Memory Impairment Allied to Temporal Lobe Epilepsy and its Deterioration by Phenytoin: A Highlight on Ameliorative Effects of Levetiracetam in Mouse Model

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Abstract

Background/Objectives Memory impairment (MI) and epilepsy go hand in hand, mainly in conditions of temporal lobe epilepsy (TLE). This disease comorbidity has been reported to worsen upon treatment. Hence this study aims to evaluate the extent of aggravating effect of phenytoin (PHT) at normal and reduced doses on MI associated with TLE and additionally assesses the protective effect of levetiracetam (LEV) on these adverse effects.

Methods Swiss albino mice of either sex (n = 36) were used for this study in which seizures were induced by intraperitoneal administration of pilocarpine (300 mg/kg i.p.) followed by evaluation of antiepileptic activity by technique of Racine’s scale for convulsive scores. Errors (a factor denoting MI) were assessed using radial arm maze. Finally brain biochemical measures of acetylcholinesterase and glutamate along with cresyl violet staining and estimation of total neuronal number of the hippocampus were performed.

Results Exacerbation of MI by PHT was observed, where the extent of MI was found to be lesser in the reduced dose approach (PHTR: 28.50 ± 1.03; p ≤ 0.05). However, this tactic in dose reduction was interfered with the antiepileptic potential of the drug. Attenuation of MI upon combining with LEV, without an interference in the principal treatment, was observed equally in the behavioral and brain aspects of the study (PHTN + LEV: 1.33 ± 0.33 and PHTR + LEV: 1.00 ± 0.17; p ≤ 0.05).

Conclusions The promising effects of LEV could thus aid in proposing a new management remedy for TLE to minimize the adverse effect associated with it.

Introduction

Epilepsy resembles a neurodegenerative disorder, in which memory impairment (MI) is a common and undesirable factor.¹ This is distinctively an observation found in cases of patients suffering from temporal lobe epilepsy (TLE)—a form of status epilepticus (SE) that is a life-threatening situation characterized by an epileptic seizure greater than 5 minutes or more than one seizure within a 5-minute period.²³ Because the mainstay of treatment is the administration of antiepileptic drugs (AEDs) intended for the disease, reports of the same to have a negative impact on memory are not desired or accepted.⁴ Therefore, a treatment therapy for epilepsy, which is devoid of worsening cases of MI, is a necessity in today’s scenario.

The pilocarpine model of SE is one of the well-established animal models for SE and shares many of the characteristics of human TLE. It is reported that pilocarpine (muscarinic
cholinergic agonist) could induce robust limbic seizures when systemically administered to mice.\textsuperscript{5,6} Here phenytoin (PHT) is elected as the AED under evaluation, which has been approved and widely used till date as a first-line agent for the treatment of TLE, with outcomes to support its worsening states on the condition of memory.\textsuperscript{7} Because a variety of racetams (drugs sharing a pyrrolidine ring) have been commonly used as memory enhancers, the selection of LEV was based on the information that it additionally functions as an adjunct antiepileptic in the management of partial seizures\textsuperscript{8,9}. Other studies involving reports of LEV to have positive effects on cognition in cases of Tourette’s syndrome and Alzheimer’s disease were taken into consideration.\textsuperscript{10,11} An alternative rationale and benefit for the selection of LEV is its pharmacokinetic parameters that include faster absorption, negligible binding to plasma proteins, lack of enzyme induction, absence of interactions with other drugs, and restricted metabolism outside the liver.\textsuperscript{12}

Therefore, this study aims at evaluating the effect of the chosen AED on MI associated with TLE (the authors have taken mouse model of TLE as a continuation study based on the previous study involving generalized seizures and preferred when compared with other models of epilepsy due to its feasibility and ease of operation) at normal and reduced doses (not less than its effective dose) upon combination with LEV using a mouse model.

Methods

Animals

Albino mice (n = 36) of either sex weighing 30 to 35 g were procured from the Central Animal House Facility of JSS Medical College, Mysore (CPCSEA 261/PO/ReBi/2000/CPCEA, date: October 16, 2015–October 15, 2018). They were housed in polypropylene cages (mice were separated in cages based on their sex) with free access to food and water, at an ambient temperature 26°C, humidity 50 to 60%, and 12:12 light/dark cycle. All efforts were made to minimize animal suffering, and chronic animal protocols were designed to reduce the number of animals used. Experiment protocols were performed in accordance with the approval of institutional animal ethics committee (IAEC) of JSSCP Mysore (proposal number 162/2014).

Drugs, Chemicals, and Materials

Phenytoin (normal 24 mg/kg and reduced 12 mg/kg body weight, p.o.) and LEV (25 mg/kg body weight, i.p.) required for the study were procured from Shivari Pharmaceuticals, Mysore, Karnataka, India. The doses were selected with slight variation based on their effectiveness as an antiepileptic and their toxic profile from the available literature.\textsuperscript{14,15} Pilocarpine HCl (300 mg/kg body weight, i.p.) from Sigma Aldrich was used to produce repetitive limbic seizures. Laboratory-made radial arm maze (RAM) was used in determining MI activity and Sony Handycam was used to record the arm preference and errors. Acetylthiocholine iodide, cage hydrate powder of glutamate and Whatman number 1 chromatography paper (Sigma Aldrich), butanol, acetic acid, ninhydrin, and cupric sulfate of analytical grade were used, provided by the JSS College of Pharmacy, Mysore, Karnataka, India. Brain studies involving histopathological staining and total neuronal number were performed by the Medall Clumax Diagnostic, Mysore, Karnataka.

Pilocarpine Model of Status Epilepticus

In this model, systemic injection of the pilocarpine induces SE, likely through activation of M1 muscarinic receptors, followed by a seizure-free latent period and eventually the appearance of recurrent seizures that continue for the rest of the animal’s life. By monitoring the behavioral severity of each seizure, scores on a standard 0–5 Racine scale were given.\textsuperscript{16}

The animals were treated as per the schedule (– Table 1) for 64 days (this period of study was developed as acute administration of PHT could not provoke worsening in conditions of MI when observed by hippocampal staining although behavioral alterations were present). Anticonvulsant potential of PHT (24 mg/kg p.o.) and PHT (12 mg/kg p.o.) in absence/presence of LEV (25 mg/kg p.o.) was assessed every seventh day for a period of 64 days. Convolusions were induced by intraperitoneal administration of pilocarpine 300 mg/kg on every sixth day. Seizures in control animals were controlled by the administration of diazepam 5 mg/kg. LEV as well as PHT was given orally 2 and 1 hour, respectively, before induction of convulsions. Convulsive scores were finally given (based on Racine’s scale for seizures) as follows: 0: no response, 1: hypoactivity, 2: monoclonic jerks of the head and head bobbing, 3: bilateral activity of the whole body, 4: rearing of limbs, and 5: generalized tonic–clonic activity and loss of posture.\textsuperscript{17}

Memory Impairment Activity of Phenytoin by Radial Arm Maze

The animals were divided into different groups as shown in Table 1, and MI activity was evaluated every week for a period of 64 days. Each animal was trained prior to the start of experiment on a daily basis for a period of 7 days in the maze to collect the food pellets. The maze was kept well illuminated. A modification of rewards by food pellets were replaced by application of butter in the inner areas of the escape box. This was done to help the animal in finding the escape box with the aid of olfactory senses. Once trained, the process of application of butter was excluded. During the test, mice were fed once a day and their body weights were maintained at 85% of their free feeding weight to motivate the animal to run the maze. The session was terminated after 120 seconds, and the animal had to find the escape box with a minimum number of errors.\textsuperscript{18}

Estimation of Brain Acetylcholinesterase Levels

The reagents used were as per – Table 2. Mice, after 64 days, were euthanized using carbon dioxide method and brains were removed quickly and placed in ice-cold saline. Frontal cortex was quickly dissected out on a petri dish chilled on crushed ice. The tissues were weighed and homogenized in 0.1 M phosphate buffer (pH 8.0). A 0.4 mL aliquot of the homogenate was added to a cuvette containing 2.6 mL phosphate buffer (0.1 M, pH 8.0) and 100 μL of DTNB. The contents of the cuvette were mixed thoroughly by bubbling air, and
absorbance was measured at 412 nm in a spectrophotometer. When absorbance reached a stable value, it was recorded as the basal reading. Substrate, that is, acetylthiocholine (20 µL), was added and change in absorbance was recorded. Change in the absorbance per minute was thus determined.\textsuperscript{19,20}

The enzyme levels = $\Delta A \times V_t/€b \times V_s \times X$

where $\Delta A$ is change in absorbance, $V_t$ is total volume (3.1), $€$ is 13610*10$^4$, $b$ is path length (1 cm), $V_s$ is sample volume (0.4 mL), and $X$ is mg protein of brain tissue.

**Estimation of Brain Glutamate Levels**

**Preparation of Reagents**

Solvent: butanol:acetic acid:water (12:3:5): 60 mL of butanol, 15 mL of acetic acid, and 25 mL distilled water was added; 0.25% ninhydrin: 200 mg of ninhydrin was dissolved in 99 mL of acetone. To this solution, 1 mL of pyridine was added, and 0.005% CuSO$_4$ solution: 5 mg of cupric sulfate was dissolved in 10 mL 75% ethanol.

**Standards**

Solvent: 2 µM glutamate: 2.942 mg of glutamate was dissolved in 10 mL distilled water.

After the 64-day treatment schedule, different brain regions were dissected and homogenized in 80% double-distilled ethanol (for every 100 mg of the brain tissue, 2 mL of 80% alcohol was used). Homogenates were transferred to polypropylene tubes and centrifuged at 1,200 rpm for 10 minutes. One milliliter of the supernatant was then transferred into small test tubes and evaporated to dryness at 70°C in an oven. The residue was reconstituted in 100 mL distilled water, and 10 mL was used for spotting on Whatman number 1 chromatography paper. Standard solutions of glutamate at a concentration of 2 mM were also spotted using an Eppendorf micropipette; the spots were dried with a hair drier. The chromatograms were then stitched at the sides and placed in a chromatography chamber containing butanol:acetic acid:water (65:15:25, V/V) as solvent. When the solvent front reached the top of the papers, the papers were removed and dried. A second run was performed similarly, after which the papers were dried, sprayed with ninhydrin (0.25% in acetone with 1% pyridine), and placed in an oven at 100°C for 4 minutes. The portions that carried glutamate corresponding with the standard were cut and eluted with 0.005% CuSO$_4$ in 75% ethanol. Their absorbance was read against a blank in an LKB 4050 spectrophotometer at 515 nm, and the levels were expressed as µmoles per gram wet weight tissue.\textsuperscript{21}

**Calculations**

Glutamate levels = Unknown OD * Standard (3 mg) * 100/ Standard OD * Volume spotted (10 μL) * X

where $A$ is amino acid content in μmoles per gram wet weight tissue, 100 is conversion factor for gram wet weight tissue, and $X$ is weight of the tissue in gram.

**Histopathology**

Mixing Cresyl Violet for 300 mL Staining Wells

Cresyl violet stock solution: 0.2 g cresyl violet-acetate was mixed with a stir bar in 150 mL distilled water for 20 minutes.
Buffer solution pH 3.5: 282 mL of 0.1 M acetic acid (6 mL of concentrated acetic acid per 1,000 mL distilled water) was added to 18 mL of 0.1 M sodium acetate (13.6 g in 1,000 mL distilled water). Finally, 30 mL of cresyl violet stock solution was added to 300 mL of buffer and mixed for 30 minutes.

**Staining Procedure for Frozen Sections**
The well containing stain was placed in an oven or incubator for at least 1 hour at 60°C prior to staining. Sectioned tissues were mounted on slides that were loaded on to holders 20 minutes before staining to stabilize to room temperature. The holder was then placed into the wells containing the following solutions for the time indicated.

Xylene (5 minutes), 95% alcohol (3 minutes), 70% alcohol (3 minutes), deionized distilled water (3 minutes), cresyl violet acetate (8 minutes) at 60°C (oven), distilled water (3 minutes), 70% alcohol (3 minutes), 95% alcohol (1–2 minutes), and 100% alcohol (up to 10 dips to remove any streaks; one dip if no streaks were found). Care was taken not to remove all the stain. Xylene (5 minutes) was placed in next xylene well, and lid was kept closed. The slides were allowed to stay in the well until cover slipped (up to 24 hours) using a xylene-based mounting media and top-grade coverslips. For fewest air bubbles and best long-term slide storage, the slides were placed for a combined total of at least 30 minutes in xylene.22,23

**Total Neuronal Number**
The total neuronal number of subregions of CA1 and CA3 regions of the hippocampus was estimated by using the method of optical fractionator described by West et al.24

**Data and Statistical Analysis**
The values are expressed as mean ± SEM, n = 6, analyzed by one- and two-way analysis of variance (ANOVA) followed by Tukey’s post hoc test in which \( p \leq 0.05 \) was considered significant. Graph pad prism version 6 was used for statistical analysis.

**Results**

**Antiepileptic Activity**

Fig. 1 and Table 3 illustrate convulsive scores based on Racine’s scale for seizures. A lessened convulsive score was

![Convulsive Scores](image)

**Table 3** Antiepileptic activity of normal and reduced doses of PHT both alone and in combination with LEV (percentage protection)

<table>
<thead>
<tr>
<th>Day</th>
<th>PHTN</th>
<th>PHTR</th>
<th>PHTN + LEV</th>
<th>PHTR + LEV</th>
</tr>
</thead>
<tbody>
<tr>
<td>63</td>
<td>60.0</td>
<td>40.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
</tbody>
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Abbreviations: LEV, levetiracetam; PHT, phenytoin; PHTN, phenytoin normal dose; PHTR, phenytoin reduced dose.
seen in both PHTN (2.00 ± 0.21; p ≤ 0.05) and PHTR (3.00 ± 0.27; p ≤ 0.05) than the control group having a convulsive score of 5.00 ± 0.15. Addition of LEV for groups of both PHT (i.e., PHTN + LEV and PHTR + LEV) further reduced the convulsive scores to 0.00 ± 0.00; p ≤ 0.05 and 0.00 ± 0.00; p ≤ 0.05, respectively.  ►Fig. 2 shows the percentage mortality of mice for the study period of 64 days where the results of control or pilocarpine-treated group were found to be 20.00 ± 0.24; p ≤ 0.05.

**Memory Impairment Activity**

►Fig. 3 denotes error scores obtained from RAM, where the control group increased the same after a study period of 64 days by 21.20 ± 0.20; p ≤ 0.05 than the normal group (0.00 ± 0.00). A similar increase in the errors was detected in PHTN group by 35.50 ± 0.56; p ≤ 0.05, which was found to be more than that of control. Out of these, noticeable measurement of errors was revealed in the reduced dose of PHT, that is, in PHTR (28.50 ± 1.03; p ≤ 0.05), which was found to be less than that of PHTN. When looking at the combination groups involving LEV, there was an extreme decline in error scores in groups PHTN + LEV (1.33 ± 0.33; p ≤ 0.05 compared with control and PHTN) and PHTR + LEV (1.00 ± 0.17; p ≤ 0.05 compared with PHTR).

**Acetylcholinesterase Levels**

►Fig. 4 represents AChE levels, in which the control group (7.52 ± 0.71; p ≤ 0.05 µmoles/mg protein) increased the enzyme levels than the normal (2.84 ± 0.48 µmoles/mg protein). An intensified escalation of the same was found in cases of PHTN (15.78 ± 0.86; p ≤ 0.05 µmoles/mg protein) and PHTR (10.48 ± 0.50; p ≤ 0.05 µmoles/mg protein) than the control. However, a reduction in the above enzyme rise was brought about in groups of PHTN + LEV (6.58 ± 0.94; p ≤ 0.05 µmoles/mg protein) and PHTR + LEV (5.17 ± 0.87; p ≤ 0.05 µmoles/mg protein).

**Glutamate Levels**

►Fig. 5 is an indication of brain glutamate measures. These levels were found to increase in the control (8.23 ± 0.40; p ≤ 0.05 µmoles/mg wet weight tissue) compared with the normal of 2.07 ± 0.31 µmoles/mg wet weight tissue. Both sets of treatment groups of PHTN and PHTR witnessed an increment in levels of glutamate (15.78 ± 0.91 and 12.78 ± 0.83; p ≤ 0.05 µmoles/mg wet weight tissue), respectively, compared
with that of normal and control groups. In treatment groups of PHTN + LEV (7.58 ± 0.79; \( p \leq 0.05 \)) and PHTR + LEV (5.51 ± 0.53; \( p \leq 0.05 \)), a decrease in the levels of the excitatory amino acid were detected than control, PHTN, and PHTR groups.

**Histopathology**

The neurodegeneration process in areas of CA1 and CA3 (subregions of the hippocampus mainly involved in memory processes) upon cresyl violet staining has been explained in Fig. 6. Apart from this, a quantitative method called the optical fractionator was used to estimate the number of neurons in regions of CA1 and CA3 as shown and described in Fig. 7. It was found that PHTN reduced the total number of neurons by CA1: 45,521 ± 5,350 and CA3: 49,763 ± 7,701; \( p \leq 0.05 \) when compared with control (CA1: 89,564 ± 8,314 and CA3: 99,280 ± 4552; \( p \leq 0.05 \)) and normal (CA1: 164,350 ± 13,917 and CA3: 179,460 ± 17,205; \( p \leq 0.05 \)) groups. However, the reduction was less in case of PHTR (CA1: 143,933 ± 13,172 and CA3: 167,098 ± 12,518; \( p \leq 0.05 \)) in combination with LEV, the extent of reduction in total neuronal number was very much less when compared with control, PHTN, and PHTR groups.

**Discussion**

Epilepsy and MI go hand in hand, and this adverse effect tends to worsen upon AED administration. The hypothesis of...
this study, that is, to correct the worsening of the above said adverse effect by co-administering an antiepileptic memory enhancer, was achieved by using LEV.

Antiepileptic Activity
The pilocarpine mouse model shares many of the characteristics of human TLE. Interpretation of convulsive scores by pilocarpine induced SE can be described as follows: the lesser the convulsive score, the better is the AED potency. Considering Table 3, the tactic used to obtain a lesser degree of MI, that is, by dose reduction of the AED, produced a disagreeable outcome of reduced efficacy. A significant correction by LEV, when combined with PHT, was attained along with a better antiepileptic potential than monotherapy of the AED used for treatment. This could therefore support the fact that polytherapy of LEV with an AED should be preferable than monotherapy.

Memory Impairment Activity
Radial arm maze that was used for evaluating MI has been extensively used for the evaluation of working and reference memory. Here error scores from RAM and MI are in proportion to each other (i.e., increased errors are an indication of increased degree of MI and vice versa). This is the basic principle applied for interpretation of RAM observations. Increased error scores of the control group were found to be significant when compared with the normal group, thus authenticating the declaration that MI is in association with TLE. A substantial advancement in errors were noticed in both PHTN and PHTR than control. This substantiates the fact that an AED itself has a self-governing potential to impair memory in addition to exacerbate the condition of MI. The dose reduction of the AED, that is, in PHTR, brought about a significant decrease in magnitude of MI than PHTN, which supports the approach of lowering the degree of MI by dose reduction. Furthermore, the addition of LEV to the treatment regimen had a superior impact than the approach of reduced dose of AED through monotherapy that was a noteworthy observation when compared with the control group.

Brain Acetylcholinesterase Levels
The implication of acetylcholine to memory is not a current awareness. Both normal and reduced doses had a negative impact on memory, which was observed by a significant increase in AChE levels. The reason for this increment by PHT could be via oxidative stress that enhanced the AChE activity, thereby depleting the levels of acetylcholine in brain regions resulting in subsequent MI. When considering AD, cholinergic deficit is one of the factors governing the disease and use of acetylcholinesterase (AchE) inhibitors for the symptomatic treatment of AD involved cognitive dysfunction is common. Additionally, LEV has been found to be beneficial in the case of AD by improving the cognition deficits. Hence based on these reports and results of decrement in levels of AChE when combined with LEV, this combination molecule may possess AChE inhibitory activity.

Brain Glutamate Levels
Although there is a link between glutamate levels and epilepsy, no strong indication of this amino acid to be proportionate to MI due to epilepsy is available. However, memory and glutamate concentration are unique to patients suffering from multiple sclerosis where TLE is said to be its manifestation. Therefore, cognitive impairment involving MI could be a common link found in both conditions due to glutamate.
The neurotoxic property of glutamate, above normal levels, could be another causative factor of MI. Considering these evidences, LEV in combination with PHT brought about a reversal of increased glutamate levels, thereby reducing its neurotoxic profile as well as bringing about synergized effect in antiepileptic potential. This mechanism of reduction in glutamate levels by LEV may be by means of modulating presynaptic P/Q-type voltage-dependent calcium channel. Alteration in these brain biochemical measures were supported by hippocampal staining and estimation of total neuronal number.

Conclusion
The elucidations from this research findings link the cause of MI associated with TLE to be a result of interferences in cholinergic and glutamatergic pathways. Though the extent of MI was condensed upon dose reduction of the AED in focus (i.e., PHT), its potency as an AED was affected, which is not acceptable for disease management. This notion was, however, tackled where a nootropic (memory enhancer, LEV) furthermore holding an antiepileptic capability was introduced into the treatment regimen where this addition did not interfere with the major drug of choice for the treatment. As this study confines the conclusions to be centered on the end result of few behavioral and biochemical parameters, a supplementary and innovative research is of utmost importance.

Conflict of Interest
None.

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