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Original article

Hippocampal mitochondrial Ca^{++} in experimentally induced Alzheimer's disease, link to calpains and impact of vitamin D3 supplementation

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ABSTRACT

Objective: Vitamin D impact on hippocampal mitochondrial Ca^{++} and calpains was not previously investigated in Alzheimer's disease (AD). The current work aimed to assess the alteration in hippocampal mitochondrial Ca^{++}, ATP & ADP and hippocampal calpains' level in ($AlCl_3$)-induced AD model, and the effect of 2 regimens of vitamin D supplementation on these alterations. Methods: Forty male Wistar rats were randomized into 4 groups; control, AD ($AlCl_3$100 mg/kg, p.o. daily for 42 days), AD and vitamin D co-treated group ($AlCl_3$ as in AD group with vitamin D_3 400 IU/kg/day, *p.o.* for 42 days) and AD, followed by vitamin D_3 group ($AlCl_3$ was given as in AD group for 42 days, then vitamin D_3 for two weeks). AD was assessed by hippocampal levels of $A\beta_{42}$, p-tau and spatial memory assessment in Morris water maze. Hippocampal mitochondrial Ca^{++}, ATP and ADP levels besides to calpain-1 & 2 and cytochrome C were assessed in addition to CA1 histological examination. Results: AD animals showed impaired mitochondrial function as denoted by high Ca^{++} and decreased ATP and ADP and elevated calpain-1 & 2 and cytochrome C. Hippocampal CA1 region showed increased degenerated neurons and reduced thickness of its pyramidal layer. Vitamin D administration minimized the hippocampal mitochondrial impairement induced by AD and mitigated histological alterations even when supplemented post AD establishment. Conclusion: Vitamin D administration to AD rats breaks the deleterious loop in the hippocampus that involves increased Ca^{++}, calpain activation, mitochondrial failure, neuronal degeneration and AD disease progression.

1. Introduction

Alzheimer's Disease (AD) is a chronic neurodegenerative disease known to be the most causative factor in the development of dementia. It is estimated that this progressive disease affects about 46.8 million individuals worldwide and that in the year 2050, the number of AD cases will increase to four-fold value (Al-Atrache et al., 2019) Also, it is the most common dementia type in elderly (Wang C et al., 2019).

AD characteristic features include cognitive impairment and progressive memory loss together with neuronal loss, extracellular amyloid plaques and neurofibrillary tangles (Wang S. et al., 2019), which are mostly found in the hippocampus (Cheng et al., 2018). Upon chronic exposure to $AlCl_3$, it accumulates in brain regions including hippocampus, this enhances Tau phosphorylation, Aβ formation, cholinergic neurons' degeneration besides to fostering both oxidative and inflammatory status (Khan et al., 2013). Consequently, $AlCl_3$ is used to induce AD-like pathology.

Mitochondria are the powerhouses of the cells, and they produce the cell's ATP via oxidative phosphorylation (Ishii et al., 2019). It also involves calcium Ca^{++} buffering to prolong its synaptic terminals' residual levels. Thus, it performs essential roles in both the regulation and maintenance of neurotransmission and synaptic plasticity (Cai and Jeong, 2020).

Calpains are a family of intracellular proteases; they are classified according to the features of their expression into tissue-specific and ubiquitous (Hwang et al., 2020). Calpains are highly expressed in

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neuronal cytosol and synaptic terminals, and the major isoforms in the CNS are calpains 1 and 2 (Tang et al., 2020).

Calpains are abnormally increased or activated due to severe brain injury, chronic and acute neurodegeneration, and in several neuro-inflammatory diseases such as AD, stroke and Parkinson's disease (Velez et al., 2020).

Vitamin D was suggested to be involved in both neuroprotective and cognitive functions, and clinical and epidemiological data also reported that vitamin D levels in serum are linked to better performance in cognitive testing (Lin et al., 2020). Our lab previously revealed that vitamin D supplementation corrected alterations in hippocampal synaptic proteins and glutamatergic transmission in metabolic syndrome model (Alrefaie et al., 2022) and improved cortical cholinergic transmission in diabetic animal models (Alrefaie and Alhayani, 2015). Nonetheless, the mechanisms underlying the possible therapeutic effects of vitamin D supplementation in AD models still need to be further clarified.

2. Aim

As the impact of vitamin D on alteration in hippocampal mitochondrial Ca^{++} and calpains' level in Al_3Cl-induced AD-like neurotoxicity was not previously investigated, the current work was designed to assess the effect of vitamin D_3 co-administration and post-administration on hippocampal mitochondrial Ca^{++} level and function and calpain-1&2 in $AlCl_3$-induced AD-like neurotoxicity.

3. Materials and methods

The current study was approved by the Biomedical Ethics Research Committee, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia (Reference No 774-19). It was conducted at King Fahd Medical Research Center, King Abdulaziz University. The study was carried out following the animal welfare act and guide for care use of animals. Forty male Albino Wistar rats (200–225 g) were allowed one week of acclimatization before the experiment. They were kept in standard laboratory conditions, with 12 h light/dark cycle, a temperature range of 23 °C ± 3 °C and free access to food and water.

3.1. Experimental protocol

Rats were randomized into four groups (N = 10) according to the research protocol; Group I (control group). Group II (AD group ($AlCl_3$-treated rats); $AlCl_3$ (Alpha Chemika, AL0198) was given daily (100 mg/kg, *p.o.*) for 42 days to induce AD (Prema et al., 2016). Group III (vitamin D_3 co-treated AD group); $AlCl_3$ was given as in group II with vitamin D_3 (400 IU/kg/day, *p.o.*) (Castillo et al., 2012) for 42 days. Group IV (vitamin D_3 post-treated AD group); $AlCl_3$ was given as in group II for 42 days, and then vitamin D_3 was administered as in group III for two weeks after the cessation of $AlCl_3$. The $AlCl_3$ administered groups started with 15 animals in each, (while 4, 2 and 3 rats died from groups II, III and IV respectively) and 10 rats were included in the final assessments.

3.2. Morris water maze (MWM)

The MWM apparatus used in this study was a large circular swimming pool with the dimensions 150 cm × 45 cm that is divided into four quadrants. The protocol of Prema et al., 2016 was followed.

Each animal was trained on day 19 of the experiment, where the maze was filled with water up to 30 cm, its temperature was adjusted to 28 ± 1 °C, and the platform was placed 1 cm above the water level. Four trials, 15 min apart were carried out for each animal, each trial was conducted by gently placing the rat in one quadrant facing the pool wall. The rat was allowed 120 s to find the platform and stay on it for 20 s, and the time it took to reach the platform was recorded and the average of the four trials was regarded as acquisition latency. In case the animal could not find the platform, it was gently guided into it and allowed to stay there for 20 s, and its acquisition latency was recorded as 120 s. On the last day before sacrifice (day 41 for groups I, II and III and day 55 for group IV), animals were tested as previously described but with the platform below the water level and hidden by the use of non-fat milk powder.

3.3. Sampling and biochemical investigations

At the end of the experiment, rats were sacrificed after collecting a *retro*-orbital fasting blood sample, and the serum was separated to assess the levels of Ca^{++} [Arsenazo III kit] and vitamin D_3 [vitamin D ELISA kit from MyBioSource, (MBS728692)].

Following sacrifice, rats' heads were placed on ice, brain was collected, and the hippocampus was dissected from both hemispheres and washed with ice-cold saline (Au – Hagihara et al., 2009). Also, the weights of the whole brain and the hippocampi were measured.

The hippocampai from one hemisphere were used for histological studies, and assessment of mitochondrial Ca^{++} using Mammalian Mitochondria Isolation Kit for Tissue homogenate (MyBioSource- MBS-841515 ELISA). Then, the level of mitochondrial Ca^{++} was assessed using the Abcam Ca^{++} detection colorimetric assay kit, (ab1025505).

The hippocampai of the other hemisphere (N = 10) were stored at −80 °C and later used for assessing the levels of $A\beta_{42}$, p-tau, cytochrome c, calpain-1, calpain-2, ATP and ADP using ELISA technique.

3.4. Histological and morphometric examination

For histological examination, 10 % neutral buffered formalin was used to fix dissected hippocampi for 24 h, followed by dehydration with serial grades of 99.7–100 % ethanol (BDH, 28304). Xylene (Lab-scan analytical science, A3523) was used to clear the samples. Then they were impregnated in paraffin (Leica biosystems, 39602004) at 56 0C for 24 h, followed by paraffin blocking in hard paraffin. Four-micron sections were stained with haematoxylin and eosin (H&E) (Ahmed, 2016).

For morphometric analysis, the thickness of hippocampal subregion CA1 and the number of degenerated dark stained cells were estimated. The thickness was measured in three non-overlapping visual fields in four random sections from each rat. Image Pro Plus software (Media Cybernetics, Silver Spring, USA, version 4.5) connected to a Brightfield Microscope (Olympus Corporation, Tokyo, Japan, BX51TF) and a digital camera (Olympus Corporation, Tokyo, Japan, DP-70) was used.

3.5. Statistical analysis

The data obtained during the study were analyzed utilizing IBM SPSS Statistics for Windows, version 23 (IBM SPSS, IBM Corp., Armonk, N.Y., USA). Shapiro – Wilk test was utilized to evaluate normal value distribution. Collected values were presented as mean +/- standard deviation for normally distributed data and median and 25 and 75 percentile for abnormally distributed parametric values. Statistical comparisons were made by Kruskal-Wallis test followed by Mann Whitney for abnormally distributed data and OneWay ANOVA test followed by Tukey's test for normally distributed data. P value < 0.05 was considered statistically significant.

4. Results

To confirm the development of the AD model, the following parameters were investigated; the escape latency in the Morris Water Maze, brain and hippocampus weight, hippocampal $A\beta_{42}$, and p-tau levels (Fig. 1).

Regarding the memory testing using the MWM, the time needed for the rats to reach the escape platform was significantly prolonged in the AD group (43 % increase) compared to the control group. Both vitamin D treatment regimens didn't significantly affect the escape latency,

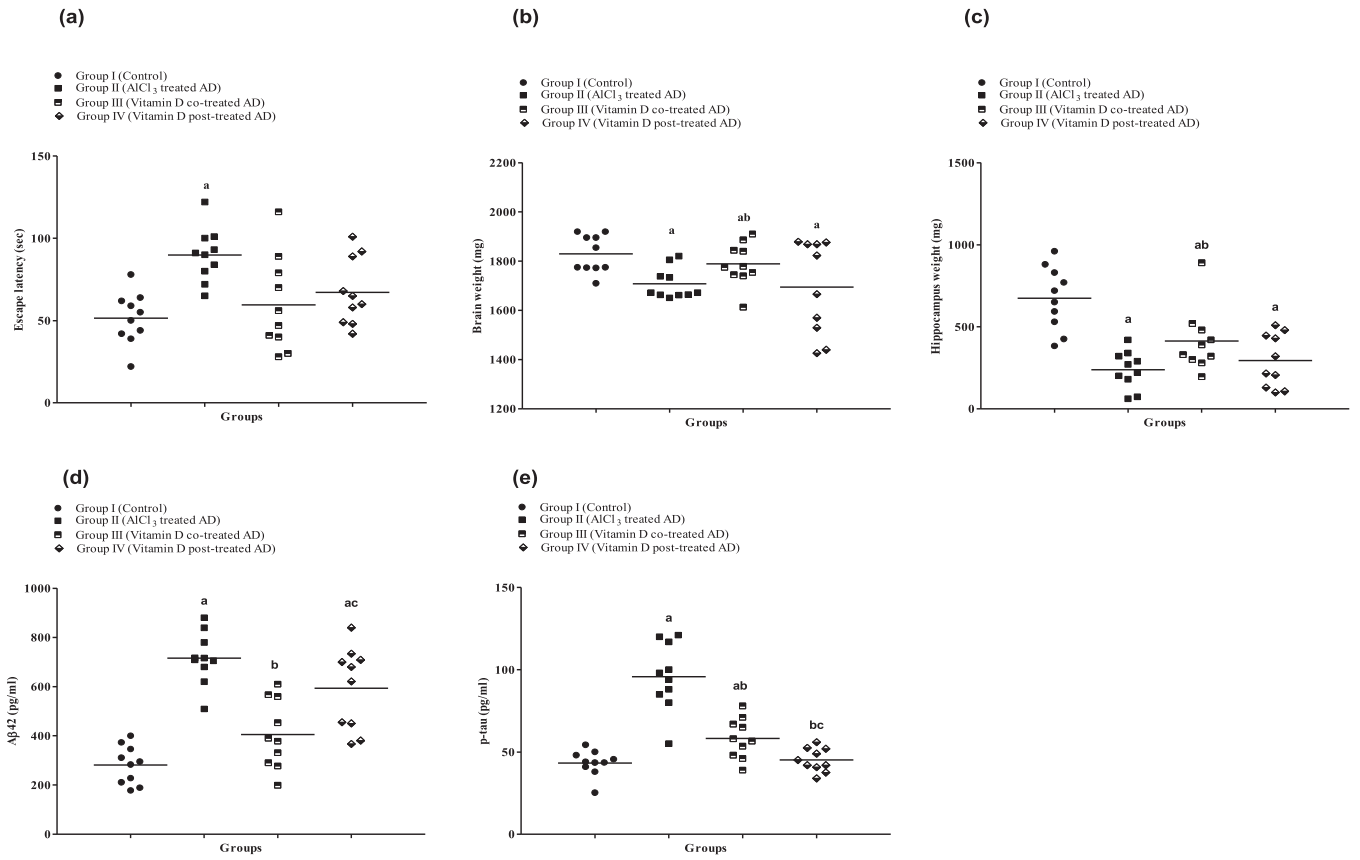

Fig. 1. MWM escape latency [sec] (panel a), brain and hippocampus weights [mg] (panel b & c), $A\beta_{42}$ and p-tau [pg/ml] (panel d & e) in the different studied groups (N = 10). Data were expressed as mean ± standard deviation. [a]: significance versus group I, [b]: significance versus group II, [c]: significance versus group III. Significance was made using the One-way ANOVA test followed by Tukey's Test.

however the time needed to reach the platform was decreased by 33 % and 24 % in vitamin D co-treated and post-treated groups respectively when compared to the AD non treated group.

Rats treated with $AlCl_3$ showed significantly lower brain and hippocampal weight (7 % and 65 % respectively) than the normal rats, while the rats co-treated with vitamin D during the induction of AD revealed significantly higher both brain and hippocampal weight (5 % and 74 % respectively) compared to the AD group. It's obvious that the alteration in the weight of hippocampus by either AlCl3 or vitamin D was more prominent than the changes in the total brain weight. Looking at the weight of brain and hippocampus in the group post-treated with vitamin D, shows non-significant minor difference on comparing it to AD group.

Hippocampal $A\beta_{42}$ and p-tau levels were found to be significantly elevated (approximately 1.5 folds) in the AD rats compared to control rats, and this increase was significantly mitigated by vitamin D co-treatment with nearly half fold decrease in the level of both $A\beta_{42}$ and p-tau in comparison with non-treated AD group. It is to be noted that vitamin D post-treated group showed only significant reduction in p-tau level compared to AD non-treated group.

To monitor the safety of vitamin D dose used in the current work, Serum Ca^{++} and vitamin D levels were assessed (Table 1). As expected, serum Ca^{++} and vitamin D levels in $AlCl_3$ treated rats were significantly decreased (25 % and 80 % respectively) than in normal rats. Both groups treated with vitamin D either co or post-treated, did not show significant alteration in serum Ca^{++} level compared to the AD non-treated group. On the contrary, both vitamin D supplementation regimens resulted in a significantly higher vitamin D level compared to the AD non-treated rats (4 fold increase in co-treated group and 4.5 fold increase in post-treated group).

Table 1
The serum levels of Ca^{++} and vitamin D_3 in the different studied groups (N = 10).

Parameters	Groups			
	Group I Control	Group II AlCl3 treated AD	Group III Vit. D co-treated AD	Group IV Vit. D post-treated AD
Serum calcium (mg/dl)	8.49 ± 0.75	6.52 ± 0.68[a]	7.14 ± 1.15[a]	7.44 ± 0.64[a]
Serum vit D (ng/ml)	19.76 ± 2.07	3.36 ± 0.77[a]	17.42 ± 2.88[ab]	19.43 ± 3.24[b]

Data were expressed as mean ± standard deviation. [a]: significance versus group I, [b]: significance versus group II, [c]: significance versus group III. Significance was made using the One-way ANOVA test followed by Tukey's Test.

To investigate the hypothesis of the current work, hippocampal mitochondrial Ca^{++} and ATP and ADP, hippocampal calpain-1 & 2 in addition to cytochrome c, were assessed in all groups (Fig. 2).

Current results demonstrated that mitochondrial Ca^{++} in the hippocampi of the AD group was significantly increased compared to the control one (2.5 fold). On the other hand, its level got significantly reduced in both groups treated with vitamin D (60 % in co-treated and 65 % reduction in post-treated groups respectively).

Hippocampal calpain-1 and 2 levels were significantly higher (1.5 fold and 1 fold respectively) in the $AlCl_3$-treated rats than in normal rats. Moreover, both methods of vitamin D treatment significantly reduced both calpains' level compared to AD rats (46 % decrease in calpain 1 & 34 % decrease in calpain 2 in co-treated group and 61 % lowering in calpain 1 & 53 % lowering in calpain 2 in post-treated group). Interestingly, calpains' level was significantly reduced with vitamin D

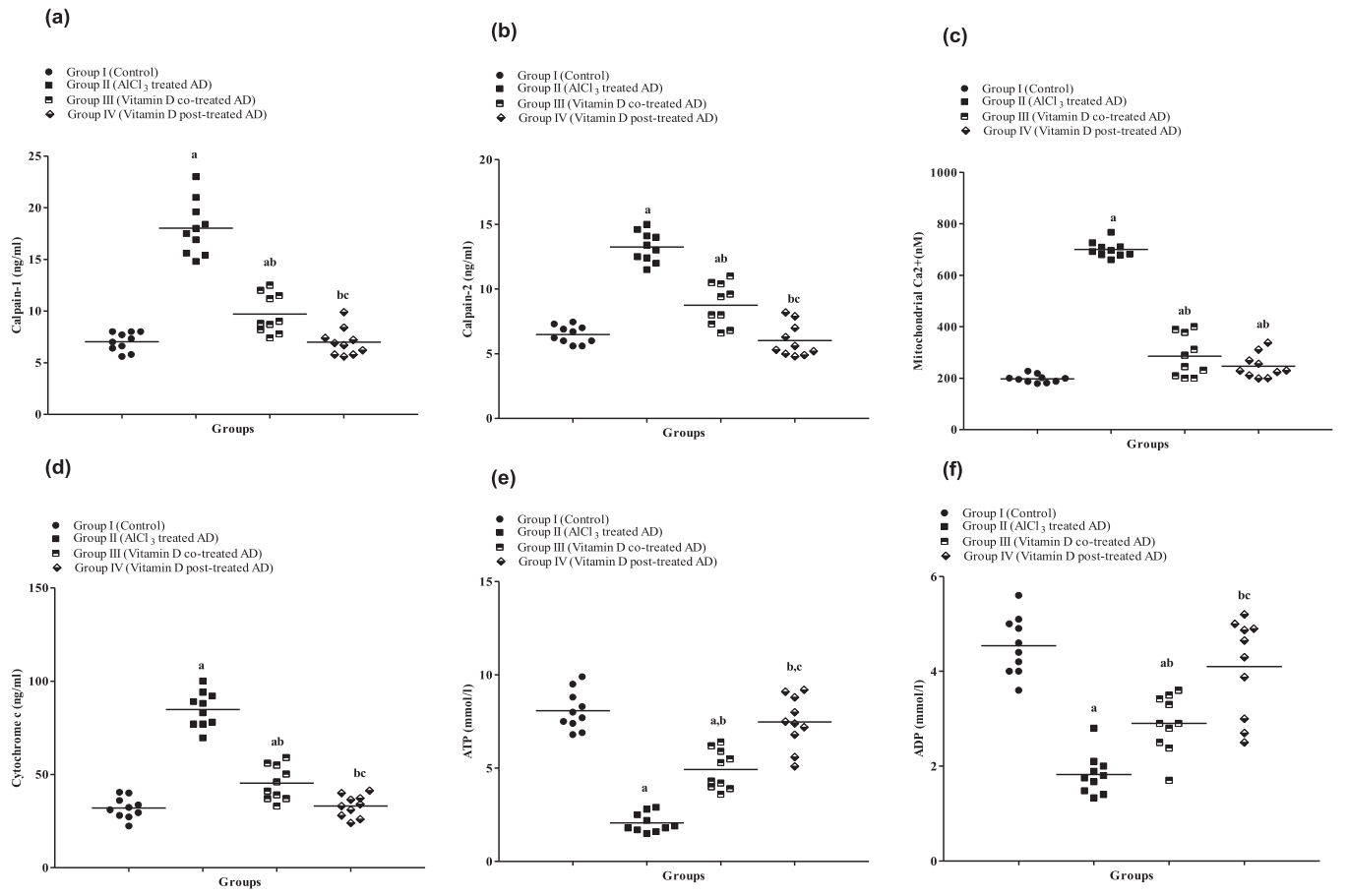

Fig. 2. The hippocampal calpain-1 &2 [ng/ml] (panel a & b), mitochondrial Ca^{++} [nM] (panel c), cytochrome C [ng/ml] (panel d), ATP and ADP [mmol/l] (panel e & f) in the different studied groups (N = 10). Data were expressed as mean ± standard deviation. [a]: significance versus group I, [b]: significance versus group II, [c]: significance versus group III. Significance was made using the One-way ANOVA test followed by Tukey's Test.

treatment post AD induction rather than with vitamin D co-administration with $AlCl_3$.

Regarding the level of hippocampal cytochrome c, it was shown to be significantly elevated in AD rats compared to normal rats (1.5 fold). This elevation was significantly reduced with both vitamin D supplementation regimens. with more significant reduction observed in rats treated with vitamin D after AD development.

In the present study, hippocampal mitochondrial energy production was impaired in AD rats compared to control rats as denoted by the diminution of hippocampal ATP and ADP levels (75 % & 60 % respectively). On the contrary, treatment with vitamin D has significantly increased the levels of ATP and ADP compared to the non-treated AD group, both co-treated (140 % & 60 % increase respectively) and post-treated regimens (250 % & 126 % increase respectively).

Histological and Morphometric Results (Fig. 3 and Table 2). The thickness of hippocampal region CA1 was significantly increased in vitamin D co-treated group (26 %) and post-treated group (30 %) in comparison with non-treated AD group, while the changes in the number of the degenerated neurones didn't reach significance in AD rats compared to control rats, neither in vitamin D treated groups compared to AD non-treated group.

5. Discussion

The current study revealed a promising finding by vitamin D administration to rats with $AlCl_3$-induced AD-like pathology, that is the mitigation in the alteration of mitochondrial Ca^{++}, energy production and calpains' level. This highlights a new possible mechanism for the protective effect of vitamin D against that model of AD. Noteworthy that, the supplementation of AD rats with vitamin D post induction of the disease significantly corrected the mitochondrial alterations compared to the non-treated AD model, and this points to the importance of vitamin D supplementation to AD patients even with the disease established. The current findings propose a hypothesis regarding the link between hippocampal calpains, mitochondrial Ca^{++} and AD pathogenesis and further illustrate the impact of vitamin D supplementation.

Calpains are Ca^{++} activated proteases that modulate postsynaptic scaffolding proteins, spectrin and b-catenin in the postsynaptic density and many other substrates. Under normal conditions calpains are activated by micromolar Ca^{++} levels and inhibited by calpstatin, and calpain upregulation was linked to AD (Mahaman et al., 2019). Calpains activate cyclin-dependent kinase 5, CDK5 (Lee et al., 2000), which increases BACE1 enzyme expression that generates Aβ from its precursor protein (APP), fostering the Aβ plaques formation (Wen et al., 2008). Calpain also favors tau phosphorylation and neurofibrillary tangles formation, another AD hallmark, through activation of CDK5 and glycogen synthetase kinase 3 (Hanger et al., 1992), while inhibition of calpain by calpeptin abolished neurofilament phosphorylation in the study of Veeranna et al., 2004. Moreover, elevated calpains were associated with gliosis, and A-705053, a calpain inhibitor, decreased microglial activation in transgenic mice model of AD (Medeiros et al., 2012). Calpain-2 activation enhanced the degradation of acetylcholine receptor α4 with impairment of cholinergic transmission in in hippocampal neuronal cultures (Yin et al., 2016).

It is noteworthy that high amount of calpain-cleaved spectrin was also detected in AD patients' cerebrospinal fluid further confirming the association of calpains to AD pathogenesis (Higuchi et al., 2012).

The implication of calpain in the pathophysiology of AD led the

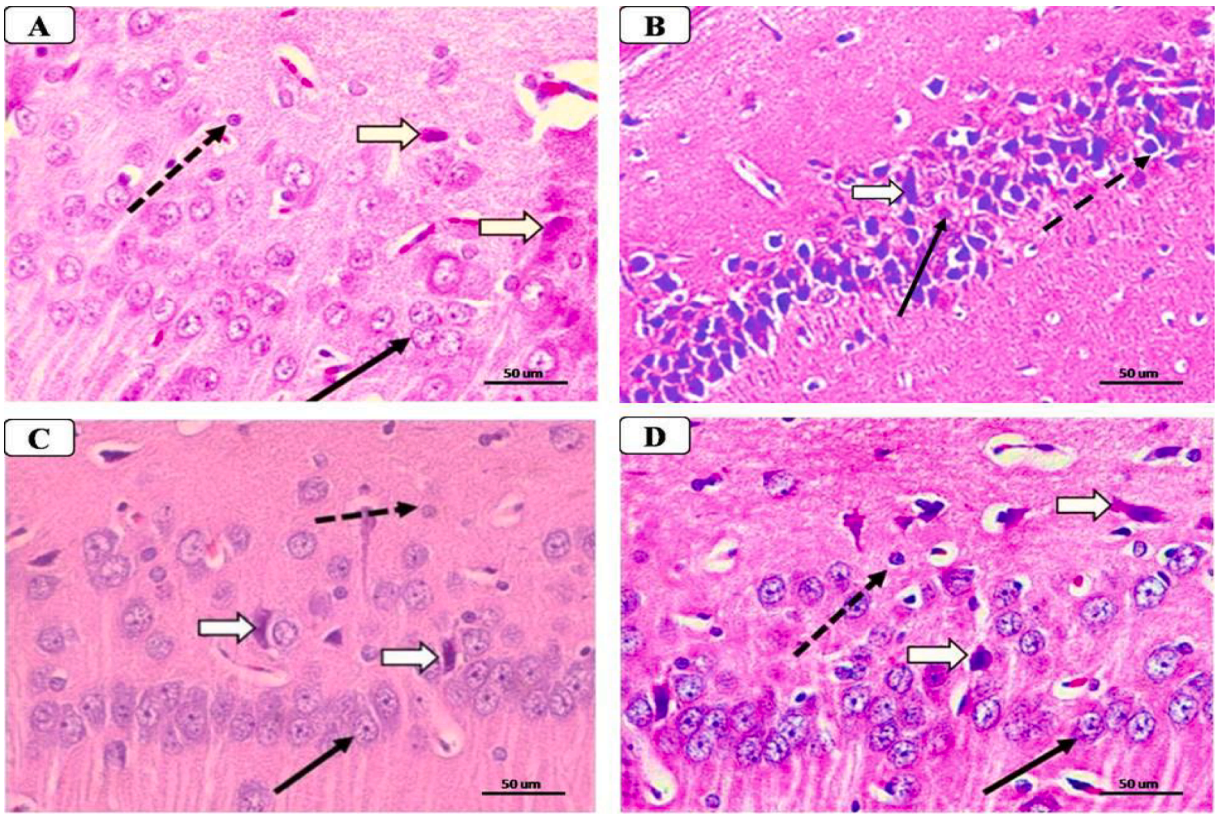

Fig. 3. Paraffin sections from CA1 region of rat hippocampus stained by H&E (N = 10). A. (control group): showing normal layers of viable pyramidal cells, with large euchromatic active nuclei and prominent nucleoli (black arrow). Few sporadic dark-stained degenerated cells could be seen (white arrows). Glial cells with their small dark nuclei are infrequent among the neurons (dotted arrow). B. (AD group): Pyramidal cells looked deformed with darkly stained cytoplasm (white arrow) masking the degenerated pyknotic nuclei. The nuclei of the rest of the neurons looked abnormal and showed vacuolated or reticulated nucleoplasm (black arrow), and Glia cells (dotted arrow). C. (AD vitamin D co-treated group): showing a decrease in the number of degenerated cells (white arrows) compared to the AD group. Viable neuronal cells showed normal vesicular active nuclei (black arrow), and Glia cells (dotted arrow). D. (AD vitamin D post-treated group): showing also decrease in the number of degenerated cells (white arrows). Viable cells showed cytoplasm and nuclei (black arrow), which looked more like the control group with Glia cells (dotted arrows).

Table 2
The hippocampal morphometric results in the different studied groups (N = 10).

Parameters	Groups			
	Group I Control	Group II AlCl3 treated AD	Group III Vit. D co-treated AD	Group IV Vit. D post-treated AD
Thickness of hippocampal region CA1 (μm) in the different studied groups				
CA1 thickness	54.87 ± 7.85	52.51 ± 14.34	67.27 ± 18.15[b]	69.23 ± 6.51[b]
The number of degenerated neurons of the hippocampal region CA1 in the different studied groups				
CA1 Median (25–75 %)	3.00 (2.00–3.00)	10.50 (3.75–12.75)	4 (4.00–4.00)	4.00 (2.50–5.50)

[a]: significance versus group I, [b]: significance versus group II, [c]: significance versus group III. Statistical comparisons were made by OneWay ANOVA test followed by Tukey's test for CA1 thickness (mean ± SD) and Kruskal-Wallis test followed by Mann Whitney for CA1 number of degenerated neurons (median and 25–75 percentile).

researchers to study the effects of calpain inhibition or calpstatin over-expression on AD symptoms. Promising results were shown in animal and in vitro cell models, but unfortunately these trials were not promoted to clinical ones, because of issues related to molecule selectivity, kinetics or cell entry. This highlights the importance of the current findings concerning the calpain 1& 2 mitigating effect of vitamin D supplementation in the $AlCl_3$-induced AD like pathology.

The current findings showed a significant elevation in hippocampal mitochondrial Ca^{++} level in AD group. Previously, amyloid beta oligomers were found to induce Ca^{++} permeable channels in cultured hippocampal neuronal membrane and to interact with voltage and ligand-gated Ca^{++} channels, including ionotropic glutamate receptors, increasing Ca^{++} influx (Alberdi et al., 2010). It was recently observed that incubation of cortical neurons with tau, inhibited mitochondrial calcium efflux via the mitochondrial Na^{+}/Ca^{++} exchanger (Britti et al., 2020), proposing an explanation for the abnormal elevated mitochondrial Ca^{++} in AD.

Elevated Ca^{++} in the mitochondrial impairs its function as it triggers the formation of mitochondrial permeability transition pores allowing the release of cytochrome C into the cytoplasm (Pahrudin et al., 2020). Upon release, cytochrome C activates caspases that ultimately causes neuronal apoptotic cell death (Prema et al., 2016), and this was evident in the current study by the elevated hippocampal cytochrome C and the number of degenerated neurons in hippocampal CA1 of AD group of rats.

Impaired mitochondrial function was also evident in the current work by the significant reduction in hippocampal ATP and ADP in AD group compared to control rats.

It can be postulated from the current findings that increased calpains fosters Aβ plaques formation, which consequently increases neuronal Ca^{++} level that further increases calpains generating a deleterious circuit that underlies the disease progression. It is also apparent that high mitochondrial Ca^{++} critically impairs its function and predisposes to neuronal degeneration.

Findings of the current work revealed that vitamin D significantly lowered hippocampal calpains 1&2, mitochondrial Ca^{++}, cytochrome c and increased energy production which possibly breaks this circuit and limits the disease worsening.

The diminution of hippocampal mitochondrial Ca^{++} and the improvement in ATP and ADP levels in $AlCl_3$-induced AD models by vitamin D were not previously demonstrated in the literature. Thus, the current findings shed the light on a novel neuroprotective mechanism for vitamin D in the AD model and the importance of its administration to already established cases of AD, as the post-treated group showed significant improvement in all mitochondrial altered parameters.

Conclusion: Considering the widespread vitamin D deficiency and/or insufficiency, especially in old aged population, the findings of our lab recommend its supplementation and ensuring its adequate level to guard against the development and progression of AD, and propose a new possible mechanism for its neuroprotective role in the $AlCl_3$-induced AD model. It's also recommended to extend the research to Vitamin D deficient animals to get better understanding of its possible protective mechanisms.

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Ethical Approval

The current study was approved by the Biomedical Ethics Research Committee, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia (Reference No 774-19). The study was carried out following the animal welfare act and guide for care use of animals.

Availability of data and materials

Data and material of the manuscript are available at request.

CRediT authorship contribution statement

Zienab Alrefaie: Conceptualization, Data curation, Funding acquisition, Investigation, Writing – original draft, Writing – review & editing. **Jana Bashraheel:** Data curation, Investigation, Writing – original draft. **Hossam A. Hammad:** Conceptualization, Data curation, Investigation, Writing – original draft. **Soad S. Ali:** Tissue examination and interpretation on the histological results, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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