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The Effects of Prenatal Exposure of Rabbit to Valproic Acid

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ABSTRACT

The present study was conducted to determine the prenatal effects of sodium valproate (VPA), an antiepileptic drug, in pregnant rabbits. Ten adult female rabbits were classified into treatment and control groups. The drug was administrated to the treatment group as oral doses of 400mg/kg of VPA for 15 days starting from the 6th day after mating until the 20th day of pregnancy, while the control group received water at the same volume and period. The pregnant rabbits were euthanized on the 29th day of pregnancy. The fetuses were collected, and the crown rump length and weight were taken. No gross or microscopic abnormalities were seen in the control group. Gross examination of the treatment group showed reduction in size and length of the fetuses and resorption of fetuses as well as retarded ossification, abnormal growth of the ribs and missing sternebrae. However, no abnormalities were seen microscopically. It was found that the use of VPA during pregnancy resulted in intrauterine growth retardation manifested by decreased fetal body weight, length, and skeletal abnormalities.

Key words: Fetus, Prenatal, Rabbit, Valproic acid.

INTRODUCTION

Valproic acid (VPA) has been widely used as an anticonvulsant drug against both generalized and partial seizures (Peterson and Naunton 2005). VPA significantly reduced body weight, crown-rump length and skull dimensions as well as axial and appendicular bones development in albino rats (Abdel Salam and Allam 2015). In humans prenatal use results in spontaneous abortions, stillbirths and spina bifida (Eadie and Vajda 2005). The risks of developing congenital malformations and neurodevelopment disorders have led to the restriction of the use of VPA for treating epilepsy or bipolar disorder in pregnant women by the European Pharmacovigilance Risk Assessment Committee (PRAC) (García-Portilla et al. 2017). A dose-effect relationship with fetal malformations and exposure to VPA was also observed in humans, where, as the dosage increased, the rate of fetal malformations also increased in the first trimester (Vajda et al. 2004). The administration of a single VPA dose (300mg/kg) resulted in weight and length loss in rat fetuses (Baran et al. 2006), while treatment with 1200mg/kg Valproic acid given daily resulted in a high rate (100%) of spina bifida occulta (Ceylan et al. 2001). VPA is also neurotoxic to the Purkinje cells of the cerebellar cortex in rats (Shona et al. 2018). In rabbits, a study revealed that there was an increased incidence of fetal resorption and major malformations when given 350mg/kg of Valproic acid on days 6-18 of gestation. In another rabbit study, Sodium valproate when used at a dose of 315mg/kg, there was an increase in renal and vertebral defects and intrauterine deaths (Whittle 1976).

Animal models are essential in preclinical anti-epileptic drug research (Jagannatha 2015). The rabbit is the most sensitive and suitable laboratory animal for studying the teratogenic potential of different chemicals because developmental patterns in humans and rabbits are similar (Beaudion et al. 2003) and the rabbit's extra-embryonic membranes are similar to that of humans (Foote and Carney 2002). Gross changes are easier to recognize on the rabbit fetus due to its size compared to that of rats or mice, therefore the rabbit was used as the model of choice. The aim of this study was to investigate the possible side effects in rabbits exposed to VPA (400mg/kg) from 6th to 20th day of gestation.

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MATERIALS AND METHODS

Animals

This study was conducted after ethical approval (CEC 149/03/17) from the Campus Ethics Committee of the University of the West Indies, St. Augustine, Trinidad, and Tobago. Ten sexually mature, virgin, apparently healthy New Zealand and California doe rabbits were used. The rabbits aged between 5-6 months, with weights ranging between 2.8-3.4kg, were housed in standard cages (Fig. 1A) in a room maintained at 27℃, with 12-hrs light/dark cycles. They were acclimated for two weeks with access to water and a balanced commercial diet ad libitum.

Experimental Protocol

The rabbits were later placed into two groups. The treatment group contained six rabbits and the control group contained four rabbits. Does were mated with a fertile buck of the same strain. The female was housed with a male for a two-hour period in the afternoon and since ovulation occurred 10-12hrs after coital stimulation this was considered day 1 of pregnancy (Bahat et al. 2005). Sanitary conditions were maintained throughout this study; the catch pans were cleaned daily. The feed was measured daily, and the weight of rabbits was taken periodically until day 29 of gestation.

As the VPA intoxication occurred during organo-genesis which was from 6^{th} to 18^{th} days of gestation (Stanley and Bower 1986; Petrere et al. 1993). Sodium valproate (Epilim®) syrup, 200mg/5ml was orally administered once a day, from day 6 through day 20 of gestation at 12:00 hr. Enteric coated tablets (Epilim®), 200mg were used for 8 weeks when there was a supply shortage of the syrup. Tablets were crushed, mixed with banana into a solution and given orally once a day (Fig. 1B). Dose administration was calculated based on per kg body weight per day to mirror the human therapeutic doses range of Valproic acid 400mg/kg per day (Reagan-Shaw et al. 2008). Rabbits were monitored for signs of toxicity. The control group received the same volume of distilled water orally. Manual abdominal palpation was performed on day 10 of gestation until confirmed by abdominal ultrasound (Fig. 1C) by day 14 of pregnancy for all does.

Sample Collection and Fetal Measurements

All pregnant rabbits were euthanized on day 29 of pregnancy to maintain the same age for all fetuses. The abdominal cavities of the pregnant does were incised, the uteri were exteriorized, and the fetuses were extracted from their sacs (Fig. 1D&E). The umbilical cords were clamped and cut. A total of 53 rabbit fetuses were collected (39 from the treatment group and 14 from the control group). Fetuses were observed for external abnormalities such as cleft palate and facial and limb deformities (Goyal et al. 2016). Crown rump length (CRL) was measured with a measuring tape, starting from the crown of the forehead along the dorsum of the fetus to the tail base. A weighing scale was used to obtain fetal weights.

Skeletal Staining Technique

Rabbit fetuses from the control and treatment groups were sacrificed to be used for this study. They were skinned and eviscerated, placed in 95% ethanol for fixation by dehydration from 4-7 days, rinsed by distilled water, put in pure acetone for 1-3 days, put in 1% potassium hydroxide solution from 1-3 days until the skeleton became clearly visible. Then they were put in a freshly prepared 0.001% Alizarin Red S solution (Fig. 1F) for skeletal staining for 24 hours until the skeleton became red. The skeletons were cleared by putting the fetuses in a mixture of 1% Potassium hydroxide and 20% glycerol for two days. Finally, they were stored in a 1:1 mixture of 95% ethanol and glycerol. The stained axial and appendicular skeletons were examined under a Stereomicroscope for abnormalities. The gross photos were taken for documentation using a digital camera (Sony 12 MP). The level of ossification of the skeletons of the rabbit fetuses was estimated according to the degree of stain coloration with Alizarin Red-S, with the more highly ossified parts had a deeper red color while the incompletely ossified parts had a less red color. No color was seen in un-ossified parts (Mohamed 2018).

Histopathological Examination

Tissue specimens which included the brain, heart, lungs, liver, kidneys, forelimbs and hindlimbs, ribs and vertebrae were immediately fixed in 10% formalin following evisceration from the fetuses. After 48 hours, the specimens were dehydrated in ascending grades of ethanol concentrations, cleared in xylene, and embedded in paraffin wax. Sections of 4-6μm thickness were then deparaffinized in xylene, hydrated in a series of descending ethanol concentrations and stained with hematoxylin and eosin stain for histological examination (Alturkistan et al. 2016). Stained sections were viewed under a light microscope (Olympus BX40 with an Olympus DP74 digital camera, Japan) at different magnifications and photomicrographs were taken.

Statistical Analysis

The obtained data (lengths and weights of each litter as well as the litter size) were expressed as Mean ± and analyzed using the Pearson correlation, the one-way ANOVA and Bonferonni post hoc.

RESULTS

Gross Findings

The gross abnormalities detected in the treatment group were underdeveloped fetus, low birth weight and fetal death (Table 1). Four dead fetuses were found with a crown-rump length of 15 to 20 mm indicating that they died around the 3^{rd} week of gestation. (Fig. 2A&B). The stained skeletons by Alizarin red- S in the treatment group showed malformed ribs (8^{th} to 13^{th} ribs) in four rabbit fetuses (Fig. 2C) and the fourth sternebra appeared to have not developed ossification in three rabbit fetuses.

The Pearson correlation of the weight to the length of the fetuses was not significant enough to show a relationship between the two. Therefore, it can be assumed the length of the fetuses had no influence on their weight and this correlation would not affect the analysis of the Sodium valproate on each of the individual values. It also shows, it affected the rabbits in either length or weight more than the other value. The one-way

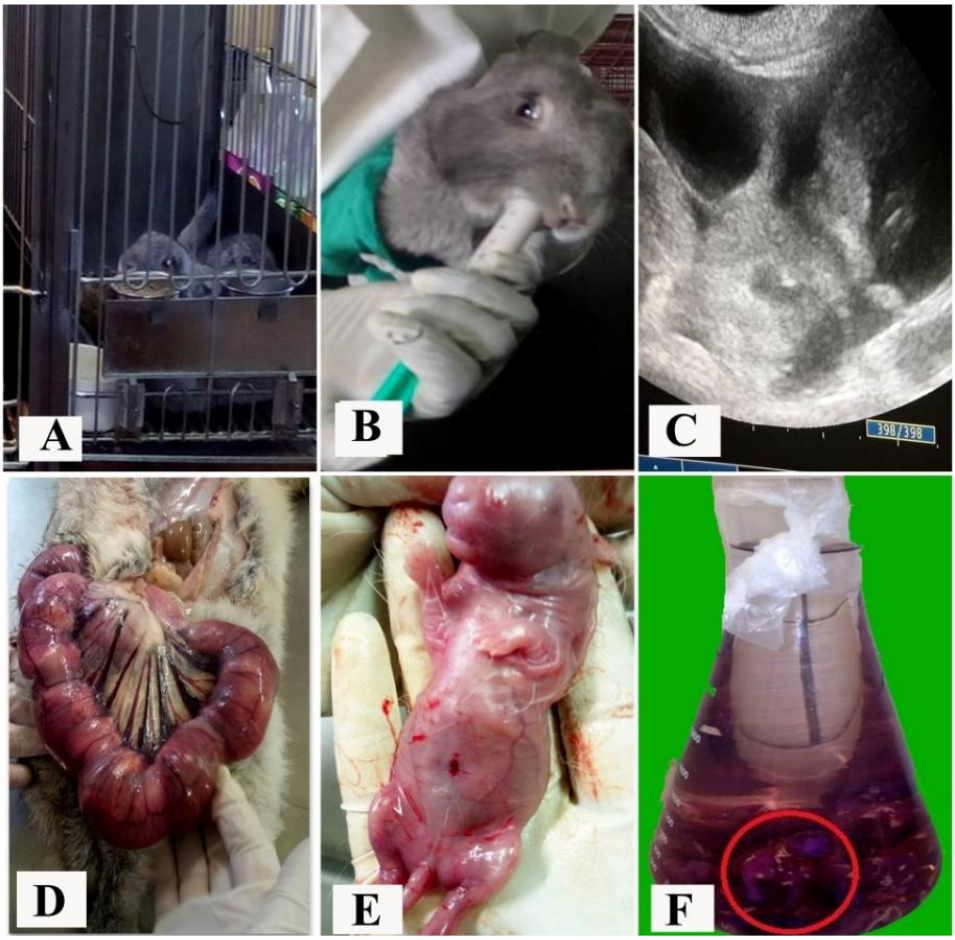

Fig. 1: A photograph showing the breeding of rabbit (A), drug administration (B), pregnancy diagnosis abdominal ultrasound (C), retrieving the fetus (D&E) and Alizarin red S staining (E).

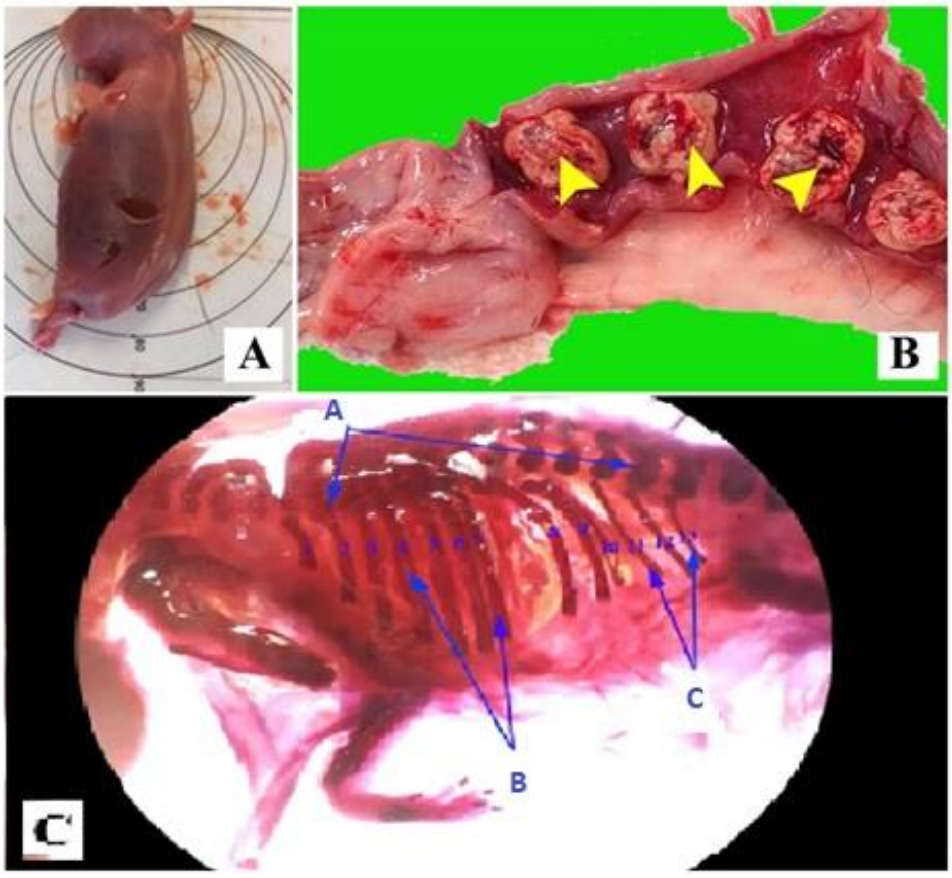

Fig. 2: A photograph showing underdeveloped fetus (A), fetal resorption (B- yellow arrowheads) and rib malformations (C) Note: A- Thoracic vertebrae, B. Normal Ribs and C- Abnormal ribs: 11-13).

Table 1: Litter values for treatment (T) and control groups (C) indicated low birth weight and length in the treatment group

Rabbit Number	Litter Size	Average Fetus Length (cm)	Average Fetus Weight (g)
T1	8	8.4	35.3
T2	8	7.4	30.7
T3	2	10.0	33.4
T4	8	9.0	37.2
T5	6	12.0	32.7
T6	7	8.5	26.4
C7	5	13.5	39.2
C8	2	9.0	44.8
C9	7	9.5	28.7

Table 2: Still births as well as change in the weight and length of the fetuses in the treatment group (T)

Parameter	T	C
Total	39	14
Still births	2	0
Mean length/cm	9.22	10.68
Mean weights/g	32.6g	37.5g

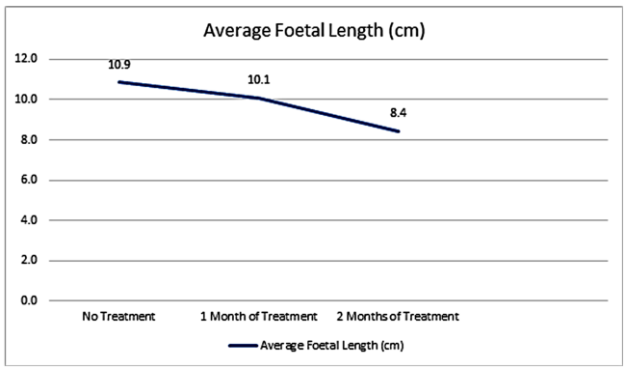

Fig. 3: The average fetal crown to rump lengths amongst treatment groups 1 month, 2 months, and no treatment.

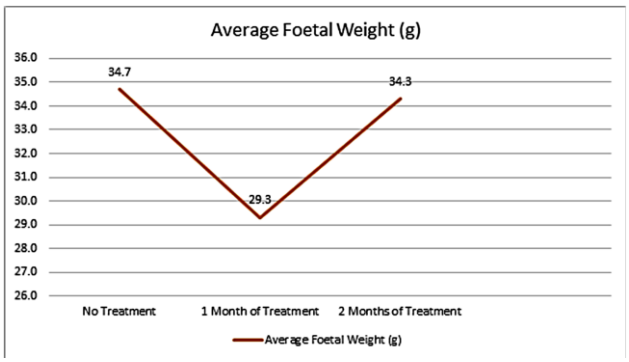

Fig. 4: The average fetal weights amongst treatment groups 1 month, 2 months and no treatment.

ANOVA showed that the Sodium valproate had an effect on the lengths of the fetus in the treatment groups for the drug ($F=9.675$, $P<0.001$), however for the weight there was not a significant correlation ($F=3.167$, $P=0.052$). The Bonferonni post hoc comparison was used to determine which treatment category was statistically significant from another. For mean length, it was observed that for the two treatment groups there was a significant difference between both ($P=0.33$), and no treatment for 2 months ($P<0.001$). There was no significance between treatment for 1 month and no treatment ($P=0.794$). Therefore, when the rabbits had more Sodium valproate in their system (2 months period), there was a significant change in the length of the fetus (Figs. 1-5; Table 2).

Histopathological Findings

There was no observable, histological differences between the treated and untreated fetuses. The femurs and thoracic region of the vertebral column were within normal limits (Fig. 6A and B). Hepatic architecture and renal tubular development were also within normal limits (Fig. 6C). Normal hematopoietic function of the neonatal liver was noted (Fig. 6D). The eyes were within normal limits (Fig. 6E). The left and right ventricles of the heart wer also within normal limits (Fig. 3F). The nasal septum was intact to allow the separation of the left and right nasal cavities. Lesions in the placenta indicating foetal resorption were observed (Fig. 6G). No abnormalities were detected in the brains when compare the treated to the non-treated groups (Fig. 6H).

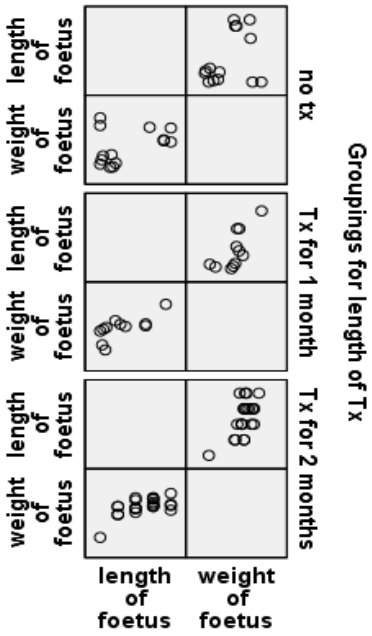

Fig. 5: A comparison of the weight and length of the fetuses amongst treatment groups month, 2 months, and no treatment.

DISCUSSION

The first scientific preference to determine the potential teratogenic effects of VPA on human health is through animal experiment. The current results showed the effects of using VPA during the pregnancy of rabbits manifested as a significant reduction ($P<0.05$) in crown rump length. There was not a significant reduction in weight. There was a significant difference in the mean length of the fetuses ($P=0.033$) between those that received treatment for two months when compared to those that received treatment for one month. Therefore, it can be noted that the longer the animal is exposed to the drugs during pregnancy, the greater the effect on the foetal crown rump length. This finding is similar to those, in pregnant albino rats, where exposure to VPA significantly ($P<0.05$) reduced all body measurements including crown-rump length, skull dimensions and body weight (Ingram et al. 2000).

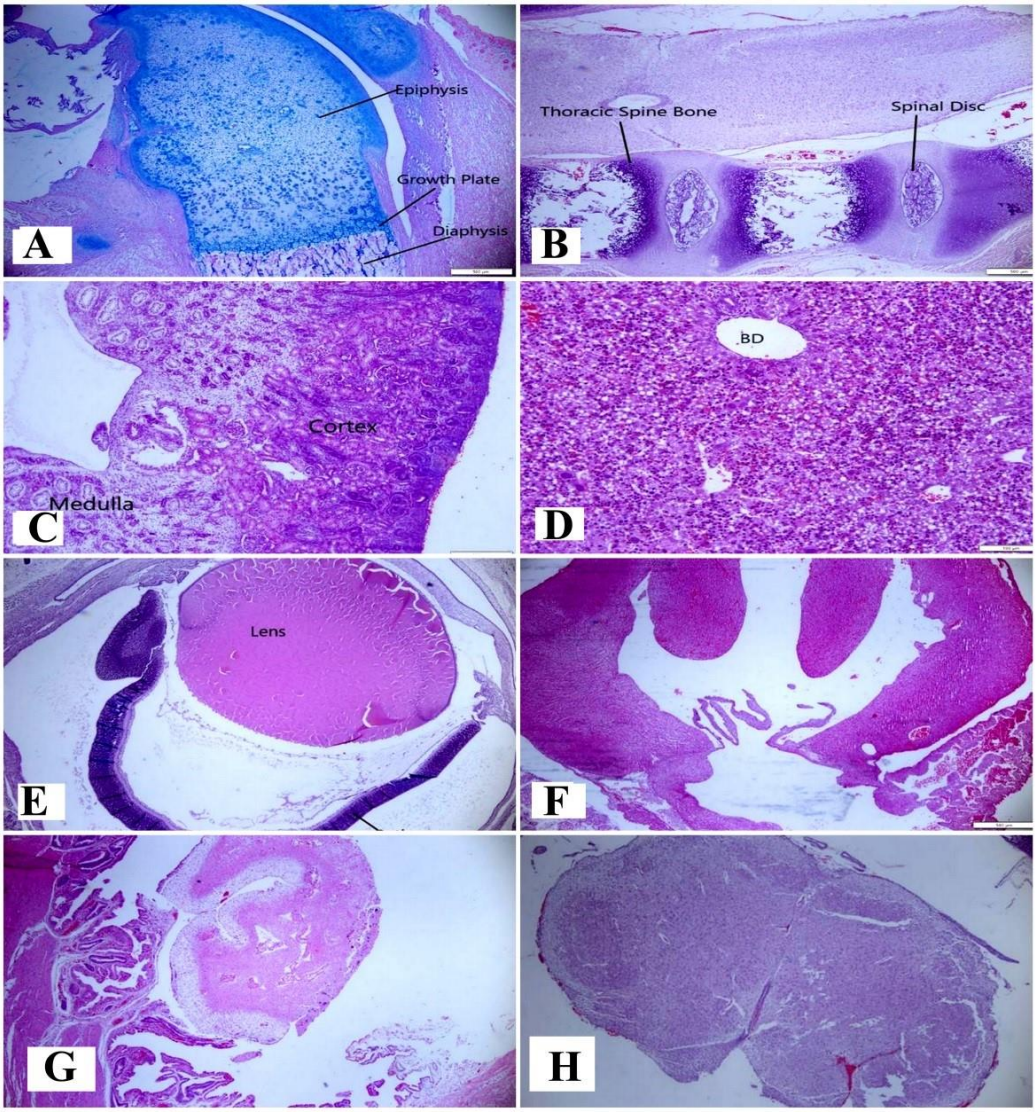

Fig. 6: A photomicrograph from the fetuses of the treatment group showing the head of the femur (A), thoracic region of the spine (B), kidney (C), liver (D), eye €, heart (F), placenta (G) and forebrain (H). A: Alcian Blue. B to H: H&E. All at X40.

Sodium valproate in this study caused a decreased crown-rump length, rib malformations, fetal resorption, underdeveloped fetuses, missing sternebrae and stillbirths. These findings were similar to calcium valproate effects such as malformed ribs and post implantation loss at 350mg/kg in rabbits (Petrere et al. 1986), malformations and loss of one sternebrae in rats (Menegola et al. 1998) as well as wavy ribs and abnormal position in the ribs (Erik-Aghaj et al. 2015) in rats. Other studies showed that the administration of Valproic acid on days 10 and 13 to pregnant rats resulted in a reduction in the number and size of offspring at parturition (Komariaha et al. 2017). The axial skeletons of rats and mice are very sensitive to the teratogenic effect of Sodium valproate especially at a dose of 300mg/kg (Menegola et al. 1996).

The current results showed that the internal organs of the treated and untreated fetuses were morphologically normal; a similar result was found with Valproic acid (500-600mg/kg) in the mouse (Emmanouil-Nikoloussi et al. 2004). Petrere et al. (1986) stated that there were no teratogenic effects in rabbit treated with Calcium valproate at 50mg/kg, however, this is below the dose required treatment. Valproic acid also causes hepatotoxicity in developing rats (Khan et al. 2011; Shakya et al. 2012) and mice (Ibrahim 2012), cardiac abnormalities in mice (Wu et al. 2010), and open brain folds at a dose 300mg/kg in mice (Menegola et al. 1996). It has a prominent neurotoxic effect on the cerebellar cortex of the adult male albino rats (Shalaby and Sarhan 2008), decreases hippocampus volume in rats (Anna Petrenko et al. 2014) and reduces cerebellar volume in rats (Ingram et al. 2000).

Conclusion

The long-term use of VPA as antiepileptic drugs during pregnancy resulted in decreased crown-rump length, rib malformations, fetal resorption, under-developed fetuses, missing sternebrae and stillbirths. Therefore, the use of VPA should be carefully decided for pregnant females and should not be used as a first line therapy in woman of childbearing unless it is an only option.

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Authors' Contribution

All authors contributed to the materials, analyzed the data and wrote the manuscript.

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