



Pregabalin does not Cause Midline Closure Defect But is not as Innocent as It is Thought

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ABSTRACT

AIM: To investigate the effects of pregabalin on neural tube closure, and other potential effects on other organ systems in a chick embryo model.

MATERIAL and METHODS: Fertilized chicken eggs were divided into groups and different doses of pregabalin were administered. All embryos were harvested on the 8th day of incubation and investigated both macroscopically and microscopically against any developmental malformations caused by Pregabalin.

RESULTS: Macroscopically no malformations were detected but macrosomia was statistically significant in medium and high dose groups. Microscopically, vertebral lamina ossification was delayed in some embryos in the high dose group but not interpreted as midline closure defect and also not statistically significant. Decrease in the number of renal glomeruli and increase in tubular damage were statistically significant in medium and high dose groups. Cardiomegaly was also found in some embryos in the middle and high dose groups but not statistically significant.

CONCLUSION: The use of pregabalin does not cause neural tube closure defect in the embryo unless it does not exceed the recommended maximum dose. Causing macrosomia instead of developmental retardation by Pregabalin is in conflict with the literature. This study revealed that Pregabalin causes fetal nephrotoxicity and macrosomia. These findings indicate that the use of Pregabalin in pregnancy still needs to be accounted as suspicious.

KEYWORDS: Neural tube, Pregabalin, Pregnancy, Avian embryo, Nephrotoxicity

INTRODUCTION

Drug use during pregnancy is a difficult issue because of the potential risks for fetuses. A particularly difficult situation is when women with chronic diseases must continue using a medication during pregnancy (3). Epilepsy is a chronic disease and approximately half of epileptic patients are women (11,16). Therefore, the number of children exposed to antiepileptic drugs (AEDs) during the intrauterine period is considerable (12). It is well known that the use of antiepileptic drugs during pregnancy adversely affects fetal development.

Although children of epileptic women are usually born without defects, they still have an increased risk of fetal malformations (2,18). There are many studies that have shown the main cause of fetal malformations in children of epileptic pregnant women is not epilepsy itself or convulsions, but the ingestion of antiepileptic drugs by the mother (2,13,17).

It is already known that there is a correlation between congenital malformations and the use of traditional antiepileptic drugs, such as phenobarbital, phenytoin, carbamazepine, and valproate, during pregnancy (13,17). A new generation of

antiepileptic drugs has been developed and claims have been made that they offer fewer risks; however, other studies have shown that they may not be as safe as previously considered.

Pregabalin can be classified as both an analgesic and anticonvulsant drug and it is widely prescribed in neurosurgery, neurology, and psychiatry. It is generally used as combination therapy for partial-onset seizures, neuropathic pain, and, in some countries, generalized anxiety disorder (23). Pregabalin is a derivative of gamma-aminobutyric acid (GABA) and it binds to the alpha2-delta ($\alpha 2-\delta$) site of voltage-gated calcium channels in the central nervous system, rather than directly binding to GABA receptors (20). It is believed that this binding results in pregabalin's nociceptive, anticonvulsant, and anxiolytic effects (21). Voltage-dependent calcium channels consist of transmembrane proteins called alpha, beta, gamma and delta proteins, with each divided into subunits. In this structure, the alpha2 ($\alpha 2$) subunit is located outside the membrane in a sensor position (22).

The delta (δ) subunit is located in the cell membrane and is connected to the $\alpha 2$ subunit by disulfide bonds. The function of alpha1 and gamma in the cell membrane and beta subunits in the cytoplasmic part of the membrane are regulated by the $\alpha 2-\delta$ complex ($\alpha 2-\delta$) (8). Gabapentin and pregabalin are thought to act mainly by binding to the $\alpha 2-\delta$ receptor. In particular, binding to $\alpha 2-\delta 1$ and $\alpha 2-\delta 2$ complexes is responsible for their effects. Studies with mice that cannot express these proteins showed gabapentin's analgesic and anticonvulsant effects were blocked (10).

However, similar studies have not been performed for pregabalin. Gabapentin binds to $\alpha 2-\delta 1$ and $\alpha 2-\delta 2$ receptors with high affinity but its affinity is too low to $\alpha 2-\delta 3$ and $\alpha 2-\delta 4$ receptors (6). Pregabalin, however, is not subtype selective. Its interaction with alpha1 ($\alpha 1$) in the brain is still unknown (7).

When pregabalin and gabapentin bind to the subunits, the function of voltage gated calcium channels are altered, especially at the synaptic junctions. This calcium channel modulation may reduce the release of many neurotransmitters but there is not a complete blockade of calcium channels (20).

According to some animal studies, pregabalin is not teratogenic when administered during organogenesis at doses up to 2,500 mg/day (14,15). An increased incidence of fusion of the jugal bone and maxilla occurred in fetuses from females treated with 1,250 mg/day doses, and an increased incidence of fusion of the nasal sutures occurred at 2,500 mg/day. It is claimed that a 2,500 mg/day dose is 5 fold higher than the maximum recommended dose (MRD) for humans. Thus, pregabalin was not teratogenic in rats at doses less than 2,500 mg/day (14,15). Some other animal studies have suggested reproductive toxicity for this agent, with skeletal malformations, neural tube defects, increased rates of spontaneous abortions, growth retardation, and behavioral anomalies reported (5,19,23). There are no studies that evaluated pregabalin use during pregnancy. Pregabalin is classified as a pregnancy-risk category C, which is defined as either animal studies showed adverse effects to the fetus with no controlled studies in pregnant animals, or studies in human

and animals are not available. Thus, pregabalin should only be used in pregnancy if the potential benefits outweigh the risks to the fetus (23).

Our study aimed to develop an avian embryo model for evaluating the teratogenic effects of pregabalin using lower doses to help resolve the conflicts and uncertainties in the literature.

■ MATERIAL and METHODS

Experimental Setup and Chemicals

Fertile and pathogen free Hubbard Broil eggs that were previously stored at 18°C to prevent embryonal development were obtained (N=50; Civkur, Adiyaman, Turkey). Eggs were stored at 18°C for the same reason until the day of the study. Thus, all embryos used were in the same embryonal stage (called day 0). Pregabalin (Abdi İbrahim İlaç, İstanbul, Turkey) was provided commercially. An orally disintegrating 75-mg capsule of pregabalin was dissolved in 2 ml of 0.1 M dimethyl sulfoxide (DMSO), sonicated, and vortexed prior to being diluted to appropriate concentrations. Eggs were weighed (mean weight 50 g \pm 2 g) and divided into five equal groups (n=10). Groups I to V were designated as control, vehicle/solvent, 2 mg/kg pregabalin, 4 mg/kg pregabalin and 8 mg/kg pregabalin, respectively. According to the literature, the maximum recommended dose is 600 mg/day for humans (14, 15). Therefore, dose calculation was done to mimic the doses of 150 mg/day/75 kg per person [2 mg/kg (0.1 mg/50 g egg)], 300 mg/day/75 kg per person [4 mg/kg (0.2 mg/50 g egg)] and 600 mg/day/75 kg per person [8 mg/kg (0.4 mg/50 g egg)]. The doses of the chemicals are given in Table I.

Pregabalin Administration and Incubation

The temperature, humidity, and repositioning time interval values of the incubator were set one day before the experiment to 37°C (\pm 0.2°C), 50% (\pm 5) humidity, and moved 45 degrees in the opposite direction every six hours. On the day of the experiment, eggs were cleaned with ethanol. A 0.5 mm hole was drilled on the top of the air sac with a needle. Predetermined volumes and doses of chemicals (drug and vehicle) were administered to the air sac of each egg with a 26 G needle (1). Eggs were transferred to the incubator after sealing with sterile tape and checked for temperature and humidity values two times per day. On the 8th day, all embryos were harvested, and the chorioallantoic membranes, blood vessels, yolks, and egg whites of the fertilized eggs were carefully removed. The embryos were successively rinsed several times with saline. The embryos were then dissected and investigated under a dissecting microscope. Then, embryos were photographed and transferred to a formaldehyde solution. One day after formaldehyde fixation, embryos were reexamined regarding weight, craniocaudal length, head diameter, abdominal diameter, and any obvious developmental malformations such as spina bifida, encephalocele, anencephaly, extremity abnormalities, and gastroschisis. All results are summarized in Table II.

Table I: Experimental Setup, Chemicals and Dose Table according to the Groups are Listed Above. The Calculated Volume Vehicle (DMSO) was 0.02 ml for Each Experiment Groups, Thus at Vehicle Group (Group II) 0.02 ml of DMSO was Taken for Each Egg and Diluted to 0,3 ml to Achieve the Same Injection Volume of Experiment Groups

Group / n	Explantation	Chemical	Dose	Volume	Extrapolation to human
Group I / n= 10	Control	None	None	None	0 mg/day
Group II / n= 10	Vehicle	DMSO	0.02 ml/ egg	0.3 ml	0 mg/day
Group III / n= 10	Experiment	Pregabalin	2 mg/kg (0.1 mg/egg)	0.3 ml	150 mg/day
Group IV / n= 10	Experiment	Pregabalin	4 mg/kg (0.2 mg/egg)	0.3 ml	300 mg/day
Group V / n= 10	Experiment	Pregabalin	8 mg/kg (0.4 mg/egg)	0.3 ml	600 mg/day

Table II: Number of Fertilized Eggs, Early Embryo Deaths, Developmental Retardation and Measurement Results of all Embryos according to Groups

Group	Explanation	Fertilized egg	Early embryo death	Severe Retardation	Number of measured embryos	Mean Cranio caudal lenght (cm)	Mean Head diamater (cm)	Mean Abdomen diamater (cm)	Mean Embryo Weight (gr)
Group I	Control	9	0	0	9	2.888	3.377	2.466	0.995
Group II	Vehicle	9	1	0	8	2.175	2.875	1.937	0.630
Group III	2 mg/kg	10	0	0	10	2.490	3.320	2.240	0.771
Group IV	4 mg/kg	9	1	0	8	3.037	3.725	2.575	1.169
Group V	8 mg/kg	10	0	1	9	3.122	3.788	2.655	1.259

Histopathological Examination

All embryos were macroscopically sampled in 2–3 mm thick transverse sections from the pelvic to cervical regions. In addition, the head was sampled with transverse sections passing through the middle of the eyeball to examine the upper half of the brain. The specimens were fixed in 10% buffered formalin for 48 h, dehydrated, cleared in xylene, and embedded in paraffin. Tissue sections (4 µm in thickness) were stained with hematoxylin and eosin (H&E) for general morphological analysis. All the sections were photographed with an Olympus BX51 microscope (Olympus Corp., Tokyo, Japan). One pathologist examined all tissue sections in a blinded fashion.

More damage was seen in renal tissue, therefore renal tubules and glomeruli were investigated in a different manner. Glomeruli were counted in two microscopic areas under 20x magnification. To evaluate tubular damage, dilatation of tubules (0: non, 1: mild dilatation, 2: severe dilatation), loss of the tubule epithelium and degeneration (0: non, 1: mild, 2: severe) were evaluated and scores were calculated. Thus, tubular damage scores were determined. The severity of tubular damage was grouped according to the scores (0: no damage; 1 and 2: mild damage; 3 and 4: severe damage).

Statistical Analysis

Continuous variables were expressed as the mean±standard deviation. The distributions of the groups were analyzed with Shapiro–Wilk tests. If a group showed a normal distribution, parametric statistical methods were used to analyze the data. A one-way ANOVA test was performed and post hoc multiple comparisons with Bonferroni's test were made using least-squares differences. A Kruskal–Wallis test and Mann–Whitney U test were used for the groups with an abnormal distribution. A p value of <0.05 was considered to indicate statistical significance. All the statistical analyses were performed using the Statistical Package for the Social Sciences, Version 21.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

In total, three eggs were not fertilized (one egg in Group I, one egg in Group II, and one egg in Group IV). In Group I, mild developmental retardation was seen in one embryo representing the 5th day of incubation [according to Hamburger and Hamilton (H&H) staging system: stage 26] (4,9). All other embryos were at H&H stage 33–34 and were coherent with the incubation day (4,9). In all embryos that were investigated in Group I, and the mean craniocaudal length, mean weight of the embryo, mean head diameter, and mean abdomen

diameter values were 2.888 cm (min. 2.0 cm, max. 3.2 cm), 0.995 g (min. 0.65 g, max. 1.34 g), 3.377 cm (min. 2.3 cm, max. 4.2 cm, and 2.466 cm (min. 2.3 cm, max. 2.9 cm), respectively. For the retarded embryo, the values were 2.0 cm, 0.65 gr, 22 mm, and 2.3 cm, respectively.

In Group II, one early embryo death was detected (H&H stage could not be verified).

Therefore, measurements could not be performed. All other embryos were at H&H stage 33–34 and were coherent with the incubation day (9). In all embryos investigated in this group, the mean craniocaudal length, weight of the embryo, head diameter, and abdomen diameter values were 2.175 cm (min 1.8 cm, max. 3.3 cm), 0.630 g (min 0.65 g., max. 0.72 g.) 2.875 cm (min 2.4 cm, max. 2.9 cm), and 1.937 cm (min. 1.8 cm, max. 2.2 cm), respectively.

In Group III, all embryos were at H&H stage 33–34 and were coherent with the incubation day (9). In all embryos investigated in this group, the mean craniocaudal length, weight of the embryo, head diameter, and abdomen diameter values were 2.490 cm (min 2.3 cm, max. 2.8 cm), 0.771 g (min. 0.65 g., max. 0.85 g.), 3.320 cm (min 3.0 cm, max. 3.6 cm), and 2.240 cm (min. 2.1 cm, max. 2.4 cm), respectively.

In group IV, early embryo death was seen in one embryo (H&H stage could not be verified). Therefore, measurements could not be performed. All other embryos were at H&H stage 35–36 and were not coherent with the incubation day (9). In all embryos investigated, the mean craniocaudal length, weight of the embryo, head diameter, and abdomen diameter values were 3.037 cm (min 2.9 cm, max. 3.2 cm), 1.169 g. (min 0.98 g., max. 1.93 g.), 3.725 cm (min. 3.2 cm, max. 3.9 cm), and 2.575 cm (min. 2.4 cm, max. 2.7 cm), respectively.

In group V, severe developmental retardation was seen in one embryo (H&H stage could not be verified) but no early embryo death was seen. Therefore, measurements could not be performed. All other embryos were at H&H stage 35–36 and were not coherent with the incubation day (9). In all investigated embryos, the mean craniocaudal length, weight of the embryo, head diameter, and abdomen diameter values were 3.122 (min 2.9 cm, max 3.3 cm), 1.259 g (min 1.20 g max 1.45 g), 3.788 (min 3.6 cm, max 4.0 cm), and 2.655 cm (min 2.4 cm mm, max 2.9 cm), respectively. Summary of all investigated parameters and their results are given in Table II and Figure 1.

Additionally, all embryos were inspected for any obvious developmental malformations such as spina bifida, encephalocele, anencephaly, extremity abnormalities, and gastroschisis under a 4x surgical microscope. None of these malformations were detected.

Embryonic development was evaluated macroscopically according to the H&H classification and it was found that control, vehicle, and low dose pregabalin groups were at stage 33–34 (representing incubation day 8). Mid-dose and high dose pregabalin groups were at stage 35–36 (representing incubation day 9 to 10). During histopathological examination, the Atlas of Chick Development guidelines were followed (4).

In contrast with the macroscopic findings, all samples were at stage 34 microscopically, which was coherent with incubation day 8. There were no differences among the groups regarding the results for brain, lung and liver. All samples were in the normal range of development. Cardiomegaly was found in three embryos (one in group IV and two in Group V) but there was no statistically significant difference among the groups. In the spinal cord and vertebral sections, there were no differences among the groups regarding spinal cord development; however, one embryo in group IV and two embryos in group V had uncompleted neural arch (laminae) development but found not to be statistically significant (Figure 2A-D). In urogenital system investigation, renal development was at the mesonephron stage and both kidneys were separated. In kidney sections, the number of glomeruli were evaluated and tubular damage (cystic dilatation in tubules and degeneration and spillage of tubule epithelium) was assessed (Figure 2A-D). The Kruskal–Wallis test was applied as a non-parametric test because the numbers of glomeruli were not normally distributed.

The difference between the groups in terms of the number of glomeruli was statistically significantly different. When the groups were compared with each other using Bonferroni's corrected Mann–Whitney U test ($p < 0.01$), there was no statistically significant difference between group I and group II as well as group III and group IV. However, the difference between Group I and Group V was statistically significant. When Group II (vehicle group) and experimental groups (Group III, Group IV and Group V) were compared, the difference was statistically significant only for Group V.

There was a statistically significant difference between the groups in terms of tubule damage ($p = 0.02$; for tubule damage, see Table III). When subgroup statistical analysis was performed, there was no significant difference between the control group (Group I) and vehicle group (Group II) and low dose group (Group III; $p = 0.006$ and $p = 0.007$, respectively). However, the control group (Group I) was statistically different from the middle and high dose groups (Group IV and Group V; $p = 0.001$).

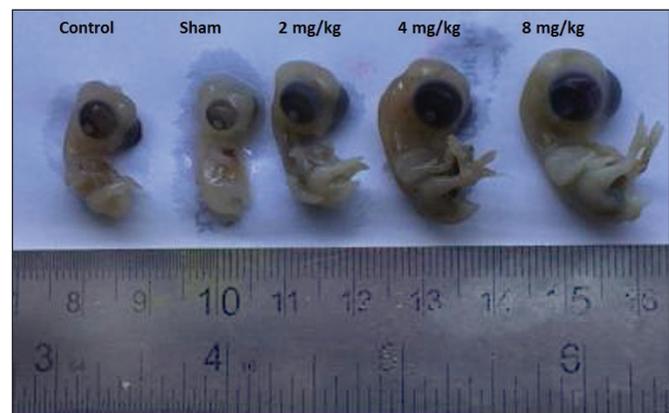


Figure 1: Difference between embryonic development according to the groups. Macrosomia is clearly seen in both medium and high dose pregabalin groups.

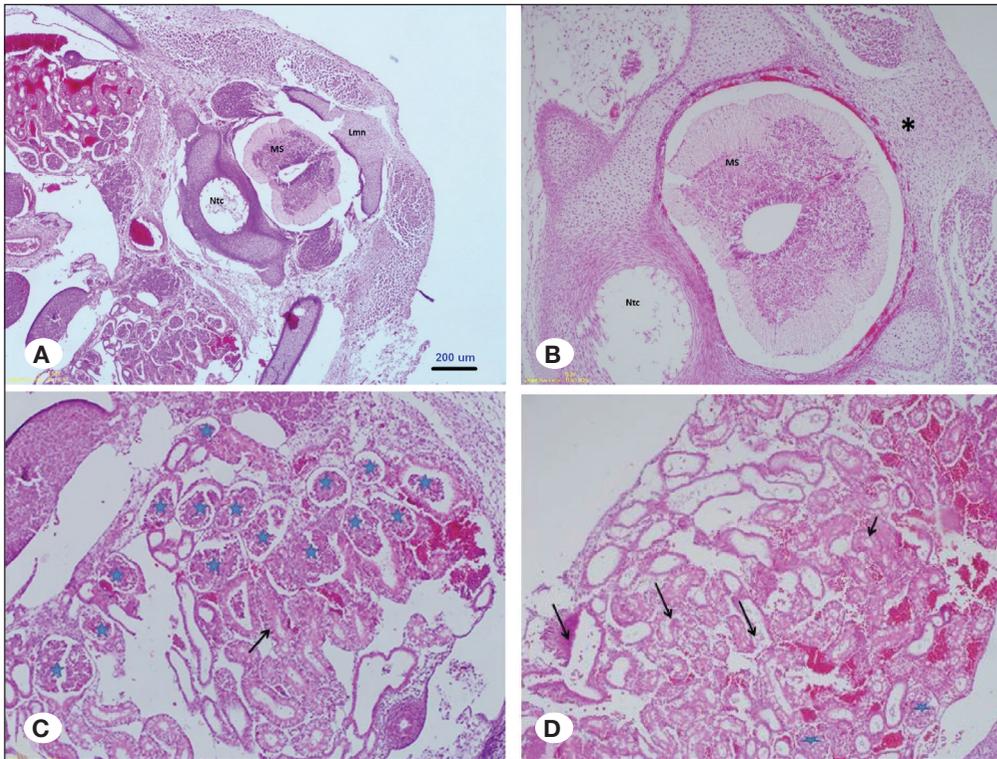


Figure 2: Developmental effects of pregabalin. Development of normal medulla spinalis (A) and normal renal tissue (C) in an embryo of the control group. Lamina development defect which is detected in an embryo of the pregabalin group (B). In this example, it can be clearly seen that the cartilage tissue, which must be organized to form the lamina, turns into the midline soft tissue and does not contain cartilage elements. Tubular damage and decrease number of glomeruli can be seen in high dose pregabalin group (D). (MS: Medulla spinalis, Ntc: Notocord, Lmn: normal lamina, (*): defective lamina, (stars): normal glomeruli, arrows: dilated tubules). (H&E, x40).

Table III: Tubular Damage is Summarised in the Table Above. Control, Vehicle, Low Dose, Medium Dose and High Dose are Representing Group I, Group II, Group III, Group IV and Group V Respectively

	Tubular damage			Total
	No damage	Slight damage	mild/severe damage	
Control	7	2	0	9
Vehicle	1	5	2	8
Low dose	4	5	1	10
Medium dose	1	4	3	8
High dose	0	4	5	9
Total	13	20	11	44

DISCUSSION

There is a limited number of experimental studies on the intrauterine effects of pregabalin and all of these studies were performed on rodents except for one study (19). Ours is the second study that was performed with an avian embryo model to assess the embryonal toxicity of pregabalin.

Both clinical trials and experimental studies on rodents claimed that pregabalin affects organogenesis and results in developmental retardation (5,23). Generalized ossification defects, including calvarial bones, have also been reported. Most abnormalities have been observed in the development of the maxillofacial skeleton (5,14,15).

Some studies have claimed that these abnormalities are seen in rodents only if the MRD (600 mg/kg/day) is exceeded (14,15).

However, there have been some reports offering conflicting results when lower doses were used (20–80 mg/day) (5). Morse et al. found that 1,250 mg/day of pregabalin resulted in embryonal defects, such as developmental retardation, reduced ossification and reduced fetal weight due to maternal toxicity (15). Additionally, some recent clinical trials called for concern about the reproductive and developmental toxicity of maternal pregabalin usage (23).

In the literature, there is only one study that focused on pregabalin use during pregnancy possibly causing a midline closure defect in embryos (19).

In this study, it was claimed that pregabalin causes alterations in neural arch development by increasing autophagy at a supra-therapeutic dose (1,200 mg/day) even though macro-

scopically or microscopically midline closure defects or major congenital malformations were not detected.

In the present study, pregabalin did not cause this type of problem. The difference between the two studies may be due to the experimental setups. In a previous study, the recommended daily maximum dose (600 mg/day) and supra-therapeutic dose (1,200 mg / day) were used, but therapeutic doses were not used.

In the aforementioned study, the pregabalin injection method was called the “windowing technique”. In this technique, injection is done subblastodermally and some albumen is discharged before the injection. Both techniques will increase embryonal stress (it is not clear whether the same procedure was applied to the control group in the study). On the other hand, it is known that autophagy becomes more pronounced in the case of stress.

The probability that the results obtained in the study are related to the experimentally created embryonal stress cannot be ruled out. However, it is important that the findings obtained were more obvious at a dose of 1,200 mg/day pregabalin, which still makes this drug questionable.

Autophagy and apoptosis are already active mechanisms in the normal embryonal development process. Cells that undergo autophagy or apoptosis are destroyed by macrophages and become undetectable after a while. If the embryos were examined on the 8th day (the end of the 1st trimester) or later, perhaps cells that underwent autophagy would not be detected.

In the present study, autophagy or apoptosis was not investigated, but no findings (e.g., neural tube development defect) that could be attributed to increased autophagy or apoptosis were encountered. In the aforementioned study, pregabalin may have accelerated the already existing autophagy and apoptosis at a nonpathological level, because in the present study, it was observed that the embryos given pregabalin were macrosomic; that is, cell division and embryo growth were increased. This may have happened in the aforementioned study as well, and due to pregabalin, the embryo may have skipped to a stage where autophagy will already increase, and this may have been well tolerated by the embryo. It is not possible to achieve a definitive judgment because there is not enough information on autophagy in chicken embryos. In brief, although pregabalin alters early neural tube development, this alteration seems to be tolerated by the embryo in the ongoing developmental process.

One of the reasons for the different findings between the two studies may be variations in the doses. The maximum dose (600 mg/day) in the present study was the same as the minimum dose in the study previously mentioned, and in both studies, no significant neural tube closure defect was detected at this dose. Even if pregabalin has the potential to cause neural tube closure defects, it seems not do this at the recommended daily maximum dose or below.

Although there are some studies that claimed pregabalin causes fetal developmental retardation, the results of the present study were totally opposite even at lower doses.

In the present study, the daily recommended maximum dose of pregabalin (600 mg/day) resulted in a statistically significant increase in fetal weight and craniocaudal length and a mild increase in the head and abdomen diameter. This phenomenon needs additional studies. This conflict with other studies may be due to differences among the animal models. According to the literature, the effects of pregabalin may vary between mice, rabbits, and monkeys (2,17,23). The design of the present study was much more distinct because avian embryos were used instead of mammals; thus our results may be due to the selected species.

Our findings suggest that pregabalin causes macrosomia in the fetus in a dose-dependent manner. Table II indicates there is an increase in the physical measurements of the embryos in parallel with dose increases. However, histopathological examination has revealed that this increase was not healthy, because physical measurements were compatible with 9–10 day embryos, but organ development was found to be compatible with an eight day incubation period.

As mentioned before, pregabalin shows its effects by binding to $\alpha 2\text{-}\delta 1$ and $\alpha 2\text{-}\delta 2$ subunits of voltage-dependent calcium channels (8,22). Binding to other subunits does not cause similar effects. This issue has been recognized especially for the nervous system (10). It is not yet clear how other tissues and organs rich in these subunits respond to pregabalin binding.

Considering that these subunits also exist in skeletal muscles, bones, and heart muscle, the detected macrosomia (and cardiomegaly, even if not found to be statistically significant) become more meaningful. Physiology- and pharmacology-based studies are needed to eliminate doubts about the subject.

It is known that the excretion of pregabalin occurs via the kidney. On the other hand, kidneys are rich in $\alpha 2\text{-}\delta$ subunits (8). It is not clear whether detected glomerular and tubular defects are due to this renal excretion or the presence of pregabalin binding subunits or both factors together. This suggests that the binding of tissues other than the nervous system of pregabalin to $\alpha 2\text{-}\delta$ subunits may not be as innocent as previously considered.

■ CONCLUSION

This study showed that the use of pregabalin does not cause neural tube closure defects, provided it does not exceed 600 mg/day during pregnancy. Higher doses are still questionable. However, the use of pregabalin during pregnancy may not be entirely safe. Fetal health risks are potential macrosomia and renal tubule damage.

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