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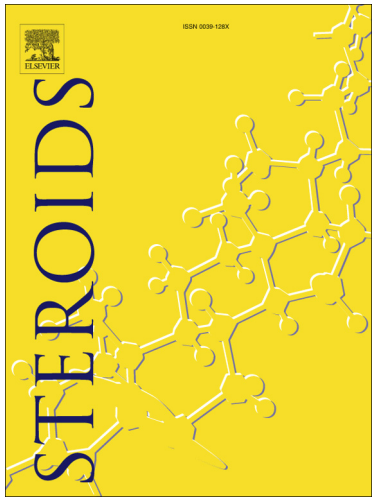

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Active Form of Vitamin D Analogue Mitigates Neurodegenerative Changes in Alzheimer's Disease in Rats by Targeting Keap1/Nrf2 and MAPK-38p/ERK signaling pathways

Shimaa Saad El-Din[1]*; Laila Rashed[1]*; Engy Medhat[1]*; Basma Emad Aboulhoda[2]*; Ahmed Desoky Badawy[3]*, Asmaa Mohammed Shamseldeen[4]*; Marwa Abdelgwad[1]*

[1] The Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Cairo University, Egypt.

[2] The Department of Anatomy, Faculty of Medicine, Cairo University, Egypt.

[3] The Department of Physiology, Faculty of Medicine, October 6 University.

[4] The Department of Physiology, Faculty of Medicine, Cairo University.

Abstract:

Background: The nuclear factor erythroid2-related factor2 (Nrf2), a chief transcriptional regulator of antioxidant response element (ARE), is considered a promising target for the prevention of Alzheimer's disease (AD). Vitamin D has been recognized to have a crucial role in improving AD cognitive functions. The present study was conducted to evaluate the effects of active vitamin D analogue, Maxacalcitol, on Keap1-Nrf2 signaling pathway in experimental Alzheimer's disease in rats.

Materials and Methods: The study was conducted on thirty female white albino rats divided equally into 3 groups: control group, Alzheimer group induced by Lipopolysaccharide and Alzheimer group treated with active vitamin D3 analogue, Maxacalcitol. The following parameters were assessed in rat brain tissues: Gene expression of Nrf2, Keap1 and MAF by RT-PCR, protein levels of phosphorylated MAPK-38p and ERK1/2 by Western Blot Technique, estimation of HO-1, Amyloid β, p-Tau levels and serum levels of TNFα, IL-10 and total 25-hydroxyvitamin D, serum calcium levels, GSH and MDA levels were also estimated in addition to cognitive function tests and histo-pathological examination of rat brain tissues.

Results: In Alzheimer group, there was a significant deficit in cognition along with down-regulation of gene expression of Nrf2 and the protein levels of its downstream antioxidant effectors (HO-1 and GSH) with increased levels of the lipid peroxidation biomarker MDA. Also, there was increased neuro-inflammation

as evidenced by increased levels of TNFα and decreased levels of IL-10. Moreover, there were increased amyloid β load and enhanced levels of phosphorylation of MAPK-38 and ERK1/2 leading to hyperphosphorylation of Tau protein. In addition, there were decreased serum levels of both total 25-hydroxyvitamin D and calcium. Treatment with vitamin D3 analogue, Maxacalcitol significantly improved cognitive dysfunction and histopathological picture of the brains of Alzheimer rats. Also, Vitamin D analogue significantly increased expression of Nrf2 and its downstream effectors (HO-1 and GSH), improved serum levels of total 25-hydroxyvitamin D and calcium, decreased neuro-inflammation and Amyloid β load as well as hyperphosphorylation of MAPK-38, ERK1/2 and tau proteins were also observed. Therefore, these data suggest that vitamin D analogue, Maxacalcitol could be used as a therapeutic agent in treatment of Alzheimer disease.

Key words: Keap1–Nrf2 pathway, Active vitamin D analogue (Maxacalcitol), Alzheimer's disease.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease which is considered the most common cause of dementia in the aging population **[1,2].** It causes brain cells to waste away gradually and eventually die, leading to progressive deficits of memory and other cognitive functions causing complete incapacity and death within 3–9 years of diagnosis **[3].** Pathological hallmarks of AD include structural changes induced by the extracellular deposition of amyloid β peptides forming senile plaques and intracellular neurofibrillary tangle of hyper-phosphorylated tau proteins in the brain **[4].**

Increasing evidence supposed that down-regulation of the cell protective transcription factor Nuclear Factor E2-Related factor 2 (Nrf2) may promote neuronal susceptibility to oxidative stress and molecular damage **[5,6],** and that its activation may grant neuronal protection **[7].** The Nrf2 transcription factor is tightly regulated by the repressor protein, Keap1 (Kelch-like ECH-associated protein 1), in the cytoplasm, which has crucial role in Nrf2 degradation by the ubiquitin–proteasome pathway **[8,9].**

Under oxidative stress, Nrf2 dissociates from Keap1, translocates to the nucleus and upregulates several cytoprotective antioxidant genes to combat the oxidative stress **[10,11].** The antioxidant defense system includes a number of enzymes, such as heme oxygenase-1 (HO-1), superoxide dismutase, glutathione peroxidase, glutamate–cysteine ligase, NADPH: quinone oxidoreductase 1 (NQO-1), catalase,

thioredoxin and glutathione S-transferase **[12,13].** The genes of these antioxidant enzymes are transcriptionally regulated by their upstream antioxidant response element (ARE) **[14].** Nrf2 is considered the key ARE-binding transcription factor **[15].** Therefore, it is crucial to figure out new therapeutic agents targeting the Keap1–Nrf2–ARE signaling pathway to overcome the oxidative stress-mediated diseases such as AD **[16,17].**

There is increasing evidence that Vitamin D deficiency can lead to a decrease in neurological functions such as cognition and can substantially increase the risk of AD **[18].** It may contribute to aging process through dys-regulation of redox cell signaling pathways **[19].** Emerging evidence supports the pivotal role of vitamin D in attenuating oxidative stress via increased Nrf2 and up-regulation of the expression of genes encoding antioxidant enzymes, as well as adjusting the levels of ROS through controlling the cellular antioxidants **[20,21].** Here, we aim to evaluate the effect of vitamin D in the regulation of Keap1–Nrf2 pathway and its importance as a potential therapeutic drug for AD.

MATERIALS AND METHODS

All animals were purchased from the animal house of research institute of ophthalmology (RIO). The study protocol was approved by Institutional Review Board approval and the animal house of RIO. The study was conducted in Unit of Biochemistry and Molecular Biology at the Medical Biochemistry Department, and Physiology Department, Faculty of Medicine, Cairo University. The rats were housed in standard cages under room temperature (25 ± 2 ^{o}C) and humidity.

Preparation of experimental animal model:

This study included thirty female adult albino rats, inbred strain (Cux1: HEL1) of matched age and weight (6months-1year and 120-150gm)**.** Rats were maintained according to the standard guidelines of Institutional Animal Care and Use Committee. Animals were maintained in an air conditioned animal house with specific pathogen free conditions. They were divided equally into 3 groups each contains 10 rats. Group 1: Negative control group (normal healthy rats), group 2: Alzheimer's rat model (pathological control) and group 3: Alzheimer's rats received intraperitoneal injection of active vitamin D3 analogue, maxacalcitol, (at a dose of 1 µg per kg of body weight) twice a day for 4 weeks by an insulin syringe **[22,23].**

-Experimental Alzheimer's rat model was induced by intraperitoneal injection of 0.8 mg/kg of Lipopolysaccharide (LPS) once a week for three weeks **[24].** Then, Alzheimer's disease was confirmed after histo-pathological examination of the hippocampus. Thereafter we started treatment with vitamin D for group 3.

-Cognitive examination using T- maze test: At the planned time (7 weeks), Cognitive functions of the rats were assessed using an enclosed elevated T-shaped apparatus. All studied rats were tested for the total time consumed and the percentage of spontaneous alternation **[25].**

Then, animals were sacrificed by cervical dislocation and venous blood was collected from the retro-orbital veins from rats of all groups for assessment of serum levels of calcium, total 25-hydroxyvitamin D, IL-10 and TNFα. The brains were immediately removed from the euthanized animals and divided along the mid sagittal plane. One hemisphere was fixed in formal saline for 24 hours, washed in serial dilutions of alcohol for dehydration then specimens from the hippocampus were cleared in xylene and embedded in paraffin at 56^{o} in hot air oven for 24 hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 µs thickness by slidge microtome, collected on glass slides, deparaffinized and stained by H&E stain for histopathological examination. From the other hemisphere, parts of the hippocampus were kept frozen at -80°C until assessment of the following: quantitative measurement of gene expression of NRF2, Keap1 and MAF by QRT-PCR, estimation of protein levels of MAPK-38p and ERK1/2 BY western blot technique, assessment of protein levels of Amyloid β, Tau protein by ELISA and assessment of MDA, GSH and HO-1 protein levels by colorimetric methods.

Quantitative real time (QRT)-PCR gene expression of NRF2, Keap1 and MAF in rat brain tissues:

-Total RNA was extracted from brain tissues and homogenization was done by the RNeasy Purification Reagent **(Promega, Madison, WI, USA)** according to the manufacturer's protocol. The extracted RNA was quantified by spectrophotometry **(JENWAY, USA)** at 260 nm.

-The extracted RNA was reverse-transcribed into cDNA by a Reverse Transcription System Kit **(#K1621, Fermentas, USA).** The cDNA was generated from 5 µg of total RNA extracted with 1 µL (20 pmol) of antisense primer and 0.8 µL of superscript AMV reverse transcriptase for 60 min at 37°C.

-The relative abundances of the mRNA species were assessed by the SYBR® Green method and an ABI Prism7500 Sequence Detector System Applied Biosystem with software version 3.1 **(StepOne™, USA).** The PCR primers used were designed with Gene Runner Software **(Hastings Software Inc., Hastings, NY, USA)** from RNA sequences in GenBank **(Table 1).**

Table 1. Primers Sequences of Nrf2, Keap1 and MAF

Parameter	Forward and Reverse Primers	Gene bank accession number
Nrf2	Forward primer: 5′ CACATCCAGACAGACACCAGT 3′ Reverse primer: 5′ CTACAAATGGGAATGTCTCTGC 3′	NM_031789
Keap1	Forward primer: 5′ GGACGGCAACACTGATTC 3′ Reverse primer: 5′ TCGTCTCGATCTGGCTCATA 3′	NM_057152
MAF	Forward primer: 5′ AAGGAGGAGGTGATCCGACT 3′ Reverse primer: 5′ CTGGTTCTTCTCCGACTCCA 3′	NM_019318.1
GAPDH (House keeping)	Forward primer: 5′ AGGTTGTCTCCTGTGACTTC 3′ Reverse primer: 5′ CTGTTGCTGTAGCCATATTC 3 ′	NM_017008

-All of the primer sets had a calculated annealing temperature of 60°C. Quantitative RT-PCR analysis was performed in duplicate in a 25-µL reaction volume consisting of 2× SYBR Green PCR Master Mix **(Applied Biosystems, USA)**, 900 nM of each primer, and 2–3 µL of cDNA. The amplification conditions were 2 min at 50°C, 10 min at 95°C, and 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 10 min. Data from the real-time assays were calculated by Sequence Detection Software version 1.7 **(PE Biosystems, Foster City, CA, USA)**. The relative expression levels of Nrf2, Keap1 and MAF were calculated by the comparative Ct method as stated by the manufacturer recommendations **(Applied Biosystems, USA).**

Estimation of protein levels of phosphorylated MAPK-38p and ERK1/2 in rat brain tissues by Western Blot Technique (usingV3 Western Workflow™ Complete System, Bio-Rad® Hercules, CA, USA):

-Homogenization of 5 mg of brain tissue in RIPA buffer was done, then centrifugation at 12,000 rpm for 20 minutes. Thereafter, we determined the

protein concentration for each cell lysate using Bradford assay. Equal amounts of protein (20-30 µg of total protein from cell lysate) were separated by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. The membrane was blocked in TBS buffer containing 5% skim milk and 0.1% Tween 20 at room temperature for 1 h and incubated with the primary antibodies for MAPK-38p and ERK1/2 supplied by Thermoscientific overnight at pH 7.6 at 4 °C with gentle shaking. After washing, we added peroxidase-labeled secondary antibodies and the membranes were incubated at 37 °C for 1 h. Band intensity was analyzed by ChemiDoc™ imaging system with Image Lab™ software version 5.1 (Bio-Rad Laboratories Inc., Hercules, CA, USA). Beta actin were used to normalize the protein levels of MAPK-38p and ERK1/2.

Estimation of HO-1 levels (Enzo Life Sciences, ANN Arbor, MI), Amyloid β and p-Tau levels in rat brain tissues by ELISA (MyBiosource sunny Southern California, San Diego, USA) according to the manufacturer`s recommendations**.**

Estimation of TNF-α and IL-10 serum levels by ELISA (Q&D system, Quatin, USA) according to the manufacturer`s recommendations.

Estimation of serum levels of total 25-hydroxyvitamin D by ELISA (Cat.No: DEIA2219, Creative Diagnostics, NY, USA) according to the manufacturer`s recommendations.

Estimation of serum calcium levels by colorimetric assay kit (BioVision Incorporated, CA, USA) according to the manufacturer`s recommendations.

Estimation of the levels of MDA and GSH in rat brain tissues by routine colorimetric methods:

-For measurement of MDA, we added thiobarbituric acid (TBA) in 2 Mol. sodium sulfate, 2.5 ml of 20% trichloroacetic acid and 1 ml of 0.67% TBA to 0.5 ml of tissue homogenate, then the mixture is heated in a boiling water bath for 30 min. The resulting chromogen is extracted with 4 ml of N butyl alcohol and the absorbance of the organic phase is determined at the wave length of 530 nm.

-As regards GSH, brain tissues were homogenized in 5–10 ml cold buffer (i,e, 50 mM potassium phosphate, pH 7.5. 1 mM EDTA) per gram tissue. The homogenate was centrifuged at 100,000 x g for 15 minutes at 4 °C. Then, the supernatant was removed for assay and stored on ice. Reduction of 2-nitrobenzoic acid with GSH

produced a yellow compound. The reduced chromogen is directly proportional to GSH concentration and its absorbance can be measured at 405 nm.

Statistical Analysis

Data were coded and entered using the graphpad prism version 7. Data was summarized using mean ± standard deviation. Comparisons between groups were done using analysis of variance (ANOVA) with Tukey's multiple comparisons test when comparing more than 2 groups **[26].** P-values less than 0.05 were considered as statistically significant**.**

RESULTS

Assessment of inflammatory and oxidative biomarkers

-As shown in **table (2),** regarding the serum levels of the neuro-inflammatory cytokine TNFα, there was a significant increase in Alzheimer group compared to the control group. However, there was a significant decreased levels in vitamin D analogue (Maxacalcitol) treated group compared to the Alzheimer group.

-Regarding the anti-inflammatory cytokine IL-10 serum levels, there was a significant decrease in Alzheimer group compared to the control group, while there was a significant increase in Maxacalcitol treated group compared to Alzheimer group.

-As we show here, there was a significant decrease in the antioxidant levels, HO-1 and GSH in Alzheimer group compared to the control group. Whereas, there was a significant increase in their levels in Maxacalcitol treated group compared to Alzheimer group. Whereas, the protein level of malondialdehyde (MDA), a marker of lipid peroxidation, in rat brain tissues showed a significant increase in Alzheimer group as compared to the control group. While there was a significant decrease in Maxacalcitol treated group compared to Alzheimer group. These data support that active vitamin D analogue, Maxacalcitol, decreased the oxidative stress biomarker and increased the antioxidant biomarkers.

Table (2): Comparison of IL-10, TNFα, HO-1, GSH and MDA levels in all studied groups

	Group		
	Control	**Alzheimer**	**Alzheimer+Active Vitamin D analogue**
TNFα (pg/mL)	16.15± 2.204	122.9± 5.168 *	66.2± 12.04 *#

IL-10 (pg/mL)	129± 18.17	69.31± 7.702 *	96.2± 4.911 *#
HO-1 (mmol/mg protein)	851.6± 123.7	383.7± 72.15 *	701.5± 69.74 #
GSH (mmol/mg protein)	58.03± 5.389	22.33± 3.137 *	41.23± 6.715 *#
MDA (nmol/mg protein)	7.675± 1.778	71.28± 16.81 *	29.1± 6.527 *#

Values are presented as mean ± SD. Statistically significant compared to corresponding value in: control group (*), Alzheimer group (#). ($P<0.05$)

Effect of active vitamin D analogue, Maxacalcitol, on Nrf2–Keap1-ARE pathway

-There was a significant decrease in the gene expression of Nrf2 in Alzheimer group compared to the control group. Whereas, there was a significant increase in their levels in active vitamin D analogue treated group compared to Alzheimer group. Also, the gene expression of MAF which has a crucial role in binding of Nrf2 to the ARE in DNA was significantly decreased in Alzheimer group compared to the control group, but there was no significant difference between Maxacalcitol treated group and Alzheimer group.

-However, there was a significant increase in gene expression of the repressor protein Keap1 in Alzheimer group compared to the control group. While there was a significant decrease in Maxacalcitol treated group compared to Alzheimer group. Thus, active vitamin D analogue, Maxacalcitol, ameliorated dysregulation of Nrf2-Keap-1 pathway in Alzheimer' s disease **(figure 1).**

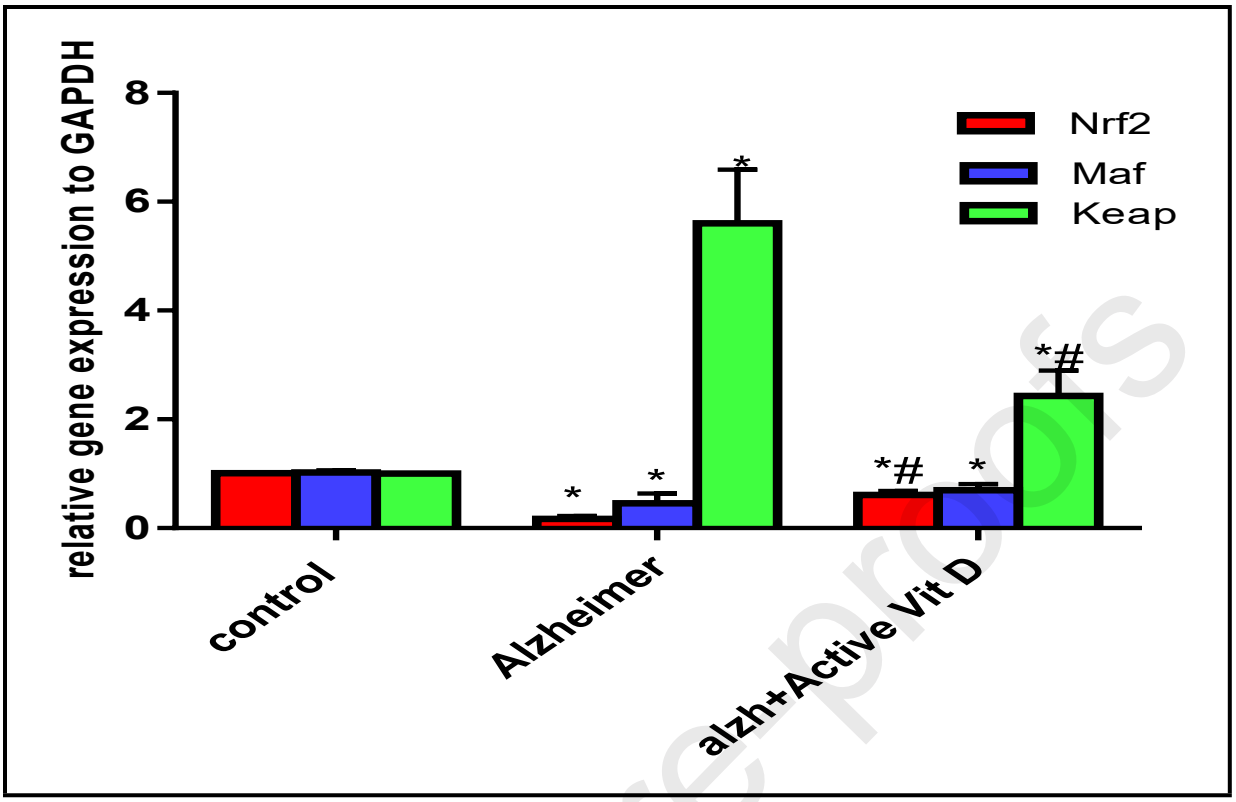


Figure 1. Gene expression of Nrf2, MAF and Keap-1 in rat brain tissues of all studied group. Results are expressed by mean ± SD as relative gene expression to the house keeping gene. Statistically significant compared to corresponding value in: control group (*), Alzheimer group (#). ($P<0.05$).

Active vitamin D analogue, Maxacalcitol, decreased the amyloid beta and hyperphosphorylated Tau protein levels via down-regulation of MAPK-38p and ERK1/2 expression

-There was a significant increase in the protein levels of both amyloid beta and p-Tau in Alzheimer group compared to the control group. Whereas there was a significant decreased levels in vitamin D analogue treated group compared to Alzheimer group **(Figure 2)**.

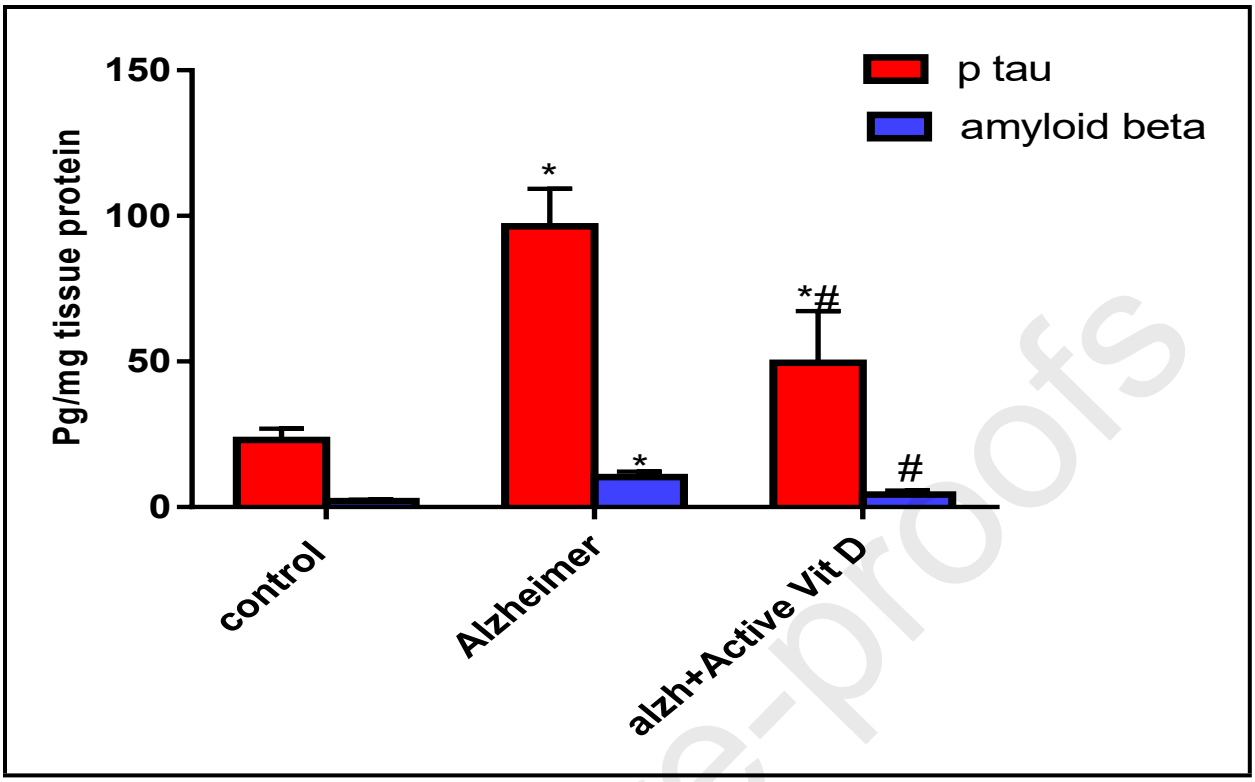
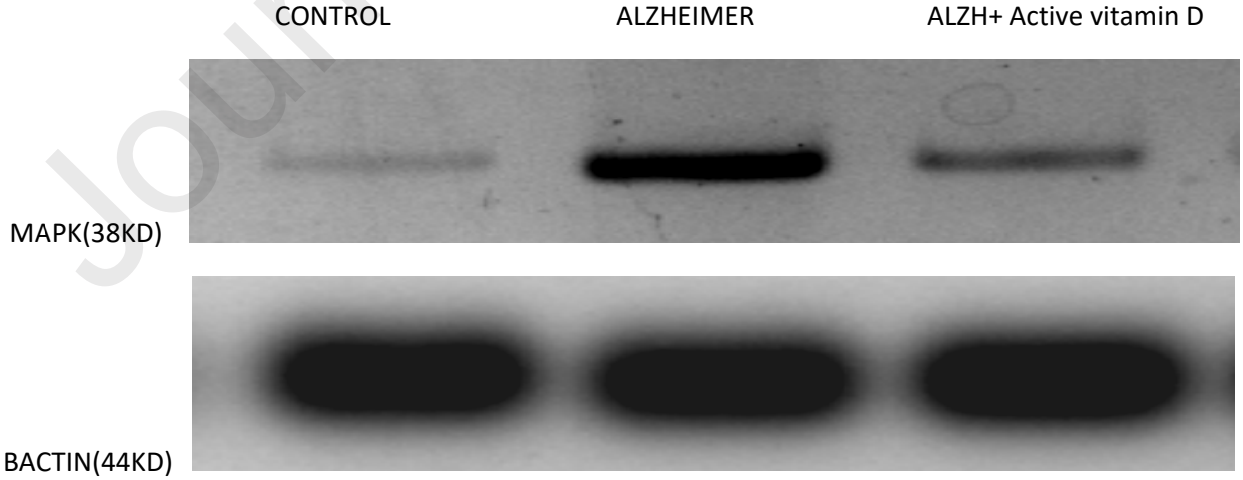


Figure (2): levels of Aβ protein (pg/mL) and p-Tau (pg/mL) in rat brain tissues in all studied groups. Results are expressed by mean ± SD. Statistically significant compared to corresponding value in: control group (*), Alzheimer group (#), (P<0.05).

-Regarding the protein levels of MAPK-38p **(figure 3)** and ERK1/2 **(figure 4),** there was a significant increase in both MAPK-38p and ERK1/2 protein expression in Alzheimer group as compared to the control group, while there was a significant decrease in vitamin D analogue treated group compared to Alzheimer group.



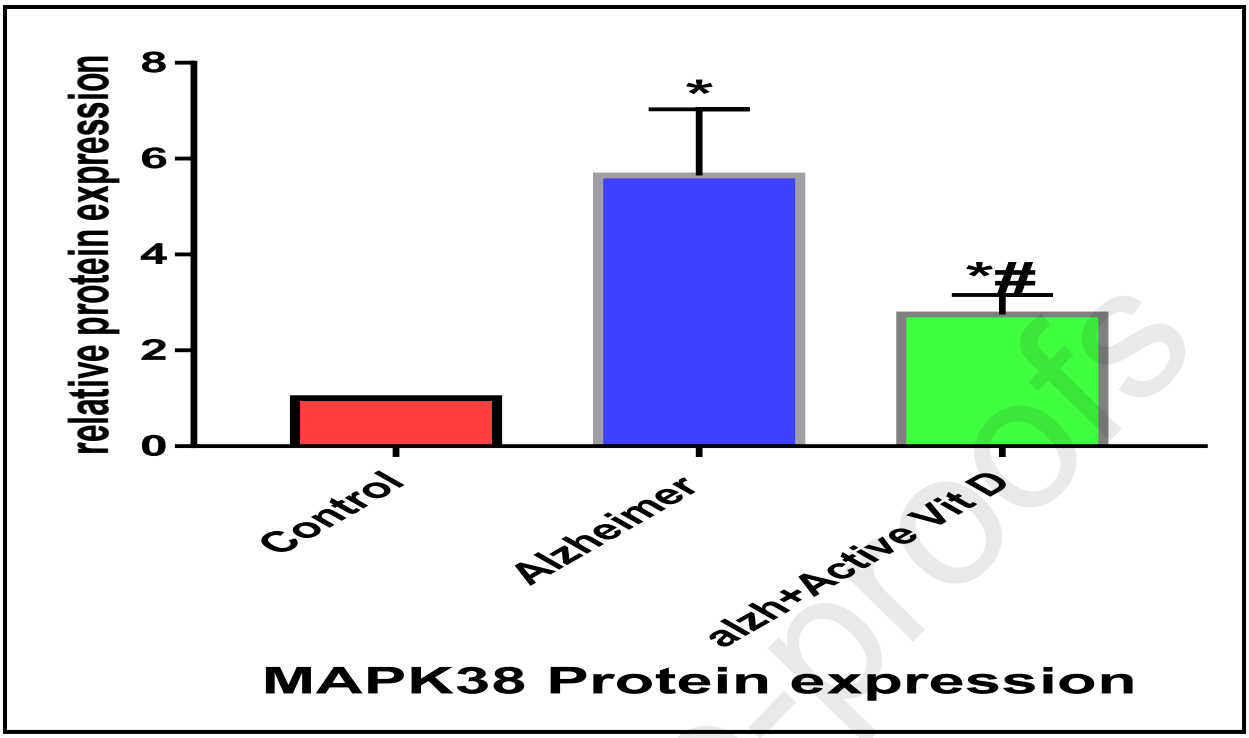
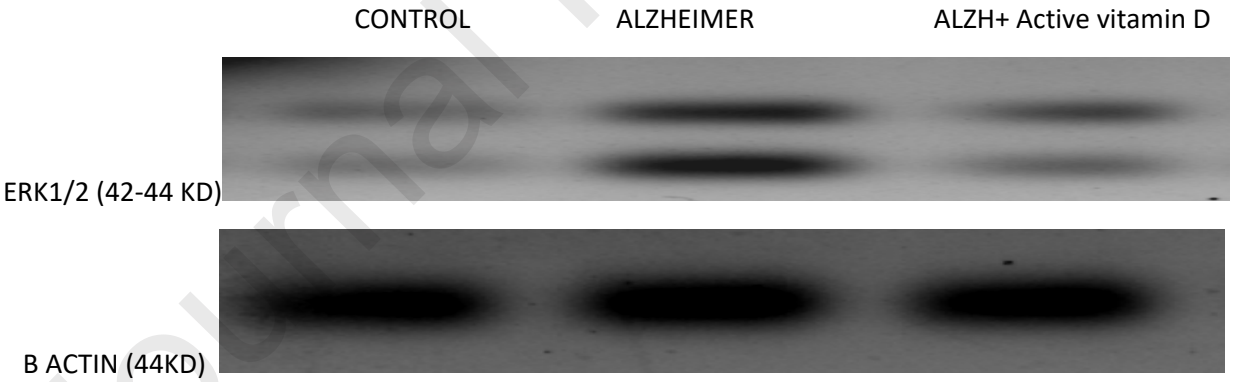


Figure (3): MAPK-38p protein levels in rat brain tissues of the studied group. Results are expressed by mean ± SD as relative expression by normalization to the loading control protein. Statistically significant compared to corresponding value in: control group (*), Alzheimer group (#), (P<0.05).



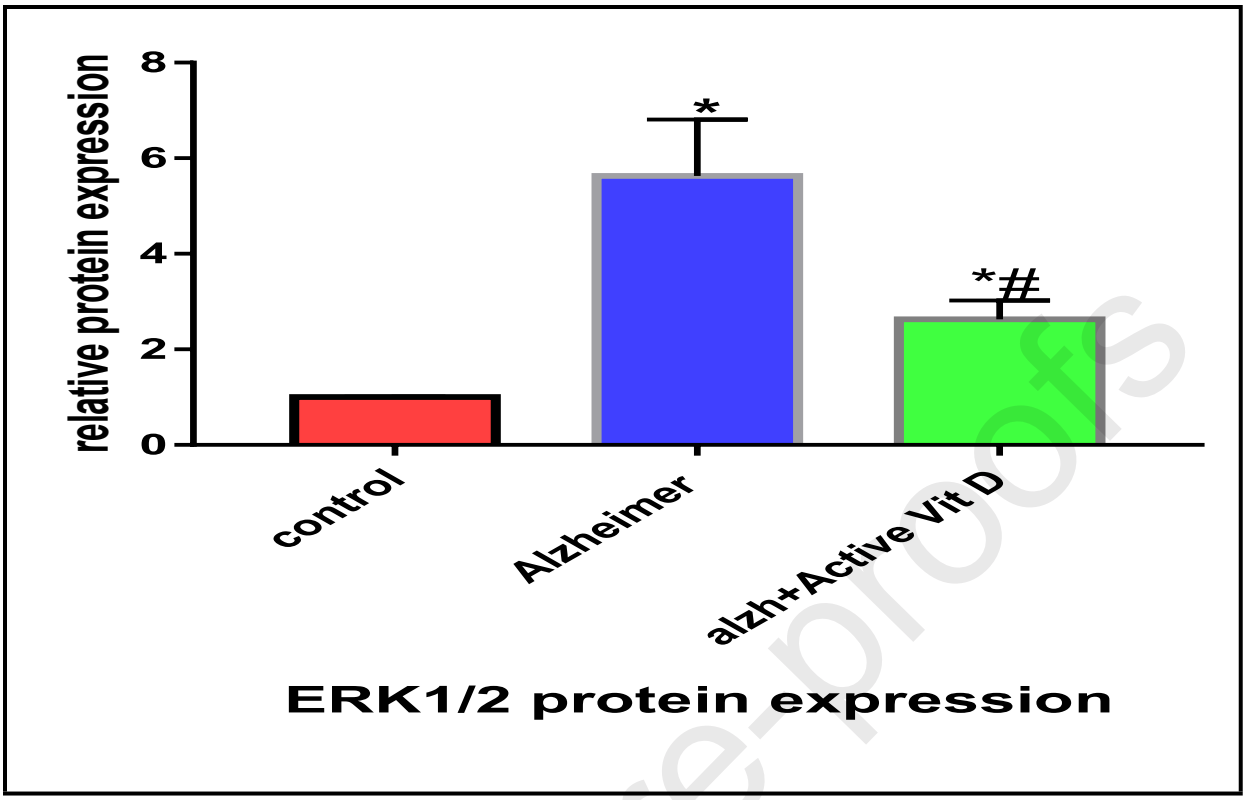


Figure (4): ERK1/2 protein levels in rat brain tissues of the studied group. Results are expressed by mean ± SD as relative expression by normalization to the loading control protein. Statistically significant compared to corresponding value in: control group (*), Alzheimer group (#), (P<0.05).

Baseline and end of study serum calcium and total 25-hydroxyvitamin D levels in all studied groups

-There was a significant decrease in serum levels of both total 25-hydroxyvitamin D and calcium in Alzheimer group as compared to the control group. While intake of vitamin D analogue (Maxacalcitol) resulted in correction of serum 25-hydroxyvitamin D and its levels showed significant increase in maxacalcitol treated group compared to Alzheimer group **(table 3).**

Table (3): Comparison of serum levels of calcium and total 25-hydroxyvitamin D in all studied groups

	Group		
	Control	**Alzheimer**	**Alzheimer+Active Vitamin D analogue**
Calcium (mg/dl)	10± 0.41	9.5± 0.26 *	10± 0.51

25(OH)vitamin D (ng/ml)	50± 5.2	22± 2.3 *	48± 2 #

Values are presented as mean ± SD. Statistically significant compared to corresponding value in: control group (*), Alzheimer group (#). (P<0.05)

Histopathological examination of the hippocampus of rat brain tissues (Figure 5)

-We found that most pathological changes were found in the CA1 region of the hippocampus of Alzheimer's rats where numerous disfigured darkly-degenerated pyramidal nerve cells with lost nuclear details were found. Administration of vitamin D analogue improved the histopathological features with restoration of the normal architecture which agree with the molecular findings of gene expressions of Nrf2-Keap1-ARE pathway. These data suggest that active vitamin D analogue, Maxacalcitol, could be used as a therapeutic drug for treatment of Alzheimer's disease.

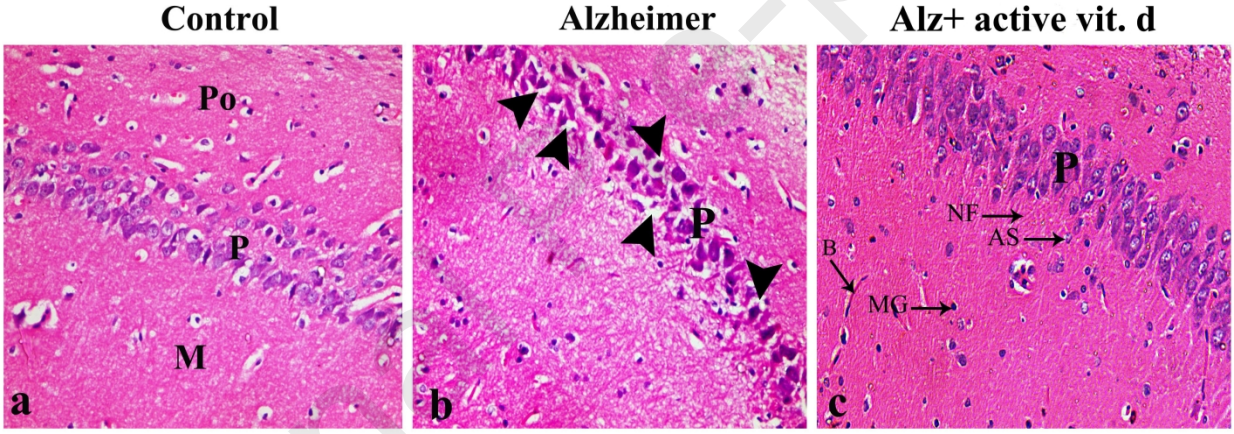


Figure (5): H&E stained sections of (a) control group showing CA1 area formed of outer polymorphic layer (Po), middle pyramidal (P), and inner molecular (M) layers. (b) Alzheimer group showing numerous disfigured darkly-degenerated pyramidal nerve cells (P) with lost nuclear details. The shrunken neurons appear surrounded by pericellular unstained halos (arrow heads). (c) Vitamin D analogue-treated group showing restoration of the normal architectural pattern of the CA1 hippocampal region. The pyramidal cells (P) appear densely-packed with vesicular nuclei and prominent nucleoli. Note: healthy astrocytes (AS), microglia (MG), blood capillaries (B) and nerve fibers (NF) (magnification x400).

Behavior study through percent of alternation and time consumed in T-maze test

-There was a significant increase in time consumed in T-maze test in Alzheimer group compared to the control group. Whereas, there was a significant decrease

in vitamin D analogue treated group compared to Alzheimer group. However, there was a significant decrease in percent of alternation in T-maze test in Alzheimer group compared to the control group. While there was a significant increase in vitamin D analogue treated group compared to Alzheimer group **(figure 6).** These data represent improvement of the cognitive functions of the brain of Maxacalcitol treated rats.

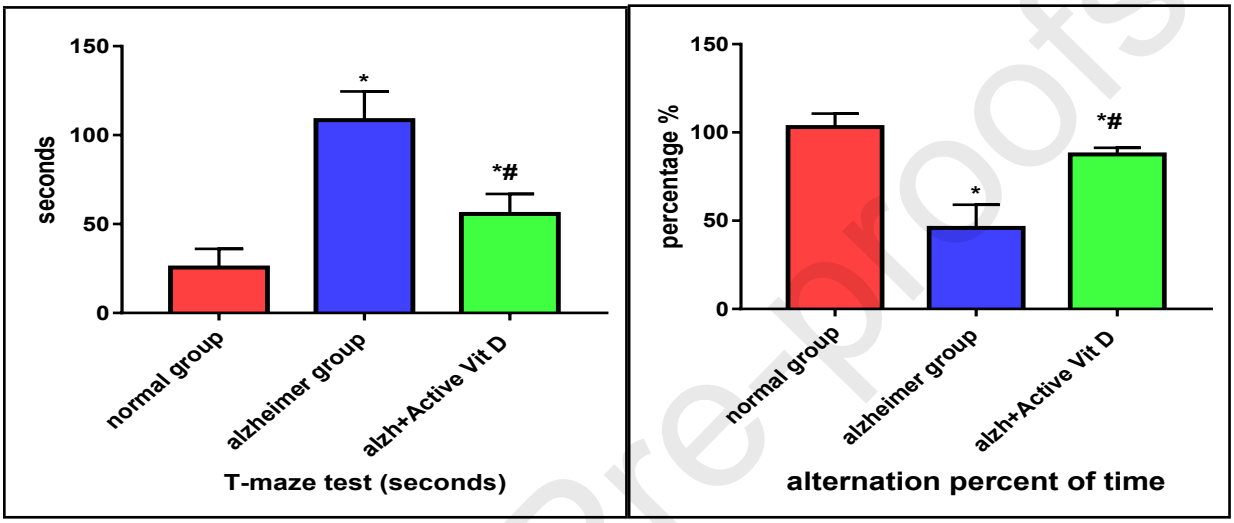


Figure (6): Time consumed and percent of alternation in T-maze test in all studied groups. Results are expressed by mean ± SD. Statistically significant compared to corresponding value in: control group (*), Alzheimer group (#), ($P<0.05$).

DISCUSSION

Nrf2 is considered an optimistic target for the prevention of several neurodegenerative diseases, but its activation must be tightly regulated to have beneficial effects **[27,28].** Exploring of the mechanisms mediating Nrf2 inhibition in neurodegenerative diseases may therefore direct the design of drugs targeted for the prevention of these diseases with minimal side-effects. The present study was conducted to evaluate the effects of vitamin D on Keap1-Nrf2 signaling pathway in experimental Alzheimer disease in rats.

The results of the present study demonstrated that treatment of Alzheimer rats with active vitamin D analogue, Maxacalcitol, has improved the condition as evidenced by the histo-pathological examination and the molecular findings of gene expression of Nrf2-Keap1 signaling pathway and the protein levels of the downstream ARE-mediated transcriptional effectors, HO-1 and GSH. These results

were consistent with the findings of **Ramsey et al., [29]** who stated that many of the deleterious effects of vitamin D deficiency in AD were due to a decline in the expression of Nrf2 in the brain of Alzheimer patients. They assumed that dys-regulation of the vitamin D/ Klotho/ Nrf2 regulatory network results in the elevated neuronal ROS levels that seems to be responsible for the onset of AD. Also, the results obtained by **Kerr et al., [30]** in Drosophila, confirmed previous reports that Nrf2 target genes were downregulated in the brain of AD mouse models **[31,32,33].** Their study further confirms that this inhibition may be mediated by naturally derived Aβ42 peptide in vivo, while other studies suggested that inhibition may be mediated by Aβ42 directly, with exogenous, synthetic, Aβ42 peptide reducing Nrf2 target gene expression in primary mouse neurons **[31],** and blocking Nrf2 nuclear translocation following injection into the hippocampus of rat **[32]** and mouse **[33]** brain.

Behavioral impairment and functional disconnection have common association with AD. With the apparent sensitivity of T-maze test for diagnosis of AD **[34,35]**, the current results showed marked affection of spatial working memory in AD group compared to control which suggests the rats couldn't remember the first arm that was visited, while maxacalcitol treated group showed marked improvement of cognition.

In AD, neuropathological changes start in the entorhinal cortex and hippocampal formations, later spreading into other association cortices. Appearance of poorly myelinated limbic neurons is related to memory and learning areas. Thus destruction of the hippocampal formation makes the storage of new memories impossible **[36].**

Nrf2 promotes re-myelination of the peripheral nerves after injury, in addition Nrf2/Keap1/ARE pathway is the primary regulator over 100 genes regulating oxidative stress and cell survival **[37].** vitamin D deficiency in AD could result in a decline in the expression of its two collaborators Nrf2 and klotho. On the other hand, Nrf2 over-expression in the hippocampus of AD transgenic mice resulted in a marked improvement in cognition **[38].**

Our findings also coincided with those reported by **Berridge [39]** which confirmed the protective function of vitamin D in aging and age related diseases by attenuating oxidative stress via increasing Nrf2 and up-regulation of the expression of genes encoding antioxidant enzymes, γ-glutamyl transpeptidase, glutamate cysteine ligase, glutathione reductase, glutathione peroxidase and

glucose-6-phosphate dehydrogenase. These effects were reflected on improved cognitive ability of AD group treated with vitamin D.

Our study also showed that there was altered oxidative profile in Alzheimer's disease evidenced by significant increased levels of the oxidative stress biomarker MDA and significant decreased levels of GSH. After treatment with active vitamin D analogue, maxacalcitol, in AD rats, there was a significant decrease in MDA and significant increase in GSH and HO-1 levels. These results are consistent with **Gastaw-Rothenberg et al., [40]** who stated that the level of MDA was significantly higher in both the newly diagnosed AD patients and in those with longer lasting neurodegenerative process in comparison with controls. Also, **Alatawi et al., [41]** agreed with our results as they confirmed the beneficial effects of vitamin D in reducing lipid peroxidation and oxidative stress observed in STZ-induced diabetic rats by the reduction of MDA levels and improvement of the activities of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase.

Several researches suggested that dysregulation of Nrf2 pathway could aggravate Aβ load in AD resulting in consequent upregulation and/or activation of phosphorylation of MAPKs especially MAPK-38p and ERK1/2. These changes trigger the hyperphosphorylation of Tau protein, which in turn contributes to the formation of neurofibrillary tangles causing the development of synaptic dysfunction and impairment of cognitive functions in AD **[42,43].** The present study confirmed the previous results as regards the significant increase in Aβ and hyperphosphorylated Tau protein levels and also showed significantly increased protein expression of MAPK-38p and ERK1/2 in Alzheimer group as compared to controls. After administration of vitamin D analogue, there was a significant decrease in Aβ, p-Tau and both MAPKs as compared to Alzheimer group. The results of histopathological examination of the hippocampus also supported these molecular findings suggesting that vitamin D could be a potential therapy for Alzheimer's disease.

Grimm and his colleagues [44] agreed with our results as they substantiated that vitamin D and its analogues could be an approach to prevent or treat AD by attenuating Aβ generation and accelerating Aβ degradation and reduction of the pro-inflammatory cytokines such as TNF-α. Another study done by **Pierucci et al., [45]** demonstrated the neuroprotective effect of vitamin D3 analogue ZK191784

against Aβ-induced cytotoxicity both in vivo and in neuroblastoma cell lines through modulation of MAPK-38p phosphorylation.

The results of our study agreed with those reported by **Briones and Darwish [46],** in which they showed decreased age-related Tau hyperphosphorylation after vitamin D supplementation. On the contrary, other researchers demonstrated no significant differences in the levels of Tau protein induced by vitamin D supplementation **[47,48].** Taken these data together, vitamin D may apparently do not affect Tau protein levels, but might have beneficial effects with respect to Tau phosphorylation.

Our results regarding serum levels of 25-hydroxyvitamin D showed significant decrease in Alzheimer group as compared to the control group. While treatment with vitamin D analogue, Maxacalcitol resulted in improvement of its level, and showed significant increase in treated group as compared to Alzheimer group. The explanation of this increase was unclear and needs further investigations. **Chen and his colleagues [49]** agreed with our results. In their meta-analysis study, they demonstrated that 25-hydroxyvitamin D was inversely associated with the risk of dementia and Alzheimer's disease. Also, they found a linear dose-response relationship showing that a 10 nmol/L increase in 25(OH)D level may lead to a 5% decrease in dementia and 7% decrease in Alzheimer's disease.

Also, a study performed by **Sato et al., [50]** confirmed our results regarding serum calcium levels. They suggested that lower serum calcium levels may be associated with conversion of mild cognitive impairment to early Alzheimer's disease in Japanese cohorts. This correlation couldn't be explained and further investigations needed to clarify this point.

In conclusion, active vitamin D analogue, Maxacalcitol, ameliorated dysregulation of Nrf2-Keap-1 pathway in Alzheimer' s disease and improved the histopathological picture of the brains of Alzheimer rats. These data suggest that potent vitamin D analogues could be used as a therapeutic agent in treatment of Alzheimer disease.

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Effect of active vitamin D analogue, Maxacalcitol, on Nrf2–Keap1-ARE pathway

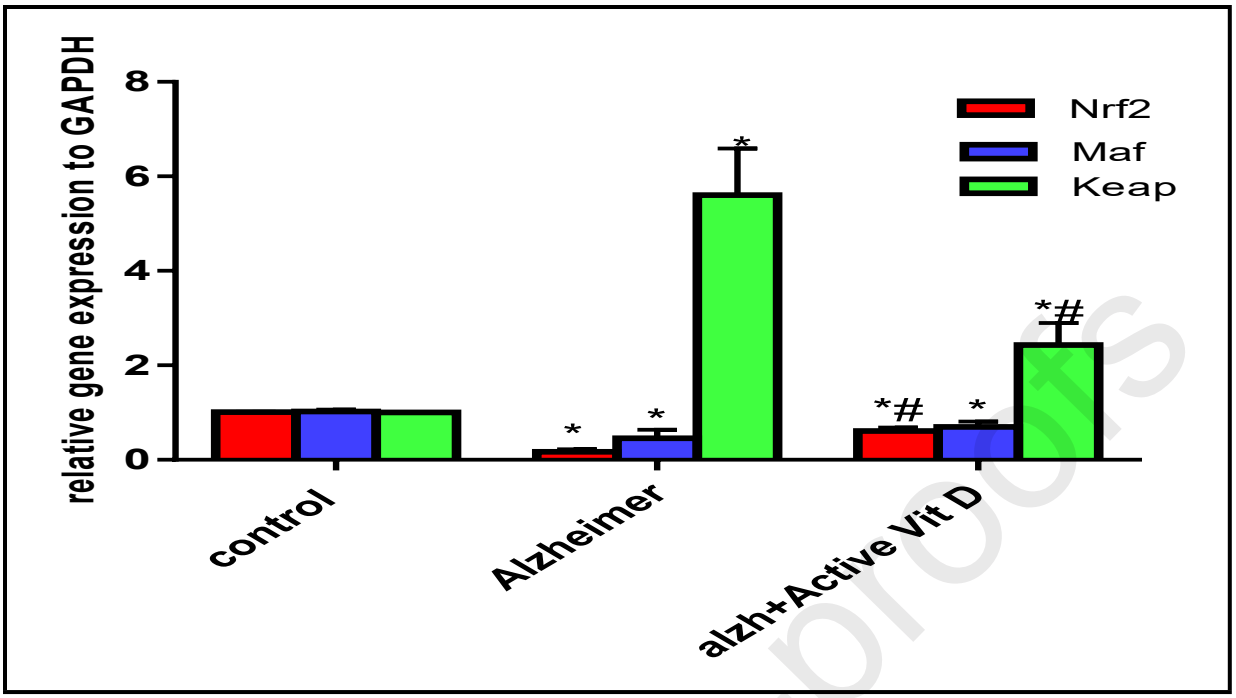


Figure 1. Gene expression of Nrf2, MAF and Keap-1 in rat brain tissues of all studied group. Results are expressed by mean ± SD as relative gene expression to the house keeping gene. Statistically significant compared to corresponding value in: control group (*), Alzheimer group (#). ($P<0.05$).

Vitamin D analogue, Maxacalcitol, decreased the amyloid beta and hyper-phosphorylated Tau protein levels via downregulation of MAPK-38p and ERK1/2

expression

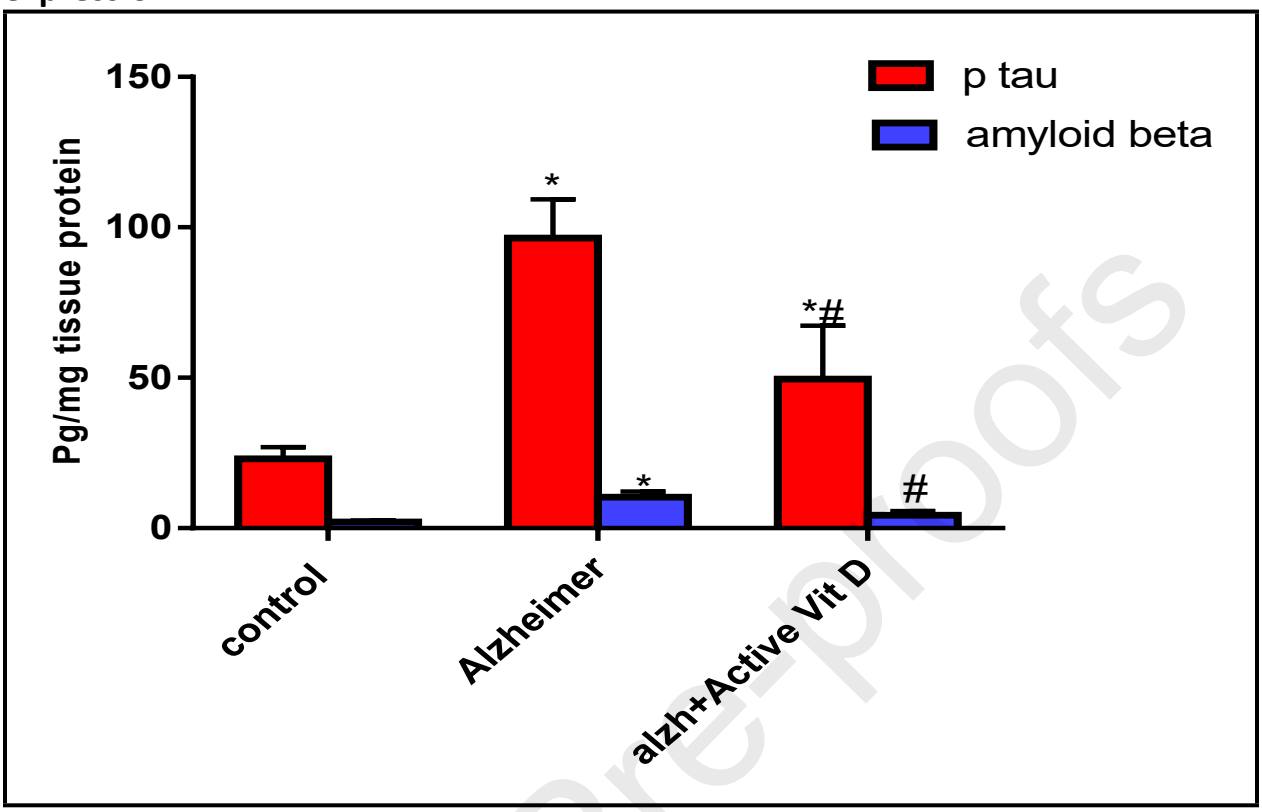


Figure (2): levels of Aβ protein (pg/mL) and p-Tau (pg/mL) in rat brain tissues in all studied groups. Results are expressed by mean ± SD. Statistically significant compared to corresponding value in: control group (*), Alzheimer group (#), (P<0.05).

CONTROL ALZHEIMER ALZH+ Active Vitamin D

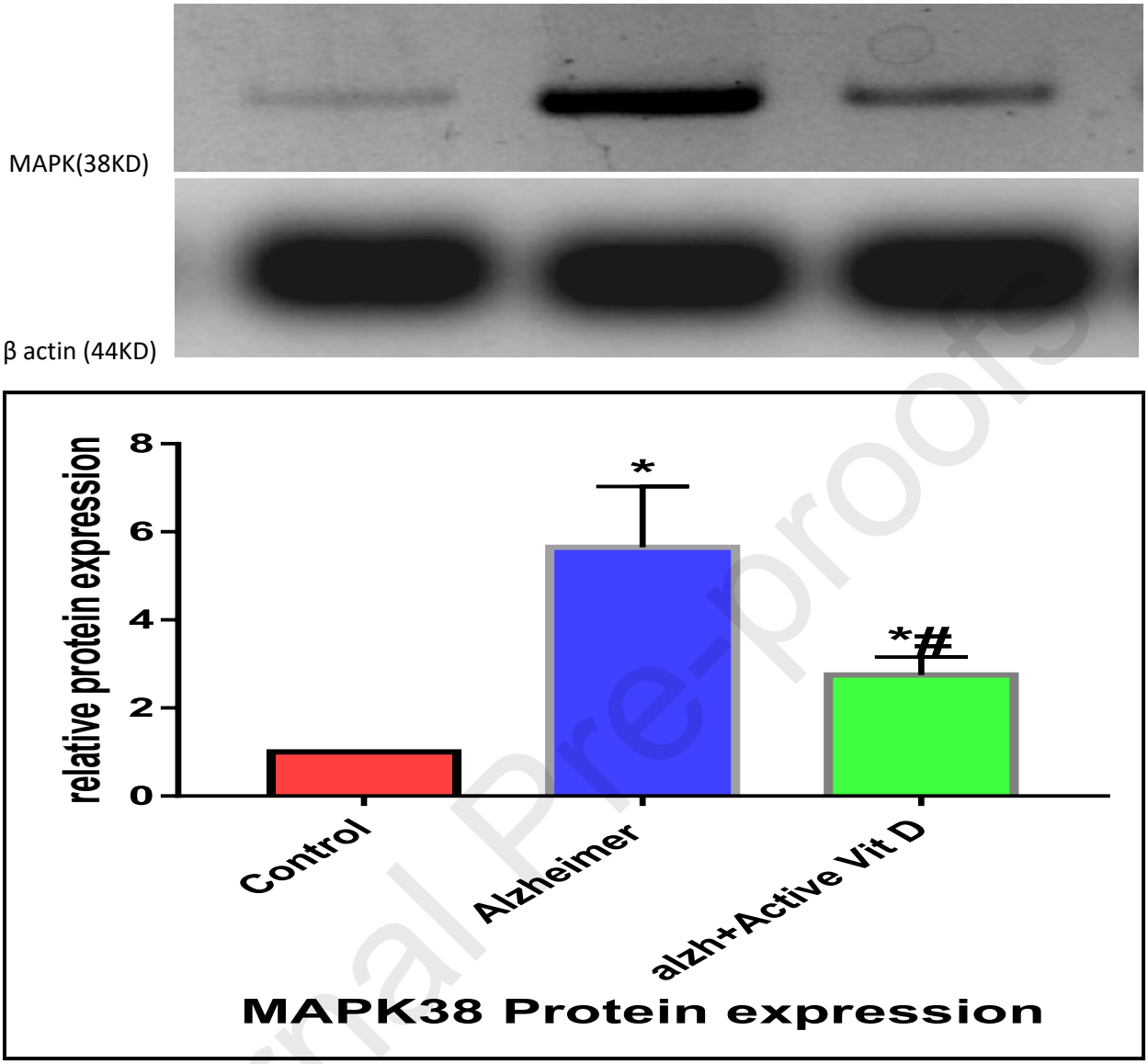
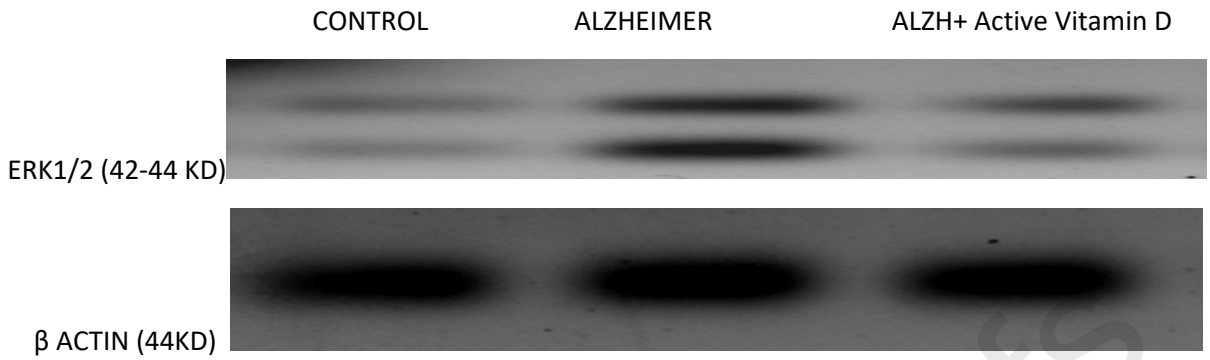


Figure (3): MAPK-38p protein levels in rat brain tissues of the studied group. Results are expressed by mean ± SD as relative expression by normalization to the loading control protein. Statistically significant compared to corresponding value in: control group (*), Alzheimer group (#), ($P<0.05$).



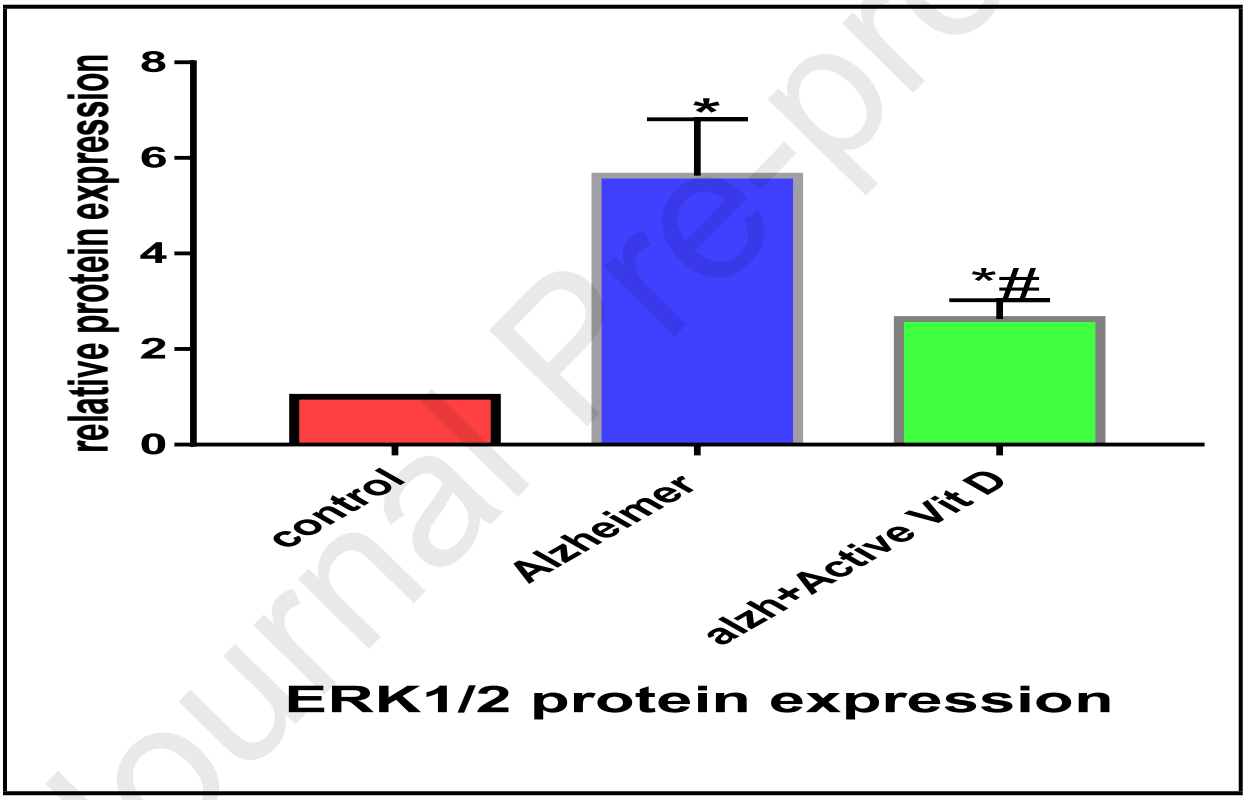


Figure (4): ERK1/2 protein levels in rat brain tissues of the studied group. Results are expressed by mean ± SD as relative expression by normalization to the loading control protein. Statistically significant compared to corresponding value in: control group (*), Alzheimer group (#), ($P<0.05$).

Histopathological examination of the hippocampus of rat brain tissues

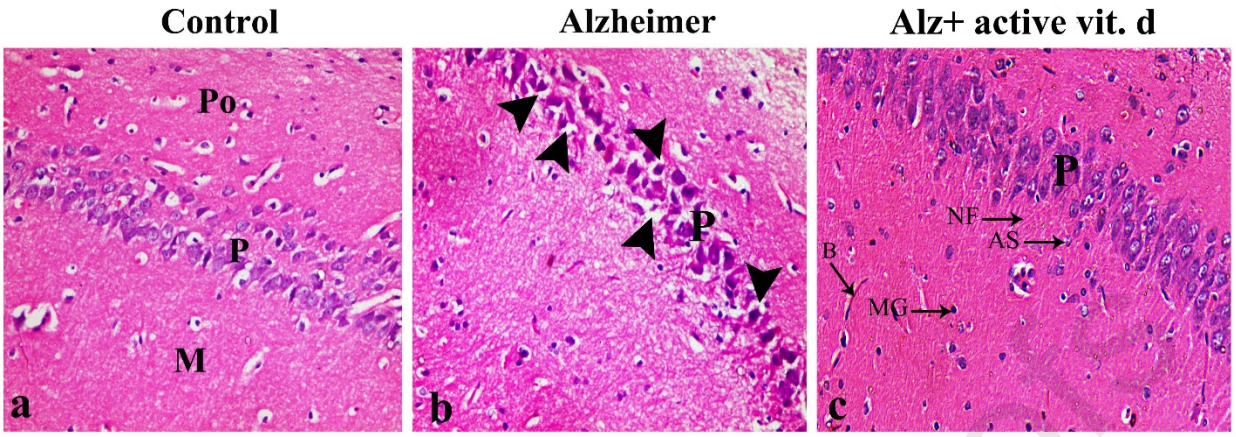


Figure (5): H&E stained sections of (a) control group showing CA1 area formed of outer polymorphic layer (Po), middle pyramidal (P), and inner molecular (M) layers. (b) Alzheimer group showing numerous disfigured darkly-degenerated pyramidal nerve cells (P) with lost nuclear details. The shrunken neurons appear surrounded by pericellular unstained halos (arrow heads). (c) Vitamin D analogue-treated group showing restoration of the normal architectural pattern of the CA1 hippocampal region. The pyramidal cells (P) appear densely-packed with vesicular nuclei and prominent nucleoli. Note: healthy astrocytes (AS), microglia (MG), blood capillaries (B) and nerve fibers (NF) (scale bar 50µm).

Behavior study through percent of alternation and time consumed in T-maze test

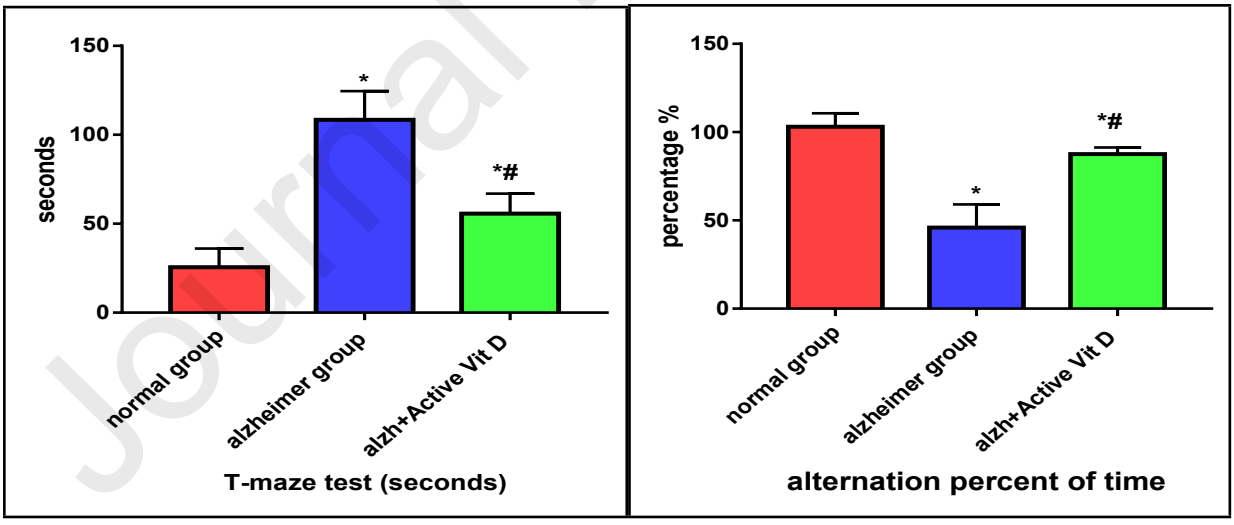


Figure (6): Time consumed and percent of alternation in T-maze test in all studied groups. Results are expressed by mean ± SD. Statistically significant compared to corresponding value in: control group (*), Alzheimer group (#), ($P<0.05$).

Table 1. Primers Sequences of Nrf2, Keap1 and MAF

Parameter	Forward and Reverse Primers	Gene bank accession number
Nrf2	Forward primer: 5′ CACATCCAGACAGACACCAGT 3′ Reverse primer: 5′ CTACAAATGGGAATGTCTCTGC 3′	NM_031789
Keap1	Forward primer: 5′ GGACGGCAACACTGATTC 3′ Reverse primer: 5′ TCGTCTCGATCTGGCTCATA 3′	NM_057152
MAF	Forward primer: 5′ AAGGAGGAGGTGATCCGACT 3′ Reverse primer: 5′ CTGGTTCTTCTCCGACTCCA 3′	NM_019318.1
GAPDH (House keeping)	Forward primer: 5′ AGGTTGTCTCCTGTGACTTC 3′ Reverse primer: 5′ CTGTTGCTGTAGCCATATTC 3 ′	NM_017008

Table (2): Comparison of IL-10, TNFα, HO-1, GSH and MDA levels in all studied groups

	Group		
	Control	**Alzheimer**	**Alzheimer+ Active Vitamin D analogue**
TNFα (pg/mL)	16.15± 2.204	122.9± 5.168 *	66.2± 12.04 *#
IL-10 (pg/mL)	129± 18.17	69.31± 7.702 *	96.2± 4.911 *#
HO-1 (mmol/mg protein)	851.6± 123.7	383.7± 72.15 *	701.5± 69.74 #
GSH (mmol/mg protein)	58.03± 5.389	22.33± 3.137 *	41.23± 6.715 *#
MDA (nmol/mg protein)	7.675± 1.778	71.28± 16.81 *	29.1± 6.527 *#

Values are presented as mean ± SD. Statistically significant compared to corresponding value in: control group (*), Alzheimer group (#). ($P<0.05$)

Table (3): Comparison of serum levels of calcium and total 25-hydroxyvitamin D in all studied groups

	Group		
	Control	**Alzheimer**	**Alzheimer+ Active Vitamin D analogue**
Calcium (mg/dl)	10± 0.41	9.5± 0.26 *	10± 0.51
25(OH)vitamin D (ng/ml)	50± 5.2	22± 2.3 *	48± 2 #

Values are presented as mean ± SD. Statistically significant compared to corresponding value in: control group (*), Alzheimer group (#). (P<0.05)

- Treatment of Alzheimer' s disease with active vitamin D analogue, Maxacalcitol, significantly improved cognitive dysfunction and histopathological picture of the brains of Alzheimer rats.
- Also, Maxacalcitol significantly increased the expression of Nrf2 and its downstream effectors (HO-1 and GSH), decreased neuroinflammation, deceased Amyloid β load and decreased hyperphosphorylation of MAPK-38, ERK1/2 and Tau proteins.
- Therefore, these data suggest that potent vitamin D analogues could be used as a therapeutic agent in treatment of Alzheimer disease.