Evaluation of prenatal administration of valproic acid on the cerebellum of albino rat offspring: A model of autism

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Abstract—Introduction: Autism is a severe neurodevelopmental disorder of poorly understood etiology; which may be genetic, epigenetic or environmental. Valproic acid (VPA), the most widely used antiepileptic drug, has been reported to increase the risk of autism among the offspring of human mothers who are medicated with it during early pregnancy. Aim: The current work aimed to study the biochemical and histological changes in the cerebellum of the offspring of prenatally VPA treated rats. Materials and Methods: Twelve pregnant female albino rats were divided into two groups; control and VPA treated (50 mg/kg/day orally). The cerebellar sections of male offspring rats were subjected to biochemical tests (brain tissue Malondialdehyde (MDA), Superoxide dismutase (SOD), Tumor necrosis factor-alpha (TNF-α) and glutamate), histological examination, immunohistochemical analyses for the expression of glial fibrillary acidic protein (GFAP) and Bcl-2 associated X protein (Bax), and electron microscopical studies. Results: VPA caused significant elevation in the brain levels of oxidative stress marker MDA, proinflammatory cytokine TNF-α and excitatory neurotransmitter glutamate, with significant reduction in the level of brain antioxidant marker SOD, compared to...
In the control group, it produced neurodegeneration with significant decrease of the molecular layer (ML), internal granular layer (IGL) thickness and the number of Purkinje cells (PCs). Immunohistochemically, there was a significant upregulation of GFAP, a marker for reactive astrocytes, and Bax, a marker for apoptosis. Conclusion: VPA induced cerebellar neurodegenerative changes through oxidative stress, neuroinflammation and neurotransmitter disturbances. Therefore, the VPA-induced rodent model of autism might be a reliable tool to study the idiopathic autism cases of environmental/epigenetic etiology, investigate the neurobiology underlying autistic behavior and screen for novel therapeutics.

**Keywords**—cerebellum, valproic acid, autism, immunohistochemistry.

**Introduction**

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder characterized by cognitive, sensorimotor and social interactions impairment with (repetitive & restricted) behaviors [1]. Its prevalence is dramatically increasing in Egypt and worldwide over the past decade affecting approximately 1–2% of the population globally, resulting in significant economic burden on education, health, and social services [2]. It is 5% more common in males than females [3]. Autism affects multiple brain regions including the cerebellum, hippocampus, prefrontal cortex, amygdala and basal ganglia [4]. The cerebellum is one of the target brain areas affected in autism as it has a critical role in the higher-order brain functions such as cognitive function, learning, and behavior [5]. In addition, it has the greatest number of neurons and synapses in the central nervous system [6]. Moreover, it is particularly vulnerable to developmental and environmental insults due to its long developmental schedule [7]. Therefore, cerebellum dysfunction plays a pivotal role in the pathogenesis of autism [8].

The etiology of autism has not been fully understood yet; however, it may be attributed to genetic or environmental factors such as prenatal exposure to infections and drugs (as valproic acid (VPA), propionic acid (PPA) and thalidomide) [9]. Therefore, experimental animal models of autism can help to understand its etiology, explore various supplements and examine the potential impact of pharmacological treatments prior to human testing [10]. VPA was used to induce rat model of autism as it is the most widely used antiepileptic drug due to its great influence on several seizure types [11]. In addition, it can cross the placenta, when used during pregnancy, accumulating in the fetal circulation with higher concentration than that in the maternal blood, causing toxicity, teratogenicity and increasing the autism rate among the offspring [12]. However, it could not be excluded in epileptic pregnant women because noncontrolled epileptic attacks during pregnancy produce high risk of injury to both mother and foetus [13].

Autism has a multifaceted nature; therefore various studies were reported to understand its pathophysiology. It produces neuroinflammation, oxidative stress elevation and neurotransmitter abnormalities resulting in neurodegeneration [14].
Consequently, the current study was designed to investigate the VPA induced cerebellar neurodegeneration based on biochemical, histological, immunohistochemical and electromicroscopical studies.

**Materials and Methods**

**Drugs and chemicals**

Valproic acid (as sodium salt), a product of Sanofi company, Egypt, was obtained from El-Ezaby pharmacy, Shebin El-Kom, Menoufia, Egypt, in the form of oral solution (Depakene syrup, 250 mg/5 mL solution).

**Animals**

Twelve adult sexually mature female albino rats and other twelve adult male albino rats (for mating), of Sprague Dawley strain, were used in this study. They were obtained from El helw animal house, Tanta, Egypt, with average weight of 200-250 g. They were housed in the animal house of the Faculty of Medicine, Menoufia University and kept in metallic cages, under good hygienic conditions, standard laboratory temperature (25±2°C), relative humidity of approximately 50%, and illumination (12 h light/dark) throughout the experiment. The animals were provided with free access to food and water. The procedure was approved by the ethics committee on animal experiment of the Faculty of Medicine, Menoufia University, Egypt, 82021 ANAT3 in accordance with the international regulations on care and use of laboratory animals.

**Experimental design**

Female rats were copulated with male rats in the proportion of 1:1 [9]. Fertilization was confirmed by vaginal smear examination every morning. The presence of vaginal plug and microscopic detection of sperms in the smears were designed as day one of gestation [13]. Pregnant females were caged separately and divided into two groups; each group consisted of six pregnant rats:

**Group I (Control group):** The rats were kept without any treatment all over the experimental periods and they were served as control group for all experimental groups.

**Group II (VPA induced autism group):** Each rat received VPA (50 mg/kg/day about 10 mg /rat) orally by gastric tube from day one of pregnancy till delivery [13].

Six male offspring rats from each group were sacrificed at 21\textsuperscript{th} day after birth.

**Tissue preparation**

At the end of the experiment, rats were anaesthetized by diethyl ether inhalation. Then, the skull of each rat was opened and the cerebellum was dissected out and examined.
**Evaluation methods**

**Death rate measurement**

Death rate was recorded on day 21.

**Body weight measurement**

Body weight measurements were recorded for all rats on day 21.

**Biochemical analysis of tissue homogenate**

The right cerebellar hemispheres were homogenised (10% w/v) in 10 mM of ice-cold Phosphate Buffer Saline (PBS), pH 7.4. The homogenate was centrifuged at 4000 rpm for 15 minutes at 4°C. The pellet was discarded, and the whole supernatant was obtained and stored at -80°C for assessment of MDA (QuantiChrom™, BioAssay Systems, USA) [15], SOD (Shimadzu-UV, USA) [16], TNF-α ELISA kit (ELab Sciences, Wuhan, China) [17], and Glutamate (HPLC-ECD Waters, USA) [1] using manufacturer’s protocol.

**Histological examination**

The left cerebellar hemispheres were fixed for 24 hours in 10% neutral buffered formalin then dehydrated in ascending grades of alcohol, cleared and embedded in paraffin blocks. After deparaffinizing, serial tissue sections were cut 5 μm thick and stained with haematoxylin and eosin [18].

**Immunohistochemical examination**

The 5-μm brain paraffin sections were deparaffinized then rehydrated in descending grades of alcohol. Endogenous peroxidase was blocked by inserting the sections in 3% hydrogen peroxide (H₂O₂). A protein blocker was used to block nonspecific binding sites then the primary antibody anti-Bax (rabbit polyclonal, Sigma-Aldrich, Cairo, Egypt) and anti-GFAP (Rabbit polyclonal, ab7260, 1:300, Midco Trade, Giza, Egypt) were added with overnight incubation. After that, biotinylated goat-polyvalent secondary antibody at a concentration of 2% (Vector, Peterborough, UK) was applied for 10 minutes (37°C) then the avidin-biotin-peroxidase complex (Vector) was added [19].

**Semi thin and Transmission Electron Microscopic (TEM) examination**

Small portions of the cerebellum (1mm³) were immediately fixed in 5% glutaraldehyde for 20 h, and then washed with cacodylate buffer and fixed in 1% osmium tetraoxide. Semi thin sections were obtained by glass knives, mounted on glass slides, stained with toluidine blue and examined by light microscope. Ultrathin sections of 50 nm were cut by an ultramicrotome, stained with uranyl acetate and lead citrate and examined by a transmission electron microscope (JEOL JSM35) at an acceleration voltage of 20 kV at the EM unit in Faculty of medicine, Alexandria University [19].
Morphometric analysis

For histological and immunohistochemical assessment of the ML, IGL thickness, number of the purkinje cells, area percentage of GFAP immunoreaction and percent of Bax positive cells. Image J version 1.47v software (National Institutes of Health, USA) was used. The sections from at least six animals/experimental group were examined. Five non-overlapping fields (400x) per section were randomly captured by a Leica Microscope DML B2/11888111 equipped with a Leica camera DFC450. The collected data was subjected to statistical analyses. This was done in Anatomy and Embryology Department, Faculty of Medicine, Menoufia University.

Statistical analysis

The collected data were presented as mean ± standard deviation. Data analysis was performed using SPSS (Statistical Package for Social Science) (Inc., Chicago, IL, USA) version 22.0 on IBM compatible computer. The obtained data were analyzed using Mann Whitney U test. The significance of the results was expressed by the P value (probability of chance): The results were considered statistically significant and nonsignificant when the p-values were <0.05 and >0.05 respectively.

Results

Death rate results

A significant elevation was observed in rats' death rate in the VPA group as compared to the control one (12.63±0.93 vs. 0.6±0.02, P<0.001) (Figure 1A).

Body weight results

A significant reduction was observed in rats' body weight in the VPA group as compared to the control one (19.54±5.93 vs. 54.17±9.53, P<0.001) (Figure 1B).

Fig 1. (A) Mean rats’ death rate (%), (B) Mean rats’ body weight (g). ** P<0.001 as compared with the control group
Biochemical results

Brain SOD activity

A significant reduction was observed in brain SOD activity in the VPA group as compared to the control one (0.93±0.05 vs. 2.13±0.18, P < 0.001) (Figure 2A).

Brain MDA level

A significant elevation was observed in brain MDA level in the VPA group as compared to the control one (2.21±0.01 vs. 1.05±0.02, P < 0.001) (Figure 2B).

Brain glutamate (Glu) level

A significant elevation was observed in brain glutamate level in the VPA group as compared to the control one (138.72±0.83 vs. 53.64±0.8, P < 0.001) (Figure 2C).

Brain TNF- α level

A significant elevation was observed in brain TNF- α level in the VPA group as compared to the control one (64.87±1.35 vs. 26.83±1.2, P < 0.001) (Figure 2D).

Fig 2. (A) Mean brain SOD activity (U/mg), (B) Mean brain MDA level (nmol/mg), (C) Mean brain glutamate level (ng/mg), (D) Mean brain TNF- α level (pg/mg).

** P<0.001 as compared with the control group
**Histopathological results**

Examination of H&E-stained sections, the cerebellum of the control group showed deep branching folia with narrow fissures in between. Each folium was composed of outer cortex, covered by the pia matter and consisted of three layers; the molecular (ML), purkinje cell (PCL) and internal granular (IGL) layers, and inner medulla. The ML showed intact clear neuropil containing many basket and stellate cells. The PCL consisted of one row of well-organized pyriform-shaped purkinje cells (PCs), with central rounded vesicular nuclei, prominent nucleoli and arborizing apical dendrites toward the ML, and neuroglia cells in-between. The IGL showed well-defined tightly packed granular cells, with dark rounded nuclei, and lightly stained non-cellular areas (cerebellar islands) in-between (Figure 3). Semi-thin sections stained by toluidine blue showed the three cortical layers. The ML showed many normal fibers and neurons. The PCs appeared normal and surrounded with astrocytes, with pale nuclei and pale cytoplasm. The IGL showed many rounded granule cells, with dark stained nuclei and granular chromatin, with pale-stained astrocytes and normal cerebellar islands in between (Figure 6).

The VPA group showed disrupted cerebellar architecture. The cerebellar fissures were wide with hemorrhage and congested blood vessels. There was meningeal detachment and persistent vacuolated external granular layer (EGL). The ML showed vacuolated fragmented neuropil with few neurons. PCs were haphazardly arranged with multilayer deposition. Some of them were degenerated, others showed hyalinization and some were lost leaving empty spaces. The IGL showed few small sized granular cells, with wide vacuolated cerebellar islands in-between, and highly congested elongated blood vessels (Figures 4, 5). Semi-thin sections showed vacuolations and congested blood vessels within the ML, PCL and IGL. Some PCs appeared distorted with ill-defined nuclei and others had pyknotic nuclei with darkly stained vacuolated cytoplasm. The IGL showed few small deeply stained granule cells with wide vacuolated cerebellar islands and pale astrocytes between them (Figure 6).

Statistically: The thickness of both ML and IGL was significantly decreased in the VPA group compared with the control one (137.8±7.9 vs 269.2±3.2; 102.38±5.09 vs 164.25±2.54 respectively, P <0.001). In addition, the normal PC number was significantly decreased in the VPA group compared with the control one (5.38±1.6 vs 13.2±2.06, P <0.001) (Figure 4).
Fig 3. (A, B, C, D) H&E staining of cerebellar sections of the control group showing: (A) deep branching cerebellar folia (F) with narrow fissures (FI) in between (Scale bar = 200 µm). (B) Each folium is composed of outer cerebellar cortex (C); which is composed of three layers (outer molecular layer (ML), middle purkinje cell layer (PCL) and internal granular layer (IGL)), and inner medulla (M) (Scale bar = 100 µm). (C) The three cerebellar layers; ML, IGL and PCL, composed of one row of well-organized purkinje cells. Notice: Pia matter (green arrow) covers the cerebellar cortex (Scale bar = 50 µm). (D) Molecular layer (ML) has clear intact neuropil (n), containing numerous basket (B) and stellate (S) cells. The PCL shows one row of pyriform-shaped purkinje cells (PC) with rounded vesicular nuclei, prominent nucleoli and apical dendrites (arrow head) arborizing toward the ML. Neuroglia cells (notched arrow) are detected between PCs. The IGL shows numerous well-defined tightly packed small granular cells (red arrow), with dark rounded nuclei, and cerebellar islands (yellow arrow) in-between (Scale bar = 20 µm).
Fig 4. (A,B,C) H&E staining of cerebellar sections of the VPA group showing: (A) cerebellar folia with wide fissure (FI) in between. Each folium is composed of outer cerebellar cortex (C); which is composed of three layers (outer molecular layer (ML), middle Purkinje cell layer (PCL) and internal granular layer (IGL)), and inner medulla (M). Hemorrhage (Hge), within the cerebellar fissure and under the meningeal covering, and many congested blood vessels (BV) can be noticed. (B) Multilayered disturbed arranged Purkinje cells (circle) of the PCL. Notice: detachment of the meningeal covering (red arrow) (Scale bar = 100 µm). (C) Vacuolated EGL, marked reduction in the thickness of ML, multilayered degenerated Purkinje cells of PCL with disturbed arrangement, Hemorrhage (Hge) and coalesce of two IGL with ill-defined medulla could be observed (Scale bar = 50 µm). (D) Mean thickness of the ML (Um), (E) Mean thickness of the IGL (Um), (F) Mean number of the normal PCs. ** P<0.001 as compared with the control group.
Fig 5. (A, B, C, D) H&E staining of cerebellar sections of the VPA group showing persistent external granular layer (EGL) with vacuolations (V) and reduced molecular layer (ML) thickness (up down arrow) and neuron number. Its neuropil (n) is vacuolated and contains migratory cells (arrow head), many fibers (blue arrow), few pyknotic glial cells with pericellular halos (notched arrow), and elongated congested blood vessels (BV). The purkinje cell layer (PCL) shows multilayer disorganized distorted irregular shaped purkinje cells (PCs). Some PCs are degenerated (arched arrow), with acidophilic cytoplasm, small deeply stained nuclei, lost dendrites and surrounded by pericellular spaces; while others with thin dendrites (black arrow); and some show hyalinization (green arrow); while others were lost leaving empty spaces (SP). The internal granular layer (IGL) contains widely separated small deeply stained cells (red arrow) with wide vacuolated (V) cerebellar islands (double arrows) in-between. Notice: hemorrhage (Hge) can be noticed within the cerebellar fissure and IGL (Scale bar = 20 µm).
Fig 6. Photomicrographs of semi-thin sections (stained with toluidine blue) in the cerebellar cortex showing its three layers: the molecular (ML), Purkinje cell (PCL) and internal granular (IGL) layers. (A) The control group showing many neurons (arrow head) in the ML. The Purkinje cells (PC) appear pyriform shaped having large central vesicular nuclei (N), prominent dark nucleoli (curved arrow) and thick apical dendrites (notched arrow). Astrocytes (yellow arrow), with pale nuclei and cytoplasm, surround PC. The IGL has many rounded granule cells (red arrow), having rounded heterochromatic nuclei and pale scanty cytoplasm, with cerebellar islands (double arrows) and pale-stained astrocytes (yellow arrow) in between. (B), (C) The VPA group showing many vacuolations (V), degenerated areas (D) and congested blood vessels (BV) within the ML, PCL and IGL. The Purkinje cells show disturbed linear organization with irregular astrocytes (yellow arrow) in between. Some PCs are distorted with ill-defined nuclei (black notched arrow), others (red notched arrow) have darkly stained nuclei and vacuolated cytoplasm, some show hyalinization (green notched arrow) and others are shrunken (curved arrow). The granular cells (red arrow) appear deeply stained with dark nuclei with wide vacuolated cerebellar islands (double arrows) and astrocytes (yellow arrow) in between. Migrating Purkinje cell (MPC) can be noticed in IGL. (Scale bar = 10 µm).

**Immunohistochemical results**

GFAP immunostaining of cerebellar sections from the control group showed few small star-shaped faint brown astrocytes with short processes dispersed among the different cortical layers. The VPA group showed a significant elevation in area % of GFAP immunoreactivity compared to the control one (P<0.001) (Figure 7). Bax immunostaining of cerebellar sections from the control group showed
negative reaction. The VPA group showed a significant elevation in % of Bax positive cells compared to the control one (P<0.001) (Figure 7).

![Image](image_url)

**Fig 7.** Representative micrographs of the two experimental groups showing significant up regulation of the GFAP (B) and Bax (E) in the VPA group compared to the negative control (A), (D) respectively (Scale bar = 20 µm). (C) Mean area percentage of GFAP immunoreactivity (F) Mean percent of Bax positive cells. **P<0.001 as compared with the control group.**

**Electron microscopic results**

Electron microscopic examination of ultrathin sections of the cerebellar cortex of control rats showed normal PCs each had large rounded euchromatic nucleus, prominent nucleolus, regular nuclear envelope, well-defined cell membrane and homogenous cytoplasm with many normal mitochondria, rough endoplasmic reticulum (rER) and well-organized Golgi apparatus (Fig. 8). The IGL showed numerous normal tightly packed granule cells with central oval to rounded heterochromatic nuclei, with condensed chromatin, surrounded by thin rim of cytoplasm that contained normal mitochondria and rER (Fig. 9). The ML showed normal glial cells and regular nerve fibers surrounded by intact myelin sheaths and contained normal mitochondria in their axoplasm (Fig. 10).

Electron microscopic examination of ultrathin sections of the cerebellar cortex of VPA treated rats showed marked degenerative changes in the three cortical layers compared to the control group. The PCs were markedly degenerated. Each one had shrunken highly electron dense nucleus with indented corrugated nuclear envelope, irregular cell membrane and rarefied vacuolated cytoplasm contained degenerated mitochondria, dilated rER and disrupted dilated Golgi apparatus.
The surrounding neuropil was severely lysed with large interstitial spaces (Fig. 8). The granule cells showed different degrees of degeneration. Some of them had euchromatic nuclei with faint chromatin, irregular ill-defined cell membranes and vacuolated cytoplasm. Others appeared apoptotic with shrunken irregular dark nuclei. Congested blood capillaries and wide interstitial spaces filled with tissue fluid were observed (Fig. 9). The ML showed degenerated glial cells and nerve fibers surrounded by splitted myelin sheaths with unusual myelin protrusions and contained degenerated or swollen mitochondria in their axoplasm (Fig. 10).

Fig 8. Transmission electron micrographs of ultrathin sections in the PCL: (A) Control group showing intact purkinje cell (PC) with large rounded euchromatic nucleus (N), prominent nucleolus (Nu), regular nuclear envelope (arrow head), homogenous cytoplasm (C), well-defined cell membrane (arrow) and normal axon (Ax) (Scale bar = 0.5 μm). (B) Higher magnification showing part of intact purkinje cell with euchromatic nucleus (N), well defined nucleolus (Nu), intact perinuclear membrane (arrow head) and homogenous cytoplasm containing numerous normal mitochondria (M), parallel strands of cisternae of rough endoplasmic reticulum (yellow arrow), well organized Golgi apparatus (circle) and free ribosomes (red arrow). Glial cells (star) surround the purkinje cell (Scale bar = 0.2 μm). (C) VPA group showing markedly degenerated purkinje cell (DPC) with an irregular cell membrane (arrow), degenerated shrunken nucleus (N) with irregular nuclear envelope (arrow head) and rarefied vacuolated (V) cytoplasm (C). Lysed granular cells (G) can be noticed (Scale bar = 0.5 μm). (D) Higher magnification showing part of degenerated purkinje cell with irregular highly electron dense nucleus (N) having indented corrugated nuclear membrane (arrow head). The cytoplasm contains polymorph degenerated mitochondria with ruptured cristae (M), dilated...
rough endoplasmic reticulum (rER), disrupted dilated Golgi apparatus (circle), vacuoles (V) and phagosome (P) (Scale bar = 0.2 µm).

Fig 9. Transmission electron micrographs of ultrathin sections in the IGL: (A) Control group showing normal granule cells (G) with central oval heterochromatic nuclei (N) surrounded by thin rim of cytoplasm (C). Mossy rosettes (circle) can be seen containing many round mitochondria (M) and synaptic vesicles (V) (Scale bar = 0.5 µm). (B) Higher magnification showing normal granule cells with large oval or rounded heterochromatic nuclei (N) with coarse central and peripheral chromatin clumps (red arrow) and thin rim of cytoplasm (C) containing numerous normal small spheroid mitochondria (M) and normal rough endoplasmic reticulum (blue arrow). Tight junctional complexes (yellow arrow) between the cells can be observed (Scale bar = 0.2 µm). (C) VPA group showing some granular cells (G) with faint euchromatic nuclei (N) and other degenerated cells (G1) having small nuclei (N1). One granule cell (G2) appears apoptotic with small dark nucleus (N2), another one (G3) with irregular nucleus (N3). Dilated congested blood capillary containing RBCs (BC) and neuropil spaces (SP) are observed (Scale bar = 0.5 µm). (D) Higher magnification showing parts of degenerated granular cells with euchromatic nuclei (N) with faint chromatin (red arrow), ill-defined cell membranes and massive lysis of their cytoplasm (C) which contains swollen mitochondria with destroyed cisternae (M). Wide interstitial spaces (SP) filled with tissue fluid are also observed. Note: loss of junctional complexes between the cells (Scale bar = 0.2 µm).
Fig 10. Transmission electron micrographs of ultrathin sections in the ML of cerebellar cortex of different groups. (A), (B) Control group showing regular nerve fibers (F) in which axons (green arrow) are wrapped in intact myelin sheaths (yellow arrow) of varying sizes and thickness with compact arrangement of the myelin lamellae with normal mitochondria (M) in their axoplasm. Normal neuroglial cell euchromatic nucleus (Ng) surrounded with thin rim of cytoplasm (C) can be noticed (Scale bar = 0.5 µm, 0.2 µm). (C), (D) VPA group showing splitting (red and yellow arrows) with unusual myelin protrusions (circle) of myelinated nerve fibers (F) containing degenerated or swollen mitochondria with disrupted cisternae (M) in their axoplasm. Degenerated neuroglial cell nucleus (Ng) surrounded with rim of cytoplasm (C) containing multiple degenerated mitochondria (m) can be noticed (Scale bar = 0.5 µm, 0.2 µm).

Discussion

Autism produces neurodevelopmental impairment of multiple brain regions including the cerebellum [2]. Human-like autistic features can be induced in rodent models by exposing animals to certain chemicals, such as VPA and PPA [12]. VPA model is considered one of the best models of autism as the VPA exposed rodents express similar behaviors and clinical features as people with autism, and can predict clinical treatments for ASD [10]. Therefore, our study reported the neurodegenerative changes in the cerebellum of the offspring prenatally treated with VPA.

A strong association has been reported between maternal gestational VPA administration and the later development of autism in the child [10]. This was
supported by Nicolini and Fahnestock [20] who stated that in utero VPA exposure in rodents might represent the idiopathic autism cases of environmental origins. Also, Mirza and Sharma [21] clarified that prenatal VPA exposure induced ASD in both human and rodents.

Prenatal VPA model was preferred in our study than the postnatal one as the development of human cerebellum, extending from the early embryonic period up to the first 2 years after birth, makes it vulnerable to developmental disorders, especially during the early fetal period [22]. This was in agreement with Elnahas et al. [23] who stated that prenatal VPA model causes more autistic-like behaviors and less mortality than the postnatal one. Also, Fereshetyan et al. [24] found structural changes in the brain of prenatally VPA-treated groups, and their absence in the postnatally treated ones. Controversially, Sunand et al. [25] stated that the postnatal method is most suitable to evaluate the potency of many therapeutic agents.

In the current study, the VPA treated rats showed significantly increased death rate compared to the control group. This was in agreement with Elnahas et al. [23]. Few studies have examined the body mass status of autistic patients with different outcomes due to the different VPA administration protocols. In the present study, there was a significant reduction in rats' body weight in the VPA group as compared with the control one. This may be attributed to VPA induced maturational delay. Similar findings were obtained by Al-Askar et al. [26]. This was in agreement with Schneider and Przewlocki [27], Yang et al. [28] and Jazayeri et al. [29] who stated that VPA exposed pups showed significant reduction in body weight, suggesting delayed growth by VPA. Controversially, Favre et al. [30] found no effect of VPA on offspring body mass gain, and explained this by being unclear if body weight observations in different VPA model versions are directly linked to autism-like symptoms or specific to rat strain or VPA dosage.

In the current study, the VPA treated rats showed significant elevation in the level of oxidative stress marker (brain MDA) and reduction in the antioxidant marker (SOD) activity, as compared with the control group. Similar findings were conducted by Morakotsriwan et al. [31]. This may be attributed to VPA induced formation of reactive oxygen species (ROS), resulting in peroxidation of lipids with MDA production that destroys the developing neurons. This was in agreement with Al-Askar et al. [26], Arafat and Shabaan [6] and Al-Gholam and Ameen [15] who found a significant elevation in oxidative stress markers in the offspring of VPA treated rats. In addition, Sunand et al. [25] found that antioxidant SOD level was declined with increase in MDA level in the autistic group.

Biological abnormalities including irregularities in neurotransmitters and enzyme activities were reported in autism. In the current study, the VPA group showed significant elevation in brain glutamate level (excitatory neurotransmitter) as compared with the control one, indicating neuronal excitotoxicity. This was in line with Al-Askar et al. [26] who reported that VPA increased glutamate levels with glutamate receptor activation in rats. The VPA group showed significant elevation in the level of the proinflammatory cytokine TNF-α, as compared with the control group. This may be due to neuroinflammation during brain development. Similar
findings were reported by Elnahas et al. [23] who stated that inflammatory cytokines and oxidative stress markers were elevated in prefrontal cortex and hippocampal homogenates of VPA exposed rats. Also, Saghazadeh et al. [32] showed that TNF-α levels increased significantly in individuals with ASD.

In the present study, Light microscopic examination (H&E and toluidine blue stained semi-thin sections) of the control group cerebellar cortex showed that it consisted of three layers: ML, PCL and IGL with absent EGL. The ML contained many basket and stellate cells within intact neuropil. The PCL consisted of single row of well-organized pyriform shaped PCs, with large rounded vesicular nuclei, prominent nucleoli and arborizing dendrites toward the ML. The IGL was formed of well-defined rounded granule cells with narrow cerebellar islands in between. This was in agreement with El-kholy et al. [33] and Mohammed et al. [34].

This was confirmed ultra-structurally; normal PC had large rounded euchromatic nucleus, prominent nucleolus, regular nuclear and cell membranes, and homogenous cytoplasm with many normal mitochondria, rough endoplasmic reticulum (rER) and well organized Golgi apparatus. The IGL showed normal tightly packed granule cells with central oval to rounded heterochromatic nuclei, with condensed chromatin, surrounded by thin rim of cytoplasm containing normal mitochondria. The ML showed regular nerve fibers surrounded by intact myelin sheaths and contained normal mitochondria. This was in agreement with Shona et al. [13].

Arrangement of PCs in one row forming PCL can be explained by the pressure exerted on the growing PCs from below by expanding IGL and the barrier formed above these cells by the parallel fibers, as reported by Ghosh et al. [35]. Mohammed et al. [34] explained absent EGL by the essential role of granule cells in final maturation of PCs responsible for stopping signal of migration. In the current study, the VPA group showed marked degenerative changes in the three cerebellar layers mostly manifested on PCs that were haphazardly arranged and degenerated pyknotic, mostly apoptotic which was supported by a significant up regulation of Bax. Some PCs were swollen, others were lost leaving empty spaces and others showed hyalinization. This was confirmed ultra-structurally; PCs had irregular shrunken nuclei, deformed nuclear membranes and vacuolated cytoplasm containing degenerated mitochondria, destroyed rER and dilated Golgi apparatus. Similar findings were reported by Shona et al. [13].

This may be attributed to neuronal oxidative stress, neuroinflammation and neurotransmitter disturbances, resulting in neurodegenerative features and cell death. This was in agreement with El-Bermawy and Salem [36] and Morakotsriwan et al. [31]. This was confirmed in our study by the significant elevation in the level of oxidative stress markers that led to activation of Bax pathway that might be responsible for the significant decrease in the number of PCs. This was supported by Sunand et al. [25] who referred the decrease in PC density in VPA treated rats to high vulnerability of neuronal cells, at the early stages of development, to the effect of ROS, which interfered with the neurodevelopmental process by damaging lipids and proteins in cellular membranes and DNA in the cells. Also, Al-Gholam and Ameen [15] clarified that elevation of inflammatory cytokines in VPA group modified both excitatory and
inhibitory synaptic formations resulting in disturbed synaptic transmission, neurotoxicity and neuronal death.

Another explanation was reported by Galaly et al. [37] who stated that increased MDA level reduced the growth of neural progenitor cells impairing the neurogenesis. In addition, Abdel Mohsen et al. [38] explained PCs degeneration by release of cytochrome c from stressed mitochondria into cytoplasm activating caspase-3 and subsequently inducing cell death. Multilayered disarrangement of PCs could be explained by their delayed migration leading to disturbance of their normal linear organization. This was in agreement with Shona et al. [13] and Ghosh et al. [35]. Also, Mohammed et al. [34] referred it to the neurodegenerative changes. In addition, Abdel Mohsen et al. [38] and Soliman and Ali [39] explained the disturbed PCs linear organization by prolonged neuronal insult resulting in adaptive mechanism in the form of PC crowding in other areas as a trial to reestablish synapsis with other nerve cells to achieve their functions.

Loss of the pyriform shape of PCs could be explained by the ability of VPA to interact with cytoskeletal elements. Nuclear pyknosis can be attributed to programmed cell death or apoptosis due to DNA damage as a result of production of ROS. This was in line with Main and Kulesza [8] and Soliman and Ali [39]. Dark PCs nuclei and cytoplasm might be attributed to failure of the antioxidant system leading to oxidative stress with subsequent accumulation of denatured proteins. This was in agreement with Ibrahim et al. [40]. Also, Galaly et al. [37] stated that dark neurons might reflect a certain phase of apoptosis.

Prominent perineural spaces with separated neuropil around PCs were attributed to shrinkage of the cells and withdrawal of their protoplasmic processes resulting from disintegration of the cellular cytoskeletal elements. This was in agreement with Abdel Mohsen et al. [38] and Ibrahim et al. [40]. Loss of some PCs leaving empty spaces could be explained by neuronal degeneration, necrosis, chromatolysis and axonal demyelination as referred by El-azab et al. [41] who stated that PCs are the most sensitive cortical neurons to neuronal toxicity which reacted to toxic elements by undergoing degeneration and loss from their place in PCL.

Regarding the ML and IGL, the VPA group showed significant reduction in their thickness. The ML showed vacuolated fragmented neuropil containing few pyknotic neurons, and congested blood vessels. This was supported by De Zeeuw and Hoogland [42]. Ultra-structurally, it showed degenerated nerve fibers with splitted myelin sheaths containing degenerated mitochondria. Similar findings were reported by Abdel Mohsen et al. [38] who explained myelin damage by altered osmotic conditions by toxic agents, thus water and electrolytes were drawn into myelin sheath leading to edema and myelin splits, decreased myelin-associated glycoprotein and axonal degeneration.

The IGL showed few apoptotic granule cells with irregular dense heterochromatic or faint euchromatic nuclei and vacuolated cytoplasm, sparsed with wide vacuolated cerebellar islands. This was supported by Shona et al. [13] who stated that VPA exacerbated the damage of granular cells with amalgamation and loss of most of cortical architecture. This was confirmed ultra-structurally. Similar
results were obtained by Kamal and Kamal [43] who stated that these changes might be a feature of apoptosis.

These degenerative changes were secondary to those of PCs as degenerated PCs failed to make normal contact with granule cells, impairing their regulatory role with loss of synchronism between them. This explanation was in agreement with Galaly et al. [37], Ibrahim et al. [40] and Ghosh et al. [35]. Another explanation was reported by Mohamed et al. [44] who related the granule cells changes to their late maturation which makes them vulnerable to the stressful stimuli. Reduced ML and IGL thickness can be explained by cellular degeneration as reported by Shona et al. [13] who referred it to disrupted cyclin-dependent kinase system which controls cellular apoptosis, division, differentiation and function. Also, Allam et al. [45] stated that reduced ML thickness was due to decreased its neuron number and size of PC arborization due to loss of PCs. Decreased IGL thickness could be explained by neuronal loss as reported by Abdel Mohsen et al. [38].

Cytoplasmic vacuolations observed within the three layers might be due to increased lipid peroxidation and ROS production attacking the cell membrane causing altered permeability and its destruction leading to cell death. This was in agreement with Yahyazadeha and Altunkaynakb [46] and Soliman and Ali [39] who explained vacuolations by marked disturbances in lipid inclusions within their cytoplasm. Another explanation was reported by Eluwa et al. [47] who referred the vacuolated cytoplasm detected in both purkinje and granule cells to loss of many cellular components within these cells.

Persistent EGL was observed in the VPA treated rats, attributed to the delayed differentiation and migration of its cells through PCL to form the IGL. This was in agreement with Allam et al. [45] who mentioned that at PND 21, the EGL was still well represented in diseased group. In the present study, VPA exposure caused significant up regulation of proapoptotic Bax. This can be attributed to VPA induced oxidative stress and neuroinflammation causing cell death via apoptosis. This was in agreement with El-Gerbed [48] who stated that neuroinflammation induced cytotoxicity through overproduction of ROS, which in turn damages the cellular components such as protein, lipids and DNA. This was supported by Sanaei and Kavoosi [49] who stated that VPA induced apoptosis via activation of the intrinsic mitochondrial apoptotic pathway, thus up regulated Bax and down regulated Bcl-2 (anti-apoptotic). Also, Zhou et al. [50] and Binabaj et al. [51] showed that VPA induced significant increase in Bax and decrease in Bcl-2 protein levels with increase in the Bax/Bcl-2 ratio. In addition, Frandsen and Narayanasamy [52] found increased Bax and decreased Bcl-2 activity in the brain tissues of ASD patients.

In this study, the significant increase in the area percentage of GFAP immunoreactive cells in the cerebellar cortex of the VPA treated rats was in line with Arafat and Shabaan [6]. This may be attributed to the astrocyte activation as a compensatory mechanism after VPA induced neurodegeneration (oxidative stress induced reactive gliosis). This explanation was in agreement with Shona et al. [13]. Also, Ibrahim et al. [40] showed significant increase in GFAP expression (astrogliosis) indicating proliferation and hypertrophy of astrocytes and other gial
cells responsible for repairing the brain injuries, as a compensatory neuroprotective process after neurodegeneration. In addition, Mohammed et al. [34] reported that GFAP expression and astrocytic dysfunction compromised the neuronal survival and were concomitant with damaging conditions in the CNS.

Moreover, Ramesh et al. [53] added that astrocytes represent the most abundant glial cell type in the CNS. Once these cells undergo reactive activation, they produce cytokines involved in inflammatory response contribute to expand brain damage. This was supported by Lawrence et al. [54] who proposed that following brain injury, astrocytes might act as stem cell initiating repair process. Also, reactive astrocytes produce and secrete lower levels of neuroprotective neurotrophic factors, which are important for survival of neurons, while releasing higher amounts of neurotoxic cytokines and chemokines, which play a role in neurodegeneration.

**Conclusion**

The results of this study confirm the neurodegenerative effects of VPA on the rat cerebellar cortex. Therefore, rodent VPA model may be suitable to examine future therapeutic interventions for autism.

**References**


