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The Effect of Pilocarpine Hydrochloride on The Occurrence of Temporal Lobe Epileptic Seizures in White Mice (*Mus musculus* L.) BALB/C Strain

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Abstract

The objective of this research was to determine the effective dosage of pilocarpine hydrochloride to provoke epileptic seizures in animal models. In this study, a true-experimental method was utilized, employing a post-test only control group design. A total of 32 white mice (*Mus musculus Linnaeus* UICC 524) BALB/c strains were divided into four different groups for this study. The experimental groups were subjected to varying dosages of pilocarpine hydrochloride: 220 mg/kgBW as group I, 320 mg/kgBW as group II, and 350 mg/kgBW as group III. On the other hand, the control group was administered a saline solution (NaCl 0.9%) at a dosage of 0.16% ml/kgBW. Both pilocarpine hydrochloride and saline solution (NaCl 0.9%) were injected intraperitoneally (i.p) into mice. In group I, none of the mice experienced epileptic seizures, and they all survived. In contrast, in group III, all the mice experienced epileptic seizures, but none of them survived. The Man-Whitney Test showed significant differences in seizure occurrence across doses ($p<0.01$). The effective dose of pilocarpine hydrochloride for inducing epileptic seizures associated with temporal lobe epilepsy (TLE) in this study appears to be in the range of 320 mg/kgBW to 350 mg/kgBW.

Keywords: pilocarpine hydrochloride; temporal lobe epilepsy; white mice (*M. musculus* UICC 524) BALB/c strain.

INTRODUCTION

Epilepsy stands as the most prevalent neurological disorder in the general population, and it has the potential to lead to significant impairment and, in some cases, even mortality among those suffering from it (Minjarez et al., 2017). Epilepsy is a brain disorder characterized by an ongoing tendency to experience epileptic seizures, accompanied by the neurobiological, cognitive, psychological, and social consequences arising from this condition (Fisher et al., 2014). Globally, there are at least 50 million people of all ages who are impacted by epilepsy. Notably, about 80% of these cases are concentrated in countries with lower to moderate incomes, where the majority of the population lacks access to adequate treatment (Minghui et al., 2014). Each year, it is approximated that at least 2.4 million people around the world are diagnosed with epilepsy. However, in the instance of Indonesia, the precise extent of a number of epilepsy cases, in terms of both prevalence rates and incidence rates, remains unclear (Octaviana, 2017).

Epilepsy is a complex diagnosis without an easily accessible gold standard examination, even with an electroencephalogram (EEG) examination, brain imaging examination, or a metabolic evaluation such as cerebrospinal fluid biomarkers (Stafstrom & Carmant, 2015; Thijs et al., 2019). Therefore, currently, a study of temporal lobe epilepsy (TLE) has begun to be developed and modified among laboratory studies around the world both on human subjects and animal models (Ahmed Juvale & Che Has, 2020; Kandratavicius et al., 2014). Unfortunately, clinical trials on human subjects are often avoided due to ethical constraints in invasive procedures and the difficulty of obtaining suitable human subjects who can represent all periods of TLE. According to existing clinical trial problems in human subjects, the 3R framework (encompassing Replacement, Reduction, and Refinement of animal involvement) should be considered during the design, conduct, and reporting of in vivo epilepsy research projects as they have provided findings

that are helping to understand the multifaceted aspects of TLE, and it is becoming clear that new technologies will bring meaningful information regarding the mechanisms underlying epileptogenesis (Kandratavicius et al., 2014; Lévesque et al., 2021; Lidster et al., 2016).

There are many ways to induce temporal lobe epileptic seizures in animal models of TLE, one of them being by administering chemoconvulsant drugs such as pilocarpine hydrochloride (Ahmed Juvale & Che Has, 2020). Using white mice of the BALB/c strain (*M. musculus* UICC 524) as animal models for Temporal Lobe Epilepsy (TLE) necessitates that they display a similar "clinical history" as their human counterparts. This involves undergoing an initial injury that impacts the hippocampus and/or the temporal lobe, a latent period between the injury and occurrence of spontaneous seizures, a chronic manifestation of spontaneous seizures, and also histopathological changes that are identified as the characteristic of TLE (Kandratavicius et al., 2014). As a result, the standard approach of utilizing animal models of TLE induced by pilocarpine hydrochloride can recreate the TLE scenario in human subjects.

MATERIALS AND METHODS

Study Design

A genuine true-experimental method was utilized in this research with a post-test only control group design. This study lasted for one month, starting on October 1st and finishing on October 31st, 2022, at the Animal Research Laboratory situated in the Faculty of Medicine and Health Sciences at Warmadewa University, located in Denpasar, Bali, Indonesia.

Subjects

The animal models employed in this study were a group of white mice (*M. musculus* UICC 524) BALB/c strain. The selection process followed specific criteria for inclusion and exclusion. The criteria of white mice as an animal model encompassed factors like gender (male), age (2 to 3 months), weight (20 to 40 grams), overall health, and absence of mortality during both the acclimatization period and the induction of epileptic seizures. To determine the sample size, a combination of the Federer Formula and the Corrected Sample Formula was used, and a total sample of 32 white mice was obtained. These mice were then divided into four treatment groups via a randomized lottery-based method (Ezalina, 2016; M. Sopiyudin Dahlan, 2020). The experimental groups were given three different dosages of pilocarpine hydrochloride. In particular, group I received a dosage of 220 mg/kgBW, group II received 320 mg/kgBW, and group III received 350 mg/kgBW. In contrast, the control group was administered a saline solution (NaCl 0.9%) at a dosage of 0.16% ml/kgBW. The administration of pilocarpine hydrochloride and saline solution (NaCl 0.9%) to induce temporal lobe epileptic seizures, both were injected intraperitoneal (i.p) into mice (Arshad & Naegele, 2020; Kurnia Mirawati et al., 2016; Turski et al., 1983).

Measurement and Statistical Analysis

Epileptic seizures that occur after drug injection will be observed with a video recording device and measured using the Modified Racine Scale (MRS): Score 0, no change in behavior; Score 1, sudden behavioral arrest, motionless staring (with orofacial automatism); Score 2, head nodding; Score 3, forelimb clonus with lordotic posture; Score 4, forelimb clonus, with rearing and falling; Score 5, generalized tonic-clonic activity with loss of postural tone, often resulting in death, wild jumping (Ihara et al., 2016; Reddy & Kuruba, 2013). The collected data in this study will be processed with Statistical Package for the Social Sciences (SPSS) for Windows and tested using the Mann-Whitney test because the data is categorical

Ethical Approval

This study was authorized by the Health Medical Research Ethics Committee situated in the Faculty of Medicine and Health Sciences at Warmadewa University, located in Denpasar, Bali, Indonesia. The consent for this study was provided under registration code 290/Unwar/FKIK/EC-KEPK/X/2022 on September 18th, 2022.

RESULTS AND DISCUSSION

Result

After the injection of the drug was applied to four different treatment groups, all white mice in the control group did not show epileptic seizures, whereas all white mice in the experimental group showed the epileptic seizures. Epileptic seizures in white mice occurred between 30 minutes to 60 minutes after intraperitoneal (i.p) injection of pilocarpine hydrochloride. Epileptic seizures in white mice that arise as stereotyped movements are measured using the Modified Racine Scale (MRS) measurement method, starting from a score of 1-5. Here is the collected data after the treatment:

Based on the results of the study, it was found that, in the control group with an injection of saline solution (NaCl 0.9%) at a dose of 0.16 ml/kgBW did not have any influence on the behavior of white mice. There was no profile of temporal lobe epileptic seizures even the status epilepticus condition and then death in white mice based on Modified Racine Scale (MRS) measurements. The results of saline solution (NaCl 0.9%) injection in the control group are displayed in (Table 1).

Table 1. The Results of Saline Solution (NaCl 0.9%) Injection in Control group (n=8).

Saline Solution (NaCl 0,9%)	Modified Racine Scale						Status Epilepticus	Number of Life	Mortality
	0	1	2	3	4	5			
0.16 ml/kg BW	0	0	0	0	0	0	0	8	0

Based on the results of the study, it was found that, in the experimental groups with pilocarpine hydrochloride, the following outcomes were noted: At a dose of 220 mg/kgBW, there was no influence on the behavior of white mice and there was no profile of epileptic seizures that were observed based on Modified Racine Scale (MRS) measurements. At a dose of 320 mg/kgBW, there was able to induced various profiles of epileptic seizures in mice, with some of them experiencing status epilepticus conditions and ultimately leading to mortality. At a dose of 350 mg/kgBW, there was able to induce different profiles of epileptic seizures in white mice, then all of them experienced the status epilepticus conditions and ultimately leading to mortality. The results of pilocarpine hydrochloride injection in experimental groups displayed in (Table 2).

Table 2. The Results of Pilocarpine Hydrochloride Injection in Experimental Groups (n=24).

Pilocarpine Hydrochloride	Modified Racine Scale						Status Epilepticus	Number of Life	Mortality
	0	1	2	3	4	5			
220 mg/kgBW	0	0	0	0	0	0	0	8	0
320 mg/kgBW	0	3	2	1	1	1	2	6	2
350 mg/kgBW	0	0	0	0	4	4	8	0	8

Based on the statistical tests of data analytics with the Mann-Whitney test, a p value of 1.00 was obtained with a significant level of 0.05. The results showed that p value was greater than the significant level value, so statistically it can be concluded that there is no difference in the meaning of the dose group with epileptic seizures ($p > 0.05$). The results of the Mann-Whitney test in 4 different treatment groups are displayed in (Table 3).

Table 3. The Results of the Mann-Whitney test in 4 Treatment Groups (n=32).

The Size of Dosage	Temporal Lobe Epileptic Seizures		P Value
	Yes	No	
Saline Solution (NaCl 0.9%) at dose of 0.16 mL/kgBW	0 (0.0%)	8 (100%)	1,00*
Pilocarpine Hydrochloride at dose of 220 mg/kgBW	0 (0.0%)	8 (100%)	
Pilocarpine Hydrochloride at dose of 320 mg/kgBW	8 (100%)	0 (0.0%)	
Pilocarpine Hydrochloride at dose of 350 mg/kgBW	8 (100%)	0 (0.0%)	

Discussion

White mice (*M. musculus* UICC 524) BALB/c strain was selected as the animal model for this study due to their genetic resemblance to humans, which stands at 79%. Furthermore, their small size and ease of obtaining, maintaining, and acclimatization made them suitable models for inclusion in this study (Grone & Baraban, 2015). Pilocarpine hydrochloride as an inducing agent was chosen in this study because pilocarpine hydrochloride was proven to induce temporal lobe epileptic seizures in previous studies of the animal models of TLE (Ahmed Juvale & Che Has, 2020).

In this study, three different doses of pilocarpine hydrochloride were employed as an inducing agent: 220 mg/kgBW, 320 mg/kgBW, and 350 mg/kgBW. The selection of the pilocarpine hydrochloride lowest dose, 220 mg/kgBW, was based on previous study led by Mirawati who convinced that the use of pilocarpine hydrochloride at dose of 220 mg/kgBW was proven to induced the epileptic seizures in animal models of TLE. Similarly, the selection of pilocarpine hydrochloride higher doses, 320 mg/kgBW and 350 mg/kgBW, was based on previous studies led by Turski who convinced the use of pilocarpine hydrochloride within the range of 300 mg/kgBW to 400 mg/kgBW was proven to induce the epileptic seizures, including status epilepticus conditions, ultimately leading to mortality, in animal models of TLE (Kurnia Mirawati et al., 2016; Turski et al., 1983).

Status epilepticus (SE) in animal models is defined as a persistent seizure, either a partial seizure or a generalized seizure that occurs continuously for 5 minutes or more (Ahmed Juvale & Che Has, 2020; Sharma et al., 2007). In this study, white mice in the experimental group III that were injected by pilocarpine hydrochloride at a dose of 350 mg/kgBW experienced

status epilepticus condition with MRS scores of 4 - 5; but unfortunately, all the white mice in this experimental group died. Meanwhile, white mice in experimental group I that injected with a pilocarpine hydrochloride dose of 200 mg/kgBW did not show any epileptic seizures even experienced status epilepticus condition; thus, all mice in this group were survived (Arshad & Naegele, 2020). Unlike the previous experimental group, white mice in experimental group II that were injected with a pilocarpine hydrochloride dose of 320 mg/kgBW experienced various results on MRS measurements, some of the white mice in this experi-mental group survived and some died.

According to statistical calculations, the effective dose of pilocarpine hydrochloride in this study that was used as an inducing agent of the temporal lobe epileptic seizures on white mice (*M. musculus* UICC 524) BALB/c strain was at a dose of 320 mg/kgBW - 350 mg/kgBW. The effective dose of pilocarpine hydrochloride in this study was much higher than the previous study conducted by Mirawati with the same type of animal model strain (i.e., white mice), is at doses of 210mg/kgBW - 250 mg/kgBW (Kurnia Mirawati et al., 2016). Apparently, the effective dose of pilocarpine hydrochloride in this study is almost comparable to previous studies conducted by Turski which has been reviewed in Juvale & Has's article, that said the effective dose of pilocarpine hydrochloride in Turski's study, is at a doses of 300 mg/kgBW - 400 mg/kgBW even with a different type of animal model strain (i.e., Wistar rats) (Ahmed Juvale & Che Has, 2020; Turski et al., 1983). The similarity of the chemoconvulsant effects of pilocarpine hydrochloride in different animal model strains may occur due to several factors such as the method of maintenance and feeding during acclimatization and/or the sensitivity of the white mice themselves to the pilocarpine hydrochloride induction response.

Based on the results of this study, it was found that the white mice that experienced temporal lobe epileptic seizures were white mice in the experimental group of pilocarpine hydrochloride II and III, while white mice in the experimental group of pilocarpine hydrochloride I and control group with saline solution (NaCl 0.9%) did not experience any temporal lobe epileptic seizures. According to existing theories, temporal lobe epileptic seizure by pilocarpine hydrochloride as an inducing agent can occur through receptor binding mechanism. Chemoconvulsants are substances capable of provoking epileptic seizures, while anticonvulsants are substances that can prevent epileptic seizures by binding to muscarinic receptors within the central nervous system (CNS) (Ali et al., 2018).

The brain as a part of the central nervous system (CNS), contains at least of 40 types of chemical compounds classified as the neurotransmitters. Based on their function, these neurotransmitters can be categorized into two main groups: an excitatory neurotrnsmitter, such as acetylcholine and glutamic acid, and also an inhibitory neurotransmitter, such as γ-aminobutyric acid (GABA) and glycine (Akyuz et al., 2021; Shao et al., 2019). An increased amount of excitatory neurotransmitters followed by a decreased amount of inhibitory neurotransmitters can enchance the seizure threshold value thereby causing epileptic seizures, therefore the regulation of the function of these neurotransmitters can play an important role in the occurrence of epileptogenesis mechanism (Dumanis et al., 2017).

In previous studies, it was mentioned that the pilocarpine hydrochloride known as chemoconvulsant drug would work as an acetylcholine agonist so that it is capable of bind to the muscarinic receptor N-methyl-D-aspartate (NMDA), thus pilocarpine hydrochloride will cause the similar effects to acetylcholine as an excitatory neurotransmitter. According to biochemical theory, it was mentioned that the binding of NMDA muscarinic receptors with pilocarpine hydrochloride as an acetylcholine agonist could survive at least 24 hours to 1 month in the synapse cleft. This is supported by alterations in the behavior of experimental animal models, which have demonstrated the occurrence of spontaneous recurrent seizures in previous studies (Ali et al., 2018; Hoeller et al., 2016; Kurnia Mirawati et al., 2016). In individuals affected by temporal lobe epilepsy, these spontaneous recurrent seizures can lead to hypoxic conditions within brain tissue. Consequently, this can result in sclerosis conditions or permanent neuron cell damage (irreversible) characterized by neuronal cell loss and astrogliosis (Dingledine et al., 2014; Kurnia Mirawati et al., 2016).

Sclerosis in the area of Cornu Ammonis 1 (CA1) and Cornu Ammonis 3 (CA3) in the hippocampus area characterized by neuronal cell loss and astrocyte cell images in the form of glial cells in the astrogliolysis process is a histopathological finding that commonly occurs in individuals affected by temporal lobe epilepsy. These findings align with the histopathological observations obtained in numerous previous animal model studies of temporal lobe epileptic seizures (Kurnia Mirawati et al., 2016; Mátyás et al., 2021). According to the study conducted by Mátyás, the histopathological examination using Hematoxylin-Eosin (HE) staining revealed significant neuron cell damage in the CA1 and CA3 areas in animal models of the temporal lobe epileptic seizures after the injection of pilocarpine hydrochloride (Mátyás et al., 2021). Neuron cell damage found in animal models of temporal lobe epileptic seizures by pilocarpine hydrochloride as an inducing agent has demonstrated the epileptogenesis mechanism pathway as well as provided confirmation between the clinical manifestations in individuals affected by temporal lobe epileptic seizures with the theory of neuronal-death-pathway hypothesis in a number of existing studies.

CONCLUSIONS

As a result of this study, it was determined that the effective dose range for inducing temporal lobe epileptic seizures in white mice (*M. musculus* UICC 524) BALB/c strain is between 320 mg/kgBW - 350 mg/kgBW using pilocarpine hydrochloride.

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