

## Effect of carbofuran insecticide administration to fetal weight and length of mice (*Mus musculus*)

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### Abstract

**Introduction:** Carbofuran could decrease cholinesterase levels are significantly associated with reduced gestation duration of and the risk of low birth weight and length. This study aims to determine a decrease in the fetal weight and length of pregnant mice exposed to carbofuran.

**Objective:** This study used 18 female mice aged 10 weeks old with a weight of 25-30 grams. Female mice were mated with 18 male mice aged 12 weeks old which were synchronized using the hormones of Pregnant Mare Serum Gonadotrophin (PMSG) and Human Chorionic Gonadotrophin (HCG). This study was divided into three treatments, namely control (C), carbofuran at a dose of 0.0417 mg/mouse (T1), and carbofuran at a dose of 0.0208 mg/mouse (T2). Each pregnant mouse was treated with carbofuran for 10 days from day 6 up to day 15 of gestation when vaginal plugs were seen. Fetal collection was carried out on day 17 to measure the fetal weight and length. Data were analyzed using the ANOVA test and Duncan's test (significance 5%).

**Results:** The results showed that the treatment groups (T1 and T2) had a decrease in the fetal weight and length compared to the control (C) ( $p < 0.05$ ), nevertheless the treatment groups (T1 and T2) did not have significant difference ( $p > 0.05$ ).

**Conclusion:** It can be concluded that administration of carbofuran to pregnant mice could decrease the fetal weight and length.

**Keywords:** Carbofuran; Fetal weight; Length of mice; Gestation; Pesticide stress.

### 1. Introduction

Agricultural environmental pollution in Indonesia is caused by the use of agricultural chemicals, namely insecticides. These insecticide residues accumulate in agricultural and plantation products. The use of insecticides to support government programs to increase agricultural production can cause acute and chronic poisoning in animals and humans [1]. Carbofuran is one of the carbamate insecticides which is often used in agriculture because it has a broad spectrum in controlling insects and nematodes. Humans and animals can be contaminated with carbofuran through water and food. Residues of the carbofuran in food can harm organisms that are not the target of the insecticide [2]

Carbofuran and the organophosphate group work to inhibit cholinesterase (ChE). However, the decrease in ChE inhibited by organophosphates is irreversible, whereas carbamate is reversible. Pesticides from the carbamate group are relatively easier to decompose in the environment [3]. A decrease in ChE levels due to carbofuran causes failure in the hydrolysis of acetylcholine into choline and acetic acid, resulting in nervous incoordination which ultimately results in failure of cellular respiration in cells [4]. ChE activity has been studied histochemically during early embryonic

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development in sea urchins, amphibians, chicks and mice. ChE activity appears at a very early stage in embryonic development [5]. A decrease in ChE levels is significantly associated with a reduction in the gestation duration and the risk of low birth weight [6].

Oral administration of carbofuran has been proven to stimulate reactive oxygen species (ROS) in the rat brain. Carbofuran administered at a dose of 1 mg/kg for 28 days orally can increase malonyldialdehyde (MDA), which is a metabolite that can damage cell membranes [7]. The presence of excessive ROS can cause macromolecular changes and disrupt cell function [8]. The presence of ROS can trigger the formation of hydroxyl radicals (OH<sup>•</sup>) which can break DNA chains or cause changes in the nucleotide sequence in DNA which results in cell death [9].

Repeated administration of carbofuran has been proven to increase oxidative stress as the dose increases. Oxidative stress is the result of an imbalance between prooxidants and antioxidants. This increase in oxidative stress can reduce the activity of antioxidant enzymes and can reduce protection against free radicals. Increased oxidative stress during gestation can contribute to birth defects and abnormal growth in the fetus, so it can be stated that ROS are involved in teratogenicity [10].

The two main metabolites of carbofuran that cause toxicity and can penetrate the placental barrier are 3-hydroxycarbofuran and 3-ketocarbofuran which cause serious damage to the maternal unit of the fetal placenta [11]. Fetal toxicity is shown by decreased weight of the fetus and it does not survive. Weight is often used as supporting evidence in assessing teratogenicity and toxicity. Susceptibility to teratogens varies according to the developmental stage at the time of exposure, the most sensitive period for causing birth defects is organogenesis period. A decrease in fetal weight and length is a manifestation of growth abnormalities in both humans and experimental animals [12]

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## 2. Material and methods

This research used a Completely Randomized Design (CRD), using 18 females in 3 groups and 6 replicates per group. This research includes several stages: firstly, exploration of the teratogenic dose of carbofuran. Next, the estrous cycle of mice was synchronized using the hormones of Pregnant Mare Serum Gonadotrophin (PMSG) and Human Chorionic Gonadotrophin (HCG). After that, examination of mouse gestation was carried out by observing the vaginal plug. The following stage, Carbofuran was administered orally using a sonde. Then, the fetuses were collected and finally the weight and length of the fetuses were measured.

### 2.1. Materials

Materials used in this research: carbofuran insecticide (Furadan 3GR MDL number MFCD00041819), complete chicken feed CP 593 (PT Charoen Pokhphand Indonesia) and PDAM drinking water, husks, ether / chloroform, physiological NaCl 0.9%, cotton, alcohol 70 %. Instruments used in this research: mouse cages, 3 ml and 10 ml syringes, bowls for feed and drink of mice, surgical equipments (scalpel, tweezers, surgical scissors), large petri dishes, sondes, test tubes, gloves, masks, anesthesia jars, 70% alcohol spray, Fisher ruler to measure fetal length, analog scale to measure fetal weight, parchment paper, fetal data list, pen and tissue.

### 2.2. Population and sample

The experimental animal population used in this research was 18 female Balb/C strain mice (*Mus musculus*) aged 10 weeks with a body weight of 25-30 grams and 18 male mice aged 12 weeks which were obtained from Pusat Veterinaria Farma Surabaya Indonesia. Mice were housed in plastic cages equipped with wire covers. The bottom of the cage was lined with husks and the husks were replaced every day. The mice were kept by providing unlimited food and water.

### 2.3. Method

#### 2.3.1. Synchronization of the estrous cycle using PMSG and HCG

Ten week old female mice (*Mus musculus*) with a weight of 25-30 grams underwent environmental adaptation for 7 days. On the 8th day, PMSG was injected at a dose of 5 IU/mouse and HCG was injected on the 10th day at a dose of 5 IU/mouse, then they were mated with 12 week old male mice. The mice were then kept in cages and were given unlimited food and drink (ad-libitum).

### 2.3.2. Gestation examination

On the 11th day, gestation examination was carried out. If a vaginal plug was visible on the female mouse's vulva, then that day was considered as day 0 of gestation. The pregnant mice were then grouped into cages consisting of 6 mice each.

### 2.3.3. Determination of teratogenic dose and administration of carbofuran

In this study, the LD<sub>50</sub> approach of carbofuran was used between 1 - 2.5 mg/Kg in rats (California Environmental Protection Agency, 2000). Based on this dose reference, a LD<sub>50</sub> value of 0.5 mg/Kg BW was obtained in mice. The teratogenic doses administered are based on the fractions of the LD<sub>50</sub> that the doses do not kill the fetus and have the potential to cause teratogenic effects, namely 1/12 LD<sub>50</sub> (0.0417 mg/mouse) and 1/24 LD<sub>50</sub> (0.0208 mg/mouse). This study used 3 groups, namely: the control group (C) which was administered with 0.9% physiological NaCl, the T1 group which was administered with carbofuran dose solution of 0.0417 mg/mouse and T2 group was administered with carbofuran of 0.0208 mg/mouse. Oral administration of carbofuran was conducted on each mouse from day 6 up to day 15 of gestation, which started when vaginal plug was seen. On day 17 of gestation, the mice were sacrificed and then the fetuses were collected and lastly the measurement on the fetal weight and length was carried out.

### 2.3.4. Measurement of the fetal weight and length

Measurement of the fetal weight and length was carried out on each fetus, and the measurement of fetal weight was carried out using an analog scale in grams. Analog scale was covered with parchment paper which was always replaced every time a fetus was measured and the results were recorded. Fetal weighing was carried out when the fetus was still fresh and was free from extra embryonic membranes. The measurement of fetal length was carried out using a Fisher ruler in centimeters. Length measurements were obtained by measuring the distance from the forehead to the base of the tail by projecting the length of the fetus's body on parchment paper. The projection of the fetus's body length was carried out by marking the forehead and base of the tail using a ballpoint pen, then these marks were measured with a Fisher ruler.

## 2.4. Data Analysis

Statistical analysis of fetal weight and length were carried out using Analysis of Variance (ANOVA). If it had significant difference of 5%, then Duncan's multiple range test was carried out.

## 3. Results and discussion

Weighing the fetus was carried out when the fetus was still fresh and was free from extra embryonic membranes. The data on the fetal weight and length can be seen in table 1.

**Table 1** The fetal weight and length of pregnant mice exposed to carbofuran from day 6 up to day 15 of gestation.

Group	C (Mean ± SD)	T1 (Mean ± SD)	T2 (Mean ± SD)
Weight	0.8829 ± 0.1803 <sup>a</sup>	0.6371 ± 0.1872 <sup>b</sup>	0.6183 ± 0.2015 <sup>b</sup>
Length	2.2129 ± 0.2411 <sup>a</sup>	1.8786 ± 0.2220 <sup>b</sup>	1.8667 ± 0.2530 <sup>b</sup>

Notes: The same superscript letters show that they are not significantly different ( $p > 0.05$ ), C= Control treated with physiological NaCl 0.9%, T1= treated with carbofuran at a dose of 1/12 LD<sub>50</sub> (0.0417 mg/mouse), T2= treated with carbofuran at a dose of 1/24 LD<sub>50</sub> (0.0208 mg/mouse)

After the normality and homogeneity tests were conducted, it showed a significant difference of more than 0.05 ( $p > 0.05$ ), so Analysis of Variance (ANOVA) was carried out. Based on the statistical results using ANOVA, the calculated F result was 4.129 with a significant difference of 0.035 ( $p < 0.05$ ). Based on the Duncan Test, the two treatment groups (T1 and T2) had significant differences compared to the control group, but there was no significant difference between the group treated with 1/12 LD<sub>50</sub> (0.0417 mg/mouse) and that of 1/24 LD<sub>50</sub> (0.0208 mg/mouse).

Carbofuran can cause toxicity and teratogenic effects on the fetus. It affects the fetuses by inhibiting the fetal growth which could be seen from a decrease in the fetal weight and length in T1 group treated with carbofuran at dose of 0.0417 mg/mouse and T2 group treated with carbofuran at dose of 0.0417 mg/mouse. By contrast, the control group had significant difference. The results showed that the means of fetal weight and length of the treatment groups (T1 and T2) were lower than those in the control group.

Carbofuran has toxicity that can penetrate the placental barrier if contaminated orally. The working mechanism of cholinotoxics such as insecticides, ethanol and nicotine (hereinafter referred to as antiChE) inhibit ChE activity by binding to ChE to form complex bonds and closing ACh receptors, both nicotinic (N-cholinoreceptor) and muscarinic (M-cholinoreceptor) receptors [13]. Nicotinic receptors receive ACh stimulation from striated muscle nerve endings, autonomic nerve ganglions and a little of the CNS, while muscarinic receptors receive ACh stimulation from smooth muscle nerve endings, exocrine and endocrine glands [14]. A decrease in ChE activity causes a buildup of ACh in the synapse and synaptic flow will be disrupted, this condition causes the individual to get hyperactive and then paralyzed and die.

Carbofuran can inhibit the activity of the cholinesterase enzyme and will bind the cholinesterase enzyme (ChE) so that ChE becomes inactive and acetylcholine accumulates. This inhibited mechanism of cholinesterase action will result in disruption of the proliferation mechanism in the early growth period, which can result in impaired fetal growth and development [15]. Decreased cholinesterase levels are significantly associated with reduced gestation duration of and the risk of low birth weight [16]

Oral administration of carbofuran has been proven to stimulate reactive oxygen species (ROS). ROS activity is required in the phagocytosis process in the immune system. An increase in excessive amounts of ROS will be very dangerous for the body and can trigger the formation of highly reactive hydroxyl radicals (OH<sup>o</sup>). Hydroxyl radicals are one of the most dangerous free radicals for the body and can damage DNA, proteins and unsaturated fatty acids which are important components of phospholipids that make up cell membranes.

Hydroxyl radical attacks on cell membranes can cause lipid peroxidation which results in the breaking of fatty acid chains into various compounds that are toxic to cells, such as malonyldialdehyde (MDA) and various hydrocarbons which can cause severe damage to cell membranes and are dangerous for cell life. Cell membrane damage caused by MDA will be followed by apoptosis and necrosis which is the cause of a decrease in fetal weight and length [7].

Decreased cholinesterase levels in pregnant mice can affect fetal growth and potentially affect the fetal weight and length. Cholinesterase is an enzyme that plays a role in destroying the neurotransmitter acetylcholine in the central and peripheral nervous system. When cholinesterase levels decrease, acetylcholine can remain in the nervous system longer, and this can affect nervous system function in pregnant mice. The following are possible mechanisms in the relationship between decreased cholinesterase levels and their effect on fetal growth: decreased cholinesterase levels can affect blood flow and nutrition to the fetus. Acetylcholine plays a role in controlling blood vessel contractions. When acetylcholine levels are high due to low cholinesterase, this can cause vasoconstriction (narrowing of blood vessels), which can limit blood flow to the placenta and nutrients provided to the fetus. This condition can interfere with fetal growth [17].

Decreased cholinesterase levels can increase oxidative stress in pregnant mice. Oxidative stress occurs when the production of free radicals in the body exceeds the body's ability to eliminate them. This can damage the body's cells and tissues, including the placenta. Oxidative stress in the placenta can disrupt placental blood flow, which can affect the supply of oxygen and nutrients to the fetus. Decreased cholinesterase levels can also be caused by exposure to carbofuran compounds. This exposure can have direct toxic effects on the fetus and placenta, which can disrupt fetal growth and development [18].

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#### **4. Conclusion**

Administration of carbofuran insecticide exposed to pregnant mice at a dose of 0.0417 mg/mouse and at a dose of 0.0417 mg/mouse was able to reduce the fetal weight and length ( $p < 0.05$ ).

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#### **Compliance with ethical standards**

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##### *Disclosure of Conflict of interest*

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: 2011/111-KE).

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## Author's short biography

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**Authors Name:** Rika Rahmawati

Rika Rahmawati, DVM, whose nickname is Rika, is a veterinarian who graduated from the Faculty of Veterinary Medicine, Universitas Airlangga in 2013. Work experience as a veterinarian in wildlife, and has been a judge of cat show or cat competition and has been a speaker at cat events. Currently active as business owner in the veterinary clinic sector which name is M-PET animal care in Gresik, East Java, and also active as Committee of Cattery Organization (Indonesia Cat Association) in Gresik, East Java



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Dr. Maslichah Mafruchati, DVM, M.Kes, Academic career: Doctorate Ilmu Kedokteran, Pasca Sarjana, Universitas Airlangga, 2015. Master, Immunology, Pascasarjana, Universitas Airlangga, 2003. Undergraduate, Veterinary Medicine, Universitas Airlangga, 1987. Employment: Senior Lecturer, Faculty of Veterinary Medicine, Universitas Airlangga. Research and development projects: Reproductive Efficiency of lemousin Cow in animal Health technical area Gresik period 2016-2017. The potential of propolis as a regenerator of spermatogenesis in testicular disorder due to exposure to cigarette smoke 2017- 2018.



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**Authors Name:** Widjiati Widjiati

Prof. Dr. Widjiati Widjiati, MSc., DVM a professor at Universitas Airlangga (UNAIR) delivered a embryo transfer program (TE) as a solution for breeding cattle in Indonesia. Prof. Widjiati said that it is time to think of other assisted reproductive technologies besides artificial insemination to overcome the slow growth of the cattle population. Prof. Widjiati studied Bachelor at Universitas Airlangga, Master at IPB-Bogor, and Doctoral at Universitas Brawijaya Indonesia.



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Prof. Dr. Epy Muhammad Luqman, MSc., DVM is a Professor in the Field of Veterinary Developmental Toxicology. This man, who was born in Surabaya, 13 December 1967, studied for a Bachelor's degree at the Faculty of Veterinary Medicine, Universitas Airlangga and graduated in 1991. Then, he continued his Master's degree in Reproductive Biology at the Universitas Airlangga Postgraduate Program. Then, PhD education in the Doctoral Science Program, Faculty of Medicine, Universitas Airlangga. So far, he has published 37 Scopus indexed publications.

