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Possible protective effect of vitamin D on age related cognitive impairment and synaptic dysplasticity in rats

Mona K.Alawam[1], Wessam A.Abouhammad[1*], Mona M.Allam[1] , Ahmed I.Agamy[1], Hala M.Anwer[1]

[1]Department of Physiology, Faculty of Medicine, Benha University, Qalubiya, Egypt.

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- Brain aging
- Amyloid β
- Vitamin D, oxidative stress
- Synaptic plasticity

Abstract

Background: Brain agingis a natural process that affects all populations, leading to declines in both cognitive and biological functions. Consequently, promoting healthy brain aging has become a major public health priority. Vitamin D (Vit.D), a crucial hormone involved in calcium regulation, plays diverse roles across various tissues, including the brain, and has been implicated in mitigating age-related cognitive decline.**Aim:** To investigate the possible neuroprotective effects of Vitamin D in alleviating cognitive dysfunction in aged rat models.**Method:** Fortymale albino rats were divided into 5 groups consisting of 8 rats in each group; control,D-galactose induced brain aging group,Vit.D100,1000,10000 IU/kg groups respectively. Upon aging induction with D-galactose, Vitamin D therapy was sustained for eight weeks, and cognitive function was measured through the Novel Object Recognition Test (NORT) and the Barnes test. At the study's endpoint,biochemical markers were measured;amyloid β (Aβ), MDA and GSHin hippocampal tissue.**Results**: Rats that received Vit.Dat doses 1000 &10000 IU/Kg showed significant decrease in Aβ level and oxidative stress state and also showed improvement in memory impairment that was confirmed by histopathological examination of hippocampal tissue and rats'performance in memory tasks. The same doses of Vit.D also improved synaptic plasticity which was confirmed by immunohistochemistry staining of synaptic proteins; post synaptic density protein 95 (PSD-95) and synaptophysin. **Conclusion**: Vit. D administration in high doses has a neuroprotective effect on cognitive impairment in aged rats throughan antioxidant mechanism andby modulating synaptic plasticity.

Corresponding author: Wessam Abdelsamea Abouhammd, Physiology Department, Faculty of Medicine, Benha University, Qalubiya, Egypt., E. Mail: wesam.abdallah.fmed.bu.edu.eg. Mobile: 002 01276415202

Introduction

Aging is a naturally occurring, multifaceted process marked by structural and functional decline in organisms, progressing spontaneously and inevitably over time[1]**.** As aging progresses, diminished internal homeostasis and decreased stress tolerance can increase susceptibility to various illnesses, especially degenerative diseases affecting the central nervous system[2].

Cognitive decline accompanies brain aging, manifesting as impairments in attention span, information processing, working memory, and both short- and long-term memory retention[3].Dementia represents the foremost cause of disability in aging populations, highlighting the significance of supporting brain health to lessen the impact of age-related disabilities [4].

While the precise pathological mechanisms contributing to cognitive decline in aging remain uncertain, oxidative stress has been identified as a key factor[5]**.**With advancing age, oxygen free radical metabolism becomes dysregulated[6]**.** As a consequence, oxidative free radicals damage cellular membranes, leading to lipid peroxidation, a rise in malondialdehyde (MDA) levels, and a concurrent decline in glutathione (GSH) activity(6)**.**The brain's susceptibility to oxidative injury stems from its elevated oxygen consumption, insufficient antioxidant defenses, and high levels of unsaturated lipids[7].

Excessive D-galactose (D-gal), a naturally occurring reducing sugar, leads to the accumulation of galactitol, reactive oxygen species (ROS), and osmotic stress, thereby accelerating the aging process. As for brain tissues, D-gal accumulation causes neurotoxicity followed by cognitive deficits [8]**.**Given its established effects, chronic D-gal exposure has been widely recognized as a reliable model for examining aging-associated neurodegeneration [9].

Vitamin D has been recognized for its crucial role in supporting memory and cognitive function [10]**.** Inadequate Vitamin D levels, especially in older adults, are associated with declines in learning ability, memory retention, and overall cognitive performance[11]**.** By influencing neurotrophin and neuromediator synthesis, reducing neuroinflammatory processes, and counteracting oxidative damage, Vit. D exerts a vital neuroprotective function in the brain [12]**.** Insufficient levels of vitamin D have been reported to attenuate the activity of critical antioxidant enzymes, including GSH peroxidase and superoxide dismutase (SOD), which play a pivotal role in mitigating oxidative stress [13]. Alternatively, Vitamin D has been found to enhance the phagocytic function of microglia and macrophages, aiding in the clearance of amyloid plaques [14]**.**

There is a strong correlation between degenerative changes in the brain with aging and Amyloid beta (Aβ) plaques [15]. Aβ can be produced from the Amyloid Precursor Protein (APP) which is a glycoprotein that plays a significant role in maintaining neuronal homeostasis like signaling, neuronal development, and intracellular transport. In normal brain physiology, APP undergoes cleavage by beta- and gamma-secretase

enzymes, generating soluble APP fragments that pose no harm to neurons, are secreted extracellularly, and are rapidly degraded[16]**.** There are many evidences that the synthesis and accumulation of Aβ causes neuroinflammation, neurodegeneration, and memory loss [17]**.**

Synapses are the fundamental units of information transfer and memory storage in the brain [18]. Plasticity of synaptic structure and function plays an essential role in neuronal development, cognitive functions, and degenerative diseases [19]. The synaptophysin protein is one of the most abundant synaptic vesicle proteins.It is critically involved in the generation of synaptic vesicles, the establishment of fusion pores between vesicles and the plasma membrane, and the processes of endocytosis and synaptic vesicle recycling[20]. The postsynaptic density (PSD) comprises a diverse array of scaffolding proteins, receptors, and signaling molecules positioned adjacent to the postsynaptic membrane. PSD proteins are fundamental to synaptic plasticity[21]. PSD-95 is the most important and abundant anchoring and scaffolding synaptic protein on the postsynaptic membrane.It orchestrates synaptic transmission, modulating their strength and plasticity [22]. Deficiency in synaptophysin and PSD-95 could disrupt hippocampal connectivity, potentially impairing cognitive processes such as memory and learning [23].

This study aims to determine the neuroprotective effects of Vit. D in preventing cognitive deficits in aged rats and to explore its potential contribution to synaptic plasticity enhancement.

2. Materials and Methods:

This investigation was carried out at the Physiology Department, Faculty of Medicine, Benha University, adhering to ethical animal research guidelines, with authorization from the Ethical Research Committee, Faculty of Medicine, Benha University (Approval No. MD12.11.2022).

2.1. Animals:

A cohort of forty adult male albino rats was acquired from the Faculty of Agriculture, Moshtohor, Egypt, for this trial. Rats aged 8-10 weeks and weighing 200 ± 10 g. Five groups, each consisting of eight rats, were formed, with four rats accommodated separately in each cage. Rats were accommodated in cages set at 25°C, with an alternating 12-hour light and dark cycle. The animalswere fed a standard diet and had free access to water ad libitum. After study completion animals were disposed ofat the incinerator of the Benha University Hospital.

2.2. Drugs and chemicals:

A white powdered form of D-gal, with a purity greater than 99%, was procured from LOBA Chemie Pvt. Ltd., Mumbai, India.Vit. D was available in the form of cholecalciferol injection (Memphis Pharmaceutical & Chemical Industry). Each ampoule of 2 ml contains: cholecalciferol (Vitamin D3) 5mg (equivalent to 200.000 I. U). It was diluted in 18 ml. saline [24][14] to get a solution with a concentration of 10000 IU/ml then further diluted to get other concentrations 1000 IU/ml and 100 IU/ml.

2.3. Study design:

After 10 days acclimatization period based on laboratory settings, the rats were stratified into five distinct groups, each consisting of eight animals (n = 8 per group), as follows:

1) **Group (I):** (**control group)** all the control rats were given (normal saline **1 ml/kg/day)** by intraperitoneal injection (IP) for 8 weeks
2) **Group (II):** (**D-gal injected group**): D-gal was administered to the rats in this group through intraperitoneal injection at a dose of 120 mg/kg/day for eight weeks[10].A total of 12 grams of D-galactose was dissolved in 100 mL of saline to prepare a solution with a final concentration of 120 mg/mL. Then the rats were given 1 ml (120 mg)/kg/day.
3) **Group (III):Injected D-gal as in group II +1 ml of vit. D100 IU/kg/day** by IP injection for 8 weeks [25][14].
4) **Group (IV): Injected D-gal as in group II + 1 ml of vit. D1000 IU/kg/day** by IP for 8 weeks[25][14].
5) **Group (V): Injected D-gal as in group II +1 ml of vit. D10000 IU/kg/day** by IP for 8 weeks[25][14][26]**.**

2.4. Behavioral tests for Memory assessment:

2.4.1. Novel Object Recognition Test (NORT)[27]

- **Purpose:** Evaluates recognition memory based on rats' natural preference to explore novel objects over familiar ones.
- **Test Arena:**

An open box (square area with high surrounding walls).

Procedure: (Figure 1)

a-During habituation phase b-During sample phase c-During choice phase

Figure 1 Novel object recognition test (NORT): Rat in the open box

- **HabituationPhase:**

Rats explored the open box for 5 minutes/day over 5 days to become familiar with the arena.

- **Sample Phase:**

Twenty-four hours after habituation, rats were exposed for 3 minutes to two identical objects. Their exploration was video recorded, then the arena and objects were cleaned with 70% alcohol to remove olfactory cues.

- **Choice Phase:**

Following a fifteen-minute interval, the rats were returned to the arena, which contained a familiar object alongside a novel object that varied in both shape and color. Exploration behavior was recorded for 3 minutes.

Measurements:

- **Exploration Time:** Time spent exploring each object (nose within 2 cm or touching counts as exploration) [28].
- **Recognition Index (RI):** Calculated as (time spent with the novel object / total exploration time) × 100.
- A minimum exploration time of 20 seconds is required for inclusion in the analysis.

2.4.2. Barnes Maze Test [29]

- **Purpose:**

  Assesses spatial memory by exploiting rats' aversion to bright lights and open spaces.
- **Maze Description:(Figure 2)**
- A 100 cm-wide raised circular platform was utilized, featuring 20 holes, each 5 cm in diameter, positioned around its perimeter. An escape box is positioned under a designated target hole.
- **Procedure:(Figure 3)**

- **Habituation Phase:**

  Rats were placed in the escape box for 1 minute, then allowed to explore the maze until they enter the escape tunnel or 5 minutes pass.

- **Training Phase:**

Conducted over 4 days with one acquisition trial per day (3 minutes per trial). Rats started from different quadrants (using a start chamber for 10 seconds before release), and the escape latency (time taken to reach the escape tunnel) was recorded.

- **Probe Test:**

On the day after the last training session, the escape tunnel was removed for a 3-minute trial. Starting from the center, the rats' time in the target quadrant was recorded as an indicator of reference memory.

- **Post-Trial Cleaning:**

The maze and escape box are cleaned with 70% alcohol after each session to eliminate olfactory cues.

- **Measurements:**

- **Escape Latency Time:** Time taken by the rat to reach the escape tunnel.
- **Time in Target Quadrant:** Used during the probe test to evaluate spatial memory.

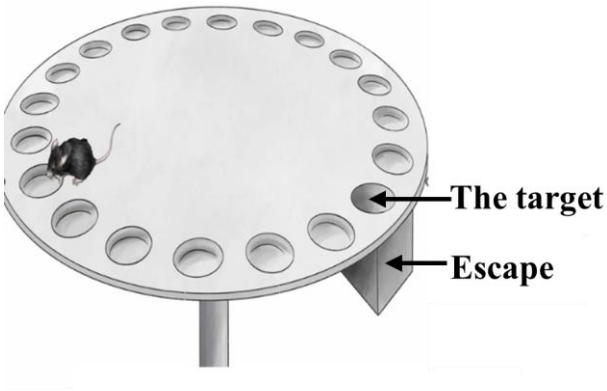

Figure 2: Barnes maze

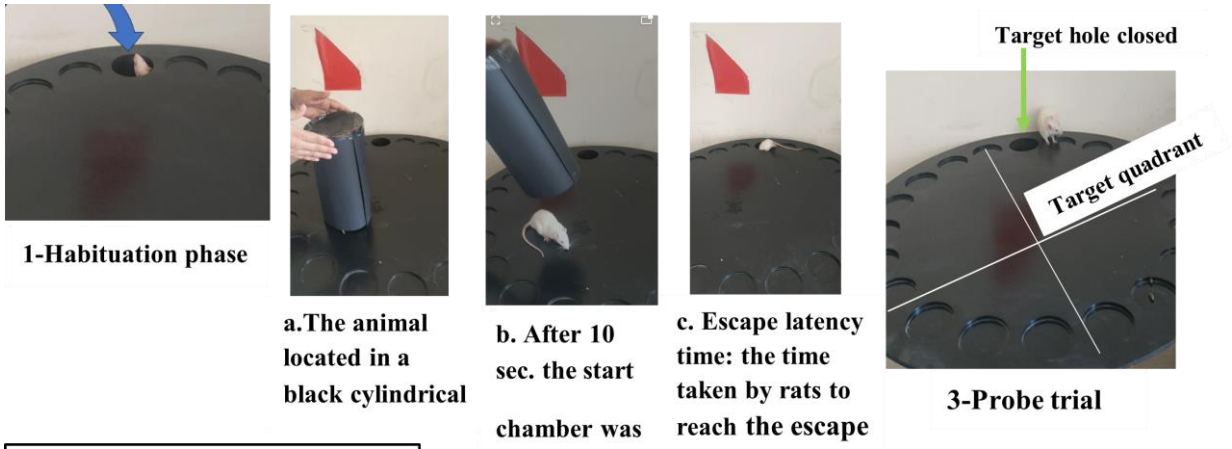

Figure 3:Barnes maze test

2.5. Tissue sampling

Following the completion of behavioral assessments, the rats were euthanized via decapitation after receiving an intraperitoneal injection of thiopental sodium (40 mg/kg). Their brains were promptly extracted, and the hippocampi were dissected according to the protocol outlined by Spijker[30].Each hippocampus was dissected into two halves, one half stored at −80°C for later biochemical analysis andthe other half was preserved in 10% buffered formalin (pH 7.8) for histopathological and immunohistochemical analysis.

2.6. Biochemical analysis

- Enzyme-linked immunosorbent assay (ELISA) was employed to assess the following parameters in hippocampal tissues, adhering to the manufacturer's guidelines.
- Amyloid beta: Using kits supplied by (Kamiya Biomedical company Cambridge Science Park, Cambridge, UK).
- The oxidative stress markers: MDA indicating lipid peroxidation was determined using MDA colorimetric kits, provided by (Abcam biochemicals, Cambridge Science Park, Cambridge, UK and GSH was determined using GSH colorimetric kit (Kamilya Biomedical Company Seattle, U.S.A).

2.7. Histopathological examination.

Hippocampi samples were fixed in 10% formalin, dehydrated, and embedded in paraffin, and 5-µm-thick sections were cut with a microtome, mounted on glass slides, and stained with hematoxylin and eosin (H&E) for examination by light microscopy. Using H&E staining, neuronal injury in the hippocampus was systematically assessed based on a predefined scale: level 0 (absence of damage), level 0.5 (<10% neuronal damage), level 1.0 (10%–30%, indicating mild damage), level 1.5 (30%–50%, classified as mild-to-moderate damage), level 2.0 (50%–70%, denoting moderate damage), level 2.5 (70%–90%, indicative of moderate-to-severe damage), and level 3.0 (>90%, severe neuronal injury) [31].

2.8. Immunohistochemistry examination of hippocampal synaptophysin and PSD-95:

Paraffin-embedded sections were deparaffinized in xylene and rehydrated through graded ethyl alcohol (100%, 95%, 70%), followed by antigen retrieval using the citrate antigen retrieval Vectastain ABC kit and quenching of endogenous peroxidase with 3% H_2O_2. The sections were then incubated overnight with primary polyclonal antibodies for PSD-95 and synaptophysin (Abcam, Cambridge, UK) at a 1:100 dilution in PBS, followed by a 30-minute application of biotinylated secondary antibody and an additional 30 minutes with a preformed avidin-biotinylated horseradish peroxidase complex. Visualization was achieved by a 5-minute incubation in 3,3'-diaminobenzidine solution and counterstaining with hematoxylin[32]. The expression of synaptophysin and PSD-95 was assessed based on staining intensity 0 (no staining) , + (weak staining), ++ (medium staining), +++ (strong staining) and ++++ (very strong staining)(32).While the percentage of stained cells, categorized as (+) 1–10%, (++) 11–50%, (+++) 50–90%, and (++++) >90% [33][32][33].

2.9. Statistical analysis.

Statistical Package for Social Sciences version 20 (SPSS Inc, Chicago, IL, USA) was used toanalyse all data, which were demonstrated as the mean ± standard deviation (SD). Intergroup differences were assessed using one-way ANOVA, followed by the Least Significant Difference (LSD) test for pairwise comparisons. A p-value of less than 0.05 was considered statistically significant. Pearson's correlation coefficient (r) was applied to evaluate correlations, using a two-tailed test with a predetermined significance threshold of $p < 0.05$.

3.Results

3.1. Effect of vit. D on rat performance in behavioural tests:

Relative to control group, rats exposed to D-gal for 8 weeks demonstrated a notable decline in RI and target quadrant time ($p < 0.01$), alongside a substantial increase in escape latency ($p < 0.01$). Administration of Vit. Dat a dose 100 IU/kg for eight weeksin group III resulted in a non-significant change regarding RI, time spent in the target quadrant and escape latency times.

In contrast, eight weeks of Vitamin D supplementation (1000 IU/kg) in Group IV resulted in a notable improvement in RI and target quadrant time, alongside a significant reduction in escape latency ($p < 0.01$) relative to D-gal group. Interestingly, administration of vit. Dat a dose 10000 IU/kg for eight weeks in (group V) resulted in the same results as administration of vit. D in a dose of 1000 IU/kg for eight weeks with no substantial difference between the effect of the two doses (**Table 1**).

3.2. Effect of vit. D on oxidative stress and amyloid beta:

In rats administered D-gal for eight weeks (Group II), tissue levels of Aβ and MDA showed a significant increase ($p < 0.01$), whereas GSH levels were markedly reduced ($p < 0.01$) relative to control group. Vitamin D at a dose of 100 IU/kg for eight weeks (Group III) did not induce significant changes in

hippocampal Aβ, MDA, or GSH levels. Conversely, an eight-week regimen of Vit. D at 1000 IU/kg (Group IV) was associated with a substantial decline in Aβ and MDA levels, while GSH levels were significantly elevated ($p < 0.01$) compared to the D-gal group. Notably, Vitamin D supplementation at a higher dose of 10,000 IU/kg for eight weeks (Group V) produced results comparable to those observed with the 1000 IU/kg dose, with no substantial variations between the two treatment regimens (**Table 2**).

Table (1): Effect of vit. D treatment on behavioral test results amongst the study groups.

		Group I (Control)	**Group II (D-gal)**	**Group III (D-gal + Vit. D 100 IU**	**Group IV (D-gal + Vit. D 1000 IU**	**Group V (D-gal + Vit. D 10000 IU**
Barnes test results	Escape latency time Day 1 (seconds)	29.5±3.58	37.38±4.13	35.13±3.39	33.25±3.61	30.75±2.12
	Escape latency time Day 2 (seconds)	20.75±4.7	35.38±5.42	33.38±5.44	28.63±2.66	26.5±5.78
	Escape latency time Day 3 (seconds)	14.38±2.56	33±2.97 *	31.38±3.85 *	22.63±6.76 * + #	20.63±6.94 * + #
	Escape latency time Day 4 (seconds)	8.63±1.84	29.75±2.25 *	30.25±3.95 *	13.63±3.11 * + #	11.63±1.59 * + #
	Time spent in target quadrant (seconds)	43±5.65	9.5±1.6 *	10.37±3.24 *	28.5±3.07 * + #	30.62±5.44 * + #
NORT results	RI(%)	74.09±5.74	20.10 ± 3.97 *	23.54±8.67 *	59.58±11.33 * + #	64.65±7.22 * + #

RI: Recognition index, Data are displayed as mean ± SD (n = 8). Statistical analysis was performed using one-way ANOVA followed by the LSD post hoc multiple comparisons test, with significance set at $P < 0.01$. Comparisons include $P < 0.01$ vs. control group, $+P < 0.01$ vs. D-gal group, and $\#P < 0.01$ vs. D-gal + Vit. D 100 group.

Table (2): Effect of vit. D treatment on hippocampal amyloid beta level and oxidative stress amongst the study groups:

	Group I (Control)	**Group II (D-gal)**	**Group III (D-gal + Vit. D 100 IU**	**Group IV (D-gal + Vit. D 1000 IU**	**Group V (D-gal + Vit. D 10000 IU**
Amyloid beta (pg/mg protein),	3.68±0.82	16.87±2.49 *	15.67±1.97 *	7.23±1.19 * + #	5.67±1.20 * + #
MDA (nmol/mg protein)	3.46±1.21	10.57±1.95 *	9.43±1.34 *	5.96±1.35 * + #	5.10±0.97 * + #
GSH (nmol/mg protein)	1.56±0.40	0.40±0.19 *	0.53±0.24 *	1.17±0.31 * + #	1.22±0.27 * + #

MDA: Malondialdehyde, GSH: Glutathione, mean ± SD values are used to represent the data (n = 8). Statistical significance ($P < 0.01$) was assessed through one-way ANOVA, followed by LSD post hoc multiple comparisons test. Statistical differences were observed as follows: $P < 0.01$ vs. the control group, $+P < 0.01$ vs. the D-gal group, and $\#P < 0.01$ vs. the D-gal + Vit. D 100 group.

3.3. Effects of vit. D on histopathological changes of hippocampal tissue.

The neurons in group I (control group) were arranged neatly with no degeneration observed and the pyramidal neurons exhibited a regular arrangement with intact nuclei (Figure4a).While in group II (D-gal group) there was shrunken neuronal cell size with densehyperchromatic nuclei, decreased thickness of pyramidal layer of

hippocampusand each neuron has acquired clear space around itself due to retraction of the cell body(Figure 4.b). Treatment with vit. D (100 IU/kg) in group III showed no improvement in the pathological changes of group II (Figure4.c). In Groups IV and V, administration of Vitamin D (1000 and 10000 IU/kg) resulted in pathological amelioration, characterized by a higher count of intact neuronal cells, a reduction in perineuronal clear spaces—indicative of decreased neuronal necrosis—and greater pyramidal layer thickness in the hippocampus (Figure 4d & e). Neuronal damage in the hippocampus was measured and categorized using a damage score system ranging from 0 to 3. The results showed that after injection of D-gal in group II there was a notableelevation ($p < 0.01$) in the neuronal damage score compared to control group. However, with vit. D treatmentat a dose 1000 & 10000 IU/kg, hippocampal damage score significantly decreased compared to the aging group II group with no significant difference between the effect of the two doses **(Figure7).**

3.4. Effects of vit. D on synaptic proteins (synaptophysin and psd95 immunohistochemical staining of hippocampal tissue.

Hippocampal sections from the control group exhibited strong immunohistochemical staining for synaptophysin and PSD-95, indicating robust expression in hippocampal cells (Figures 5.a & 6.a). In contrast, the rats injected with D-gal for eight weeks showed notable decrease ($P < 0.01$) in the density of stained cells (weak staining) and this indicated loss of synaptic plasticity in aging rats(Figures5.b,6.b& 8).In Group III, Vitamin D administration at 100 IU/kg for eight weeks did not lead to a significant alteration in stain density (Figures 5.c, 6.c & 8). Conversely, treatment with higher doses (1000 and 10000 IU/kg) for the same duration resulted in a significant elevation in stain density ($P < 0.01$) when compared to aging rats in Group II. Interestingly, the two higher doses exhibited comparable effects, with no substantial variation between them (Figures 5.d, e, 6.d, e & 8) (Table 3)

Table (3): The correlation between RI, synaptophysin, PSD-95 and Amyloid beta

Parameter	**RI**	
	Rs	**P value**
Amyloid Beta.	**-0.918***	**P < 0.01**
PSD-95	**0.862***	**P < 0.01**
Synaptophysin	**0.899 ***	**P < 0.01**

Rs= rs for spearman correlation coefficient*
Correlation is significant at the 0.01 level (2-tailed).

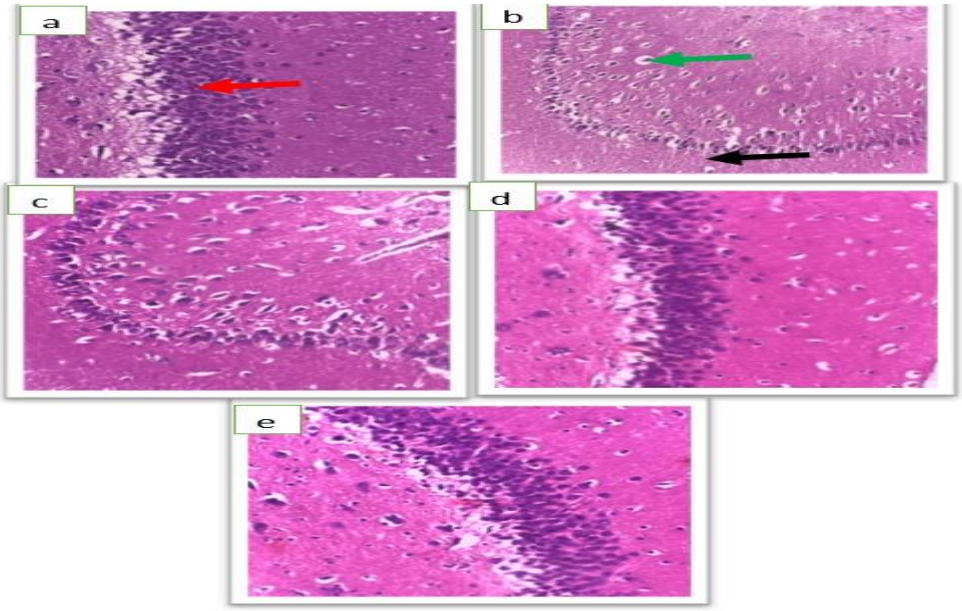

Figure 4. light microscopic representative pictures of H&E-stained hippocampal tissues in the 5 studied groups from (a-e) magnification X400).

(a)Group I (Control group) neurons arranged neatly with no degeneration observed. (b)Group II (D-gal group) showing decreased thickness of pyramidal layer of hippocampus (black arrow) with clear spaces around neurons (green arrow). (c) Group III (D-gal + Vit. D100) showing no improvement of pathological changes in group II. (d) Group IV (D-gal + Vit. D1000) showing improvement of pathological changes (e)Group V (D-gal + Vit. D10000) showing more improvement of pathological changes.

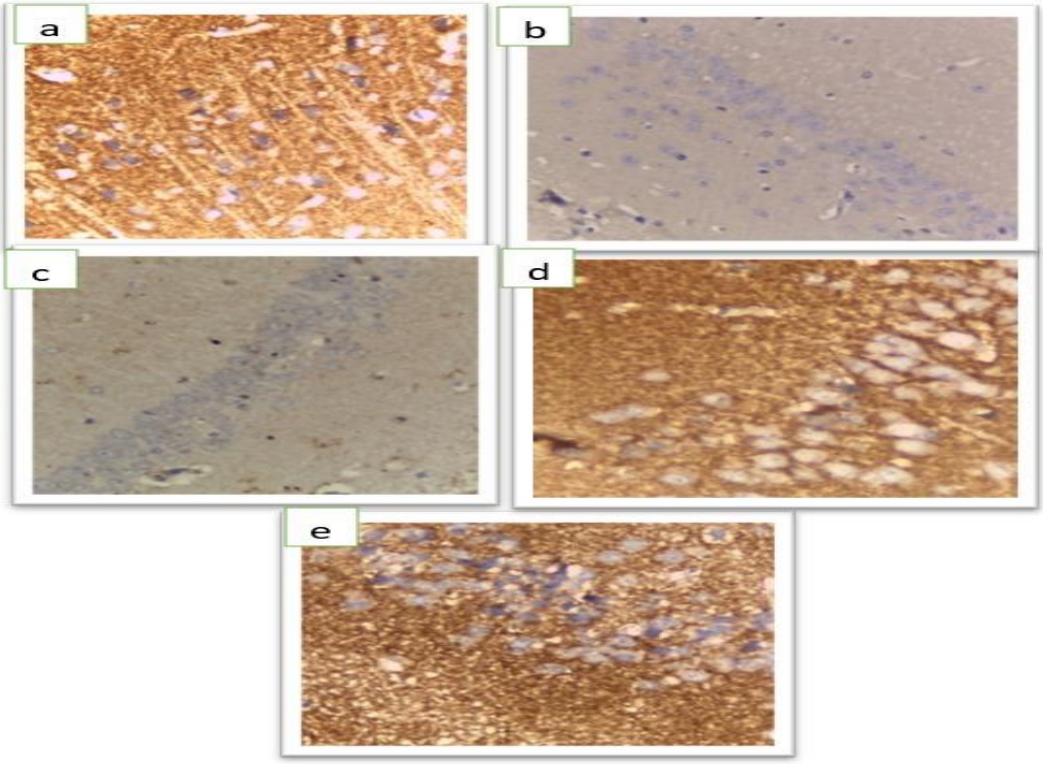

Figure.5 immunohistochemical staining of hippocampal tissue of synaptophysin marker

(a) Group I (Control group), (b)Group II (D-gal group), (c) Group III (D-gal + Vit. D100), (d) Group IV (D-gal + Vit. D1000), (e)Group V (D-gal + Vit. D10000).

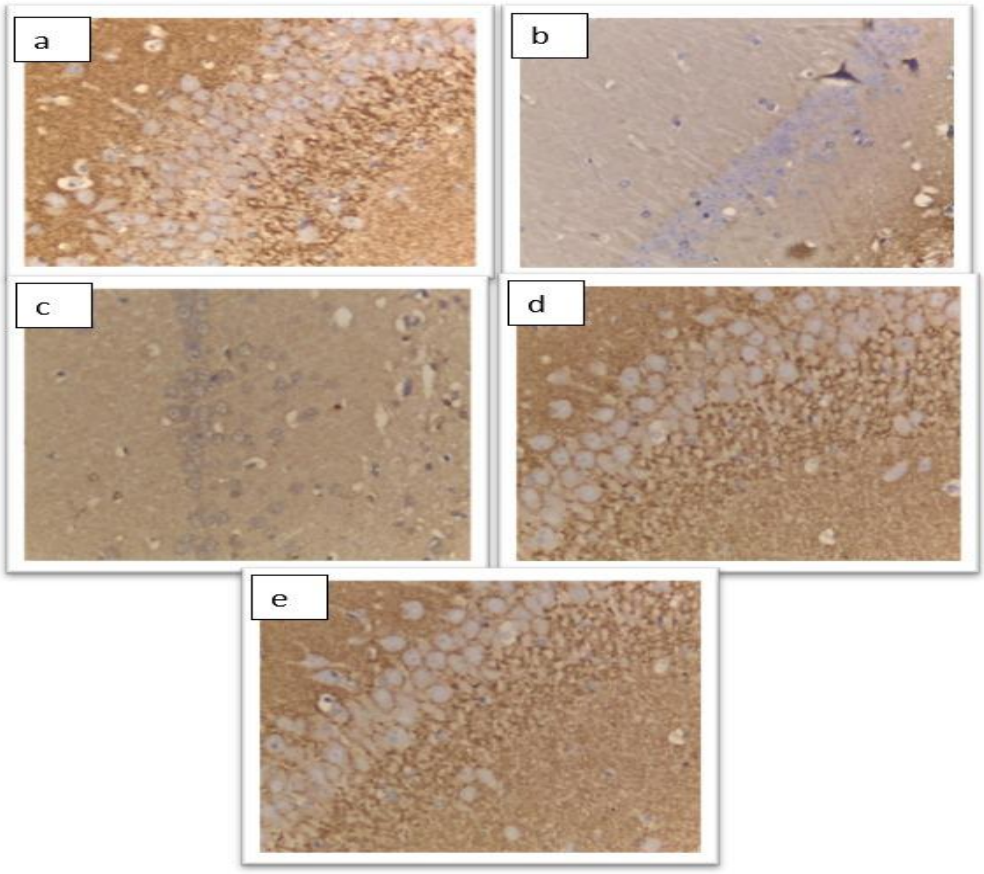

Figure. 6 immunohistochemical staining of hippocampal tissue of PSD-95 marker

(a) Group I (Control group), (b)Group II (D-gal group), (c) Group III (D-gal + Vit. D100), (d) Group IV (D-gal + Vit. D1000), (e)Group V (D-gal + Vit. D10000).

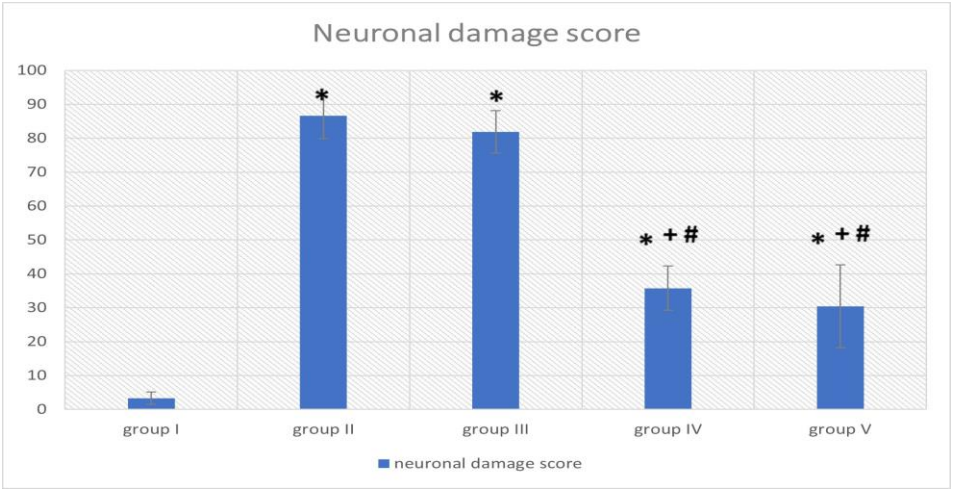

Figure. 7 neuronal damage score

(a) Group I (Control group), (b)Group II (D-gal group), (c) Group III (D-gal + Vit. D100), (d) Group IV (D-gal + Vit. D1000), (e)Group V (D-gal + Vit. D10000).

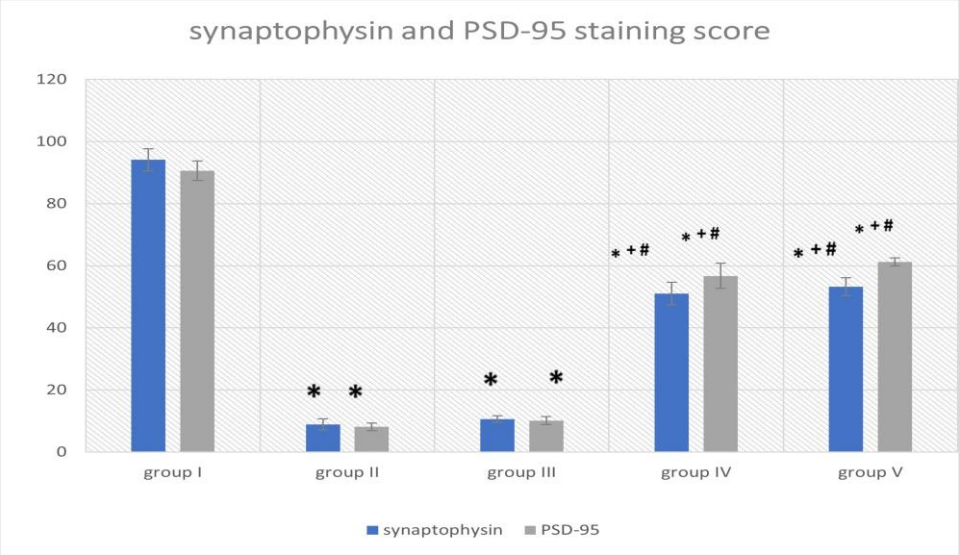

Figure. 8 synaptophysin and PSD-95 staining score

(a) Group I (Control group), (b)Group II (D-gal group), (c) Group III (D-gal + Vit. D100), (d) Group IV (D-gal + Vit. D1000), (e)Group V (D-gal + Vit. D10000).

4. Discussion

As aging progresses, biological homeostasis is disturbed, causing impairments in cognitive, physical, and social functions. While it occurs in all organs, the aging of the brain is of paramount importance [34]**.** The aging brain undergoes substantial behavioral changes, impaired neuronal connectivity, oxidative damage, mitochondrial dysfunction, and synaptic remodeling, ultimately leading to neurodegeneration and neuronal death [35].

This work was carried out to assess the possible protective effect of vit. D on cognitive impairment in D-gal induced brain aging in rats and to explore its role in improvement of synaptic dysplasticity. In the current study, long-term administration of D-gal for 8 weeks led to a novelty cognition impairment of exploratory behavior in rats. There was a significant decreasein RI in the D-gal group compared to control group. Additionally, spatial memory was compromised in D-gal administrated rats as the escape latency times on the 3rd and 4th days of training sessions during the Barnes test had shown a significant increase compared to rats in control group. Reference memory deficits were observed in the D-gal injected group, indicated by a notably shorter duration spent in the target quadrant relative to control group.

Our findings are consistent with previous studies indicating that chronic intraperitoneal administration of D-gal induces memory impairments in both the NORT and Barnes maze tests[36][37].

The underlying mechanisms of D-gal neurotoxic effects was reported by aberrant accumulations of ROS and Advanced Glycation End Products (AGEs), which are linked to aging-related memory loss [38]. Nam et al.,2019[39] documented that continuous D-gal administration led to a decline in adult

hippocampal neurogenesis, which was associated with memory impairment.

As evidenced by our results, Group III, which received Vitamin D at 100 IU/kg, did not exhibit a significant improvement in NORT and Barnes test scores compared to the D-gal group. No discernible improvement in the RI, escape latency times, or time spent in the target quadrant. These findings were supported by **Patel and Shah., 2021**[40]**.**

However, rats received higher doses of vit. D (1000 and 10,000 IU/kg) in group IV and V respectively performed better in the Barnes maze test and the NORT in compare to D-gal group , there was a significant increase in the RI and the time spent in the target quadrant in both groups with a significant decrease in the escape latency times and these results were in harmony with aprior study by **Mansouri et al., 2021**[14]indicated thatmemory and learning impairments induced by scopolamine were alleviated by pretreatment with Vitamin D at higher doses (1000 and 10,000 IU/kg). Also, **Gáll et al.,2024**[41] mentioned in their study that Vit. D supplementation at doses 1000 & 10000 IU/kg improved the cognitive performance in vit. D-deficient rats. Furthermore, vit. D corrected the cognitive impairments caused by a high-fat diet (HFD) in rats by lowering nuclear factor kappa beta (NF-κB), raising Brain-derived neurotrophic factor (BDNF) concentrations, and altering the permeability of the blood-brain barrier (BBB) in the rat hippocampal region [42][43].

There is a strong correlation between degenerative changes in the brain with aging and Aβ plaques [15]. There are many evidences that the synthesis and accumulation of Aβ causes neuroinflammation, neurodegeneration, and memory loss [17]**.** According to the findings of the current study, chronic D-gal injection in the (D-gal group) significantly increased the amount of Aβ in the hippocampus tissue and this was consistent with the cognitive impairment observed in rats in the same group.These results came in agreement with[44].

Moreover, the lower dose of vit. D used in group III did not show a significant improvement in the levels of Aβ. While treatment with the high doses of vit. D (1000 &10000 IU/kg) notably decreasedAβ burden in the hippocampus. Consistent with earlier research, our findings reaffirm that, Vit. D supplementation correlates with a decline in Aβ protein levels[45][46].

The observed decline in Aβ protein levels could be due to Vitamin D's role in promoting Aβ phagocytosis[47]and enhancing its permeability across the BBB[46**].**Moreover, Patel and Shah (2021) [40]demonstrated that Vitamin D reduces Aβ accumulation by inhibiting secretase enzyme activity and downregulating APP protein expression.

MDA is considered an aging marker and an excellent indicator of oxidation status. It can cause damage to proteins, DNA, and other components within the cell, which can result in a number of health issues, including cognitive impairment [48]**.** Conversely, GSH plays a crucial role in the antioxidant defense system, effectively eliminating free radicals produced within cells and organelles, including mitochondria[49]. GSH depletion fosters

oxidative stress within the cell, contributing to mitochondrial impairment, cellular injury, and eventual neuronal cell death[34]**.** In our study, chronic D-gal administration in group II showed a significant elevation in the level of MDA and a significant reduction in the level of GSHin the hippocampal tissue compared to control group. These were in agree with earlier studies **Mohammadi et al.2018**[50] and **Sun et al. 2022**[51].Lower dose of Vitamin D (100 IU/kg) in Group III failed to elicit a significant improvement in MDA and GSH levels. In contrast, Groups IV and V, which received higher doses of Vitamin D (1000 and 10,000 IU/kg), exhibited a marked reduction in MDA levels alongside a significant elevation in GSH levels relative to D-gal group, confirming the antioxidant efficacy of Vitamin D. Our observations parallel those of **Mansouri et al.2021and Bayat et al. 2021** [11]**.**

Indeed, Vitamin D exhibits strong antioxidant, anti-apoptotic, and cell-protective properties[52].The antioxidant mechanism of vitamin D involves the inhibition of inducible nitric oxide synthase (iNOS) and glutamyl transpeptidase, the latter being a key enzyme in GSH metabolism[53]. Vitamin D exerts anti-inflammatory effects by blocking NF-κB activation and suppressing its downstream targets, IL-1β and TNF-α, which in turn mitigates D-gal-induced cognitive dysfunction[35].

Regarding histopathological analysis of hippocampal tissue, rats in D-gal injected group showed degenerative changes as well as shrinkage in the neuronal cell size with dense hyperchromatic nuclei, decreased thickness of pyramidal layer of hippocampus and showed a notable elevation in neuronal death score relative to control group and this was in harmony with **Younis et al. 2024** [54] who reported that chronic D-gal administration led to similar degenerative changes in the hippocampus. D-Gal can also promote neuronal apoptosis while impairing the repair mechanisms of degenerated neurons [55]. Rats received the lower dose of vit. D 100 IU/kg in group III did not show a significant improvement in neuronal death score however, rats received high doses of vit. D (1000 &10000 IU/kg) in group IV and V, showed a significant decrease in the neuronal death score and near normal cytoarchitecture. A similar neuroprotective role of vit. D in rats was reported previously [40][56].

Learning and memory are represented by largely interconnected neural circuits, which are mediated by synapses.Synaptic plasticity, defined as the ability of synapses to modify their structure and function, allows the synapse to respond dynamically to a range of stimuli [57]. As a key process in neural adaptation, synaptic plasticity serves as the neurobiological foundation for learning and memory[39].

Widely accepted as markers of synaptic plasticity, synaptophysin and PSD-95 play a crucial role in detecting synaptic modifications. In our study, the expression of synaptophysin and PSD-95 in the hippocampus was detected by immunohistochemistry. Chronic D-gal administration in group II markedly decreased the expression of synaptophysin and PSD-95 proteins. Our

findings are similar to those published by[58]. Moreover, our immunohistochemical results proved that vit. D administration in high doses (1000&10000 IU/kg) significantly enhanced expression of both pre-and post-synaptic related proteins (synaptophysin and PSD-95). Our results are in harmony with the studies conducted previously by [59].Furthermore, our results revealed that there was a negative correlation between RI and Aβ level in the hippocampus and these data were in a line with **Shallie et al. 2020** [60] and **Li et al. 2021** [61]**.** On the other hand, there was a positive correlation between RI and synaptophysin and PSD-95 these data were in a line with several studies which reported significant positive correlations between synaptophysin and PSD-95 levels as more synaptophysin and PSD-95levels were associated with better spatial memory performance in memory tasks[62][63]**.**

Conclusion

This study demonstrated that vit. D administration in high doses has a neuroprotective effect on cognitive impairment in aged rats. Vit. D's protective effects may stem from its antioxidant properties and its ability to regulate synaptic proteins, such as synaptophysin and PSD-95, which are crucial for synaptic plasticity.

Limitations of the study:

Despite the promising outcomes of this research, our study has some inherent limitations:

1-The results showed that the dose of vitamin D at 100 IU/kg has a non-significant effect while dose 1000 IU/kg has a relatively highly significant effect, more studies using lower doses than 1000 IU/kg are needed to find out the dose of vit D that gives the optimum cognitive protection with minimal side effects.

2-In our trial to investigate the possible mechanisms of the neuroprotective effect of vitamin D we found that its protective effects may be related to its antioxidant properties and its role in modulation of synaptic proteins (synaptophysin and PSD-95) which are essential for synaptic plasticity, more studies are needed to explore additional mechanisms for the neuroprotective effects of vitamin D especially those at the molecular level.

Data availability

Raw data and videos for behavioral tests for memory assessment (Novel object recognition test and Barnes maze test) are available upon request.

Conflictofinterest

None.

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