ORIGINAL ARTICLE

Effect of Folic Acid in Prenatal Pregabalin-Induced Cerebellar Changes in the Swiss Albino Mice

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ABSTRACT

Background: The first report that antiepileptic drugs (AEDs) may have teratogenic effects dates back over 40 years. Pregabalin (PGB) is approved as new AEDs, in July 2004. Folic acid supplementation during organogenesis is associated with a decrease in the incidence of nervous system malformations. The objective is to detect the morphometrical and histological effect of PGB on the early embryogenesis of the cerebellum and to detect the prenatal effect of folic acid on PGB-induced cerebellar changes in Swiss albino mice.

Materials and Methods: Twenty-four pregnant female mice were divided into four groups, which received folic acid, PGB, and PGB with folic acid at 0.1 mg; 600 mg/kg b.wt/day, respectively, and one control group. Mice were allowed to deliver naturally; on PND 31–34, they were sacrificed. Slides of cerebellar section were prepared and sent for histopathological examination.

Results: Sections of PGB group showed disruption of the architecture in the Purkinje cell layer along with pyknotic nuclei. Mild vacuolization was seen in the granular layer. Compression and hemorrhage were seen in focal area of white matter.Cerebellar sections of folic acid and PGB showed restoration of the cerebellar architecture. A significant difference ($P < 0.05$) is noted when the brain weight of PGB-treated group is compared with PGB and folic acid-treated group. Mean diameter of Purkinje neurons of PGB-treated group when compared with PGB and folic acid-treated groups showed a significant difference ($P < 0.001$).

Conclusions: The study confirmed the protective role of folic acid against cerebellar neurotoxic effects of PGB prenatal exposure.

Key words: Cerebellum, folic acid, pregabalin, Purkinje cells

INTRODUCTION

The cerebellum is the second largest part of the brain and anterior part of the hindbrain lies behind the pons and medulla oblongata. 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. 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).[1]

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, which is composed of a single layer of Purkinje cell bodies, and the deepest layer is the granular layer, which contains densely packed granule cells.[2]

A community-based survey in Morang district, Nepal, showed that the prevalence of epilepsy was 7.3/1,000 populations.[3]

The first report that antiepileptic drugs (AEDs) may have teratogenic effects dates back over 40 years. Since that time, considerable evidence has accumulated demonstrating that AED use is associated with an increased risk of congenital malformations and may have long-term effects on intellectual development during childhood. AEDs have the potential to affect the fetal development from early in the first trimester through birth. Although the risks of teratogenicity of AEDs are most often discussed in reference to patients with epilepsy, there are currently more women taking AEDs for other indications such as psychiatric disturbances, fibromyalgia, and sleep disorders.[4]

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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. Pregabalin (PGB) is the latest compound that joins the list of approved new AEDs, European 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.[5]]

Investigation has shown that folic acid supplementation during the critical period of pregnancy is associated with a decrease in the incidence of skeletal, craniofacial, and nervous system malformations and decreasing folic acid during pregnancy results in a range spectrum of malformations. Neural-tube defects are a worldwide problem, affecting an estimated 300,000 or more fetuses or infants each year. It is reported that ingestion of 400 µg of folic acid alone per day during the periconceptional period prevents neural-tube defects.[6]

MATERIALS AND METHODS

Female Swiss albino mice weighing 20–25 g were obtained from the departmental animal house of BPKIHS. Mice were housed in standard plastic cages under room temperature (22–24°C) with a 12-h light/dark cycle (lights on 6:00 a.m.). They were fed on pellets and were given drinking water *ad libitum* throughout the experiments. Animals were allowed to acclimatize to the laboratory conditions for 7 days before the experimental procedures. Females were kept 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. The presence of the vaginal plug indicated pregnancy and was designated as day "0" of gestation.

The 24 female mice were divided into four groups; three treated group each consisting of six pregnant mice which received folic acid 0.1 mg/kg b.wt/day, PGB600 mg/kg b.wt/day, and both folic acid 0.1 mg/kg b.wt/day and PGB600 mg/ kg b.wt/day, respectively, and one control group that received distilled water orally through polyethylene orogastric tubes connected to a hypodermic syringe throughout the organogenesis period (i.e., 6–15 days of gestation).

The treated and control mice were allowed to deliver naturally and their pups were allowed to reared with their biological mothers till postnatal day 30 (PND-30).

At the age of PND 31–34, they were euthanized and sacrificed by cervical dislocation. The head of mice was cut and brain was taken out and blotted dry and weighed individually. Mid-sagittal sections of the cerebella were obtained and left immersed in the 10% formalin to continue fixation 2 more days. Then, the specimens were processed for the preparation of paraffin blocks. Paraffin sections (7 µm) of cerebellar hemispheres were cut longitudinally, prepared, and stained with hematoxylin and eosin stain. The prepared slides were coded and sent for examination to the histopathologist.

RESULT

Microscopic Observation of the Cerebellum

Histological examination, with hematoxylin and eosin stain, of the specimens of the control group, presented the normal architecture of the cerebellar cortex. Architecture of the molecular layer and the granular layer was maintained. There was no disarray of Purkinje cells as well as necrosis and pyknosis were not seen in Purkinje cell layer. White matter fibers were normal and chattering effect was noted [Figure 1a and b].

Cerebellar sections of treated group with PGB 600 mg/ kg b.wt/day showed a slight decrease in the thickness of molecular as well as in the granular layer. Granular layer cell shows intercellular edema and intracellular vacuolization. There was mild disruption of the architecture in the Purkinje cell layer where the Purkinje cells were not aligned. Pyknotic nuclei were noted within the Purkinje cells as well as granular cell layer. Hemorrhage in the focal area of white matter was observed which had caused focal area of white matter to be compressed [Figure 2a-c].

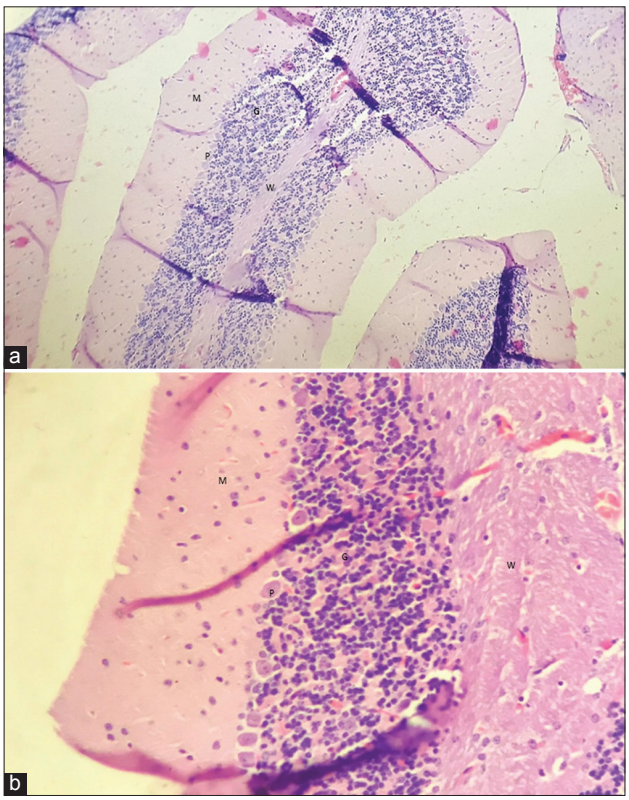

Figure 1: (a) Control Group: ×100, M - molecular layer, P- Purkinje cell layer, G - granular layer, W - white matter fibers, (b) control Group: ×400, M - molecular layer, P - Purkinje cell, G - granular layer, W - white matter fibres

Sagittal and mid-sagittal histological sections of treated group with folic acid 0.1 mg/kg b.wt/day also showed the normal architecture of the cerebellar cortex which was no different than that of the control group.

The cerebellar histological section of a treated group with both folic acid 0.1 mg/kg b.wt/day and PGB600 mg/kg b.wt/day showed restoration of the cerebellar architecture where there was no disarray of Purkinje cells layer; the cells showed normal-shaped cells, vesicular nuclei, and granular cytoplasm. The granular layer contained normal granular cells, and the white matter fibers are normal [Figure 3].

Brain Weight Measurement

The average brain weight of the pubs of the three treated experimental groups, i.e. folic acid, PGB, and PGB + folic acid group were 0.361, 0.316, and 0.349, respectively, whereas the average brain weight of the control group was 0.344 g. One-way ANOVA showed that there was a significant difference ($P < 0.05$) in the brain weight between the groups [Table 1].

Post-hoc test showed that there was no statistically significant difference in the brain weight of control group when compared with the brain weight of other three experimental groups, i.e., folic acid, PGB, and PGB with folic acid group. However, the brain weight difference between folic acid-treated group and PGB-treated group was found to be statistically significant ($P < 0.001$) as well as significant difference ($P < 0.05$) was noted when the brain weight of PGB-treated group is compared with PGB + folic acid-treated group [Table 2].

Measurement of the Diameter of Purkinje Neuron

The mean determine of Purkinje neurons was calculated by measuring the major (a) and minor (b) axis of randomly selected Purkinje neuron, using the formula: $D = \sqrt{a.b}$

Where D: Mean diameter of neuron; a: Long axis of neuron; b: Short axis of neuron.[7]

The mean diameter of Purkinje neurons of the folic acid, PGB, and PGB with folic acid experimental groups was 13.17 µm, 11.91 µm, and 13.55 µm, respectively, whereas the mean diameter of Purkinje neurons was 13.32 µm in the control group. One-way ANOVA showed that there is significant difference ($P < 0.001$) in the diameter of Purkinje neurons between the groups [Table 1].

Post hot analysis demonstrated that there was a significant difference in mean diameter of Purkinje neurons of cerebellum of PGB-treated group when compared with control, folic acid, and PGB with folic acid-treated groups ($P < 0.001$), whereas there is no significant difference ($P > 0.05$). Mean diameter of Purkinje neurons when compared among control, folic acid, and PGB with folic acid-treated groups [Table 3].

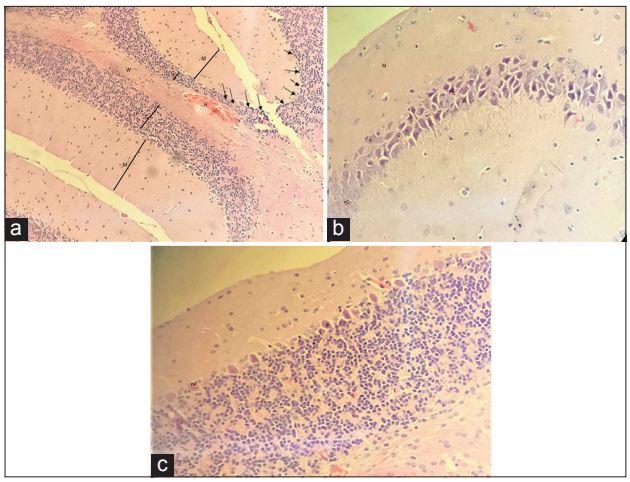

Figure 2: (a) Pregabalin (PGB) treated Group: ×100, decreased thickness of molecular layer and granular layer, H-hemorrhage causing compression of white matter, → disruption of the architecture in the Purkinje cell layer, (b) PGB treated Group: Tecture - molecular layer, P - Purkinje cell, n G - granule cells, pG - pyknotic granule cells and G - granular layer showing intercellular edema with vacuolization, (c) PGB-treated Group: ×400, nP - normal Purkinje cells, P - Pyknotic Purkinje cells

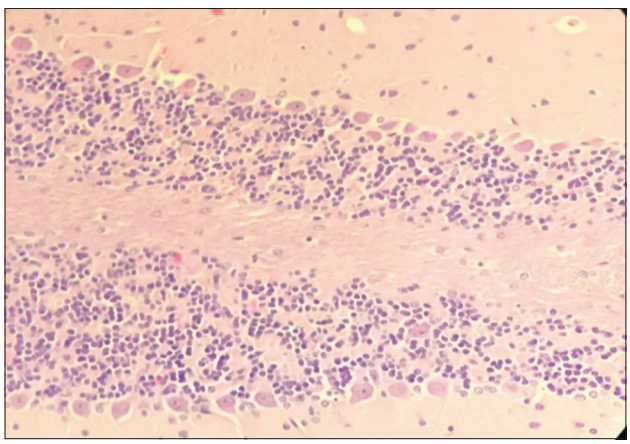

Figure 3: Pregabalin with folic acid treated Group: ×100, showing restoration of the cerebellar architecture

DISCUSSION

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.[8]

AEDs are one of the most important drugs that are used in many pregnant women all over the world. The importance of these drugs in embryological toxicology is due to the need of continuous use during the life of the patients.

PGB has been shown to rapidly pass the blood–brain barrier and placenta easily in preclinical studies conducted

Table 1: Mean±SD of the whole brain weights (g) and diameter of Purkinje neuron of mice

Groups	Brain weight (g)	Diameter of Purkinje neuron (µm)
Control	0.344±0.022	13.32±1.26
Folic acid	0.361±0.018	13.17±1.33
PGB	0.316±0.007	11.91±1.27
PGB+folic acid	0.349±0.019	13.55±1.48
F value	6.930	14.899
P value	0.002	0.001

PGB: Pregabalin, SD: Standard deviation

Table 2: Group wise comparison (*post hoc* tests) of brain weight

Group comparison	*P* value
Control versus folic acid	0.390
Control versus PGB	0.053
Control versus PGB+folic acid	0.968
Folic acid versus PGB	0.001
Folic acid versus PGB+folic acid	0.654
PGB versus PGB+folic acid	0.020

PGB: Pregabalin

Table 3: Group-wise comparison (*post hoc* tests) of diameter of Purkinje neuron

Group comparison	*P* value
Control versus folic acid	0.941
Control versus PGB	0.001
Control versus PGB+folic acid	0.821
Folic acid versus PGB	0.001
Folic acid versus PGB+folic acid	0.477
PGB versus PGB+folic acid	0.001

PGB: Pregabalin

in mice, rats, and monkeys, so this of obvious importance for a drug influences central nervous system during the development.[5]

This study revealed that the mice treated with PGB 600 mg/kg b.wt/day showed the degenerative effect in the developing cerebellum. Similar findings were reported by Sahil *et al.* which revealed different degrees of degenerative changes and necrosis in granular layer, Purkinje layer cells and thickening in the external granular layer of the metencephalon anlage. Faber *et al*., 1981; Bittgau, 2002; Jevtovic, 2003; and Kim, 2007 also revealed the neurotoxic effect of many AEDs in rodents and showed the majority of these drugs cause necrosis, neurodegeneration in the developing rat brain.[5]

In the study done by Xu *et al*., where they investigated the changes of the fetal brain proteome, found that some essential enzymes, which were modified by prenatal alcohol exposure, were protected by FA supplementation. In this study too, the folic acid supplementation has shown to restore the normal architecture cerebral cortex.[9]

In the present study, PGB treatment caused reduction in the weight of the whole brain which is not significant ($P > 0.05$) when compared with the control group. PGB and folic acid caused significant increment ($P < 0.05$) in the weight of the whole brain when compared with PGB treated group.

The insignificant reduction in the brain weight of PGB-treated group compared to control is due to neurotoxic effect of many AEDs in rodents and the significant increment in brain weight of PGB + folic acid group compared to control is due to beneficial role of folic acid in preventing a range of birth defects, especially neurological abnormalities.[9]

Similarly, the study "folic acid deficiency and methyl group metabolism in rat brain: Effect of L-dopa" by Ordonez and Wurtman where the rats were divided into three groups, i.e., control, folic supplemented, and folic deficient, respectively, showed that the brain weight of folic deficient group decreased and folic supplemented group increased compared to control group, but the difference was not significant among the three experimental groups.[10]

There was a significant difference in mean diameter of Purkinje neurons of PGB-treated group when compared with control, folic acid, and PGB with folic acid-treated groups ($P < 0.001$), whereas there is no significant difference in mean diameter of Purkinje neurons when compared among control, folic acid, and PGB with folic acid-treated groups ($P > 0.05$).

A study done by Sobaniec-Lotowsk found that long-term intragastric administration of the AED sodium valproate showed morphological evidence of encephalopathy, manifested by numerous nonspecific changes within Purkinje cell perikarya and their dendritic processes. Purkinje cells appeared as "dark" ischemic neurons, with focally damaged cellular membrane and features of disintegration.[11]

CONCLUSIONS

The present study confirmed the protective role of folic acid against the cerebellar neurotoxic effects of PGB prenatal exposure.

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