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ORIGINAL PAPER

Neuroprotective effect of levetiracetam on hypoxic ischemic brain injury in neonatal rats

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Abstract
Purpose Hypoxic-ischemic brain injury that occurs in the perinatal period is one of the leading causes of mental retardation, visual and auditory impairment, motor defects, epilepsy, cerebral palsy, and death in neonates. The severity of apoptosis that develops after ischemic hypoxia and reperfusion is an indication of brain injury. Thus, it may be possible to prevent or reduce injury with treatments that can be given before the reperfusion period following hypoxia and ischemia. Levetiracetam is a new-generation antiepileptic drug that has begun to be used in the treatment of epilepsy.
Methods The present study investigated the effects of levetiracetam on neuronal apoptosis with histopathological and biochemical tests in the early period and behavioral experiments in the late period.
Results This study showed histopathologically that levetiracetam reduces the number of apoptotic neurons and has a neuroprotective effect in a neonatal rat model of hypoxic-ischemic brain injury in the early period. On the other hand, we demonstrated that levetiracetam dose dependently improves behavioral performance in the late period.
Conclusions Based on these results, we believe that one mechanism of levetiracetam's neuroprotective effects is due to increases in glutathione peroxidase and superoxide dismutase enzyme levels. To the best of our knowledge, this study is the first to show the neuroprotective effects of levetiracetam in a neonatal rat model of hypoxic-ischemic brain injury using histopathological, biochemical, and late-period behavioral experiments within the same experimental group.

Keywords Hypoxic-ischemic brain injury · Neonatal · Levetiracetam · Apoptosis · Antioxidant enzymes · Behavioral experiment

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Introduction

Hypoxic-ischemic brain injury (HIBI) is one of the leading causes of death, cerebral palsy, mental retardation, and epilepsy that occur in the neonatal period worldwide. The immature brain is more susceptible to hypoxia and ischemia [1–3]. Hypoxia affects selected areas such as the thalamus, periventricular white matter, and the hippocampus [4]. Cell death may occur through necrosis, apoptosis, or both processes in HIBI [5]. The reperfusion period that follows hypoxia and ischemia is a period during which the most important injuries occur. Thus, it may be possible to prevent or reduce HIBI through treatments that are administered before the start of this period [6].

Levetiracetam (LEV) is a new-generation antiepileptic drug that has begun to be used in the treatment of epilepsy. Although its antiepileptic mechanism of action is not fully understood, it has been reported to act by reducing calcium in intracellular stores by partially inhibiting active N-type high-voltage calcium channels and/or by specifically binding to synaptic vesicle protein 2A (SV2A) [7–9]. The neuroprotective effect of LEV and its antiepileptic effects have been shown in animal studies [10, 11]. Hanon and Klitgaard showed that brain injury due to the ligation of the middle cerebral artery is reduced by LEV therapy in adult rats [11]. Kim et al. created a neonatal rat model of status epilepticus and histopathologically showed that LEV prevents apoptosis in the cerebral tissue at high doses [12]. Kilicdag et al. showed that LEV administration after hypoxia reduces neuronal apoptosis [13]. However, there are no animal studies showing the late-period effectiveness of LEV in HIBI. There have been a few recent clinical studies regarding its use in neonatal seizures [14, 15]. However, its effectiveness in reducing HIBI in the neonatal period and its effect on neuronal apoptosis are not fully understood. To the best of our knowledge, there are no studies that show the effect of LEV with behavioral experiments in a neonatal rat model of HIBI.

Hence, this study aimed to evaluate the effect of LEV on neuronal apoptosis with histopathological and biochemical tests performed in the early period and behavioral experiments in the late period.

Experimental procedures

This study was carried out by the Mersin University School of Medicine, Division of Pediatric Neurology and the Physiology, Pathology, and Biochemistry Departments. The study was carried out at the Mersin University School of Medicine Experimental Animals Research Laboratory and the Physiology Department Behavioral Experiments Laboratory. Approval of the Mersin University Animal Experiments Ethical Board was obtained for the study.

Induction of hypoxic-ischemic brain injury

Seven-day-old male Wistar rat pups were used in the study. Rats included in the study were divided into four groups as follows:

Group 1 (sham group) (n=27): After a median neck incision was made, ligation was not performed and no hypoxia was experienced performed. Four rats in this group died during the procedure.

Group 2 (saline treatment group) (n=27): Saline (0.2 ml) was injected intraperitoneally immediately after hypoxia.

Group 3 (100 mg/kg LEV treatment group) (n=27): The rat pups were intraperitoneally administered with 100 mg/kg of LEV dissolved in saline immediately after hypoxia.

Group 4 (200 mg/kg LEV treatment group) (n=27): The rat pups were intraperitoneally administered with 200 mg/kg LEV dissolved in saline immediately after hypoxia.

The animals were monitored at the Mersin University School of Medicine Experimental Animals Laboratory until the behavioral tests were completed. First, the rat pups were anesthetized via halothane inhalation for less than 5 min, and HIBI was induced according to the Levine-Rice model [16]. Then, incisions were made in the middle of the rats' necks, and the right common carotid artery was dissected and ligated with a 6–0 silk suture under microscopic magnification. The wound was sutured, and the animals were allowed a 2-h recovery and feeding period. Following this period, the rats, except for the sham group, were placed in a plastic chamber and exposed to a continuous flow of 8 % oxygen and 92 % nitrogen for 2 h. After the hypoxic period, the rats were allowed a 2-h recovery period with the mother rats in an open chamber without any supplemental oxygen. The sham group rats were taken, with their mothers, to an open chamber for the same intervals. After these procedures were completed, blood specimens were taken from nine rats from each group for malondialdehyde (MDA), glutathione peroxidase (GPO), superoxide dismutase (SOD), and catalase measurements by intracardiac intervention. The rats were then decapitated to evaluate early period neuronal apoptosis after the application of euthanasia and cervical dislocation. The brains were then removed for pathological evaluation. The brain tissue was removed, and apoptosis was evaluated histopathologically with the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and caspase-3 methods. The other remaining rats were subjected to a Morris water maze (MWM) test in the 14th week. In the 16th week, all rats were decapitated, and their bodies and brains were weighed.

Histopathological evaluation

Serial sections from paraffin-embedded coronal brain sections were deparaffinized in xylene. Next, the sections were dehydrated through graded concentrations of ethanol. The sections were heated in 0.01 mol/l citrate buffer in a microwave oven for 20 min following the blocking of endogenous peroxidase activity with hydrogen peroxide.

Apoptotic cell counting Apoptotic cell counting was performed in subthalamic nuclei, hippocampus, and parietal cortex of both the right and left hemispheres. In evaluating numeric density, total TUNEL and caspase-3-positive stained neurons were calculated in five high-power fields (5×400) under a light microscope. Microscopic examinations were made by a single pathologist (A.P.) who was unaware of the treatment groups.

TUNEL method The TUNEL method (executed with the in situ Apoptosis Detection Kit, Biogen, USA) was selected to investigate DNA fragmentation in the rats' neurons. After the sections were deparaffinized and rehydrated, they were processed with proteinase K for 15 min at room temperature. After this procedure, endogen peroxidase activity was quenched with 2 % H_2O_2. Slices were then incubated at 37 °C for 60 min in a moist chamber with 50 μl of TdT buffer. Finally, the reaction was visualized with a streptavidin-biotin-peroxidase complex and diaminobenzidine. TUNEL-labeled slides were counterstained with 1 % methyl green.

Caspase-3 method Sections were incubated for 1 h with a caspase-3 polyclonal antibody (dilution 1/100, Neomarkers, RB-1197-B0, USA) at room temperature in a humidified chamber. The sections were then stained using the avidin-biotin complex (ABC; Labvision, Fremont, USA) immunoperoxidase technique with a commercially available reagent. The sections were counterstained with Mayer's hematoxylin and mounting media (Labvision, Fremont, USA).

Biochemical evaluation

Blood specimens were taken by intracardiac intervention from rats in which HIBI had been induced on the seventh day following birth and were centrifuged at 3,000 rpm for 10 min and prepared as plasma. The specimens were then stored at −70 °C until they were measured. On the day of the measurement, the plasma was allowed to reach room temperature and was then analyzed. During the measurement process, MDA, GPO, SOD, and catalase levels were detected via the enzyme-linked immunosorbent assay (ELISA) method; MDA levels were measured with a Rat MDA ELISA Kit (catalog no.: CSB-E08558r, Cusabio Biotech Company, PRC); SOD levels were measured with a Superoxide Dismutase Assay Kit (catalog no.: 706002, Cayman Chemical Company, USA); catalase levels were measured with a Catalase Assay Kit (catalog no.: 707002, Cayman Chemical Company, USA); and GPO levels were measured with a Glutathione Peroxidase Assay Kit (catalog no. 703102, Cayman Chemical Company, USA).

Morris water maze test

The rats were subjected to the MWM test after they had completed their 14th week of life. Two days before the start of the test, the rats were brought to the behavioral laboratory and allowed to spend a time that was equal to the duration of the MWM test in that environment to ensure that all rats were exposed to the same stressors and were acclimated to the environment. Throughout the entire experiment, the same investigator wore the same color outfit and stood in the same spot in the room. Behavioral experiments were carried out on all groups between 0900 and 1400 h.. The tests were performed over five consecutive days. The 42-cm-deep maze was filled with 22 °C water. In the image that was transferred to the computer screen, the maze was divided into four equal quadrants: west, north, east, and south. On the first 4 days of the experiment, a 15-cm-diameter platform was set to a height of 40 cm so that it was hidden under water and placed in the middle point of the eastern quadrant. On the first day of the experiment, all rats, starting from the western quadrant and proceeding clockwise, were placed in the water once in each quadrant with their heads turned toward the wall of the water maze. On the second, third, and fourth days of the experiment, the first placements were in a different quadrant each day, proceeding clockwise from the first placement of the first day. The rats were allowed to swim for 60 s during each trial, and the rats that climbed onto the platform and stayed there for 5 s were considered to have completed that stage of the experiment and allowed to stay on the platform for 15 s. The rats that were unable to find the platform within 60 s were manually directed to the platform and allowed to stay on the platform for 15 s. The times required by the rats to find the platform were recorded for each trial every day for 4 days. The daily average time required to find the platform was calculated for each group. These times were compared between days within each group and between groups for each day. The differences in the times required to find the platform between the first and fourth days were compared among groups [17].

On the fifth day of the experiment, the hidden platform in the eastern quadrant was removed. All rats were placed in the water in the western quadrant once and recorded for 60 s. The time spent in the eastern quadrant, where the platform was previously located, was recorded in seconds [17].

At 16 weeks of age, the rats were decapitated, and their brains were taken out without distorting their

integrity. The brain tissues that were removed were macroscopically examined.

Statistical methods

The normalities of continuous measurements were examined with the Shapiro-Wilk test after data were entered into the SPSS 11.5 program package; all measurements were determined to be normally distributed. Within-group differences in MDA, SOD, GPO, catalase, body weight, and brain weights were tested with one-way ANOVAs. The homogeneities of the variances were controlled for with Levene's tests, and paired comparisons were performed with Tukey's tests. Paired sample *t* tests were used to compare TUNEL and caspase-3 data from the left and right hemispheres. Both the differences between the groups and the differences between the days within each group were analyzed with two-factor repeated-measures ANOVAs to compare water maze data from the first, second, third, and fourth days. On the fifth day of the water maze, repeated-measures ANOVAs were used to compare differences in the times spent in the northern, southern, eastern, and western areas. Moreover, paired sample *t* tests were used to examine differences between the average times spent in the northern, southern, and western areas. The average and standard deviation values are provided as descriptive statistics. A threshold of $p<0.05$ was adopted to indicate statistical significance.

Results

Ten of the 108 7-day-old neonatal rats included in the study died for various reasons. A total of 36 rats, 9 from each group, were decapitated in the second hour after HIBI. Sixty-two rats survived for the MWM test: 17 of these rats were from the sham group, 14 were from the saline group, 13 were from the 100 mg/kg LEV group, and 18 were from the 200 mg/kg LEV group.

Histopathological findings

Apoptotic cell numbers in the right and left hemispheres of the brain as detected with the TUNEL method after HIBI are shown in Table 1. The right-hemisphere apoptotic cell numbers in the saline group were found to be significantly higher than those of all other groups ($p<0.001$ for all three groups). Additionally, when the average right-hemisphere apoptotic cell numbers of the sham group were compared with those of the 100 mg/kg LEV and 200 mg/kg LEV groups, they were found to be significantly lower ($p<0.001$ and $p<0.021$, respectively) (Table 1). The right-hemisphere apoptotic cell numbers of the 200 mg/kg LEV group were significantly lower than those of the 100 mg/kg LEV group ($p=0.001$) (Table 1).

Table 1 The number of TUNEL-positive apoptotic neurons by group

Groups	Right	Left	P_0
Sham ($n=9$)	3.56±0.73	3.11±0.78	0.055
Saline ($n=9$)	10.33±1.12	6.33±1.94	*<0.001*
LEV 100 ($n=9$)	6.67±1.00	4.56±1.01	*0.001*
LEV 200 ($n=9$)	4.89±0.78	3.33±1.12	*<0.001*
P_1	*<0.001*	*0.004*	
P_2	*<0.001*	*0.019*	
P_3	*0.021*	*0.96*	
P_4	*<0.001*	*0.046*	
P_5	*<0.001*	*0.007*	
P_6	*0.001*	0.112	

Italicized values are significant ğ values which are lower than 0.05

P_0 the comparison between the number of apoptotic neurons in the left and right brain, P_1 sham-saline, P_2 sham-LEV 100, P_3 sham-LEV 200, P_4 saline-LEV 100, P_5 saline-LEV 200, P_6 LEV 100-LEV 200

The left-hemisphere apoptotic cell numbers of the sham, 100 mg/kg LEV, and 200 mg/kg LEV groups were significantly lower than those of the saline group ($p=0.004$, $p=0.046$, and $p=0.007$, respectively). The left-hemisphere apoptotic cell numbers of the sham group were significantly lower than those of the 100 mg/kg LEV group.

While no statistically significant differences were detected between apoptotic cell numbers in the right and left hemispheres of the sham group ($p=0.055$), the right-hemisphere apoptotic cell numbers of the saline, 100 mg/kg LEV, and 200 mg/kg LEV groups were significantly higher than those of the left hemispheres ($p<0.001$ for all three groups) (Table 1, Figs. 1 and 2).

The apoptotic cell numbers of the right and left hemispheres of the brains as detected with the caspase-3 method are shown in Table 2. The right-hemisphere apoptotic cell numbers of the saline and 100 mg/kg LEV groups were significantly greater than those of the sham group ($p<0.001$ for both). Furthermore, the right-hemisphere apoptotic cell numbers of the saline group were significantly greater than those of the 200 mg/kg LEV group ($p=0.003$) (Table 2, Fig. 3).

The average left-hemisphere apoptotic cell numbers of the saline group were significantly higher than those of the sham, 100 mg/kg LEV, and 200 mg/kg LEV groups ($p=0.005$, $p=0.043$, and $p=0.032$, respectively).

While no statistically significant differences were detected between apoptotic cell numbers in the right and left hemispheres of the sham group ($p=0.447$), the right-hemisphere apoptotic cell numbers of the saline, 100 mg/kg LEV, and 200 mg/kg LEV groups were significantly higher than those in the left lobe ($p=0.022$, $p=0.001$, and $p=0.006$, respectively) (Table 2, Figs. 3 and 4).

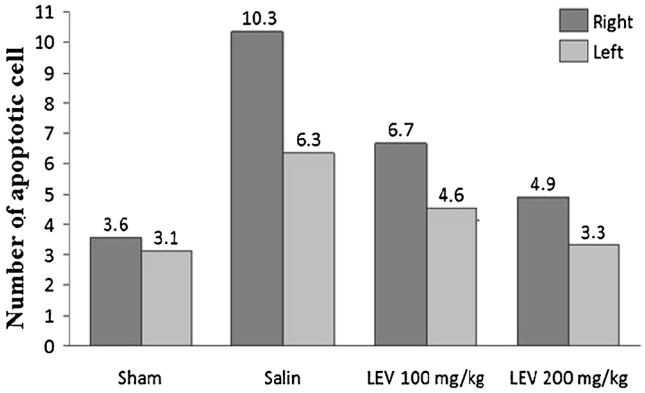

Fig. 1 The number of apoptotic neurons in the right and left sides of the brains by group as detected by the TUNEL method (group 1, $p=0.055$; group 2, $p<0.001$; group 3, $p=0.001$; and group 4, $p<0.001$)

Biochemical findings

MDA, catalase, GPO, and SOD levels were detected in the samples taken by intracardiac intervention after HIBI. MDA levels were significantly higher in the saline group than in the sham group ($p=0.02$). When other groups were compared to each other, no statistically significant differences were detected. In the intergroup comparisons of catalase levels, no statistically significant differences were detected. However, GPO levels in the 200 mg/kg LEV group were found to be statistically higher than those in the sham, saline, and 100 mg/kg LEV groups ($p<0.001$, $p=0.003$, and $p=0.013$, respectively). When the other groups were compared to each other, no statistically significant differences were detected. The SOD levels of the 200 mg/kg LEV group were found to be statistically higher than those of the sham, saline, and 100 mg/kg LEV groups ($p=0.033$, $p=0.030$, and $p<0.001$, respectively).

Table 2 The number of caspase-3-positive apoptotic neurons by group

	Right	Left	P_0
Sham ($n=9$)	3.89±0.78	3.67±0.71	0.447
Saline ($n=9$)	9.89±3.10	6.56±1.81	*0.022*
LEV 100 ($n=9$)	8.44±2.01	4.78±0.97	*0.001*
LEV 200 ($n=9$)	6.33±1.00	4.22±1.30	*0.006*
P_1	*<0.001*	*0.005*	
P_2	*<0.001*	0.062	
P_3	*0.056*	0.682	
P_4	0.41	*0.043*	
P_5	*0.003*	*0.032*	
P_6	0.121	0.737	

Italicized values are significant ğ values which are lower than 0.05

P_0 the comparison between the number of apoptotic neurons in the left and right brain, P_1 sham-saline, P_2 sham-LEV 100, P_3 sham-LEV 200, P_4 saline-LEV 100, P_5 saline-LEV 200, P_6 LEV100-LEV 200

When other groups were compared to each other, no statistically significant differences were detected.

Morris water maze learning and memory experiment

In Table 3, the average times required in finding the platform on the first, second, third, and fourth days of the MWM test and the statistical comparisons between the groups across to days are shown. The average times required to find the platform were gradually decreasing for the rats in the sham, 100 mg/kg LEV, and 200 mg/kg LEV groups, and this decrease was statistically significant ($p<0.001$). Examination of the average times required to find the platform across the first 4 days revealed that the rats in the sham and 200 mg/kg LEV groups were fasted to find the platform (Table 3).

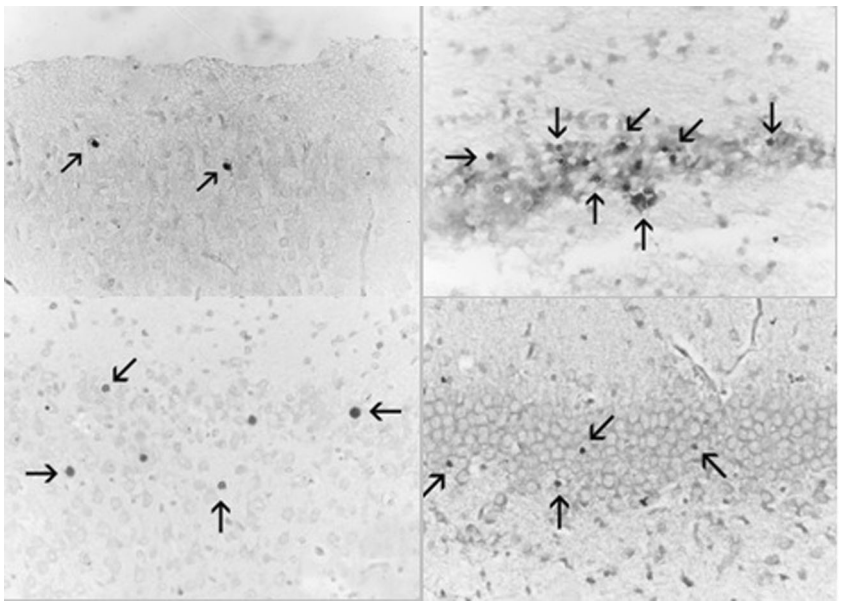

Fig. 2 TUNEL-positive apoptotic neuron by group. *Arrows* indicate the apoptotic neurons

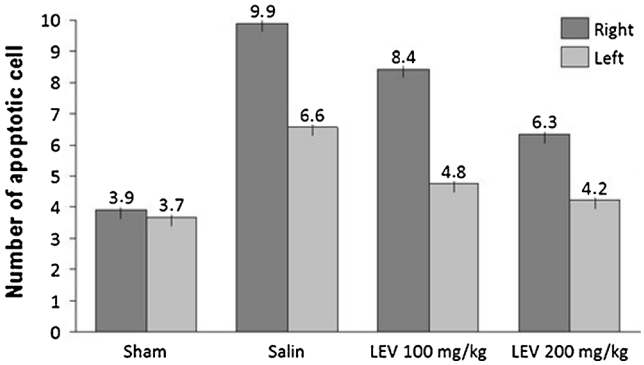

Fig. 3 The number of apoptotic neurons in the right and left sides of the brain by group as detected by the caspase-3 method (group 1, $p=0.447$; group 2, $p<0.022$; group 3 $p=0.001$; and group 4, $p=0.006$)

The differences between the times required in finding the platform on the first, and fourth days and the intergroup comparisons are shown in Table 3. No significant differences were detected between the sham and 200 mg/kg LEV groups; these groups exhibited the greatest reduction in the times required to find the platform. Moreover, statistically significant differences were found when these groups were compared to the saline and 100 mg/kg LEV groups ($p<0.001$ between sham, and saline and 100 mg/kg LEV; $p=0.001$ between 200 mg/kg LEV and saline; $p=0.015$ between 200 mg/kg LEV and 100 mg/kg LEV) (Table 3).

Rats in the sham and 200 mg/kg LEV groups were found to have spent the most time in the eastern quadrant of the maze on the fifth day of the MWM test. For both of these groups, the time spent in the eastern quadrant was significantly greater than the time spent in the other quadrants ($p<0.001$). In the saline and 100 mg/kg LEV groups, no statistically significant differences between the time spent in the eastern quadrant and the time spent in other quadrants were detected ($p=0.577$ for saline, $p=0.105$ for 100 mg/kg LEV).

Average body and brain weights of the rats

While the brains of the sham group were confirmed to be normal, the right hemispheres of the rats in the saline, 100 mg/kg LEV, and 200 mg/kg LEV groups (i.e., the groups in which HIBI was induced on the seventh day after birth) were observed to have contracted. The extent of this contraction was greater in the right hemispheres of the rats in the saline and 100 mg/kg LEV groups, and the borders of the hemispheres had more irregular appearances.

No statistically significant differences in brain weight were detected between the sham and 200 mg/kg LEV groups; these groups exhibited the highest brain weights. There was a statistically significant difference in average brain weight between these groups and the saline and 100 mg/kg LEV groups (Table 4). The rats in the sham group exhibited the greatest average body weight, and the lowest average body weight occurred in the 100 mg/kg LEV group. The average body weight of the sham group was statistically significantly greater than that of the 100 mg/kg LEV group ($p=0.045$) (Table 4). The highest average brain-to-body weight ratios among the groups were detected in the sham and 200 mg/kg LEV groups (0.74±0.07 and 0.7±0.1, respectively). The average brain-to-body weight ratios of both of these groups were significantly different from those of the saline and 100 mg/kg LEV groups ($p=0.001$ for both between group 1 and groups 2 and 3, $p=0.023$ for both between group 4 and groups 2 and 3) (Table 4).

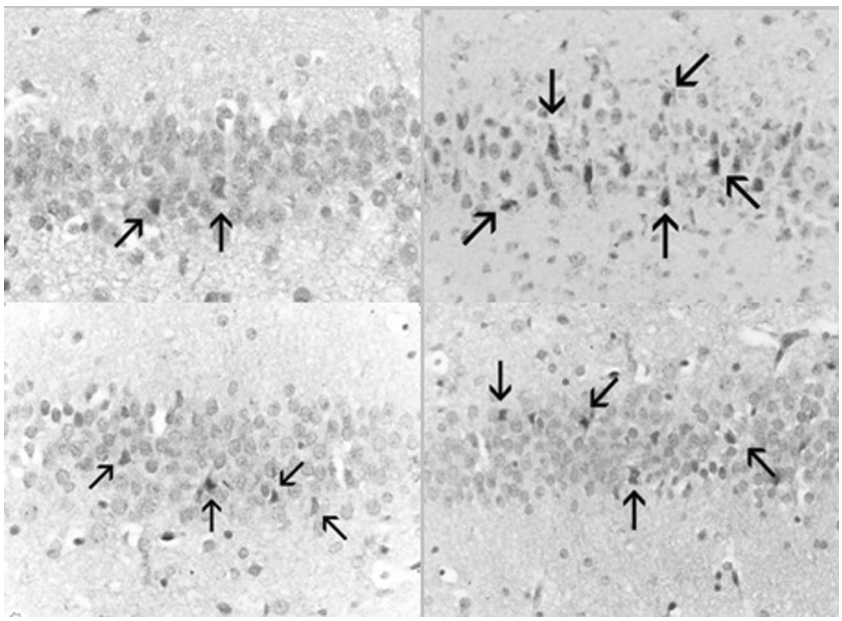

Fig. 4 Caspase-3-positive apoptotic neuron by group. *Arrows* indicate the apoptotic neurons

Table 3 The average time required to find the platform on the first, second, third, and fourth days, the difference between the times required to find the platform on the first and fourth day; and the intergroup comparisons

Groups	1st day (sn)	2nd day (sn)	3rd day (sn)	4th day (sn)	1–4-day difference (sn)
Sham (n=17)	48.82±20.64	32.08±21.73	18.52±16.88	12.29±12.22	36.53±2.89
Saline (n=14)	54.76±15.11	48.80±20.04	41.74±22.88	40.25±22.60	14.50±3.42
LEV 100 (n=13)	55.56±13.48	48.80±20.04	45.37±19.82	34.19±24.95	21.37±3.45
LEV 200 (n=18)	54.20±16.12	39.81±24.78	25.17±21.61	24.42±22.32	29.78±2.97
P_1	0.257	*<0.001*	*<0.001*	*<0.001*	*<0.001*
P_2	0.141	*0.001*	*<0.001*	*<0.001*	*<0.001*
P_3	0.322	0.207	0.181	*0.001*	0.451
P_4	0.991	0.781	0.814	0.552	0.063
P_5	0.997	*<0.001*	*<0.001*	*0.001*	*0.001*
P_6	0.956	0.616	*<0.001*	0.118	*0.015*

Italicized values are significant ğ values which are lower than 0.05

P_1 sham-saline, P_2 sham-LEV 100, P_3 sham-LEV 200, P_4 saline-LEV 100, P_5 saline-LEV 200, P_6 LEV 100-LEV 200

Discussion

HIBI and associated complications during the neonatal period are leading causes of neonatal death and disabilities throughout the world, despite developments in diagnostics and treatments [1–3]. Until recently, there has been no treatment option for HIBI other than support therapy, but there are now ongoing studies of many treatment methods, including allopurinol, magnesium sulfate, cooling therapy, stem cell transplantation, and antiepileptic drugs because the understanding of the physiopathology of HIBI is improving [1, 2, 6, 18–23]. This study evaluated the neuroprotective effects of LEV, an antiepileptic drug, with histopathological and biochemical tests in the early period and with the MWM test in the late period in a neonatal rat model of HIBI.

Evaluation of apoptosis

The neuroprotective effects of antiepileptic drugs in a neonatal rat model of HIBI have been reported by various studies [24–28]. The number of apoptotic neurons in neonatal rat models of HIBI has been shown to decrease significantly after the administration of valproic acid in the study of Kabakus et al. and after the administration of a combination of topiramate and memantine in the study of Liu et al. [24, 25]. Kilicdag et al. showed that LEV administration after hypoxic ischemia results to a significant decrease in the number of apoptotic cells in a neonatal rat model of HIBI [13]. Similar to the results of Kilicdag et al., we showed that apoptotic neuron numbers were significantly reduced in the 100 mg/kg LEV and 200 mg/kg LEV groups using the TUNEL method. Also, the caspase-3 indicated that, compared to the saline group, apoptotic neuron numbers were reduced only in the 200 mg/kg LEV group. These results histopathologically demonstrate that LEV reduces apoptotic neurons in a dose-dependent fashion in a neonatal rat model of HIBI.

Lipid peroxidation and antioxidant enzymes

In recent years, a parallel relationship has been found between increases in plasma MDA levels, which indicate oxidative stress, and the intensity of HIBI [29, 30]. In a study by Kumar et al., in neonatal rat model of HIBI, serum and BOS MDA levels and BOS/serum MDA ratios were found to be elevated compared to a control group [31]. Oliveira et al. found that in a pilocarpine-stimulated seizure model, the serum MDA levels of a group to which LEV was administered were significantly reduced [32]. Furthermore, in a study by Marini et al., LEV was shown to have the neuroprotective effect of reducing MDA levels after kainic acid-induced neurotoxicity [33]. In our study, plasma MDA levels were highest in the group that received saline after HIBI, and these levels were significantly different compared to those in the sham group (p=0.02). Plasma MDA levels were lower in the groups that received LEV compared to those in the group that received saline; however, there were no statistically significant differences

Table 4 The body and brain weights and the brain weight/body weight ratios of the rats by group

	Sham (n=17)	Saline (n=14)	LEV 100 (n=13)	LEV 200 (n=13)
Body weight (g)	254.52±20.58	236.16±23.30	232.69±21.49	246.85±22.72
Brain weight (g)	1.86±0.128	1.448±0.229	1.449±0.164	1.716±0.197
Brain weight/body weight	0.74±0.07	0.62±0.12	0.63±0.07	0.7±0.1

between the groups. These results showed that LEV decreased MDA levels, which are an indicator of oxidative stress, in rats in which HIBI was induced, although this decrease was not significant. Further studies are needed to speculate further on this issue.

In recent years, various studies have demonstrated a relationship between HIBI and antioxidant enzymes [26, 28, 32, 34]. Kumar et al. found that GPO, SOD, and catalase levels were elevated compared to a control group in a neonatal rat model of HIBI. This study suggested that the increases in these antioxidant enzymes are a mechanism of cell defense against the harmful effects of SOR in a neonatal rat model of HIBI [31]. Some recent studies have reported that LEV mediates its neuroprotective effect by increasing antioxidant enzyme levels [32, 35]. Oliveira et al. found that LEV decreases nitrite levels, prevents glutathione loss, and protects catalase activity in the hippocampus during seizures triggered by pilocarpine in adult rats. At the end of that study, the authors suggested that LEV mediates its neuroprotective effect by decreasing lipid peroxidation and hippocampal oxidative stress [32]. Ueda et al. reported that LEV mediates its neuroprotective action by decreasing glutamate levels and increasing the antioxidant effect [35]. Our results also showed strong similarities with the studies of Oliveira and Ueda. GPO and SOD enzyme levels were significantly elevated in rats that received 200 mg/kg LEV compared to the other three groups. We think that LEV may have induced this effect by increasing the levels of SOD and GPO, which are antioxidant enzymes. Moreover, this supposition is supported by the fact that LEV decreased MDA levels.

Behavioral experiments

The neuroprotective effects of antiepileptic drugs during the late period have been evaluated with behavioral experiments in neonatal rat models of HIBI in various studies [23–28, 36]. Noh et al. found that the times required to find the platform were similar for groups that had received topiramate and a sham group [28]. Although a few studies have shown that LEV reduces apoptosis in HIBI rat models and status epilepticus, the effects of this drug on the behavior during the late period have not been investigated. In our study, the results of the sham group and the group that was administered with 200 mg/kg LEV were similar in terms of the times required to find the platform over the first 4 days in the MWM test. However, the differences between the times required in finding the platform between the first and fourth days and the times spent in the eastern quadrant on the fifth day were different between these groups. There were also significant differences between these groups and the saline and 100 mg/kg LEV groups. Based on these findings, we conclude that LEV contributed favorably and dose dependently to the learning abilities of the rats. Moreover, the findings suggest that the use of LEV contributes favorably to both the management of seizures and, directly or indirectly, to learning abilities during the treatment of HIBI.

Brain and body weight

The greatest brain weights and ratios of brain to body weights were detected in the sham and 200 mg/kg LEV groups, and these values were found to be significantly higher than others. LEV was found to have positive and dose-dependent effects on brain and body weights in HIBI-induced rats. This finding suggests that feeding difficulties and malnutrition associated with HIBI also decrease as the severity of HIBI decreases.

In recent clinical studies, LEV has been shown to manage neonatal seizures without triggering significant side effects [14, 15, 37, 38]. Kim et al. reported that MK-801, phenytoin, and valproic acid cause apoptosis in neonatal rat models of status epilepticus and showed that LEV did not cause apoptosis doses much higher than the treatment dose [12]. In our study, LEV was also found to have a more marked neuroprotective effect at high doses (200 mg/kg). Based on these results, we believe that LEV may be useful for the treatment of neonatal seizures and HIBI because its neuroprotective effects are present across a wide range of doses, including high doses.

Moreover, this study used the TUNEL method to histopathologically show that LEV had neuroprotective effects that decreased the number of apoptotic neurons in the groups that received 100 or 200 mg/kg LEV. However, based on the caspase-3 method, neuroprotective effects were demonstrated only for the 200 mg/kg LEV group. We also found that LEV dose dependently positively contributed to behavioral performance in the late period. The present study showed that one of the neuroprotective mechanisms of LEV action may mediated by increases in antioxidant enzymes such as GPO and SOD that are formed during the hypoxic-ischemic and reperfusion periods. These results suggest that LEV may have potential for safe use in neonates with HIBI both to prevent injuries associated with ischemic hypoxia and to manage seizures. However, further studies of this issue are needed. To the best of our knowledge, this is the first study to show the neuroprotective effects of LEV in a neonatal rat model of HIBI using histopathological, biochemical, and late-period behavioral experiments within the same experimental groups.

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Conflict of interest The authors declare that they have no potential conflicts of interest.

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