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Vitamin D3 Attenuates the Teratogenic Effects of Gabapentin on Rat Fetal Skeletal Systems

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ABSTRACT

Background: The antiepileptic drugs (AEDs) used to control epileptic seizures, the developing fetuses of pregnant women with epilepsy who are treated with AEDs might be subjected to an increased risk of major congenital malformations. Few studies drew our attention to the possible genotoxicity or/and mutagenicity of gabapentin in treated mothers and their fetuses. **Objectives:** To evaluate the effects of gabapentin administration on the skeletal systems of albino rat fetuses. **Results:** Both experimental groups showed similar disorders, including increased fetal resorption and skeletal malformations. The macroscopic malformations included limb defects and skeletal malformations, such as delayed ossification and mandibular hypoplasia. Fetal resorption was significantly higher in the gabapentin-treated group than that in either the control group or the combined gabapentin and vitamin D3 group. **Conclusions:** Gabapentin is a teratogenic agent. Decreased Crown-rump length (CRL) and diminished biparietal diameter (BPD) were observed in the fetuses of the female albino rats that received the drug during pregnancy. Additionally, the drug produced skeletal malformations by inhibiting the ossification of bones, although vitamin D3 attenuated its teratogenicity.

INTRODUCTION

Epilepsy is a common health problem in pregnant women, and it represents the second most common chronic neurological disorder and one of the most important neurological syndromes affecting women during the reproductive period. Epilepsy is characterized by sudden recurrent episodes of sensory, motor or autonomic disturbance associated with abnormal brain electrical activity, with or without the loss of consciousness or convulsions (Sridharan, 2002).

Many of these women must continue taking antiepileptic drugs (AEDs) to control epileptic seizures, and although treating a pregnant woman who has epilepsy with AED presents high risks associated with the increased occurrence of complications, it may be dangerous to stop or even change the AED regimen during pregnancy because of the frequency and severity of the underlying epileptic disorder. Thus, prescribing AEDs during pregnancy is a challenge to clinicians (Ozyurek *et al*., 2010; Evan *et al*.,2013). The majority of antiepileptic drugs are trans-placental xenobiotics (Ohman *et al*., 2000). Moreover, the developing fetuses of pregnant women with epilepsy who are treated with AEDs might be subjected to an increased risk of major congenital malformations (MCMs), post-natal developmental anomalies, developmental delay, fetal death or adverse pregnancy outcomes (Chen *et al*., 2009; Nowiska *et al*., 2012).

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Vitamin D deficiency has been shown to reduce mating success and fertility in female rats. Furthermore, female rats fed a vitamin D-deficient diet are capable of reproduction, although their overall fertility is reduced, including the probability of impregnation, and they present an increased risk of pregnancy complications. These complications are not corrected by normalizing the hypocalcemia in vitamin D-deficient female rats but require treatment with 1,25(OH)2D3 (Kwiecinksi *et al*.,1989).

In diabetic rats, 1,25(OH)2D3 treatment has a protective effect on alloxan-induced damage in the reproductive system by enhancing testosterone and 17b-estradiol levels, which protects the animals from oxidative stress and cellular toxicity by scavenging free radicals, inducing antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase, and preventing oxidative damage in humans (Hamden *et al*.,2008).

The results of an in vivo study showed that 1,25(OH)2D3 significantly increases uterine weight and induces decidual reaction (Halhali *et al*.,1991), suggesting a physiological role in the differentiation of endometrial cells into decidual cells, which is a crucial step in the process of blastocyst implantation. However, high doses of 1,25(OH)2D3 result in a reduced corpus luteum, reduced progesterone levels, and alterations in the estrous cycle in rats (Horii *et al*.,1992).

These findings drew our attention to the possible genotoxicity or/and mutagenicity of gabapentin in treated mothers and their fetuses (Quintero, 2017). Therefore, the present study was performed to determine the potential genotoxic and mutagenic effects of gabapentin in the skeletons of treated pregnant rats and their fetuses during pregnancy.

MATERIAL AND METHODS

Animals:

This study was conducted on thirty female albino rats of a uniform strain and weight that were obtained from the animal house in the Faculty of Medicine, Umm-AlQura University, Saudi Arabia. All of the procedures involving rats followed the specifications recommended in the Guide for the Care and Use of Laboratory Animals (NRC, 1996)and guideline of Umm-AlQura University research ethical committee. Animals were housed 2 per cage at a temperature of 24°C and a relative humidity of 60%. The light cycle was 12 h light/12 h dark. The animals were allowed free access to tap water.

Experimental Design:

Thirty pregnant rats at 11-13 w old (275±24 g body weight (b.w.)) were selected for the present study, and vaginal smears were prepared every morning and examined under a light microscope according to the method of Snell, (1956) for 5 days to select rats in proestrus (Table I).

Two females with regular estrous cycles were selected in the proestrus stage (Table I) and caged together with one male overnight under controlled temperature, humidity and light conditions. The first day of gestation was determined by the presence of sperm in the vaginal smear (McClain and Becker, 1975).

The pregnant rats were divided randomly into 3 groups of ten each, including 2 experimental groups that received gabapentin intragastrically daily at 9:00 a.m. (±30 min) from the first day to the 20th day of gestation.

Group A received 324 mg/kg (equivalent to the maximum human therapeutic dose, 3600 mg/kg) of gabapentin alone, and Group B received 324 mg/kg (equivalent to the maximum human therapeutic dose, 3600 mg/kg) of gabapentin and vitamin D3 (0.3 mg/kg body weight/day). Group C (control group) received normal saline by the intragastric route.

Materials:

- Gabapentin (GBP) powder was obtained from neurontine capsules containing 400 mg GBP (Pfizer,Park Davis, Pharmaceutical, Ltd, NY,USA). Dilution was conducted with normal saline.
- Cholecalciferol (vitamin D) was purchased from Sigma (St. Louis, MO, USA).

Stock solutions of vitamin D were prepared fresh for each 3-d subcutaneous injection cycle at a concentration of 4.3 mmol/L in 7% emulphor (alkamuls EL-620; Rodia, Cranbury, NJ) and then placed in foil-wrapped containers and stored at 4°C as previously described.

The present study was performed on healthy (non-epileptic) pregnant rats. The rats were weighed daily, and the doses of GBP were adjusted according to their body weight.

On the 20th day of gestation, all of the pregnant rats in the study groups (A, B, and C) were euthanized under chloroform vapor and sacrificed by decapitation after being fasted overnight. The uterine horns were properly exposed, and the number of living fetuses and the fetal resorption were detected. The fetuses were removed from their membranes and then separated from their placentas. External observation was conducted with a stereo research microscope (SZX, Olympus, Tokyo, Japan).

Australian Journal of Basic and Applied Sciences, 11(10) July 2017, Pages: 60-66

Fixation:

Up to the 15th day of age, the small size of the embryo increased the difficulty of shelling manipulations and elevated the risk of damage; therefore, the embryos were fixed in situ using the perfusion of the mother. Older fetuses were removed from the uterine horns, and their placentas were removed after opening the surrounding membranes, and then their abdominal walls were opened by a scalpel. Whole fetuses were immersed in 10% formalin for two days, after which the kidneys could be dissected alone or with part of the posterior abdominal wall.

Perfusion technique:

The thorax of the anaesthetized pregnant female rat was opened by a left parasternal incision from the xiphoid process to the first rib, with care taken to prevent injury to the internal mammary artery. Another transverse incision was made in the fourth intercostal space to widen the thoracic opening. The thoracic flaps were retracted, and the thymus gland was displaced to expose the heart and aorta. A loose ligature was applied around the ascending aorta, and the left ventricle was then snipped. A cannula for perfusion was inserted into the ascending aorta, and the ligature was tightened around it, and then the right atrium was opened. The perfusion started at a pressure of 120 mm/Hg; saline was used initially until it exited out of the right atrium as a clear fluid, and then 10% formalin was allowed to pass for approximately 10 minutes. Subsequently, the animal exhibited generalized rigidity, which was apparent in the tail and indicated that all of the tissues were fixed in situ. After perfusion, the uterine horns were removed, and the embryos were excised and immersed in 10% formalin for another day.

Estimation of the age of the rat embryo:

1 The vaginal smear method, including vaginal cytology.

The presence of a copulation plug and spermatozoa in the vaginal smear indicated the beginning of pregnancy as previously mentioned.

2 The length-age relationship, which indicates the relationship between the length and gestational age, was applied according to Hanse's equation: $L = DT + F$

where:

L = length;

T = time of gestation; and

D and F = constants for the species.

The equation applied in the present work was as follows:

$R = 0.3D - 3.4$

where:

R = crown-rump length;

D = gestation in days; and

0.3 and 3.4 = constants for rats.

3. The weight-age relationship.

For the rat, this relationship was calculated as follows:

$W = (0.15D — 1.7)^3$

where

D = gestation in days; and

W = weight in grams.

In the present work, previously described methods, such as the vaginal smear method and length-age relationship equation, were used to estimate the age of the embryo.

Fifteen female rats were isolated to ensure that they were not pregnant, and they were then placed with males in separate cages until they became pregnant as confirmed by vaginal smears. The time of pregnancy was calculated and at the proper day, and the embryos were collected daily from 9 days to full term.

The embryos up to the age of fifteen days were fixed in situ by perfusion of the mother.

Embryos above that age were removed from the uterine horns and fixed in 10% formalin, and then the perfusion method was used.

Transverse and longitudinal sections at five microns thick were obtained and stained with hematoxylin and eosin.

Fetuses were assessed as either alive or dead, and exteriorized uterine horns were inspected for fetal resorption using yellow ammonium sulfide. Then, each fetus was weighed by a sensitive electronic balance.

All live fetuses were measured and examined externally for gross malformations or deviations from normal growth. Fetuses with skeletal malformations were chosen for double staining.

The brain was extracted as follows: the scalped skull was opened carefully using fine scissors starting laterally from the outer ear foramen and moving forward. Using forceps, the roof of the skull bones was carefully removed in pieces. The brain was finally separated from the skull base after cutting all of the cranial nerves.

Parts of the liver and brain tissues of pregnant rats and fetuses of different groups were stored at -20˚C for further investigations. Malformations were detected and photography was performed.

Results:

Reproductive performance:

Examinations of the uteruses on the 20th day of gestation revealed that significant fetal resorption occurred in 37% of the rats in the GBP alone group (group A), whereas resorption occurred in 4% of the rats in the negative control group (C) and 5% of the rats in the GBP and vitamin D3 group (group B). Significant differences in fetal weight were not observed, whereas significant differences in the CRL and BPD of the fetuses were observed among the different groups (Table II).

Clinical signs of toxicity:

Mortality was not observed in any of the treated groups. All of the female rats dosed with the control substances or with GBP alone (324 mg/kg) or in combination with vitamin D3 (0.3 mg/kg body weight/day) appeared normal throughout the experiment. Furthermore, GBP did not cause miscarriage, and intragastric GBP exposure at the tested doses did not affect the duration of gestation.

The frequency of the total number of fetuses, live fetuses, and their weights in the experimental groups A and B and the control group C showed that significant differences did not occur in the mean number of live fetuses among the different studied groups (Table II).

Skeletal anomalies:

Fetuses obtained from the negative control and both treatment groups showed normal skeletal structures when examined from the dorsal surface (Figures 1). The rat fetuses exposed to GBP appeared smaller in size than the combination therapy and control groups (Figure 2).

These deformities included malrotation and delayed development in the upper and lower limbs, which appeared as micromelia in 13% of fetuses in group A. Additionally, 7% of animals in group B had limb deformities (Figure 3) (Table II).

Mandibular hypoplasia and delayed ossification were observed. Significant mandibular hypoplasia was observed in the GBP alone group (Figures 4-5). Significant delayed ossification was observed in the metacarpus and metatarsal bones, although primary ossification centers in these bones and phalanges were not observed (Table II).

The density of compact bones of the forearm and leg decreased, and the calvaria also showed delayed ossification (Figure 6) (Table II).

Insert Figure 10 near here

Discussion:

This investigation showed that GBP administered at doses of 324 mg/kg (comparable to doses in human patients) during the implantation and organogenesis stages can cause fetal resorption, brachygnathia, limb anomalies, mandibular hypoplasia, and delayed ossification. These findings contradict the observations by Petrere and Anderson, 1994, who studied the teratogenic effects of GBP in mice with different doses of 500 up to 3,000 mg/kg on gestational days 6-15 and concluded that GBP exposure during pregnancy was not associated with an increased risk of adverse maternal and fetal events. This contradiction might be related to the different mouse strains used in their study. In this study, a significant increase in resorbed fetuses was observed in the experimental groups compared with number of resorbed fetuses in the control group. Similar findings have been reported by Afshar and Golalipour, 2008 after injection of GBP intraperitoneally at doses of 1400 mg/day and 1800 mg/day from gestational day (GD1 to GD10) in pregnant mice. Prakash *et al*., (2008) injected GBP at doses of 1800 mg/day and 3600 mg/day intraperitoneally in the mid GD (7-12) and late GD (13-17) and reported similar results. Additionally, the mean weight of the fetuses in the treated groups was significantly less than that of the control group, which is similar to the findings of Afshar and Golalipour , 2008.

Furthermore, Prakash *et al*. (2008)'s study [15] also assessed the length of the fetuses and reported that the crown-rump distance decreased significantly in the treated groups receiving GBP in the mid and late GDs compared with the corresponding control group. In their study, small bodies were observed in the experimental groups Prakash *et al*. (2008). All of these data are similar to our results, which are consistent with those of Wide and Winbladh, 2000, who reported that the use of antiepileptic drugs during pregnancy affected the mean value of the birth weight CRL and head circumference compared with the control groups. The Montouris, 2003 Registry study , which was performed on 39 pregnant women taking GBP, showed that GBP exposure does not carry an increased risk of malformation, fetal loss, low birth weight babies, or maternal complications. Brachygnathia was the most prevalent observed anomaly in the fetuses of experimental groups I and II, and this finding was also similar to the results of Prakash *et al*., 2008.

In our study, bone double staining showed that brachygnathia was caused by mandibular hypoplasia (Table I). Another observed macroscopic malformation in the treated groups was limb defects that appeared as abnormal growth and malrotated limbs. Such anomalies have been reported by Prakash *et al*.,2008 and Afshar and Golalipoor, 2008.

Therefore, the present study confirms that relative GBP exposure can produce limb anomalies. Deformities in the vertebrae were another anomaly that appeared as abnormal curvatures along the vertebral column similar to scoliosis. Deviation in the vertebral column was also reported in a previous study McClain and Becker, 1975. In Prakash *et al*., 2008.'study, shortening of the neck was reported . Anomalies of the calvaria were the most common skeletal anomalies observed in this study. Defects of the calvaria were more obvious in the frontal and parietal bones and appeared as overlapping defects in the formation of these bones. This finding has not been reported in previous studies. In macroscopic studies of the fetal skeleton, delayed ossification was observed and appeared predominantly in the metacarpus and metatarsal bones. The long bones in the forearm and leg also showed a decrease in the density of compact bone tissue, and primary ossification centers did not appear in these bones or in the phalanges. The calvaria also showed delayed ossification. In a study in which GBP was administered orally in doses of 1000-3000 mg/day during the organogenesis period, delayed ossification was observed in the calvaria, humerus and forearm bones Briggs *et al*., 2008, which is consistent with our findings.

The exact mechanism or mechanisms of the teratogenic effects of GBP are not fully understood and require further investigation. However, alterations in GABA neurotransmitter concentration may be an important factor in the probable teratogenic mechanisms Prakash *et al*., 2008.

Thus it was concluded that GBP has structural similarities with GABA and is an analog of GABA; therefore, the teratogenic effects of GBP, especially the production of neural tube defects (NTD), may be related to changes in GABA concentration or an influence on GABA metabolism and musculoskeletal defects and anomalies in the number of digits. Vitamin D3 attenuates the teratogenicity of GBP and more studied are required to document the effect of GBP in different species to further prove this hypothesis.

Table I: The rat estrous cycle occurs typically every 4-5 days and can be described in 5 stages

Stage	Superficial genitalia	Vaginal smear	Uterus	Ovary	Duration
1	Lips slightly swollen, vagina dry	Epithelial cells only	Increased distension fluid	Growth follicles enlarged	12 hours
2	Lips swollen, vagina dry	Cornified cells only	Minimum distention, early regression	Large follicles, maturation of eggs	12 hours
3	Lips still swollen, regression a cheesy mass in the vagina	Cornified cells only	Epithelium degeneration	Ovulation	15-18 hours
4	No swelling, mucosa moist	Cornified cells and leucocytes	Regression begins	Eggs in oviduct	6 hours
5	No swelling, mucosa moist	Leucocytes & epithelial cells	Epithelium regeneration	Corpus lutea formed	57—60 hours

Table II:

Parameters	Group A (GBP alone)	Group B (GBP & vitamin D3)	Group C (Control group)
No. of dams bred	10	10	10
No. of dams pregnant	8	9	9
Resorption, %	37	5	4
No. of live fetuses	27	83	84
BPD	7.43	9.57	9.77
CRL	32.2	36.8	37.6
Limb deformities, %	13%, p=0.0001	7%	1%
Brachygnathia, N/T	10/15, p=0.0001	4/15	0/15
Mandibular hypoplasia, N/T	7/15 , p=0.001	3/15	1/15
Delayed ossification, N/T	11/15, p=0.0001	9/15	1/15

Fig. 1: Normal skeletal structures.

Fig. 2: Fetuses smaller in size.

Fig. 3: Malrotation and delayed development.

Fig. 4: Mandibular hypoplasia.

Fig. 5: Mandibular hypoplasia.

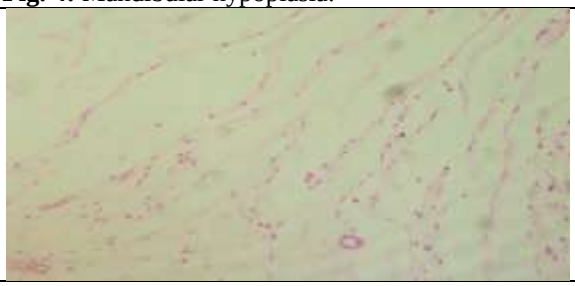

Fig. 6: Delayed ossification.

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REFERENCES

Afshar, M., M.J. Golalipour, 2008. Teratogenic effects of gabapentin on neural tube and limb development in mice. Neurosciences, 13: 321-323.

Briggs, G.G., R.K. Freeman, S.J. Yaffe, 2008. Drugs in pregnancy and lactation: a reference guide to fetal and neonatal risk. 8th ed. Lippincott Williams & Wilkins, Philadelphia, PA.

Chen, Y.H., H.Y. Chiou, H.C. Lin, H.L. Lin, 2009. Affect of seizures during gestation on pregnancy outcomes in women with epilepsy. Arch Neurol, 66: 979-984. doi: 10.1001/archneurol.2009.142.

Evan, G., J.M. Kimford, 2013. Antiepileptic drugs in women with epilepsy during pregnancy. Ann Neurol, 74: 223-231.

Halhali, A., G.M. Acker, M. Garabedian, 1991. 1,25-Dihydroxyvitamin D3 induces in vivo the decidualization of rat endometrial cells. J Reprod Fertil, 91: 59-64. doi: 10.1530/jrf.0.0910059.

Hamden, K., S. Carreau, K. Jamoussi, F. Ayadi, F. Garmazi, N. Mezgenni, A. Elfeki, 2008. Inhibitory effects of 1alpha, 25dihydroxyvitamin D3 and Ajuga iva extract on oxidative stress, toxicity and hypo-fertility in diabetic rat testes. J Physiol Biochem, 64: 231-239. doi: 10.1007/BF03216108.

Horii, I., S. Takizawa, T. Fujii, 1992. Effect of 1,25-dihydroxyvitamin D3 on the female reproductive system in rats. J Toxicol Sci., 17: 91-105. doi: 10.2131/jts.17.91.

Kwiecinksi, G.G., G.I. Petrie, H.F. DeLuca, 1989. 1,25-Dihydroxyvitamin D3 restores fertility of vitamin D-deficient female rats. Am J Physiol, 256: E483-E487.

McClain, R.M., B.A. Becker, 1975. Teratogenicity, fetal toxicity, and placental transfer of lead nitrate in rats. Toxicol Appl Pharmacol, 31: 72-82. doi: 10.1016/0041-008X(75)90053-8.

Montouris, G., 2003. Gabapentin exposure in human pregnancy: results from the gabapentin pregnancy registry. Epilepsy Behav, 4: 310-317. doi: 10.1016/S1525-5050(03)00110-0.

Barbara Nowiska, Joanna Folwarczna*, Agnieszka Dusi£o, Maria Pytlik, Leszek Aliwiski, Urszula Cegie£a, Ilona Kaczmarczyk-Sedlak, Wojciech Pietryka, Tomasz Hanke and Henryk i. Trzeciak, 2012. Effects of vigabatrin on the skeletal system of young rats. Acta Poloniae Pharmaceutica ñ Drug Research, 69(2): 327-334.

NRC., 1996. Guide for the care and use of laboratory animals. National Research Council, National Academies Press, Washington, DC.

Ohman, I., S. Vitols, T. Tomson, 2000. Lamotrigine in pregnancy: pharmacokinetics during delivery, in the neonate, and during lactation. Epilepsia, 41: 709-713. doi: 10.1111/j.1528-1157.2000.tb00232.x.

Ozyurek, H., A. Bozkurt, S. Bilge, E. Ciftcioglu, F. Ilkaya, D.B. Bas, 2010. Effect of prenatal levetiracetam exposure on motor and cognitive functions of rat offspring. Brain Dev, 32: 396-403. doi: 10.1016/j.braindev.2009.05.003.

Petrere, J.A., J.A. Anderson, 1994. Developmental toxicity studies in mice, rats, and rabbits with the anticonvulsant gabapentin. Fundam Appl Toxicol., 23: 585-589. doi: 10.1006/faat.1994.1144.

Prakash, Prabhu, L.V., R. Rai, M.M. Pai, S.K. Yadav, S. Madhyastha, R.K. Goel, G. Singh, M.A. Nasar, 2008. Teratogenic effects of the anticonvulsant gabapentin in mice. Singapore Med J, 49: 47-53.

Gabriel C Quintero, 2017. Review about gabapentin misuse, interactions, contraindications and side effects. Journal of Experimental Pharmacology, 9: 13-21.

Snell, G.D., 1956. Biology of the laboratory mouse. Dover Publications, New York.

Sridharan, R., 2002. Epidemiology of epilepsy. Curr Sci., 82: 664-670.

Wide, K., B. Winbladh, T. Tomson, B. Kallen, 2000. Body dimensions of infants exposed to antiepileptic drugs in utero: observations spanning 25 years. Epilepsia, 41: 854-861. doi: 10.1111/j.1528-1157.2000.tb00253.x.