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RESEARCH ARTICLE

TERATOLOGICAL EFFECT OF PREGABALIN DRUG ON THE PRENATAL DEVELOPMENT OF THE CEREBELLUM IN THE ALBINO RATS

Lina A.Salih[1*], Freal A. Al-mahdawi[1] and AnamR.Al-salihi[2]

[1]Department of Biology, College of Science, University of Baghdad, Aljadria, Iraq- Baghdad
[3]Department of Anatomy, College of Medicine, University of Al-Nahrine, Iraq-Baghdad

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ABSTRACT

The cerebellum is the second largest part of the mammalian brain; the cerebellum cortex has a striking morphology consisting of folia and fissures and variety of cells morphologically and functionally, so it considered an ideal system to study the development of the central nervous system in the mammals.

The ontogenesis of the cerebellum in the mammals takes place along long period of the development from the embryogenesis to the postnatal time after delivery to reach to the mature form morphologically and physiologically, therefore these stages are very critical and sensitive to the growth of the cerebellum.

The antiepileptic drugs which are used in the control and treatment many neurological disorders in the infants, children and pregnant women like epilepsy are one of the most drugs that have harmful effects on the growth and development of the nervous system; Pregabalin (PGB) (Lyrica®) is the last generation of the Antiepileptic medications. The study was aimed to deal with the ontogenesis of the white rat cerebellum during normal neurogenesis and determine the histopathological changes in the cortex of the developing cerebellum prenatally after administrated with three routine therapeutic antiepileptic doses of Pregabalin drug from the first day of gestation until the 21 postnatally.

The present study was done on 80 pregnant rats which divided into three prenatal group of 60 pregnant rats 30 in each control group and 10 pregnant rats of each of the three treatment groups which exposed to three antiepileptic doses of Pregabalin drug (150, 300 and 600 mg/kg b.wt / day) were administrated to the pregnant rats during gestation period from the first day to the day of normal delivery.

The results showed that that the lower antiepileptic dose of the PGB (150mg/kg b.wt/day) has not any histopathological effects on the cerebellum along the embryogenesis period. While the effect of the other doses (300 and 600 mg/kg b.wt/day) revealed different degree of degenerative changes and necrosis in both granular and primitive purkinje layer cells and thickness in the external granular layer of the metencephalon anlage and low differentiation in the fibrous layer of the cortex, these degenerative changes were more obvious along the progress of the embryonic stages of the fetuses and specially in the (600mg/kg b.wt/ day) treated group.

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INTRODUCTION

The cerebellum is the second largest part of the brain, anterior part of the hindbrain lies behind the pons and medulla oblongata; the embryogenesis of the mammalian cerebellum is very striking histological event during the development of the central nervous system, therefore the cerebellum is an ideal useful model for studying many aspects of neural development, because each stage of development has a distinct morphology and special histological features with different types of cells , as well as the process of cerebellar ontogeny and development is not complete during gestation only (prenatal development) but continues after birth to maturation of the cerebellum (postnatal development) (Eilers *et al*., 2001)

The mature cerebellum has three distinct layers and contains five major types of neurons, the outermost layer is called the molecular layer, which contains few nerve cells with a finely punctuate appearance in transverse section, the middle layer also called the purkinje cell layer, is composed of a single layer of purkinje cell bodies. The deepest layer is the granular layer, which contains of densely packed granule cells (Ajibada *et al*., 2009)

The neurotoxic effects of AEDs have been recognized since 1970s, the mechanisms are not clearly understood, but it's known that AEDs target ion channels and neurotransmitter system in the brain; these targets are responsible for regulation of processes essential for the development of the brain. There is a continuous requirement to the AEDs, because the patients may have to take these drugs along their life, thus there is a need for newer and better AEDs (Trojnar *et al*., 2002; Ikonomidou, 2010), Pregabalin (PGB) is the latest compound that joins the list of approved new AEDs, European

* Corresponding author: **Lina A. Salih**
Department of Biology, College of Science, University of Baghdad, Aljadria, Iraq- Baghdad

Commission granted Pfizer Company the approval for PGB in July 2004 for the treatment of peripheral neuropathic pain and as an adjunctive therapy for epilepsy Food and drug Agency (FDA) in (2004) Bansal *et al*., (2009) approved the PGB in December 2005 as an add-on therapy for epilepsy, have an adverse effect on the fetus and there are no adequate and well-controlled studies in humans. The study aims to detect the morphometical and histological effect of the three therapeutic dose of the PGB on the early embryogenesis of the cerebellum of the white rats.

MATERIALS AND METHODS

Animals

This study was carried out80 Female wistar rats weighing 200-250 gm were selected, The rats were maintained in a controlled room temperature (25±2oc) on 12 hr light/dark cycle (lights on 7.00 am) with free access to sterile food and water ad libitum.. The rats were placed in poly propylene cages with three animals per cage and were allowed to acclimatize with the laboratory conditions one week prior to treatments.

Mating the animals and timing of pregnancy

The isolated females put in breeding cages each 2 females with one mature male and left overnight. Early in the next morning, copulation was confirmed by examining the females. In this work the gestational day zero was defined as the day when spermatozoa were observed in a smear of the vaginal contents and/or a copulatory plug observed *in situ.* The 80 females were divided into four groups, three treated group each consist of 20 pregnant rats received three doses of PGB and 20 pregnant rats in one control group.

Drug

In this study Pregabalin capsules (Lyrica®) has obtained from the pharmaceutics and used in the three effective doses: 150,300,600mg/kg, according to the doses used in several clinical cases in human, depend on the recommended document of the Pfizer company, and the local pharmacies, these doses were administrated to the pregnant rats from the first day post coitus (dpc); day after the positive vaginal plug, or sperms in the vaginal swabs, until the day of the delivery; 20- 21 day**.** Each concentration was diluted with appropriate volume of D.W to obtain the equivalent concentration in rats with according to the body weight. The drug solution was given with a volume of 1-2 ml. orally using polyethylene orogastric tubes connected to a hypodermic syringe. The dosage was in milligram per kilogram body weight (mg/Kg) given twice a day (twelve hourly), D.W was used as the vehicle. The control group was administrated with the same volume of distilled water.

Retrived of Embryos

In this study starting from 13dpc to 21dpc, two pregnant rats were anesthetized by intramuscular IM injection of a mixture of Ketamine (90 mg/kg body weight) and Xylazine (10 mg/kg body weight). Abdominal midline incision was performed, the two uterine horns were exposed, the embryos and fetuses were extracted from the placental sacs by hysterectomy, and the extra-embryonic membranes were then removed, rinsed in normal saline, then each embryo was examined under the dissecting microscope. The treated animals were sacrificed twelve hours after the last administration of the drug.

The embryos at embryonic day E13, E14 transferred immediately to the Bouin's solution, embryos at E15, E16 and fetuses at E17 the whole heads only were used, the rest fetuses at E 18,19,20,21 the skulls were removed and the cerebellum was isolated from Hemispheres and brain stem carefully by incision along the dorsal aspect, under the dissecting microscope, blotted dry with filter paper and weighed.

Some of the samples were fixed in the Bouin's solution, and the others were fixed in 10% formal saline at 24-48h.then transferred to 70% ethanol until the time of the histological sectioning technique (Butler and Jurrlink, 1987)

Determination of the Chronological Age

No standard development staging system for rodent embryos is founded, investigators were chose a varieties of systems that differ significantly (M Fuhinaga, N A Brown, J M Baden 1992).

So in this study we used the E-designation system (Al-Salihi, 1995). This system is used to standardized embryological material, depending on the previous scientific developmental staging systems.

This system uses the letter E to describe the embryological age of rat, as well as the main morphological characteristics of the external features of the embryos; the E-designation includes several parameters to detect the exact chronological age of the embryos.

Histological study

The preparation of histological sections depends on the standard methods of Allen & Cameron (2004), the sections were stained with H&E stain.

Statistical Analysis

The Statistical Analysis System- SAS (2004) was used to find the effect of differerent factors (concentrations & days) in study parameters during the development, in this study the three treated groups in the embryos were compared to their dependent control group**.**

RESULTS

Histopathological examination of cerebellum sections of normal control group showed normal cellular architecture at all the embryogenesis period of the study. The metencephalon anlage rostrally consists of compact layer of neuroepithelium that borders the lateral recess of the fourth ventricle; this layer is well developing ventrocadually on E13-E15 and considered the germinal zone of the cerebellum ; slight necrosis and degeneration and loss the architecture of the primitive purkinje layer cells, and thickening in the external granular layer of the metencephalon anlagewere observed in the treated pregnant in both 300 and 600mg/kg b.wt/day doses at E13-14 (Fig.1 A,B and C).

The serial sagittal and midsagittal histological sections of the 300&600mg/b.wt groups showed degeneration and necrosis in both granular cells and primitive purkinje cells, with low differentiation in the fibrous layer of the cortex. Also an obvious more thickness in the external granular layer when compared with the control group at E16 (Fig.2 A,B and C) While at E19-E21the serial sagittal and midsagittal sections were revealed normal to less thickening in the external granular

layer, with increased necrosis in its cells; however the cortical layers were not distinguished clearly. The molecular layer was damaged and approximately disappeared, increased atrophy in the purkinje cells and degeneration in the fibers in this layer (Fig3 A, B, C, D and F)

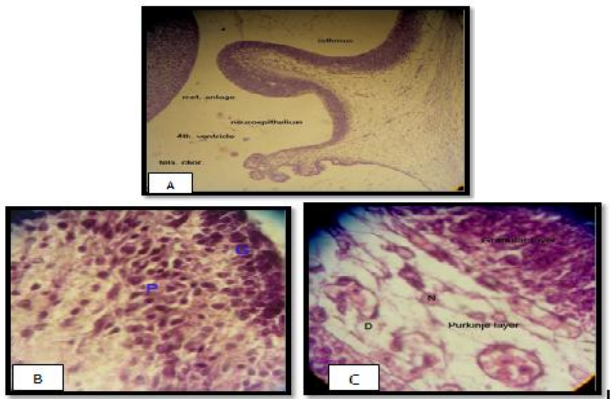

Figure (1) Midsagittal sections in the met encephalon anlage A: midsagittal sections at (40x) (H&E) B: magnified part showing G: Granular layer P: Purkinje layer at (1000x) (H&E) C: sagittal section of the met encephalon anlagen at E13 of the treated group with (300&600mg/kg) of Pregabalin (1000x) (H&E) D: degeneration N: necrosis

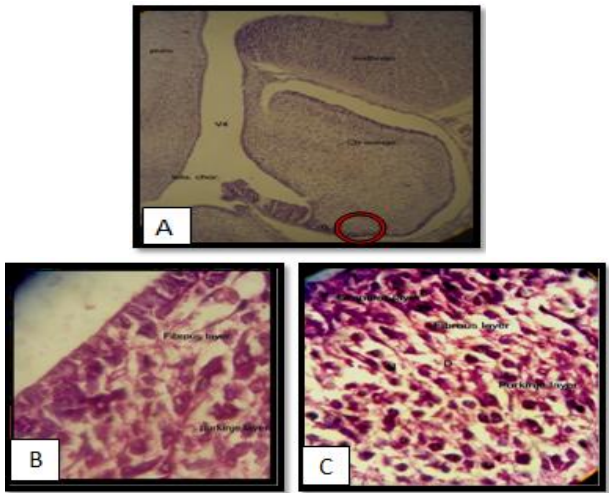

Figure (2) midsagittal sections of the met encephalon anlage at E16of the control group A: at (40x) B: magnified section for the area in the red circle in A at (1000x) (H&E) C: sagittal section of the met encephalon anlage at E13 of the treated group with (300&600mg/kg) of Pregabalin (1000x) (H&E) D: degeneration N: necrosis

DISCUSSION

The embryogenesis of the mammalian cerebellum is very striking histological event during the development of the central nervous system, therefore the cerebellum is an ideal useful model for studying many aspects of neural development, because each stage of development has a distinct morphology and special histological features with different types of cell (Eilers *et al*., 2001)

The cerebellum is very sensitive to the abnormal changes during the embryological development in its histological structure; this may be due to the maternal exposure to chronic or acute diseases or exposure to certain chemicals (drugs or toxicants) during early term of pregnancy (Qin, *et al*., 2006) Antiepileptic drugs were one of the most important drugs that used in many pregnant women all over the world, about 20-30% of pregnant women are with epilepsy having increased risks of seizures, and these drugs used in prevention and control of the epileptic seizures in epileptic women and some neurological pains like diabetic neuropathy, anxiety, and headache, the importance of these drugs in embryological toxicology is due to the need of continuous using during the life of the patients especially if there is no other choices. There are several antiepileptic drugs, but the latest one was added to the list is the Pregabalin (Gajraj, 2007; Kluger and Meador, 2008)

Pregabalin has been shown to rapidly pass the blood-brain barrier and placenta easily in preclinical studies conducted in mice, rats, and monkeys, so this of obvious importance for a drug that influence central nervous system during the development (Menachem, 2004). Therefore the rat is a classic mammalian animal model for a wide range of embryonic studies aimed to address congenital abnormalities; rat physiology is considered to be more complex and informative than that of the mouse because it is more close a mammalian model to human regarding neurobehavioral, toxicology and organ manipulation studies (Larina*et al*., 2009)

The study reveals a demarcated cerebellar anlage at dorsal part of the metencephalon on E13 old rat embryo; 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. The penetration of drugs to their sites of action, lead to metabolic transformation by specialized enzymes, which determine the plasma level of drug, these metabolic processes still undifferentiated functionally during the embryonic development and the level of the drug in the blood was not controlled, therefore theses defects may diffuse the histogenesis stage of the nervous system. This present theory was agreed with Meyer *et al*., 2000, the more striking mechanisms which control the passage of the drug to the brain are the blood-brain barrier (BBB), which prevents many metabolites and chemicals to pass through the brain. In the early development the neuroepithelium of the brain and the endothelial of the blood vessels in the BBB still immature functionally, this due to cross many chemicals and metabolites of the drugs through the brain membranes this proved by (Marchi *et al*., 2009)

Fisher and David,2006 and Wells *et al.*,1997) were pointed that the direct delivery of the AEDs to the central nervous system theoretically provides for the possibility of a therapeutic-toxic ratio greater than that found with systematic drug delivery and the side effects resulting from toxicity within the (CNS) might still remain The exposure to antiepileptic drugs during the critical period in brain development, the toxic effects due to the accumulation of some active intermediates like free radicals (FR) release from the metabolism action.

These active materials contribute to oxidize the biological molecules in the cells as, DNA, RNA, proteins and lipids which have critical role in the early organogenesis. The results of the necrosis and degenerative effect of the PGB in the developing cerebellum were in agreement with this previous study. this theory was improved by experimental data of the study of (Jacobson, 1991; Hatten and Heintz, 1995) on the rodents, they indicated that the proliferation and migration of granular cells and dendritic growth of purkinje cells in the developing mouse cerebellum, would be susceptible to neurotoxic agents, because external granular layer of the cerebellum is the site with the

most intense proliferative and migratory activity in newborn mammals.

The ion channels and neurotransmitters systems in the brain are the most important targets of the antiepileptic drugs because they are responsible for brain development (Ikonomidou, 2010). The results of haematoxylin and eosin stain of the treated groups with Pregabalin (PGB) in this study, showed degenerative changes in the cerebellar cortex of the treated groups, and cell death may result from necrosis, pathological or accidental death and could result from extrinsic insults to the cells such as osmotic or toxic traumatic effect of the PGB as improved by (Faber *et al* .,1981and Bittgau, 2002 ; Jevtovic, 2003; Kim, 2007) they revealed the neurotoxic effect of many antiepileptic drugs in rodents and showed the majority of these drugs cause necrosis neurodegeneration in the developing rat brain.

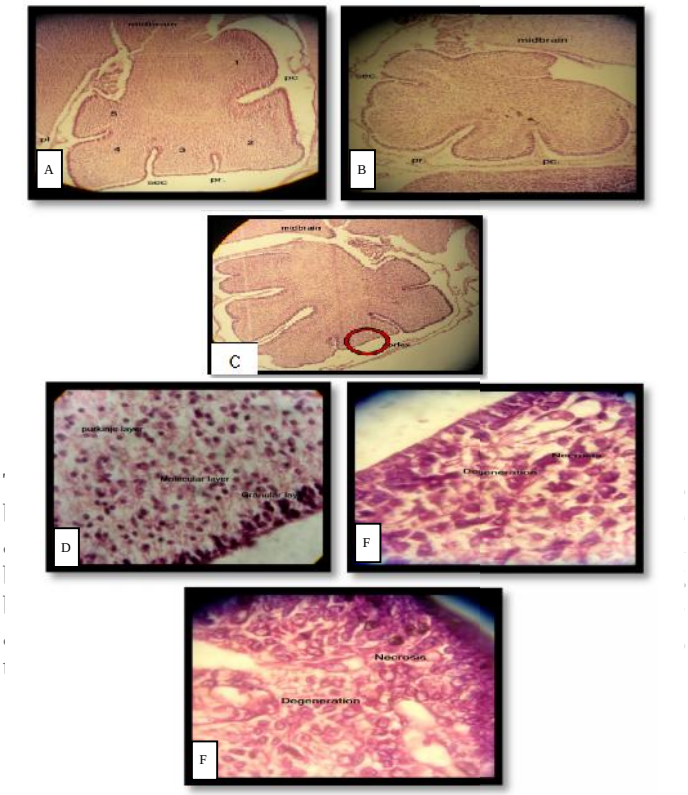

Figure (3) Midsagittal sections in the developing cerebellum in the control groupA: E 19 (40x) (H&E) Pc: preculminate, Pr: primary, Sec: secondary,Pl: posterolateral fissure, cardinal lobes are designated as; 1: anterobasal,2: anterodorsal, 3= central lobes 4: posterior lobes 5: inferior lobes. B: E20 (40x) (H&E) Pc: preculminate, Pr: primary Sec: secondary C: E21 (40x) (H&E) D: magnified part for the area in the red circle at (1000x) (H&E)showing: 1-purkinje layer 2-molecular layer 3-granular layer E: Sagittal sections in the developing cerebellum of the embryos treated with 300 mg/kg of Pregabalin F: with 600 mg/kg of Pregabalin (H&E) (1000X)

Many growth factors control the ontogenesis of the nerve cells, as well as reduce the level of the active phosphorylated forms of the extracellular signal regulated kinase (ERK1/2) and protein kinase B (AKT). These kinases are key players in two major survival-promoting pathways. These pathways are activated by tyrosine kinase receptors upon binding of growth factors (Morrow, 2006)

CONCLUSION

As it seen from the results the use of Pregabalin can be safe and dose not have any harmful effects on the cerebellum when given to the pregnant rats in a dose of 150 mg/kg b.wt / day, however when was given in higher doses 300 and 600 mg/kg/b.wt/day the results were proved loss of cellular components; distortions of cerebellar cortical layers that may be effect on the physiological functions of the cerebellum.

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