



Experimental Evaluation of Vitamin - D Supplementation on Memory and Learning Using Different Animal Models in Albino Rat

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Authors' contributions

This work was carried out in collaboration among all authors. Author NRP carried out the experiments analyzed the data and wrote the manuscript. Author YSK supervised the experimental design and laboratory analysis. Author UAB corrected the manuscript. Author PD looked after all the animal-related help. All authors read and approved the final manuscript.

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ABSTRACT

Aim: We conducted this study with the aim to investigate the effect of vitamin D3 on spatial learning and memory in healthy young albino rat.

Study Design: Experimental evaluation.

Place and Duration of Study: Department of Pharmacology, Smt. Kashibai Navale Medical College and General Hospital (SKNMCGH), Pune, between October 2019 to February 2020.

Methodology: All the pharmacological experiments were conducted using Wistar albino rats (n=6), weighing between 100 g – 150 g. Total 18 animals (9 male and 9 female) were screened and marked into 3 different groups (6 in each group) Control (Normal saline 10 ml/kg), Vitamin D (Cholecalciferol 1000 IU/kg) and standard (Piracetam 200 mg/kg). Drugs were administered per os for 21 days. Elevated Plus Maze (Transfer Latency), Open Field Test (Rearing, Locomotion), Radial Arm Maze (Working and Reference memory) were used as amnesic models and their parameters for evaluation of this study.

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Results: After 21 days of treatment among all the three groups, Transfer Latency ($p=9.55$) in elevated plus maze, Working memory ($p=0.454$) and Reference memory ($p=0.929$) observed in radial 8 arm maze were non significant. In open field apparatus pellets count was significant (0.010), rest all parameters were non significant.

Conclusion: The result of study suggests that no significant beneficial effect of Vitamin D was seen on various learning models as assessed by Elevated Plus Maze, Radial Arm Maze, Open Field Test.

Keywords: Vitamin D; elevated plus maze; open field test; radial 8 arm maze; nootropic effect.

1. INTRODUCTION

Memory is a complex function of the brain and is considered as the ability to store, retain, recall information. It plays a critical role in “the process of thought” which is known as cognition. Learning is defined as the acquisition of information and skills, while subsequent retention of that information is called memory. Spatial memory is one of the crucial abilities for everyday living and survival that decline with aging, in rodents, non-human primates, and humans [1].

Research is going all over the world to explore the neurobiology of learning and memory and to investigate agents that prevent the progression of memory loss or to improve the existing capacity.

Recent studies also suggest that specific nutritional factors may be linked with brain function and as such with cognitive decline [2]. Since pharmacological treatment of cognitive decline and dementia is currently far from effective at the moment, knowledge on the effect of modifiable dietary factors may bring us a step closer toward preventive solutions. Vitamin D deficiency is one of the suggested modifiable factors for brain function [3].

Vitamin D a lipid-soluble vitamin, synthesized in our skin in the presence of sunlight. Vitamin D receptors are widespread in brain tissue, and vitamin D's biologically active form (1,25(OH)₂D₃) has shown neuroprotective effects including the clearance of amyloid plaques, a hallmark of Alzheimer's disease. Recent studies have confirmed an association between cognitive impairment, dementia, and vitamin D deficiency [4].

Currently, large numbers of mostly elderly individuals suffer from vitamin D deficiency due to the decreased cutaneous synthesis, and dietary intake of vitamin D. Therefore, preventing and/or treating vitamin D deficiency may be

relevant, easy, publicly accessible, and cost-effective strategy to improve long-term mental health [5]. Cognitive outcomes in vitamin D supplementation trials have been unclear, and this may be due to previous studies using cross-sectional designs or the use of a single short-term dose.

We conducted this study intending to investigate the effect of vitamin D₃ on spatial learning and memory in the healthy young albino rat.

2. METHODOLOGY

2.1 Experimental Animals

All the pharmacological experiments were conducted using Wistar albino rats ($n=6$), weighing between 100 g – 150 g. They were procured from the Central Animal Facility of Smt. Kashibai Navale Medical College, Pune. The animals were maintained under standard controlled environmental conditions. The care and use of laboratory animals were strictly by the guidelines prescribed by CPCSEA, India. The animals were shifted to the laboratory one hour before the experiment.

A total of 18 animals (9 male and 9 female) were screened and marked into 3 different groups (6 in each group) Control (normal saline 10 ml/kg body weight) [6], Vitamin D (Cholecalciferol 1000 IU/kg), and standard (Piracetam 200 mg/kg). Baseline readings were recorded in all animals. Drugs were dissolved in distilled water and administered per os for 21 days. Alternate day exploratory sessions of 3-5 mins for each animal on all the models were maintained. All the tests were repeated after 21 days

2.2 Models Employed for Evaluation of Memory Enhancing Activity in Rat

2.2.1 Elevated plus maze

The Elevated Plus-Maze (EPM) has been used to test anxiety and memory in rats and mice.

The plus-maze was made of dark Plexiglas and consist of two open arms (50 × 10 cm) and two enclosed arms (50 × 10 × 40 cm) arranged such that two open arms are opposite to each other. The arms are connected by a central platform (10 × 10 cm). The apparatus is shaped like a “plus” sign and is elevated to a height of 50 cm above the floor [7,8,9].

3 groups were taken for the study (each group contains 6 rats). The animals were placed individually on the maze at the end of the open arms facing away from the center and the time to move from the open arm to one of the closed arms was recorded as transfer latency (TL). After 21 days of drug administration, diazepam was administered on the 22nd day and transfer latency was measured again after 45 minutes.

A longer transfer latency (TL) period to reach the closed arm on the 22nd day indicated poor retention [1] which was suggestive of impairment of spatial memory.

2.2.2 Spontaneous locomotor activity using a digital photoactophotometer [10]

Spontaneous locomotor activity was evaluated by using a Digital Photoactophotometer, described by Dews P.B. (1953). Photoactophotometer is provided with a digital counter, photocell and a light source were used to measure locomotor activity (horizontal movement) of animals. The spontaneous locomotor activity of each rat was recorded individually for 10 minutes using the Digital Photoactophotometer. The basal activity score of all animals was recorded. Following test drugs administration, all animals were re-tested after 21 days for 10 mins.

Animal when exposed to a new environment (neophobia) have a natural tendency to get anxious so there is decreased locomotor activity or the animal stands still at one place but when an animal is exposed repeatedly for 21 days there is increased locomotor activity, this is suggestive of retention memory.

2.2.3 Radial arm maze [11,12]

The radial maze is the prototype of a “multiple- \square solution problem” task. The radial arm-maze used in the present study consists of eight arms, numbered from 1 to 8 (48 × 12 cm), extending radially from a central area (32 cm in diameter). The apparatus was placed 40 cm

above the floor and surrounded by various extra-maze visual cues placed at the same position during the study. At the end of each arm, there was a food cup that has a single 50 mg food pellet.

Before the actual training began, three or four rats were simultaneously placed in the radial maze and allowed to explore for 5 min and take food freely. The food was initially available throughout the maze but gradually restricted to the food cup. This was done for at least 4 days before taking the baseline readings. To evaluate the basal activity of rats in radial eight arm-maze, the rats were given one training trial per day to run to the end of the arms and consume the bait. The training trial continued until all the five baits had been consumed or until 5 min has elapsed.

21 days after the administration of the test drugs, each animal was placed individually in the center of the maze and subjected to working and reference memory tasks, in which the same 5 arms (no. 1, 2, 4, 5, and 7), were baited for each daily training trial. The other 3 arms (no. 3, 6, and 8) were never be baited. An arm entry was counted when all four limbs of the rat were within an arm. This calculation was made of the number of errors in working memory (entering an arm containing food, but entered previously), reference memory errors (entering an arm not baited)

Revisits are scored as errors (i.e., as failures to remember that an arm was already visited on that trial). Because the test measures trial-dependent memory, it is an assessment of working memory and, more specifically, spatial working memory because the principal cues to guide arm choices are outside the maze [13].

2.2.4 Open field experiment [14]

Open Field Habituation consists of exposing an animal to an open arena, a new environment without any aversive or appetitive stimuli, and let explore it freely for a fixed amount of time. The reduction in exploratory behaviors during re-exposures was interpreted in terms of remembering the characteristics of the environment. This test was used to examine short term spatial memory and/or long term spatial memory. The open field apparatus was designed as described by Gray and Lalji (1971) with few modifications. Dimensions were 60 cm x 60 cm x 40 cm made up of plywood open from

the top; the surface was divided into 25 equal squares i.e. 9 central and 16 peripheral.

The rodents were placed in the center of the open field for 5 min session. Evaluation was done on basis of the behavior of the animal as determined by Vertical activity, Center time, Corner time, ambulation (total number of squares entered), and defecation was recorded.

Rearings are innate exploratory postures of small rodents, and the newer the environment, the more rearings the animal will exhibit. Crossings also express exploration, of course, and have the advantage to measure basal motility. Normal memory retention is indicated by a reduction of the number of rearings and / or crossings; this reduction means that the animal has learned correctly the task; defecation boli, a more controvertible anxiety indicator, for which no agreement is achieved to this point [15].

3. RESULTS

3.1 Elevated Plus-Maze

Table 1 represents observations of transfer latency in control group (89.99 ± 31.15), piracetam (82.61± 21.05) and vitamin D (79.52 ± 21.07) with *p* value = 0.955, This difference was not statistically significant

3.2 Photoactometer

Table 2 represents locomotor activity score observed by digital photoactometer. There was no statistically significant difference observed among control group (181.2 ± 17.28), piracetam (192.5 ± 19.15) and vitamin D (157.7 ± 23.2) with *p* value = 0.473.

3.3 Radial 8 Arm

Table 3 represents working memory errors observed in control group (2.333 ± 0.5578), piracetam (2.333 ± 0.3333) and vitamin D (1.667 ± 0.3333) with *p* value =0.454.

Table 1. Observations in elevated plus maze considering the transfer latency

Groups	Mean ±SEM	P-value
Control	89.99 ± 31.15	0.955
Piracetam	82.61 ± 21.05	
Vitamin D	79.52 ± 21.07	

Each group consists of six animals. Values are mean ± SEM; **P* =0.05. Significance was determined by a One-way ANOVA test

While reference memory errors observed in control group (2.667 ± 0.6667), piracetam (2.5 ± 0.4282) and vitamin D (2.833 ± 0.7032) with *p* value =0.929, as observed there is no statistical difference among all groups.

Table 2. Locomotor activity score in photoactometer method

Groups	Mean ± SEM	P-value
Control	181.2 ± 17.28	0.473
Piracetam	192.5 ± 19.15	
Vitamin D	157.7 ± 23.2	

Each group consists of six animals. Values are mean ± SEM; **P* =0.05.

Significance was determined by One-way ANOVA test

3.4 Open Field Apparatus

Table 4 represents various observations of open field method ,Vertical activity in control group (7.667 ± 0.6667) piracetam (7.833 ± 0.8333) and vitamin D (8.167 ± 0.6009) with *p* value = 0.879, Corner time in control group (1.275 ± 0.06292) piracetam (1.188 ± 0.1573) and vitamin D (1.53 ± 0.1915) with *p* value =0.266, Center time in control group (3.725 ± 0.06291) piracetam (3.812 ± 0.1573) and vitamin D (3.47 ± 0.1915) with *p* value = 0.266 , Total distance in control group (88.33 ± 4.264) piracetam (83.33 ± 3.947) and vitamin D (85.67 ± 4.828) with *p* value = 0.725, number of pellets or boli in control group (4.5 ± 0.7638) piracetam (2.667 ± 0.7149) and vitamin D (1.167 ± 0.4773) with *p* value = 0.010* which is statistically significant.

Table 3. Observations in RADIAL 8 Arm

GROUP	WORKING		REFERENCE	
	Mean ± SEM	P value	Mean ±SEM	P-value
Control	2.333 ± 0.5578	0.454	2.667 ± 0.6667	0.929
Piracetam	2.333 ± 0.3333		2.5 ± 0.4282	
Vitamin D	1.667 ± 0.3333		2.833 ± 0.7032	

Each group consists of six animals. Values are mean ± SEM; **P* =0.05. Significance was determined by One-way ANOVA test

Table 4. Mean score using open field performance method

Parameters	Groups	Mean \pm SEM	P-value
VERTICAL ACTIVITY	Control	7.667 \pm 0.6667	0.879
	Piracetam	7.833 \pm 0.8333	
	Vitamin D	8.167 \pm 0.6009	
CORNER TIME	Control	1.275 \pm 0.06292	0.266
	Piracetam	1.188 \pm 0.1573	
	Vitamin D	1.53 \pm 0.1915	
CENTER TIME	Control	3.725 \pm 0.06291	0.266
	Piracetam	3.812 \pm 0.1573	
	Vitamin D	3.47 \pm 0.1915	
TOTAL DISTANCE	Control	88.33 \pm 4.264	0.725
	Piracetam	83.33 \pm 3.947	
	Vitamin D	85.67 \pm 4.828	
PELLETS	Control	4.5 \pm 0.7638	0.010*
	Piracetam	2.667 \pm 0.7149	
	Vitamin D	1.167 \pm 0.4773	

Each group consists of six animals. Values are mean \pm SEM; *P = .05. Significance was determined by One-way ANOVA test

4. DISCUSSION

Cognitive impairment and dementia are significant public health problems, especially with aging [16].

EPM and the radial arm maze model a widely accepted paradigm to study learning and memory processes in rodents. The radial arm maze helps to determine spatial reference and working memory processes in the rat. Reference memory was regarded as a long-term memory for information that remains constant over repeated trials (memory for the positions of baited arms), whereas working memory was considered a short-time memory in which the information to be remembered changes in every trial (memory for the positions of arms that has already been visited in each trial. The problem with RAM is that animals may solve the maze in ways other than relying on spatial working memory. use of chaining or a serial strategy (i.e., entering each arm successively in a systematic order) [13]. In our study pre-treatment with Vitamin D for 21 days did not protect the animals from memory deficits produced by diazepam in EPM Model as well as no change in reference and working memory in the radial arm maze.

The results we observed are consistent with Brouwer-Brolsma EM et al. [5] who studied the effects of AVD status in healthy, adult male C57BL/6J mice. After 10 weeks on diets that varied in vitaminD3 (deficient, control, elevated), serum levels of 25(OH)D3 were altered, but this was not associated with alterations in learning

and memory or neurochemical processes in the hippocampus.

Numerous studies showed no effect of vitamin D status on several behavioral domains in the EPM (Elevated Plus Maze), HPT (Hot Plate Test), or the FST (Forced Swim Test) that may affect performance on APA (Active Place Avoidance [16]. Although spontaneous locomotion in a novel open field has been observed in some rodent models of developmental and adult vitamin D deficiency [17,18] the effect is not consistent between studies [19,5].

By contrast, few studies demonstrated improvement in learning and memory with supplementation of vitamin D in rodents. This disparity in results can be due to considerable variation in the duration of vitamin D exposure, administration of vitamin D, levels of dietary vitamin D, age of rodents, rodent strain, behavioral tests used, and brain regions tested.

In one study, AVD (Adult Vitamin D) deficient BALB/c mice exhibited an imbalance between excitatory and inhibitory neurotransmitters, showing the reduction in glutamate, glutamine, and GAD65/67 levels and elevations in GABA. In rats, vitamin D treatment resulted in elevated dopamine in the brain stem and homovanillic acid in the hypothalamus and striatum [20].

This neurotransmitter change may affect the behavioral status of animal-like anxiety and depression which can affect results in various memory models.

The other important factor which gives different results is the age of the animal. The age of the behavioral assessment varied from 3 to 20 months, and the duration of the intervention varied from 2 to 20 weeks. Numerous studies have shown that there is a more pronounced effect of vitamin D supplementation on cognitive function in aged mice. [21,22] and only a subtle effect in young, healthy adult mice [23]. Furthermore, there has been limited evidence to suggest that AVD-deficiency significantly affects spatial performance and neurochemistry [5,19] in young, healthy rodents. In our study, we used 3-month-old rats. This can be one of the reasons for the negative results observed in our study.

Animal models do not mimic the most common clinical condition. In vitamin D deficiency usually slowly develops over time, mainly because the ability of the human skin to synthesize vitamin D under the influence of ultraviolet-B light exposure decreases while aging. Thus, like age-related depression and cognitive decline, vitamin D deficiency in humans often just becomes manifest at an older age. Which is not in the case of animals.

There are few human studies which concluded that vitamin d supplementation improves memory but there is no proven mechanism of the same so we planned to find out if we get the same results in animals.

As shown in Table 2 there was no change in locomotor activity amongst all the groups. These results are inconsistent with observations in other tests where vitamin D did not alter any effect on other behavioral models for the given dose and duration in our case.

This study demonstrated that Vit D had no significant nootropic effect. The only change is seen in Pellet count is changed in both Vit D and Piracetam group. This may be related to the gastrointestinal effect of drugs /incomplete absorption and digestion of drugs.

5. LIMITATION

Animal models are not reliable or inconclusive and many environmental factor timing, starving, noise can affect results. While learning the RAM rats learn both spatial and associative aspects of the task because of the internal structure of the maze and olfactory cues, thus learning is slower as more information is available, longer learning times, and nonspatial learning components lead

to the interpretation of RAM data challenging [13]. Animals aging factor should be considered, the results may be different with aged rats. Estimation of vitamin D level was not done in our study. We had given drugs for 21 days, further experiment required with a longer duration of treatment

6. CONCLUSION

According to literature there were controversial results reported of Vitamin D on learning and memory in both animal and humans. The future perspective of this study was to find out mechanism of Vitamin D in improvement of memory. As the current study did not show favourable improvement in spatial learning and memory in healthy young albino rat as assessed by Elevated Plus Maze, Radial Arm Maze, Open Field Test after administration of vitamin D by oral route for 21 days, hence more similar studies need to be conducted with modifications with respect to species, age, dose, duration etc.

CONSENT

Not Applicable

ETHICAL APPROVAL

All experiments have been examined and approved by the institutional animal ethics committee (SKNMC&GH/IAEC/APP/2019/17).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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