p<0.001 compared to DC rats; $ - p < 0.05$, $ - p < 0.01$, $ - p < 0.001$ vs. NC rats.
Figure 4

(a) Effect of vitamin D supplementation on the freezing time during contextual analysis of fear avoidance test after 21 days of treatment. (b) Effect of vitamin D supplementation on the freezing time during conditioned avoidance analysis of fear avoidance test after 21 days of treatment. NC: Normal control rats receiving normal saline solution; DC: Disease control rats treated with scopolamine (2 mg/kg, single dose, \( \text{ip} \)); CAL 2.5: Alzheimer's disease induced rats treated with vitamin D (2.5 µg/kg/day, po); CAL 5:
Alzheimer’s disease induced rats treated with vitamin D (5 µg/kg/day, po); DPZ: Alzheimer’s disease induced rats treated with donepezil (5 mg/kg/day, po). Data expressed as means ± SEM (n=6 animals per group). *- p<0.05, **-p<0.01, ***-p<0.001 compared to DC rats; $ - p<0.05, $ - p<0.001 vs. NC rats.

Figure 5

Alterations in the Alzheimer’s disease markers produced due to vitamin D supplementation in the brain homogenates of scopolamine induced Alzheimer’s disease in rats (a) Aβ1-40 concentration (b) Aβ1-42 concentration (c) p-Tau (hyperphosphorylated tau protein) concentration (d) β-secretase 1 (BACE1) activity (e) IDE (Insulin degrading enzyme) levels (f) NEP (Neprilysin) levels. NC: Normal control rats receiving normal saline solution; DC: Disease control rats treated with scopolamine (2 mg/kg, single dose, ip); CAL 2.5: Alzheimer’s disease induced rats treated with vitamin D (2.5 µg/kg/day, po); CAL 5: Alzheimer’s disease induced rats treated with vitamin D (5 µg/kg/day, po); DPZ: Alzheimer’s disease induced rats treated with donepezil (5 mg/kg/day, po). Data expressed as means ± SEM (n=6 animals per group). *- p<0.05, **-p<0.01, ***-p<0.001 compared to DC rats; $ - p<0.05,$ - p<0.001 vs. NC rats.
Figure 6

Alterations in the inflammatory markers produced due to vitamin D supplementation in the brain homogenates of scopolamine induced Alzheimer’s disease in rats (a) IL-1β concentration (b) IL-6 concentration (c) interferon-γ concentration (d) TNF-α concentration (e) NF-κβ concentration. NC: Normal control rats receiving normal saline solution; DC: Disease control rats treated with scopolamine (2 mg/kg, single dose, ip); CAL 2.5: Alzheimer’s disease induced rats treated with vitamin D (2.5 µg/kg/day, po); CAL 5: Alzheimer’s disease induced rats treated with vitamin D (5 µg/kg/day, po); DPZ: Alzheimer’s disease induced rats treated with donepezil (5 mg/kg/day, po). Data expressed as means ± SEM (n=6 animals per group). *- p<0.05, **-p<0.01, ***-p<0.001 compared to DC rats; $- p<0.05,$ - p<0.001 vs. NC rats.
Figure 7

Representative photomicrographs of H & E stain (100 x) of hippocampus in which (a) Normal control rats showing normal morphology of neurons (b) Disease control rats showing neurodegeneration and loss of intercellular spaces (c) Alzheimer’s induced rats treated with vitamin D (2.5 µg/kg/day, po) (d) Alzheimer’s disease induced rats treated with vitamin D (5 µg/kg/day, po) (e) Alzheimer’s induced rats treated with donepezil (5 mg/kg/day, po). All the three treatment group rats showed much less neurodegeneration and reversal of normal morphology.
Figure 8

Representative photomicrographs (400x) of hippocampus synaptophysin in which (a) Normal control rats showing indicating healthy cells as no stain was taken up (b) Disease control rats indicating loss of synaptic plasticity as most of the cells have taken the stain (c) Alzheimer’s disease induced rats treated with vitamin D (2.5 µg/kg/day, po) showing structural disruption in some cells with some few healthy cells (d) Alzheimer’s disease induced rats treated with vitamin D (5 µg/kg/day, po) showing slightly stained cells indicating cell viability after 21 days of treatment (e) Alzheimer’s disease induced rats treated with donepezil (5 mg/kg/day, po) showing moderately stained cells indicating functional presence of cells with structural integrity of the hippocampal cells.

Supplementary Files

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