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(RESEARCH ARTICLE)

The Influence of Carbofuran on Purkinje cells death in the fetal cerebellum of mice (*Mus Musculus*)

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Abstract

The objective of this research was to assess the extent of damage to cerebellar Purkinje cells in fetal mice caused by exposure to the insecticide carbofuran during the embryonic period. The study involved eighteen pregnant female mice (*Mus Musculus*) exposed to carbofuran from gestational days 14 to 17. The female mice were divided into three groups: P0 (treated with physiological NaCl solution), P1 (treated with carbofuran at 0.0208 mg/Kg/day), and P2 (treated with carbofuran at 0.0417 mg/Kg/day). The data were analyzed using ANOVA and followed by the BNJ test. The results indicated a significant difference, with an increase in Purkinje cell necrosis corresponding to the dose of carbofuran.

Keywords: Carbofuran; Purkinje cell; Necrosis; Pesticide stress

1. Introduction

Pesticides can become a problem in agriculture due to farmers' habits of sometimes not following recommended usage guidelines. Apart from exceeding recommended dosages, farmers often mix various types of pesticides to enhance their effectiveness against crop pests. Such practices are detrimental as they can lead to increased environmental pollution from pesticide residues [1]. Negative effects of these chemicals have been reported, including disturbances in the central nervous system and teratogenic or embryotoxic effects in both animals and humans [2].

Insecticides are classified into three groups: organochlorines, organophosphates, and carbamates. Carbamates, such as carbofuran, are widely used in agriculture because their toxic effects are relatively lower compared to organochlorine and organophosphate insecticides. Carbofuran is frequently used in agriculture due to its broad spectrum in controlling insects and nematodes. Exposure to carbofuran residues through food or drink consumption by humans and animals can result in neurotoxic, neurobehavioral, and neuropsychological effects [3].

High levels of carbofuran residues can be found in plant parts such as stems, leaves, roots, and rice grains. Carbofuran residues have been detected in crops like potatoes, corn, sunflowers, cotton, sugarcane, cloves, pepper, and grapes because these crops are often treated with pesticides by farmers to protect them from pest infestations [4]. Carbofuran has maximum residue limits (MRLs) allowed in food ranging from 0.05 to 0.5 mg/Kg body weight, with MRLs of 0.05 mg/Kg body weight for livestock products like meat and fat, 0.2 mg/Kg body weight for rice, and 0.5 mg/Kg body weight for potatoes [5].

When carbofuran is present in the human or animal body, its main targets of toxicity are the brain, liver, muscles, and heart [6]. Carbofuran toxicity in mammals is high when consumed orally, as its toxicity is higher than that of other carbamates, and it has similar properties to organophosphate insecticides, known to stimulate the production of reactive oxygen species (ROS) [7].

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In Ecuador in 2001, in an area contaminated with carbofuran insecticide, cases were reported where babies born exhibited reduced reflexes and motor skills. In children, developmental brain function disorders, including decreased memory and concentration, were observed [8].

The cerebellum consists of approximately 30 million functional units, almost identical to each other, with Purkinje cells being the central functional units. These cells play a crucial role in transmitting signals to the cerebellar nuclei. Purkinje cells also serve as inhibitory signals that help dampen or stop excessive muscle movements. Damage to Purkinje cells due to ROS can lead to an imbalance between excitatory and inhibitory signals, resulting in motor function disorders in the affected animals [10].

Therefore, research is needed to understand the impact of carbofuran exposure during specific gestational periods, in line with the critical brain development period, and to assess the histopathological damage to Purkinje cells in the cerebellum of mice fetuses at twenty days of gestation.

2. Material and methods

2.1. Materials

The materials used in this study included the insecticide carbofuran (Furadan 3GR, MDL number MFCD00041819), complete chicken feed CP 593 (PT Charoen Pokhpand Indonesia), tap water (PDAM), chloroform, distilled water (aquadest), 70% alcohol, physiological NaCl, 10% formalin, and cotton. The instruments used in this research included mouse cages, syringes, surgical equipment (forceps, scalpel, scissors), a sonde, masks, gloves, pipettes, Erlenmeyer flasks, test tubes, test tube racks, Petri dishes, and containers for mouse feed and water.

2.2. Sample

In this study, experimental animals used were female Balb/C mice (*Mus Musculus*) aged 10 weeks with a body weight of approximately 25-30 grams, and male mice aged 12 weeks, obtained from the Veterinary Center of Farma (PUSVETMA). The number of mice used was in a 1:1 ratio, with one female and one male in each cage. In general, this study consisted of several stages as follows: Exploration of the teratogenic dose of carbofuran. Synchronization of the estrous cycle in mice using Pregnant Mare Serum Gonadotrophin (PMSG) and Human Chorionic Gonadotrophin (hCG). Examination of mouse pregnancy through vaginal plug observation. Oral administration of carbofuran. Counting of Purkinje cells in the cerebellum of 20-day-old mouse fetuses.

2.3. Determination of LD_{50} Dose

In this study, the LD_{50} dose of carbofuran ranged between 1 - 2.5 mg/Kg in rats [11]. Based on this dosage reference, after preliminary research, it was found that this dose still caused more than 50% mortality in female mice, resulting in an LD_{50} value of 0.5 mg/Kg body weight in mice. The teratogenic doses administered were based on fractions of this LD_{50} that did not cause fetal mortality but had the potential to induce teratogenic effects, namely 1/12 LD_{50} (0.0417 mg/Kg body weight) and 1/24 LD_{50} (0.0208 mg/Kg body weight) [12].

2.4. Methods

2.4.1. Synchronization of the Estrous Cycle Using PMSG and hCG Hormones

Synchronization of the estrous cycle in mice can be achieved by injecting gonadotropin hormones, PMSG, and hCG sequentially. The most suitable timing for injecting PMSG is in the late afternoon, followed by hCG injection 46-48 hours later. The mechanism of action of PMSG is analogous to Follicle Stimulating Hormone (FSH), which plays a role in follicle growth and slightly contributes to corpus luteum formation. Human Chorionic Gonadotropin (hCG) functions similarly to Luteinizing Hormone (LH), which is responsible for maturation and accelerating ovulation. After PMSG and hCG injections, ovulation is expected to occur in the evening, resulting in fertilization occurring 8-20 hours after copulation. The female mice are then kept in cages and provided with food and water ad libitum. Female mice aged 10 weeks with a body weight of 25-30 grams undergo a 7-day environmental adaptation period to reduce stress and allow them to acclimatize to the research environment. On day 1 after the 7-day adaptation period, PMSG is injected at a dose of 5 IU per mouse, followed by hCG injection 48 hours after PMSG injection, and then they are mated with male mice aged 12 weeks.

2.4.2. Pregnancy Examination

On the 3rd day, pregnancy is examined. If a vaginal plug is visible in the female mice's vulva, that day is considered day 0 of pregnancy. The female mice are then grouped into individual cages, one per cage.

2.4.3. Treatment

Preparation of Experimental Animals Mice (*Mus Musculus*) are randomly selected and divided into three treatment groups (P0, P1, and P2), each with six replicates. These mice are then kept in cages and provided with food and water ad libitum. Treatment: Control group (P0) receives physiological NaCl 0.9%. Treatment groups are each given carbofuran dissolved in 10 ml of physiological NaCl 0.9% according to carbofuran doses of 0.0208 mg/Kg body weight (P1) and 0.0417 mg/Kg body weight (P2). Each pregnant mouse receives a daily dose of 0.5 ml given orally on gestational days 14-17, starting from the appearance of the vaginal plug. The pregnant mice are kept until they give birth, and the brains of 20-day-old mice fetuses are collected after birth.

2.4.4. Histopathological Examination

Euthanasia was performed on 20-day-old mice fetuses using diethyl ether, followed by surgery to extract their brains. The extracted brains were then placed in containers filled with 10% formalin, and histological preparations were made using Hematoxylin Eosin (HE) staining. Microscopic observations of the histological preparations of mice fetal brains were conducted using a microscope at magnifications of 100x and 400x. Subsequently, the count of Purkinje cells undergoing necrosis was conducted in five different fields of view.

2.4.5. Observed Variables

In this study, the variable observed was the count of Purkinje cells in the cerebellum of fetal mice brains undergoing necrosis. This was determined by examining cell membrane damage, resulting in the visualization of necrotic Purkinje cells (pyknosis, karyorrhexis, and karyolysis). Necrotic Purkinje cells exhibit darker nuclei (pyknosis), ruptured Purkinje cell nuclei (karyorrhexis), the absence of Purkinje cell nuclei (karyolysis), and a wrinkled and homogeneous appearance.

2.5. Data Analysis

The research design employed was a Completely Randomized Design (CRD). Data analysis involved the use of Analysis of Variance (ANOVA) statistical tests, followed by the BNJ test using the Statistical Programs for Social Scientists (SPSS) program.

3. Result and Discussion

The assessment of necrosis was conducted microscopically using histopathological preparations stained with H.E. from the brains of Balb/C mice (*Mus Musculus*). There were three treatments: P0 (Control), P1 (carbofuran 1/24 LD_{50}), and P2 (carbofuran 1/12 LD_{50}). The evaluation was conducted on Purkinje cells undergoing necrosis and observed in five different fields of view using an Olympus® CX-41 microscope at 400x magnification. Quantitative data were obtained from the observations and assessments of carbofuran exposure in each treatment. The analysis results revealed significant differences ($p<0.05$), which were then followed by the BNJ test.

Table 1 Mean Purkinje Cells Necrosis Scores in the Cerebellum of Mice Fetuses after Carbofuran Exposure during the Embryonic Period

Group	**Necrosis Score (Mean ±SD)**
P0 (Control)	0.16 ± 0.04^{a}
Treatment 1 (Carbofuran 1/24 LD_{50})	0.80 ± 0.33^{b}
Treatment 2 (Carbofuran 1/12 LD_{50})	0.92 ± 0.01^{c}

Note: Different superscripts in the same column indicate significant differences ($P<0.05$).

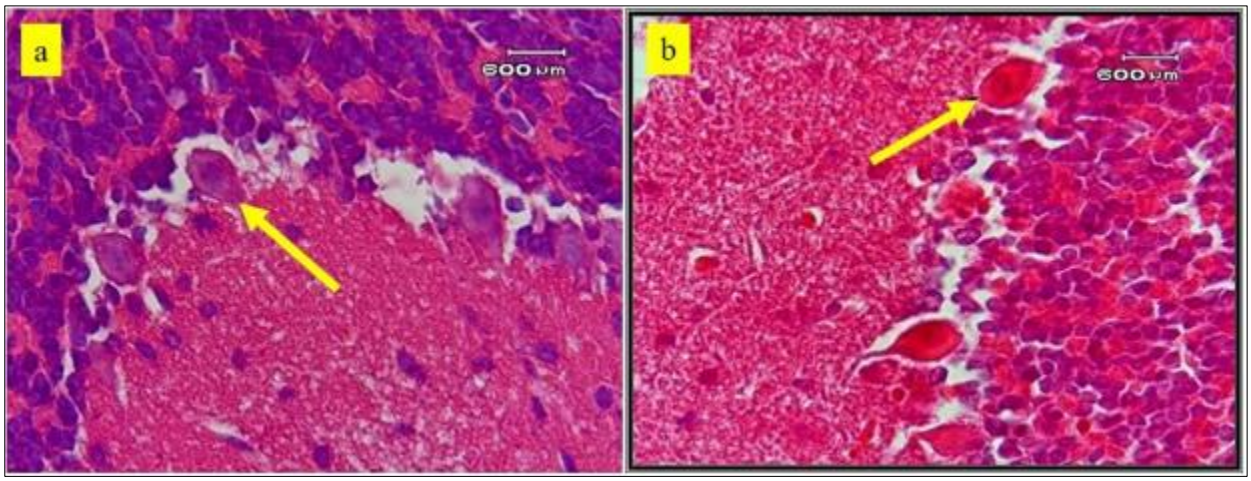

Figure 1 Histopathological image of fetal brain exposed to carbofuran during the embryonic period. Yellow arrows indicate normal Purkinje cells with clear nuclei (a=Control). Yellow arrows indicate necrotic Purkinje cells with fragmented nuclei (b=carbofuran-exposed treatment). H.E. staining; 400x magnification; Olympus CX-41 microscope

The research findings revealed significant differences among the treatment groups P0 (control), P1 (carbofuran exposure at 0.0208 mg/kg BW), and P2 (carbofuran exposure at 0.0417 mg/kg BW). There was an increase in the number of Purkinje cells undergoing necrosis between treatment P1 and treatment P2. Based on the statistical analysis results, the calculated F value was 1010.20. This result indicates a highly significant difference between the control group (P0) and the treatment groups (P1, P2) (Table 1).

Based on the results of the ANOVA test, followed by the BNJ test with a significance level of 5%, to obtain more accurate results regarding which dosage differs among the treatment groups. The average number of cells undergoing necrosis in this study follows a normal distribution, and the variance data is homogeneous, allowing the use of ANOVA. The ANOVA test indicates a significant difference between the control group and the treatment groups. Carbofuran exposure at dosages of 0.0208 mg/Kg (1/24 LD_{50}) and 0.0417 mg/Kg (1/12 LD_{50}) increases the number of necrotic Purkinje cells. Necrotic Purkinje cells exhibit fragmented nuclei (Figure 1). This demonstrates that higher dosage levels lead to a higher level of response.

Increased carbofuran dosage can result in more Purkinje cell deaths because higher doses of carbofuran cause greater damage. The increase in carbofuran dosage causing an increase in Purkinje cell necrosis is a common principle in toxicology known as the "dose-response" [13]. Higher carbofuran dosages can lead to increased Purkinje cell deaths because high doses can cause physical or chemical damage to the cells. Higher doses can result in more severe damage, including to Purkinje cells [14].

Exposure to carbofuran at a dosage of 0.0208 mg/Kg (1/24 LD_{50}) resulted in 80.28% necrosis, while a dosage of 0.0417 mg/Kg (1/12 LD_{50}) resulted in 92.41% necrosis. This exceeds the normal embryonic neuron cell death threshold of 15% [12]. The analysis results indicated significant differences among the treatments.

The increase in the number of cells undergoing necrosis due to carbofuran intoxication can lead to increased Reactive Oxygen Species (ROS). ROS continuously forms during oxidative metabolic processes, comprising inorganic molecules like anions, superoxide (O_2-), hydrogen peroxide (H_2O_2), hydroxyl radicals (-OH), and organic molecules such as alkoxyl and peroxyl [12]. Carbofuran-induced intoxication induces ROS, leading to cell membrane damage. This membrane damage is caused by lipid peroxidation, a complex process resulting from the reaction of unsaturated fatty acids composing cell membrane phospholipids with reactive oxygen compounds. Membrane damage leads to reduced sodium-potassium pump activity, cellular volume dysregulation, and intracellular calcium increase, resulting in cell death (necrosis) [15].

Physiologically, the cerebellum plays a role in regulating body balance, skeletal muscle tone, and controlling conscious muscle activities [16]. The cerebellum has a crucial role in rapid muscle activities such as running, typing, playing the piano, and even speaking. If the nervous system within the cerebellum experiences disturbances due to the damage of Purkinje cells, which are the central functional units within the cerebellum, it can result in incoordination of movements across all the mentioned activities, even though muscle paralysis may not occur [16].

Carbofuran can trigger oxidative stress, resulting in an imbalance between the production of free radicals (damaging molecules) and cellular antioxidant defenses. Higher doses of toxic substances can increase oxidative stress, which can damage Purkinje cells. Higher carbofuran doses can also disrupt the metabolism of cells, including Purkinje cells. This disruption can interfere with the normal function of these cells and lead to cell death [17]. High carbofuran doses can damage cellular structures, including cell membranes and organelles. This can inhibit the normal function of cells and lead to necrosis or cell death [7]. It is important to note that the impact of carbofuran (a toxic substance) on cells depends on the type of toxic substance, dosage, exposure duration, and the type of cells involved [18].

4. Conclusion

Based on the analysis of this study, it can be concluded that the administration of carbofuran insecticide during the embryonic period can cause necrosis of Purkinje cells in the cerebellum of mice fetal brains (*Mus Musculus*). The necrosis of Purkinje cells in mice fetal brains increases with the dosage of carbofuran administered.

Compliance with ethical standards

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Conflict of interest statement

No conflict of interest to be disclosed.

Statement of ethical approval

The study was approved by the Faculty of Veterinary Medicine Animal Ethics Committee of Universitas Airlangga. All variables were considered in accordance with the Ethics Committee related to the animal handling to ensure no discomfort or pain was caused to the animals during sampling(certificate registration number: 2012/112-KE).

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