

Taurine supplementation to anti-seizure drugs as the promising approach to treat pharmacoresistant epilepsy: A pre-clinical study



Sandeep Kumar, Rajesh Kumar Goel*

Department of Pharmaceutical Sciences & Drug Research, Punjabi University, Patiala, India

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ABSTRACT

Background: Pharmacoresistance leads to severe, irreversible disabilities and premature death in ~30% cases of epilepsy despite adequate and appropriate treatment with available anti-seizure drugs (ASDs) without any underlying cause. In light of the large body of evidence which suggests the anti-seizure action of taurine in experimental animals and its wide safety margins in human, supplementation of this inhibitory amino-sulfonic acid to available ASDs seems promising to treat pharmacoresistant epilepsy. **Methods:** We examined the anti-seizure effect of lamotrigine (15 mg/kg), levetiracetam (40 mg/kg), carbamazepine (40 mg/kg), phenytoin (35 mg/kg) & taurine (50, 100 & 200 mg/kg) in lamotrigine pre-treated pentylenetetrazole-kindled mice (LPK) which mimic core features of pharmacoresistant epilepsy, either alone ASDs or in combinations whereby three different doses of taurine were supplemented with tested ASDs.

Results: Both, the ASDs and the taurine were failed to suppress generalized tonic-clonic seizures in LPK mice. However, taurine supplementation clearly restored the anti-seizure effect of tested ASDs. Further neurochemical studies revealed that higher levels of taurine in the hippocampus and cerebral cortex restored the imbalance between major excitatory neurotransmitters glutamate & its inhibitory counterpart GABA.

Conclusions: These findings emphasize that supplementation of taurine with ASDs may be useful to treat pharmacoresistant epilepsy. Thus, further clinical validation is encouraged.

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1. Introduction

Persistent seizures is a characteristic feature of pharmacoresistant epilepsy as clear from ~30% of 65 million total worldwide cases of epilepsy, despite adequate and appropriate treatment with available ASDs.^{1–3} It leads to developmental delay, severe irreversible disabilities and premature deaths, suggesting that seizure control is important.⁴

In order to limit severity and frequency of persistent disabling seizures, ASD combinations possessing multiple mechanisms of actions can be considered¹ however; serious adverse effects and drug interactions often limit their usage.⁵ Moreover, chronic polytherapy further aggravates epilepsy associated comorbidities such as depression and memory impairment.⁶ On the other side, available non-pharmacological alternatives such as neurosurgery, central & peripheral neurostimulation are clinically underutilized, either due to ineffectiveness or inappropriateness.^{7–10}

Thus, epilepsy field suffers from pharmacoresistance despite intensive epilepsy research over the years and clinical availability of more than ten leading ASDs. Thus, much interest is in the development of safe and effective treatment approaches, with an emphasis to restore drug response in pharmacoresistant epilepsy.

The precise pathological mechanisms underlying pharmacoresistance in epilepsy stay elusive.³ However, disturbance in regulatory roles of excitatory and inhibitory amino acids are thought to lead neuronal hyperexcitability in epilepsy.^{11–13} In this context, uses of neuroactive amino acids have been recently drawn in for treatments of pharmacoresistant epilepsy.^{14,15} Another believed disturbance is the failure of neuronal regulation by taurine, an inhibitory amino sulfonic acid.¹³

In parallel to this, supplementation of taurine to available ASDs seems an promising approach to treat pharmacoresistant epilepsy, considering favourable effects of this inhibitory amino sulfonic acid such as neuroprotection from glutamate induced excitotoxicity,^{16,17} direct agonistic action at GABA_A receptor complex,^{18,19} enhancement of GAD activity and GAD-positive neurons^{20,21} as well proven anti-convulsant effect^{22,23} with wide margin of clinical safety²⁴ made the choice convincing.

* Corresponding author.

E-mail address: goelrkpup@gmail.com (R.K. Goel).

The Epilepsy Therapy Screening Program of National Institute of Neurological Disorder & Stroke (NINDS) offer a battery of well-established rodent seizure models to screen promising molecules. Among these, lamotrigine resistant seizures in Swiss albino mice mimics core features of pharmacoresistant epilepsy.^{25–28} Therefore, this study was aimed to evaluate taurine supplementation to known ASDs, as promising approach to treat pharmacoresistance on LPK mice model of pharmacoresistant epilepsy.

2. Materials and methods

2.1. Animals

Experiments were performed on total forty-two adult male Swiss albino mice (obtained from a breeder, Lala Lajpat Rai University of Veterinary and Animal Science, Hisar, Haryana, India). Mice were kept in plastic cages (6 mice/cage) in the animal house condition, at controlled room temperature ($22 \pm 3^\circ\text{C}$), humidity ($50 \pm 5\%$) and light-dark cycle (12 h light: 12 h dark, lights on at 8:00 am) with free access to food (standard pellets for rodents) and water (ad libitum), except during experimental schedules. The experimental protocol was duly approved by the Institutional Animal Ethics Committee (protocol approval no. 107/99/CPCSEA/2014-08). Experiments were carried out as per guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India. The cages were cleaned regularly. Mice were acclimatized to the laboratory for a week, before these experiments. For pentylenetetrazole kindling model, $n = 10$ mice were used, termed as PK mice. For lamotrigine pre-treated pentylenetetrazole kindling model, $n = 32$ mice were used, termed as LPK mice. For both the models, mice were of twelve-week-age and individual body weight 25–28 g at the time of seizure induction.

2.2. Pentylenetetrazole kindling model

The pentylenetetrazole kindling is a well-established rodent seizure model that mimics core features of pharmacoresponsive epilepsy and widely employed in the screening of potential molecules.²⁵ It involves a progressive increase in seizure susceptibility of rodents due to repeated pentylenetetrazole treatments.^{29–31} For this, $n = 10$ mice were treated with a sub-convulsive dose of pentylenetetrazole (40 mg/kg) on alternate days. Pentylenetetrazole (Sigma-Aldrich, USA) was dissolved in normal saline and administered via i.p route at every 48 ± 2 h intervals. After every injection, mice were placed individually in transparent plexiglass cages ($20 \times 20 \times 30$ cm) and convulsive seizures were recorded visually for a time period of 30 mins, as per modified Racine's scale which is mentioned as: Stage 0: no response; Stage 1: hyperactivity, restlessness and vibrissae twitching; Stage 2: head nodding, head clonus and myoclonic jerks; Stage 3: unilateral or bilateral limb clonus; Stage 4: forelimb clonic seizures; Stage 5: generalized tonic-clonic seizures with falling; Stage 6: hind limb extension. Pentylenetetrazole treatments continue for each mouse until it has achieved the criterion of 3 consecutive stage 5 seizures, whereby it is considered in a "stable kindled state".^{29–31} Mortality was observed in 2 out of 10 mice.

2.3. Lamotrigine pre-treated pentylenetetrazole kindling model

The lamotrigine pre-treated pentylenetetrazole kindled mice mimic core features of pharmacoresistant epilepsy and described in detail.^{25,26} Briefly, this model has modified the traditional kindling protocol with the addition of lamotrigine pre-treatments during kindling acquisition phases, which does not inhibit kindling acquisition, but leads to the subsequent development of pharmacoresistant seizures in kindled animals. For this study, $n = 32$ mice received lamotrigine pre-treatments (5 mg/kg suspended in 0.5% methylcellulose, i.p) at 45 min before every pentylenetetrazole

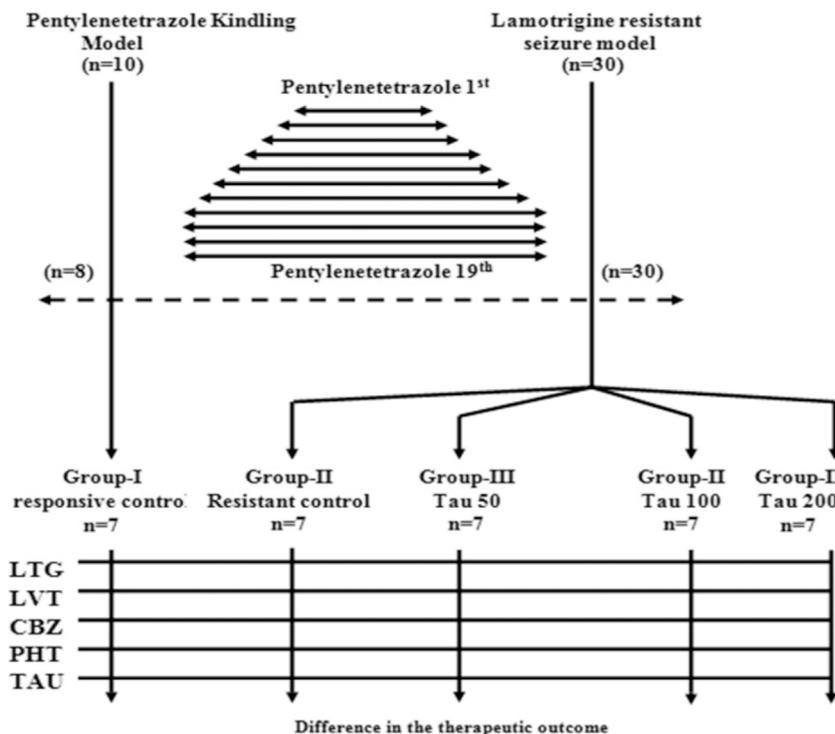


Fig. 1. Schematic illustration of the study design.

treatment (40 mg/kg at 48 ± 2 h intervals) till stable kindled state.²⁶ Mortality in this kindling protocol was low (2/32).

2.4. Drug testing

Starting 48 h after the kindling protocols, standard drugs were tested with v/s without taurine supplementations: 7 PK mice and 30 LPK mice were used for these experiments (Fig. 1). For this, PK mice were assigned as (i) pharmacoresponsive control group-I in which $n = 7$ PK mice received standard anti-seizure drug treatment without taurine supplementation. Another side, the LPK mice were randomly assigned into 4 groups (ii) pharmacoresistant control group-II in which $n = 7$ LPK mice received standard drug treatment without taurine supplementation (iii) Group-III in which $n = 7$ LPK mice received 50 mg/kg i.p dose of taurine 1 h before standard drug treatment (iv) Group-IV in which $n = 7$ LPK mice received 100 mg/kg i.p dose of taurine 1 h before standard drug treatment (v) Group-V in which $n = 7$ LPK mice received 200 mg/kg i.p dose of taurine 1 h before standard drug treatment.

ASDs were tested at well-established therapeutic efficacious doses, in ascending order of their elimination half-life's i.e. 15 mg/kg lamotrigine suspended in 0.5% methylcellulose, i.p (obtained from Cadila Healthcare, India), 40 mg/kg levetiracetam in normal saline, i.p, $t^{1/2} \sim 1.5$ h (Torrent, India), 40 mg/kg carbamazepine in 0.5% methyl cellulose, i.p, $t^{1/2} \sim 2$ h (Sun, India) and 35 mg/kg phenytoin in normal saline, $t^{1/2} \sim 12$ h (Troikaa, India) with adequate washout period of 48 h for each drug except 96 h for phenytoin.^{32,33,34} In all five groups, mice were challenged with pentylenetetrazole (40 mg/Kg, i.p) with the time gap of 45 mins after standard drug treatment. The severity of convulsive seizures was scored according to modified Racine's scale. Additionally, anti-seizure effect of taurine *per se* was studied in all five groups, at an i.p dose of 200 mg/kg.

2.5. Neurochemical analysis

The levels of taurine, glutamate & GABA in cerebral cortex and hippocampus areas of mice brain were estimated by the HPLC-ECD method.³⁵ After therapeutic evaluation, the animals were decapitated. Cerebral cortex and hippocampus were dissected,³⁶ weighed

and homogenized in freshly prepared ice-cold 10% w/v perchloric acid followed by centrifugation at 14,000g for 30 min at 4 °C (REMI C-24BL, cooling centrifuge, REMI, India) to get clear supernatant for rapid analysis of taurine, glutamate & GABA.^{35,37}

Briefly, chromatographic separations were made using a Waters HPLC system (Milford, USA) consisted of 515 binary pumps (Waters, USA) and Rheodyne manual injector (20 μ l) with Hamilton syringe. Separations were achieved on reverse phase column (250 mm \times 4.6mm \times 5 μ m; Sun fire, USA) at 35 ± 1 °C temperature followed by electrochemical detection (2465, Waters, USA). The acquired data was processed as an area under the curve (AUC) using Empower Pro-III Operating System (Waters, Milford, USA).

The mobile phase consisted of 100 mM sodium dihydrogen phosphate: methanol (60:40) and 36 mg EDTA per liter. The solution was adjusted to pH 7.8 with o-phosphoric acid and filtered through a 0.45 μ m membrane (Millipore, USA) and degassed (TransonicT 570/H, Elma, Germany). The flow rate was set at 1.2 ml/min. Standard GABA was obtained from central drug house (New Delhi, India), glutamate from S.D Fine Chem. Ltd. (India) and taurine from Hi-media laboratory, Mumbai (India).

2.6. Statistics

Statistical analysis was performed using Graph Pad Prism software version 7 (Graph-Pad Software Inc., San Diego, CA, USA). The nonparametric one-way ANOVA followed by Student Newman Keuls multiple comparison post hoc tests were used for statistical evaluation of data generated from drug testing experiments & neurochemical analysis. The data is expressed as mean \pm standard error mean. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Induction of kindling

No significant differences were observed in the mean seizure severity score of PK mice as compared with that of LPK mice, at any point of time throughout the kindling (independent Student's *t*-

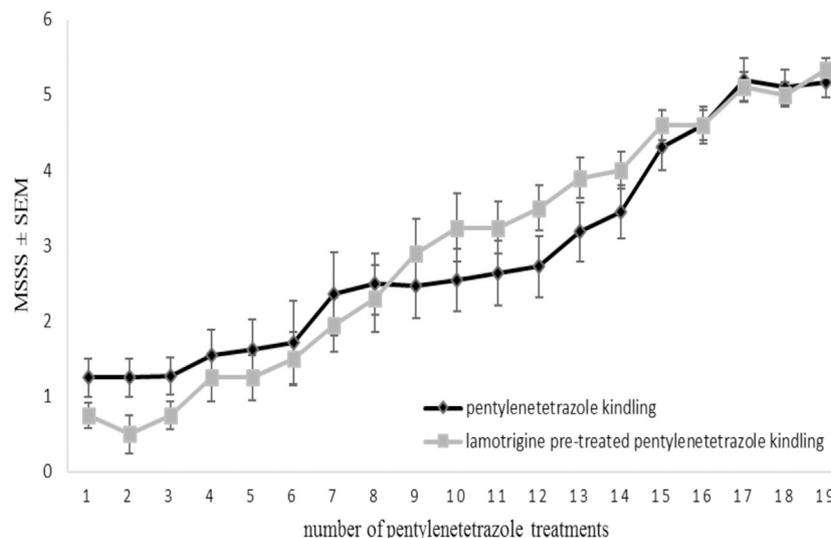


Fig. 2. Kindling development in pentylenetetrazole kindling model ($n = 10$) v/s lamotrigine pre-treated pentylenetetrazole kindling model ($n = 32$). Convulsive seizures are expressed as a mean seizure severity score \pm standard error mean on y-axis v/s number of pentylenetetrazole treatments on the x-axis. Differences were considered significant at $P < 0.05$ (Independent Student's *t*-test).

test; $P > 0.05$). Mice were kindled within 17 ± 2 pentylenetetrazole treatments in both the models (Fig. 2).

3.2. Development of resistance to anti-seizure drugs

An acute lamotrigine treatment (15 mg/Kg, i.p) completely blocked the generalized convulsive seizures on PK mice i.e. group-I (mean seizure severity score 1.2, $n = 6$; Fig. 3). In contrast, similar treatment by lamotrigine was found to be ineffective on LPK mice i.e. group-II (mean seizure severity score 4.1, $n = 6$). This significant difference in the mean seizure severity scores (1.2 v/s 4.2, $P < 0.05$; Independent Student's *t*-test) represents the lamotrigine resistance in group-II.

Furthermore, differences in mean seizure severity scores of group-I v/s II after: levetiracetam (40 mg/kg) was 1.0 v/s 3.5 ($P < 0.05$; Independent Student's *t*-test), carbamazepine (40 mg/kg) 1.2 v/s 4.0 ($P < 0.05$; Independent Student's *t*-test), phenytoin (35 mg/kg) 1.3 v/s 3.7 ($P < 0.05$; Independent Student's *t*-test) & taurine (200 mg/kg) was 1.9 v/s 4.1 ($P < 0.05$; Independent Student's *t*-test). These results represent that in group-II resistance was not limited to lamotrigine but extended to levetiracetam, carbamazepine, phenytoin & taurine as well.

3.3. Restoration of anti-seizure effect by taurine

A significant difference in the anti-convulsant effect of lamotrigine ($F_{(4,27)} = 10.9$, $P < 0.0001$), levetiracetam ($F_{(4,27)} = 16.2$, $P < 0.0001$), carbamazepine ($F_{(4,27)} = 7.5$, $P < 0.0001$) & phenytoin ($F_{(4,27)} = 16.0$, $P < 0.0001$) was observed between group I to V

The anti-seizure effect of lamotrigine, levetiracetam, carbamazepine & phenytoin was significantly ($P < 0.05$) restored in taurine treated animals (group-IV & V) as compared to control pharmacoresistant animals (group-II). The difference in the mean seizure severity score of group-II v/s III, IV & V after lamotrigine treatment was 4.1 v/s 3.8, 3.0, 2.1 ($P < 0.05$ except II v/s III), levetiracetam treatment was 3.2 v/s 2.0, 1.4 & 1.0 ($P < 0.05$), carbamazepine treatment was 4.1 v/s 3.7, 2.8 & 2.2 ($P < 0.05$ except II v/s III) and phenytoin treatment was 4.1 v/s 3.6, 2.4 & 2.1, ($P < 0.05$ except II v/s III).

3.4. Neurochemical changes

In taurine treated animals, taurine levels were found to be significantly enhanced in the cerebral cortex ($F_{(5,30)} = 26.6$; $P < 0.001$) and hippocampus ($F_{(5,30)} = 77.1$; $P < 0.001$) in comparison to control animals that received drug treatment only (group-IV & V v/s I & II, in Fig. 4a).

The control pharmacoresistant animals (group-II) showed significantly reduced GABA levels in comparison to control pharmacoresponsive animals (group-I; Fig. 4b). However, taurine treatment in pharmacoresistant animals (group-V) significantly up-regulated the GABA levels in cerebral cortex ($F_{(5,30)} = 9.90$; $P < 0.001$) & hippocampus ($F_{(5,30)} = 10.9$; $P < 0.001$).

The control pharmacoresistant animals (group-II) showed significantly elevated glutamate levels in comparison to control pharmacoresponsive animals (group-I Fig. 4c). However, taurine treatments in pharmacoresistant animals (group-V) significantly down-regulated the glutamate levels in cerebral cortex ($F_{(5,30)} = 25.3$; $P < 0.001$) & hippocampus ($F_{(5,30)} = 77.1$; $P < 0.001$).

4. Discussion

Disturbance in the regulatory roles of neuroexcitatory and inhibitory amino acids are thought to lead neuronal hyperexcitability in epilepsy.^{11–13} In this context, uses of neuroactive amino acids have been recently drawn in for treatments of pharmacoresistant epilepsy.^{14,15} Parallel to this, another disturbance that is believed to occur is the failure of neuronal regulation by taurine,¹³ an inhibitory amino sulfonic acid.

A large body of evidence support to the anti-seizure effect of taurine^{22,23,38,39} is based on the agonistic action of taurine on GABA_A receptors^{18,19} as well as its ability to enhance GAD (glutamic acid decarboxylase) number & activity in the brain^{20,21} that potentiates GABAergic neurotransmission, in addition to its ability to protect neurons from glutamate induced excite-toxicity.¹⁷

However, in this first of its kind study several novel findings have been made on (i) anti-seizure effect of taurine on lamotrigine pre-treated pentylenetetrazole-kindled mice that mimic core features of pharmacoresistant epilepsy rather than its previously

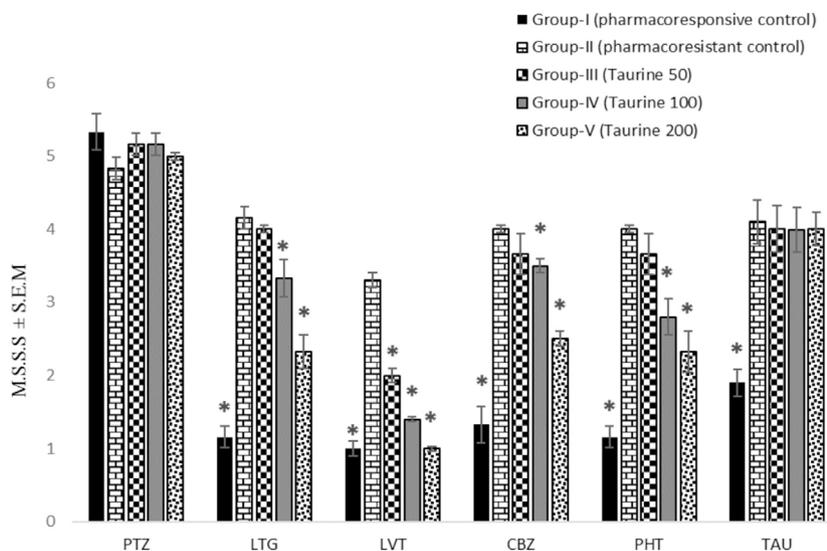


Fig. 3. Anticonvulsant effect of lamotrigine (LTG; 15 mg/kg), levetiracetam (LVT; 40 mg/kg), carbamazepine (CBZ; 40 mg/kg), phenytoin (PHT; 35 mg/kg) & taurine (200 mg/kg) in control group-I v/s II; ($n = 6$) & with taurine supplementation in group-III, IV, V; ($n = 6$). Each value is expressed as mean seizure severity score \pm standard error mean (y-axis). *: compared to pharmacoresistant control group for corresponding drug. Differences were considered significant at $P < 0.05$ (One-Way ANOVA with Student Newman Keuls multiple comparison post hoc).

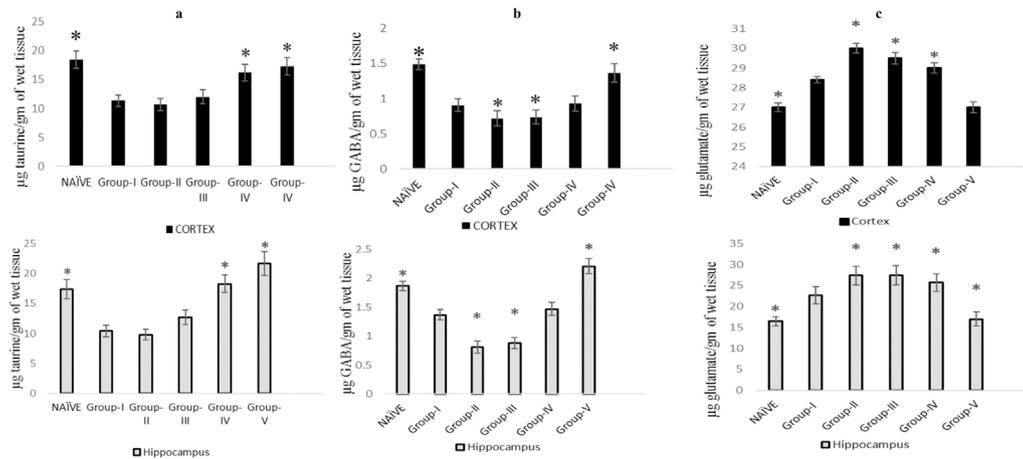


Fig. 4. The levels of taurine (a), GABA (b) & glutamate (c) in $\mu\text{g/g}$ of wet tissue on y-axis in the cerebral cortex and hippocampus of control groups (I, II; $n = 6$) and taurine treated groups (III, IV, V; $n = 6$) on x-axis. Each value is expressed as a mean seizure severity score \pm standard error mean. *: compared to pharmacoresponsive control mice. The naïve group is secondary data of $n = 6$ mice (same lab). Differences were considered significant with $P < 0.001$ (One-Way ANOVA with Student Newman Keuls multiple comparison post hoc).

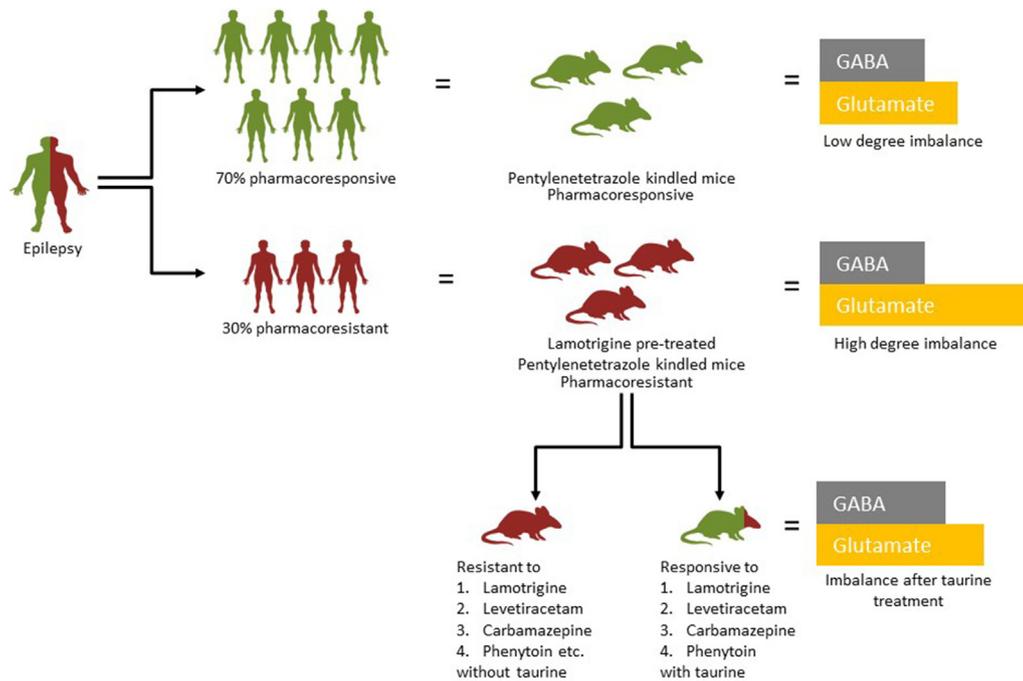


Fig. 5. Schematic illustration of the findings.

evident actions on pharmacoresponsive rodent seizures (ii) anti-seizure effect of standard drugs after taurine supplementation (iii) and interplay between the levels of taurine and major excitatory & inhibitory neurotransmitters i.e. GABA & glutamate, in cerebral cortex and hippocampus regions of LPK mice (Fig. 5).

In similar to the anti-seizure effect of standard drugs (Fig. 3) the taurine suppressed generalized tonic-clonic seizure on pentylenetetrazole-kindled animals that mimic core features of pharmacoresponsive epilepsy²⁵ in line to the earlier reports suggesting its anti-seizure potential.^{22,23} It seems to be due to a direct agonistic action of taurine on GABA_A receptors.^{18,19} However, seizure suppression was poor as compared to that of standard anti-seizure drugs justifying their clinical preference over the taurine.

However, unlike to expected anti-seizure effect of taurine as clear from the large body of evidences^{22,23,38} and our observations with taurine on pentylenetetrazole-kindled animal in Fig. 3,

significant ($P < 0.05$) resistance to anti-seizure effect of taurine was observed on LPK mice that mimic core features of pharmacoresistant epilepsy. It suggests the possible inability of GABA_A receptors mediated seizure suppression by taurine in LPK mice as well as the failure of standard anti-seizure drugs in the clinics.

In addition to the direct agonistic action of taurine on GABA_A, indirect potentiation in the GABAergic neurotransmission by taurine is well reported.^{20,21} Moreover, its wide margin of clinical safety further prompt us to use it as a supplement with available ASDs. Here, we found that anti-seizure actions of lamotrigine (15 mg/kg), levetiracetam (40 mg/kg), carbamazepine (40 mg/kg) & phenytoin (35 mg/kg) were significantly ($P < 0.05$) improved after taurine supplementation with these drugs, at 100 and 200 mg/kg, i. p. dose of taurine (Fig. 3).

It is likely that taurine mediated improvement in the anti-seizure effect of tested standard drugs may be due to indirect

potentiation of GABAergic neurotransmission, as evident that the taurine up-regulate GAD-positive neurons and as well as the activity of this key enzyme responsible for the conversion of excitatory neurotransmitter glutamate into inhibitory neurotransmitter GABA.^{20,21} Therefore, we estimated the levels of taurine, glutamate and GABA in cerebral cortex and hippocampus regions in mice brain.

According to results of this study, higher levels of taurine in the brain were strongly associated with significantly ($P < 0.05$) up-regulated GABA and down-regulated glutamate levels in taurine treated animals (Fig. 4) as compared to that of animals without taurine treatment. These neurochemical findings are favorable in justifying observed improvements in the anti-seizure effects of tested drugs after taurine supplementation, as one of the possible reason. Furthermore, significant lack of the anti-seizure effect of *per se* taurine reflects the true reversal of pharmacoresistance rather than additive effects of taurine and standard drugs.

Although, precise mechanism underlying poor anti-seizure response of standard drugs in LPK mice stay elusive,^{26–28} however in light of neurochemical findings of this study (Fig. 4) one testable hypothesis is that resistance is due to degree of imbalance between the inhibitory neurotransmitter GABA and excitatory neurotransmitter glutamate in LPK mice as compared to relatively lesser degree of imbalance in PK mice, and is further supported intrinsic severity hypothesis of pharmacoresistant epilepsy.⁴⁰ Further studies need to explain secondary structural and functional changes associated with severity of this imbalance between excitatory and inhibitory neurotransmitters.

Conflict of interest

The authors have none to declare.

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