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ORIGINAL ARTICLE

Evaluation of apoptotic cell death on liver and kidney tissues following administration of levetiracetam during prenatal period

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Abstract

Objective: Levetiracetam is a new generation antiepileptic drug used in treatment of patients with epilepsy and has adverse effects on different tissues. We aimed to evaluate the apoptotic effects of levetiracetam exposure during pregnancy on liver and kidney tissues of rat pups.
Methods: We analyzed the newborn rat pups exposed to levetiracetam during prenatal period. Fifteen pregnant female rats were divided into three groups. The group 1 and 2 rats were treated with different doses of levetiracetam (25 mg/kg/d and 50 mg/kg/d, respectively) from gestational days 1–22 during pregnancy. Group 3 (control group) was treated with the same volume of saline. Apoptosis was evaluated by the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) method. Liver and kidney tissues from rat pups were used for investigation.
Results: The percent of TUNEL positive apoptotic cells in group 1 were 22 and 17.5 for kidney and liver, respectively. The percent of TUNEL positive apoptotic cells in group 2 were 20.9 and 20.9 for kidney and liver, respectively. The percent of TUNEL positive apoptotic cells in group 3 were 18.4 and 17.1, respectively, for kidney and liver. The apoptotic index was the same in kidney and liver tissues of all groups.
Conclusion: Our results demonstrate that the prenatal exposure of levetiracetam has no apoptotic effects on liver and kidney of rat pups and, it has biosafety in pregnancy in terms of apoptosis. The first study evaluating the apoptotic effects on liver and kidney tissues following administration of levetiracetam during prenatal period.

Keywords

Apoptosis, kidney, levetiracetam, liver, prenatal exposure, TUNEL assay

History

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Introduction

Epilepsy is one of the common neurological disorders during pregnancy, and the antiepileptic drugs (AEDs) must be used. In general population, 0.3–0.4% of the women suffer from epilepsy while the pregnancies of epileptic women constitute ~1% of all pregnancies. The fetus of treated mothers with antiepileptic drugs are at increased risk in terms of major congenital malformations (MCMs) and fetal death [1,2]. The fetus is exposed to a variety of environmental agents and drugs, including AEDs, that passing through the placenta and can induce DNA damage [3]. It is of value to say passing through placenta than penetrating the placenta [4,5]. Pregnancies of woman with epilepsy is at the high-risk because of the more frequent complications, such as for MCMs and postnatal developmental anomalies [6–11]. Additionally, the risk of MCMs in newborn children of women with epilepsy treated with AEDs during pregnancy is higher than the 1–2% frequency in the general population [12]. In women with epilepsy, stopping the AED treatment during pregnancy may be dangerous [12], but the genotoxic effects of drugs must also be considered by clinicians on the newborn. Some defects and anomalies were observed in newborn whose mothers were treated with AEDs, especially in poly-therapy with 6% risk. On the other hand, the MCMs risk occurred 3.5% in the newborns of untreated epileptic woman during pregnancy [13–15].

Levetiracetam is a new generation AED initially approved as an adjunct treatment for patients with partial seizures [16]. Levetiracetam is a pyrrolidine derivative antiepileptic that binds to the synaptic vesicle protein SV2a, which is expressed throughout the brain. LEV binding to SV2a prevents neurotransmitter release and transport within the neuron [17]. SV2a receptor appears to be important in both partial and generalized seizure disorders [18]. While the loading doses of levetiracetam, which is effective in neonates are 10–20 mg/kg, the maintenance dose range of 10–80 mg/kg/day [19]. A low risk for MCM has been suggested with levetiracetam use in pregnancy [20], but there are limited data about the teratogenic and genotoxic effects of levetiracetam. Based on a

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number of studies, increased seizure risk during pregnancy was suggested with use of lamotrigine and oxcarbazepine, both of which are glucuronidated for elimination [21,22]. So far, to our knowledge, it has not published any study related to apoptotic effects of levetiracetam administration in pregnancy on the fetus.

Cells undergoing apoptosis are characterized by some morphological events that resulted in phagocytosis of these cells such as cell contraction and retraction from other peripheral cells, blebbing of the membrane, cellular and DNA fragmentation in case of apoptotic bodies. These alterations are subcellular changes, such as nuclear condensation and DNA fragmentation in apoptotic cells. The regular control of apoptosis required for normal renal development. Additionally, it has specified that the genetic and epigenetic mechanisms (such as heritable changes in gene activity that are not caused by DNA sequence changes) are playing a role for the regulation of apoptosis in nephron progenitors. Also, there are various environmental factors that influences apoptosis in the early kidney development [23]. TUNEL is a common and important method for detecting DNA fragmentation in apoptotic process. The method relies on the presence of nicks in the DNA that can be identified by terminal deoxynucleotidyltransferase (TdT), an enzyme that will catalyze the addition of dUTPs that are secondarily labeled with a marker. It may also label cells that have suffered severe DNA damage [24]. This study aimed to evaluate the apoptotic effects of levetiracetam exposure during pregnancy on liver and kidney tissues of rat offspring.

Material and methods

Animals

Sexually mature 12- to 14-week-old female Sprague–Dawley rats (Weighing 250–300 g) were obtained from Ondokuz Mayis University Laboratory Animal Research Center (Samsun, Turkey). Animals were housed in a quiet, temperature (22 °C)- and humidity-controlled room (50–60%) in a 12-h light/dark cycle, receiving food and water *ad libitum*. All procedures and protocols were conducted in accordance with the Guide for the Care and Use of Laboratory Animals [25]. The study protocols were approved by the Committee for Animal Experimentation of Ondokuz Mayis University (Approval number 011.43).

Experimental design

Female rats in oestrous were mated with one fertile male rat, respectively, and pregnancy was confirmed by the presence of a vaginal plug. And, this day was accepted as first gestational day. Fifteen pregnant female rats were divided into three groups of five and each rat was housed separately. Groups 1 and 2 were treated with different doses of levetiracetam (Abdi Ibrahim, Istanbul, Turkey) (25 and 50 mg/kg/d intragastrically, respectively) by gavage through a polyethylene tube during 1–22 gestational days until parturition. Group 3 (control group) was treated with the same volume of saline (1 ml/kg/d) by the same route. From first gestational day, until parturition, each presumably pregnant female was checked routinely for difficulties. The day of parturition was defined as postnatal day (PND) 1. On the 5th day of PND, the 10 pups in each group (two pups from each pregnant rat at random) were analyzed. In the analysis of rat pups on 5th day of PND, it was seen that the range of body weights were between 7–11 g.

TUNEL assay

Kidney and liver tissues were fixed in 10% neutral buffered formalin, embedded in paraffin wax. The tissue sections (5 μm) were mounted on a slide, dewaxed and rehydrated in graded alcohol solutions. DNA fragmentation for apoptosis was detected by the modified TUNEL method as described by Tuncdemir et al. [26] and following the manufacturer's instructions. TUNEL staining was performed using an apoptosis detection kit (Roche Diagnostics GmbH, Mannheim, Germany). On the slides, kidney and liver cells with brown nuclear labeling were considered as TUNEL-positive. Staining was evaluated using Olympus light microscope under 400X magnification.

Staining specificity controls

Thymus tissue sections from dexamethasone-treated rats (5 mg/kg, i.p.) were used as positive control [26,27]. For negative controls, distilled water was used instead of TUNEL reaction mixture.

Apoptotic index

Apoptotic index shows the ratio of the number of viable cells by apoptotic cells and this refers to the severity of apoptosis. On each slide, 15 Welds were randomly selected. To quantitate the extent of apoptosis, we recorded numbers of apoptotic cells (TUNEL-positive cells) in sections from the three groups. Apoptotic index was calculated according to the formula: $AI = (AC/AC + NAC) \times 100$. (Apoptotic index: AI, Apoptotic cell number: AC, Non-Apoptotic cell number: NAC) as expressed by Tuncdemir et al. [26]. We totaled all TUNEL-positive and intact cells in those Welds, and then calculated apoptotic index by means of an average count per slide.

Statistical analysis

Statistical analyses were performed using SPSS version 15.0 (Chicago, IL) for Windows. The number and percent of positive and negative apoptotic cells between three groups were compared by Chi-Square test. 95% confidence intervals (CIs) were calculated. p Values 0.05 or less were considered statistically significant.

Results

In the analysis of newborn pups, no significant gross external malformations were observed. Also, number of offspring were not different from controls in parturition. When apoptotic index values for groups treated with levetiracetam at 25 mg/kg/d and 50 mg/kg/d were compared with those of the control group, it was observed that levetiracetam did not significantly alter apoptotic index in the prenatal period (even at different doses). The percent of TUNEL positive apoptotic cells in group 1 (LEV 25 mg/kg/d) were 22 and 17.5, respectively, for kidney and liver. The percent of TUNEL positive apoptotic cells in group 2 (LEV 50 mg/kg/d) were 20.9 and 20.9,

Table 1. TUNEL positive apoptotic cells and negative cells in LEV administered groups and control group.

Assays	Groups	Group 1 (LEV 25 mg/kg/d) $n = 10$	Group 2 (LEV 50 mg/kg/d) $n = 10$	Group 3 (Control) $n = 10$	p values
Kidney	Positive apoptotic cells (%)	22	20.9	18.4	>0.05
	Negative apoptotic cells (%)	78	79.1	81.6	>0.05
Liver	Positive apoptotic cells (%)	17.5	20.9	17.1	>0.05
	Negative apoptotic cells (%)	82.5	79.1	82.9	>0.05

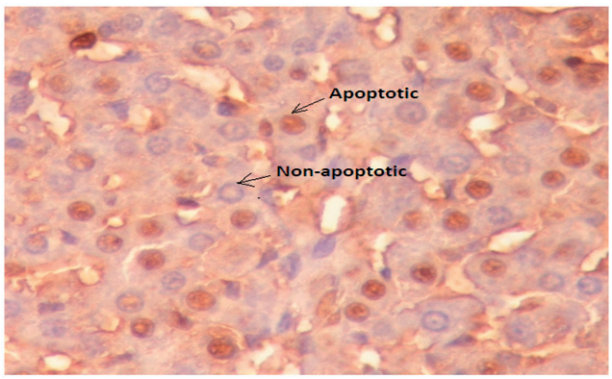

Figure 1. TUNEL-positive apoptotic liver cells and TUNEL negative (non-apoptotic, normal) liver cells.

respectively, for kidney and liver. The percent of TUNEL positive apoptotic cells (%) in group 3 were 18.4 and 17.1, respectively, for kidney and liver (Table 1 and Figure 1 and 2). The apoptotic index in liver and kidney tissues were the same in all groups. TUNEL-positive apoptotic cells and TUNEL-negative non-apoptotic cells are seen in Figure 2.

Discussion

Our study demonstrates that prenatal exposure to levetiracetam has no significant apoptotic effects on liver and kidney tissues of rat offspring. Levetiracetam also did not cause miscarriage in mother rats. There are limited data about the teratogenic effects of levetiracetam in the literature. The teratogenic potential of levetiracetam on organogenesis period was tested by different doses and, any gross external malformations were not observed in mice [28]. Currently, one of the most frequently used methods is TUNEL assay which is cost and easily applicable to detect apoptosis. Apoptosis is a physiological, programed process for the elimination of cells from living organisms. It has provided valuable information about apoptosis in various tissues. It is characterized by a number of distinct morphological alterations, such as chromatin condensation and marginalization, cell shrinkage and plasma membrane blebbing, which are accompanied by biochemical features such as DNA fragmentation, membrane alterations and degradation of specific cellular proteins, as a result of the massive activation of a large number of intracellular proteases and endonucleases. In the latest stages, the dying cell is fragmented into membrane bound vesicles containing relatively intact organelles and chromatin residues named apoptotic bodies [29].

In previous studies, Genton et al. determined that different doses of levetiracetam did not have embryo-toxic or teratogenic effects during the period of organogenesis in mice [30]. In rats, decreased fetal weight and minor skeletal abnormalities were seen at a dose of 3600 mg/kg/d, and in rabbits an increase in embryo-fetal mortality, an increase in minor fetal skeletal abnormalities and a decrease in fetal weight were observed at doses of 600–1800 mg/kg/d [30]. In the study of Ozyurek et al., the physical, motor and cognitive teratogenic effect of levetiracetam on prenatal period was tested by administering doses of 25, 50 and 100 mg/kg/day to rat. They showed that levetiracetam had only a transient impact on reflex maturation and no impact on physical and cognitive function in the offspring of rats exposed to the drug during pregnancy. They suggested that levetiracetam may become a promising candidate for the treatment of epileptic women in pregnancy, which our results support [16]. Also, there is no study about apoptotic effects on liver and kidney tissues of used levetiracetam on pregnancy in the literature.

Spengler et al. [31] reported that the levetiracetam may have potentially dangerous effects on renal function and they said that the epileptic patients should be closely monitored for changes in renal function while using levetiracetam. Therefore, it is important to investigate treated epileptic patients with levetiracetam and their offspring in terms of renal failure. Kılıcdag et al. [32] demonstrated that LEV administration after hypoxia reduces neuronal apoptosis and they proposed that LEV, due to such effects, may be a novel approach regarding control of seizure activity in neonates with hypoxic-ischemic brain injury. When all the results are evaluated together, levetiracetam is safe for many tissues. Tuncdemir et al. [26] investigated the protective effect of losartan as an AT1 receptor antagonist by evaluating the expression of apoptosis-regulatory genes that contribute to the progressive damage in the renal tubules of hyperoxaluric rats and they suggested that losartan may provide a beneficial effect against tubulointerstitial damage and decrease renal tubular apoptosis. In our previous study [33], we reported that levetiracetam exposure did not alter sister chromatid exchange and micronuclei frequencies on bone marrow in the prenatal period. Levetiracetam has been recommended by pediatric neurologists for the treatment of neonatal seizure due to these advantages [19].

Conclusion

Our results showed that the levetiracetam have no potentially dangerous effects on kidney and liver cells. This also means that levetiracetam taken by pregnant women with epilepsy does not evoke apoptotic effects on fetus. This study indicates fetal biosafety after prenatal exposure to levetiracetam to apoptotic effect in kidney and liver tissues. The first study evaluating the apoptotic effects of prenatal levetiracetam

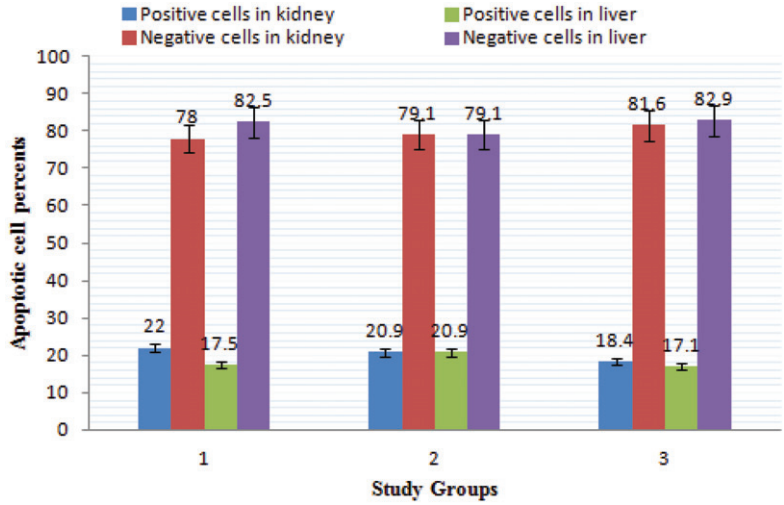

Figure 2. TUNEL positive and negative apoptotic cells in study groups.

administration on liver and kidney tissues of rat offspring. However, further work is required to confirm these findings in different study groups.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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