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Does Gabapentin Affect Neural Tube Development? Experimental Study Using an Early Stage Chick Embryo Model

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ABSTRACT

AIM: To determine whether using gabapentin (GBP), especially in the first maternal trimester, would affect the neural tube development of embryos in an early stage chick embryo (ESCE) model.

MATERIAL and METHODS: One hundred fertile specific pathogen-free (SPF) chick eggs were used to investigate neurulation; they were divided into four groups of 25 eggs (Groups A, B, C, and D including control, subtherapeutic, therapeutic, and supratherapeutic dose subjects, respectively). After 30 hours of incubation, all eggs reached the ninth stage of embryonic development, as defined by Hamburger and Hamilton. GBP was administered through the subblastoderm, and the eggs were incubated for 72 hours. The embryos were macroscopically and histopathologically investigated with hematoxylin eosin following incubation and extraction.

RESULTS: In the 72nd hour of the study, a total of 6 eggs showed no embryo development. We detected 1 (4.34%), 13 (59.09%), 15 (65.21%), and 18 (81.81%) neural tube defective embryos in groups A, B, C, and D, respectively. Statistically, the differences between the groups were significant, especially in the comparisons of all GBP groups to the control group ($p<0.001$). However, there was no significant difference between groups B, C, and D. Additionally, we suggest that at all doses, GBP could cause neural tube defects in the ESCE.

CONCLUSION: Based on these results, we concluded that GBP use at any dose led to midline closure defects in ESCEs. This is the first report in the literature on GBP using an ESCE model. However, further investigations with a larger sample size are required to assess its effect at lower doses and to determine the mechanisms of embryonic damage.

KEYWORDS: Neural tube defect, Early chick embryo, Neurulation, Gabapentin

ABBREVIATIONS: GBP: Gabapentin, **ESCE:** Early stage chick embryo, **SPF:** Specific pathogen-free

INTRODUCTION

The four major steps of human embryological development are proliferation, differentiation, adhesion, and migration (22). Any disruption to the neural tube closure process during the gestational period can cause neural tube defects (NTDs). Abnormal neurulation can cause serious medical problems in the prenatal and postnatal periods and can even lead to death. This congenital malformation has both financial and social effects (31). Since neural tube closure is a vulnerable process, external factors play an important role in the aetiology of NTD. In the first trimester, maternal drug use can cause a wide variety of embryonic malformations. However, the majority of congenital abnormalities are caused by chromosomal abnormalities or translations, as well as environmental exposure and nutritional deficiencies (30).

Furthermore, several chemicals that induce NTDs have been previously studied using different methods (28,30). Gabapentin (GBP), a gamma-aminobutyric acid (GABA) analogue agent, is listed in the safety chart as "*category C*". It is used for the treatment of postherpetic neuralgia, refractory partial-onset seizures, diabetic neuropathy, and other neuropathic pain conditions. Considering the increasing

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number of pregnant women who may be taking GBP during pregnancy and the paucity of information regarding the safety of this medication in pregnancy, more information regarding foetal safety is required. The objective of our study was to determine whether GBP exposure during pregnancy causes NTDs. To our knowledge, there is no study in the literature on the effects of GBP on neural tube development in an early stage chick embryo (ESCE) model.

MATERIAL and METHODS

This study was conducted at the Istanbul Medipol University Medical School, with the cooperation of the Neurosurgery, Pathology, and Research Laboratory, and all experiments were conducted in accordance with the animal research protocol of the Medipol University Ethics Committee (No. 38828770-772.02-E.61220). Fertilised specific pathogen-free (SPF) Leghorn chicken eggs were supplied by the Ministry of Agriculture and Forestry İzmir / Bornova Veterinary Control Institute. GBP (*Nepitin*®) was supplied by Ali Raif, Inc. Istanbul, Turkey.

The GBP doses for adults vary from 900 to 3600 mg/day. In addition, the doses were arranged according to standard SPF egg weighing. GBP solutions of three concentrations were prepared as low (group B: 0.9 mg/d or 0.9 mg/0.1 ml), high (group C: 3.5 mg/d or 3.5 mg/0.1 ml), and overdose (group D: 7 mg/d or 7 mg/0.1 ml) doses.

We designed the study to include four groups, with 25 eggs per group. Group A comprised the control group. However, six infertile (undeveloped) and four sacrificed eggs resulted in differences in the number of subjects enrolled in the study groups (Table I). One hundred eggs (mean ± SD, 65 ± 5 g) were incubated at 37.2 °C ± 0.1°C and 60%–70% humidity for 30 hours. Each egg was rotated on its longitudinal axis. One egg in each group was sacrificed at random to verify the Hamburger Hamilton stage 9 (11) (Figure 1 A–B). At this stage of the study, according to New's technique, 0.1 ml GBP was injected under the discs after each egg was opened at 4X optical magnification (23).

Incubation was continued for 72 hours after all the eggs were sealed with sterile adhesive strips. They were reopened using microsurgical procedures and the water-floating technique. At this stage, the presence of a heartbeat was used to ascertain whether the embryos were viable. The embryos were transported to a Petri dish to dissect the vitellin membranes. All embryos were subsequently immersed in a 5% formalin solution for 24 hours before being sent for histopathological analysis (Figure 1C–D). Briefly, after dehydration of the embryos, the embryos were incubated in xylene and then transferred into a paraffin embedding mixture. Afterward, the samples were prepared to be cut axially into 5 µm-thick serial sections from paraffin blocks with rotary microtoms, oriented at an angle of 7°; following this important step, the specimens were stained with hematoxylin eosin (HE). The sections were

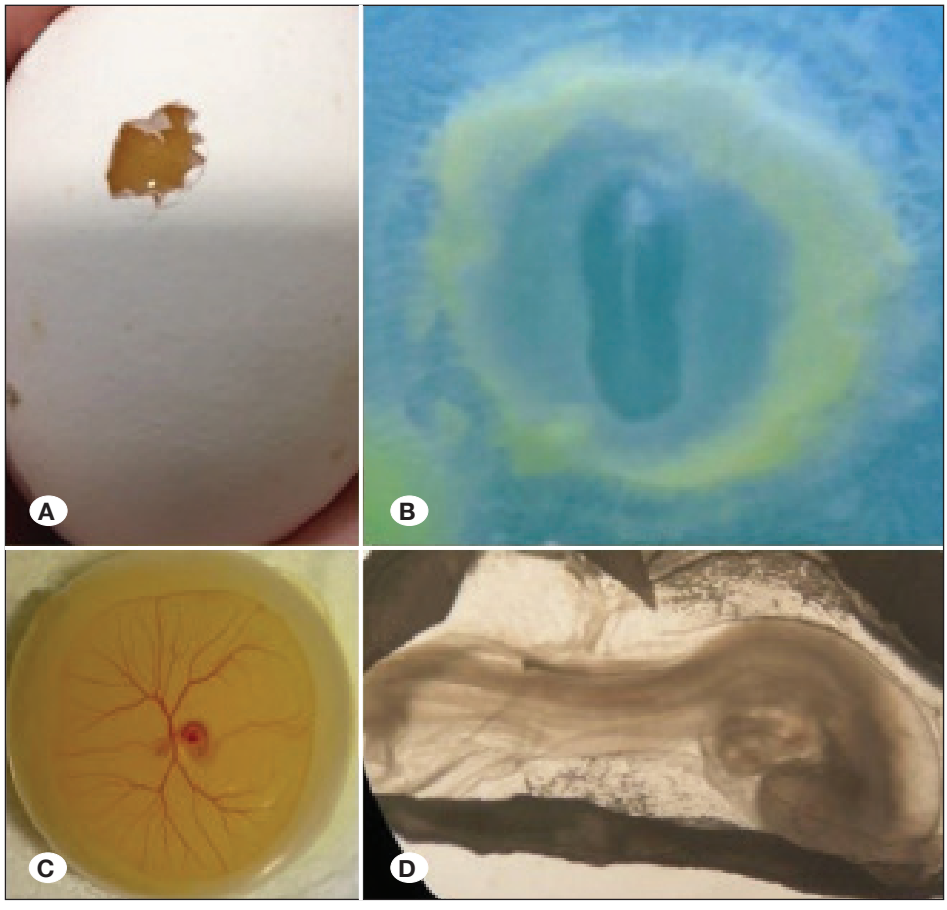

Figure 1: A) An opened window in an eggshell during the 30th hour test. **B)** An embryo in Hamburger-Hamilton stage 9. **C)** An embryo in Hamburger-Hamilton stage 18 and its omphalomesenteric (vitellin) vascularization observed during the 72nd hour test. **D)** An embryo in Hamburger-Hamilton stage 18 seen under a light microscope (40X magnification).

examined under a light microscope (Nikon Eclipse Ni-U, Japan) (Figure 1D). Both macroscopically and microscopically, the structural continuity of neural tubes and somites was investigated. A neural tube closure defect was identified as any interruption in somite or neural tube continuity (Figure 2A–D).

The Statistical Package for the Social Sciences (SPSS v 23.0, SPSS Inc., Chicago, IL, USA) was used to analyse the raw data. Pearson's chi-square test was used to determine the difference between groups, with a value of $p<0.05$ indicating statistical significance.

RESULTS

The quantity of omphalomesenteric vascularization was observed macroscopically. In group A (control group), vascularization quality and quantity were found to be within the physiological range. However, a decrease in vascularization was observed in all GBP groups (B, C, and D). In addition, the absence of embryo development was observed in six eggs, regardless of the gradually increasing doses.

NTDs were detected in 1 (4.34%) embryo in Group A, 13 (59.09%) embryos in Group B, 15 (65.21%) embryos in Group C, and 18 (81.81%) embryos in Group D (Figure 2A–D).

All subjects are summarised in Table I (see Figure 2). When we compared all groups using Pearson's chi-square test, we found a statistically significant difference ($p<0.001$). After using a post-hoc test (Bonferroni method) to determine the difference, we observed that there was a difference between the control group and the other groups, but there was no difference between the GBP-injected groups. Based on our findings, GBP could cause NTDs and abnormalities in early chick embryos, regardless of dose.

DISCUSSION

NTDs are major congenital anomalies, as they can cause death as well as medical, economic, and social difficulties as a result of inappropriate neurulation during the embryonic stage (31). After congenital heart anomalies, central nervous system anomalies are the most common. Approximately 300,000 children are born with NTDs annually. In the United States, approximately one in 1000 newborns is affected by NTDs, which vary from spina bifida to anencephaly. In other words, the global NTD incidence is 1–10 per 1000 births (30). Chemical exposure, medications, trauma, maternal infection, and folic acid insufficiency are among the well-known causes of neural tube non-closure diseases (4,11,14,20). Moreover, environmental and nutritional factors play a crucial role in the

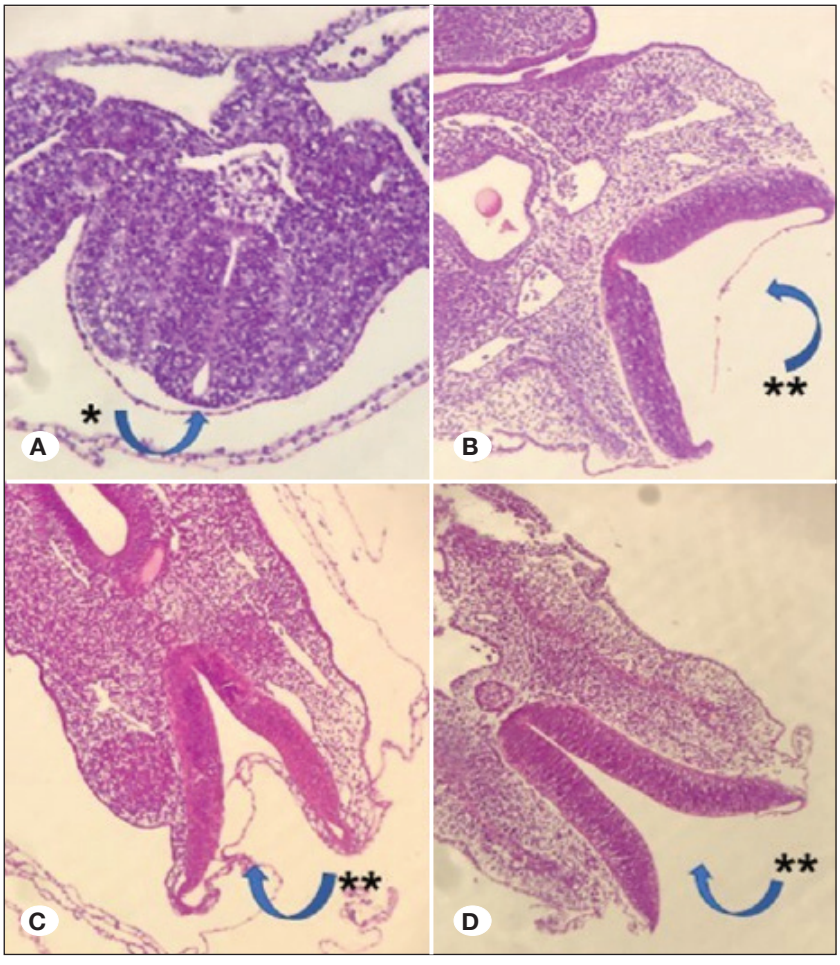

Figure 2: Neural tube sections stained with HE (x200) [(*) A closed neural tube subject from group **A**. (**) Neural tube closure defects from the groups **B, C and D**].

Table I: Distribution of the Subjects in the Groups. A Total of 90 Eggs were Enrolled in the Study

Groups	A n (%)	B n (%)	C n (%)	D n (%)
Total	25	25	25	25
Sacrificed*	1	1	1	1
Undeveloped**	1	2	1	2
NTD	1 (4.34)	13 (59.09)	15 (65.21)	18 (81.81)
Intact	22 (95.65)	9 (40.90)	8 (34.78)	4 (18.18)

(*) *Sacrificed eggs to ensure that the subjects were in Hamburger-Hamilton stage 9 at the 30th hour.* ***(**)*** *No embryo development at the 30th hour.*

aetiology of these, including well-known factors such as folic acid supplementation, maternal diabetes, and anticonvulsion therapy (4,8,15). The first month of pregnancy, known as gastrulation, is a crucial period since it coincides with the dates when the woman becomes aware of her pregnancy. The embryological progress is exceedingly prone to external variables and chemicals during its early phases. Multisystem congenital malformations can be triggered by changes that affect the germ layers, which are the precursors of future tissues and systems. All of the factors that cause NTDs will generate these effects, particularly during the mid-gestational stage once neural tube closure occurs.

The first month of embryonic development in mammals is represented by an early chick embryo model (31). It is also well suited to investigate the effects of chemicals on the development of embryos, but not the mechanism, since the study design does not allow it. The importance and necessity of apoptosis in neural tube closure was previously determined using the ESCE model (6,28,32). Moreover, folic acid antagonists, antiepileptics, local anaesthetics, other drugs, and MRI applications have all been shown in experimental studies to cause neural tube closure defects in ESCE, predominantly by affecting the cell membrane Ca^{2+} ion channels (5,16,20,26,27,30,33,35). Although it is technically impossible to determine the mechanism by which any active ingredient causes NTDs in the ESCE model, we believe that GBP causes NTDs potentially via the same Ca^{2+} ion exchange mechanism.

GBP (Nepitin®, Ali Raif, Inc. Istanbul, Turkey) is a GABA analog that is used to treat postherpetic neuralgia and partial seizures. It is also commonly used off-label to treat diabetic neuropathy and neuropathic pain. Other off-label and investigational uses for GBP have been described in the literature (1,3,21,36). Hahn et al. suggested the use of vigabatrin or GBP, particularly in patients with acute porphyria and seizures (10). GBP received approval from the US Food and Drug Administration as a anticonvulsant therapy in 1993 and subsequently for neuropathic pain in 2002. It shows its anticonvulsant, antinociceptive, and possible anxiolytic effects through inhibition of neuronal signalling by binding to the α2-δ subunit of voltage-gated calcium channels in the central nervous system (7,29). In fact, Oka et al. stated that GBP causes activity-dependent Ca^{2+} channel blocking by different mechanisms, which are associated with their cerebroprotective actions, and that they are dependent on the severity of hypoxic insults (25). Contrary to theoretical knowledge, Karkar et al. ascertained that glycine receptor potentiation does not contribute to the antiepileptic action of GBP on hippocampal neurons (18). GBP is typically excreted in the urine (19). The wide usage of GBPs leads to huge consumption. In fact, Henning et al. reported that GBP was detected in Germany surface water, ground water, and potable water with the highest concentrations of 3.2 mg/ L, 1.3 mg/L and 0.64 mg/L, respectively (13). Moreover, He et al. found that GBP could cause neurotoxicity and immune toxicity in zebrafish embryos at realistic environmental concentrations (12). Additionally, GBP neurotoxicity has been proven in clinical research (17). Although there were no reports of any adverse effects in six neonates whose mothers took GBP throughout pregnancy, Ohman et al. reported that there is likely an active transplacental transportation mechanism for GBP when it accumulates in the foetus, which could be due to the specific L-type amino acid transporter that is expressed in the placenta (24).

In another comparative study, Wilton et al. stated that there were no congenital anomalies detected in the 11 babies born to women who used GBP during the first trimester of pregnancy out of 3100 GBP-administered patients. In this article, there were five spontaneous abortions, excluding one pregnancy in which GBP administration was stopped before the last menstrual period; there were also 4 pregnancies that were terminated out of 19 pregnancies. Additionally, of the 11 women who gave birth, nine were taking GBP before their last menstrual period and continued the treatment, including a prematurely born baby. We believe that these 11 babies, including cohort, are insufficient to determine whether GBP affects neural tube development (34). Additionally, it was reported that GBP use in pregnancy does not appear to increase the risk of major complications, except for the increased risk of low birth weight and preterm birth (p=0.033 and p=0.019, respectively). In this manner, we found a decrease in vitellin vascularization in all GBP groups. Fujii et al. reported that there were seven major complications in 223 pregnancies, including in the GBP group, and none of the major complications were NTDs (9). However, their study included a small numbers of patients, considering that there are probably thousands of women of childbearing age taking

this drug worldwide. Consequently, it would be difficult to ascertain whether NTDs are caused by GBP. In this regard, we believe that further studies with larger sample sizes are required in order to determine whether GBP affects neural tube development.

To the best of our knowledge, this is the first experimental study to date analysing the outcomes after exposure to GBP in an ESCE model. Therefore, many clinical reports and experimental studies have been conducted in the literature concerning the effects of GBP. In a study of 30 subjects on mouse foetuses, Afshar et al. emphasised that GBP can induce severe malformations during the early stages of pregnancy (2). However, this was a preliminary study consisting of 20 mice in two drug groups. In our study, we found that GBP could cause NTDs and abnormalities in early chick embryos, regardless of dose.

CONCLUSION

GBP is a drug used for a variety of purposes, predominantly neuropathic pain and partial epilepsy. Our results on ESCE revealed that the administration of GBP at all doses caused NTD. This study is the first to demonstrate this effect. Although we do not know exactly which step of neurulation is affected by GBP, the drug can block Ca^{2+} ion exchanges and thus lead to defects in neural development. Realistically, considering the widespread usage of GBP, it is potentially responsible for the birth of children with NTDs, even at environmental concentrations. However, further investigations with a larger sample size are required to assess its effect at lower doses and to determine the potential mechanisms of embryonic damage.

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