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Expression profile of apolipoprotein E in Alzheimer rat animal model: Modulation effect of vitamin D and mushroom on the behavioral deficits and neuropathological alterations

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A close relationship has been found between the central nervous system and vitamin D in which its deficiency leads to neurological and psychological disorders. The current study is focused on utilizing mushrooms as an important dietary source of vitamin D2 against AD. Male Wister rats (n=70) were divided into several groups, supplemented with vitamin D and mushroom and exposed to $ALCI_3$ to induce AD. Expression of apolipoprotein E (ApoE4) biomarker of AD and neuropathological alterations were conducted in brain hippocampus. Additionally, behavioral analysis using Y Maze Spontaneous Alternation Test was performed. The results showed that AD group of rats exhibited high expression levels of ApoE4 gene associated with neuronal cell loss. Additionally, an elevation in number of degenerated neurons in the hippocampal sub-regions coincided with low spontaneous alternation percentage (SA%) were observed in AD group. Conversely, pre-supplementation of rats exposed to $ALCI_3$ with vitamin D and mushroom mitigated the severe neurotoxicity resulted from $AlCl_3$ inducing AD rats. The beneficial effects of vitamin D and mushroom consumption on improving memory in AD mice could be due to the interaction between other bioactive compounds and vitamin D2 inhibiting the oxidative stress in brain hippocampus.

Keywords: Alzheimer's disease, Apolipoprotein E biomarker, Neuropathological alterations, Behavioral deficits, Vitamin D, Mushroom

INTRODUCTION

AD is a worldwide epidemic that is a major cause of morbidity and higher death rate in the elderly population (GBD 2019). The global number of AD patients is estimated by the World Health Organization (WHO) and Alzheimer's Association to be more than 35 million individuals (Tarawneh 2020). Numerous national and international attempts were made to discover successful methods to either inhibit or slow AD progression but still no cure exists for AD (Tarawneh 2020).

The hippocampus is a region of the brain that is crucial for the creation and storage of semantic declarative and episodic memories. Its synaptic plasticity aids in the process of memory consolidation and long-term memory storage. Thus, this area is vital for learning as well as the new memories acquisition and their retention (Avchalumov and Mandyam 2021). In AD's early stages, the neurogenesis of the hippocampus is impaired and is believed to aid in the early decline of cognition (Morello *et al*. 2018).

As in the case that increasing reliance on healthy food that contains a little extra fat, salt and sugar is useful for reducing the incidence of chronic diseases such as diabetes, cancer and cardiovascular diseases, it was also found that paying attention to diet leads to reducing the appearance of dementia and its symptoms (Bennett et al. 2013). On the other hand, some studies have shown that there is a need for other factors besides adherence to the diet to treat cognitive impairment or Alzheimer's (Gardener *et al.* 2012). On the other hand, there is evidence demonstrating a positive role for the beneficial effects of micronutrient cocktail supplementation on memory in Alzheimer's patients, which have been shown to significantly reduce amyloid load such as consumption of curcumin extract (Yang et al. 2005, Garcia-Alloza et al. 2007), grape seed (Wang et al. 2009) and fish oil (Lim et al. 2005).

Vitamin D is a member of vitamins that are soluble in

fat (Aguilar-Shea 2021). It has several biological effects including skeletal and non-skeletal functions (Kumar et al. 2021). Sources of vitamin D include diet, sunlight and medicinal supplementation (Charoenngam and Holick 2020) and its deficiency is revealed by accumulating evidences to be a global health problem (Szymczak-Pajor et al. 2020). Also, vitamin D is suggested by mounting evidence to have positive effects on the risk of the development of neurodegenerative diseases (Maretzke *et al.* 2020), including AD (Mokry et al. 2016). Vitamin D acts on tissues and cells that possess its receptor. Vitamin D receptors (VDR) are expressed in several brain areas in both humans and animals, including the hypothalamus, thalamus and the hippocampus (Uberti et al. 2016), where its expression is among the highest areas (Moretti *et al.* 2018).

Vitamin D and its metabolites have the ability of crossing the blood brain barrier (BBB). VDR were also reported to be expressed in glial and neuronal cells which could indicate a possible influence of vitamin D on the functions of the central nervous system (CNS) (Lauer et al. 2019). Moreover, several reported actions of vitamin D in the brain include modification of the growth and differentiation of neurites as well as the modulation of synaptic plasticity and neurotransmission (Banerjee et al. 2015).

One of the most important food types in eastern cultures is the consumption of edible mushrooms for many years, which are considered beneficial for health in general. Moreover, it has been found in Japan that the mushroom (Hericium Erinaceus) is used in the treatment of elderly Japanese men and women with moderate cognitive disabilities to improve cognitive function (Mori *et al.* 2009). It was found that the bio-elements in these mushrooms, along with vitamin D, were responsible for the effect and increased the possibility that the mushrooms rich in vitamin D would act synergistically with other components.

Therefore, mushrooms can be considered a good source of vitamin D by exposing the mushrooms to UV rays to stimulate vitamin D synthesis (Koyyalamudi *et al.* 2009) to produce a bioavailable food source of vitamin D (Jasinghe *et al.* 2006, Ozzard *et al.* 2008). Besides, it was found that mushrooms have the ability to increase mineral density and enhance calcium absorption in the bones of mice (Lee *et al.* 2009). So the increased vitamin D levels in mushrooms, along with other nutritional benefits for cognition, make vitamin D-rich mushrooms a good target for efficacy testing on brain function. Some studies have shown that dietary supplementation with vitamin D in Alzheimer's disease model mice (Yu *et al.* 2011) has cognitive benefits for vitamin D, but to date the effects of vitamin D combination and the assumed additional bioactivities in mushrooms have not been extensively studied.

MATERIALS AND METHODS

Chemicals

Aluminum chloride (AlCl3, MW 133.34.) which was used to induce AD, was purchased from Sigma (USA). Memantine (Ebixa) used a reference drug for treatment AD, was obtained from Merz Pharma. The kits and fine chemicals for molecular biological analysis were obtained from Invitrogen (Germany).

Mushroom Sampling

Fresh *Agaricus bisporus* were bought from private Markets in Saudi Arabian. The mushroom samples were identified and a voucher specimen (No. D231) has been deposited in the herbarium of the king Abdelaziz University, Suadi Arabian.

Experimental setup

A total of 70 (over 15 months) male Wister rats weighing approximately 250 g was used in this study. Animals were housed in polypropylene cages with dust free rice husk with control environment of temperature (23) °C and humidity (40%-60%).

Rats randomly divided into seven groups as follows: Group 1 (Negative control): Rats were given by oral administration of water saline (100 mg/kg /day) daily for one month. Group 2 (positive control): Rats were injected intraperitoneal with AlCl3 for 4 weeks at a dose of 100 mg/kg per day as a reference drug (Lim et al. 2022). Group 3: Rats were administrated orally with vitamin D for 3 months at a dose of 1000 IU/ kg per day (Hayes et al. 2019). Group 4: Rats were fed daily with diet containing 20% Agaricus bisporus; Monterey Mushroom, Temple, PA) for 3 months (Agunloye and Oboh 2022). Group 5: Rats were given orally vitamin D for 3 months at a dose of 1000 IU and then injected with ALCI3 for 4 weeks as mentioned in group 2. Group 6: Rats were given mushroom for 3 months and then injected with ALCI3 for 4 weeks. Group 7: Rats were injected with ALCI3 for 4 weeks and then treated with memantine (10 mg/kg/day) for 6 weeks (Cabuk et al. 2011).

Expression analysis of Apolipoprotein E (ApoE4) gene

RNA isolation and reverse transcription reaction

To isolate total genomic RNA from brain tissues of all treated groups, TRIzol® chemical extraction (Invitrogen) was utilized. The obtained RNA pellets were stored in DEPC treated water after completion of the isolation procedures. The isolated RNA pellet was processed using an RNAse-free DNAse kit (Invitrogen, Germany) to digest potential DNA residues. The RNA aliquots were stored at -20 °C or used immediately for reverse transcription (Salem *et al.* 2018).

Via reverse transcription reaction (RT), the First Strand cDNA Synthesis Kit (RevertAidTM, MBI Fermentas) was utilized to synthesize the cDNA copy from brain tissues. To obtain the cDNA copy of brain genome, RT reaction program of 25°C for 10 min, then one hour at

42 °C then 5 min at 95°C was used. Finally, tubes of reaction containing cDNA copy were collected on ice up to use for cDNA amplification (Hamed et al. 2021).

Quantitative Real Time-PCR

To perform the qRT-PCR analyses, SYBR® Premix Ex TaqTM kit (TaKaRa, Biotech. Co. Ltd.) was utilized using the synthesized cDNA copies from brain tissues. A melting curve profile for each reaction was built. The quantitative values of the housekeeping gene (Table 1) were used to normalize the quantitative values of the target genes. The 2−ΔΔCT method was utilized to determine the quantitative values of the specific genes to the reference gene (Hamza *et al.* 2015).

Table 1: Primer sequences used for qPCR

Gene	Primer sequence (5'–3'	References/ NCBI
ApoE4	F: caa cag ctg gga atg gga ac	NM_001270683.1
	R: tga tct gtc acc tcc agc tc	
GAPDH	F: aac gac ccc ttc att gac ct	XM_039107008.1
	R: ccc cat ttg atg tta gcg gg	

ApoE4: Apolipoprotein E; GAPDH: Glyceraldehyde-3-phosphate dehydrogenas

Behavior analysis

Y Maze Spontaneous Alternation Test

The behavioral test for the rats under study consisted of a memory test known as the Y Maze spontaneous alternation (Momeni *et al.* 2015) (Fig. 1).

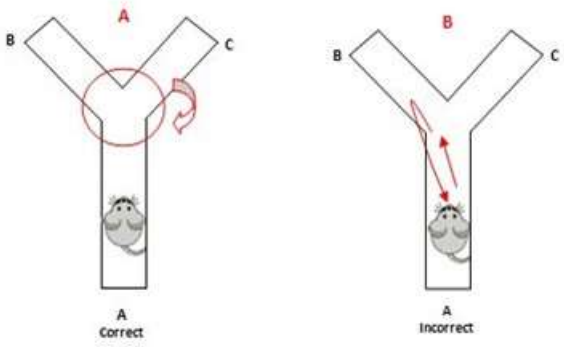

Figure 1: Schematic design showing model (A) as a correct and model (B) as an incorrect alternation in the Y-maze test.

This behavioral test is based on the animals' natural curiosity for exploration and is therefore called the "spontaneous rotation of the Y maze". This test is valid (A) when the animals usually tend to explore a new arm of the maze and the test is invalid (B) when the animals begin to return to the arm they started from and visited earlier. It is known that there are many parts of the brain that are responsible for performing this task, including the septum, hippocampus, prefrontal cortex, and basal forebrain.

Histopathological Analysis

Determination of the changes that occur in the normal or abnormal state of living tissues is known as histopathology. These changes are identified with a microscope where they cannot be observed with the naked eye. In this study, autopsy samples were taken from the brain of rats from all different groups under study. Samples were fixed in saline 10% formalin for 24 h. Brain samples were then washed with tap water followed by dehydration of the samples using serial dilutions of alcohol (methyl, ethyl and absolute ethyl) for drying. After the drying process, the samples were wiped with xylene and then placed in paraffin at 56 degrees in a hot air oven for a whole day (24 h). After obtaining paraffin wax tissue blocks, the tissues were cut at 4 µm by a slide microtome. Obtained tissue sections were collected on glass slides, decaffeinated and stained by hematoxylin and eosin stains (Bancroft and Gamble 2008) for histological examination. Therefore, brain sections were examined and visualized using a Nikon camera to examine tissue pathology.

Statistical analysis

General Liner Models (GLM) of Statistical Analysis System (SAS 1982) was utilized to analyses the data of RT-PCR, values of spontaneous rotation of the Y maze and data of degenerated cells of hippocampus. Subsequently, Scheffé-test was utilized to determine the significant differences between tested groups. All significance statements were based on probability of $P < 0.05$.

RESULTS

Expression alteration of ApoE4 biomarker gene

The expression changes in brain tissues of control, AD and AD treated with vitamin D and mushroom as well as reference drug memantine are illustrated in Figures 2 and 3. Figure 2 and 3 show that expression levels of ApoE4 gene was highly expressed with significant differences ($P<0.02$) in AD group compared to those in control rats. In contrast, the expression levels of ApoE4 gene in the groups treated with treated with vitamin D and mushroom were relatively very close to those in control group. Moreover, pretreatment of rats with vitamin D and then exposed to ALCI3 to induce AD decreased significantly ($P<0.05$) the expression levels of ApoE4 gene in comparison to those in rats exposed to ALCI3 only. Furthermore, pretreatment of rats with mushroom and then exposed to ALCI3 declined considerably ($P<0.05$) the levels of ApoE4 gene in comparison to those in rats exposed to ALCI3 only and to the group of rats pretreated with vitamin D then exposed to ALCI3. On the other hand, the expression levels of ApoE4 gene in AD rats treated with memantine drug inhibited significantly the expression

levels to be the lowest levels compared with all other groups.

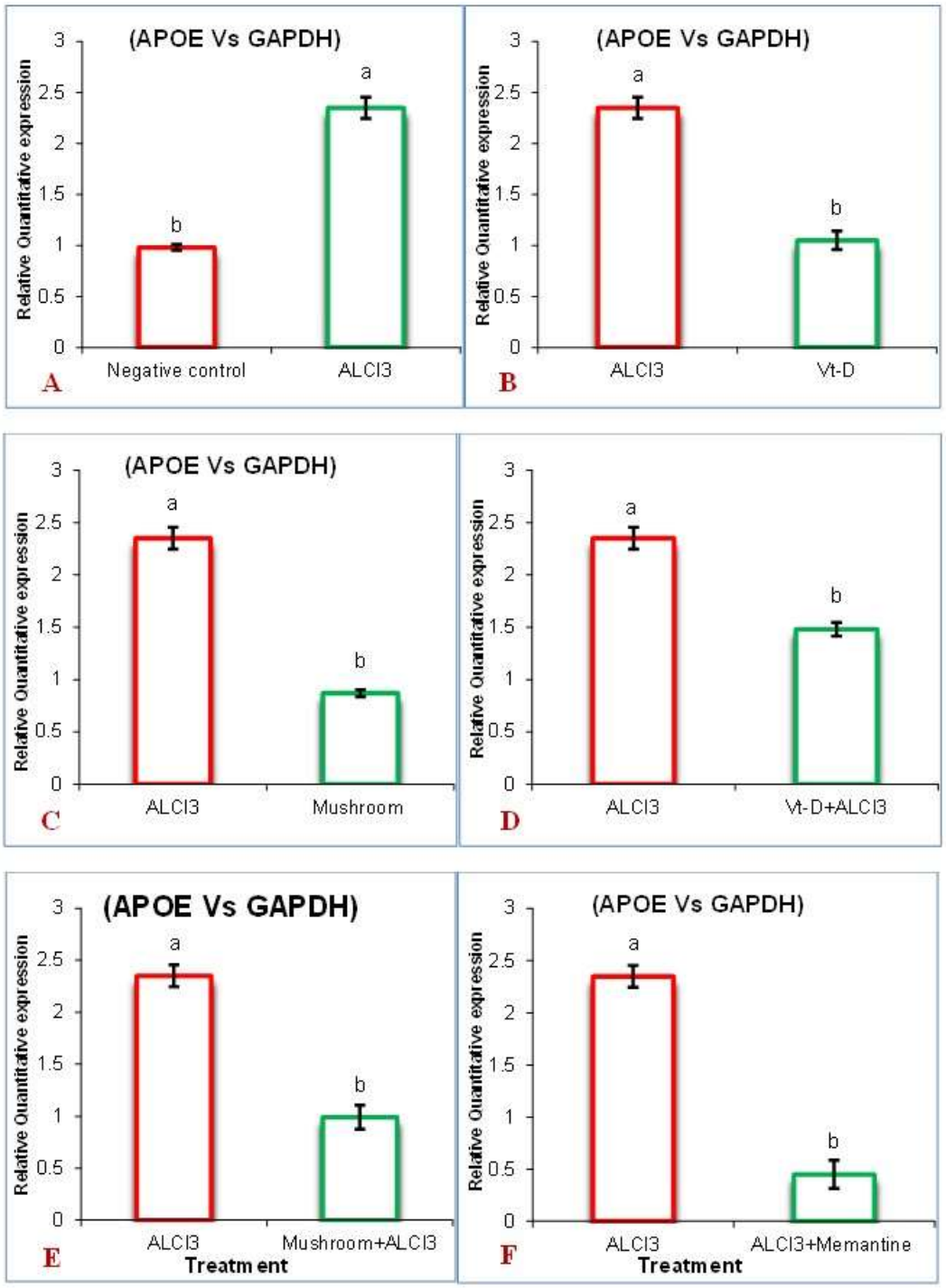

Figure 2: Effect of different treatments (A-F) on the expression alterations of the ApoE4 gene in brain tissues exposed to ALCl3 and/or vitamin D and mushroom. Data are presented as mean ± SEM. a,b: Mean values within tissue with unlike superscript letters were significantly different (P< 0.05).

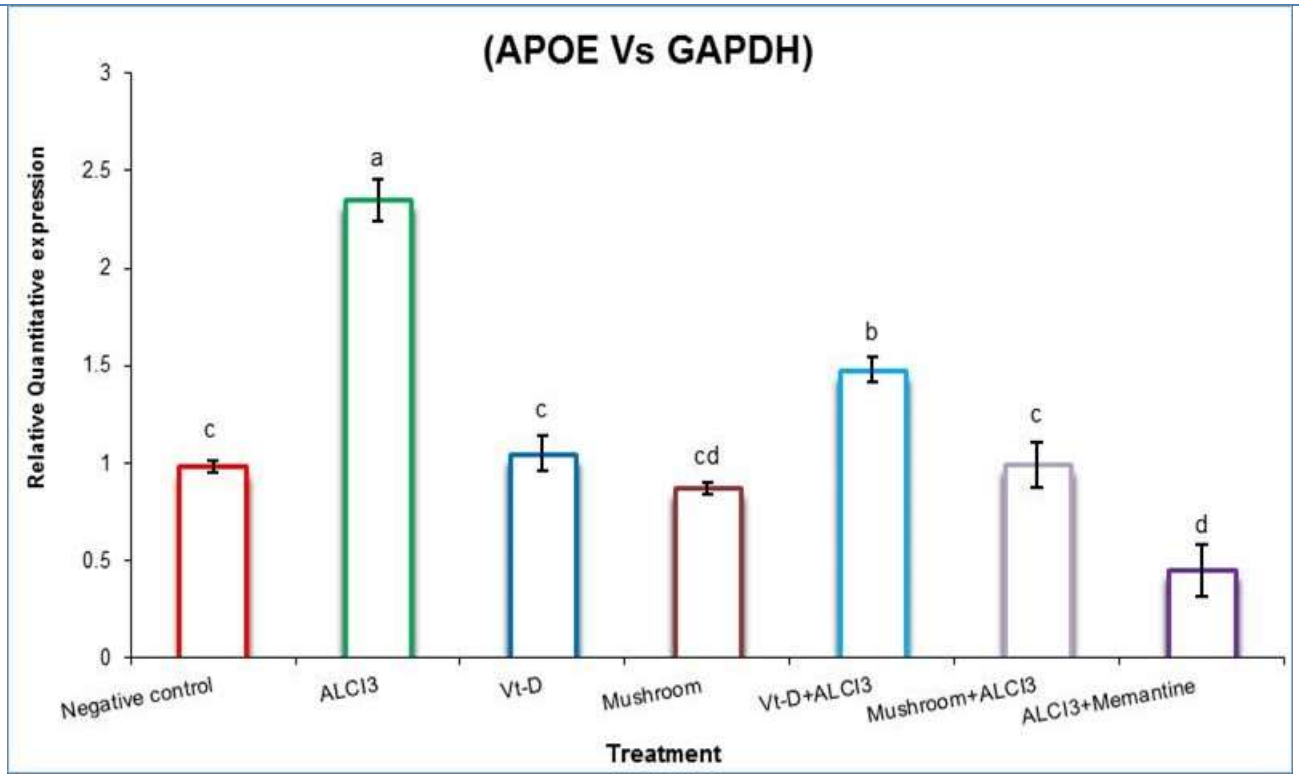

Figure 3: Expression patterns of *ApoE4* in seven different treatments demonstring the changes in the *ApoE4* biomarker in brain tissues. [a,b,c,d:] Mean values within tissue with unlike superscript letters were significantly different (*P*< 0.05)

Behavioral testing

The test of behavior was conducted on rats before induction of AD in which the rats were given orally vitamin D or mushroom for 3 months and then injected with ALCI3 for 4 weeks without any extra treatment. Additionally, this test was performed on rats after induction of AD in which the rats were given orally vitamin D or mushroom for 3 months and then injected with ALCI3 for 4 weeks and continued in treatment with vitamin D or Mushroom for extra 3 months.

The mean values of spontaneous alternation percentage (SA%) of the control and ALCI3 groups were 79.94±7.4 and 48.4±10.0, respectively (P<0.001) (Table 2 and Fig. 4a). For vitamin D treatment, the mean values of SA% of the pretreatment with Vt-D+ALCI3 (Group 5) and positive control group (ALCI3) were 64.56± 2.18 and 48.37±10.02, respectively (P<0.05) (Table 2 and Fig. 4b). Additionally, the mean values of SA% of the post-treatment with Vt-D+ALCI3 (Group 5) and positive control group (ALCI3) were 70.39±2.74 and 48.37±10.02, respectively (P<0.01) (Table 2 and Fig. 4c). However, the mean values of SA% of the pretreatment with Vt-D+ALCI3 and post-treatment with Vt-D+ALCI3 were 64.56± 2.18 and 70.39±2.74, respectively (P>0.05) (Table 2 and Fig. 4d).

Table 2: Behavioral testing of control, $AlCl_3$ (AD) and pre-Vt-D+ALCI3 and post-Vt-D+ALCI3 groups determined as SA percentage

Groups	No of Rats	%SA (Mean±SD)
G1: Control	5	79.98 ± 7.37 [a]
G2: $AlCl_3$	5	48.37 ± 10.02[c]
G5: Vt-D+ALCI3 before	5	64.56 ± 2.18 [bc]
G5: Vt-D+ALCI3 after	5	70.39 ± 2.74[b]

[a,b,c:] Mean values within tissue with unlike superscript letters were significantly different (*P*< 0.05).

For mushroom treatment, the mean values of SA% of the pre-treatment with mushroom+ AlCl3 (Group 6) and positive control group (ALCI3) were 59.58 ± 4.44 and 48.37 ± 10.02, respectively (P<0.05) (Table 3 and Figs. 5&7). Additionally, the mean values of SA% of the post-treatment with Mushroom+ AlCl3 (Group 6) and positive control group (ALCI3) were 66.49 ± 8.26 and 48.37 ± 10.02, respectively (P<0.01) (Table 3 and Figs. 5&7). However, the mean values of SA% of the pretreatment with Mushroom+ AlCl3 and post-treatment with Mushroom+ AlCl3 were 59.58 ± 4.44 and 66.49 ± 8.26, respectively (P<0.01) (Table 3 and Figs. 5&7).

On the other hand, the mean values of SA% of the pretreatment with ALCI3+Memantine (Group 7) and positive control group (ALCI3) were 70.46 ± 3.07 and 48.37 ± 10.02, respectively (P<0.01) (Table 4 and Figs. 6&7). Additionally, the mean values of SA% of the post-treatment with ALCI3+Memantine (Group 7) and positive control group (ALCI3) were 74.22 ± 4.24 and 48.37 ± 10.02, respectively (P<0.001) (Table 4 and Figs. 6&7). However, the mean values of SA% of the pretreatment with ALCI3+Memantine and post-treatment with ALCI3+Memantine were 70.466 and 74.220, respectively (P >0.05) (Table 4 and Figs. 6&7).

Table 3: Behavioral testing of control, $AlCl_3$ (AD) and pre-mushroom+ALCI3 and post-mushroom+ALCI3 groups determined as SA percentage

Groups	No of Rats	%SA (Mean±SD)
G1: Control	5	79.98 ± 7.37[a]
G2: $AlCl_3$	5	48.37 ± 10.02[c]
G5: Mushroom+ALCI before	5	59.58 ± 4.44[b]
G5: Mushroom+ALCI3 after	5	66.49 ± 8.26[b]

[a,b,c:] Mean values within tissue with unlike superscript letters were significantly different (*P*< 0.05).

Table 4: Behavioral testing of control, $AlCl_3$ (AD) and pre-treatment with ALCI3+Memantine and post-treatment with ALCI3+Memantine groups determined as SA percentage

Groups	No of Rats	%SA (Mean±SD)
G1: Control	5	79.98 ± 7.37[a]
G2: $AlCl_3$	5	48.37 ± 10.02[c]
G5: Memantine+ALCI3 before	5	70.46 ± 3.07[b]
G5: Memantine+ALCI3 after	5	74.22 ± 4.24[b]

[a,b,c:] Mean values within tissue with unlike superscript letters were significantly different (*P*< 0.05).

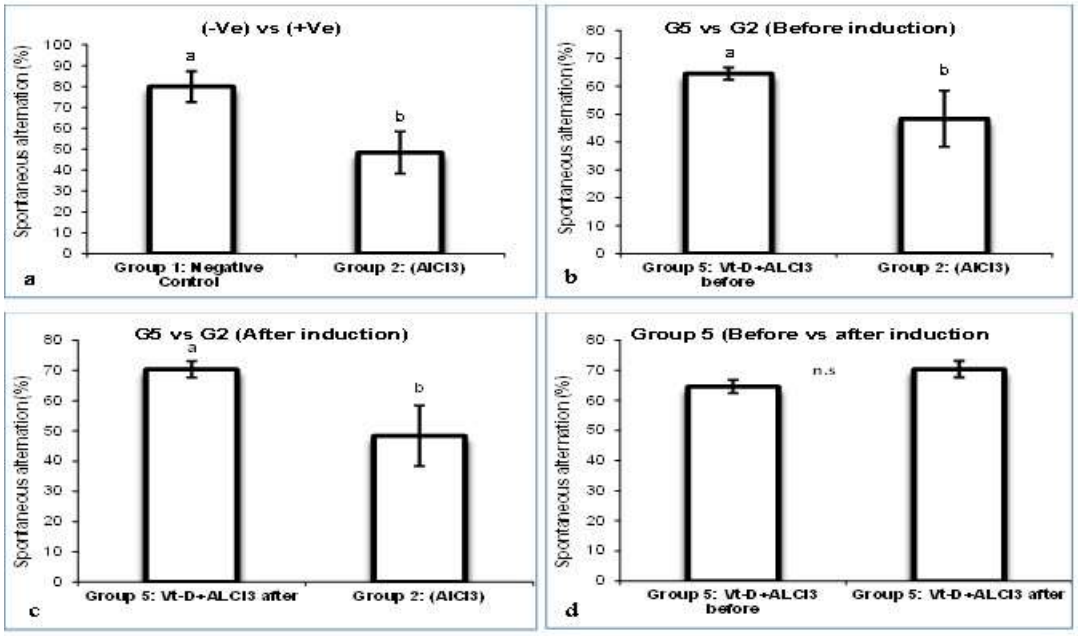

Figure 4: The comparison between spontaneous alternation percentage of control, AD, pre-Vt-D+ALCI3 and post-Vt-D+ALCI3 groups of rats. [a,b:] Mean values within tissue with unlike superscript letters were significantly different (*P*< 0.05).

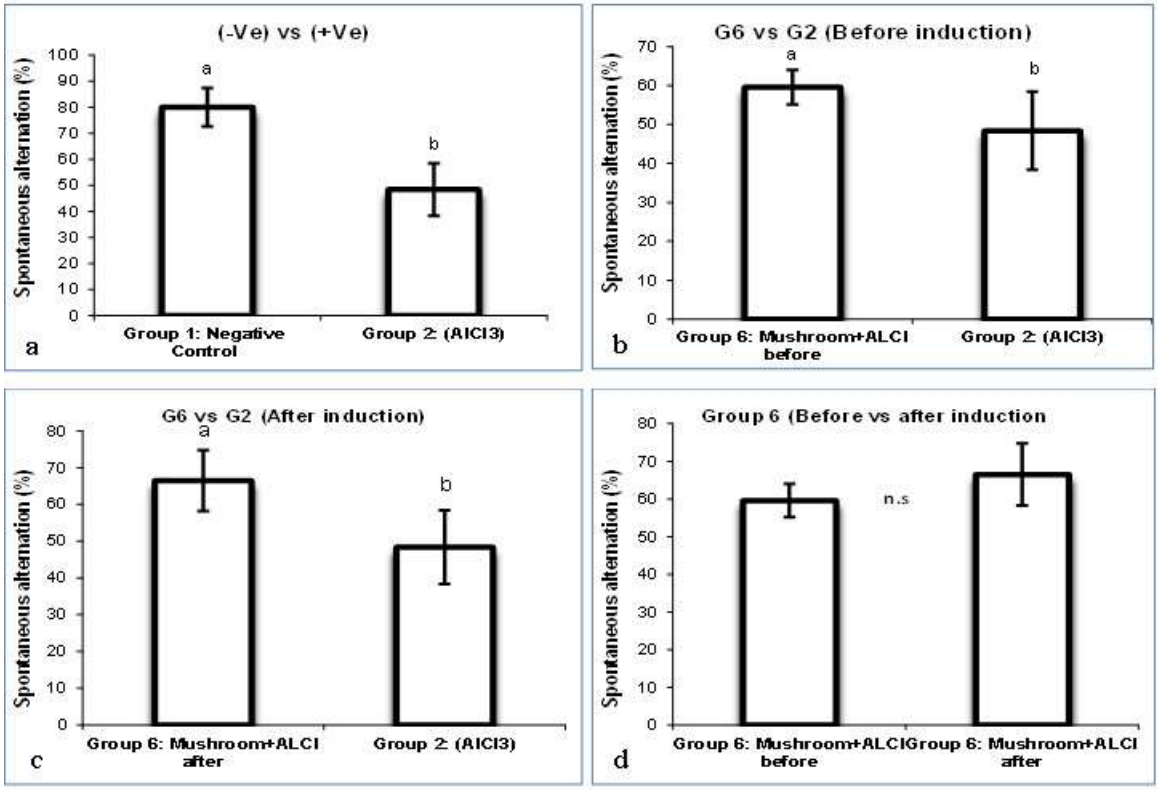

Figure 5: The comparison between spontaneous alternation percentage of control, AD, pre-mushroom+ALCl3 and post-mushroom+ALCl3 groups of rats. [a,b:] Mean values within tissue with unlike superscript letters were significantly different (P< 0.05).

Table 5: Comparison of number of degenerated cells of hippocampus at different areas of Cornu Ammonis (CA1, CA2 and CA3) in different treated groups

Treatment	Degenerated neurons (Mean±SEM)		
	CA1	CA2	CA3
Control	3.01±0.24^{c}	2.34±0.48^{c}	3.36±0.58^{c}
$AlCl_3$	9.03±0.97^{a}	28.67±2.03^{a}	17.60±3.02^{a}
Vt-D	3.62±0.18^{bc}	3.95±0.14^{c}	3.82±0.09^{bc}
Mushroom	3.14±0.53^{c}	3.48±0.28^{c}	3.36±0.71^{c}
Vt-D+$ALCl_3$	4.67±1.02^{b}	6.73±1.08^{b}	5.40±1.05^{b}
Mushroom+$ALCl_3$	4.32±0.72^{bc}	5.59±1.21^{b}	4.77±0.65^{b}
ALCl3+Memantine	4.11±0.94^{bc}	5.44±1.01^{b}	4.13±0.86^{bc}

[a,b,c:] Mean values within tissue with unlike superscript letters were significantly different (P< 0.05).

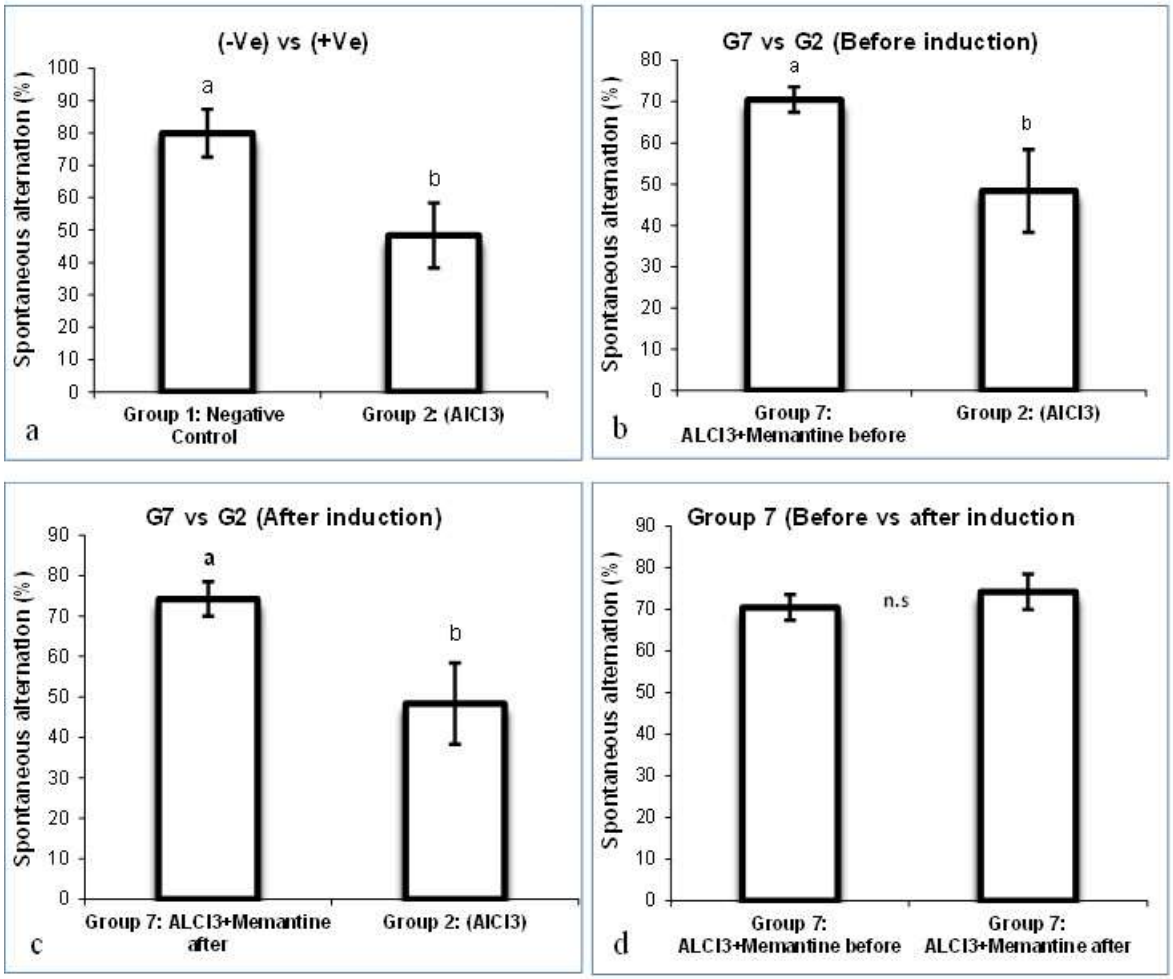

Figure 6: The comparison between spontaneous alternation percentage of control, AD, pre-treatment with ALCl3+Memantine and post-treatment with ALCl3+Memantine groups of rats. [a,b:] Mean values within tissue with unlike superscript letters were significantly different (*P*< 0.05).

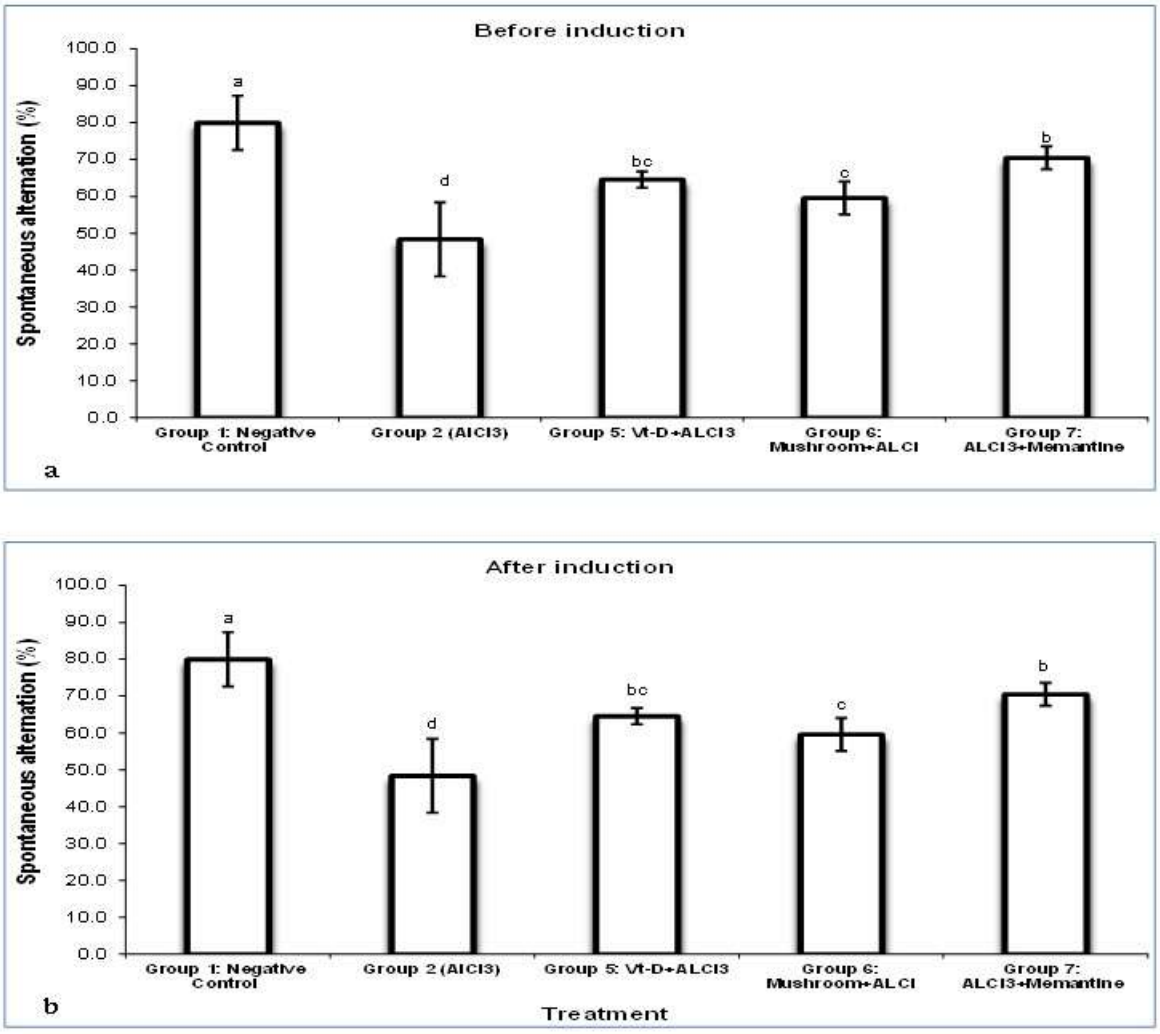

Figure 7: The comparison between spontaneous alternation percentage of all treatment groups before (a) and after (b) induction. [a,b,c,d:] Mean values within tissue with unlike superscript letters were significantly different (*P*< 0.05).

Histopathological examination in brain hippocampus

Different treatment groups including AlCl3, vitamin D, mushroom, AlCl3+ vitamin D; AlCl3+ mushroom and AlCl3+ memantine were used to determine its effects on improving the histopathological alterations in brain hippocampus of male rats (Fig. 8). The results proved that the hippocampal region in control group showed normal architecture of neuronal cell (Fig.8A). Glial cells with its small dark nuclei were infrequently observed and white matter was homogenously stained. Also, the brain sections showed viable pyramidal cells having large vesicular (euchromatic), active nuclei with prominent nucleoli.

In the $AlCl_3$ group, hippocampus showed some neuronal cell loss with mild degeneration and necrotic cells when compared with controls. Shrunken deeply stained pyknotic cells were observed (Fig. 8B). White matter among the cells looked rarified and reticulated with deposition of filamentous material which known as tangles. Nuclei of glial cells were also be seen among neurons

Sections from group received Vitamin D or mushrooms showed nearly neurons cells (Fig. 8C and 8D, respectively). Fig. 8C shows brain tissues treated with Vitamin D which had nearly normal neuronal cell. The viable neuronal cells showed normal vesicular active nuclei. The glial cells could be identified by their smaller nuclei. Fig. 8D shows brain tissues treated with Mushrooms which also had nearly normal neuronal cells. Viable cells showed cytoplasm and nuclei which looked more similar to control group. White matter and glial cells were also observed.

In the group received $AlCl_3$ plus Vitamin D showed less degenerative changes with pyknotic nuclei (Fig. 8E). The brain tissues showed less neurodegeneration with

few pyknotic nuclei. The number of degenerated cells was less compared to AD group treated with $AlCl_3$. White mater looked also less affected compared to non-treated AD group. Viable neuronal cells showed normal vesicular active nuclei. Glial cells were identified by their smaller nuclei.

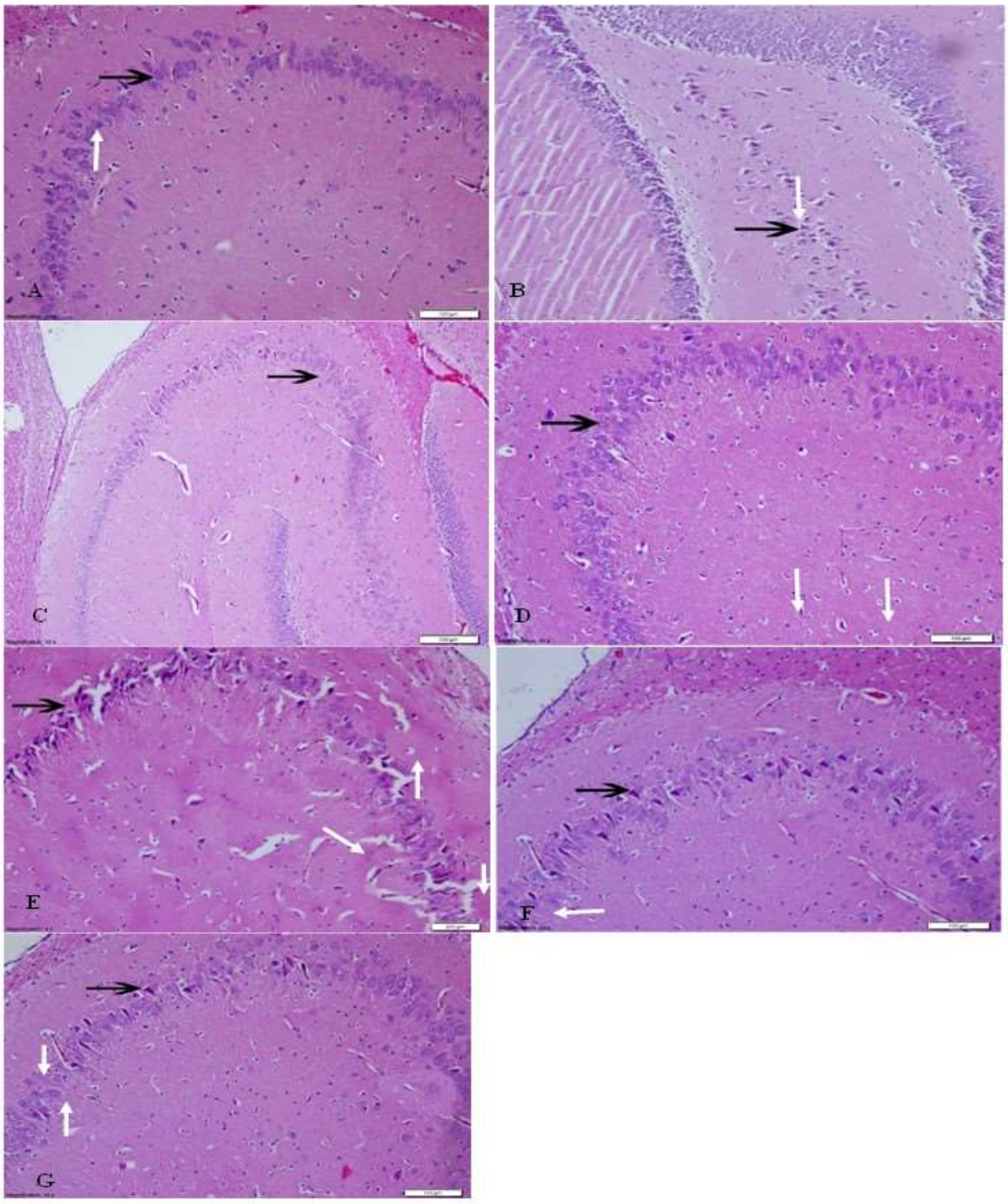

Figure 8: Photomicrograph of hippocampus of different treatments: (A) control group showing normal hippocampus architecture of neuronal cell (arrow); (B) AlCl3 group showing some neuronal cell loss with mild degen¬eration and shrunken, darkly stained pyknotic nuclei (arrow); (C) Vitamin-D group showing nearly normal neuronal cell (arrow); (D) Mushrooms group showing nearly normal neuronal cell (arrow); (E) AlCl3+Vitamin-D group showing less neurodegeneration with few pyknotic nuclei (arrow); (F) AlCl3+Mushrooms group showing noticeable improvement with slight pyknotic nuclei (arrow); (G) AlCl3+Memantine group showing remarkable improvement with few pyknotic nuclei (H & E).

However, in the group received $AlCl_3$+ memantine showed remarkable improvement in the neuronal cells (Fig. 8G). Moreover, some pyknotic cells were noticed in the group of $AlCl_3$+ memantine (Fig. 8G). There was also apparent decrease in the number of degenerated cells. Viable cells showed cytoplasm and nuclei which looked relatively similar to control group. Additionally, white matter and glial cells were also showed.

Assessment of the number of degenerated neurons in the hippocampal sub-regions Cornu Ammonis (CA1, CA2 and CA3)

The number of degenerated neurons in the hippocampal sub-regions (CA1, CA2 and CA3) in the tested groups is demonstrated in Table 5. It was observed that the numbers of degenerated neurons which were deeply stained in CA1, CA2 and CA3 of AD group was increased significantly ($P<0.01$) compared with control group. However, the numbers of degenerated neurons in CA1, CA2 and CA3 of the groups of rats treated with vitamin D and mushroom were quite close to those in control without significant differences. Furthermore, pretreatment of rats-induced with AD with vitamin D decreased significantly ($P<0.05$) the number of degenerated neurons in CA1, CA2 and CA3 compared with those of rats exposed to $AlCl_3$ (AD). In the same line, pretreatment of rats-induced with AD with mushroom (mushroom+ $AlCl_3$) or treatment of AD rats with Memantine ($AlCl_3$+Memantine) declined significantly ($P<0.05$) the number of degenerated neurons in CA1, CA2 and CA3 compared with those of rats exposed to $AlCl_3$ (AD). The efficiency of the tested materials which inhibited number of degenerated neurons in CA1, CA2 and CA3 has been arranged as follows Memantine > mushroom > vitamin D (Table 5).

DISCUSSION

The attention on the implication of vitamin D_3 deficiency as a risk factor of AD is among the interests of scientists in brain research (Littlejohns et al. 2014). The neuroprotective effects of vitamin D_3 and its capability in preventing the oxidative stress consequences were also reviewed by Kalueff et al. (2004) and Panza et al. (2021). The present study was designed to evaluate the possible protective and possible therapeutic efficacy of vitamin D_3 and mushroom (as good source of vitamin D after its exposure to UV light) on the expression of AD biomarker (ApoE4 gene), behavioral deficits and neuropathological in the hippocampus of the AD model in rats induced by $AlCl_3$.

The results proved that expression levels of ApoE4 gene was over-expressed significantly in AD group compared to those in control rats. However, pretreatment of rats with vitamin D or mushroom and then exposed to ALCI3 to induce AD decreased significantly the expression levels of ApoE4 gene in comparison to those in rats exposed to ALCI3 only.

In consistent with our findings, Dorey el al. (2017) reported that over-expression of ApoE4 enhances the AD neuroinflammation in in-vivo and in-vitro systmes. Additionally, they found that Vitamin D relieves the AD neurodegeneration or/and neuroinflammation through activating the vitamin D receptor (VDR). Moreover, Fedotova et al. (2019) showed that vitamin supplementation reduced the manifestations of anxiety-like appearance in old ovariectomized rats. So, Vitamin D is effective in treating anxiety-like conditions in older people with long-term estrogen deficiency. Also, Vitamin D_3 supplementation with vitamin E was more effective in reducing the oxidative stress in AD rats (Mehrabadi and Sadr 2020). They proved also that vitamin D_3 and E and their combination could improve memory, reduce neuronal loss, learning deficits and oxidative stress in an Alzheimer's disease model.

Moreover, Alrefaie and Alhayani (Alrefaie and Alhayani 2015) reported that diabetic rats with complication in brain cortex showed a much longer time exploring new things than the control animals. Treating diabetic mice with vitamin D_3 significantly reduced the impairment caused by diabetes so that the animals again spent much less time exploring the new things (Alrefaie and Alhayani 2015). In agreement with these observations our results found that the mean values of SA% of the control were higher than those in $ALCI_3$ group indicating that AD rats spent much more time coincided with incorrect way to exploring the new things. However, vitamin D pre-treatment plus AD (Vt-D+ALCI3) exhibited SA% higher than those in AD alone. Alrefaie and Alhayani (Alrefaie and Alhayani 2015) indicated that acetylcholinesterase (ache) activity was significantly increased in AD-animals treated with vitamin D compared to control animals. It was found that treatment with vitamin D attenuated the changes caused by diabetes with degenerative brain cells in enzyme activity in the prefrontal cortex of the brain (Campanari et al. 2014). So, our results suggested that vitamin D could improve cognitive function in AD which might be produced by enhancing the cholinergic transmission of the prefrontal cortex.

Ergosterol, of which mushrooms are a rich source, can be converted into vitamin D2 by UV treatment. So mushrooms are a new and convenient food source for Vitamin D2 (Bennett et al. 2013). Exposure of mushrooms to ultraviolet irradiation is increasing Vitamin D2 synthesis (Koyyalamudi et al. 2009, Jasinghe et al. 2006, Ozzard et al. 2008) and enhancing the mineral density which increased calcium uptake in mice bones (Lee et al. 2009). Therefore, the high level of vitamin D2 in mushrooms makes mushrooms rich in vitamin D an exciting target for testing its efficacy on brain function and cognition. Some studies in mice of the Alzheimer's disease model have shown the ability of vitamin D3 to reduce cognitive decline and improve brain cells (Yu et al. 2011). Furthermore,

mushroom extracts have been shown to attenuate cognitive dysfunction in chemical-induced Alzheimer mouse models (Lee et al. 2011).

Feeding AD mice 0.5% mushrooms remembered the location of the core system by showing better learning from one experience than AD mice without mushroom feeding, demonstrating an improvement in spatial working memory. This finding is in line with the study by Nurk et al. (2010), where there was a positive association between improved sensory perception and mushroom consumption with improved semantic memory and executive function in the elderly. The beneficial effects of mushroom consumption on improving memory in AD mice could be due to vitamin D2 supplementation or the interaction between other bioactive compounds (such as selenium and vitamin C) in mushrooms and vitamin D2 (Cardwell et al. 2018).

Histological examination combined by quantitative morphometric studies were used to evaluate the efficacy of vitamin D_3 supplementation to ameliorate the alteration in rat hippocampal sub-regions (CA1, CA2 and CA3), that results from $AlCl_3$-induced insult. The hippocampus of rat is composed of several regions, and includes the hippocampus proper (with its three sub-regions: CA1, CA2 and CA3), the dentate gyrus and subiculum (Kamsu *et al.* 2013).

$AlCl_3$ is an identified neurotoxin that has been linked to AD since it leads to neuronal inflammation, worsen the oxidative injury of the brain and triggers the deposition of Aβ, which impairs the working memory (Aboelwafa *et al.* 2020). $AlCl_3$ was also reported to possess the ability of crossing the blood brain barrier (BBB) and the accumulation in many brain regions (Haider *et al.* 2020).

In the present study, histopathology sections demonstrated the presence of marked histological alterations in all hippocampal sub-regions especially in CA2 in rats treated with $AlCl_3$. Histological changes included neuronal shape deformity, shrinkage and dark cytoplasmic staining with degenerated pyknotic nuclei. Our data are in consistence with the results reported by Zhao et al. (2020), in which the neurons in the hippocampi of rats treated with $AlCl_3$ (100 mg/kg./b.wt) for 60 days were shrunken and had hyperchromatic nuclei.

The results are also consistent with the study of Haider *et al.* (2020) in which neuronal cytoplasm in the hippocampi of rats treated with $AlCl_3$ (150 mg/kg/b.wt) and D-galactose (300 mg/kg/b.wt) for one week, was reported to be darkly stained. The present results also go in hand with that of Khalaf *et al.* (2020), which reported the presence of several apoptotic bodies in the hippocampi of Sprague-Dawley rats treated with $AlCl_3$ (100 mg/kg. b.wt, p.o.) for 42 days. Furthermore, the results of the current study are also similar to those previously reported by Aboelwafa *et al.* (2020), who demonstrated marked alterations in the hippocampi of rats treated with $AlCl_3$ (17 mg/kg.b.wt) for 15 days. The presence of acidophilic extracellular neurofibrillary tangles (NFT) among dark stained degenerated neurons could be explained in view of the study done by Haider *et al.* (2020). They reported that changes in the morphology of the neurons are the most common reported characteristic of $AlCl_3$ toxicity which causes functional and structural alterations in cytoskeletal proteins, the buildup of hyperphosphorylated proteins that leads to NFT formation and the breaking of axons and dendritic system with subsequent neurodegeneration.

In the present study, counting the number of degenerated (apoptotic) neurons was used to evaluate the neurotoxic degenerative effects of $AlCl_3$ and the possible preventive or therapeutic role of vitamin D_3 against this effect. $AlCl_3$ administration was found to result in a significant increase in the number of dark stained degenerated neurons in CA2 and CA3 in comparison to normal. Their number was also increased in CA1 in comparison to normal. The results of this study are consistent with the one reported by Auti and Kulkarni, (Auti and Kulkarni 2019), in which the induction of AD in male Albino Wistar rats, with $AlCl_3$ (100 mg/kg, p.o.), for 42 days resulted in neurodegeneration in their hippocampi neurons. These results are also consistent with the results of Chiroma *et al.* (2018) in which the pyramidal cells of all three hippocampus areas were shown to be markedly degenerated in male Albino Wistar rats treated with $AlCl_3$ (100 mg/kg, p.o.) and D-galactose for 10 weeks. Ravi *et al.* (2018), also reported neurodegenerative alterations in CA1 and CA3 sub-areas of male Albino Wistar rats treated with $AlCl_3$ (300 mg/kg, p.o.), for 60 days. When, $AlCl_3$ enters the brain, it affects slow and fast axonal transport, causes structural abnormalities and inhibits long-term potentiation, thereby causing neurodegeneration. Other $AlCl_3$ effects include the depletion of the brain's cell number as clarified by Chiroma *et al.* (2018), $AlCl_3$ also fastens the process of lipid peroxidation and enhances the production of free radicals, which in turn causes severe neurotoxicity (Aboelwafa *et al.* 2020). AlCl3 was also reported to result in AD-like symptoms in animals as it led to depletion of neuronal elements in the hippocampal region. The neurotoxic effect of $AlCl_3$ was reported to be via enhancing formation of free radicals (ROS) with subsequent lipid peroxidation and neuronal degeneration (Zhang et al. 2020).

Moreover, when the AD rats treated with vitamin D_3 were compared with the AD model, it was found that vitamin D_3 treatment significantly decreased the number of degenerated neurons in CA1, CA2 and CA3. Antioxidant vitamins including vitamin D are considered to exert potent neuroprotective effect (Lee *et al.* 2020) and vitamin D deficiency especially in elderly people is implicated in AD development and cognitive impairment (Mohajeri *et al.* 2015). In experimental animals, vitamin D was reported to improve cognition via the stimulation and improvement of neurogenesis (Mohajeri *et al.* 2015).

CONCLUSION

Over-expression of the ApoE4 biomarker, behavioral deficits and neuropathological combined with number of degenerated neurons provided an evidence for $AlCl_3$ neurotoxic degenerative effects that simulate what occurs in AD disease in human. So, the severe neurotoxicity resulted from $AlCl_3$ inducing AD rats could be attributed to the enhancement of oxidative stress stimulating free radicals generation. Such changes were much ameliorated with vitamin D_3 and mushroom supplementation given as both protective during the induction of AD rat model or as therapeutic effects after establishment of the model. The results of this study may support the proposal for further research to elucidate the exact underlying mechanism and optimal doses of vitamin D and mushroom supplementation and to design clinical trial plans in Alzheimer's patients.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

All the authors declare to have made substantial contributions to the conception, or design, or acquisition, or analysis, or interpretation of data; and drafting the work or revising it critically for important intellectual content. All authors have read and approved the final version of the manuscript.

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