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Assessment of Cholecalciferol's Therapeutic Efficacy on the Cerebellum in an Autistic Male Pup Model

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ABSTRACT

Background: Autism spectrum disorder (ASD), neurodevelopmental disorder, is found to be linked to pathological changes in the cerebellum. Interestingly, vitamin D is reported to be deficient in autistic kids.

Objective: This study aimed to demonstrate the effect of cholecalciferol administration on impaired autophagy in the cerebellum of male rat pups in a postnatal valproate (VPA)-induced model of autism.

Materials and methods: A total of 15 Sprague-Dawley male rat pups were used to assess the possible therapeutic effects of vitamin D (cholecalciferol) on the cerebellum of autistic rats through autophagy augmentation. The rats were divided equally into three groups. The control group received saline on the 14th postnatal day, while valproate (VPA) group received single dose of 400-mg/kg VPA on same day. The cholecalciferol-treated group received a single dose of VPA on the 14th postnatal day followed by a single intramuscular injection of cholecalciferol on the 21st postnatal day. At the assigned time, the animals were sacrificed, and cerebellar tissue samples were taken for biochemical, histological, and immunohistochemical analyses.

Results: VPA treatment increased the oxidative stress marker malondialdehyde (MDA) significantly. Histological examination revealed disrupted cerebellar architecture and the light chain3-II (LC3-II) immunohistochemical expression dropped significantly in VPA group. Co-treatment with cholecalciferol significantly reversed the previous changes.

Conclusion: Cholecalciferol has neuroprotective activity on the cerebellum of autistic rats due to its antioxidant mechanisms as well as its ability to improve autophagy.

Keywords: Autism (ASD), Cerebellum, Purkinje cell, Valproate (VPA), Cholecalciferol, Autophagy.

INTRODUCTION

The main symptoms of ASD involve limited interests, repetitive stereotypical behaviors, and poor social interaction [1]. Unluckily, there is no curative therapy for ASD. Children with ASD exhibit inadequate social adaptation, imposing significant emotional and economic expenses on both society and families, hence garnering considerable attention [2]. Unfortunately, till now, the actual cause and pathophysiology of ASD are not identified. On the other hand, there is a strong relationship between genetic and environmental factors [3].

Multiple animal models are utilized to induce behaviors similar to autism, aiding in the understanding of ASD and the creation of innovative treatments; VPA is the most widely studied among them [4].

Being a common target brain area affected in ASD, the cerebellum is considered as an excellent model to study autism since it has a crucial role in motor coordination, balance and higher-order brain functions which include cognitive functions, learning, emotion, and behavior [5]. These functions are achieved through the cerebellar connectivity with cortical and subcortical regions. Numerous studies have demonstrated reciprocal connections among the cerebellum, prefrontal cortex, and amygdala, enabling the cerebellum to control reactivity in those predisposed to anxiety [6].

Cerebellar changes is a common features in cases with ASD, according to numerous postmortem investigations [7]. As well, Purkinje cell loss is the most common neurohistopathological finding in nearly all postmortem autistic brains. This loss is widely distributed across the folia and is noticed in the vermis, in particular in the cerebellar hemispheres [6].

Autophagy is a well-preserved physiological cellular decomposition mechanism that recycles long-lived proteins and broken organelles. Autophagy commences with the creation of U-shaped isolation membranes, which then expand and seal to create autophagosomes; a process that entails the processing of LC3-II [8].

The growth of the brain depends on vitamin D. According to reports, vitamin D insufficiency is frequently present in children with ASD [9]. Vitamin D could adjust the inflammatory processes by promoting autophagy in different tissues. It has been displayed that active vitamin D promotes chondrocyte autophagy to reduce osteoarthritis. It also protects against myocardial injury, apoptosis, and inflammation by promoting autophagy. Additionally, vitamin D may have reno-protective benefits in diabetic nephropathy through mTOR gene expression down-regulation, autophagy promotion, and anti-inflammatory and antioxidant properties [10]. Although, vitamin D has been demonstrated to affect the autophagy pathway in a number of experimental diseases, its impact on the autophagy pathway in an autistic model hasn't yet been assessed. Here, we aimed to demonstrate the effect of cholecalciferol administration on impaired autophagy in

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the cerebellum of male rat pups in a postnatal VPA-induced model of autism.

MATERIALS AND METHODS

Experimental animals: Fifteen of seven-day old albino rats weighing 5 to 10 gram were utilized. The animals were housed in standard cages under controlled situations (21 ˚C ± 2 and a 12 h light/dark cycle) and were fed regularly. This experiment was carried out in the Medical Experimental Research Center (MERC), Mansoura University.

Drugs and chemicals: Valproic acid sodium salt (Powder, five grams in a glass bottle) was obtained from ThermoFisher Scientific Company, USA (Catalog number: 271830050, lot number: A0436861). Cholecalciferol (Vitamin D3) was purchased from pharmacy in the form of Devarol-s ampoules of Memphis Company. Each 2 ml contains 5 mg chole-calciferol equivalent to 200000 IU. MDA was purchased from Biodiagnostic Company, Elomraniya, Egypt. The rabbit polyclonal anti-LC3-II antibody was purchased from Abca, UK, No. ab48394. The biotin-labelled secondary antibody rabbit anti-rat IgG was purchased from Boster Biological Technology, USA, No. BA1005.

1. **Experimental design:** Fifteen male albino rat pups were divided haphazardly into three groups, each contains five rat pups:
- **Group 1 (control group):** each animal received 0.1 ml/kg of normal saline subcutaneously on the 14th postnatal day as single dose [11].
- **Group 2 (Valproate (VPA) group):** Each animal received 400-mg/kg subcutaneously valproate dissolved in saline at a concentration of 0.1 mL/kg at the 14th postnatal day as single dose [11].
- **Group 3 (Cholecalciferol-treated group):** Each animal received a single dose of valproate at the 14th postnatal day followed by single intramuscular injection of cholecalciferol of 80,000 IU/kg (Diluted into 3000 IU per ml with saline) on the 21st postnatal [9].
2. **Specimen's collection:** On the 35th postnatal day, the rats were sacrificed by intra-peritoneal injection of sodium thiopental (120 mg/kg) [12]. The cerebella were extracted after the brains were dissected. Each cerebellum was then split in half sagittal. One half was prepared for light microscopic evaluation after being fixed in ten percent neutral buffered formalin for histopathology and immunostaining studies. The other half was used to quantify MDA, a biomarker of oxidative stress, in cerebellar tissue homogenate.

Biochemical studies for the assessment of oxidative stress: Cerebellar tissues were homogenized to detect the tissue levels of MDA as described by **Babiuch *et al.* [13].**

Histopathological Studies: After being gradually dehydrated with ascending graded alcohol concentrations, the cerebellum specimens were cleaned in xylene and imbedded in soft and then hard paraffin wax in preparation for paraffin sectioning [14]. In addition, they were processed into 3-5µ paraffin sections for the light microscopic assessment by the H & E stain for histopathological alterations and immunohistochemical stain (IHC) for the autophagy marker light chain3-II (LC3-II).

Immunohistochemical studies: To inhibit endogenous peroxidase activity, 0.03% H_2O_2 was added to the produced paraffin sections after they had been deparaffinized with xylene and rehydrated. Sections were treated in PBS to prevent non-specific binding after being boiled in 0.01 M citrate buffer (pH 6) to display the antigenic site. The diluted primary antibodies for LC3b (1:400) were added and incubation was performed overnight at 4 ˚C. Following incubation and washing in PBS, the 2nd biotin-labeled anti-rabbit IgG was added. Incubation of the sections were conducted by using DAB for five min. Lastly, washing of the slides were performed by using PBS and counterstained with hematoxylin [15].

Morphometric analysis: Counting the Purkinje cell in calibrated areas was conducted by utilizing the software Image J program (Version 1.46i, NIH) in Hx & E-stained sections. The analysis was conducted at a magnification of ×400. Ten sections of each animal were chosen and the mean value was measured [16].

The optical density (OD) of the LC3-II was assessed per field (magnification: x400, area: 312.46 µm x 221.43 µm =0.069 mm^2) in DAB images and for data collection also by using Image J program. Each animal's mean was determined after 5 or more non-overlapping fields from all slides were analyzed. Immunoexpression manifested as the unique brownish coloring [15]. The colour deconvolution plugin was utilized to isolate the color components of each image by quantifying the brown hue in immunostained sections. The mean gray value was reported, and OD was measured by the next formula: OD= log (maximal intensity/mean intensity), in which max intensity = 250 for 8-bit images [17].

Ethical approval: The study was conducted following the ethical guidelines of the use of animals for research purposes and it was approved by Mansoura University Animal Care and Use Committee (MU-ACUC).

Statistical analysis

Data were collected and analysed using the SPSS version 22 (mean ± SD or median). One-way ANOVA test was utilized to compare between three or more groups of parametric data followed by Kruskall–Wallis test to compare between at least three groups of non-parametric data followed by U test for multiple comparisons. P-value ≤ 0.05 was considered significant.

RESULTS

Biochemical assessment of malondialdehyde (MDA) as oxidative stress marker:

Valproate group MDA level was raised significantly (P≤0.05) compared to the controls. On the other hand, MDA level of the cholecalciferol-treated group diminished significantly (P≤0.05) in relation to valproate group **(Fig.1).**

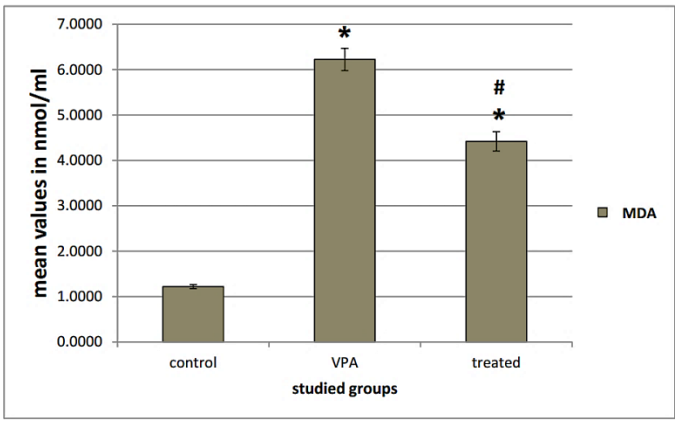

Figure (1): Histogram showed the mean ± SD values of malondialdehyde (nmol/ml) (MDA) among different groups.

* Significant difference compared to the controls. # Significant difference compared to valproate group.

Hematoxylin and eosin staining: The control group displayed normal architecture of the cerebellum consisting of folia separated by deep fissures covered by a very thin layer of pia mater. Each folium was composed of an outer cerebellar cortex and inner cerebellar medulla (Figure 2A). Three layers comprised the cerebellar cortex: Outer molecular layer (OML), middle Purkinje layer, and inner granular layer (GL). Numerous nerve fibers and a smaller number of dispersed, tiny cells made up the outer molecular layer. One row of Purkinje cells (PCs), with big pear-shaped somas, central vesicular nuclei with apparent nucleoli, and dendrites pointing toward the molecular layer, made up the Purkinje cell layer (PCL). Small, spherical granular cells were closely packed throughout the GL (Figure 3A).

Examination of cerebellar sections of valproate (VPA) rats also formed of folia and fissures in between covered with pia mater. Congested blood vessels were noticed (Figure 2B). The cerebellar cortex of the folium displayed discontinuity of the PCL. The number of PCs revealed a significant decrease (Figure 4). The remaining Purkinje cells lost their normal architecture. They had an irregular shrunken outlines with deeply stained cytoplasm and pyknotic nuclei. Perineural spaces appeared around cells in the three cortical layers (Figure 3B).

Compared with valproate group, cerebellar sections of cholecalciferol-treated group, revealed few congested blood vessels (Figure 2C). PCL was restored. The number of Purkinje cells elevated significantly (Figure 4). While, some Purkinje cells began to regain their typical flask-shaped appearance with vesicular nuclei, others had pyknotic nuclei and some vacuoles in between (Figure 3C).

LC3-II stained sections: Positive expression of LC3-II was noticed in the cell cytoplasm as brown staining. The cerebellar sections of the controls revealed positive high-intensity cytoplasmic reactions for LC3-II among the cerebellar cortex layers (Figure 5A). While, cerebellar sections of valproate group displayed a low-intensity LC3-II cytoplasmic reactions in the cerebellar cortex layers (Figure 5B). Additionally, relative to the control group, there was highly statistically significant reduction in LC3-II immunostaining optical density in VPA rat's cerebellum (Figure 6). Cerebellar sections of the treated group exhibited a moderate positive immune reaction for LC3-II in Purkinje cells and in few cells of molecular layer (Figure 5C). It was a statistically significant elevation in contrast with the VPA group (Figure 6).

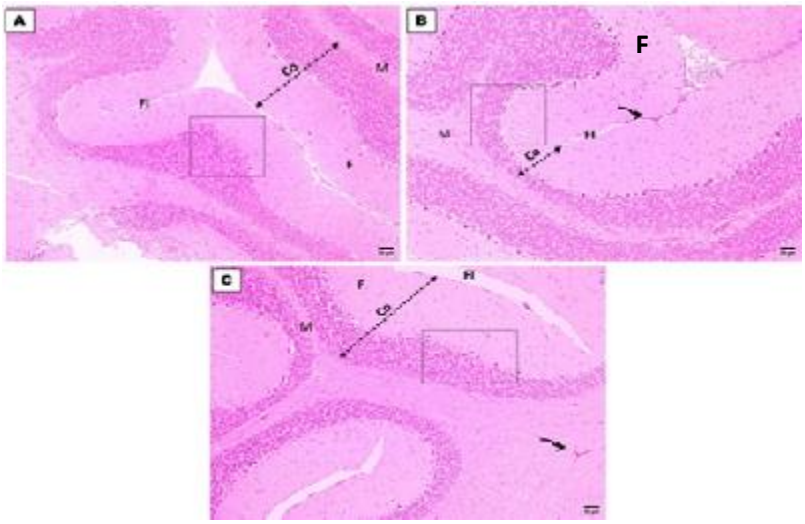

Figure (2): Photomicrographs of cerebellum stained with H & E showed a normal appearance in the control group section **(A)** consisting of cerebellar folia (**F**) with narrow fissure (**FI**) in between, all covered with a very thin layer of pia mater. The folium is composed of an outer cerebellar cortex (**Co**) and inner medulla (**M**). While, in VPA group section **(B)** highly congested blood vessels are noticed (curved arrows). Few congested blood vessels (curved arrows) are present in treated group section **(C)** (Magnification X 100).

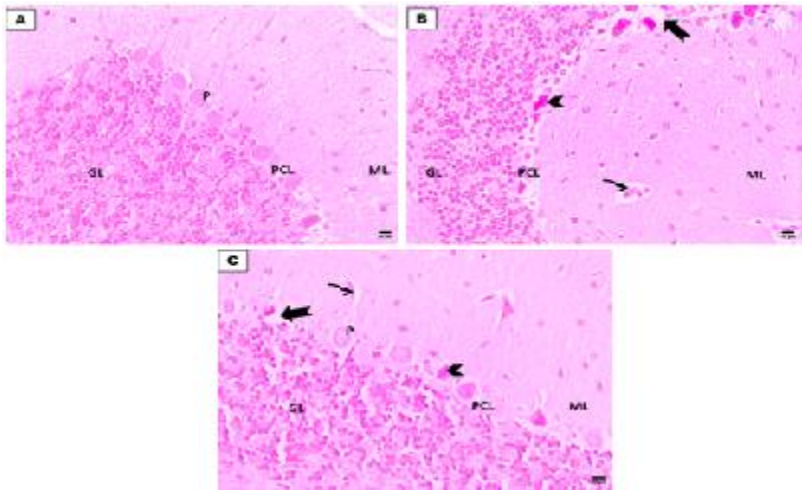

Figure (3): A higher magnification of squared parts of (Figure 2). Control group section **(A)** exhibiting typical cerebellar cortex structure consisting of three layers; the OML (**ML**) with small, scattered cells, the middle Purkinje layer (**PCL**) displaying large pyriform Purkinje cells (**P**) with vesicular nuclei and apparent nucleoli, and inner **GL** full of well-defined tightly packed small granular cells. The VPA group **(B)** showing disturbed continuity of PCL. There were loss of the pyriform shape and shrinkage of Purkinje cells (black arrowhead**)** with deeply stained cytoplasm and ill-defined pyknotic nuclei. There are perineural spaces or halos (notched arrows around cells of the three layers with the presence of congested blood vessels (curved arrows). Cholecalciferol-treated group section **(C)** revealed restoration of PCL continuity. Some of Purkinje cells **(P)** had the normal pyriform shape with vesicular nuclei and prominent nucleoli. Other Purkinje cells remain irregular in shape (black arrowhead) with deeply stained cytoplasm and pyknotic nuclei. Halos (notched arrows) are still present around cells of the three cerebellar layers. Few areas of blood vessels congestion (curved arrows) are noted (Magnification X 400).

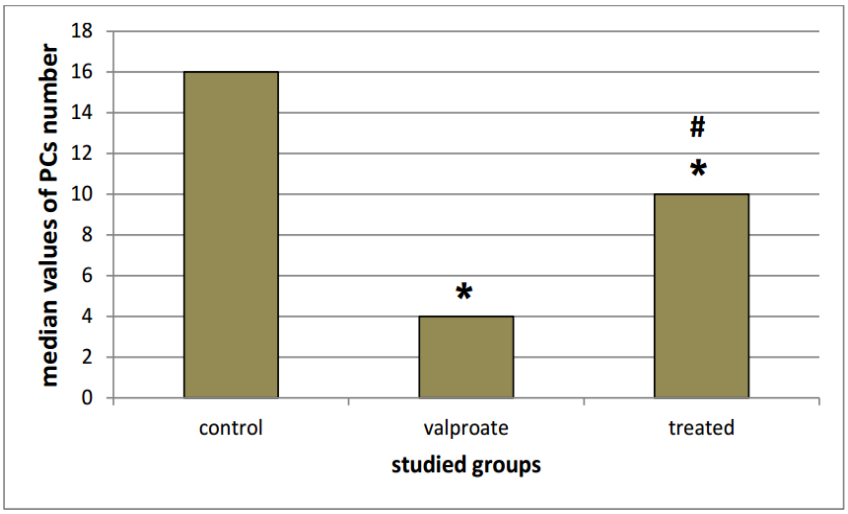

Figure (4): Histogram showing the median values of Purkinje cell count among different groups.
* Significant difference compared to the controls. # significant difference compared to valproate group.

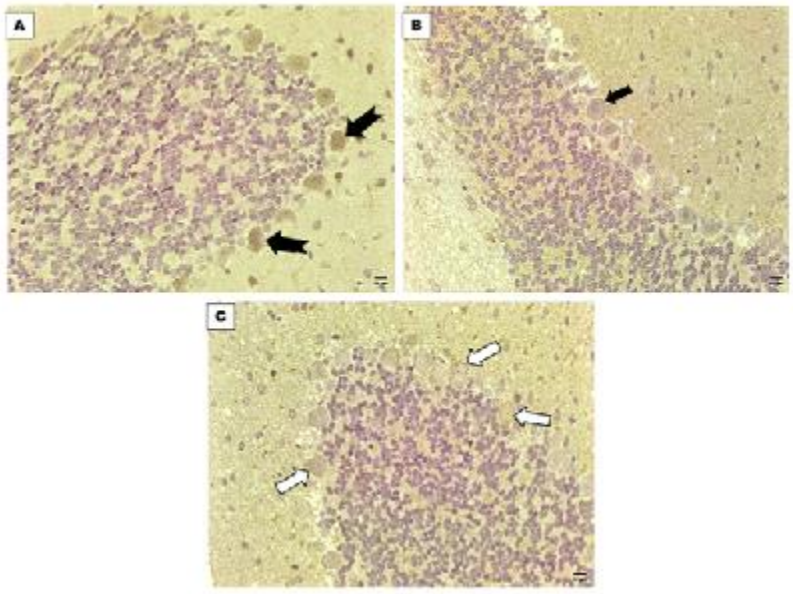

Figure (5): Rat cerebellar LC3-II immunohistochemical stain of **(A)** control, **(B)** VPA, **(C)** cholecalciferol treated groups respectively. (Notched black arrows) indicate strong immune reactivity of the cytoplasm of neurons in the controls. (Black arrow) indicates weak intense immune reactivity of the cytoplasm of neurons in the valproate group. (White arrows) indicate moderate intense immune reactivity of the cytoplasm of neurons in
the treated group (Magnification X 400).

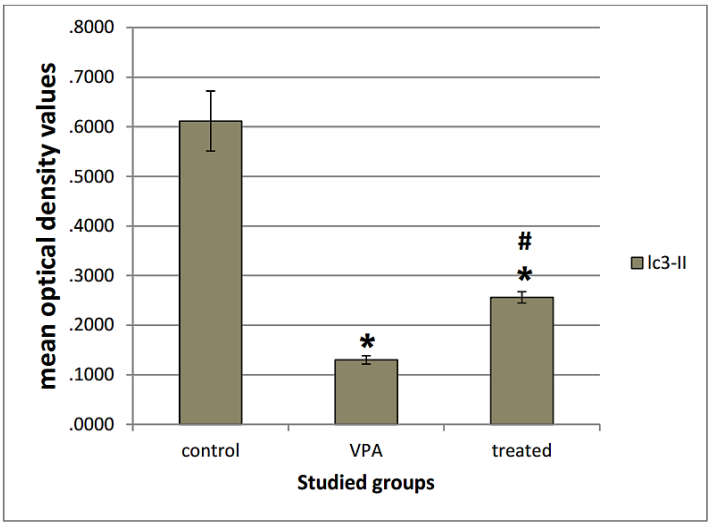

Figure (6): Histogram showing the mean ± SD values of LC3-II optical density among different groups.
* Significant difference compared to the controls. # Significant difference compared to valproate group.

DISCUSSION

Autism has no biomarker to confirm the diagnosis because the exact mechanism behind it is still unknown [18]. Numerous researches have linked ASD to cerebellar dysfunction. On top of that, it has been demonstrated through research on the experimental VPA model of ASD results in a significant drop in the size of the cerebellar hemispheres and the quantity of cerebellar Purkinje cells as cerebellar development proceeds after birth [19].

Purkinje cells, serving as the cerebellar output neurons, assimilate intricate inputs inside the cerebellum and transmit signals to other brain regions. These denote substantial cerebellar connections linking the limbic system and the cerebral cortex. In the postnatal developing phase, Purkinje cells are post-mitotic and in the early stages of differentiation. The behavioral symptoms of autism spectrum disorders are thought to be mediated by a decreased number of Purkinje cells [20]. This coincides with our experiment outcomes, where the H & E-stained cerebellar sections of VPA rats revealed marked degenerative changes mostly manifested on Purkinje cells. A reduction in Purkinje cell number was seen, along with regions of Purkinje cell loss and disarray. The mean number of Purkinje cells dropped significantly compared to the controls. Many cells seemed shrunken, irregular in size and shape and had condensed nuclei. Similar findings were reported by **Arafat & Shabaan** [21] and **Mokhtar Tawfeek *et al.*** [6].

Hussein *et al.* [12] clarified that oxidative stress brought on by the suppression of oxidative phosphorylation in Purkinje cell mitochondria could be the cause of the notable loss of Purkinje cells and the degenerative changes in remaining ones. In addition, **Sunand *et al.*** [22] observed that in the initial stages of development, when neuronal cells are more susceptible to the effects of elevated reactive oxygen species (ROS), oxidative stress has an essential role in neuronal cell degeneration. Proteins can be oxidized by oxidative stress, which deactivates enzymes and receptors, causing endothelial dysfunction and vascular changes including blood vessel dilatation and congestion in areas like the cerebellum [23]. This is confirmed in our study as congested blood vessels observed in VPA cerebellar sections.

It is clear from the current study that valproate-induced oxidative stress was a main factor in the histological alterations in the cerebellum of the rats in the valproate group, based on the current finding that VPA rats exhibited a significant increase in the level of oxidative stress marker (cerebellar MDA) relative to the control group rats. Equivalent outcomes were found by **Eid *et al.*** [7] who explained that VPA induced the formation of ROS, causing peroxidation of lipids with MDA formation eventually destroying the neurons. This also agrees with **Arafat & Shabaan,** [21] who displayed a significant rise in oxidative stress markers in VPA treated rats. Additionally, **Sunand *et al.*** [22] declared that the brain tissue MDA level was markedly raised in the autistic groups.

Yin *et al.* [24] conveyed that mitochondrial functional deficits, raised intracellular ROS levels, and neuronal damage in the hippocampus of autistic rats resulted from VPA-induced stimulation of mTOR signaling which is the main inhibitory pathway of autophagy leading to autophagy impairment. The impairment of autophagy in the present experiment was evidenced in valproate group by the examination of LC3-II immune stained cerebellar sections in addition to their optical density, statistical analysis showed significantly decreased LC3-II cytoplasmic expression in the three layers of valproate cerebellar cortex sections compared to the controls. Likewise, **Zhang *et al.*** [25] reported decreased LC3-II expression in the VPA group relative to the controls, suggesting inactive autophagy in the baby rats with ASD.

Researchers have discovered that intervention with vitamin D during early neonatal life may enhance the development, and behavioural performance of ASD rats, as cholecalciferol has cerebral anti-inflammatory actions, comprising decreasing hazardous cytokines and neuroinflammation induced by oxidizing agent and toxic compounds [10].

The significant reduction in cerebellar MDA level in our work suggests depressed oxidative stress in the cerebellum on giving cholecalciferol to autistic rats. This is similar to study of **Abbarin *et al.*** [26] who found that vitamins D reduced valproic acid-induced oxidative stress in the cortical layer of autistic rat brain. Authors stated that the activation of the vitamin D receptor (VDR) leads to an elevation in antioxidants, which are crucial in

mitigating excessive reactive oxygen species (ROS) formation [27].

Appropriate calcium levels are essential because too much calcium in the neurons may drive ROS development and damage the neurons. By encouraging the synthesis of calcium binding proteins, cholecalciferol has been shown to directly protect neurons [28]. This is consistent with our experiment finding, where the majority of the cerebellar architecture was restored in the H & E cerebellar sections of the cholecalciferol treated rats as well as the significant elevation in Purkinje cell number. The cerebellar cortex was roughly the same as in the control group, except for a small number of cells with deeply stained cytoplasm and few pericellular spaces as well as few congested blood vessels. The current findings are supported by **Samad *et al.*** [29] who found that vitamin D improved the cerebellar cortex degenerative alterations in repeated noise stress model.

The VDR has been considered a main transcriptional regulator of autophagy, according to **Tavera-Mendoza *et al.*** [30]. They found that by upregulating the LC3 protein level and increasing the amount of autolysosomes, vitamin D administration causes autophagy in the murine mammary gland. Consequently, decreased damage in autistic rat cerebellum in the current study can be linked to stimulated autophagy that was evidenced in our work where the examination of immune stained cerebellar slides of LC3-II and their optical density statistical analysis in treated rats demonstrated the significant improvement of autophagy in response to cholecalciferol administration in autistic rats. **Magdy *et al.*** [15] reported an upregulation of LC3 gene and protein expressions, in the midbrain of parkinsonian rats after giving vitamin D indicating a stimulatory action of vitamin D on autophagy pathways.

CONCLUSION

In conclusion, cholecalciferol treatment improves VPA-induced cerebellar injury most probably through enhancement of autophagy and also by its antioxidant effect. Therefore, in order to maintain cerebellar structure and function, early cholecalciferol supplementation is advised as an adjuvant treatment for autistic patients.

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