



The Effects of Levetiracetam on Neural Tube Development in the Early Stage of Chick Embryos

Levetirasetamın Erken Dönem Cıvcıv Embriyosunda Nöral Tüp Gelişimine Olan Etkisinin Araştırılması

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ABSTRACT

AIM: This study aimed to investigate the effects of a new generation antiepileptic agent, levetiracetam, on the neural tube development in a chick embryo model that corresponds to the first month of vertebral development in mammals.

MATERIAL and METHODS: Forty-five Atabay® breed fertilized chicken eggs with no specific pathogens were randomly divided into 5 groups. All of the eggs were incubated at 37.8±2°C and 60±5 % relative humidity in an incubator. Group A was control group. The other eggs were applied physiological saline and drugs at a volume of 10 µL by the in ovo method at the 28th hour of the incubation period. Group B was given distilled water; Group C, physiological saline; Group D, Levetiracetam (L8668) at a dose equivalent to the treatment dose for humans (10 mg/kg), and Group E, Levetiracetam (L8668) at a dose of 10 times the treatment dose. The embryos in all of the groups were removed from the shells at the 48th hour and morphologically and histologically evaluated.

RESULTS: Of the 45 embryos incubated, neural tubes of 41 were closed and the embryos displayed normal development.

CONCLUSION: Levetiracetam, at a dose equivalent to human treatment dose and 10 times the treatment dose, was shown not to cause neural tube defects in chick embryos.

KEYWORDS: Levetiracetam, Chick embryo, Neural tube defect

ÖZ

AMAÇ: Bu çalışmada yeni bir antiepileptik ajan olan levetirasetamın memelilerde embriyonel omurga gelişiminin ilk ayına uyan erken dönem civciv embriyo modelinde nöral tüp gelişimi üzerine olan etkilerinin incelenmesi amaçlanmıştır.

YÖNTEM ve GEREÇLER: Çalışma için spesifik patojen içermeyen 45 adet Atabay® cinsi döllenmiş tavuk yumurtası rastgele 5 gruba ayrıldı. Tüm yumurtalar 37.8±2°C sıcaklık ve % 60±5 nem oranında kuluçka makinasında inkübe edildi. Grup A kontrol grubu olarak ayrıldı. Diğer yumurtalara ise inkübasyonun 28. saatinde 10 µL hacim içerisinde in-ovo yöntem ile serum fizyolojik ve ilaç uygulamaları yapıldı. Grup B'ye distile su, Grup C'ye serum fizyolojik, Grup D'ye insanda kullanılan tedavi dozuna (10 mg/kg) eşdeğer dozda levetirasetam (L8668) ve Grup E'ye bu tedavi dozunun 10 katı yükseklikte levetirasetam verildi. Tüm gruplardaki embriyolar 48. saatte yumurtadan çıkarılarak morfolojik ve histolojik olarak incelendi.

BULGULAR: İnkübe edilen 45 adet embriyonun 41'inde nöral tüpün kapalı olduğu ve embriyonun normal gelişim gösterdiği görüldü.

SONUÇ: Levetirasetam'ın insan tedavi dozuna eşdeğer dozda ve bu değer 10 katı yükseklikte verilen dozlarda civciv embriyosunda nöral tüp defekti oluşturmadığı gözlemlenmiştir.

ANAHTAR SÖZCÜKLER: Levetirasetam, Civciv embriyosu, Nöral tüp defekti

INTRODUCTION

Congenital central nervous system anomalies are the second most common anomalies following congenital cardiovascular anomalies (23). Neural tube defects (NTD) are heterogeneous and complex congenital anomalies of the central nervous system. Varying incidence rates (0.3-0.58%) for NTD have been reported for Turkey (33). Environmental contaminants, infectious agents, maternal hyperthermia, usage of certain antiepileptic drugs (phenytoin, valproic acid) during

pregnancy, deficiency of nutritional components, the intake of which are definitely required, and chronic diseases of the mother (diabetes mellitus, etc.) increase the incidence of NTD (27). In addition, the risk of major malformation associated with prolonged use of antiepileptic drugs is known to be 2-3 folds of the normal (8).

Levetiracetam, a new generation antiepileptic drug, is used in the management of focal and generalized epilepsy. Although Levetiracetam has a different action mechanism than that

the other anti-epileptic drugs, its adherence mechanism by which it recognizes the cell membranes of the central nervous system has not been clearly defined (6,26).

This study aimed to investigate the effects of a new generation antiepileptic agent, Levetiracetam, on the neural tube development in a chick embryo model that corresponds to the first month of vertebral development in mammals. Thereby, it was also aimed to determine potential results with its use during pregnancy.

MATERIAL and METHODS

Fertile, specific pathogen free eggs of the domestic fowl (Atabey®, Gallus gallus, Poultry Research Institute, Ankara, Turkey) were selected for the present study. The eggs were incubated at 37.5°C and 75% relative humidity until the embryos reached stage eight of development according to Hamburger and Hamilton. At this stage, the eggs were divided into five groups that consisted of 9 eggs per group: Eggs in Group A were not subjected to any injection and named the sham group. Group B and C were injected with 10 µL of distilled water and saline by in ovo method respectively. In Group D, Levetiracetam was administered at a dose (0.6 mg/kg) equivalent to the therapeutic index dose (10 mg/kg). In Group E, Levetiracetam was administered at a dose equivalent to 10 times the therapeutic index dose (100 mg/kg) (Table I).

Therapeutic dose index dosage of Levetiracetam is 10 mg/kg for humans. An equivalent dose for a chick embryo is calculated according to the weight of the egg. The equivalent dose for study of Levetiracetam (Sigma-Aldrich catalog number: L8668, St. Louis, Missouri, USA) was calculated to be 0,6 mg/kg per egg. The stock solution was prepared to dissolve Levetiracetam in distilled water and then the calculated dose of Levetiracetam was diluted in 10 µL for Group D. For the high dose (ten times) Levetiracetam group (Group E), 6 mg/kg of Levetiracetam was injected in the same volume of distilled water.

At the eighth stage of development, the eggs were washed with 70% alcohol and properly labeled on the outer shell. A hole was made on the blunt pole of the eggs with a sharp and thick needle under laminar flow. Using a sterile Hamburger® syringe, 10 µL of fluid was injected from the blunt end under the embryonic disc. The holes were sealed with paraffin. The eggs were then placed in an incubator. Embryo collection:

The eggs were opened at 50 hours of incubation. They were cracked open and the outer shell was chipped out to create a wide opening for visualization of the embryo. The viability of the embryos was assessed by the heartbeat. The embryos were transferred to a petri dish by careful dissection along the allantoic stalk and other embryonic structures. All the embryos were fixed with 10% formalin and examined under stereomicroscope to assess any gross developmental abnormalities. Then, embryos that passed Hamburger Hamilton stage 12 were embedded into paraffin. Sections of five micron thickness were prepared and stained with hematoxylin–eosin for light microscopic examination. Slides were examined with Leica DM 4000 (Germany) photo-light microscopy.

RESULTS

Group A: Of the 9 chick embryos, 8 were in the stage they were expected to be according to Hamburger-Hamilton embryonic classification, and one of them has neural tube defect (Figure 1).

Group B: All of the 9 chick embryos that were given distilled water were found to be in the embryonic stage where they were expected to be according to Hamburger-Hamilton classification. The neural tube was closed in all of them. No malformation or developmental retardation was observed (Figure 2A,B).

Group C: In one of the chick embryos (n=9) that were given physiological saline, the embryonic vertebra was not developed and the embryo was dead. The remaining 8 embryos sustained normal development and were in the embryonic stage where they were expected to be according to Hamburger-Hamilton classification. In all of the embryos, the neural tube was closed. No malformation or developmental retardation was noted.

Group D: The nine chick embryos that were given an equivalent dose of Levetiracetam within therapeutic index (10 mg/kg/day) sustained their normal development and were in the embryonic stage where they were expected to be according to Hamburger-Hamilton classification. The neural tube was closed in all of the embryos. No malformation or developmental retardation was observed.

Group E: Seven of the 9 chick embryos that were given 10 times the equivalent therapeutic dose of Levetiracetam (100

Table I: Study Groups

Group A	No intervention was made on the embryos.
Group B	The embryos were administered 10 µl distilled water.
Group C	The embryos were administered 10 µl physiological saline.
Group D	The embryos were administered Levetiracetam in 10 µl distilled water at a dose equivalent to the dose for the human therapeutic index.
Group E	The embryos were administered Levetiracetam in 10 µl distilled water at a dose equivalent to 10 times the dose for the human therapeutic index.

mg/kg/day) sustained their normal development and were in the embryonic stage where they were expected to be according to Hamburger-Hamilton classification. The neural tube was closed in all of these 7 embryos. No malformation or developmental retardation was observed (Figure 3A,B). In the remaining 2 embryos, embryonic vertebra was not developed and the embryo was dead. In one embryo, however, the embryonic development was normal under the stereomicroscope; the neural tube was closed, but the sample of the same embryo that was stained with hematoxylin-eosin, the neural tube was observed to be open. In this embryo, the folding of the neural plate was completed. Nevertheless, the ends of the neural plate coincided in the midline but not closed (Figure 4).

DISCUSSION

Neural tube defects (NTD) are congenital anomalies of the central nervous system. NTD is among most severe congenital anomalies and causes important social, economical and medical problems (34). The period from appearance of neural plaque up to closure of palate, i.e. the period between 18th day and 60th day of pregnancy, is the period where the possibility of congenital anomaly is highest. These anomalies originate from insufficiency in neural tube formation (36) or re-opening after formation of neural tube (10). Environmental factors that would influence development of fetus may cause congenital anomalies at this period. In this study, neural tube closure defects that may appear on fetus in epileptic women in case of levetiracetam use during pregnancy process have been investigated on chick embryo model.

Genetic factors (trisomy 13, 18, 21), geographical factors, mother's age, socio-economic factors, diseases of folic acid metabolism, diabetes mellitus, high fever on mother during first month of pregnancy, alcohol use during pregnancy and use of some antiepileptic drugs can be specified in etiology of neural tube closure defects (16, 19, 25, 33). The studies have been demonstrated in early stage chick embryos in

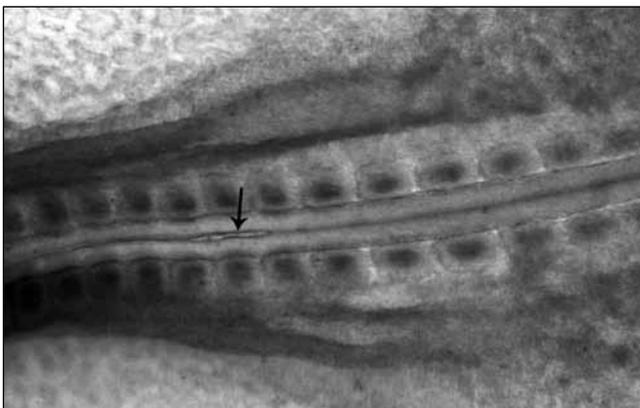


Figure 1: The complete image of the chick embryo from Group A that was not subjected to any intervention and opened at the 48th hour of incubation. The arrow indicates the opening at the neural tube.

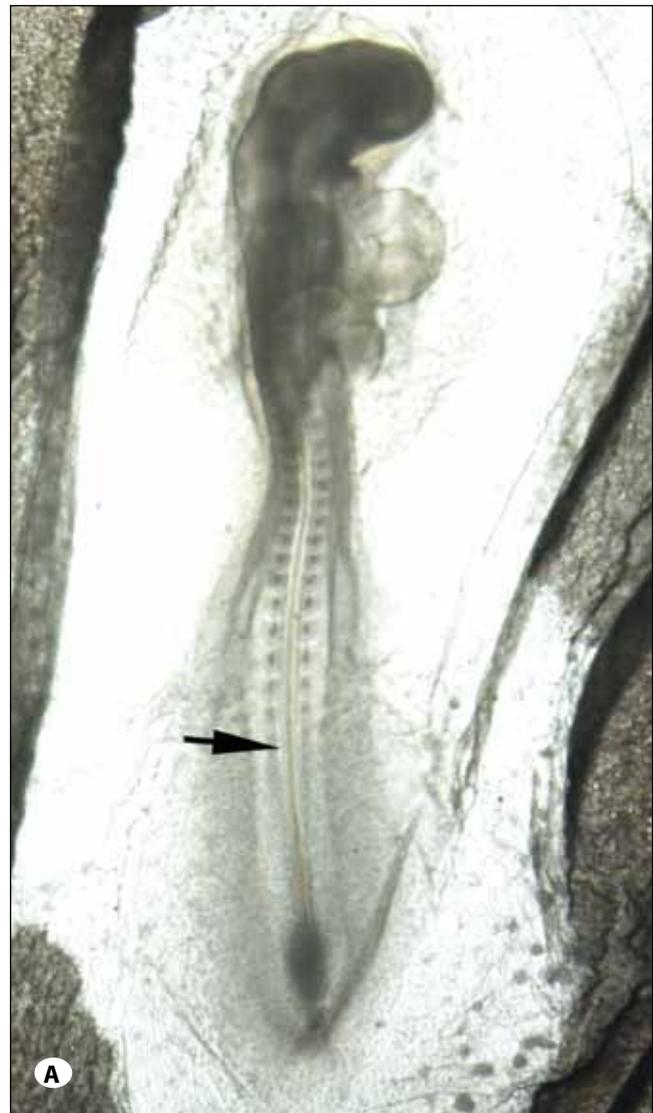


Figure 2: The neural tube in the chick embryo from Group B that was given distilled water in ovo and opened at the 48th hour appeared to be closed in the complete image under stereomicroscope (A). A section was obtained at the level indicated by the arrow as seen in Figure 2A, stained with Hematoxylin-eosin. The thick arrow points at the neural tube that was closed, while the thin arrow points at the notochord (stain: H.E. x40 magnification) (B).

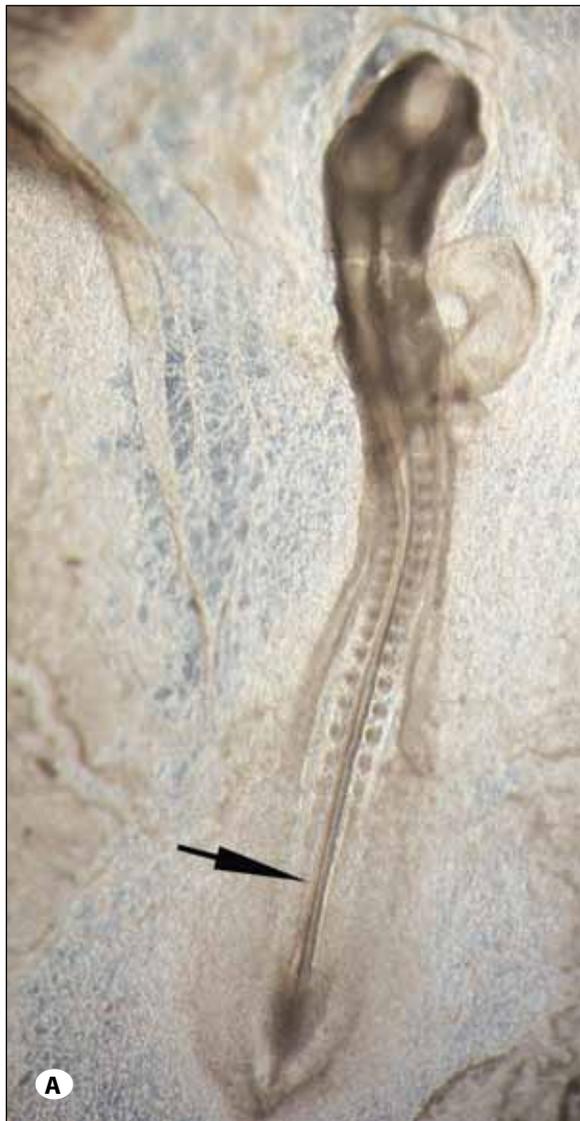


Figure 3: In the complete image of the chick embryo from Group E that was given in ovo 100 mg/kg/day Levetiracetam and opened at the 48th hour, the neural tube was closed under the stereomicroscope (A). A section was obtained at the level indicated by the arrow as seen in Figure 3A, The thick arrow points at the neural tube that was closed, while the thin arrow points at the notochord (stain: H.E., x40 magnification) (B).

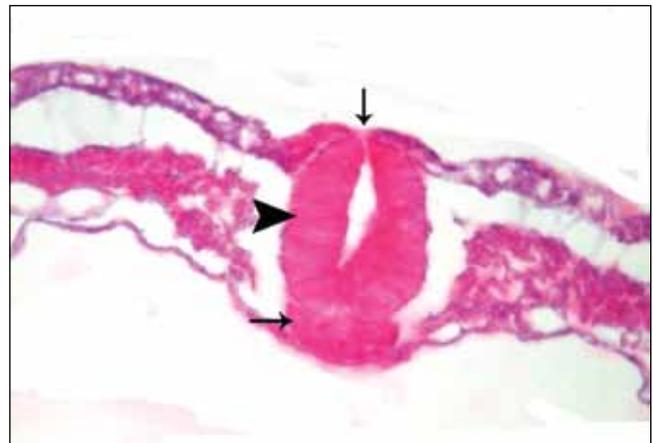


Figure 4: The image of the chick embryo that was administered in ovo 100 mg/kg/day Levetiracetam and opened at the 48th hour. In the section that was stained with Hematoxylin-eosin, the arrow above shows the opening in the neural tube; the neural tube is shown with the thick arrow, and the thin arrow below points at the notochord (stain: H.E., x40 magnification).

experimental studies that ethanol, high dose meloxicam, high dose progesterone, a folic acid antagonist methotrexate, cotinine included in cigarette causes neural tube closure defect (1,3, 5, 7, 35).

Our subject, which is a greatest problem in epileptic pregnant women all over the world and on which many studies are being conducted, was the teratogenic effect of antiepileptic drugs on embryo. Pregnancy increases the frequency of seizures in 20% of epileptic women (32) and leads to a change in serum concentration and clearance of the antiepileptic drug in pregnancy. It may be needed to use epileptic drugs in higher doses or in combination with other antiepileptic drugs in pregnancy. Raised blood level of antiepileptic drugs is also associated with high risk. It is known that risk of major malformation increases 2-3 times than normal due to use of long-term antiepileptic drug use (8). In addition, it has been shown that they have also behavioral teratogenicity as well as causing anatomic malformation because cognitive disorders occurred in animal and human studies performed (9).

One of mechanisms causing appearance of side effects of antiepileptic drugs is neuronal apoptosis during late gestational and prenatal period. It has been seen that valproic acid, phenobarbital and phenytoin have increased neuronal apoptosis in brains of newborn rats during two weeks (2). It is indicated in a study of Temiz et al. that phenytoin caused less teratogenic effect than expected when given in therapeutic concentration to chick embryo but teratogenic effect on neural tube development appeared clearly when high dose phenytoin concentration is given (31). It has been displayed based on several studies that anatomic and behavioral defects increased with use of phenobarbital (15, 30). Valproic acid taken during pregnancy causes appearance of anatomic defects on fetus in first three months of pregnancy (8) and behavioral defects in last three months of pregnancy (9). It

has been reported that risks are less in Carbamazepine and lamotrigine but both caused formation of cleft lip and palate (14).

Action mechanism of levetiracetam has not been understood clearly yet (37). It is thought in some studies related to the action mechanism of levetiracetam that it regulates high-voltage N-type calcium channels and potassium flow (22). Recent data suggest that the primary action mechanism occurs by increasing GABAergic efficiency by regulating protein 2A, a synaptic protein included in vesicular exocytosis (4, 21).

Studies on levetiracetam have reported that its teratogenic effect risk is low in case it is used in pregnancy, but they are limited (17). On the other hand, levetiracetam use during pregnancy has caused fetal skeletal system anomalies, growth retardation and increase of incidence of fetal mortality. Fetal skeletal anomalies are seen when it is given 350 mg/kg/day to rats during gestation, and skeletal anomalies are seen when dose given during organogenesis is 3600 mg/kg/day. Ratio of appearance of defects in the skeletal system increases when rats are treated with a dose of 600 mg/kg/day during organogenesis period in rabbits (11). It is seen that doses given in these studies were very high values above the therapeutic index.

In our study, possible adverse effect of levetiracetam on the neural tube was investigated in chick embryo model. The early stage chick embryo model corresponds to first one month of mammalian spine (vertebra) development and this is a suitable example for investigating effects of chemicals (12, 13, 28). It was seen in the study we conducted that the development of 41 embryos out of a total 45 embryos was normal and neural tube was closed. Neural tube defect was shown on one embryo in Group A that any intervention was not made to embryo during incubation. Also there was one embryo death in Group C where saline was given. In Group E, there is one embryo death and one embryo has been neural tube defect where we administered high dose levetiracetam. The most effective factor on embryo development is temperature. Taking eggs out of incubator for purpose of injection to embryo at 28th hour of incubation causes occurrence of temperature changes and increases risk of developmental defect on embryo. Seeing neural tube defect on one embryo in each of control group and high-dose Levetiracetam may because of this. It has been observed in our study that when levetiracetam was given in recommended starting dose of 10 mg/kg and given as maximum dose of 100 mg/kg. Neural tube defect and death in embryos of Group E have originated from factors independent of levetiracetam.

On the study of J. Kim et al. on postnatal 8 days-old rats, levetiracetam alone did not cause cell death in the rat brain when given in therapeutic doses and did not interact with other drugs as well. In this frame, it has been indicated that the therapeutic index of levetiracetam is higher than 5 times and possibly about 10 times higher (20). It has also been indicated in study of Manthey et al. that levetiracetam does

not cause neuron death when given alone at a dose of 100 mg/kg/day (24).

Initial reports published related to Levetiracetam indicate that the use of this drug during pregnancy is safe (17). Studies conducted on mice have shown that use levetiracetam and its major metabolite on humans during pregnancy is safer than all of other first generation antiepileptic drugs (18). It has been indicated that levetiracetam can be a good candidate in monotherapy and polytherapy of epilepsy during pregnancy period (29). According to our study, developmental anomalies were not seen on low dose of levetiracetam in an early chick embryo model, and this finding may indicate that it may not developmental anomalies during pregnancy.

CONCLUSION

Neural tube defects have not been seen with the therapeutic dose range given to embryos. However, a larger number of subjects and further studies related to use in high doses are needed. Levetiracetam can be a safe candidate drug for the aspect of occurrence of neural tube defect when it is used in epileptic women during pregnancy at doses in the therapeutic range in line with these studies.

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