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Original Article

Sedative Effect of Intraperitoneal Diazepam in Mice

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Abstract

Objective: To determine the effectiveness of Diazepam in comparison with Phenobarbital.

Methods: Twenty-seven male Swiss Webster mice were used and randomly divided into three groups of negative control (NS), positive control (Phenobarbital), and diazepam group. Two tests were performed on these group: the traction Test and the Fireplace Test. Pupillary diameter was also observed.

Results: A significant difference based on the Kruskal - Wallis statistical test was observed between the positive control and the diazepam group (<0.05) in the traction test, which was also true for the fireplaced test ($p<0.05$). The pupillary diameter in the test animals in the positive control and diazepam group was not statistically significant ($p>0.05$).

Conclusion: Diazepam has a better sedative effect than Phenobarbital. The sedative effect produced by Diazepam is stronger, with faster onset and longer half-life than the Phenobarbital the positive control. However, different test methods and comparisons should be sought to support this conclusion.

Keywords: Diazepam, fireplace test, phenobarbital, traction test

Introduction

Induction of anesthesia is the administration of drugs approximately 1-2 hours before anesthesia to assist the process of anesthesia. Pre-medication can be given before general or local anesthesia by intravenous (IV), intramuscular (IM), or subcutaneous (SC). Pre-medication drugs are classified into sedatives (diazepam and phenobarbital) and non-sedative drugs such as atropine.[1] Generally, phenobarbital and diazepam can be delivered by IM injection. Meanwhile, the most frequently used injections for solutions or suspensions were SC, IP, and IV injections. IP injection is a simple technique because the large surface area of the peritoneal cavity and many blood vessels allow for rapid absorption.[2]

Phenobarbital is a derivative of the barbiturate group for hypnotic therapy and the treatment of epilepsy. Phenobarbital has a long-acting period, used as general anesthesia in rats with a small dose.[3] Diazepam has the same use as phenobarbital and can be used as an anesthetic induction and antiepileptic therapy. Diazepam is widely used for anxiety disorder, short-term relief of anxiety symptoms, alcohol withdrawal, and insomnia.[4] Therefore, diazepam has largely replaced phenobarbital for anxiety and sleep disorders treatment due to fewer side effects. However, diazepam and phenobarbital have the same general side effect, sedation.[4] Diazepam was also used frequently as a positive control in study behavior in mice. Meanwhile, phenobarbital is commonly used as a positive control in study anesthesia in rodents.[3,5,6] The phenobarbital side effect was hepatotoxicity, which may affect the use of anesthesia that assess the effects on the liver.[7,8] These may limit the use of phenobarbital as a positive control. The alternative positive control needs to be studied.

Marina's study showed a diazepam sedative effect rather than an anxiolytic effect in mice.[9] This finding may use as basic information to develop diazepam use in experimental animal research. The study regarding the sedative

effect of diazepam in experimental animals is still limited. Otherwise, diazepam is potentially used as an alternative positive control in study anesthesia and as a pre-medication anesthesia in the animal study. The route of administration is a critical factor for the availability of the drugs in plasma and could affect pharmacodynamics. In experimental animal research, IP injection used frequently because the simplicity of technique and minimal stress for the animal study.[10]

The study regarding diazepam's effectiveness in various injection routes is also still limited. At the same time, it can be informative to other researchers especially in experimental animal research. Further study needs to be conducted to assess how the effectiveness of diazepam sedative effect in experimental animals (mainly mice and rats) by various routes of administration. Therefore, this study aimed to determine the duration and effectiveness of diazepam sedative effect by IP injection in mice.

Methods

The experimental study was conducted in the Faculty of Medicine Universitas Muhammadiyah Prof. DR. HAMKA. This study used male Swiss-Webster mice (mus musculus) aged 3-6 months and weighed 20-30 grams. The mice are cared for under standard conditions, by the temperature of 26 – 28 °C, 12-hour light/dark cycle, and humidity. Animal acclimatization was carried out for ten days before the test, had free access to food and water in their cages. The animal experiments were carried out under the supervision of veterinarians to ensure their health during acclimatization until the tests of study. The animal experiments were eligible to undergo several study tests. The procedures of study were approved by Animal Ethics Committee, Universitas Muhammadiyah Prof. Dr. HAMKA (Protocol No.KEPKK/FK/024/01/2022).

This animal research applies ethical principles 3R (replacement, reduction, and refinement). The ethical principles also refer to the 5F (Five freedom) consist of freedom from hunger and thirst (free from hunger and thirst), freedom from discomfort (free from discomfort), freedom from pain, injury, and disease (free from pain, injury, and illness), and freedom to express normal and natural behavior. The number of samples was calculated with the Federer formula, resulting in 27 mice for three groups (n=9) by random sampling. The groups were group I (normal saline as negative control), group II (phenobarbital as positive control), and group III (intervention with diazepam). All the interventions by IP injection.

The dosage of 0.325 mg phenobarbital for 20 g mice is equivalent to 100 mg phenobarbital in humans and adjustable as the weight. Diazepam dosage use 0.0325 mg as for 20 g mice, equivalent with 10 mg of diazepam in humans. The animals performed several tests such as traction test, fireplace test, and examination of pupil diameter. The traction test was conducted first after the acclimatization and ensured all the animals were eligible for the study. The test was started when the mice was confirmed under sedation effect by touching the mice and showed less or no response. The onset of the drugs may vary each other. The traction test aimed to observe muscle relaxation activity by placing the mice with the anterior body position facing a wire stretched horizontally, then the tail is pulled up. The animal tests that fail to make a re-establishment of at least one of its posterior limbs to reach the wire are considered under a sedative effect. The duration of mice to make re-establishment were recorded by stopwatch.

The fireplace tests were conducted three days after the fireplace tests were finished. The animal tests considered under sedation effect when showed no response or less response. The fireplace test was used to observe decreased activity and sensitivity toward the environment. The test was conducted by placing the mice individually in a cylindrical tube to assess the time mice get out quickly. The duration of mice attempting to escape the cylindrical tube was recorded by stopwatch. The pupillary reflex is used to determine the sedation effect by observing the pupil diameter. The normal pupil diameter in mice was 2 mm. This test is conducted during the fireplace test.

The statistical product and service solution (SPSS) program was used to analyze the results. The Kruskal–Wallis test was performed to measure the significance of differences among groups. A value of $p<0.05$ is considered a statistically significant difference.

Result

The animal tests weighed during the study period presents in **Table 1**. The weight of animal tests was not varied among groups because there was no significant elevated body weight during the study. The pupil diameter before tests were homogenous among groups

Table 1 General Characteristics of Mice

Parameters	Groups		
	Negative Control (NS)	Positive Control (Phenobarbital)	Diazepam
n	9	9	9
Body Weight (g)	32.9	33.2	32.8
Pupil Diameter (mm)	2	2	2
Onset:			
Traction Test (min)	0	15	5
Fireplace Test (min)	0	10	3

Table 2 Parameter Tests Results of Sedation Effect

Parameter Test	Group		
	Negative Control n=9	Positive Control n=9	Diazepam n=9
Traction Test	2 seconds ± 0.5	5 seconds ± 1.5	7 seconds ± 1.8
Fireplace Test	2 seconds ± 0.5	80 seconds ± 72.8	500 seconds ± 141.4
Pupil Diameter	2 mm	1 mm	1 mm

with normal-sized diameter pupil for mice (2 mm). There were no significant differences statistically in the body weight between groups. Table 1 also showed the onset of the drugs (phenobarbital and diazepam) in traction and fireplace tests were not far different. The onset of the drugs also may vary each other's.

The animal test undergoes several interventions based on their group test. Several test parameters, such as traction test, fireplace test, and assessment of diameter pupil, collected the data results. Table 3 presents the results of those several parameters test. The tests were conducted to determine the effect of sedation in animal tests by the following results.

The negative control group took an average of two seconds in the Traction and Fireplace Test. Meanwhile, the positive control group took longer by an average of five seconds in the Traction test and 80 seconds in the Fireplace test. The Diazepam group showed a more prolonged sedation effect than the positive control by an average of seven seconds in the Traction Test and 500 seconds in the Fireplace Test (Table 2). The assessment of the diameter pupil in the negative control group, positive control group, and diazepam group, respectively, 2 mm, 1mm, and 1 mm (Table 2).

The statistical analysis presents data results that were not distributed normally ($p<0.05$) by the Shapiro - Wilk test. Table 3 shows the traction test result's significant difference between the positive control and the diazepam group ($p<0.05$). Moreover, the Fireplace test also performed a statistically significant difference between the positive control and the diazepam group ($p<0.05$) by the Kruskal – Wallis test. Pupillary diameter in

Table 3 Statistical Analysis Results in Several Parameter Tests

Groups	Traction Test	Fireplace Test	Pupillary Diameter
	p	*p*	*p*
NS with Diazepam	0.000	0.000	0.000
Phenobarbital with Diazepam	0.015	0.001	1.000

the animal test was not statistically different between the positive control and diazepam groups ($p>0.05$). The negative control and diazepam groups showed a statistical difference in pupillary diameter (Table 3).

Discussion

A significance different among parameter tests between positive control and diazepam groups indicates a decrease in the activity of the animal's test. These may show the occurrence of Central Nervous System (CNS) suppression.[11] Mostly, the pharmacokinetics of sedative drugs are fat soluble, well absorbed, and distributed to the brain. High lipid solubility drugs can quickly enter the CNS. Sedative drugs are metabolized by liver enzymes and excreted by the kidney. However, there are different metabolic rates in each drug class. Diazepam belongs to benzodiazepine groups with anxiolytic action, hypnotic, muscle relaxation, and anti-convulsion, used in anesthesia and insomnia conditions.

Diazepam elevated GABA potential action by opening chloride channels, leading to hyperpolarization membrane, inducing CNS depression and sedative activity. The traction and fireplace tests were used as assessment parameters for the sedative effect in mice.[12,13] The traction test showed the length of time in mice to turn around and fall. The sedative effect becomes significant if the mice take longer to turn around. Moreover, the fireplace test showed the length of time mice jumped out of the tube. The sedative effect gets stronger if the mice take longer to jump out. Pupillary diameter changes may show decreased spontaneous activity in mice as a sedative effect.[14]The onset of the drugs varied among groups and could be affected by the route of administration. The administration by intraperitoneal injection was to avoid the potential degradation or modification of the drugs.[2] Meanwhile, the IV injection was hard to use in rodents. The IP injection was minimally used in clinics but preferred in animal studies. Durk and colleagues' studies showed that IP administration resulted in lower Tmax and higher Cmax than SC injection.[15]

The diazepam group's traction and fireplace test results showed longer than the positive control (phenobarbital) group. These may cause the benzodiazepine group (diazepam) to become an active metabolite with a long half-life resulting in a more prolonged effect sedative than the positive control group. Meanwhile, barbiturates, especially phenobarbital, are partly excreted in the urine, and some undergo extensively metabolized.[16]

Sadanandan's study shows Diazepam has a long duration of sleep than Ganaxolone.[17] Diazepam is frequently used as a comparator drug in studies related to sedative effect and anesthesia (intravenously). In used to anesthesia, diazepam is mainly combined with other agents.[16] In experimental animal models, benzodiazepines and older sedative-hypnotic drugs can exert an anti-anxiety effect. However, not all sedative effects drugs have it.[16] The sedative-hypnotic drug group are dose-dependent and induce sleep in high dose. The specific drug and administration frequency could affect the sleep stages in the sedative-hypnotics effect.[16] Phenobarbital has a long duration of action and long half-life (80-120 hours), although the traction and fireplace test results showed a shorter duration of sedative effects in phenobarbital than diazepam. Phenobarbital has low lipid solubility, protein binding to albumin at approximately 55%, and a long onset delay.[18] Meanwhile, diazepam has a 20-80 hours half-life, highly lipid soluble and highly protein bound, and easily crosses the blood-brain barrier. Diazepam is considered a better choice because of its rapid onset and long half-life compared to phenobarbital or in the benzodiazepine group.[19]

Based on the traction and fireplace test results, this study concluded that diazepam has a better sedative effect than phenobarbital. The sedative effect produced by diazepam is more prolonged compared to phenobarbital as a positive control. These findings may be helpful to other researchers as an alternative agent to anesthesia the animal study or as a positive control in an anesthesia study. However, there were still several areas for improvement in this study, as we did not collect the blood sample to measure the drug plasma concentration or collect the organ to assess molecular parameters regarding the sedative effect. These could be used as complementary data to resulting comprehensive results. Despite that, this study followed the ethical principle of animal welfare, the sample size counted based on the Federer formula, the animal study health was under expert supervision, and used established methods. Therefore, suggestions for further research to be performed with different test methods, parameters, and comparisons.

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