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Original article

Unfavorable effect of levetiracetam on cultured hippocampal neurons after hyperthermic injury

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ABSTRACT

Background: The aim of this study was to examine the viability of neurons and the putative neuroprotective effects of second-generation antiepileptic drug, levetiracetam (LEV), on cultured hippocampal neurons injured by hyperthermia.
Methods: Primary cultures of rat's hippocampal neurons at 7 day *in vitro* (DIV) were incubated in the presence or absence of LEV in varied concentrations under hyperthermic conditions. Cultures were heated in a temperature of 40 °C for 24 h or in a temperature of 41 °C for 6 h. Flow cytometry with Annexin V/PI staining as well as fluorescent microscopy assay were used for counting and establishing neurons as viable, necrotic or apoptotic. Additionally, the release of lactate dehydrogenase (LDH) to the culture medium, as a marker of cell death, was evaluated. Assessment was performed after 9DIV and 10 DIV.
Results: Incubation of hippocampal cultures in hyperthermic conditions resulted in statistically significant increase in the number of injured neurons when compared with non-heated control cultures. Intensity of neuronal destruction was dependent on temperature-value. When incubation temperature 40 °C was used, over 80% of the population of neurons remained viable after 10 DIV. Under higher temperature 41 °C, only less than 60% of neurons were viable after 10 DIV. Both apoptotic and necrotic pathways of neuronal death induced by hyperthermia were confirmed by Annexin V/PI staining.
Conclusions: LEV showed no neuroprotective effects in the current model of hyperthermia *in vitro*. Moreover, drug, especially when used in higher concentrations, exerted unfavorable intensification of aponecrosis of cultured hippocampal neurons.

Introduction

Febrile seizures (FS), usually observed in patients in the course of infection with a fever above 38.5 °C, are the most common form of convulsive phenomena in children under 5 years old [1]. The International League Against Epilepsy (ILAE) defined FS as "a seizure in association with a febrile illness in the absence of a central nervous system infection or acute electrolyte imbalance in children older than 1 month of age without prior afebrile seizures" [2].

FS affect 2% to 10% of the general pediatric population [3]. FS are divided into simple and complex depending on the severity and duration of the seizures. Complex febrile convulsions are defined by the presence of prolonged seizures (lasting more than 15 min), with partial and multiple seizures occurring during the same day.

A number of studies have shown a significant relationship between a history of FS, particularly recurrent and prolonged FS, in childhood and the presence of hippocampal sclerosis and symptomatic temporal lobe epilepsy [4–6]. The incidence of such intractable epilepsy varied from 2% to 10% [7–9].

Histologically, hippocampal sclerosis is characterized by loss of nerve cells, pathologic neural network remodeling, and glial reaction (gliosis) [10]. Such changes are considered pathognomonic for the epileptogenesis process [11]. Loss of neurons, particularly in areas of the vulnerable CA1 and CA3 sectors of the hippocampus, was observed in animal experimental models of FS [12,13] as well as in patients with epilepsy and history of FS [14]. The most recent animal study performed by Saeedi Borujeni et al. indicates that FS disturbed the expression of both Bcl2 and Bax proteins, resulting in apoptosis of pyramidal neurons in the hippocampi of young rats [15].

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On the other hand, other authors found the opposite, that prolonged FS did not cause loss of neurons in the hippocampus, just its excessive neuronal excitability resulting from a reorganization of the hippocampal microstructure (mossy fiber sprouting) induced by fever [16].

Many authors suggest that FS alone are insufficient for the appearance of epilepsy and additional coexisting factors are necessary, e.g. genetic mutations in neuronal ion channels or focal cortical dysplasia (see [17], for review). According to the latest retrospective analysis by Asadi-Pooya et al., which included 262 patients with drug-resistant temporal lobe epilepsy resulting from hippocampal sclerosis, as many as 92 patients (31.5%) had a history of FS [18]. Thus, a statistical correlation between FS and hippocampal sclerosis seems obvious. Results of numerous volumetric magnetic resonance studies performed in patients with a history of FS confirm a correlation between early childhood prolonged FS, the severity of hippocampal atrophy, and temporal lobe epilepsy. The authors concluded that complex FS can produce acute hippocampal injury that evolves into hippocampal atrophy and subsequent unprovoked seizures in the future [14,19,20]. In a cohort study performed by Annegers et al. [21], the risk of developing epilepsy by age 20 was about 6% for all children who had experienced FS. However, this risk figure consisted of a combination of 2.5% of children without complex FS, and 17% of those with complex FS. The findings of an electroencephalographic study also supported the clinical observations [22].

Prophylactic treatment of FS is still controversial. Heretofore used drugs, such as diazepam or phenobarbital, are indeed effective in preventing FS recurrence, but permanent prophylaxis with phenobarbital may be associated with side effects, such as a decrease of cognitive functions [23]. Despite the fact that pharmacological prophylaxis of FS is currently not recommended, such treatment must be taken into account in some children.

Levetiracetam (LEV) is a second-generation antiepileptic drug (AED) with a unique mechanism of action that involves interactions with the synaptic vesicle protein 2A [24]. LEV has favorable, dose-proportional pharmacokinetics in children and a relatively rapid onset of action [25]. The drug has demonstrated good tolerability and efficacy against seizures as adjunctive therapy or monotherapy in children, including children aged 1 month to <4 years [26,27].

Data from the literature showed both favorable [28,29] and unfavorable [30,31] effects of LEV on nerve cell survival in varied models of neuronal injury.

In our previous study, we showed that LEV exerted a promising neuroprotective effect on cultured hippocampal neurons after hypoxic damage. The drug improved survival of neural cells in culture in a dose-depended manner [32]. It seems that pharmacological neuroprotection can be an interesting target for the prevention of fever-induced neuronal death within the hippocampus, hippocampal sclerosis, and symptomatic epilepsy. Because FS are provoked by fever, the idea of our experiment was to induce fever-like conditions *in vitro*. Therefore, we decided to study the putative neuroprotective effect of LEV on cultured hippocampal neurons injured by hyperthermia.

Materials and methods

Culture of hippocampal neurons

Primary cultures of hippocampal neurons were prepared from embryonic day 18 Sprague–Dawley rats as described previously [32,33]. The dissected hippocampi were purchased commercially and delivered in B27/Hibernate E from Brain Bits UK. Tissues were incubated with papain (Worthington) in Hibernate E medium (BrainBits) at 30 °C for 20 min, followed by mechanical trituration with a fire-polished Pasteur pipette. The mixture was transferred into B27/Hibernate E medium and the cells were centrifuged at $200 \times g$ for 1 min. The supernatant was quickly aspirated and the cells were resuspended in 1 mL of B27/Neurobasal medium (Invitrogen) with 0.5 mM Glutamax and 25 μM glutamate. Once in suspension, the number of viable cells was determined by trypan blue exclusion using a hemacytometer. Next, the cells were plated on 24-well plates coated with poly-D-lysine (Becton Dickinson) at a density of 32×10^3 cells/2 cm^2. Cultures were grown in a humidified incubator at 37 °C, 5% CO2. Half of the medium was replaced with NbActiv4 medium (BrainBits, USA) every 3 days. Under these culture conditions, more than 95% of the cells are neurons [34].

Drug preparation

Levetiracetam (LEV) was supplied from Sigma-Aldrich. The drug was dissolved in NbActiv4 medium (BrainBits, USA) at a concentration of 1 mM as a stock solution. The solution was further diluted with NbActiv4 medium to obtain the desired concentrations in the culture well: 50 μM, 100 μM, and 300 μM. The currently used LEV concentrations were chosen according to our previous experiment with LEV [23].

Model of hyperthermic shock

The experiment was performed after 7 days of culture. LEV solutions were added directly to the culture medium 120 min before exposure to hyperthermia. Then the cells were incubated under hyperthermic conditions i.e. at 40 °C for 24 h or at 41 °C for 6 h. Control cultures were incubated at 37 °C. Immediately after cessation of heat-insult the culture medium was changed completely and the cultures were returned to the incubator at 37 °C. Quantitative assessments of neuronal injury were done at two time points: 24 h (9 DIV) and 48 h (10 DIV) after cessation of hyperthermic shock. The neuronal cells were counted and established in flow cytometry and fluorescent microscopy.

Evaluation of cell death by lactate dehydrogenase (LDH) assay

Neuronal injury was evaluated by measuring LDH activity released in the media 24 h and 48 h after hyperthermia and LEV exposure, using colorimetric assay (Roche). The experiment was performed as per manufacturer's instructions. The intensity of the red color formed in the assay and measured at a wavelength of 490 nm was proportional to LDH activity and the number of damaged cells. The data were normalized to the activity of LDH released from the control culture media (100%) and expressed as a percentage of this control. The experiment was performed three times with 3 wells per condition each time.

Flow cytometry with annexin V/PI staining

Flow cytometric analysis with a detection apoptotic kit (BD Biosciences) was used for counting and distinguishing necrotic from apoptotic cell death. Cells undergoing apoptosis were detected with the use of double staining with Annexin V-FITC/PI in the dark, according to the manufacturer's instructions. The hippocampal neurons that had been treated as mentioned above were harvested by 0.25% trypsin-EDTA, washed twice with cold phosphate buffer solution (PBS), and then resuspended in $1 \times$ binding buffer at a concentration of 1×10^6 cell/ml. To a 100-μl aliquot of the cell suspension, 5 μL of FITC-conjugated annexin V and 5 μL of propidium iodide (50 μg/ml) were added. After 15 min incubation in the dark at room temperature, the cells were analyzed within 1 h with a flow cytometer. Annexin V-FITC