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The effect of levetiracetam on neuronal apoptosis in neonatal rat model of hypoxic ischemic brain injury

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ABSTRACT

Background: Hypoxic–ischemic brain injury (HIBI) is a common cause of neonatal mortality and morbidity. The use of levetiracetam (LEV), as a potential neuroprotective in brain ischemia, receives an increasingly high attention, and it could have a crucial role in the regulation of epileptogenesis and neuroprotection. Potential effects of LEV on neuronal apoptosis in HIBI have not previously been reported in literature.
Objectives: The aim of this study is to evaluate the possible effects of LEV on neuronal apoptosis in neonatal rat model of HIBI.
Methods: Seven-day-old Wistar rat pups were subjected to right common carotid artery ligation and hypoxia (92% nitrogen and 8% oxygen) for 2 h. The pups were treated with LEV or saline after hypoxia. In sham group rats, neither ligation, nor hypoxia was performed. Neuronal apoptosis was evaluated by the terminal deoxynucleotidyl-transferase- mediated dUTP nick-end labeling (TUNEL) methods.
Results: The counts of apoptotic cells in both hippocampus and cerebral cortex were significantly higher in the saline treatment group than in the sham group. The counts of apoptotic cells in both hippocampus and cerebral cortex were similar to those in the sham group and in the LEV treatment group. The number of apoptotic cells decreased significantly in the LEV-treated group compared with the saline group.
Conclusions: These results show that LEV administration after hypoxia reduces neuronal apoptosis. Thus, we propose that LEV, as an effective antiepileptic and antiapoptotic drug, may be a viable choice for the control of seizure activity in neonates with HIBI.

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1. Introduction

Hypoxic–ischemic brain injury (HIBI) is a significant neurological problem of the perinatal period [1]. It has frequently been stated that hypoxia–ischemia is considered the most common cause associated with neonatal seizures [2–4]. Antiepileptic drugs (AEDs) are among some medications most commonly administered in neonates with HIBI. The mechanisms of such drugs, critical to controlling anticonvulsant activity, could also be conducive to neuroprotection. In other words, AEDs may not only control the seizure activity, but may also potentially suppress evolving injury independently of seizure control [5].

In brain injury of newborn animal models, topiramide (TPM) appears to be an effective antiseizure medication with evident neuroprotective properties. However, there is evidence of increased apoptosis in the white matter after high dose-TPM administration, although this was not observed in low dose-treated animals [6]. TPM confers neuroprotection by blocking AMPA/KA receptors and use-dependent Na^+ channel in developing rat brain [7].

Although controversial, results of some studies suggest that valproic acid can have therapeutic and protective effects in hypoxic–ischemic brain injury [8]. Similar studies suggest that valproic acid increases resistance against cerebral ischemia by affecting GABA receptor [9]; enhances expression of antiapoptotic genes, and inhibit DNA fragmentation in cerebellar granule cells [10].

Levetiracetam (LEV), as a pyrrolidine-derivative antiepileptic drug, is chemically different from all other antiepileptic drugs, with a new mechanism of action remaining ununderstood [11]. Hanon et al. reported that LEV possessed neuroprotective properties in the rat middle cerebral artery occlusion model of focal cerebral ischemia and that it reduced the infarct volume [12].

The effects of LEV on neuronal apoptosis in HIBI have not previously been reported.

The aim of this study is thus to evaluate this potential effect of the drug on neuronal apoptosis in neonatal rat models of HIBI.

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2. Materials and methods

Seven-day-old Wistar rat pups (n = 27) of either sex, delivered spontaneously, were used in this experimental study. The brain of the rat at this stage is histologically similar to that of a 32–34-week gestation human fetus or newborn infant, and this model has proved to be useful in many studies [13].

2.1. Animal preparation and surgical procedure

Rat pups were anesthetized by halothane inhalation. Hypoxic ischemia was induced according to the Levine–Rice model [14].

A median incision was made in the neck. Under microscopic magnification, the right common carotid artery was dissected and ligated with a 6/0 silk sutures. After the wound was sutured, the animals were allowed to have a 2 h recovery and feeding period. Except for the sham group, rats were then placed in a plastic chamber and exposed to a continuous flow of 8% oxygen–92% nitrogen for 2 h. The chambers were partially submerged in a water bath at 37 °C to maintain a constant thermal environment. After the hypoxic period, immediately saline or LEV injection was done. The doses of levetiracetam were described as a 10–80 mg/kg/day by Abend et al. These researchers' study demonstrates that dosing of 40 mg/kg/day was not associated with major adverse effects [15]. Therefore, we chose loading dose as a 80 mg/kg/day and maintenance dose as a 40 mg /kg /day. The brain growth spurt (i.e. the transient phase of rapid growth) of rats occurs postnatally with peak growth velocity on postnatal 7–10 days and ends in the third week. When hypotheses relating to brain vulnerability are formulated, this period should be taken into consideration [16]. Thus, we preferred a 7-day administration of treatment.

The pups were returned to their dams and kept in a room (12:12 h light/dark cycle) until being euthanized. Following these procedures and a seven-day LEV or saline treatment (postnatal 14th day), all pups were euthanized by intraperitoneal pentobarbital sodium injection and brains were removed for histopathological evaluation.

2.1.1. Saline treatment group (n:8)

Saline (0.5 ml) was injected intraperitoneally immediately after hypoxia and saline injection was repeated for seven days. Two rats died during the procedure.

2.1.2. Levetiracetam treatment group (n:8)

The rat pups were administered intraperitoneally loading dose of 80 mg/kg/day LEV which was dissolved in saline, and a maintenance dose of 40 mg/kg/day after hypoxia for seven days. One rat died during the procedure.

2.1.3. Sham group (n: 8)

After median neck incision was conducted, neither ligation nor hypoxia was performed.

All animal experiments follow a protocol approved by the ethical committee on animal research at Çukurova University.

2.2. Histopathological evaluation

All histopathological analyses described below were performed by a histologist and a pathologist blinded to rats' treatment. We evaluated all hippocampus and cerebral cortex.

2.3. Electron microscopy

Brain tissues for electron microscopic examination were immediately placed in 5% glutaraldehyde buffered at pH 7,4 with Millonig phosphate buffer for 3 h [17]. The tissue pieces were subsequently fixed in 1% osmic acid for 2 h. The samples were then dehydrated in graded ethanols; embedded in araldite, and processed for electron microscopy using conventional methods.

2.4. Light microscopy

The brain tissue removed from rats, submerged in a 10% formaldehyde solution, was subjected to routine follow-up procedures. The apoptotic cells were detected by using the ApopTag®Plus Peroxidase In Situ Apoptosis Detection Kit (ApopTag® S7101, Chemicon International, Inc. USA and Canada). ApopTag was applied to 5 μm histological slices prepared from paraffin tissue blocks through an immunohistochemical method. This kit detects apoptotic cells by labeling and detecting DNA strand breaks by the indirect TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling) method. Apoptotic cell counting was performed in hippocampus and cerebral cortex. In evaluating numeric density, total TUNEL positive stained neurons were calculated in 10 high power fields (10 × 400) under the light microscope [18].

2.5. Statistical analysis

All data were expressed as mean ± standard deviation (SD). Unpaired t test and ANOVA was used for statistical analysis. Multiple comparisons were made using Tukey's procedure with $p<0.05$ considered statistically significant.

3. Results

3.1. Light microscopic findings

TUNEL positive apoptotic cells of each group are shown in Figs. 1–3. The numbers of TUNEL positive apoptotic cells in both hippocampus and cerebral cortex were significantly higher in the saline treatment group than they were in the sham group ($p<0.000$). The counts of TUNEL positive apoptotic cells in both hippocampus and cerebral cortex were similar in the sham group and in the LEV treatment group (p: 0.218). The numbers of TUNEL positive apoptotic cells decreased significantly in LEV treated group compared with the saline group ($p<0.006$).

3.2. Electron microscopic findings

3.2.1. Sham group

Both in the cerebral cortex and the hippocampus, the nerve cells, the neuroglial cells, and the nerve fibers showed normal ultrastructure (Fig. 4).

3.2.2. Saline group

Both in cerebral cortex and hippocampus, most of the nerve cells exhibited clumping of nuclear heterochromatin, enlargement of granular endoplasmic reticulum cisternae, and an increase in lysosomes.

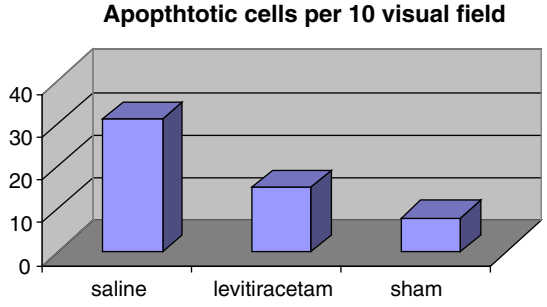

Fig. 1. TUNEL positive apoptotic cells in the cerebral cortex and the hippocampus of the sham, saline and levitiracetam treated animals. Saline vs sham, $p<0.000$ Levitiracetam vs. sham animals, $p<0.218$. Levitiracetam vs. saline treated animals, $p<0.006$.

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