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Title: Pentylenetetrazole Induced Kindling Model of Refractory Epilepsy: A Proof of Concept Study to Explore Dose and Time Range of Phenobarbital in Rats

Running title: Drug-Resistant Epilepsy Model in Rats

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Highlights:

1. Pentylenetetrazole and Phenobarbital (40 and 60 mg/kg) ameliorate the seizure severity in animal model of drug-resistant epilepsy.

2. Pentylenetetrazole and Phenobarbital (40 and 60 mg/kg) provides neuronal protection in hippocampus and restore the altered antioxidant enzymes.

3. Pentylenetetrazole and Phenobarbital (40) as a possible screening model for drug-resistant epilepsy.

Plain language summary:

Inspite of the various antiseizure drugs (ASDs) in the market, some group of patients do not respond to these ASDs. Therefore, our study makes an attempt to validate an animal model of drug resistant epilepsy (2 different time periods) to dive deep into its molecular mechanism. Furthermore, these attempts could help us to develop therapies that might overcome drug –resistance.
Abstract

**Purpose:** Drug resistant epilepsy is an unmet medical condition that impacts 30 percent of the epileptic patients. Numerous antiseizure drugs (ASDs) have already been developed but they provide only symptomatic relief and do not target the underlying pathogenesis. Preclinical models give us the opportunities to gain insights into obscure mechanisms of Drug resistant epilepsy. Current animal models possess lacunae that need rectification and validation to discover novel antiepileptic drugs (AEDs). The present study was undertaken to validate 3 different doses of Phenobarbital (PB) at 2 different time periods.

**Methods:** Pentylenetetrazole (PTZ) was given at sub-convulsive dose (30 mg/kg/day/i.p.) for 28 days to develop kindling in male Wistar rats. Further, kindled rats were divided into 4 groups: Pentylenetetrazole control, Pentylenetetrazole and Phenobarbital (20), Pentylenetetrazole and Phenobarbital (40), Pentylenetetrazole and Phenobarbital (60) and assessed at day 14 and 28 post kindling. Seizure scoring, oxidative stress, Phenobarbital plasma levels and histopathology of hippocampal neuron were analyzed.

**Results:** The results showed the combination of Pentylenetetrazole and Phenobarbital (40 and 60 mg/kg) remarkably decreased seizure score, elucidated higher antioxidant effect and prevent neuronal injury on day 14, whereas increased seizure score, oxidative stress and neuronal death was observed with chronic administration of Pentylenetetrazole and Phenobarbital in kindled rats at day 28. Moreover, Phenobarbital levels in blood were significantly increased at the end of day 28 of PB treatment as compared to day 14.

**Conclusion:** The adapted protocol with Phenobarbital 40 mg/kg dose could be of great potential in screening of antiseizure drugs in refractory epilepsy.

**Keywords:** Drug-resistance epilepsy, Pentylenetetrazole, Phenobarbital, Oxidative stress, Kindling, Hippocampus.
Introduction

Epilepsy is a multifaceted brain disorder, characterised by frequent episodes of seizures, which could range from mild muscle jerks to unstoppable convulsions (Kumar et al. 2018). Despite the enormous antiseizure drugs (ASD) available, about 30% of patients experience recurrence of seizures, a condition known as ‘drug resistance’ (Lalitha et al. 2018). According to International League against Epilepsy (ILAE), drug resistance is considered as uncontrollable seizure even after sufficient trials of two tolerated and appropriately chosen ASD schedules (whether as monotherapies or in combination) (Kwan et al. 2010). Moreover, the mechanisms underlying pharmacoresistance remains obscure due to the lack of adequate experimental models of chronic intractable epilepsy (Löscher 1997). Therefore, this prompted us to validate a model of drug-resistant epilepsy. The most laborious and widely used animal epilepsy model is kindling, induced via chronic administration of Pentylentetrazole (PTZ) (Singh et al. 2021). It acts as GABA antagonist (Ergul 2015) and when given at sub convulsive dose for a longer time period cause permanent changes in the brain (Sato et al. 1997). In addition, it causes alterations in antioxidant defence systems of the brain which leads to oxidative stress (Ilhan et al. 2004) and neuronal death in the hippocampus (Pavlova et al. 2006).

Two types of protocols are used to evaluate ASDs effect on kindling: 1) Pre-treatment with drugs, before each PTZ injection and examine their effect on kindling process (anti-epileptogenic potential) via comparing them with control group; or 2) Drugs are tested on fully kindled rats to determine their anticonvulsant potential (Saha and Chakrabarti 2014). In the present study, the second method was followed to develop drug-refractoriness in rats because of its potential merits. Firstly, the PTZ kindled rats treated with ASDs model targets the refractory neuropathology of seizure whereas the pre-treatment ASDs before PTZ kindling model is a consequence of tolerance (ASDs used at sub-therapeutic doses) (Loscher and Schmidt 2006). Secondly, the kindling time in latter model (8 weeks vs 9 weeks 5 days) is shorter as compared to prior kindling model (Singh et al. 2014). One of the essential criteria during validation of pharmacoresistant models is perhaps the maintainence of ASD effective levels (Löscher 2007). That’s why, PB was used because of its longer half-life and has been reported to maintain its adequate levels in plasma for longer period of time (Löscher and Hönack 1989).

The PB+PTZ model perfectly mimic drug-resistant epilepsy in human (McNamara et al. 1980). The model suggested that resistance to phenobarbital was initially induced as a result of epileptic seizure and
combination of both receptor desensitization and seizure at later stage (Jing X et al. 2010). Based on these findings, the present study was aimed to validate a refractory model which targets the epileptogenic process behind development of drug-resistant epilepsy, which is one of the imperative advantages of this model over other preclinical models. Therefore, we planned to study the effect of 3 different doses of Phenobarbital i.e. 20 mg (below sub therapeutic), 40 mg (sub therapeutic dose) and 60 mg (maximum tolerated dose) at 2 different time period interval in refractory model of epilepsy in experimental rats by PTZ induced kindling.

Material and Methods

Chemicals and Experimental Animals

Pentylenetetrazole (PTZ) was obtained from Sigma, India. Phenobarbital was received as a gift sample by Harman Finochem Pvt Ltd. Aurangabad for carrying out the study. It was performed in the Department of Pharmacology, PGIMER, and Chandigarh. Male Wistar rats were acclimatized for one week prior to being used at controlled environment (temperature 24°C and 12-hour light/dark cycles). All the experiments were performed according to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India, guidelines. It was approved by the Institutional Animal Ethics Committee and Institutional Biosafety Committee. PTZ was dissolved in saline and administered intraperitoneally whereas Phenobarbital was dissolved in carboxymethylcellulose and administered orally. (Approval no: JSSCP/IAEC/OT/M.PHARM/PH.COLOGY/04/17-18).

Groups

Rats were randomly divided into six groups (Fig. 1) i.e. Group 1: saline control group, Group 2: vehicle carboxymethylcellulose (CMC) treated group, Group 3: PTZ-kindled rats, Group 4: PTZ-kindled rats received Phenobarbital(20mg/kg) dose, Group 5: PTZ-kindled rats received Phenobarbital(40mg/kg) dose, Group 6: PTZ-kindled rats received Phenobarbital(60mg/kg) dose, Equal number of animals were sacrificed from each groups at day 14 and day 28.

PTZ kindling

The sub-convulsive dose of PTZ (30 mg/kg/day) was given intraperitoneally for 28 days. The rats were then observed for 30 minutes for the convulsion activity in a transparent plexi glass chamber. The seizure scale followed for seizure scoring (De Sarro A et al. 1999, Singh et al. 2017a, Singh et al. 2017b, Singh et al. 2017c, Singh et al. 2016).
0-no response;
1-mouth and facial jerks;
2-nodding or myoclonic body jerks;
3-forelimb clonus;
4-rearing, falling down, hind limb clonus and forelimb tonus
5-tonic extension of hind limb, status epileptic and/or death observed for 30 min.

Biochemical Estimation

The degree of brain oxidative stress was determined using free carbonyl protein groups as biomarkers, and its measurement was performed according to the spectrophotometric assay. The levels of reduced glutathione, catalase and superoxide dismutase were estimated in rat brain homogenate.

Estimation of Reduced glutathione levels

The (Ellman 1959) method was followed in order to estimate the levels of reduced glutathione. The proteins were precipitated by adding 10% trichloroacetic acid to the equal quantity of homogenate. The homogenate was then centrifuged for 10 min at 2000 rpm. To 100 µl of protein free supernatant, firstly 0.3 M phosphate buffer, pH 8.4 (2 ml) was added then 0.04% DTNB was prepared in 1% tri-sodium citrate and 0.5 ml was added. Thirdly distilled water (0.4 ml) was added to the supernatant. The GSH concentration was determined by running a parallel standard GSH. The concentration of reduced glutathione as µg/g-wet tissue was observed spectrophotometrically at 412 nm within 15 min.

Estimation of Superoxide dismutase levels/activity

Standard method (Kono 1978) was followed to estimate SOD levels. Supernatant (0.1 ml) was taken and the ice cold chloroform (0.25 ml) and methanol (0.15 ml) were added. The mixture was centrifuged at 3000 rpm for 10 minutes at 4°C. The supernatant was separated and to the 0.2 ml of the supernatant, 0.5 ml ethylene diamine tetraacetic acid, 1.3 ml buffer and 0.8 ml water were added in succession. The addition of epinephrine (0.2 ml) started the reaction. The absorbance was read at every 30 seconds for 3 minutes at 480 nm and then the average change in absorbance was calculated. The data was expressed as nmol/min/mg protein.

Estimation/determination of Catalase activity

The catalase activity of the brain tissue was measured as described by (Greenwald RA). Briefly, 0.05 mL of brain homogenate was used for reaction mixture. In the homogenate, 0.05 M phosphate buffer, pH 7.0 (1.95 mL) and 0.019 M hydrogen peroxide (1.0 mL) was added for the final volume make up of 3 mL. The
absorbance of the reaction mixture was calculated at 240 nm after 60-second interval for 2 min. The catalase activity was expressed in μmol/mg protein.

**Estimation of Plasma levels of Phenobarbital**

The levels of phenobarbital were estimated via HPLC analysis. Animals were sacrificed at two different time points i.e. day 14 and day 28 after kindling and blood was collected retro orbitally. The elution of phenobarbital was done from a reversed-phase column with an optimum temperature of 50°C. The mobile phase used consisted of 19/81 by vol. of acetonitrile/phosphate buffer and the flow rate was set at 3.0 ml/min. The absorption of phenobarbital occurs at 195 nm and its quantity was estimated from the respective peak height. The sample injection volume was 10 μl. The time required for each analysis was about 14 minute (Moriyama M 1999).

**Histopathology of Rat brain**

The brain tissues were kept stored in 10% neutral formalin for the histopathological analysis. The brain tissues were processed by treating them with various concentrations of alcohol to remove the water from the tissues. The brain sections were then embedded in a molten wax and thin sections were cut (5 μm) and fixed on a glass slide. Then, it is stained with haematoxylin and eosin dye. Bright field images were occupied using an Olympus upright microscope.

The neuronal injury score was determined according to the following criteria: Normal, no injury or rare isolated apoptotic neuron was given the score of 0, Rare neuronal injury less than 5 clusters as score 1, the occasional neuronal injury (5-15 clusters) was given the score of 2, the frequent neuronal injury (>15 clusters) was given the score of 3 and diffuse neuronal injury was given the score of 4 (Wyler 1992).

**Statistical Analysis**

All data were expressed as mean ± standard deviation (SD). The quantitative parameters like seizure score and oxidative stress parameters were evaluated using one-way ANOVA and bonferroni posthoc analysis. Unpaired student’s ‘t’ test was performed for comparing the plasma concentration of different doses of phenobarbital. The P<0.05 was considered to suggest statistical significance.
Results

Seizure scoring in PTZ kindled rats after administration of different doses of Phenobarbital for different time periods

The rats in the saline as well as CMC treated groups demonstrated a zero seizure score throughout the kindling period of 4 weeks. In PTZ (30 mg/kg) treated group, the seizure score was increased from 2.67 ± 0.44 to 3.3 ± 0.57 during the study period (Fig. 2). Kindled rats treated with phenobarbital (20 mg/kg), has shown no significant decline in the seizure score at the end of day 14 as well as day 28 of the study period. The phenobarbital 40 (P<0.05) and 60 mg/kg (P<0.01), showed a significant reduction in the seizure score at the end of day 14 in the PTZ kindled animals. However, the chronic treatment of Phenobarbital at the dose of 40 mg/kg and 60 mg/kg along with PTZ administration produced resistance as the seizure scores were found to be increased at the end of day 28 (P < 0.05) (Fig. 2).

Effect of Phenobarbital treatment on reduced glutathione (GSH) levels

The GSH levels in rat brain were remarkably higher (P<0.05) in phenobarbital 60 mg/kg treated group compared to PTZ treated group and no significant effects were observed at PB dose 20 mg/kg and 40 mg/kg at the end of 14th day. However, at the end of 28th day of PB treatment, no significant effects were observed in GSH levels in all the three treatment groups as compared to PTZ treated group (Fig. 3).

Effect of Phenobarbital treatment on superoxide dismutase (SOD) levels

The level of SOD in rat brain was found to be significantly reduced (P < 0.05) in PTZ treatment group as compared to vehicle, saline and treated groups at the end of both 14 and 28 days of PB treatment. The decrease in the SOD levels was alleviated (P<0.05) with the treatment of phenobarbital (40 and 60) mg/kg at the end of day 14. However, the chronic administrations of Phenobarbital along with PTZ for 28 days to the kindled rats lead to reduction in the levels of SOD (Fig. 4).

Effect of Phenobarbital treatment on catalase (CAT)

The catalase levels in rat brains were significantly decreased (P < 0.05) in PTZ treated group as compared to both the vehicle and saline treated group. The catalase levels were found to be remarkably increased (P < 0.05) with Phenobarbital treatment (40 and 60) mg/kg dose on the 14th day. The treatment of phenobarbital (40 and 60) mg/kg reduced the brain catalase levels as compared to normal control group at the end of day 28 of PB treatment. However, this decrease in CAT level was not statistically significant (Fig. 5). The brain catalase levels of phenobarbital 20 mg/kg group showed no significant effects when compared to PTZ control at 28th day of PB+PTZ treatment.
Effect on plasma levels of Phenobarbital

The concentration of different doses of phenobarbital (20, 40 and 60 mg/kg) was measured in the rat’s blood at the end of 14th day and 28th day of treatment with PB. The Phenobarbital treatment at the dose of 20 mg/kg showed no significant increment in its plasma at the end of 28 days. However, a highly significant increase ($P<0.01$) in the phenobarbital levels was observed with the treatment of 40 mg/kg at the end of day 28 as compared to day 14 of PB treatment in PTZ kindled rats. The plasma levels of PB were found to be significantly increased after 28 days of treatment at the dose of 60 mg/kg as compared to PTZ kindled rats. Moreover, the plasma concentration of phenobarbital was remarkably higher ($P < 0.05$) in the phenobarbital treatment (60 mg/kg) group in contrast to the phenobarbital treatment (20 mg/kg) group at the end of day 28 (Table 1).

Neuronal injury score

The PTZ treated group showed a remarkable higher ($P < 0.001$) neuronal injury score in comparison to the normal saline control group. The treatment of Phenobarbital (40 and 60) mg/kg significantly reduced ($P < 0.01$) the neuronal injury score as compared to the PTZ treated group at the end of day 14. However, the combination of PTZ and chronic administration of phenobarbital for 28 days increased the seizure severity and neuronal injury score leading to PB resistance. Thus, it was observed that the treatment of phenobarbital (40 and 60) mg/kg reversed the decrease ($P < 0.05$) in neuronal injury score at the end of day 28 when compared to day 14 of PB+PTZ treatment period (Fig. 6).

The PTZ treated group showed a diffuse neuronal injury as compared to normal saline group showing normal neuron with rare neuronal injury (Fig. 7). The treatment of Phenobarbital at 20 mg/kg dose showed frequent neuronal injury with (>15 clusters) at the end of 14 as well as 28 days of the study period. The occasional neuronal injury with (5-15 clusters) of neurons was observed with treatment of phenobarbital (40 and 60) mg/kg at the end of day 14 of the study period. However, at the end of day 28, the frequent neuronal injury with (>15 clusters) was observed with 40 and 60 mg/kg doses of phenobarbital treatment.

Discussion

We are the first one to elucidate and report the different parameters studied on PTZ kindled Wistar rats at two different time points with three different doses of PB to select the dose at which maximum resistance occurs with chronic period of time. The current research work reveals that phenobarbital treatment at 20 mg/kg dose showed no protective effect on seizure severity at any time point. The phenobarbital administration at both 40
mg/kg and 60 mg/kg dose showed an initial decrease in the seizure score and later developed resistance with increase in seizure severity. It might be due to increase in P-glycoprotein which gets triggered by both seizure and the drug effect thus, leading to refractoriness (Jing X et al. 2010). Moreover, the drug tolerance is also one of the phenomena which lead to resistance. For example phenobarbital undergoes pharmacokinetic tolerance which is attributed to its fast metabolism rate (Gay et al. 1983). Hence, in the current research paper dose escalation (20mg, 40mg, 60mg) method was used to combat tolerance at two (14th day, 28th day) time interval. Increased dose helps in maintaining the same antiepileptic efficacy as observed earlier with low dose treatment. The above described tolerance is detected through the plasma level monitoring (Loscher and Schmidt 2016). In accordance to previous study similar increase in trend of PB levels in plasma has been observed at day 14 & day 28 in our research report as well (Jing X et al. 2010). However, one preclinical study on Spontaneous recurrent seizures (SRS) model of drug-resistant epilepsy have also reported no differentiable comparison between plasma levels of PB responders and non-responders rodents (Brandt et al. 2004). PB epileptic brain is more prone to adverse effects because of the permanent alteration imprinted in the brain during epileptogenesis. There are studies reporting symptoms of sleepiness, dizziness, altered behaviour and depression associated side effects among people with epilepsy taking Phenobarbital at higher doses (Zhang et al. 2011). Therefore, in order to avoid the associated side effects 40mg/kg was advised over 60mg/kg, as perfect dose of PB to induce resistance model which can easily be extrapolated in the clinical scenario.

The present pharmacoresistant animal model has advantage over conventional Amygdala kindling and Spontaneous recurrent seizures (SRS) followed by Phenobarbital and Phenytoin treatment. It is less time consuming, labor intensive and could screen compounds over short period of time. Moreover, no surgical expertise and electrodes implants are required in the current animal model, which further decreases the chances of animal mortality. The major difference between our model and another promising pharmacoresistance model is difference in study period. Our research model was a 56-day chronic study which is better suited to study drug-resistant mechanism. Additionally, chronic brain alteration can also change the pharmacological response that cannot be visualized in acute models (Loscher 2017).

Oxidative stress due to free radicals plays a pivotal role in the neurobiology of epilepsy. Recurrent seizures lead to activation of oxidative defense system and production of free radicals (Shin E et al. 2011). Further, the activation of free radical leads to alteration in the levels of endogenous antioxidant enzymes, lipids and expression of DNA (Ergul 2015). Numerous studies have depicted the involvement of certain endogenous
(GSH, CAT, SOD) enzymes with neurodegenerative disorders (Cardenas-Rodriguez 2013). Previous evidences support relationship between oxidative stress and mechanisms which leads to drug-resistance (Lorigados 2018). The present study showed that the levels of antioxidant enzymes (GSH, SOD and CAT) in the phenobarbital 40 and 60 mg/kg treatment groups were decreased at the end of 28 days indicating the increase in oxidative stress, which depicts its positive co-relation with drug-resistance epilepsy.

Previous reports have suggested that majority of PB resistant rats lead to major hippocampal damage (Bethmann 2007). Based on preclinical findings it has been proposed that patient with uncontrollable seizure will have severe hippocampal damage as compared to patient with controlled epilepsy (Sloviter 1994). These clinical studies have found a progressive damage to hippocampus of the patient with drug-resistant epilepsy (Kalviainen 1998). Similar findings have been observed in our study where the combination of PTZ and chronic administration of Phenobarbital group has shown increase in the neuronal injury score at the end of 28 days contributing to the process of epileptogenesis and the development of the model of drug resistance.

Conclusion

The drug resistance is the most challenging problem in the management of epilepsy. Therefore, it is necessary to understand the multifactorial mechanisms of epileptogenesis and develop animal models that targets epileptogenesis for the better management of refractory epilepsy. Further it can be concluded that PTZ + phenobarbital (40 mg/kg) have shown the best results and it could be used to develop the refractory model of epilepsy and also to screen and study the various combination of drugs which would be helpful in preventing and treating the resistance epilepsy.

Declarations

Funding Not applicable

Conflicts of interest/Competing interests None

Availability of data and material The authors confirm that the data supporting the findings of this study are available within the article

Code availability Not applicable

Authors’ contributions Conceptualization: [BM]; Methodology: [NG, AC, BM]; Formal analysis and investigation: [ST, NG]; Writing- original draft preparation: [ST, NG, RJ]; Writing - review and editing: [NG, RJ, AC, BM]; Supervision: [AC, BM].
Ethics approval Approved by the Institutional Animal Ethics Committee and Institutional Biosafety Committee, PGIMER, Chandigarh, India.

Consent to participate Not applicable

Consent for publication Not applicable
References


Table 1: Plasma concentration of Phenobarbital in PTZ induced kindling model of rat. Data were expressed as Mean±SD, Unpaired student’s ‘t’ –test. *P <0.05, **P<0.01 compared to day 14; $P<0.05$ compared to phenobarbital 20 mg/kg at day 28.

<table>
<thead>
<tr>
<th>DRUG DOSE</th>
<th>PLASMA CONCENTRATION (14 DAY-TREATMENT)</th>
<th>PLASMA CONCENTRATION (28 DAY-TREATMENT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenobarbital 20 mg/kg</td>
<td>9.27 ± 1.12</td>
<td>10.23 ± 2.166</td>
</tr>
<tr>
<td>Phenobarbital 40 mg/kg</td>
<td>10.216 ± 1.847</td>
<td>14.48 ± 2.229 **</td>
</tr>
<tr>
<td>Phenobarbital 60 mg/kg</td>
<td>10.562 ± 3.188</td>
<td>16.25 ± 4.491 &amp;$ $</td>
</tr>
</tbody>
</table>
Fig. 1 Schematic illustration of the experimental study used to validate the drug-resistant animal model of epilepsy. Animals were sacrificed at two time intervals i.e. Day 14 and day 28 of the post kindling period.

The parameters like Seizure Scoring, brain oxidative stress, Phenobarbital levels in Plasma, brain histopathology were measured at above said time intervals.
Fig. 2 Effect of various treatments on seizure score in PTZ kindled animal model in rats. Data are expressed as Mean ± SD, one-way ANOVA followed by Bonferroni post hoc analysis. *P< 0.05 compared to saline control; *P < 0.05; **P < 0.01 compared to PTZ treated group, ₵P < 0.05 compared to phenobarbital 20 mg/kg.
Fig. 3 Effect of various treatments on brain reduced glutathione (GSH) level at day 14 and 28 of the study period. Data are expressed as Mean ± SD, one-way ANOVA followed by Bonferroni post hoc analysis. **P < 0.01 compared to saline control, *P < 0.05 compared to PTZ control, $P < 0.05$ compared to phenobarbital 20 mg/kg.
Fig. 4 Effect of various treatments on brain superoxide dismutase (SOD) levels at day 14 and 28 of the study period. Data are expressed as Mean ± SD, one-way ANOVA followed by Bonferroni post hoc analysis. *P < 0.05 compared to saline control, *P < 0.05 compared to PTZ treated group.
Fig. 5 Effect of various treatments on brain catalase levels at the end of day 14 and 28 of the study period. Data are expressed as Mean ± SD, one-way ANOVA followed by Bonferroni post hoc analysis. @ $P < 0.05$ compared to saline control, * $P < 0.05$ compared to PTZ control.
Fig. 6 Effect of various treatments on Neuronal injury score at day 14 and 28. Data are expressed as Mean ± SD, (n=6), one-way ANOVA followed by Bonferroni post hoc analysis. @@ P < 0.001 compared to saline control, **P < 0.01 compared to PTZ control, & P < 0.05 compared to day 14 of the treatment.
Fig. 7 Histopathological changes (40X) in rat brain treated with different doses of Phenobarbital at day 14 and 28 of the study period.