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The Protective Effect of Folic Acid against Valproate-Induced Neurological and Skeletal Congenital Anomalies in Mice

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ABSTRACT

Background: Valproate is a broad spectrum anti-convulsant drug used to control all types of seizures. Inspite of its effectiveness in the management of epilepsy, valproate induces skeletal and neurological malformations when used by the pregnant women. Folic acid is an essential nutrient required for normal cell development and metabolism. We aimed in our work to evaluate the possible protective role of folic acid against the valproate-induced skeletal and neurological congenital malformations.

Methods: This experiment was done in the animal house in the Faculty of Medicine, Ain Shams University using ninety adult mice "Sixty females and thirty males". The histopathological procedures were done in Histology Department, Faculty of Medicine, Al-Azhar University.

Results: Valpeoate produced numerous neurological and skeletal congenital anomalies in mice fetuses when given to the pregnant females. The concomitant administration of folic acid ameliorated the teratogenic effects of valproate.

Conclusions: Folic acid is effective in prevention of the neurological and skeletal congenital malformations induced by valproate.

Keywords:
Congenital anomalies; Skeletal; Neurological; Folic acid; Valproate

INTRODUCTION

Valproate or valproic acid (VPA) is a well-known anti-epileptic drug commonly used as the drug of first-choice for the treatment of all forms of seizures [1]. VPA also used for the management of other neurological disorders including psychological disturbances, migraine, headache, bipolar disorder and neuropathic pain [2,3].

Valproate produces its anti-convulsant action mainly through elevation of the synaptic level of gamma aminobutyric acid (GABA) that inhibits the propagation of action potentials[1]. VPA also blocks the voltage-gated sodium channels and T-type calcium channels so, it elevates the threshold for seizures [1]. The available VPA therapeutic formulations include tablets, capsules, oral solution and intravenous injectable solution. When administered orally, VPA is rapidly and completely absorbed [3].

Valproate is a short-chain branched fatty acid derived from the natural valeric acid. It is highly protein bounding with low clearance[2] and when used by pregnant women, VPA crosses the placenta barrier, so it is used with precaution during pregnancy considering its teratogenic potency as it induces a broad spectrum of congenital anomalies[4]. The congenital malformations induced by VPA include congenital limb anomalies, exencephaly "absent skull cap bones", lip and palate cleft, craniofacial cleft, fused ribs, supernumerary ribs, fused vertebrae and spina bifida[5,6].

Valproate produces its teratogenic effects by inducing pathological increase of apoptosis resulting in an imbalance between cell proliferation and cell death[7]. It decreases the cell viability through enhancement of oxidative stress with overproduction of the reactive oxygen species and mitochondrial and lysosomal dysfunction[8]. VPA also, causes intrauterine growth restriction and decreased number of live births due to postimplantation losses[9].

Folic acid (FA) is a small molecule, also known as vitamin B9. It is essential for normal metabolic and biochemical functions of the human body such as normal cell multiplication, gene activity regulation, red and white blood cells production, skin and intestinal epithelium renewal and synthesis of chemicals that modulate brain function [10].

Folic acid is needed for the normal development of tissues, particularly those associated with rapid cell division such as in embryology. So, FA is required for normal fetal growth and development hence, it is recommended for periconceptional supplementation to reduce the risk of congenital malformations [11].

We aimed in this study to evaluate the possible protective effect of FA against the skeletal and neurological congenital anomalies induced by VPA in mice.

METHODS

Animals:

Ninety healthy adult ICR (CD-1) mice "Sixty females and thirty males" were used for the experimental process [12]. The mice were obtained from the animal house (Faculty of Medicine, Ain shams University) with an average weight of 30-35 grams and of about 12 weeks age [7].

Animal husbandry and breeding procedure:

The animals were used according to the ethical guidelines. They were kept in the animal house for one week to acclimate before mating then divided randomly into three groups with twenty females and ten males in each group; Group I "control group", group II " VPA group" and group III "FA and VPA group". The mice were fed by the standard food and tap water ad libitum [13]. The animals were housed in a suitable room maintained at 22 ± 1 °C with a humidity of about 55 ± 5%. The photoperiod of the animals' room was 12 hrs of darkness and 12 hrs of artificial light [13,14]. The animals were handled according to the code of ethics of experimental research adopted by Ain Shams University ethical research guidelines with a code number in the experimental animal research unit of [RE(100)22]

Mating:

Before mating, vaginal smears were taken using plastic pipettes containing normal saline and examined microscopically to detect the oestrus phase of the uterine cycle. The oestrus phase was detected by the presence of epithelial cells predominance in the vaginal smears; each two females at the oestrus phase were then caged with one male [15]. The matted females were viewed at the end of the dark cycle (8:00 a.m.) for the presence of a vaginal plug that indicated mating. The females having vaginal plugs were considered to be pregnant at gestational day 0 (GD 0) [16].

Experimental procedure and chemicals:

Group I (Control group): The rats were fed with standard diet and tap water only.

Group II (VPA group): The females received VPA dissolved in sterile saline by intra-peritoneal injection in a dose of 500 mg/kg on the 7th, 8th and 9th days of gestation[7].

Group III (VPA and folic acid group): The females received VPA dissolved in sterile saline by intra-peritoneal injection in a dose of 500 mg/kg on the 7th, 8th and 9th days of gestation [7] and FA dissolved in sterile saline by gastric gavage in a daily dose of 3 mg/kg from the beginning of the experiment till the 14th day of gestation [17].

Caesarian Section:

At the end of gestation (GD 18), the pregnant females were euthanized by cervical dislocation then, the fetuses were collected by caesarean sections [18].

Gestational outcome and external examination:

The numbers of total, living and dead fetuses were calculated. The live fetuses were weighed in grams for determination of the mean fetal body weight of the three groups and examined for structural malformations [19]. The findings were calculated and processed for statistical analysis.

Double staining technique:

The fetuses were processed and stained according to the method described by Liao et al. The fetuses were euthanized by cervical dislocation then, the skin is gently removed and the abdominal and thoracic viscera were extracted. The skinned eviscerated specimens were put in 95% ethanol for 3-5 days then, in Alcian blue stain (800 ml of 95% ethanol, 200 ml of glacial acetic acid and 150 mg of Alcian blue) for 24 hours to stain the cartilages blue. Thereafter, the specimens were exposed to alizarin red S (50 mg alizarin red in 1 L of 2% KOH) for one hour to stain the bones red. Finally, the specimens were exposed to a series of 2% KOH + glycerin solutions (60% KOH + 40% glycerin, 40% KOH + 80% glycerin then 20% KOH + 80% glycerin) to clear the skeleton then, examined under a stereomicroscope [20].

Statistical analysis:

One-way analysis of variance (ANOVA) was done for mean comparison between the three groups. Two-way ANOVA was done for multiple comparisons. The Tukey-Kramer post-hoc test was used to test the significance between them. The value of $P<0.05$ was set as statistically significant [21].

RESULTS

Effects on the gestational outcome:

The VPA-treated group showed reduced total number of fetuses and increased percentage of dead fetuses up to 22.1% in group II (VPA-treated group) in comparison to 1.6% in group I (control group) and 5.9% in group III (FA and VPA-treated group). The control group showed no

congenitally malformed fetuses while the VPA-treated group showed many fetuses with neurological and skeletal congenital malformations in the form of exencephaly, spina bifida, facial and palatine cleft and limb defects. In group III, concurrent administration of FA has decreased the VPA-induced congenital anomalies (Table 1 and figures 1-5)

Effects on the fetal body weight:

The VPA-treated group showed reduced mean fetal body weight in comparison to the control group. The mean fetal body weight was 1.32 grams for the VPA-treated group and 1.83 grams in the control group. Co-administration of FA with VPA increased the mean fetal body weight in group III up to 1.7 grams (Table 2).

Table 1. The gestational outcome in the three groups of mice

	Group I (Control group)	Group II (VPA group)	Group III (VPA and FA group)	P value	Significance
Total number of fetuses	124	113	118	< 0.05	**S**
Number of living fetuses	122	86	110	< 0.002	**S**
Dead fetuses	2/124 (1.6%)	27/113 (23.8%)	8/118 (6.7%)	< 0.002	**S**
Fetuses with neural tube defects (exencephaly)	0	26 (30.2%)	1 (0.9%)	< 0.005	**S**
Fetuses with skeletal and limb defects	0	14 (20.9%)	2 (1.8%)	< 0.002	**S**
Fetuses with facial/palate cleft	0	21 (24.3%)	1 (0.9%)	< 0.002	**S**

P value was calculated by Independent Sample T-Test. S means significant P value

Table 2: The mean fetal body weight in the three groups of mice:

	Group I (Control group)	Group II (VPA group)	Group III (VPA and FA group)	P value	Significance
Mean fetal body weight in grams (g ± SD)	1.83 ± 0.11	1.32 ± 0.15	1.70 ± 0.01	< 0.002	S

P value was calculated by Independent Sample T-Test. S means significant P value

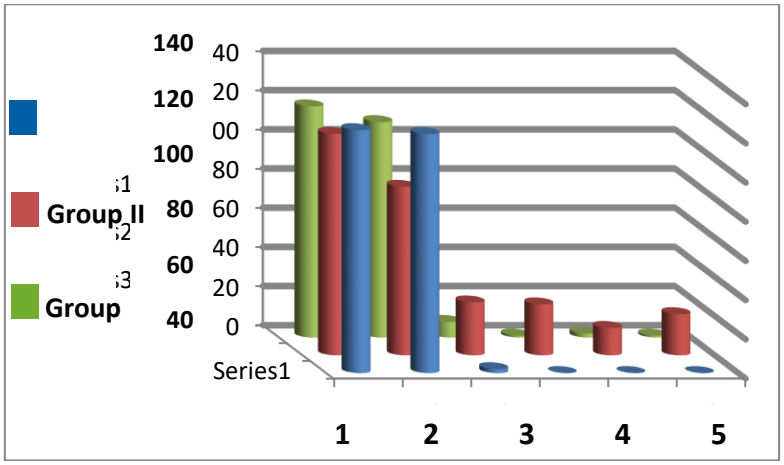

Figure 1: The number of total fetuses (1), living fetuses (2), dead fetuses (3), exencephalic fetuses (4), fetuses with skeletal and limb defects (5) and fetuses with cleft face and palate (6) in the three groups.

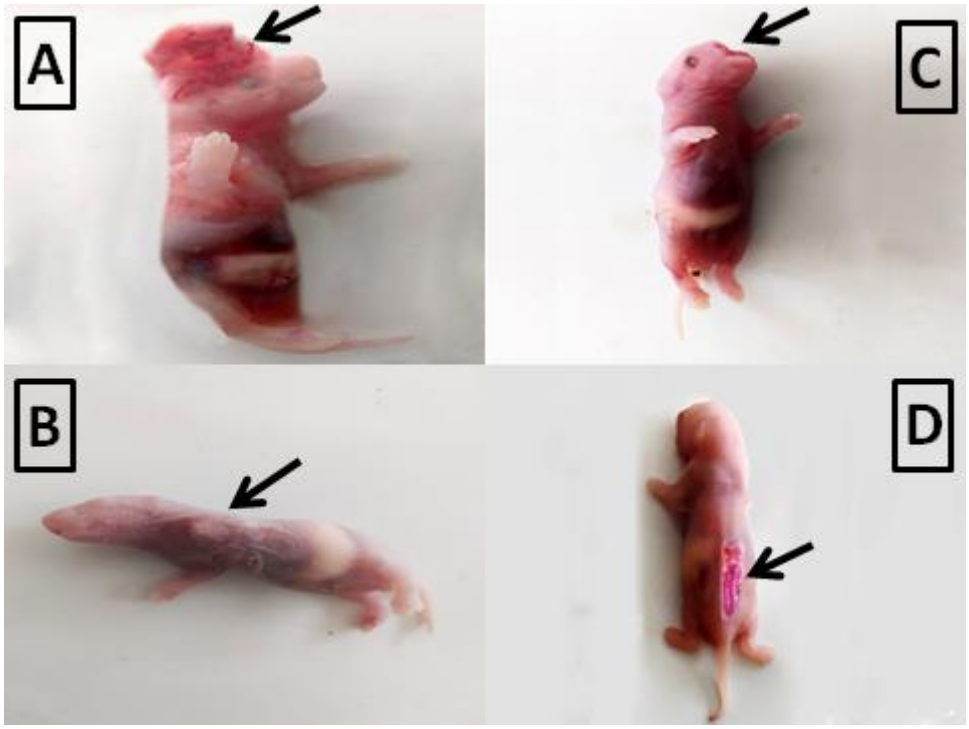

Figure 2: Photographs of four mice of the VPA-treated group (group II). Photograph (A) shows a mouse with exencephaly (arrow). Photograph B shows a mouse with amelia affecting the left forelimb (arrow). Photograph C shows a mouse with cleft face and palate (arrow). Photograph D shows a mouse with spina bifida (arrow).

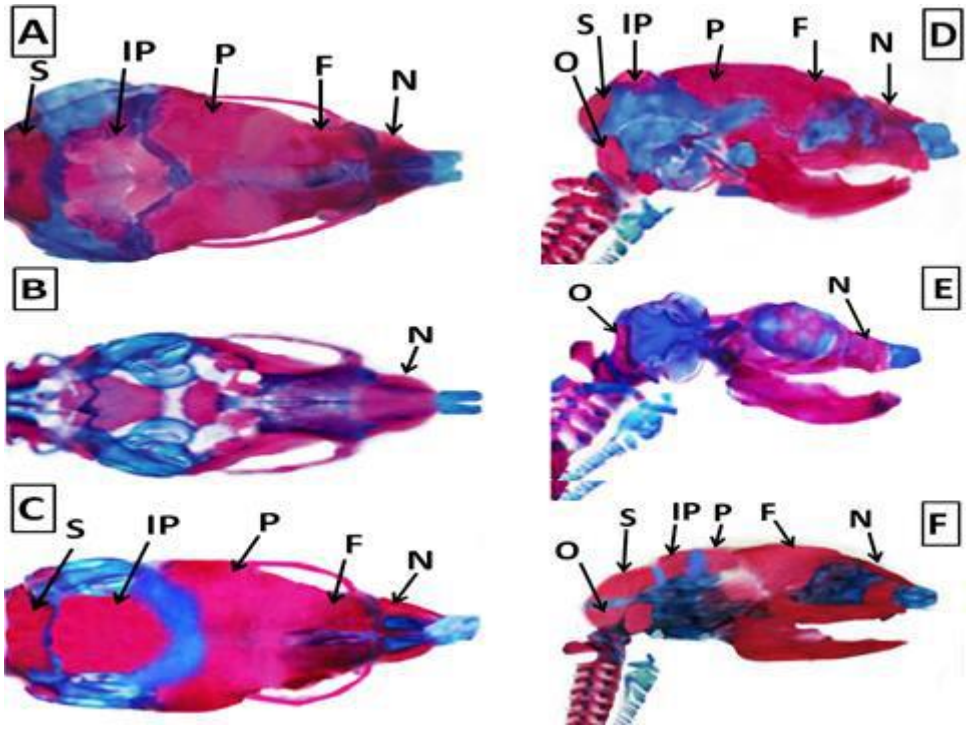

Figure 3: Stereomicroscopic photographs showing overhead (A-C) and lateral (D-F) views of the skulls of the three groups of the mice stained with alizarin red S for bone and Alcian blue for cartilage. The control group (A & D) shows the presence of all skull cap bones; nasal (N), frontal (F), parietal (P), interparietal (IP), supraoccipital (S) and occipital (O) bones and their intermediate cartilages. The skulls of the VPA-treated group mice (B & E) show the presence of the nasal (N) and occipital (O) bones only with the absence of the frontal, parietal, interparietal and supraoccipital bones and their intermediate cartilages. In the FA and VPA-treated group (C & F), the nasal (N), frontal (F), parietal (P), interparietal (IP), supraoccipital (S) and occipital (O) bones are present separated by their intermediate cartilages.

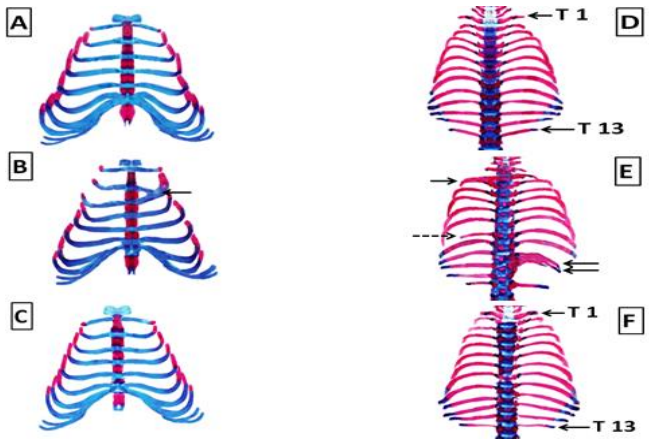

Figure 4: Stereomicroscopic photographs showing ventral (A-C) and dorsal (D-F) views of the chest wall of the three groups of the mice showing the thirteen ribs from the first (T1) to the last (T13) stained with alizarin red S and Alcian blue. The bony parts of the ribs are stained red while their costal cartilages are stained blue. In the control mice (A & D), the ventral view (A) shows the anterior ends of the upper eight ribs and the upper ten costal cartilages. The upper seven costal cartilages reach the sternum while each of the 8th-10th costal cartilages is attached to the costal cartilage above. The dorsal view (D) shows separate ribs. In the VPA-treated group (B & E), the ventral view (B) shows fused left 2nd and 3rd costal cartilages (arrow), and the dorsal view (E) shows fused left 2nd and 3rd ribs (arrow), fused right 10th, 11th and 12th ribs (double arrow) and rudimentary short left 8th rib (dashed arrow). The FA and VPA-treated group (C & F) shows separate complete ribs and their costal cartilages.

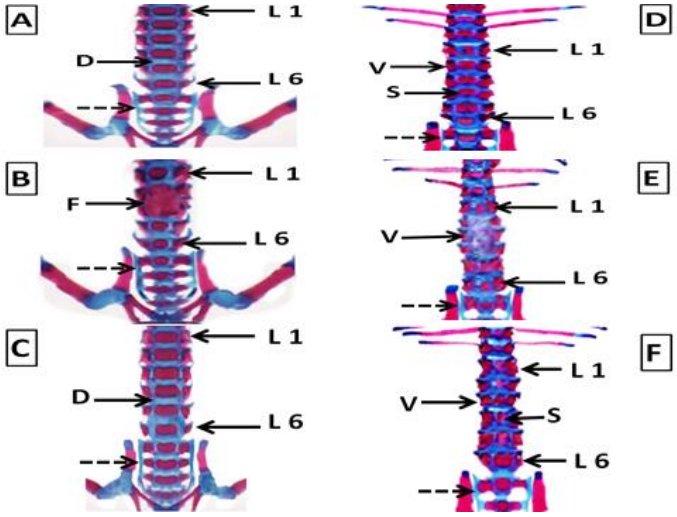

Figure 5: Stereomicroscopic photographs of the ventral (A-C) and dorsal (D-F) aspects of the lumbosacral parts of vertebral column of the three groups stained with alizarin red S and Alcian blue showing the six lumbar vertebrae from the first (L1) to the sixth (L6) and the sacrum (dashed arrows). In the control group, the ventral view (A) shows that, the six lumbar vertebrae are separated by the intervertebral discs [D] and the dorsal view (D) shows complete lumbar vertebral arches [V] with spinous processes [S] in the midline. In the VPA-treated group, the ventral view (B) shows fused 2nd, 3rd and 4th lumbar vertebrae [F] and the dorsal view (E) shows bifid vertebral arches [V] of the 2nd, 3rd and 4th lumbar vertebrae with absence of their spinous processes. In the FA and VPA-treated group (C & F), the ventral view (C) shows intervertebral discs [D] between the lumbar vertebrae and the dorsal view (F) shows complete lumbar vertebral arches [V] with spinous processes [S] in the midline.

DISCUSSION

The present study was carried out to evaluate the possible protective role of FA against VPA-induced neurological and skeletal congenital anomalies. VPA and FA were given to the pregnant mice in our study according to the doses and methods used in previous studies had demonstrated the effects of VPA and FA on the developing mice embryos during the early stages of pregnancy[7,17]. Administration of VPA resulted in structural neurological and skeletal congenital malformations. The neurological malformations were in the form of exencephaly and spina bifida while the skeletal anomalies were in the form of facial and palatine cleft, fused vertebrae, fused and supernumerary ribs and amelia. Moreover, VPA administration significantly increased the fetal lethality and

decreased the mean fetal body weight. On the other side, co-administration of FA resulted in a significance amelioration of all undesirable effects of VPA.

The VPA teratogenic potency demonstrated in the current study was attributable to its ability to cross the placental barrier and disturb the process of organogenesis of the developing fetuses[22]. According to previous results, VPA produces its teratogenic effects mainly through induction of oxidative stress, DNA damage and enhancement of apoptosis leading to defective histogenesis of the fetal tissues[22]. Also, VPA can induce teratogenicity through interference with the polycomb group proteins that are responsible for modulation of chromatin structure during normal fetal development. Interference with the polycomb group proteins results in expression of abnormal genes with subsequent structural congenital anomalies [23].

Valproate-induced exencephaly detected in the present study was in agreement with the results of previous studies demonstrated that, prenatal exposure to VPA leads to absence of the frontal, parietal, interparietal and supraoccipital bones of the skull with the presence of the brain outside the cranial cavity[7,24]. These findings were attributable to previous results proved that, VPA administration during early pregnancy leads to defective osteogenesis and chondrogenesis of the bones of the skull cap and their intermediate cartilages[24].

The spina bifida demonstrated in the VPA-exposed mice fetus in the current study was in correlation with previous studies detected that, intrauterine exposure to VPA leads to spina bifida through interference with the closure of the spinal neural groove and prevention of fusion of the two halves of vertebral arches[6,25].

In the present study, VPA induced limb reduction, fused vertebrae and fused and rudimentary ribs. These results were in agreement with the results of previous studies detected similar patterns of skeletal defects in mice fetuses born to mothers received VPA during early pregnancy[26]. These skeletal malformations were attributable to the ability of VPA to induce alterations in gene expression leading to disturbed development of the intraembryonic mesoderm with subsequent malformation of their derivatives including the limb core, vertebral column and ribs[26]. In addition, previous results proved that, VPA can lead to limb reduction through disrupting the genes responsible for chondrogenesis and osteogenesis in the limb buds in the med-organogenesis period[27].

The VPA-induced decreased mean fetal body weight and increased frequency of the fetal deaths detected in the current study were in line with previous findings[23]. When administered during early pregnancy, VPA disturbs the normal gene expression of the placental transporters leading to impaired transport of the nutrients and gases across the placental barrier with subsequent intrauterine growth restriction and increased fetal lethality[28].

The fetal protective effects of FA demonstrated in the current study were attributable to its ability to cross the placental barrier and promotes normal development of the fetus when administered during the period of organogenesis[29]. Numerous previous studies proved that, FA supplementation during early pregnancy is required to reduce the frequency of fetal malformations as it has numerous roles essential for the normal fetal development including normal cell growth, normal gene expression, immune function and angiogenesis[29]. According to previous findings, FA is critical for normal nucleotide synthesis required for DNA replication and subsequent cell growth and proliferation during the normal fetal development[30].

The ability of FA to rescue the VPA-induced congenital malformations detected in the current study was in correlation with previous results proved the effectiveness of FA in counteracting the bad effects of VPA on the gestational outcome[31]. According to previous results, FA counteracts the teratogenic effect of VPA through alleviation of the VPA-induced oxidative stress by ameliorating the inhibitory effects of VPA on superoxide dismutase (SOD) enzyme so, FA inhibits the VPA-induced accumulation of superoxide that leads to cell destruction[31]. In addition, FA ameliorates the VPA-induced inhibition of histone deacetylase enzyme (HDAC) so, FA enhances HDAC enzyme that is an essential enzyme required for normal replication of DNA during cell development[32].

The protective effect of FA against the VPA-induced exencephaly was in correlation with previous studies proved that, pretreatment of pregnant mice with FA can counteract the VPA-induced neural tube defects in mice[32]. According to previous results, FA prevents exencephaly by improving the fusion of the cranial neural folds and suppression of apoptosis of the neuroectodermal cells in the forebrain region[33].

In the present study, the protective potency of FA against VPA-induced spina bifida was in correlation with previous results[34]. According

to previous studies, this protective effect of FA was attributable to its potency to enhance the closure of the lumbosacral neural folds through stimulation of the normal neurogenesis and stem cell proliferation. FA also, prevents VPA-induced apoptosis, plays a critical role in the maintenance of neuroplasticity and neuronal integrity in the region of the developing spinal cord and promotes normal histogenesis of the developing vertebral arches of the vertebral column[35].

In the present study, the protective effects of FA against the congenital skeletal malformations induced by VPA were in correlation with previous results detected that, FA supplementation during early pregnancy successfully reduces the prevalence of limb reduction defects[19], rescues craniofacial skeletal malformations such as facial cleft and palatine cleft through inhibition of the teratogens-induced apoptosis and oxidative stress[36]. According to previous studies, the ameliorative effects of FA against the VPA-induced vertebral column and ribs malformations were attributable to its potency to improve the osteogenesis and mineralization in the developing vertebrae and ribs[37].

In the current study, FA was effective in preventing the intrauterine growth restriction induced by VPA. This was in line with previous results proved the importance of FA supplementation to improve fetal growth and to prevent the incidence of low birth weight [38]. According to previous studies, folic acid supplementation during early pregnancy improves the growth of the fetus and placenta through enhancement of cell proliferation, DNA and RNA synthesis and amino acids metabolism [39, 40].

In conclusion, the present study showed that, VPA induces skeletal and neurological congenital abnormalities in mice while co-administration of FA protects against these VPA-induced malformations.

Conflicts of interest: None

Financial disclosure: None

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