Research Article

Evaluation of Embryotoxicity Potentials of Pregabalin in Albino Rats

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ABSTRACT

Pregabalin (PGB) [(S)-3-(Aminomethyl)-5-methylhexanoic acid 3-isobutyl GABA], as a new anticonvulsant drug is structurally related to gabapentin. However, it is a novel gamma-aminobutyric acid (GABA) analog which is virtually inactive at GABA receptors. Objective: to assess the histological effects of Pregabalin on albino rat fetuses during the pregnancy period. Twenty pregnant rats (Rattus norvegicus) weighing (190-200) were divided into the two groups. The control group was administered oral dose of distilled water and the treated group was administered oral dose of PGB (61.7 mg/kg) from the 6th day to the 19th day of gestation. At 20th day of gestation the females were sacrificed. Administration of PGB significantly reduced the fetal body weight and crown-rump length in comparison with the control group. Moreover, PGB treatment caused malformations that summarized as severe growth retardation, open eyes and skeletal abnormalities. Examination of fetal liver, kidney and brain of pregnant rats treated with PGB showed histopathological changes. It has been found that gestational usage of PGB may cause fetal risk with intrauterine growth retardation, hepatic injury and nephrotoxicity. The pregnant women should be used PGB if its benefits more than the potential risks.

Keywords: Antepileptic, Pregnancy, Malformation, Skeletal Abnormalities, Fetus.

INTRODUCTION

The expression "teratogen" is usually used to characterize any substance which able to make structural or functional abnormalities in the embryo during its developmental stages.[1]

Epilepsy consider one of the most important neurological disease that strike the women during childbearing period and needs treatment constantly all over the pregnancy, since antiepileptic drugs (AEDs) have major and minor teratogenic effects, it is summarized in congenital heart disease, cleft lip/palate, urogenital defects, and neural tube defects.[2]

Many AEDs have been shown to readily cross the placenta from the apical (maternal interfacing) syncytiotrophoblast plasma membrane to the basal (fetal facing) circulation.[3]

Oxidative stress means the imponderables between free radicals and the antioxidant enzymes. Reactive oxygen species (ROS) normally generated during metabolism that occur in the cells and interact with large molecules as protein, lipid, and DNA and finallylead to cellular injury and all successive degenerative alternation.[4]

Pregabalin (PGB) [(S)-3-(Aminomethyl)-5-methylhexanoic acid 3-isobutyl GABA], is an anticonvulsant drug and has a chemical structural similar to the gabapentin. PGB is a novel gamma-aminobutyric acid (GABA) analog which is virtually inactive at GABA receptors. PGB works throughits linkage the α2δ-1 subunit of voltage dependent Ca2+ channels to decrease calcium passage through the cell membrane. [5,6]The accurate mechanism of action of PGB is yet obscure. Numerous mechanisms have been suggested in order to interpret the teratogenic effects and embryo-toxicity of antiepileptic drugs. There are some proofs which indicate the role of reactive oxygen species in the embryonic malformations caused by the antiepileptic drugs.

Very few information have been presented about teratogenic potentials of pregabalin. There was few work shows that pregabalin may be used in the absences of any other choice. In the previous study that was done in 51 infants there was not risk of fetal malformation. [7,8]The present experiment aim to prove the potential embryo-toxic effects of PGB on rat offspring through histological and physiological studies.

MATERIALS AND METHODS

Animals and Caring

Twenty adult female Wistar rats (190–200 g) and ten male were used and acclimatized for one week under the standard animal house conditions.

Animals were housed in a controlled environment (23 ± 2°C, 12:12-hr light–dark cycle. Pelleted food and tap water were provided ad libitum. Animal handling was in accordance with the guidelines for the use of laboratory animals. All the experimental protocols and procedures used in this study were approved by the Cairo University, Faculty of Science Institutional Animal Care and Use Committee (IACUC) (Egypt), (CUFS/S/Phy/48/15) and the work Does not violate the ethics working with laboratory animals. The female rats were mated with healthy untreated male rat overnight. The male rats were removed after successful mating and the female rats were continually exposed to the treatments.

Study Protocol

The Pregabalin was purchased from Chemipharm for pharmaceutical industries S.A.E. Egypt (Kemirica) ® 300 mg/tablet. The animals were divided into two groups with ten animals in each. Healthy dams were randomly assigned to two experimental groups 10 rats each, group A: Control rats received distilled water orally and group B: Treated group, rats in this group given (61.7 mg/kg b wt, Pregabalin via stomach tube). The recommended maximum dose for human is 600 mg/daily, the dose was modified to suit the weight of rats according to [9](tablet of 300 mg dissolved in 25ml distilled water to give 12mg/ml solution, each 200 gm rat given 1ml of the prepared solution using rats stomach tube. All pregnant females were examined daily throughout the gestation period for mortality, morbidity, general appearance and the fetuses from both groups were examined for external malformations and skeletal abnormalities. All pregnant females were examined daily throughout the gestation period for mortality, morbidity, general appearance.

All mothers went through Cesarean section on GD 20. The uterus was observed for the live and dead pups as well as the resorption if any. Early resorption sites were evaluated and crown rump length of all the pups was measured. Fetuses were preserved in 95% ethyl alcohol and were stained with double staining of fetal skeletons for cartilage (Alcian blue) and bone (Alizarin red) according to the method described by.[10]

Histopathological examination

At the end of the experimental period, freshly dissected fetal liver, kidney and brain were picked out, rinsed in phosphate buffer (pH 7.5) and fixed in phosphate-buffered formalin. Sections were prepared and stained with hematoxylin and eosin.[11]

Antioxidant analysis

Weighted frozen tissue from the fetus was separately homogenized in an elongated U shaped glass homogenizer with 50 mM PBS (Phosphate Buffer Saline) (pH 7.4) and 50 mM normal saline to obtain 1:10 (w/v) whole homogenates. The homogenates were then centrifuged at 10000 g for 10 min at 4˚C to discard any cell debris. The supernatant was used to measure Reduced Glutathione (GSH) according toBeutler et al.[12], the activity of antioxidant enzyme Catalase (CAT)as described byAebi[13]and lipid peroxidation (MDA) according to the methods described byOhkawa et al.[14]. The levels of GSH, CAT and MDA kits were purchased from Bio-diagnostics Company (Dokki, Giza, Egypt).

Statistical analysis

All the values were presented as means (μ) ± standard errors of the means (S.E.M.) Comparison between two different groups was carried out using the Student t-test, where $P \leq 0.05$ was considered significant. GraphPad Software InStat (version 2) was used to carry out the statistical tests.

RESULTS

Maternal Effects

There was no maternal death in the treatment group during the experiment. But there was a significant increase ($P \leq 0.05$) in the mother weight loss. As well as there was a significant reduction in the placenta weight compared with the control (Table 1). The uterus of control group showed normal distribution of the implanted fetuses between the two horns (Fig. 1) while the uterus of treated females showed normal shape and sometimes with asymmetrical distribution of fetuses in the two uteri, completely resorbed uterus also revealed (Fig. 1).

Embryonic Effects

The morphological examination of the fetuses showed that the PGB caused growth retardation represented by a significant decrease in fetal body weight and body length in comparison with the control group (Table 1).

The fetus from control animals appeared normallyin shape but treated group showed several fetuses malformation which manifested in subcutaneous hematoma in many regions as limb, face and phalanges, deformed limbs and club foot (Fig. 2).

The cleared cartilage and bone preparations of control rat fetuses have designated that in all parts of the axial skeleton skull, vertebrae and ribs as well as appendicular skeleton comprising the fore and hind limbs, pectoral and pelvic girdles, both chondrification and ossification processes have been obviously completed (Fig, 3). On the other hand, fetuses maternally treated PGB showed lack of ossification in the skull roof (frontal and parietal) and ribs abnormalities, which included wavy shape ribs, curved ribs, incomplete ossification and presence of costal separation (wide angle between ribs) (Fig. 4).

Histopathological studies

Liver

The control fetal liver revealed normal hepatic structure, appearance and organization which clear as large, polygonal hepatocytes and different types of blood forming cells, namely the lymphocytes and erythroblasts (Fig. A5). On the opposite side the fetal liver from treated mothers exhibited dilatation of central veins and detached of endothelial cells that lining the central vein wall and lumen of vein continuous with the sinusoid. Increase in number of megakaryocytes and decrease of erythroblasts number. Also some cells showed signs of pyknotic nuclei and vacuolization of cytoplasm that might be attributed to lipolytic degeneration and in some cases the histopathological alterations in fetal liver sections showed pathological responses in the nuclei of liver cells ranging from karyolysis to almost complete necrosis (Fig. B, C5).

Brain

Microscopic examination of brain tissue samples from untreated mother apparent normal histological structure of different parts of cerebral cortex with densely backed neurons without distinct layers (Fig. A6).

The fetal brain from treated mother revealed several histopathological alterations as pyknotic and degenerated neurons, fibrin deposition (fibrosis), cerebral cortex showed disorganization appearance and dilated and enlarged blood vessels (Fig. B-D6).

Biochemical observations

The antioxidant state (GSH &CAT) and lipid peroxidation level were measured in both fetal liver and brain tissues in the study groups. The liver tissue showed a significant ($p \leq 0.05$) reduction in the GSH and CAT levels and a significant increase in the MDA concentration in comparison with their levels in the control tissue. As well as the fetal brain samples revealed non-significant ($p > 0.05$) decrease in GSH activity but a significant decrease in the CAT level and non-significant elevation in the MAD concentration when compared with the untreated group (Table 2).

Table 1: Showing effect of Pregabalin on mother weight loss (MWL), placenta weight (P. WT), fetus weight (F.WT) and fetus length (F.L) at 20th day of gestation. Values are expressed as Mean ± SEM. The statistical differences were analyzed by independent samples T test. *= P ≤ 0.05 compared with control.

Group	Mother Weight Loss (MWL) g	Placenta weight (P.WT) g	Fetus weight (F.WT) g	Group Fetus length (F.L) cm
Control	22.2± 1.34	0.44± 0.007	1.99± 0.033	2.73± 0.031
Treated 61.7 mg/Kg	37.3± 4.69*	0.32± 0.018*	1.20± 0.122 *	2.54± 0.051*

Table 2: Showing effect of pregabalin on fetal liver & brain antioxidant system and MDA 20th day of gestation. GSH= reduced glutathione, CAT= Catalase and MDA= Malondialdehyde. Values are expressed as Mean ± SEM. The statistical differences were analyzed by independent samples T test. *= P ≤ 0.05 compared with control.

Group Parameter	Control	Treated (61.7 mg/Kg)
GSH Liver (mg/g)	32.23± 2.4	20.31± 2.8*
CAT Liver (U/g)	6.47± 0.19	5.46±0.26*
MDA Liver (nmol/g)	4351.07± 338.01	5083.19± 534.85*
GSH Brain (mg/g)	20.09± 4.11	16.18± 2.28
CAT Brain (U/g)	2.74± 0.36	1.58± 0.13*
MDA Brain (nmol/g)	6358.56±552.91	7587.34±769.75

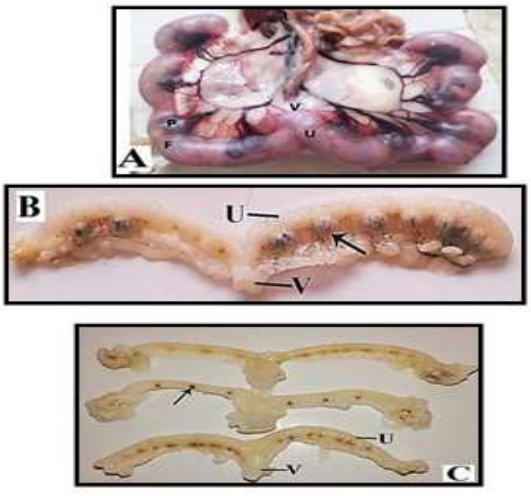

Figure 1: Photographs of uterus of pregnant rat at the 20th day of gestation.

(A) Normal symmetrical distribution of fetuses (F) in the two uteri horns.

From treated group showing:

(B) Uterine horns (U) showing clearly visible late embryonic resorption sites (R). V=Vagina, P=Placenta.

(C) Uterine horns showing pinpoint hemorrhagic implantation sites (early resorption= R).

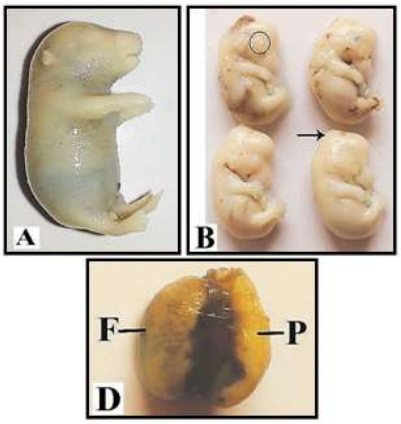

Figure 2: Photographs of the fetuses at 20th day of gestation.

Fetus of control mother showing:

(A) Normal morphology and normal length.

Fetuses of treated group showing:

(B) Fetuses with open eyes (o) and small size.

(C) Resorbed fetus (F) and small placenta on the other side (P).

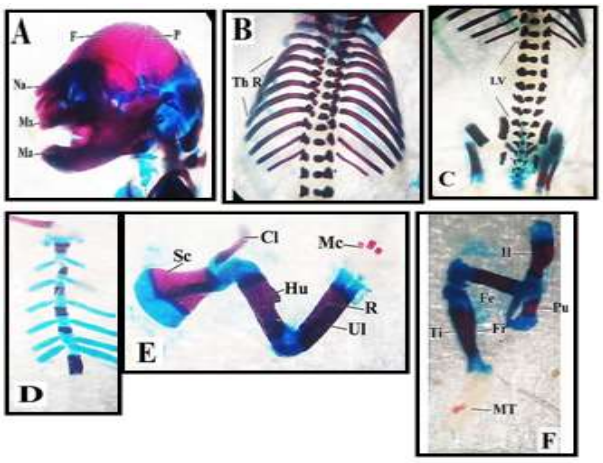

Figure 3: Photographs of the control fetal skeleton.

(A) Skull bones. Fr= frontal, Pr= parietal, N= nasal, Mx= maxilla, Ma= mandible.

(B&C) Ribs and vertebral column. C. V= cervical vertebrae, Th V= thoracic vertebrae, Th R= thoracic rib, L.V= lumbar vertebrae.

(D) Well ossified sternum

(E) Pectoral girdle and Forelimb. Cl= clavical, Sc= scapula, H= humerus, U= ulna, R= radius, MC= metacarpals.

(F) Pelvic girdle and Hind limb. I= ilium, Fe= femur, Ti= tibia, Fi= fibula and MT= metatarsus.

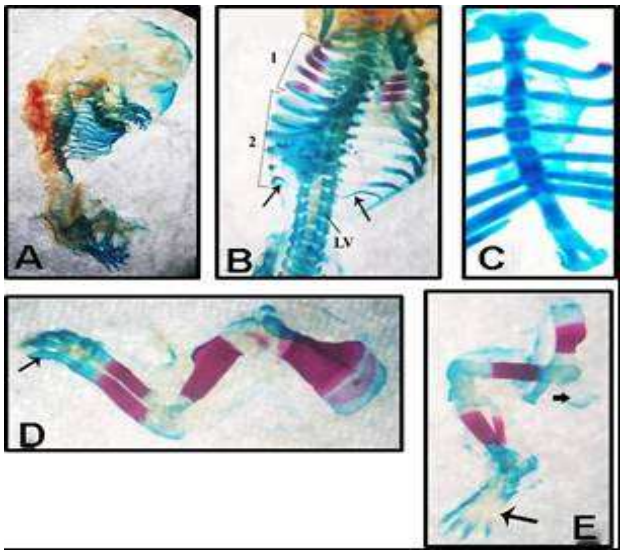

Figure 4: Photographs of the fetal skeleton from treated group.

(A)Completely unossified skeleton.

(B) Less ossified thoracic ribs (1), un-ossified ribs (2), lumbar vertebrae (LV) and curved rib (arrow).

(C) Un-ossified sternal bones.

(D) Un-ossified metacarpals bones of the fore limb.

(E) And (short arrow) metatarsals bones of the hind limb (long arrow).

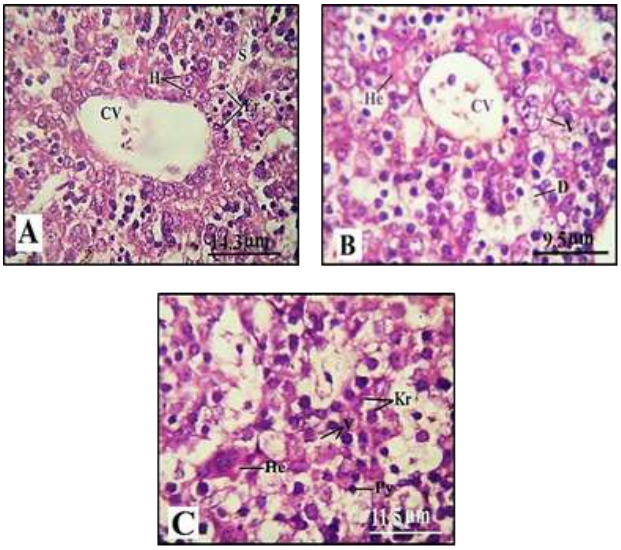

Figure 5: Photomicrographs of a section of the fetal liver. H&E stain.

From control group:

(A) Normal architecture of the liver tissue. The hepatic lobules that can be only distinguished by their central vein (CV), hepatocytes (H), and numerous erythroblasts (Er) and megakaryocyte (Me).

From treated group:

(B) Rupture of the endothelial cell that lining the wall of central vein (CV) (arrow).

C) Degenerated hepatocytes (D), cytoplasmic vacuoles (V), hemorrhage areas within hepatic cells (He), karyolysis (Kr) fragmentation of chromatin materials and pyknotic nuclei (Py).

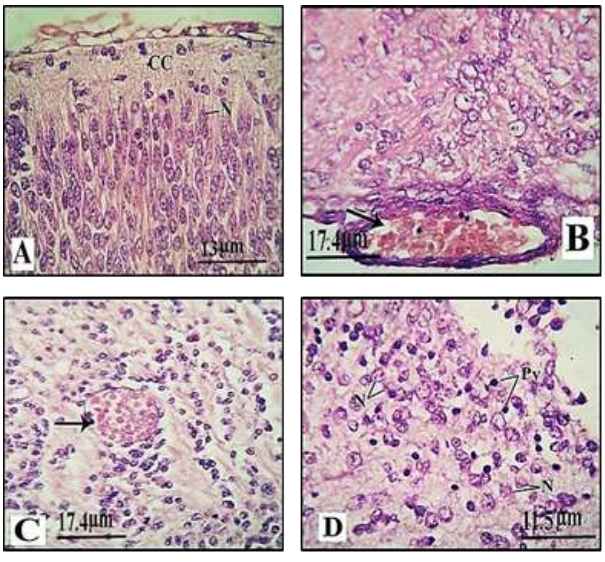

Figure 6: Photomicrographs of a section of the fetal brain from control. H&E stain.

From control group:

(A) Normal structure of brain tissue with normal appearance of neuropil and CC (cerebral cortex), neuron (N) and normal blood vessel (BV).

From treated group :

(B&C) Dilated and congested blood vessel (arrow).

(D) Disorganization of cerebral cortex appearance, vacuolization of neuropil (V), pyknotic neuron (Py) and degenerated and ghost shape nerve cell (N).

DISCUSSION

Several AEDs have the ability to transport through the placenta from the maternal face (syncytiotrophoblast plasma membrane) to the fetal face circulation. [3]In many cases the concentration of AEDs in the fetal blood becomes higher than in the maternal blood, this may be the principle cause for embryo-toxicity and elevate teratogenic potential. [15,16]

The present study was designed to evaluate the teratogenic effect of PGB on the growth and development of the fetuses of albino rats. The pregnant rats treated orally with 61.7 mg/Kg bwt of Pregabalin during gestational period showed no external signs of toxicity. No mortality cases were recorded; all the treated dams were survived to the end of study but showed a significant loss in their weight compared with the control mother. The reduction in maternal body weight may be due to reduced food intake, or as a result of developmental toxicity of the used drug as displayed by reduced weight of gravid uterus due to reduced number of alive fetuses and mean fetal weight and increased incidence of early and later resorption, or may be due to metabolic disorders in maternal body. [17]

The placental weight of all treated pregnant rats group was significantly decreased as compared to control. It can be said that, the previous prevailing phenomena of teratogenicity may attributed to toxicity of pregabalin due to accumulation of this drug in certain organs, placental barriers, endometrial layer of uteri as well as placental dysfunction.

The present experiment showed that treated pregnant rats showed asymmetrical distribution of fetuses in the two uteri horns and reduced number of fetuses, uterine horns showed clearly visible embryonic resorption sites and also showed pinpoint hemorrhagic implantation sites. Increase of resorption sites resulted in a significant reduction of uterine weight compared to the control group at the mentioned dose.

In the present work, PGB induced growth retardation represented by a decrease in fetal body weight and body length. Referring to Gerenutti et al.[18,19] the mechanism of drug toxicity during the pregnancy depends on reproductive performance of mother and drug dose. Corpus luteum has a significant function in the reproductive implementation, as it able to produce critical hormones, progesterone and 20-hydroxy progesterone, which keep fetus growing. In this study may be PGB affects corpus luteum subsequently reproductive performance finally caused intrauterine growth retardation. [20]

Malformation of fetuses considered a major part of the results of our work, PGB caused several fetal malformations that summarized in hematoma (red patches at different parts of the body), club foot and deformation in limbs and many skeletal defects have been observed included incomplete ossification of some skull bones, irregular shape ribs and incomplete ossification of ribs. Delaying of the ossification and severs skeletal anomalies may be due to mesenchymal condensation during embryonic development, or may be due to resorption of cartilage, during embryonic development, which precedes endochondral ossification.

Schaefer and collaborators reported that exposure to PGB during the first trimester in a small number of pregnant women did not induce fundamental teratogenicity. On the other hand, high doses of PGB caused skeletal abnormalities and neural tube defects (NTDs) in some animal experiments. [21]It was reported that PGB can be teratogenic in rat at higher doses of 1250-2500 mg/kg. Though, it was not teratogenic in mice or rabbits. [22]

Prakash and his team reported that the IP injection of 113, 226, or 452 mg/kg doses of gabapentin, an antiepileptic (ADE) similar to PGB, at three different gestational stages induced fetal resorptions, growth retardation and various gross malformations in all treated groups at mid gestation. [23]

The results obtained from the present study showed PGB caused various changes in liver of fetuses such as fatty changes, haemolysis of the blood in sinusoidal spaces and dilatation of central vein and pyknotic nuclei. In addition, a necrotic mass was identified in the liver tissue. This necrotic lesion may either due to progressive degenerative action of cellular enzymes of the injured cells or to a metabolic disturbance and inhibition of protein synthesis in the hepatic cells. Vacuolation of the cytoplasm was observed in the degenerated hepatocytes in the treated group. Previous authors revealed that these vacuoles represent hydropic degeneration in the affected liver and other considered the cytoplasmic vacuolization in the animal cells as a result of the breakdown of lipoprotein complexes in the affected cells. [17]

PGB administration induced several histopathological changes. Disorganization and degeneration of brain tissue, numerous dark dead densely packed and apoptotic neurons and dilation of blood vessels within brain tissue was observed in some regions.

There is evidence that several AEDs may influence brain development by inducing neural apoptosis. One study showed that therapeutic concentrations of several common AEDs (Valproic acid (VPA), phenytoin (PHT),

clonazepam, vigabatrin, and diazepam) can have effects on rat brain development. They found that all drugs caused neuronal apoptosis through the suppression of an endogenous neuroprotective system. [24]

In the present study, the effect of oral administrated 61.7 mg/Kg of Pregabalin during the gestation on the levels of reactive oxygen species markers was estimated. GSH and CAT levels in the fetal and liver tissues of the treated pregnant rats were lower than those of the control rats and the level of MDA enzyme activities was elevated as an indicator of oxidative stress.

During the period of organs formation the developing embryo was so susceptible to hug ROS levels. Despite of the mechanism of teratogenicity that mediated by ROS still ambiguous, the teratogenic and embryotoxic potentials of many drugs is depend upon their bioactivation to electrophilic and/or free radical reactive intermediates that bind to or oxidize cellular macromolecules as DNA, protein, and lipid, resulting in intrauterine fetal death or teratogenesis. [25-27]

CONCLUSION

In conclusion, depending on our results PGB may cause teratogenic effects in albino rats. It able to produce skeletal abnormalities and elevates ROS in the fetal level. So, a great caution should be taken when prescribing PGB during pregnancy.

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