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Research Article

Effect of Curcumin and Vitamin D3 on Learning and Cognition in Rat Model of Alzheimer's Disease

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder which is characterized by gradual memory loss and shrinkage of neuronal cells particularly in the hippocampus and basal forebrain regions. Loss of central cholinergic neurotransmitter and accumulation of ubiquitinated proteins in the neurons and sign of inflammation are considered important hallmarks of AD. The development of drug effective for AD widely anticipated because of the increase in the elderly population and the progressively increasing number of AD patients worldwide. The aim of this study is to determine the effect of Curcumin and Vitamin D3 for improving AD care and to design strategy for treatment and prevention of AD.

Curcumin and vitamin D3 used as neuroprotective agents against scopolamine-induced dementia in male Sprague-Dawley rats of 200 ± 25g. Donepezil at a dose (2.5 mg/kg) used as standard drug. The rats were divided into 5 groups; they were injected with scopolamine at a dose of 2.5 mg/kg. Oral Curcumin dose (herbal drug) was given to animals at a dose of 80 mg/kg. Vitamin D3 (0.0179 mg/kg) was given oral (using oral gavage). Scopolamine induced impaired cognition was observed with behavioral tasks including; rectangular maze test and locomotor activity. In scopolamine treated animals, the correct response rate during acquisition and retention period was significantly lower than the control group. Curcumin and/vitaminD3 showed improvement in cognition and learning. It was observed that the correct response rate for both tasks was significantly equal to the control group ($*p < 0.05$). H and E staining of a coronal section of rat brain at magnification 40 X (Scopolamine group) showed less number of cells (as indicated in the figure 4 picture B) and it was indicative of cell degeneration. Curcumin and/vitamin D3 groups showed significantly increased number of cells compared with the scopolamine group (showed in the figure 4 pictures C and D). No gap around neuronal cells was observed in the drug treated groups compared with control group. Immunoblotting of rat brain tissues using anti-human Paired Helical Filament demonstrated significantly presence of abnormal protein in scopolamine group when compared with drug-treated groups. Densitometry data and immunoblot image clearly showed the presence of abnormal tau protein. The presence of abnormal protein could be the reason of memory loss event in the rat brain observed during the behavioral study of scopolamine group compared with the control group. Therefore, on the basis of significant behavioral improvement in drug treated animals (Curcumin/vitaminD3) groups ($*p < 0.05$) (showed in the figure 1 and 2) and the results obtained from the histological and Immunoblotting study, we can conclude that Curcumin and/or vitamin D3 may have potential to reverse some cognitive deficits and protect brain from cell degeneration (which could be due to Aβ toxicity and presence of abnormal tau protein in the brain). Drug treatment may also improve memory and learning. Our results suggest that Curcumin and vitamin D3 could be used in a potential preventive therapy during AD progression.

Keywords: *Alzheimer's Disease; Memory Impairment, Inflammation, Scopolamine, Curcumin, Vitamin D3 And Donepezil*

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder of the elderly population. It is characterized by progressive loss of cognitive functions, amyloid β (Aβ) deposition and formation of neurofibrillary tangles (NFTs) in the brain cells. These NFTs cause shrinkage of neurons. NFTs are formed inside the cell bodies of those neurons, whose axons are projecting to the sites of neuritic plaques, specifically to

entorhinal to hippocampal-prefrontal pathways. This process would be a reason for the loss of cognition and learning [1]. Oxidative stress is considerably important for AD pathology characterized by the oxidization of the the lipid, protein and nucleic acid components of the cell to a mitochondrial dysfunction, inflammation, and Aβ deposition [2]. Low central cholinergic levels and loss of cholinergic neurons have been linked with the key events in AD [3]. The earliest degeneration occurs in basal forebrain cholinergic nuclei found at predementia stages [4]. Evidence from animal model and postmortem reports have proved that cholinergic transmission and amyloid accumulation have two ways interaction [5], and basal forebrain cholinergic neurons are specifically vulnerable to the toxicity of amyloid. The decline of cholinergic transmission is also found to be associated with increased amyloid formation [6]. Similarly, tau hyperphosphorylation can be the earliest event in abnormal processing of tau protein during AD pathogenesis [7]. Hyperphosphorylated tau involves in synaptic function specifically in long-term depression (LTD). It seems to be the earliest event in synaptic transmission [8]. Animal models have proved that loss of synaptic plasticity is key components in the neurodegenerative process of AD and tau is one of contributing factors for neurodegeneration [9].

In this study, neuroprotective effect/s of Curcumin and vitamin D3, on scopolamine-induced learning and memory impairment in animals are investigated. Scopolamine is one of a group of drugs which acts on muscarinic acetylcholine receptors. It is widely used in experimental animals. Rectangular maze test (for spatial memory), and locomotors activity are shown to assessed the memory and learning processes among control scopolamine and drug treated groups of animals. The histological study is done to find out the lesion in brain region caused by scopolamine. Immunohistochemistry was done to specify the location of presence of Aβ deposition. Immunoblotting was done to investigate the presence of (NTFs) in rat brain region.

Material and Methods

Animals

Male Sprague-Dawley rats of 200 ± 25g were obtained from the animal house (University Brunei Darussalam). They were housed individually in a cage (at an ambient temperature of 25 ± 2°C) and 45 - 55% relative humidity with 12 hours light/dark cycles. Rats were free to access food and water *ad libitum*.

All rats were handled daily for a minimum of 1 week prior to behavioral testing. One week prior to testing in the "Rectangular Maze" and throughout testing in the rectangular Maze and for locomotor activity using "Actophotometer", the food was restricted to a daily feeding of approximately 80% of their *ad libitum* consumption to maintain the weight of each rat at approximately its freely fed weight. Experiments were performed between 09:00 am to 5:00 pm to reduce the stress effect of noise and other variants. A constant temperature of 25 ± 2°C and 12:12 light-dark cycle were maintained throughout the experiments.

Experimental Design

- All experiments were conducted during the daytime between 9:00am to 5:00pm
- The procedure of drug treatment was carried out for 27 days.
- All experiments were conducted in accordance with an institutional guideline for animal care and use.

Group I	Saline-control	(0.9% saline) + behavioral test
Group II	Disease control	Scopolamine (2.5 mg/kg) i. p. + behavioral test
Group III	Experimental	Scopolamine (2.5 mg/kg) i. p. Curcumin (80mg/kg) oral + behavioral test
Group IV	Experimental	Scopolamine (2.5 mg/kg) i. p., Donepezil (2.5mg/kg) oral + behavioral test
Group V	Experimental	Scopolamine (2.5 mg/kg) i. p. Vitamin D3 (0.0179mg/kg) oral +behavioral test

Table 1.

Drug treatments

There were 30 animals divided into 5 groups of six animals in each group. All groups except vehicle group received scopolamine (2.5 mg/kg body wt.) i.p. injection for each day for 27 days to induce excitotoxicity. Donepezil (2.5 mg/kg) (serves as a standard drug) oral dose was given to experimental groups for each day for 27 days. Group 5 received vitamin D3 (0.0179 mg/kg) oral for days for 27 days. Curcumin (80 mg/kg) oral dose was given to experimental groups except for vehicle group. Vehicle group was given 0.9% saline (i.p. Injection).

Behavioral Tests

All groups of animals were given training for behavioral tests for one week before drugs induction.

Rectangular Maze Test

Learning, memory, and reasoning in animals were assessed using rectangular maze. The animal with the intact memory makes use its past experience, to find a way out in the maze [10]. Rectangular maze it is a rectangular box with the entry and reward (food) appended at opposite ends. Each animal was trained prior to drug induction with the maze. Time was taken to the animal to reached reward chamber was recorded. For each animal, five reading are taken and the average is calculated as their learning score [11].

Locomotor Activity

CNS drugs have an influence on locomotor activity in both man and animals. The equipment measures the locomotor activity of drug operates on photoelectric cells which are connected in circuit with a counter. When the ray of light falls on photocells is cut off by the animal then, a count is recorded [12]. Each animal has given 2 minutes in activity cage and activity is recorded. The count is recorded and increase or decrease in locomotor activity was calculated.

Histology

After a behavioral study on animals, they were anesthetized and decapitated. Brains were removed from the skull and stored in paraformaldehyde (4%), and then brains were embedded in paraffin and kept in the refrigerator. 5 µm coronal sections were prepared using rotary microtome stained with Hematoxylin and Eosin. Photographs were taken for each section [13].

Estimation of protein concentration

Immunoblotting

Antibodies against modified microtubule-associated protein Tau was obtained from Thermo scientific). Dissect the brain tissue of coronal area with clean tools on ice, and as quickly as possible to prevent degradation by proteases. Placed the tissue in microcentrifuge tubes and immerse in liquid nitrogen to snap freeze. Homogenized brain tissues on the ice. 1X ice-cold lysis buffer rapidly added to the tube, homogenized with an electric homogenizer, rinsed twice a rinsed twice with the same conc. of lysis buffer, and agitation was maintained for 2 hours at 4°C. Centrifugation was done for 20 mins at 12000 rpm at 4°C microcentrifuge tube was then placed on ice. The supernatant was removed and placed in a fresh tube kept on ice; discard the pellet. A small volume of lysate was taken to perform a protein quantification assay.

Boil each cell lysate in TBST buffer at 100°C for 5 min. 50 micrograms of protein were loaded into the wells of the SDS-PAGE gel. Gal was run for 1 - 2 hours at 100V. Transferred the protein onto the membrane. The membrane was blocked for 1 hour at room temperature. Membrane washed with TBST 3 times 5 mins each. The membrane was incubated with secondary antibody in blocking buffer at room temperature for 1 hour. It was washed with TBST buffer three times 5 mins.

Blocked the membrane for 1 hour at room temperature. The membrane was then incubated with 1:1000 dilution of primary antibody in blocking buffer. Washed the membrane in three washes of TBST, 5 min each. Incubated the membrane with the 1:1000 dilution of conjugated secondary antibody in blocking buffer at room temperature for 1 hour. Wash the membrane in three washes of TBST, 5 min each. Remove excess reagent and cover the membrane with the transparent plastic wrap. Acquired image using darkroom development techniques for chemiluminescence detection.

Statistical Analysis

All data are expressed as mean standard error of the mean (SEM). Transfer latency over the 27 days was analyzed of variance (ANOVA, single factor) measures. The crite¬rion for statistical significance was $p < 0.05$.

Results

Behavioral Tests

Rectangular Maze Test

The neuroprotective activity of curcumin vitamin D3 was evaluated using a rectangular maze. Donepezil was used as standard drug. The rats in Curcumin and vitamin D3 groups except for scopolamine-treated group (disease control group) showed lower transfer latency (time taken by the animal to move from open arm to coverd arm) in Figure 1. The performance of rats between groups (control, scopolamine and drug-treated groups) significant difference across all days shown in the table ($*p < 0.05$).

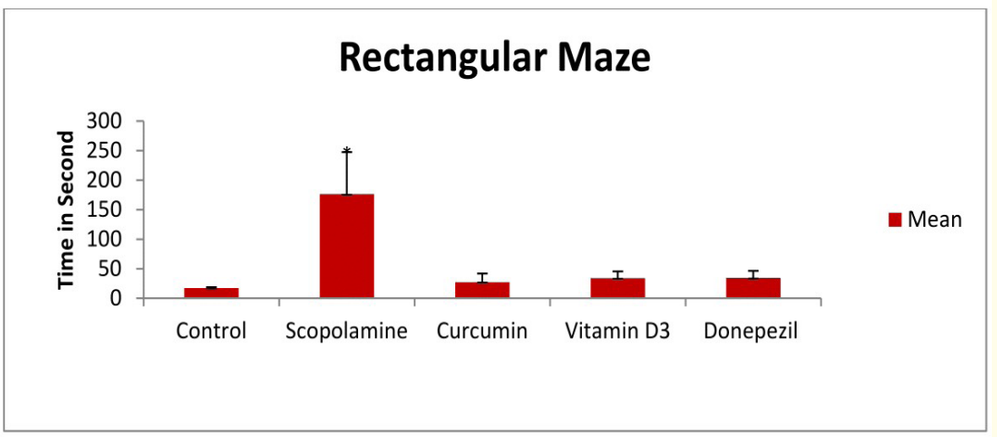

Figure 1: *Performance of rats in all groups in the rectangular maze, the Y axis represents time in second. Scopolamine group showed a significance difference (*p < 0.05). Data expressed as the mean of time taken to reach the reward chamber each alternate day for 27 days.*

Anova: Single factor						
Summary						
Groups	Count	Sum	Average	Variance		
Control	14	180.416	12.88686	13.78096		
Scopolamine	14	86.263	6.161643	45.78577		
Curcumin	14	138.76	9.911429	6.264198		
Vitamin D3	14	164.91	11.77929	15.94404		
Donepezile	14	168.93	12.06643	10.49621		
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	405.1101	4	101.2775	5.488037	0.00072	2.51304
Within Groups	1199.525	65	18.45423			
Total	1604.635	69				

Table 2: *ANOVA single factor for Rectangular maze test.*

Locomotor Activity

The locomotor activity of curcumin and vitamin D3 treated rats was evaluated using actophotoactometer. The rats showed significant transfer latency in the experimental groups except for scopolamine treated group as shown in Figure 2. The performance of the groups (control, scopolamine and treated groups) showed a significant difference across all number of days as shown in table 3 (*p < 0.05).

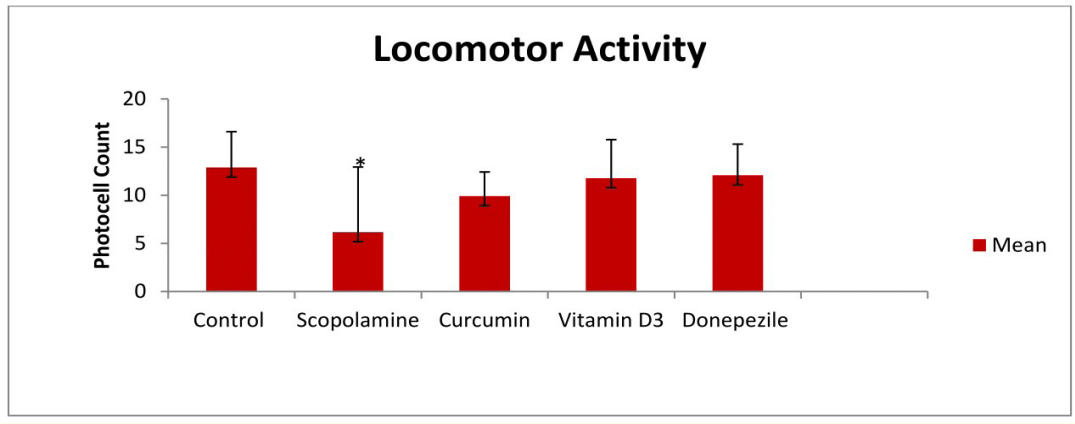

Figure 2: *Locomotor activity of all rats except scopolamine treated rats in actophotometer showed no significant difference. Data expressed as mean of photocell count (Mean.SD, n = 6) of animals each alternate days for 27 days.*

Anova: Single Factor						
Summary						
Groups	Count	Sum	Average	Variance		
Control	14	180.416	12.88686	13.78096		
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Between Groups	405.1101	4	101.2775	5.488037	0.00072	2.51304
Within Groups	1199.525	65	18.45423			
Total	1604.635	69				

Table 3: *ANOVA single factor for locomotor activity.*

Data expressed as mean, standard deviation expressed number of photocell count for each group.

Histology

It is shown in Figure 3, degeneration of cells is more prominent in the scopolamine group than in the drugs (curcumin and vitamin D3) treated groups. This degeneration was indicated by a gap arround the neuronal cells indicated by arrows and the slides after H and E staining. The drugs treated groups (Curcumin and vitamin D3) showed the almost equal number of cells and similar morphology of cells compared with control group. Figure 4 (Control group) represent important memory keeping regions in the hippocampus of the brain which was observed during histology study.

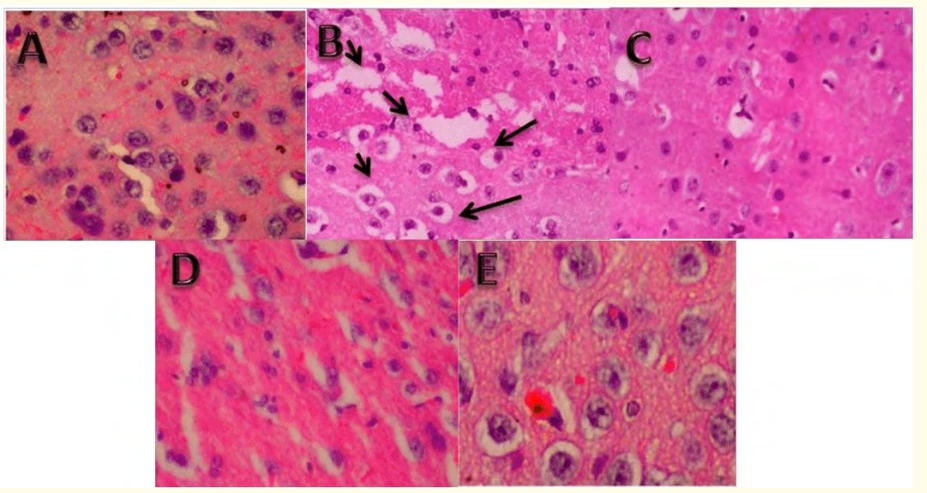

Figure 3: *Hematoxylin and Eosin staining for A control, B Scopolamine, C Curcumin, D Vitamin D3 and E Donepezil. Images show no significant difference in cellular histology in the hippocampal area found in the experimental groups (Curcumin, Vitamin D3, and Donepezil) compared with the scopolamine group. Scopolamine group shows less number of nuclei stained with H and E staining. (Arrows in scopolamine slide B indicated the gaps around neuronal cells and in the slide) Coronal sections (5 µm) at magnification 40X.*

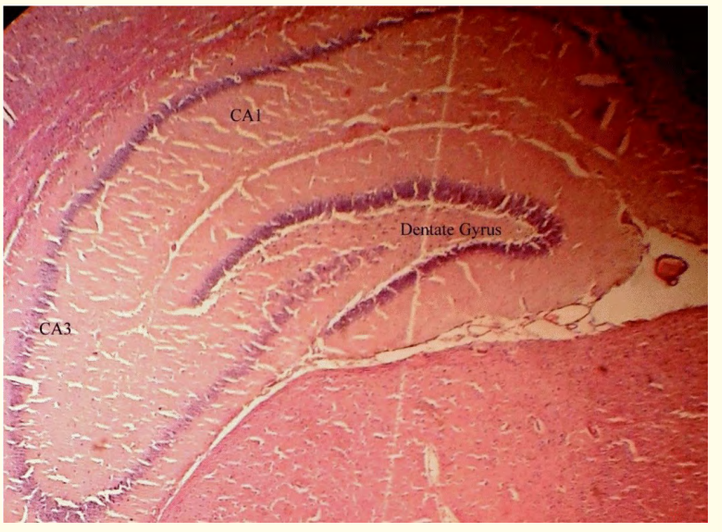

Figure 4: *Coronal section of rat brain (Control group) at 10X to represent important memory keeping regions in the hippocampus of the brain.*

Estimation of protein concentration

Immunoblotting

Curcumin and/or vitamin D3 reduce tau phosphorylation in the brains of a scopolamine-treated rat model of Alzheimer's disease. (a) Immunoblot of hippocampus homogenates from treated rats (scopolamine, vehicle, treated with Curcumin and vitamin D3) using the PHF monoclonal antibodies. Figure 6 corresponds to the densitometric analysis of the bands normalized against β-Actin.

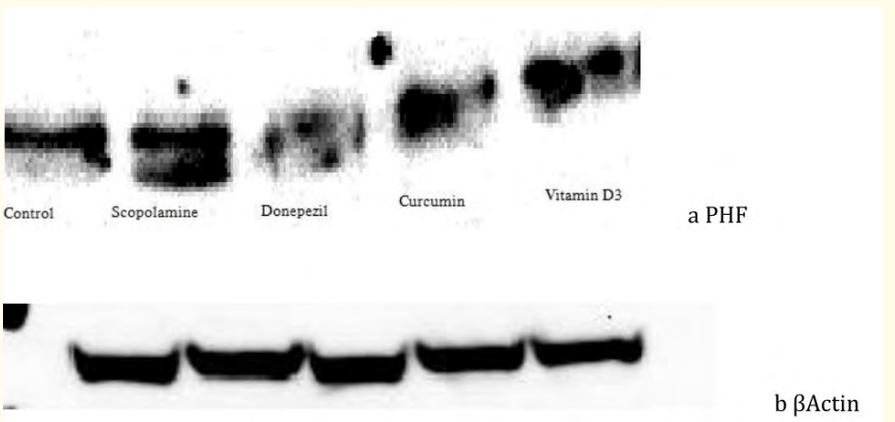

Figure 5: *Western blot image. From left to right (Control, scopolamine, Donepezil, Curcumin+ scopolamine, VitaminD3+scopolamine).*

S/no	Area	Percent	Relative density
1	13690.4	17.975	0.760653379
2	22076.4	28.986	1.484786395
3	16481.8	21.641	1.247176118
4	10292	13.513	0.493517403
5	13621	17.884	0.935649262

Table 4: *Densitometry data were obtained using image J software. The data represent the relative density of PHF in each of the samples presented in Figure 10.*

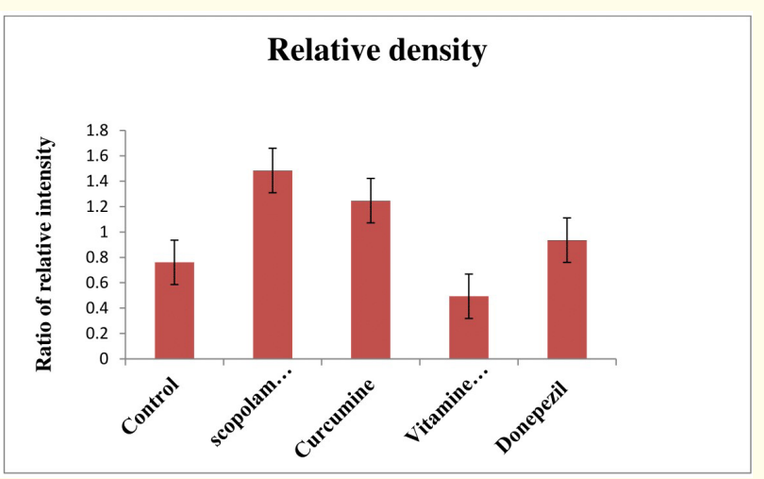

Figure 6: *Densitometry data obtained and presented as the relative density of the tau protein present in all groups. It was normalized to β actin protein.*

Discussion

In present study effect/s of Curcumin and/or vitamin D3 on an animal model of AD was investigated. Curcumin has been used as anti- inflammatory agent to relieve pain and inflammation in skin and muscles [14]. It is beneficial to improve behavioral disturbance in an animal model of AD [15]. Curcumin has proved significant improvement in behavioral symptoms when AD patients were given turmeric treatment [16]. It has demonstrated the ability to enter in the brain and reduced toxicity a study published in the journal of

Alzheimer's disease (News.vanderbilt.edu/2015/01/08/curcumin's-ability-to-fight-Alzheimer-studied/). While this news also published that use of aerosolized Curcumin is more beneficial to cortex and hippocampus than intravenous injection in a transgenic mouse model of Alzheimer's disease," Pham said. Similarly, Vitamin D has multiple biological targets can be used as an adjunct to standard anti-dementia treatments for corrections of neurological deficits in AD. From vitamin D, the most important compounds are vitamin D3 ,also it is known as cholecalciferol. Scopolamine is widely used to make an animal model of AD for screening anti-Alzheimer's drug [17]. Scopolamine acts as muscarinic cholinergic antagonist causes impairment in cognition and learning (Sunderland., *et al*. 1987). Scopolamine amnesia has shown there was here was a general decrease in the transfer latency in all experimental groups as compared to the scopolamine-treated group from the behavioral study. The memory loss effect of scopolamine is more prominent compared to the control group. The Curcumin and vitamin D3 treated group had almost equal performance compared with control group, which may indicative of the neuroprotective effect of Curcumin, against memory loss. To evaluate the effect of CNS drug such as scopolamine locomotor activity test was done on actophotometer which also indicates the improved learning ability in Curcumin and vitamin D3 treated groups (Figure 1). On the other hand, histological studies showed no significant difference in all treated groups except scopolamine treated group. Scopolamine group has shown degenerated cells observed in figure 4, there is a gap in the slide was seen when it is H and E stained. Scopolamine induced degeneration was clearly seen in previous studies [12]. While the Immunoblotting study of brain tissues using anti-human Paired Helical Filaments (PHF-Tau monoclonal antibody) (pdt.no.MN1020, thermoScientific) have demonstrated significantly presence of abnormal Tau protein in disease control group when compared with treated groups. And the presence of abnormal Tau protein could be the reason of memory loss event in rat brain observed during in behavioral study of different groups. Therefore, on the basis of behavioral improvement in treated animals and Immunoblotting results, we can be concluded that Curcumin and vitamin D3 may have potential to reverse some cognitive deficits and memory impairment induced by scopolamine.

Conclusion

In conclusion, effect/s of Curcumin and vitamin D3 on learning and memory on an animal model of AD has shown improvement. The use of Curcumin and vitamin D3 can slow down the progress of AD pathologies or delay the onset of AD. And therefore, could be used as a future drug for treatment of AD. It indicates that both agents could be beneficial for the betterment of AD patients. Vitamin D due to its multiple biological targets sides can be used as an adjunct to standard anti-dementia treatments for corrections of neurological deficits in AD. Curcumin has intensively used for the betterment of AD symptoms; inhalable curcumin and ar-turmerone on neural stem cells (NSCs) investigations are in pipeline. However, further investigations on both compounds are needed to be carried out.

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