

Effect of Valproic Acid on Pre and Postnatal Development of the Cerebellar Cortex of the Albino Rat and the Possible Protective Role of the Folic Acid

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ABSTRACT

Background: Valproic acid (VPA) is one of antiepileptic drugs that causes neurodevelopmental disorders, including the autism spectrum disorder. Folic acid is essential for DNA synthesis and its periconceptional supplementation protects against several birth defects.

Objective: To clarify the folic acid's protective potential against the valproic acid induced neurodevelopmental toxicity on rat cerebellar cortex.

Material and Methods: 60 pregnant albino rats were divided into three groups; group I (control, received saline), group II (valproic acid 50 mg/kg once daily) and group III (valproic and folic acid (400 mg/kg) once daily). The offspring rats were sacrificed at day 15 and 17 prenatally, newborn, and at day 15 and 21 postnatally. Samples of cerebellar cortex were prepared for light and electron microscopic examination, immunohistochemical GFAB study as well as for histomorphometric analysis.

Results: In group II, the rat offspring's cerebellar cortex showed marked degenerative changes, mostly in Purkinje cells. The cells appeared shrunken, irregular with vacuolated cytoplasm contained damaged organelles and nuclei. The external granular layer appeared decreased in thickness and contained less cells with deeply stained nuclei. The cerebellar cortex of the group III showed restoration of the normal architecture. GFAB immunoreaction in group III appeared comparable to control against the strong positive reaction (gliosis) in group II. All studied morphometric parameters in group III were close to the control group and markedly reduced in group II.

Conclusion: Periconceptional folic acid supplementation reduces VPA neurotoxic effects on rat cerebellar cortex development pre and postnatally.

Keywords: Cerebellar cortex, Valproic acid, Folic acid, GFAP.

INTRODUCTION

Antiepileptic drugs are important drugs that are used in many pregnant women for prevention and control of the seizures. Non-controlled epileptic attacks during pregnancy causes harmful effects to both mother and fetus. Valproic acid (VPA) is considered the most efficient and in some cases, the only successful treatment for some forms of epilepsy⁽¹⁾. VPA traverses the placental barrier and accumulates in the fetal blood causing injurious effects on the developing embryo^(2,3,4). It also transferred through the breast milk, so infants can be exposed postnatally to VPA if their mothers taking it⁽⁵⁾.

VPA produces various types of developmental defects that depend on the dose of the drug⁽²⁾. Valproic acid causes neural tube defects and skeletal malformations include limb defects, fused vertebrae, fused ribs, syndactyly, and dysplasias^(5,6). In addition to the fetal malformation, exposure to VPA in utero also causes neurodevelopmental disorders including reduced cognitive function, learning difficulties, attention-deficit disorder and increases the prevalence of children suffering from autism spectrum disorder^(3,5).

It was found that VPA causes harmful effects on the proteins and genes that play a vital role in guidance of differentiation and cell divisions during organogenesis with the production of many apoptotic pathways⁽⁷⁾. Furthermore, it was reported that VPA induced congenital anomalies and degenerative effects caused by increasing levels of free radicals in the body. It is also found that cerebellar toxic effects caused by

VPA are accompanying by obvious increase in the fetal oxidative stress^(7,8).

Folic acid (Vitamin B9) plays a vital role in the expression of genes and new protein production which is important for fetal cell differentiation and development^(7,9,10). The folic acid requirements increase during periods of rapid tissue growth as during pregnancy^(11,12). Adequate folic acid intake by pregnant women protects against several congenital anomalies⁽¹¹⁾.

Therefore, the present study was carried out to examine the possible protective effect of folic acid against the degenerative changes induced in rat cerebellar cortex due to pre and postnatal exposure to valproic acid.

MATERIAL AND METHODS

Drugs used:

1. **Valproic acid (VPA):** Sodium valproate in the form of syrup 250 mg/5 mL, produced by Sanofi Company, Egypt.
2. **Folic acid:** in the form of tablets (5 mg), a product of El-Nile Company for pharmaceutical and chemicals, Egypt. Each tablet was dispersed in 10 mL of distilled water. Each mL of the produced suspension contained 500 µg of folic acid.

Animals used:

A total number of 60 pregnant albino rats were used in the current study that obtained after each 4 adult females and one adult male rat (180-200g) were housed

separately. The animals were obtained from the Animal House at the Faculty of Medicine, Assiut University and housed in a room with 12:12hr light: dark cycle and kept under a controlled temperature. Food and water were available ad libitum. This study was carried after approval from the Committee of Animal Research Ethics at the Faculty of Medicine, Assiut University, Assiut.

Experimental design:

A vaginal smear was examined for sperm every morning which indicated positive conception (day zero). Female rats that successfully breed were caged separately. The pregnant rats were equally divided into three groups (n=20):

- **Group I (control group):** received distilled water through intragastric tube once daily.
 - **Group II (valproic acid treated group):** received VPA by intragastric tube at a dose of 50 mg/kg once daily from day one of pregnancy till the end of lactation (21 day postnatal). This is the therapeutic dose of VPA used in the treatment of epilepsy⁽⁸⁾.
 - **Group III (valproic acid and folic acid treated group):** received both VPA (50 mg/kg) and folic acid (400 mg/kg) by intragastric tube once daily from day one of pregnancy till the end of lactation (21 day postnatal)^(8, 13).

The ages used:

The offspring were sacrificed at the following ages:

1. Prenatal ages: include 15 and 17 embryonic days (n=6).
2. Postnatal ages: include new born, 14 and 21 days (n= 18).

Histological and immunohistochemical techniques:

Light microscopic examination

In prenatal ages, the pregnant rats were lightly anesthetized using diethyl ether and fetuses were extracted, fixed in Bouin's solution. Paraffin sections at 4-5 μ m thickness of each embryo head were prepared from the three studied groups and stained with hematoxylin and eosin⁽¹⁴⁾ for examination of cerebellar cortical structure.

In the postnatal ages: The cerebellum of each rat offspring at various postnatal ages was removed cautiously. Some cerebellar specimens were processed for obtaining 4 -5 μ m thickness paraffin sections in median sagittal plane and stained with hematoxylin and eosin⁽¹⁴⁾ for examination of cerebellar cortical architecture. Others were fixed in 10% neutral buffered formalin and cortical architecture. Others were fixed in 10% neutral buffered formalin and processed for GFAP immunohistochemically staining, using streptavidin–biotin–peroxidase technique. With the use of 0.9% hydrogen peroxide in absolute methanol for 10 min, the nonspecific endogenous peroxidase activity was blocked then incubated with the primary anti-GFAP antibody at 1:100 dilutions for 20 min at room

temperature. Mouse monoclonal antibody (GFAP) Ab-1 produced by (Dako Cytomation) was used. The slides then incubated with the secondary anti-mouse antibodies universal kits. Incubation with substrate chromogen 3,3'-diaminobenzidine for 5-10 min was done, which resulted in brown-colored precipitate at the antigen sites⁽¹⁵⁾. The primary antibody was omitted during staining of some slides to be used as a negative control. The nuclei were counterstained with Mayer's hematoxylin. Positive control (brown discoloration) of the glial cells mainly astrocytes denoting increase gliosis⁽¹⁵⁾.

Electron microscopic examination

The cerebellar specimens at different postnatal ages were cut into small slices (about 1mm³), fixed in 4% glutaraldehyde then washed in phosphate buffer and post-fixed in 1% osmium tetroxide and prepared for ultrathin sectioning. With the use of uranyl acetate and lead citrate, the ultrathin sections (50-60nm) were stained⁽¹⁶⁾, examined and photographed using a transmission electron microscope (JEM- 100 Cx11, Jeol, Assiut, Egypt).

Histomorphometric measurements:

The following parameters were estimated at the postnatal ages in various experimental groups, using Hematoxylin and eosin stained sections (at magnification X 400) in different random fields:

- Thickness of the cerebellar cortex.
- Count the number of Purkinje cells per definite area (13413 μ ²).
- Estimation of nuclear diameter of Purkinje cells: The longest axis of the nucleus was measured as the major (a) diameter, and crossing axis of the major diameter from its midpoint at right angle was measured as the minor (b) diameter. The mean diameter (d) was estimated by using the relationship: $d = \sqrt{(a \times b)}$ ⁽¹⁷⁾.

The measurements were obtained in non-overlapping 10 fields in 10 randomly chosen sections by using an image analyzer computer system; OLYMPUS DP27 digital camera attached to an OLYMPUS CX41 light microscope and PC running cell Sens Standard software (version 1.7).

Statistical analysis

The collected data by histomorphometric measurements from the three experimental groups (6 animals for each group) were expressed as (mean \pm SD) and statistically analyzed using (SPSS, version 22; SPSS Inc., Chicago, Illinois, USA). Histograms were constructed and a comparison of significance between the experimental groups was carried out using the analysis of variance (ANOVA) test. Statistical significance was set at p values \leq 0.05.

RESULTS

Light and Electron microscopic examination:

In the control group (group I), H&E stained sections showed a well identified cerebellar area at E15 day prenatally that represented by two zones; outer superficial fibrous layer and inner differentiating neuroepithelial cell layer. The inner layer consisted of small rounded cells with vesicular nuclei (Fig. 1a). The first appearance of the external granular layer in the primitive cerebellar cortex was evident at E17 day that expanded over the surface of the cerebellum and consisted of small, rounded cells (Fig.1b). The primitive molecular layer started to appear as thin rim deep to external granular layer. A Purkinje cell layer consisted of differentiating cells arranged in several layers were obvious under the molecular layer at this E17 prenatal age (Fig.1b).

The cerebellar cortex in new born control sections consisted of external granular layer; molecular layer; Purkinje cell layer and internal granular layer, from the pial surface inwards. The external granular layer was formed of two well-defined zones of small rounded cells that differentiated into; outer multiplying zone and inner premigratory one. The cells of outer multiplying zone were parallel to the pial surface while the cells of the pre-migratory zone were arranged in rows perpendicular to the surface. The molecular layer at this age appeared as thin rim, contained few scattered cells. The Purkinje cell layer appeared as multi laminated layer crowded with oval or rounded bodies of Purkinje cells with vesicular nuclei. The internal granular layer was ill-defined and had small rounded cells (Fig.2a).

The cerebellar cortex of fourteen days postnatally appeared with decreased in the thickness of external granular layer which stuffed with small rounded neurons. The Purkinje cells were regularly arranged in monolayer and appeared well differentiated with flask or oval shaped and rounded vesicular nuclei. This age also revealed an apparent increase in the area occupied by the internal granular layer which contained small closely packed rounded cells with vesicular nuclei (Fig.2b). The cerebellar cortex at 21 days revealed the adult architecture of cerebellar cortex, with complete

disappearance of the external granular layer (Fig.2c). The Purkinje cell in control ultrathin sections had large nucleus with uniform fine chromatin distribution, prominent nucleolus and intact regular nuclear membrane. The cytoplasm showed the presence of many healthy ribosomes, rough endoplasmic reticulum and mitochondria (Fig.3a,b,c).

The VPA treated rat cerebellum (group II) showed low differentiation and faintly stained in the superficial fibrous layer of the cerebellar area, while the neuroepithelial cell layer appeared with clumps of darkly stained degenerated cells and showed areas of necrosis (Fig.1c). The external granular layer appeared decreased in thickness and contained clumped cells with more deeply stained nuclei (Fig.1d & 2d,e). The molecular layer appeared thin with scanty deeply stained cells (Fig.1d& 2d,e,f). The Purkinje cell layer showed depletion of cells in some areas and most Purkinje cells appeared less differentiated, irregular, shrunken and degenerated with pyknotic nuclei (Fig.1d&2d,e,f). The cells of internal granular layer appeared small, darkly stained and grouped in clusters with wide intervening spaces; cerebellar islands (Fig.1d&2d,e,f).

The ultrathin sections in VPA treated cerebellum revealed that the nucleus of Purkinje cell appeared shrunken with uneven distributions of condensed chromatin and had irregular nuclear envelope. The cytoplasm contained damaged mitochondria, dilated vesicular rough endoplasmic reticulum and many vacuoles (Fig.3d,e,f).

Specimens treated with VPA and folic acid (group III) showed nearly normal architecture of the cerebellar cortex. The thickness of its layers was apparently similar to that of control and most of its neurons appeared morphologically normal, but some appeared irregular and darkly stained (Fig.2g,h,i).

Nearly normal Purkinje cells were revealed in the ultrathin sections of VPA and folic acid treated group. Their nuclei had prominent nucleoli and uniform fine chromatin distribution. The cytoplasm contained free ribosomes and a lot of normal mitochondria. The rough endoplasmic reticulum appeared normal except for some dilated ones (Fig.3,g,h,i).

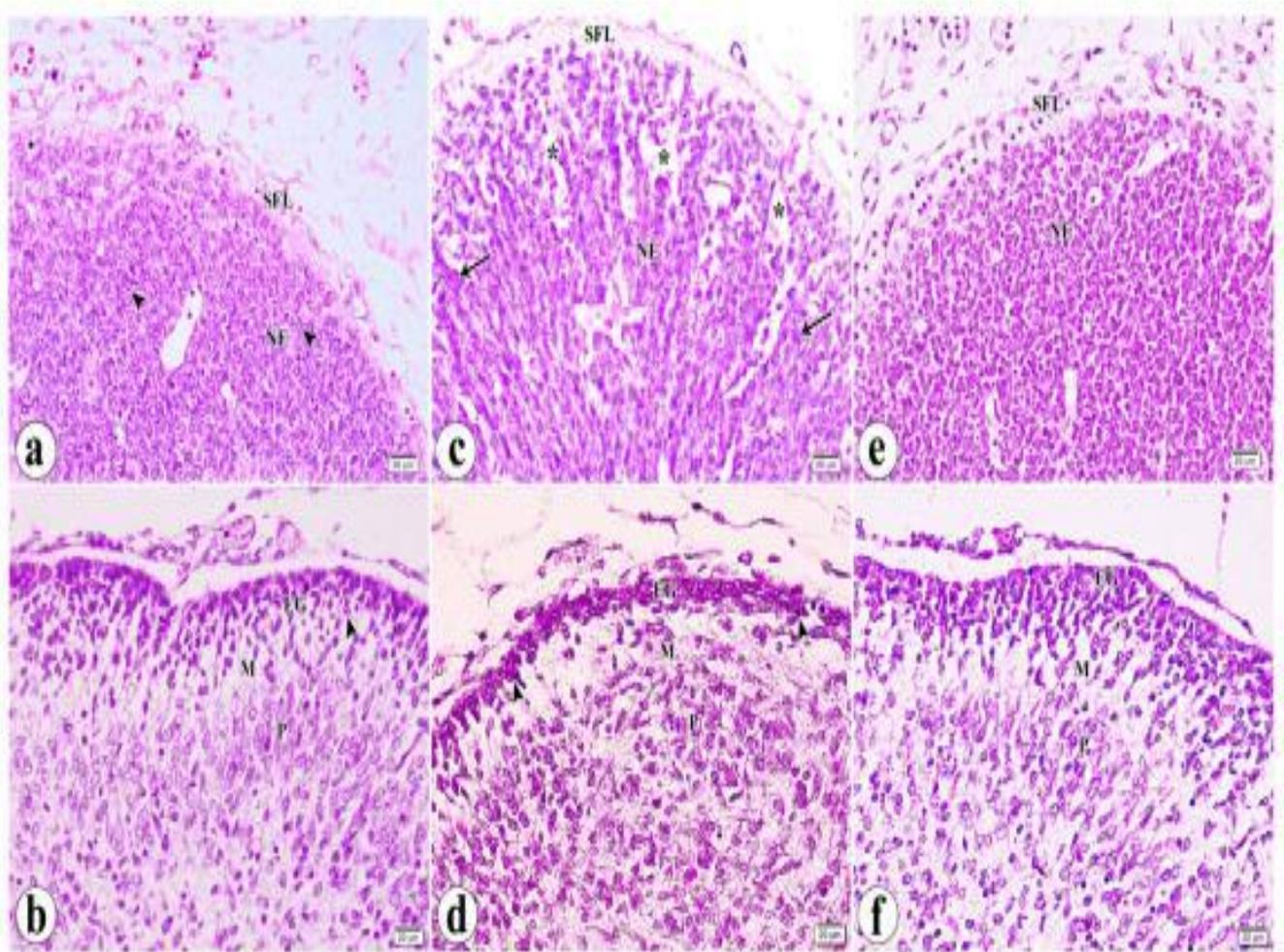


Fig. (1): A photomicrograph of cerebellar cortex at embryonic days 15 & 17: Control group (a, b) a- The cerebellar cortex at E 15 consists of superficial fibrous layer (SFL) and a zone of differentiating neuro-epithelial cells (NE) containing small vesicular cells (arrow head). b- The cerebellar cortex at E 17 shows the appearance of external granular layer (EG) that contains small rounded cells (arrow head), a thin molecular layer (M) and the differentiated Purkinje cells (P) appear to be arranged in several layers. VPA treated group (c, d) c- The cortex at E15 shows low differentiation in the superficial fibrous layer (SFL). The zone of differentiating neuro-epithelial cells (NE) shows clumps of degenerated cells (arrow) and areas of necrosis (*). d- The cortex at E17 shows apparent decrease in thickness of the external granular layer (EG) with darkly stained cells (arrow head). The Purkinje cell layer (P) appear distorted with less differentiated cell. VPA and folic acid treated group (e, f) e- Cerebellar cortex at E15 preserves its normal architecture, with its two zones; the superficial fibrous layer (SFL) and the differentiating neuro-epithelial cells (NE). f- Cerebellar cortex at E 17 shows that the external granular layer (EG) restores its thickness and structure as that of control group. The Purkinje cells (P) appear well differentiated and arranged in several layers. (H & E \times 400)

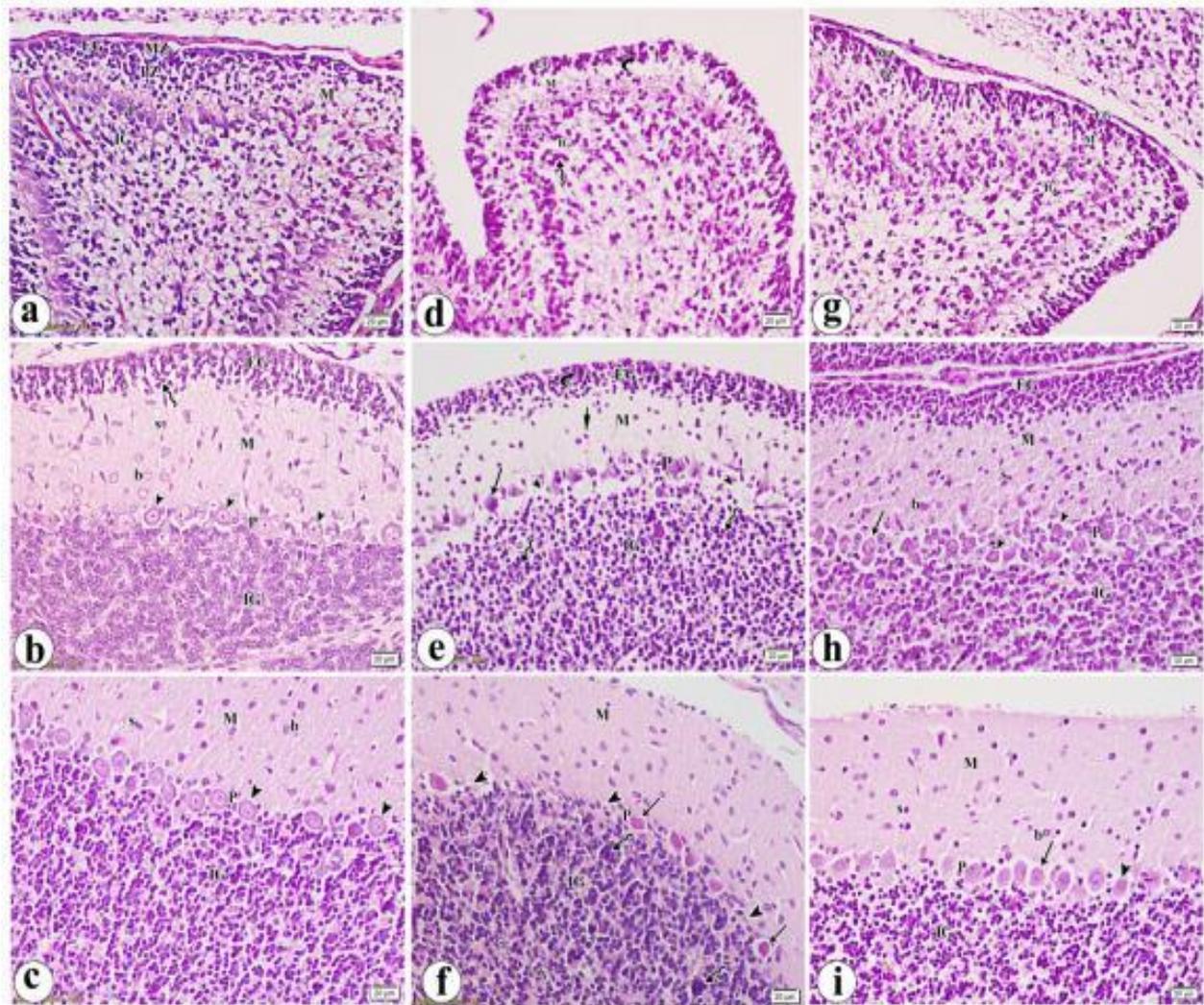


Fig. (2): A photomicrograph of cerebellar cortex at newborn, fourteen and twenty one days: Control group (a, b, c) showing differentiation of external granular layer (EG) into the multiplying zone (MZ) and the premigratory zone (PZ) in newborn rat (a). In fourteen days old rat, (b) the external granular layer (EG) consists of small rounded closely packed cells (wavy arrow), but in twenty one days old (c) it disappears. At fourteen (b) and twenty one days (c) the molecular layer (M) is well defined and containing basket (b) and stellate cells (s). The Purkinje cells (P) at newborn (a) appear small and arranged in several rows, but in fourteen (b) and twenty one days (c) arranged in one row and appear flask shaped or oval with large vesicular rounded nuclei in pale acidophilic cytoplasm (arrow head). The internal granular layer (IG) in newborn age (a) contains small rounded cells, while in fourteen (b) and twenty-one days (c) the cells are more differentiated with vesicular nuclei. VPA treated group ((d) new born, (e) 14 days & (f) 21 days) marked reduction in external granular layer (EG) and contains deeply stained cells (d&e). The Molecular layer (M) shows reduction in thickness (d, e, f) with small deeply stained cells (e) (short arrow). The Purkinje cell layer (P) contains dark stained cells in newborn (d). At fourteen and twenty one days (e,f), the Purkinje cells (P) appear degenerated (arrow) with depletion in some areas (arrow head). The internal granular layer (IG) shows densely packed darkly stained cells (wavy arrow) at different ages (d, e, f) with wide cerebellar islands (*) in twenty one days old (f). In VPA and folic acid treated group (g (new born), h (14 days), i (21 days)), the external granular layer (EG) at newborn and fourteen age (g, h) appears comparable to control similar in thickness and structure (g) with its multiplying zone (MZ) and premigratory zone (PZ). The molecular layer (M) in postnatal ages (h, i) appears well developed and contains vesicular basket (b) and stellate cells (s). Most of Purkinje cells (P) in different ages (g, h, i) improved and appear similar to control (arrow), but some cells (h, i) appear disfigured, degenerated and darkly stained (arrow head). At all ages (g, h, i) Internal granular layer (IG) appears improved and comparable to control. (H & E \times 400)

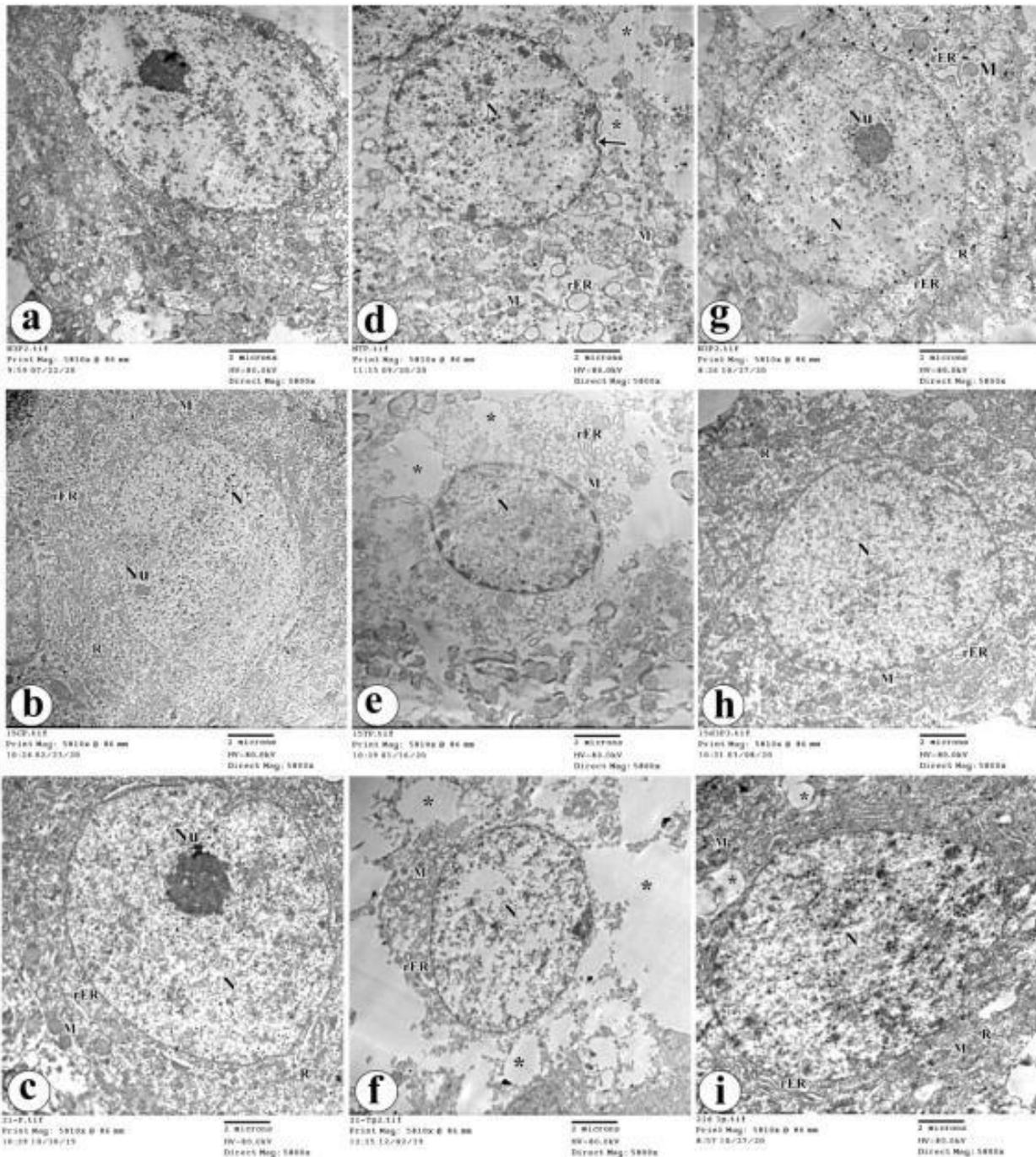


Fig. (3): An electron photomicrograph of Purkinje cell in postnatal ages (new born, 14 and 21 days). In control group (a (newborn), b (14days), c (21days)), the Purkinje cell has large nucleus (N) with prominent nucleolus (Nu), uniformly distributed chromatin and regular nuclear membrane. The cytoplasm contains mitochondria (M), rough endoplasmic reticulum (rER) and a lot of free ribosomes (R). In VPA treated group (d (newborn), e (14days), f (21days)), the nucleus of the Purkinje cell appears shrunken with peripheral chromatin condensations and surrounded by irregular nuclear membrane (arrow). The cytoplasm appears to be rarified and vacuolated (*). It contains damaged mitochondria (M) and dilated vesiculated rough endoplasmic reticulum (rER). In VPA and folic acid treated group (g (newborn), h (14days), i(21days)), the Purkinje cell has large nucleus (N) with some condensed chromatin distribution (i). The cytoplasm contains normal mitochondria (M), normal rough endoplasmic reticulum (rER) except for some dilated ones (g) and free ribosomes (R) with some small vacuoles (*) (i). (X5800)

Immunohistochemistry with GFAP:

Mild positive immune reaction of GFAP was found in different cerebellar cortical layers of control rats and rats treated with VPA and folic acid (fig.4a.b.c.g.h.i) against the strong positive GFAP immune expression in VPA treated rats (fig.4d,e,f). The presence of many immunoreactive glial cells in Purkinje cell layer and internal granular layer, in addition to many immunoreactive fibers in external granular and molecular layers, indicated the presence of gliosis in VPA treated group (Fig.4 d,e,f).

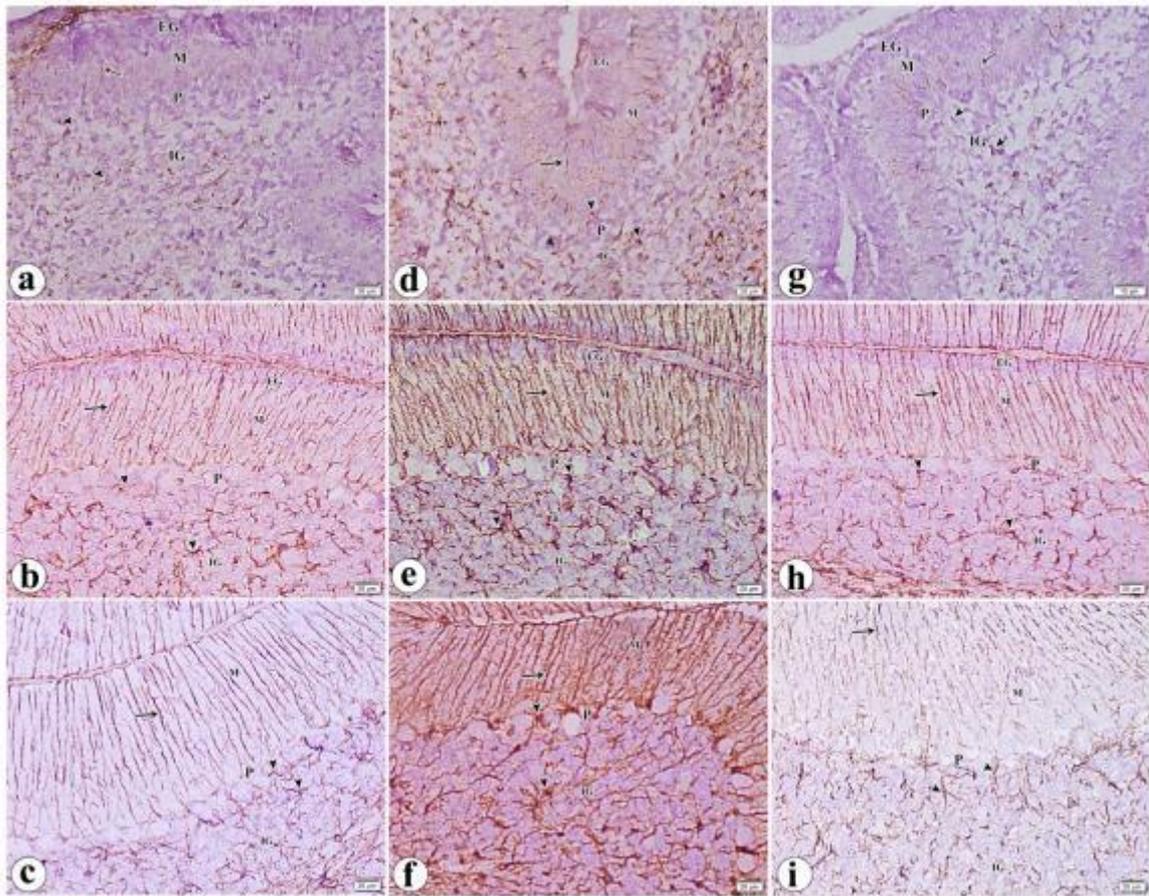


Fig. (4): A photomicrograph of cerebellar cortex in postnatal ages (new born, 14 and 21 days). Control group (a (newborn), b (14days), c (21days)) showing weak immune expression of glial fibrillary acidic protein (GFAP) in all layers of the cortex. The external granular layer (EG) and molecular layer (M) show the presence of weak immunoreactive fibers (arrow). Some glial cells (arrow head) are observed to be scattered among the Purkinje cells (P) and cells of internal granular layer (IG). VPA treated group (d (newborn), e (14days), f (21days)), showing the presence of strong positive immune expression of glial fibrillary acidic protein (GFAP) in all layers of the cortex comparable to control. The external granular layer (EG) and molecular layer (M) show extensive positive immunoreactive fibers (arrow). Many glial cells and their processes (arrow head) are observed among Purkinje cells (P) and cells of internal granular layer (IG). VPA and folic acid treated group (g (newborn), h (14days), i (21days)) showing weak positive immune reaction of GFAP in all layers of the cortex. The external granular layer (EG) and the molecular layer (M) show the presence of weak immunoreactive fibers (arrow). Some weak immunoreactive glial cells and their processes (arrow head) are present scattered among Purkinje cells (P) and cells of internal granular layer (IG). Note the apparent decrease in immunoreactivity in comparison with the treated group with valproic acid. (GFAP x400)

Histomorphometric results:

On comparing with **group I**, there was a highly significant ($p \leq 0.01$) reduction in the mean thickness of the cerebellar cortex and its layers in **group II**, while non-significant reduction with **group III**. On the other hand, the mean thickness of cerebellar cortex and its layers in **group II** shows a highly significant ($p \leq 0.01$) reduction compared with **group III** at various postnatal ages (Tables 1,2,3).

Table (1): Showing the thickness (microns) of the cerebellar cortex (mean±SD) in the newborn rat in different studied groups

	Group I (Mean ± SD)	Group II (Mean ± SD)	Group III (Mean ± SD)	P-value ¹	P-value ²	P-value ³
Cerebellar cortical thickness	91.2±7.8	70.10±6.4	87.28±5.4	0.000**	0.3 ^{NS}	0.000**

(NS) → Non-significant ($p > 0.05$).

(**) → Highly Significant ($p \leq 0.01$). P-value¹ compares group II to group I

P-value² compares group III to group I

P-value³ compares group II to group III

Table (2): Showing the thickness (microns) of the cerebellar cortex and its layers (mean±SD) in the postnatal day 14 rat in different studied groups

	Group I Mean ± SD	Group II Mean ± SD	Group III Mean ± SD	P-value ¹	P-value ²	P-value ³
Cerebellar cortical thickness	304±13	201.5±18	290.3±7	0.000**	0.1 ^{NS}	0.000**
External granular	36.3±5.6	22.8±5	31.6±2.6	0.000**	0.1 ^{NS}	0.000**
Molecular layer	101.1±13.9	73.14±4.7	92.3±2	0.000**	0.09 ^{NS}	0.000**
Internal granular layer	138±7.8	106.9±16.4	132.8±14.6	0.001**	0.5 ^{NS}	0.001**

(NS) → Non-significant (p> 0.05). (***) → Highly Significant (p≤ 0.01)

P-value 1 compares group II to group I

P-value 2 compares group III to group I

P-value 3 compares group II to group III

Table (3): Showing the thickness (microns) of the cerebellar cortex and its layers (mean±SD) in the 21 days rat in different studied groups

	Group I Mean ± SD	Group II Mean ± SD	Group III Mean ± SD	P-value ¹	P-value ²	P-value ³
Cerebellar cortical thickness	407.8±8	279±10	399±8	0.000**	0.1 ^{NS}	0.000**
Molecular layer	211.3±21	139.4±6.5	208.23±9.8	0.000**	0.7 ^{NS}	0.000**
Internal granular layer	181.9±11.5	126.8±9.5	174.7±14.6	0.000**	0.3 ^{NS}	0.000**

(NS) → Non-significant (p> 0.05). (***) → Highly Significant (p≤ 0.01)

P-value1 compares group II to group I

P-value2 compares group III to group I

P-value3 compares group II to group III

There was a highly significant reduction in the mean Purkinje cell number of **group II** as compared with **group I** and **group III**. On the other hand, the mean number of Purkinje cells of **group III** shows non-significant reduction on comparing with **group I** at different postnatal ages (Table4).

Table (4): Showing the number of Purkinje cells (mean±SD) per an area 13413μ² at different postnatal ages in all studied groups

	Group I Mean ± SD	Group II Mean ± SD	Group III Mean ± SD	P-value ¹	P-value ²	P-value ³
New born	21±3.5	10.5±3.6	18±2.8	0.000**	0.14 ^{NS}	0.000**
14 days	12.3±3.14	6.6±1.8	10±1.7	0.001**	0.1 ^{NS}	0.000**
21 days	5.4±0.5	3±0.5	4.9±0.3	0.000**	0.1 ^{NS}	0.000**

(NS) → Non-significant (p> 0.05). (***) → Highly Significant (p≤ 0.01)

P-value1 compares group II to group I

P-value2 compares group III to group I

P-value3 compares group II to group III

There was a highly significant reduction in the mean **nuclear diameter** of Purkinje cell of **group II** as compared with **group I** and **group III**. On the other hand, the mean **nuclear diameter** of Purkinje cells of **group III** shows non-significant reduction on comparing with **group I** at different postnatal ages (Table 5).

Table (5): Showing the nuclear diameter (microns) (mean±SD) of Purkinje cells at the different postnatal ages in all studied groups

	Group I Mean ± SD	Group II Mean ± SD	Group III Mean ± SD	P-value ¹	P-value ²	P-value ³
New born	4.8±1.4	2.8±0.7	3.8±1.4	0.000**	0.1 ^{NS}	0.01**
14 days	6±3.14	3.6±1.6	5.6±1.3	0.000**	0.7 ^{NS}	0.01**
21 days	10.8±2.3	7.8±1.16	10.16±1.4	0.000**	0.5 ^{NS}	0.000**

(NS) → Non-significant (p> 0.05). (***) → Highly Significant (p≤ 0.01)

P-value1 compares group II to group I

P-value2 compares group III to group I

P-value3 compares group II to group III

DISCUSSION

The cerebellum is very sensitive to the abnormal changes occurred during the embryological development as maternal exposure to certain chemicals. Therefore the cerebellum is a perfect model for studying many aspects of neural development⁽¹⁸⁾. Valproic acid (VPA) is used medically as an anticonvulsant and mood-stabilizing agent for the treatment of epilepsy and bipolar disorder respectively⁽¹⁹⁾. Although valproic acid is widely used as antiepileptic drug, a little is known about its effect in different stages of embryogenesis. Therefore, this study was carried out to investigate the histological effects in the cerebellar cortex of albino rats after valproic acid treatment and to detect any possible protective role of folic acid when given concomitantly with valproic acid.

In the present study, light microscopic examination showed that on embryonic day 15, the cerebellar anlage of control rat was formed of a plate of neuroepithelial cells covered with a fibrous layer. In line with these findings, a study stated that the cerebellar anlage was demarcated at dorsal part of the metencephalon on embryonic day 13 old rat embryo; it first appeared as a thick swelling on each side of the metencephalon and with the progress of the development the thickness of this region increased⁽²⁰⁾.

However other study in human fetus demonstrated that the earliest description of the cerebellum was on seven weeks old embryos. The cells of the superior rhombic lip and adjacent dorsal part of the dorsolateral lamina of the metencephalon proliferated to form the rudiment of the cerebellum⁽²¹⁾. The cerebellar cortex of control group on embryonic day 17 in this study consisted of external granular layer that was firstly appeared, thin rim of molecular layer and Purkinje layer contained several rows of differentiating cells.

Concomitant with the present results, other investigators **Berry et al.**⁽²²⁾ reported that during the development of rat cerebellum, the Purkinje cells were born mainly between embryonic day 13 and 16 with peak on embryonic day 14 to 15. On the other hand, a previous research **Mohammed**⁽²³⁾, reported that, the Purkinje cells began its appearance in rabbit on embryonic day 18 underneath the superficial fibrous layer, while other study **Miale and Sidman**⁽²⁴⁾ revealed that the formation of the Purkinje cells occurred on days 11-13 of embryonic period in mice. This interspecies variation in the appearance of the Purkinje cells may be due to differences in the duration of the fetal life.

In the current study, the cerebellar cortex of control group at age of new born consisted of four layers; the external granular layer (with two zones), the molecular layer, the Purkinje layer and the internal granular layer. In accordance with present results, the findings of other researchers **Altman**⁽²⁵⁾ and **Rakic and Sidman**⁽²⁶⁾ found that the granule cells were produced postnatally. These findings were also in accordance with a previous study (Schilling, 2018) that found the external granular layer was a temporary germinal matrix that

gives rise to the granule cell. This germinal matrix was composed of two regions the proliferative zones, just beneath the pia matter where the precursors of granule cells were located, and the premigratory zone which characterized by the postmitotic granule cells that would migrate across the molecular layer to settle and constituted the internal granular layer.

The cerebellar cortex of control group at age of fourteen days appeared consisted of the same four layers in the previous age. But, the external granular layer appeared decreased in thickness and the cells in the Purkinje layer arranged in one row. In harmony with present results, a study **Hafez et al.**⁽¹⁸⁾ demonstrated that the Purkinje cells showed a monocellular arrangement on the second postnatal week and then attained its final maturity by the end of the third postnatal week.

The cerebellar cortex of 21 days showed the adult architecture of cerebellar cortex with complete disappearance of the external granular layer. In accordance with that, a previous research **Altman**⁽²⁵⁾ reported that the external granular layer normally reached its maximum thickness at the age of seven days and completely disappeared at the age of twenty-one days.

Ultrastructurally, the Purkinje cells of control group in this study showed large rounded nucleus with dispersed chromatin. The cytoplasm contained mitochondria, rough endoplasmic reticulum and free ribosomes. These findings were in consistent with other investigators^(8, 27). They reported that the Purkinje cell appeared with normal nuclei surrounded by regular intact nuclear envelop and its cytoplasm contained large number of mitochondria.

In the present work, immunohistochemical study at different postnatal ages showed the presence of weak expression of GFAP in the form of immunoreactive fibers in the external granular and the molecular layer with scattered glial cells in the Purkinje cell layer and the internal granular layer. These findings were in agreement with **Schilling**⁽²⁸⁾ who found that the external granular layer originated the granule cells of the internal granular layer after their migration through Bregman radial glial fibers. Other researchers **Cerri et al.**⁽²⁹⁾ concluded that the Bregman radial glial fibers represented the mechanical force of cerebellar foliation for the achievement of the normal cerebellum architecture. GFAP was an intermediate filament protein that was expressed by numerous cell types of the CNS including astrocytes and ependymal cells⁽¹⁸⁾.

In the present study, examination of VPA treated cerebellar cortex showed its toxicity on the pre and postnatal morphology and architecture of the cortex. The prenatal exposure of the valproic acid caused degenerative changes and necrosis of the cellular content in embryonic cerebellar cortex. The external granular layer appeared less in thickness compared to the control group and contained darkly stained cells. The Purkinje cell layer appeared with degenerated cells. In accordance, some investigators found that basic research

in animals had shown that prenatal exposure to specific antiepileptic drugs caused microscopic structural abnormalities in the fetal brain. Specifically, prenatal exposure to valproic acid had been reported to inhibit the differentiation of neural progenitor cells during the early to middle phases of neurogenesis (5, 20).

At postnatal ages, all cerebellar cortical layers by light microscope showed degenerative changes especially on Purkinje cells and this was in harmony with previous studies. They recorded that the external granular layer contained pyknotic cells and the Purkinje cells showed marked depletion and surrounded with vacuolated spaces (8, 18). They added that the disarrangement of Purkinje cell layer, after VPA administration could be due to delayed migration of the cells leading to disturbance of their normal linear organization. The ultrastructural changes of Purkinje cells of offspring born to VPA treated mothers in the current study confirmed the histological changes noticed by the light microscopic examination; in the form of nuclear changes, irregular cell membranes and irregular chromatin distributions while the cytoplasm showed damaged organelles and many vacuoles. These current findings were in line with others who found that the nuclei appeared shrunken, and the cytoplasm contained dilated rough endoplasmic reticula (8, 27).

Regarding the other layers of the cerebellar cortex in the present study, the molecular layer in VPA treated group showed degenerative changes in the form of presence of deeply stained cells while the granular layer showed degenerated and shrunken cells with deeply stained nuclei. Similar observations were mentioned by previous studies that revealed this finding were due to neuroapoptosis which was caused by the valproic acid (18, 27).

Shona et al. (8) found that the nerve cell damage caused by VPA is due to suppression of oxidative phosphorylation in the mitochondria of the Purkinje cell. Additionally, there was association between increase in the free radicals and VPA cerebellar degenerative changes. Others **Reynolds and Green** (30) reported another mechanism of valproate induced neurodegeneration and malformations. They demonstrated that valproic acid interfered with one-carbon metabolism, including the transport of methylfolate into the brain and the placenta by targeting folate receptors. Valproic acid also, effected on the folate metabolic system contributed to congenital and developmental problems associated with valproate exposure. Lastly, the genetic factors contributed to the vulnerability to the valproic acid induced risks.

From this viewpoint the present study examined the protective effect of folic acid against VPA toxic effects. The neuroprotective effects of folic acid manifested by normal cerebellar cortical architecture with normal cells comparable to those of the control group. These findings were in accordance with others **Fujimura et al.** (10) and **Hafez et al.** (18) who stated that folate acted as a cofactor for enzymes involved in DNA

and RNA biosynthesis. **Dawson et al.** (31) stated that folic acid protection might be mediated by prevention of VPA-induced alterations in proteins involved in neurulation or prevented VPA-induced oxidative stress.

Other important finding of the current work was the strong positive immune-expression of GFAP in all layers of the cerebellar cortex as compared to control. This was in agree with previous study **Shona et al.** (8) that found in VPA treated group, strong positive GFAP immune reaction could be a compensatory mechanism after nerve cell damage induced by VPA. These neuroglial cells were transformed into potentially cytotoxic cells mediated by the release of toxic reactive oxygen and nitrogen species in response to neurodegeneration. The accompanying folic acid therapy with VPA in this work displays marked improvement in the GFAP immunohistochemical reaction proving the protective effect of folic acid. This was in agreement with other investigators **Shona et al.** (8) who proved that the folic acid inhibited VPA neuronal apoptosis and gliosis acting as a coenzyme in single carbon transfers essential for the amino acid and nucleic acid metabolism.

In the current work the morphometric study revealed statistically significant reduction in the thickness of the cerebellar cortex and its layers, number and nuclear diameter of Purkinje and granule cells in offspring of VPA treated mothers and improvement of all morphometric measures in the cerebellar cortex treated with both valproic acid and folic acid. This was in agreement with others **Magar et al.** (27) who stated that the cyclin-dependent kinase system which controlled cellular apoptosis, division, differentiation and function damaged by VPA and they added that the folic acid acted as a co-factor in controlling expression of gene and synthesis of protein fundamental for foetal cell differentiation and development protecting the nerve cells from VPA teratogenicity.

CONCLUSION

The present study demonstrated the VPA bad impaction and the opposing protective role of the folic acid on the cerebellar cortical growth and development, so periconceptual prophylaxis with folic acid is recommended for all women on valproic acid and counseling should also emphasize planning pregnancy to optimize folic acid supplementation to guard against such impaction.

Conflict of interest: The authors declare no conflict of interest.

Sources of funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contribution: Authors contributed equally in the study.

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