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Original Article

CHALLENGES IN REPRODUCING THE STANDARD LITHIUM PILOCARPINE MODEL OF TEMPORAL LOBE EPILEPSY IN *WISTAR* RATS: TROUBLESHOOTING STRATEGIES

Asha Rao[1], Tulika Gupta[1], Ashish Kumar Kakkar[2], Shammy Chandel[3], Ashish Jain[2], Lekha Saha[2], Ranjana Bharti[1], Ujjwaljit Kaur[1], Navneet Singla[3]

1. Department of Anatomy, PGIMER, Chandigarh, India
2. Department of Pharmacology, PGIMER, Chandigarh, India
3. Department of Neurosurgery, PGIMER, Chandigarh, India

ABSTRACT

Introduction: Animal model of temporal lobe epilepsy (TLE) provide an essential platform to study the disease pathophysiology, and disease modifying management protocols. Lithium –pilocarpine model is one of the most favored model. Many practical difficulties have been encountered while creating this model in this study as well as by other authors. We have systematically attempted to compile all issues and their possible solutions in establishment of Li-Pi model of status epilepticus in *wistar* rats, to provide a dependable protocol with improved seizure induction rates and lower mortality rates.

Materials and Methods: Young small size male wistar rats (100-150gm) were injected with lithium chloride (LiCl) (127 mg/kg, i.p.), followed by methyl scopolamine (1mg/kg, i.p.) after 18-22 hrs. Precisely after 30 minutes, dose of pilocarpine (i.p.) was administered. Post seizures, diazepam (10 mg/kg) was injected to reduce seizures severity and to increase survival rate and rats were fed glucose (10% of body weight) through oral gavage. Model creation was validated by histological examination of hippocampus. The rats which did not develop seizures, were reutilized for the model generation after drug wash-out period of 48 hours.

Results: Initial model creation was unsuccessfully attempted in 75 rats. Standard described protocol of three incremental doses of pilocarpine (10mg/kg, maximum of 30mg/kg) did not elicit epilepsy. Loading dose of 30mg/kg pilocarpine caused stage 1 epilepsy in 60% rats; second dose of pilocarpine (30mg/kg), caused stage 2 epilepsy in 70% rats. Successful TLE model creation was seen in 33 out of 57 rats, using proposed modification in which 2 incremental doses of pilocarpine (30mg/kg each) were followed by 3 doses of 10mg/kg each; 6 rats died.

Conclusions: The study provides a detailed procedure for lithium pilocarpine model creation and various pitfalls encountered. Modified pilocarpine dosage led to improved seizure induction rates (58%) and lower mortality rates (10%).

Address for Correspondence:
Dr. Tulika Gupta, Additional Professor, Department of Anatomy, PGIMER, Chandigarh, India - 160012
Email: tulikag11@gmail.com Phone: 0172 2755272

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INTRODUCTION

The World Health Organization states that epilepsy is a chronic non-communicable disease characterized by recurrent seizures, sometimes accompanied by loss of consciousness and loss of control of bowel or bladder function. Status epilepticus (SE) is a persistent seizure lasting for more than five minutes, causing long-term detrimental consequences [1]. In India, the prevalence rate for epilepsy varies from 1.2 to 11.9 per 1000 population among adults [2, 3]. Epileptic patients experience a reduced quality of life, an increased risk of psychosocial dysfunction, injuries, and premature death [4-6]. Therefore, there is an imperative need to understand the pathophysiology of epilepsy in order to develop preventive or disease-modifying therapies. This need has led to an intense research focus on epilepsy. Epilepsy research is conducted either on human tissue samples obtained from surgery on epileptic patients or on animal models of epilepsy [7-10]. Human surgical tissue has two drawbacks: it cannot be used to study acute changes during epileptogenesis as surgery is performed on chronic and drug-resistant patients, and interventional studies are not possible.

Animal models of epilepsy have proven to be of immense importance. Animal models allow for detailed study of the disease pathophysiology. Interventions can be performed for experimentation as well as validation, thus allowing testing of the efficacy, mechanism of action, and side effects of potential therapeutic agents [11]. An ideal animal model of epilepsy should have disease mechanisms as well as phenotypic features similar to human epileptic conditions; moreover, it should demonstrate predictive validity, representing the treatment responses observed clinically [12, 13].

Chemoconvulsants and Electrical (Kindling) are the two most commonly used animal models to study mesial temporal lobe epilepsy (MTLE) [14, 15]. Chemoconvulsant models of epilepsy are created by administering excitotoxic substances and include the Kainic acid model [16-22] and the Lithium-pilocarpine (Li-Pi) model of epilepsy [14, 23]. We chose the Li-Pi model to study the role of calcium channels in epilepsy-induced cell death through an interventional study.

The Li-Pi model replicates the natural history of human MTLE. Similar to human temporal lobe epilepsy, the seizure focus is localized in the limbic areas [24], interictal activity is generated in the subiculum [25], upregulation of neurotrophins has been noted in the hippocampus [26], and pathologically, granule cell dispersion, cell death, and mossy fiber sprouting are observed [27]. Lastly, cognitive and memory impairment is noted [28, 29], with an initial epileptogenic insult in the form

of status epilepticus leading to the acute phase of MTLE. In the kainic acid epilepsy model, there are certain limitations; unlike the pilocarpine model, the stages of seizures are not easily distinguishable, and the animals show behavioral changes such as anxiety and depression.

Although the Li-Pi model has become a favorable model for conducting epilepsy-related studies, it has a high mortality rate [30-33]. We encountered many difficulties while replicating the standard Li-Pi model. Similar issues have also been reported by many studies [34, 35]. Thus, here we have attempted to provide detailed steps for the creation of the Li-Pi model of TLE in *Wistar* rats. Issues faced and their possible solutions have been addressed with the aim of creating a dependable protocol with improved seizure induction rates and lower mortality rates.

Fig. 1. Acclimatization of rats (2rats/cage)

MATERIAL AND METHODS

Selection of animals

Seventy-five (n=75) male Wistar rats weighing 100-150g were obtained from the Central Animal House facility at the Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh. All experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC no. 803/116th dated 14th September 2021). Clear plastic shoebox cages containing corn cob bedding were used to house two rats per cage, under a 12-hour light: 12-hour dark cycle to acclimatize rats to the light/dark cycle, at a constant temperature (22 ± 2 °C) with free access to food and water. Rats were acclimatized for at least one week before being used in the experiments. Extreme care was taken to minimize the pain and discomfort of the animals through interventions (Fig. 1).

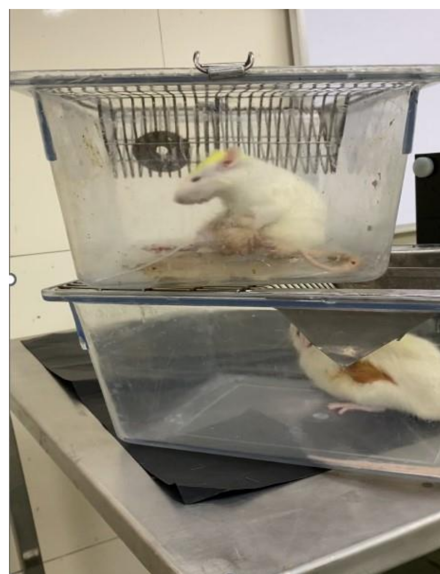

Fig. 2. Still image from video 1

LPISE rat model

For model establishment, pilocarpine hydrochloride, methyl scopolamine, lithium chloride (LiCl) were purchased from Sigma-Aldrich, and diazepam, ketamine, and xylazine were procured from a medical shop. Prior to the initiation of any experiment, the weight of the rats was recorded. Then, the rats were segregated into two groups, namely, control and epilepsy groups.

Standard Method of Model Development: As per the literature, LiCl (127mg/kg), methyl scopolamine (1mg/kg), and pilocarpine (30mg/kg) induce SE in rats via the intraperitoneal (i.p.) route [36-38]. In our study, we faithfully tried this protocol in 75 rats. LiCl was administered at a dose of 127mg/kg (18-22 hrs before pilocarpine administration), and the next day, 30 minutes before pilocarpine dosage, methyl scopolamine (1mg/kg) was given to decrease the peripheral effects of pilocarpine. During several attempts, pilocarpine was given at a dosage of 30mg/kg intraperitoneally, either as a single dose or as a breakup of a 30mg/kg dose (10+20mg/kg or 15+15mg/kg); however, SE was not observed even a single time.

Continuous video monitoring was done to observe the same. There was no mortality. Later, we came across a paper [34] in which the authors recommended the subcutaneous route for pilocarpine (25mg/kg to 75mg/kg) with low mortality (up to 1%) [34]. So, we tried to reproduce the same but achieved success only at a higher pilocarpine dose (90 mg/kg). Comparison revealed that seizures came earlier and were of greater intensity when pilocarpine was injected intraperitoneally compared to subcutaneously, at the same dose.

Modified Method of Model Development: After the failure of the method given in the literature, for model development, we used young small-sized rats (100-150g). In the epileptic group, LiCl (127 mg/kg) was injected via the intraperitoneal route. After 18-22 hrs, methyl scopolamine (1mg/kg), a muscarinic acetylcholine receptor antagonist, was injected intraperitoneally to decrease the peripheral effects of pilocarpine. Precisely after 30 minutes, two incremental doses of pilocarpine (30mg/kg each) were followed by two doses of 15mg/kg each until the occurrence of seizures (a maximum dose of 90 mg/kg of pilocarpine) via the intraperitoneal route.

The rats developed seizures at a high dose of pilocarpine (80-90 mg/kg). Once the rats started to develop seizures, diazepam (10mg/kg) was injected to reduce seizure severity. To increase the survival rate, glucose was orally fed (10% of the body weight) to rats through oral gavage, and their eyes were kept moist by swiping them with water-soaked cotton swabs to prevent dehydration in rats. In concordance with the

3Rs (Replacement, Reduction, and Refinement) of animal welfare use, rats that did not develop seizures even after the maximum dose of pilocarpine were reused for model development after a washout period of 48 hours; about half of these rats developed seizures.

Video monitoring and seizure analysis

During model development, a blinded observer rated the seizure severity using a modified Racine scale (Salem, El-Shamarka et al., 2018, [39]):

- Stage 1: Staring with mouth clonus
- Stage 2: Head nodding, automatisms (e.g., scratching, sniffing orientation)
- Stage 3: Unilateral forelimb clonus
- Stage 4: Forelimb clonus (rearing)
- Stage 5: Forelimb clonus with rearing and one fall
- Stage 6: Forelimb clonus with rearing and multiple successive falls
- Stage 7: Tonic/clonic seizures (e.g., running and jumping)

SE was assumed to occur in rats with seizures of stage 4 and above (Video 1: [link]) (Fig. 2). The time of SE arrest, and 24-hour survival rate were noted. The weight of animals was taken consecutively for 3 days; starting from the day when LiCl dosing was started, the next day before giving methyl scopolamine, and then on the third day when they were sacrificed after 24 hours of *model development. Their weight was found to be decreased by 7-10g on the third day.*

* Video link at the end of the article

Tissue harvesting

After the generation of the epilepsy model, the rats that developed seizures were selected for downstream processing. Rats were anesthetized using ketamine (100 mg/kg i.p.) and xylazine (10 mg/kg i.p.) [40] and then sacrificed by cervical dislocation. The brain was harvested, and the hippocampus was isolated and stored in 10% buffered formalin, RNA later, and frozen at -80 degrees for histology, gene expression, and protein studies, respectively. Sacrificed rats were disposed of in yellow polybags for incineration.

Histological validation of Temporal lobe epilepsy

It was done by identifying two hallmark features – Granule cell dispersion (GCD) and cell death in the hippocampal tissue of rats in Nissl-stained slides [41]. In all rat epilepsy cases (n=18), statistically significant GCD and cell death were observed in comparison to the control rats. (Fig. 3).

RESULTS

Results with standard protocol:

The standard protocol was attempted eight times (8 animals – 6 epilepsy group,

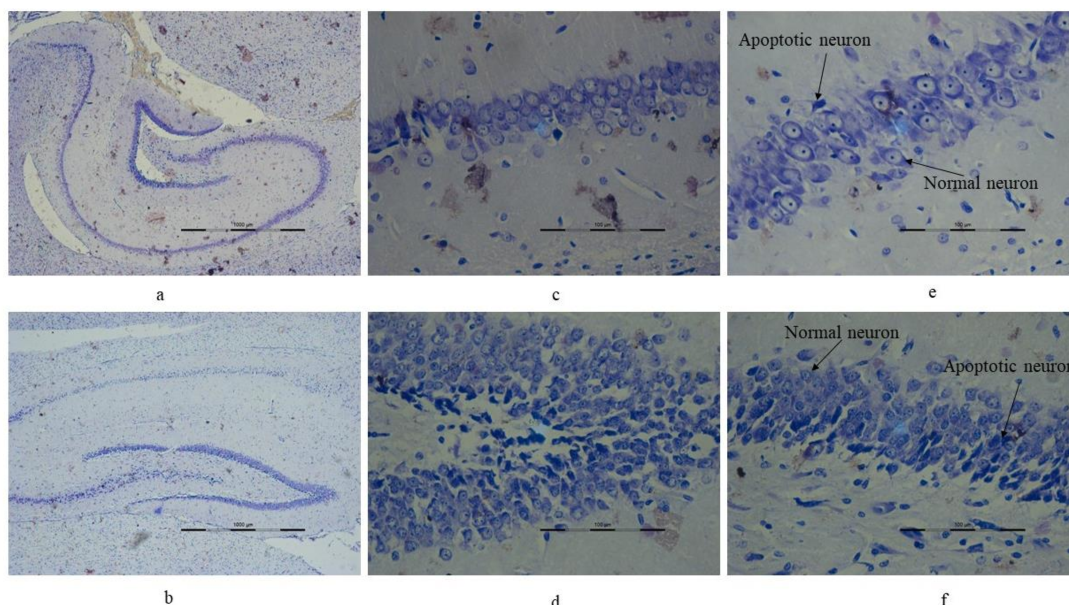

Fig. 3. Results of Nissl's staining showing- a. & b. Hippocampus at 4X in control rat (a.) and epileptic rat (b.) c. & d. GCD seen in epileptic rat (d.) as compared to the compact granule cell layer in control rat (c.), at 40X. e. & f. Extensive neuronal apoptosis seen in the epileptic rat (f.) as compared to control rat (e.) hippocampus at 40X.

2 control group) with all due precautions, but none of the rats demonstrated status epilepticus. There was no mortality. In one batch, LiCl was administered at a double dose (254mg/kg), but still, the rats did not experience seizures.

Results with modified protocol

For every batch, six rats were used for model development, and two rats were taken as controls. Most of the time, out of these 6 rats, 2 rats developed seizures of stage 4 and above, 2 rats were moderately sensitive to the increased pilocarpine dose, i.e., they showed Racine scoring stage of <4. 2 rats were completely resistant to the induction of epilepsy despite the increased pilocarpine dose (90mg/kg) (Fig. 4). A total of 75 rats were utilized for the model establishment. These rats were divided into 9 sub-groups; 18 rats were in the control group, and 57 rats were in the epilepsy group. Fate of 57 epileptic rats: 18 rats (31.57%) developed seizures of stage 4 and above; 15 rats (26.31%) were moderately sensitive (less than stage 4), remaining 18 (31.57%) rats were resistant to the increased dose of pilocarpine, and 6 rats died (10.52%). Deaths occurred within 24 hours of the model development. (Fig. 5)

Complications encountered

1.Resistance to Model Development: Some rats didn't develop seizures (31.57%). Even after the maximum dose of pilocarpine, no external change was visualized.

2. Shivering: In some batches of rats, even at a dose of 90 mg/kg, they didn't enter stage 4 and above of seizures, but they were shivering, though the same was not visible externally in terms of forelimb clonus, rearing, and falling. Shivering was not classified as any stage of seizures. Since the rats were weak and shivering, further doses

were not given to them.

3. Moderately Sensitive: On following every possible troubleshooting advice, we found that the maximum number of rats were only moderately sensitive to the dose of pilocarpine being given (90 mg/kg) and exhibited seizure severity less than stage 4.

4. Alopecia & Patchy Skin: In a single case, a rat suffered from the loss of body hair on the ventral side and yellowish patchy skin. (Fig. 6)

DISCUSSION

We attempted to generate the LPISE model of TLE to investigate the role of the mitochondrial calcium uniporter in an animal model of SE. Being a stable model of TLE, this model is useful for studying both acute and chronic epileptic changes. Moreover, studies on etiopathophysiology and management of epilepsy using various interventions can be performed on this model.

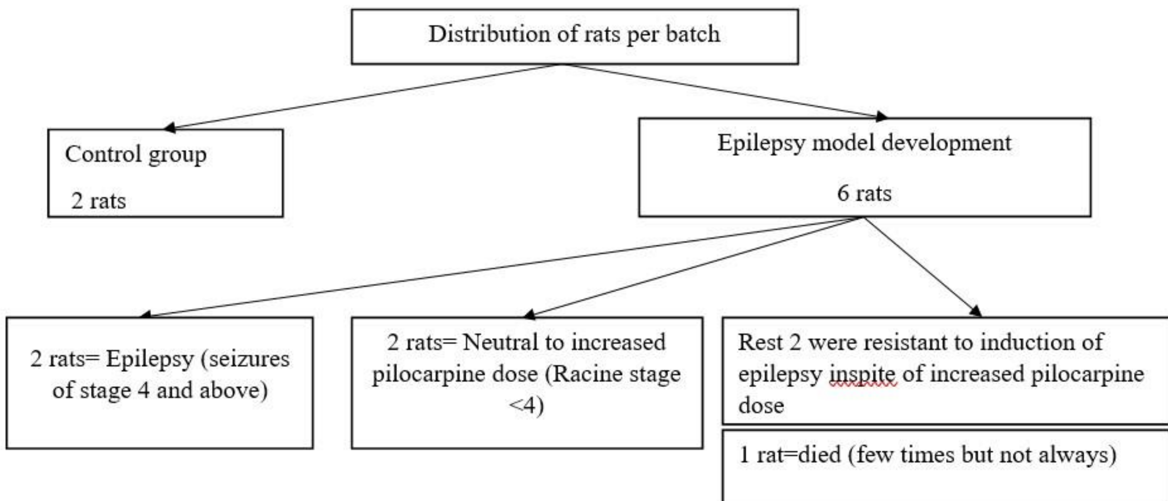

Fig. 4. Flow chart showing distribution of rats per experimental batch

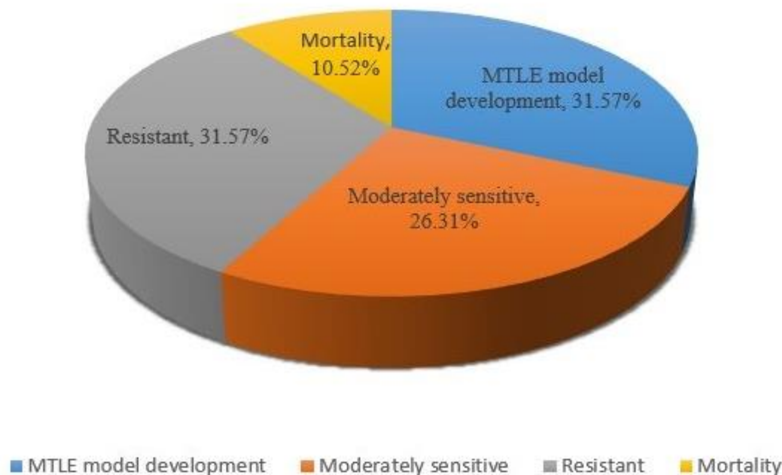

Fig. 5. Pie-chart showing response of rats towards lithium-pilocarpine

Fig. 6. Various complications encountered during model development. a: Alopecia; b: Patchy skin.

According to many reports, this epilepsy model can be created with ease [36, 37, 42]. Contrary to this, we could not achieve the desired goal of status epilepticus even in a single animal despite repeated attempts with the standard dosage. With modified pilocarpine dosage (as described above), we could achieve a 58% seizure induction rate while maintaining lower mortality rates. MTLE model creation was validated by histological examination of the hippocampus. In the current report, we discuss the difficulties encountered during the generation of this model, how troubleshooting was done, and propose a protocol based on these findings.

Animal Selection and Housekeeping

A. Age and Weight: SE was not observed in heavy rats weighing 350-400 grams even with the modified dosage; however, small Wistar rats weighing 100-150 grams showed SE generation. The increased occurrence of seizures in small rats as compared to large rats was in accordance with those of Hirsch et al., 1992 [43] and Curia et al., 2008 study [44], in which rats of P11-30 (P: postnatal) were taken, SE was induced in all small rats, and the mortality rate varied from zero% (in P11-14) to an intermediate 33% (in P15-21) spiking up to 50% (in P22-30), signifying that the mortality rate increased with the age and weight of rats. The chemicals used in the generation of the LPISE model are weight-dependent; an increased dose of pilocarpine required for seizure induction in heavy rats may lead to severe toxicity and death [45, 46].

B. Sex: Only male rats were used as female rats are more resistant to SE induced by pilocarpine due to estradiol causing relative

insensitivity to cholinergic drugs. In females, the different phases of the estrous cycle show differences in the efficacy of pilocarpine at the estradiol peak of the estrus cycle [47, 48]. Lawrence et al., 2010 reported that progesterone-derived neurosteroids (e.g., pregnanolone, allopregnanolone) affect spontaneous recurrent seizures (SRS) in female rats injected with pilocarpine [49].

C. Feed and Bedding: In an attempt to normalize all physiological and environmental conditions, AIN-76 rodent diet was used as a standard reference diet to reduce feed variability [50], and corncob bedding was provided to the animals because it inhibits the accumulation of ammonia. Good husbandry practices of changing bedding frequently, sanitation, and frequent disposal of feces were religiously followed.

Model Creation Mechanism

It has been documented that the administration of LiCl 18-22 hours prior to the injection of pilocarpine and methyl scopolamine before pilocarpine administration successfully induces SE in Wistar rats [36, 37, 42]. Lithium has been shown to potentiate the effect of pilocarpine, thereby decreasing the dose of pilocarpine by 10 times, ultimately reducing the mortality rate of rats to a greater extent.

Lithium, when administered to rats prior to pilocarpine, increases acetylcholine release in the hippocampus and results in more acetylcholine crossing the synaptic cleft and reaching the postsynaptic membrane, where it activates M1 muscarinic receptors and further decreases the dose of pilocarpine by indirectly activating T-lymphocytes and mononuclear cells by complementing the pilocarpine action [51-53]. Pilocarpine induces peripheral inflammation, enhancing blood-brain barrier (BBB) permeability, which further causes its diffusion in the brain resulting in an imbalance between excitatory and inhibitory transmission causing SE [54-56].

Pilocarpine is a muscarinic receptor (M1) agonist, thus stimulating continuous excitatory activity; this enhanced excitability leads to seizures that build up into a limbic SE [57, 58]. Methyl scopolamine does not cross the BBB and reduces the peripheral effects caused by pilocarpine [59]. The absence of methyl scopolamine results in animals displaying symptoms of peripheral cholinergic activity such as tremor, salivation, piloerection, chromodacryorrhea, and diarrhea after pilocarpine administration [60].

Structural damages and subsequent development of spontaneous recurrent seizures resemble those of human seizures. Moreover, the Li-Pi model is a good mimic of human TLE and is best suited for molecular as well as histological studies because the seizures originate from the mesial temporal

structures of the hippocampal formation, and electrical/chemical changes in brain tissue have been found to be similar to human epilepsy, and pathological changes (mesial temporal lobe sclerosis) are comparable to human pathology [14].

Mortality: When pilocarpine is administered in a single dose (30mg/kg), then mortality is reported to be 90-92% [33]. But when the same dose is split (10mg/kg each), then mortality is reduced by 50% [61]. In our present modified protocol, we have evidenced a 10% mortality. Dehydration and exhaustion are considered to be the main reasons for mortality. Diazepam helps by arresting the SE. Nutritional support in the form of regular feeding of glucose by oral gavage and moistening the dry eyes were some of the practices which we found useful.

Possible Reasons for Resistance to Model Development:

During this study, the inability to reproduce the LPISE model with the standard recommended protocol as per the literature is difficult to explain because all the factors with the potential to create variability were judiciously monitored, and multiple attempts were taken. Prior to this report, the lack of reproducibility of the LPISE model in different labs has already been documented [62-64]. Pilocarpine-resistant animals have been cited as the cause of difficulty in seizure induction.

Pilocarpine resistance could be due to a particular rodent strain, species, or gender. This variability could also be attributed to the increased expression of drug efflux transporters on the BBB. Efflux transporters have been extensively studied in relation to drug resistance in epilepsy. The main efflux transporters are Permeability glycoprotein (P-gP) and Multiresistance protein-1 (MRP-1). Their physiological role is to extrude harmful substances at the blood-brain barrier (BBB) so that the internal milieu of the brain is preserved. They have been found to be overexpressed in drug-resistant epileptic patients leading to subclinical concentrations of antiepileptic drugs at the epileptic focus.

Similarly, efflux transporters may extrude the pilocarpine and consequently reduce the bioavailability of the drug at the target site [65]. The saturation level of muscarinic receptors causing resistance of rats to further increased doses of pilocarpine might result in this failure. When the agonist concentration is high, the receptors get saturated, which may lead to receptor downregulation and ultimately lead to resistance. Thus, there is a need of standardization of protocol to develop LPISE model, considering practical problems, so that the research on translational preclinical epilepsy is comparable [62, 66-69].

Proposed method of model development:

Young small-sized rats (100-150g) should be chosen. To ensure the drug reaches the

peritoneum, we recommend using a 26G needle, which is longer (38mm) than the routinely used 24G needle. LiCl (127mg/kg, i.p.) should be injected, followed by methyl scopolamine (1mg/kg, i.p.) after 18-22 hours. Precisely 30 minutes later, injection of pilocarpine via the intraperitoneal route is recommended, starting with incremental doses from a minimum of 30 mg/kg, up to a maximum total dose of 90 mg/kg of pilocarpine. Once the rats begin to develop seizures (usually after an interval of 40-50 minutes), diazepam (10mg/kg) should be injected to reduce seizure severity and increase survival rate.

Since the rats are fatigued due to seizures and unable to eat and drink on their own, glucose (10% of body weight) should be administered to rats through oral gavage as an energy drink to endure the severity of seizures. Rats that did not develop seizures should undergo a wash-out period of 48 hours and can then be reused for model generation; this aligns with the ethics of using the minimal number of animals. It is noteworthy that a mortality rate of 10% was observed during our model generation.

CONCLUSION

The current paper highlights the various adjustments made to the original lithium pilocarpine model to address both the high mortality and low induction rates. These modifications led to improved seizure induction rates (58%) and lower mortality rates (10.52%). The protocol proposed by us meets all three criteria laid down for an ideal animal model of epilepsy [12, 13]. It demonstrates constructive validity, as evidenced by the typical histological features observed in the hippocampal tissue. Furthermore, typical stages of epilepsy were demonstrated, representing face validity, and we have observed predictive validity as it responds to the anti-epileptic drug valproic acid (Results not shared in this paper).

Limitations of the present study:

Since we intend to study acute events such as changes in calcium levels, neuroinflammation in tissue samples, oxidative stress-related changes, etc., the model development was conducted, and rats were sacrificed after 24 hours for downstream processes like histology, gene, and protein expression. The time frame of 24 hours appears to be a potential limitation for the study of complications related to model development, as longer-term issues are not addressed.

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