MSD

Medical Science and Discovery
ISSN: 2148-6832

Evaluation of anti-epileptic Lacosamide treatment on neural tube development: Chick embryo experimental model

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ABSTRACT

Objective: Even if epileptic women can give birth to healthy babies, the rates of premature birth, low birth weight, fetal and neonatal death risk, congenital malformations and growth retardation in pregnant women who use the antiepileptic drugs (AEDs) are high compared to the population. The present study aimed to examine the effects of AED Lacosamide (LCM) during pregnancy on the fetus in terms of neuronal and embryonic development in 48-hour chick embryo model.

Material and Methods: 40SPF fertilized eggs divided into 4 equal numbers. At the 28th hour, LCM was applied in the sub-blastoderm, and the experiment was terminated at the 48th hour. Embryos were evaluated morphologically by neural tube position, somite number, and cranio-caudal length. Histopathologically, hematoxylin-eosin, Caspase-3 immunohistochemistry, and TUNEL staining were performed and and assessed for cell death.

Results: LCM adversely affected neural tube position in groups 3 and 4 compared to control. In addition, it regressed embryonic development by decreasing somite number and craniocaudal length in groups 3 and 4. When evaluated in terms of apoptotic cell death, LCM increased caspase-3 immunoreactivity and the number of TUNEL-positive cells in groups 3 and 4, respectively ($p=0.002$), ($p \leqslant 0.001$).

Conclusion: LCM was caused to regression of embryonic development and impaired neural tube position in early chick embryo model, dose-dependent manner. It increased cell death and showed teratogenic effects in the early embryo model. The usage of LCM for pregnant women should be considered carefully. It is obvious that more preclinic studies are needed to demonstrate LCM effects comprehensively.

Keywords: Lacosamide, neural tube defects, teratogenicity, chicken Embryo model.

Research Article

Received 15-08-2022

Accepted 25-08-2022

Available Online: 27-08-2022

Published 30-08-2022

INTRODUCTION

Epilepsy is an important neurological disease with a frequency of 0.5%. The most characteristic feature of the disease is epileptic seizures. These seizures are mostly controlled with antiepileptic drugs. The majority of patients with epilepsy are women of reproductive age. Due to the increased frequency of seizures in women with epilepsy, the fetus is exposed to antiepileptic drugs during pregnancy (1).

Although pregnant women with epilepsy give birth to healthy children at a high rate, the rates of premature birth, low birth weight, fetal and neonatal death risk, congenital malformations and growth retardation are two times higher among the antiepileptic drug users compared to the normal population (2–4).

Major and minor congenital malformations may observe in the babies of epileptic pregnant women. The most common period of major congenital malformations is between the 3rd and 8th weeks of organogenesis, Neural tube defects, cardiac defects, urogenital malformations, craniofacial defects and skeletal anomalies are among the most common major congenital malformations.

http://dx.doi.org/10.36472/msd.v9i8.793

Lacosamide (LCM) is one of the most frequently prescribed antiepileptic drugs, which is used in the treatment of epilepsy in pregnancy. However, its effects on the fetus are not known precisely (1). LCM was approved by the FDA (Food and Drug Administration) in the USA in 2008 for the treatment of adult focal epilepsy patients; It is a 3rd generation antiepileptic agent licensed in our country in October 2012 (5,6). LCM promotes slow inactivation of voltage-gated sodium channels in neurons, and stabilization of neuronal membranes resulting in decreased long-term channel availability (7). In addition, LCM modulates neurotrophic signals, including neuronal differentiation, polarization, and axonal growth. In addition to the treatment of epilepsy, LCM has also been shown to be effective in central and peripheral neuropathic pain. It is pregnancy category C in the classification made by the FDA. Although there is no controlled study in humans, its use in pregnancy is only recommended in cases with high benefit/risk ratio. Considering the mechanisms of action of LCM, the fact that it is directly related to neuronal stability suggests that it may have an effect on the neuronal development of the embryo during pregnancy. Apoptosis is programmed cell death that occurs as a result of physiological or pathological conditions and results in biochemical changes in the cells. Therefore, it is very important to detect signs of apoptosis in the study of cellular responses or cell metabolism. The TUNEL method is a cell death determination method and based on detecting double-strand breaks in DNA (8). Activation of Caspase-3, is an intracellular marker that is indicates to activation of apoptotic pathway and triggering the caspase cleavage frequently used to driven of the cell to death. Our study was designed to investigate the effects of Lacosamid in different doses, which is widely used in the clinic as an AED, during pregnancy on the fetus in terms of neuronal development and embryonic development. Therefore, we aimed to histopathologically and morphologically examine the effects of different doses of LCM treatment on neural tube development in 48-hour embryos in a chick embryo model.

MATERIAL and METHODS

1. Experimental design

This study was carried out at Afyonkarahisar Health Sciences University, Faculty of Medicine, Department of Anatomy and Histology and Embryology. Ethics approval was obtained from Afyon Kocatepe University Local Ethics Committee in September AKUHADYEK-95-21. In our study, daily fertilized, special pathogen-free (SPF-specific pathogen-free) eggs with a weight of 60±5 g were used (Supplier; Izmir/Bornova Veterinary Control Institute).

In order to evaluate the effects of LCM (BENVIDA 10mg/ml Lacosamide IV, CIB27545A, UCB Pharma SA, Belgium) on neural tube and embryo development, the eggs were divided into four groups, three experimental and one control, as indicated below (Table 1).

According to the Hamburger and Hamilton model, neurogenesis begins at the 28th hour of incubation (stage 8) (9). In our study, different doses of LCM were administered by opening a small window over the eggs at the 28th hour (Table 1)(10). The eggs were followed for 48 hours in an incubator that provided the appropriate temperature and humidity for the development of the eggs (37.5±0.5°C and 60±5% humidity). At the end of the 48th hour, the embryos were hatched.

2. Morphological Examination

Hatched Embryos at 48th hour were fixed in 10% formalin after cleaning from the amniotic sac and membranes. At the end of the fixation, it was evaluated on the basis of the Hamburger-Hamilton Chick Embryo Classification System with a light microscope. (9). Morphological evaluations were made by determining the neural tube position (according to whether it is open/closed) (Table 2), craniocaudal length, developmental delay, and somite counts were statistically analyzed.

3. Histopathological Evaluation

Embryos were fixed for 48 hours after hatching, and underwent histological tissue processing. Hematoxylin and Eosin staining (Figure 1) for histochemical evaluation, TUNEL method for evaluation of cell death, and Caspase-3 for determination of apoptosis were evaluated by taking 5 µm thick sections.

Caspase-3 immunohistochemistry: After the histological tissue processing of the samples, paraffin blocks were formed, and 5µ thick sections were taken. Deparaffinization was completed in an oven at 65°C overnight, it was kept in xylene for 30 minutes, and then rehydration was performed by passing through decreasing alcohol series. Antigen retrieval was performed in citrate buffer for 20 minutes at 60°C in the microwave, and endogenous peroxidase activity was suppressed for 5 minutes with 3% hydrogen peroxide. After 15 minutes of protein block solution, it was incubated with anti-Caspase 3 antibody (1/200, NB100-567608, Novusbio) at +4°C for 1 night. After incubation, secondary antibodies were applied, and their immunoreactivity was visualized with DAB chromogen. Photographs were taken with a light microscope (NIKON Eclipse E600) using the Image Analysis Program (NIS elements, Japan). After immunohistochemical staining, H-Score evaluation, which is a semi-quantitative method, was performed for statistical analysis (Figure 2).

Table 1: Organization of the experimental groups

Groups	Agent and dose	Way of administration	volume	Frequency of administration	Effect duration
Group 1 (Control group)	Saline	into the sub-blastoderm area with a Hamilton injector.	0.04 ml	1 time	20 h
Group 2	200 mg/kg LCM	into the sub-blastoderm area with a Hamilton injector.	0.04 ml	1 time	20 h
Group 3	400 mg/kg LCM	into the sub-blastoderm area with a Hamilton injector.	0.04 ml	1 time	20 h
Group 4	600 mg/kg LCM	into the sub-blastoderm area with a Hamilton injector.	0.04 ml	1 time	20 h

http://dx.doi.org/10.36472/msd.v9i8.793

TUNEL method: In situ apoptosis detection kit (abcam, ab206386) was used. Sections were incubated with proteinase K for 20 min for permeabilization according to the manufacturer's instructions. Endogenous peroxidase inactivation was achieved with 3% hydrogen peroxide. It was incubated for 90 minutes at 37°C in a humid atmosphere with Equilibration buffer and then TdT enzyme for 10 minutes. It was then placed in stop/wash buffer at room temperature for 5 minutes and incubated with anti-streptavidin-peroxidase for 30 minutes. Samples were photographed by counterstaining with DAB chromogen and Methyl Green. The number of TUNEL-positive cells was determined by counting 100 cells in randomly selected areas from each sample(Figure 3) (11).

4. Statistical analysis

Before the experiments started, power analysis was performed to determine the number of samples and 10 SPF eggs were used for each group. Statistical analyzes were performed with the demo version of SPSS 25.0 package program. Neural tube position (open/closed) was evaluated with the Chi square test. Comparisons between multiple groups were evaluated with the Kruskall Wallis H test. Bonferroni correction was performed using the Mann Whitney U test for post hoc comparisons. Carino-caudal lengths, somite counts, TUNEL and IHC H score data were shown as mean±standard deviation in Table 3. Those with a P value of less than 0.05 were considered statistically significant.

RESULTS

1. Morphological effect of LCM on embryo development

Evaluation of neural tube position

The number of samples, which was determined as 10 eggs in each group, was rearranged to be 7 samples for each group due to unfertilized eggs and manipulative errors. When the groups were evaluated in terms of neural tube position, open neural tube was not seen in the control group, while the number of open neural tubes increased with increasing drug dose in the other groups (Table 2).

The groups were evaluated morphologically in terms of cranio-caudal length and somite numbers in terms of developmental delay. Compared to group 1, it was observed that the cranio-caudal length decreased as the LCM dose dependently and deformations in the axial skeleton of the embryo were observed. While the decrease in cranio-caudal length was not significant in group 2 compared to group 1, the decrease in length in group 3 ($p=0.038$) and group 4 ($p\leq 0.001$) was statistically significant.

Another criterion taken into consideration to evaluate embryos in terms of developmental delay was the somite number. When the groups were assessed in terms of somite number, it was observed that there was a decrease in somite numbers according to LCM application dose. When compared with group 1, the somite counts were not statistically significant in group 2, but significantly decreased in group 3 ($p=0.003$) and group 4 ($p=0.001$).

Effect of LCM on cell death

To evaluate the effects of LCM on cell death during embryonic development, samples were evaluated using the TUNEL method and Caspase-3 immunohistochemistry. According to TUNEL staining results, after LCM application, TUNEL positive cell numbers in NT cells and surrounding cells did not increase significantly in group 2 compared to group 1, but the number of TUNEL positive cells in group 3 ($p=0.002$) and group 4 ($p\leq0.001$) had increased significantly.

Caspase-3 immunohistochemistry was performed to evaluate the effects of LCM administration on the apoptotic cell death process in embryonic cells. The results of the staining were evaluated with H-Score. According to the H-Score results, there was no statistically significant difference in group 2 when compared to group 1, while Caspase-3 immunoreactivity was significantly increased in NT cells and surrounding cells in group 3 ($p=0.002$) and group 4 ($p\leq 0.001$). In addition, when group 2 and group 4 were compared according to the evaluation between groups, a significant increase can be mentioned ($p=0.002$).

Table 2. Distribution of neural tube position samples by groups.

Parameters	Group 1	Group 2	Group 3	Group 4
Open NTs	-	3	4	4
Closed NTs	7	4	3	3

Table 3. Mean ± Standard deviation values of the evaluation criteria of the groups.

Groups	Cranio-Kaudal Length	Somit	IHC H-Score	Tunel
Group 1	8239,00±1778,16	15,33±1,21	122,50±9,33	24,72±4,88
Group 2	7677,71±383,78	14,00±1,83	255,00±7,37	4,83±0,61
Group 3	6349,50±563,14	9,50±2,81	301,00±6,39	2,85±0,16
Group 4	4965,33±526,43	7,33±4,50	434,80±4,17	1,50±0,13

http://dx.doi.org/10.36472/msd.v9i8.793

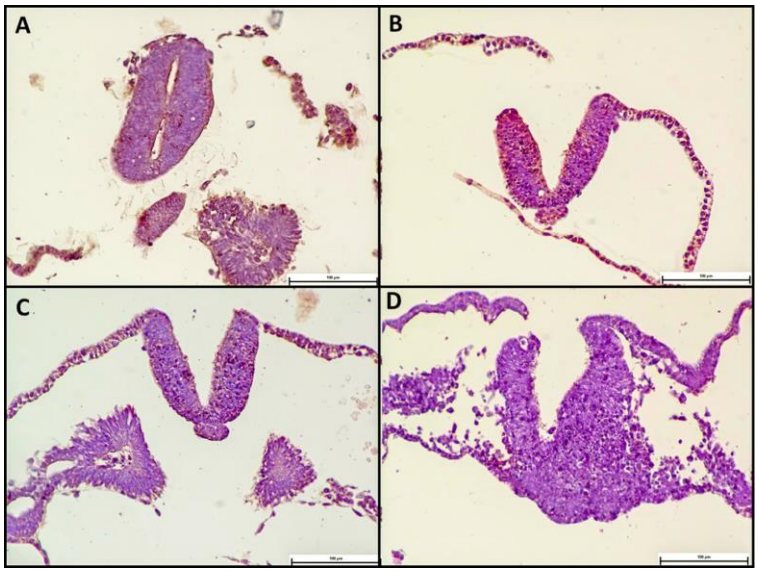

Figure 1: Hematoxylen-eosin stainings of the groups. A: Grup 1, B: Grup 2, C: Grup 3, D: Grup 4

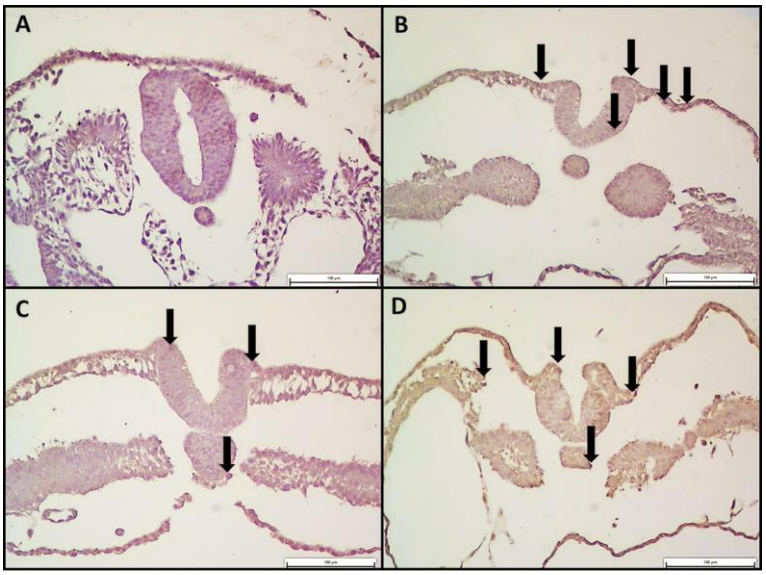

Figure 2: Caspase-3 immunohistochemistry staining of the groups. A: Grup 1, B: Grup 2, C: Grup 3, D: Grup 4. Arrow heads: positively stained cells.

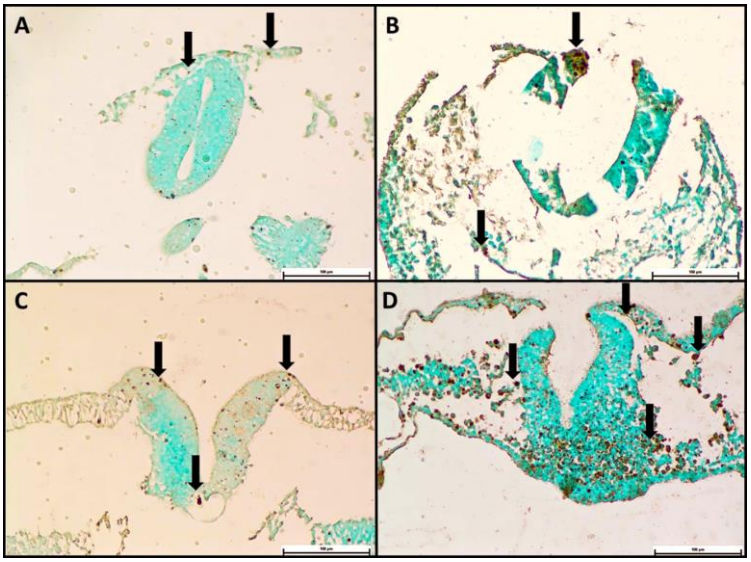

Figure 3: TUNEL staining of the groups. A: Grup 1, B: Grup 2, C: Grup 3, D: Grup 4. arrowheads: positively stained cells.

http://dx.doi.org/10.36472/msd.v9i8.793

DISCUSSION

Although the effects of AEDs used during pregnancy on the fetus are not yet fully known, malformations such as neural tube defects have increased in the babies of pregnant women using AEDs. Heart defects, facial clefts, extremity abnormalities, neural tube defects and growth retardation may also develop depending on the teratogenicity of the AEDs (12,13). Considering the teratogenic effects of drugs during epilepsy treatment, these effects can be reduced by arranging of the treatment and other preventive treatments. LCM is a new generation AED that is a CRMP-2 (collapsing response mediator protein 2) protein modulator in adults and is known to have neuroprotective effects (14). However, the lack of sufficient data on the teratogenic effects of AEDs, such as Lacosamide, which are frequently used in the clinic, brings risks associated with their use during treatment. The early chick embryo model is an ideal model that mimics the first months of embryonic development in mammals. The chick embryo model used in the study we presented has also been used in many studies to gain preclinical knowledge about the teratogenicity of drugs (10,15,16). We investigated morphologic and histopathologic effects of LCM at different doses on embryonic development in a chick embryo model for possible teratogenic effects of LCM, which considered as category C (17). In our study, three different LCM dose applications (low, medium and high doses) were calculated in kg/mg, which mimics the use in humans, and 28-hour embryos were injected to sub-blastoderm, and the experiment was terminated at the 48th hour, and the embryos were hatched. We examined morphology of hatched embryos under a light microscope by measuring cranio-caudal length, somite counts and neural position. Moreover, we evaluated the cell death rate by hematoxylin-eosin staining (TUNEL and Caspase-3 Immunohistochemical staining). As a result of the morphological evaluations, it was observed that neural tube patency was not observed in the control group, but increased in other groups depending on the LCM application doses. This increase was statistically significant compared to the control group. Similarly, comparisons of somite numbers did not show any statistically significant difference between control and low dose LCM application, but we found statistically significant differences between control medium and high dose. We couldn't observe the differences between the groups according to cranio-caudal length. Due to our morphological evaluation results, it is seen that LCM causes retardation in embryonic development at medium and high doses which adapted to the human dose. Based on the literature review, clinical and pre-clinical animal studies appear to be few in number and usually conducted within weeks of the end of organogenesis. Ylikotila et al. reported that, although it coincided with the late organogenesis period, LCM did not show teratogenicity but organogenesis was behind than healthy babies with equal birth weeks (18). Our study showed that as the LCM dose increased in the groups, the somite number and cranio-caudal length decreased, and the embryos displayed developmental delay. Studies of Ylikota et al. supports our results. Accordingly, it can be thought that LCM may cause developmental delay on the embryo.

Histopathological evaluation of the groups was made by caspase-3 immunohistochemistry and hematoxylin-eosin staining. Caspase-3 immunoreactivity intensities were evaluated with the H-Score scoring system. When the statistical data of the results were examined, it was seen that Caspase-3 immunoreactivities have increased due to LCM dose, and this increase was statistically significant in group 3 and group 4 compared to the control group. Hematoxylin-eosin staining was evaluated between groups and it was noted that the cell density with pycnotic nuclei increased with increasing doses of LCM. Although these pycnotic cells and caspase-3 positive cells in the groups were known to show cells undergoing apoptosis, it was thought that LCM drove the cells to death by following the apoptotic pathway. According to the results of TUNEL staining performed to show the presence of cell death, we found that TUNEL positive cells increased in groups with LCM doses, and this increase was significant in group 3 and group 4 compared to the control group. In the literature, Gürcü et al. showed that LCM treatment increased the number of cells with pycnotic nucleated morphology in Stage 9 embryos, according to the Hamburger Hamilton stages (19). In the study we presented, we showed that Caspase-3-stained cells and TUNEL-positive cells increased with the increase of the LCM dose. As Gürcü. et al. have shown, this increase suggests that apoptosis-mediated cell death is increased in embryos exposed to LCM (19). It can be said that LCM has a teratogenic effect during neural tube development, causes cells to die, and has a toxic effect on cells at doses of 400 mg/kg and 600 mg/kg in early chick embryo model. Although the mechanism of this lethal effect is not fully known, there are some studies in the literature that LCM inhibits neurite outgrowth in neurons and impairs tubulin polymerization in the cytoskeleton (20,21). As stated in these studies, disruption of cytoskeletal stabilization may mediate the death of cells. Mete et al. showed that LCM has neuroprotective, antiapoptotic, and antioxidative stress effects in their adult rat model study in 2021 (22). Although these results provide evidence that LCM is safe for use in adults, we think that its effects in the embryonic period are different.

CONCLUSION

According to our study, which was designed to mimic the doses used in humans in the early embryonic period, LCM appears to induce neural tube cells to die and at the same time, cause developmental delay. In order to elucidate the effects of LCM use during pregnancy, further studies designed to reveal the possible teratogenic effects of its use during pregnancy are needed. For the use of LCM during pregnancy, it may be recommended to make a decision considering the profit/loss ratio and to use it at low doses, where we think that teratogenic effects are less likely at low doses.

Acknowledgments: None

Conflict of interest: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive and specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author Contributions: FF, AB, EA, HD: Experimental duties**,** conceptualization, methodology, Data collection, formal data analysis, **FF:** writing and editing.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the institutional and/or national research committee's ethical standards and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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