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Original Article

Sedative Effects 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 groups: Traction Test and 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 fireplace 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 commonly defined as the administration of drugs approximately 1-2 hours before anesthesia to assist the process of anesthesia. Pre-medications can be given before general or local anesthesia through intravenous (IV), intramuscular (IM), or subcutaneous (SC) route. Pre-medication drugs used for this purpose are classified into sedatives, such as 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 known derivative of the barbiturate group that is used for hypnotic therapy and epiplepsy treatments. This agent has a long-acting period, and a small dose of this agent is used as general anesthesia in rats.[3] Another drug, diazepam, also has the same use as phenobarbital, and can be used as an anesthetic induction and antiepileptic therapy agent. Diazepam is widely used for anxiety disorder, insomnia, alcohol withdrawal, and short-term relief of anxiety symptoms.[4] Thus, diazepam has largely replaced phenobarbital for anxiety and sleep disorders treatment due to fewer side effects. Nonetheless, diazepam and phenobarbital have the same general side effect, sedation.[4] Diazepam is also used frequently as a positive control in behavioral studies in mice. Meanwhile, phenobarbital is commonly used as a positive control in study anesthesia in rodents.[3,5,6] The phenobarbital side effect is hepatotoxicity, which may affect the use of this anesthetics in the anesthesia that assess the effects on the liver.[7,8] These may limit the use of phenobarbital as a positive control. Alternative positive controls need to be studied.

A previous study by Marina demonstrated that diazepam has more sedative effects than anxiolytic effects in mice.[9] This finding may be

used as basic information to study diazepam use in experimental animal research. Studies regarding the sedative effect of diazepam in experimental animals are still scarce despite the fact that diazepam is potentially used as an alternative positive control in studies on anesthesia and as a pre-medication anesthesia in animal studies. The administration route is a critical factor for the availability of the drugs in plasma and may affect the pharmacodynamics. In experimental animal studies, IP injection is frequently used because of the simplicity of the technique and the minimum stress it causes for the animals under study.[10]

Studies regarding diazepam's effectiveness in various injection routes are also limited. At the same time, this type of study can be informative to other researchers, especially those involved in animal experiments. Further studies are needed to assess the effectiveness of diazepam sedative effects in experimental animals, which mainly consist of mice and rats, using various routes of administration. Hence, this study aimed to determine the duration and effectiveness of diazepam sedative effect when it was administred through IP injection in mice.

Methods

This experimental study was performed on 3-6 month old male Swiss-Webster mice (*mus musculus*) weighed 20–30 grams. This study was condocuted in the Faculty of Medicine of Universitas Muhammadiyah Prof. DR. HAMKA, Indonesia. The mice are maintained under standard conditions: a temperature of 26 to 28 °C, a 12-hour light/dark cycle, and standard humidity. Animal acclimatization was done for ten days before the test and they had free access to food and water in their cages. These animal experiments were under the supervision of veterinarians to ensure their health during the acclimatization period until the tests were performed. These experiment animals were eligible to undergo several tests in this study. The procedures of study were approved by the Animal Ethics Committee, Universitas Muhammadiyah Prof. Dr. HAMKA (Protocol No.KEPKK/FK/024/01/2022).

This animal research applied the ethical principles of 3R (replacement, reduction, and refinement), which also referred to the 5F (five freedom) of free from hunger and thirst; free from discomfort; free from pain, injury and illness; free to express normal and natural behaviors; and free from fear and distress. The sample size was calculated using the Federer formula, resulting in 27 mice for three groups (n=9) selected by random sampling. The mice were then assigned to group I (normal saline as negative control), group II (phenobarbital as positive control), and group III (intervention with diazepam). All interventions was done using IP injections.

The dose of 0.325 mg phenobarbital for a mouse weighed 20 g is equivalent to 100 mg phenobarbital dose in human and adjustable based on the weight. Thus, a diazepam dose of 0.0325 mg for 20 g mice is equivalent with 10 mg of diazepam in humans, which was the dose used in this study. 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 all of the animals were ensured to be eligible for the study. The test was started when the mice was confirmed to be under the sedation effect by touching them and observing their reponse. The sedation effect was confirmed when there was less or no response. The onset of the drugs may vary between mice.

The traction test aimed to observe muscle relaxation activities, which was performed by placing the mouse in an anterior body position, facing a horizontally stretched wire, and had its tail pulled up. The test animals that failed to re-establish at least one of its posterior limbs to reach the wire were considered under a sedative effect. The duration needed by each mouse to make re-establishment were measured using a stopwatch and recorded.

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

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

Table 1 General Characteristics of Mice

Parameter	Group		
	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 Test 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

Results

Test animals were weighed during the study period as presented in Table 1, showing no variation among the groups with no significant increase in body weight observed during the study. The pupil diameter before tests were homogenous among groups with a normal-sized diameter pupil for mice (2 mm). There was no statistically significant differences in the body weight between different groups. The onset of the drugs (phenobarbital and diazepam) in traction and fireplace tests were not far different, albeit may vary.

The animal tests performed in this study comprised of several different interventions based on the groupings. Parameters of these tests, i.e., traction test, fireplace test, and pupil diameter assessments, were measured and data on the results were collected, as presented in Table 3. The tests were conducted to determine the effect of sedation in test animals by referring to the test results.

The negative control group took an average of two seconds in the traction and fireplace tests while the positive control group took longer, by an average of five seconds and 80 seconds, respectively. The diazepam group presented 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 pupil diameter in the negative and positive controls and diazepam group demonstrated an average of 2 mm, 1mm, and 1 mm, respectively (Table 2).

The statistical analysis showed abnormally distributed data ($p<0.05$) based on the result of the Shapiro-Wilk test. Table 3 shows the significant difference between the positive

Table 3 Statistical Analysis Results in Several Parameter Tests

Group	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

control and the diazepam group ($p<0.05$) in the traction test, which was also true for the fireplace test ($p<0.05$). Pupillary diameter was not statistically different between animals in the positive control and diazepam groups ($p>0.05$). Meanwhile, the negative control and diazepam groups showed a statistical difference in pupillary diameter (Table 3).

Discussion

A significance difference in the test parameters between animals in the positive control and diazepam groups indicates a decrease in the test animal acitivities. This may be due to the Central Nervous System (CNS) suppression.[11] Most sedative drugs has a pharmacokinetics nature of fat soluble, well absorbed, and is 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, different metabolic rates is observed for each drug class. Diazepam, a benzodiazepine group drug with an anxiolytic action, hypnotic, muscle relaxation, and anti-convulsion, is used in anesthesia and for insomnia conditions.

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

The traction and fireplace test results in the Diazepam group presented longer time than the positive control (phenobarbital), which may be because the benzodiazepine group (diazepam) becomes an active metabolite with a long half-life, resulting in a more prolonged sedative effect, while barbiturates, particularly phenobarbital, are partly excreted in the urine, with some extensively metabolized.[16]

A study by Sadanandan demonstrates that diazepam produced a longer duration of sleep than Ganaxolone.[17] Diazepam is frequently used as a comparator drug in studies related to sedative effect and anesthesia (intravenously). When used in 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 drugs have this effect.[16] Furthermore, the sedative-hypnotic drug group are dose-dependent and induce sleep in a 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 when compared to diazepam. Phenobarbital has low lipid solubility, protein binding to albumin at approximately 55%, and a long onset delay[18]while 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 due to its rapid onset and long half-life compared to phenobarbital or other drugs in the benzodiazepine group.[19]

In conclusion, diazepam has a better sedative effect than phenobarbital as it produces more prolonged sedative effect. Thus, diazepam can be used as an alternative agent for anesthesia in animal studies or as a positive control in an anesthesia study. Nevertheless, futher studies are need as no blood sample was collected to measure drug plasma concentration and organ assessment was not done to assess molecular parameters of sedative effect. These could be used as complementary data to resulting comprehensive results. This study followed the ethical principle of animal welfare, with the sample size calculated based on the Federer formula, and by using established methods. The health of the study animals was also ensured by having expert supervision. Further studies should be performed with different test methods, parameters, and comparisons.

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