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Research Article

The Effect of Carbofuran Exposure on Mice During Lactation Period Against on Histopathology Description of The Heart of Mice (*Mus Musculus*) Offspring

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ABSTRACT

Carbofuran exposure has been reported to be able to induce reactive oxygen species or free radicals in the heart that cause oxidative stress. In the present study of carbofuran induced increase heart damage in histophatological analysis of mice heart. Aim: this study was to evaluate the effect of carbofuran exposure during lactation period on heart histopathological of mice offspring. Adult in lactation period BABLB/C were randomly divided into four treatment groups. Group K as control were given distilled water, group P1 were given carbofuran 1/16 LD_{50} (=0.3125 mg/kg), P2 1/8 LD_{50} (=0.625 mg/kg), and P3 1/4 LD_{50} (=1.25 mg/kg) for nine days from first day their offspring born. Twenty offspring were randomly selected each treatment group. On the ten day of experimental offspring dissected and the heart organ was taken to made histophatology sample. Samples were compared and the results showed a significant increase in the degree of cardiac damage between K, P1, P2, and P3. Results, carbofuran caused increased group P1 suffered heart damage as much as 9.28 including minor damage, group P2 suffered heart damage as much as 12.56 including moderate damage, and group P3 suffered heart damage as much as 18.04 including severe damage. Conclusion, carbofuran exposure in heart cells triggers histopathological damage to heart organs such as inflammatory cells, degeneration, necrosis, and congestion as the dose is increased.

Keywords: Carbofuran, lactation, heart, heart damage.

INTRODUCTION

Insecticide in agriculture has been used for a long time. Indonesia became the largest country in the world in the use of Chinese and Indian insecticides in 1980 [1]. Exposure to insecticides can enter the body through food, air, and water [2]. Various types of insecticides with various active ingredients are developed according to pest control. One of the active ingredients of insecticides that are widely used is the one from carbamate groups such as carbaryl, benfuracarb, carbosulfan, and carbofuran [3]. Data on the field has shown that carbamate insecticides have less toxic effect and residual residues rather than organophosphate groups which have the same working relationship with carbamate class insecticides which are more widely used by farmers [4].

A case study conducted in Curut village, Penawangan district, Grobogan district, Indonesia has proven that intensive insecticide used which carried out excessively and not on target, indirectly, can be used as a safe and secure environment [5, 6]. Carbofuran insecticide metabolites were found in all of the beef samples in Blora area, Central Java, Indonesia and had exceeded the maximum limit set by FAO [7]. The average metabolite found in the beef samples has reached 169.17 ppb (0.17 mg / Kg BW) which exceeded the maximum residual limit (BMR) of beef by 50 ppb (0.05 mg/Kg BW). Metabolics accumulation are found in fatty tissue, placenta, umbilicus, blood and on the metabolism of some animals [8, 9,].

Lactation period is a period of critical development of postpartum individuals because the function and the organ systems are still in development so that they are more prone to disease development [10]. Metabolic material in breast milk which is produced by insecticides in lactating mothers distributes toxic substances to the children [9]. The breast milk first will be absorbed by the digestive tract and then carried out by blood through the circulatory system because toxic substances in the breast milk's air can cause heart damage to body cells, including the heart.

Cell damage involving carbofuran by the forming of reactive oxygen species (ROS) [11]. According to

[12], ROS embodies the lipid membrane peroxidation so that the fatty acid chain on the cell membrane which eventually breaks down due to cells. The increased levels of MDA due to the widespread of membrane damage, including cell organelle cells which thus interfere the synthesis process of fat-binding enzymes and disruption of sodium pumps, so that the cells repair necrosis and degenerate [13].

Lipid peroxidation in the membrane has caused mitochondrial dysfunction and excessive production of ROS [14]. Cardiotoxicity due to the influence of oxidative stress of carbofuran exposure is related to histopathological changes which then form necrosis tissue [15]. Oxidative stress and free radicals which are carried out by pups due to the exposure of carbofuran which caused by inflammatory reactions in the form of inflammatory cells or myocarditis in the blood caused by lesions / carbofuran lesions can be used as an inflammatory mediator, vasodilation, possibly taken with blood dilation known to increase permeability inflammation cells and congestion can be formed [13].

Research into the effects of carbofuran induction needs to be carried out to determine the effects that can be caused on breastfeeding children, because the post-partum development period is very important. This study aims to determine the histopathology heart of pups which are exposed to carbofuran during the lactation period associated with damage to cardiac organs including infiltration of inflammatory cells, cell degeneration, necrosis, and congestion. The results of this study provide knowledge about the escalated damage of the heart organ in pups during histopathology.

MATERIALS AND METHODS

The research design used in this study was a Completely Randomized Design (CRD) with four designs and five replications. The treatment group consisted of four groups namely K, P1, P2, P3. The experimental animals consist of mother mice and pups (*Mus musculus*) Balb / C strains that were lactating aged 0-9 days. In this study, the samples obtained were based on the formula of Federer [16]: t (n-1) ≥ 15, n: larger samples in each group, t: higher number of samples in each group (n)= 5 or 20 for 4 groups. The research was carried out in the Department of Veterinary Anatomy of the Faculty of Veterinary Medicine, Universitas Airlangga, the In vitro Fertilization Laboratory, the Animal Cage of the Faculty of Veterinary Medicine, Airlangga University and the Department of Veterinary Pathology of the Faculty of Veterinary Medicine, Airlangga University, Surabaya.

The experimental animals: The experimental animals used in this study were 20 pregnant mice *(Mus musculus*) Balb / c with a body weight of approximately 25-35 grams and 10-week-old which are obtained from the Farma Veterinary Center in Surabaya. Pregnant mice *(Mus musculus)* used as experimental animals are all in healthy conditions, this can be seen based on the characteristics of clear eyes, clean fur, and normal behavior.

Research Materials: The material used in this research was carbofuran insecticide (2,3-Dihydro-2,2-dimethyl-7-benzofuranol N-methylcarbamate 98%) from Aldrich Chemistry with Bellstain Registry number 1428746, code 1000899348 Product of USA, foods given are in the form of 98% N-methylcarbamate pellets, drinking water, husks for cage tools, Aquadest as a carbofuran solvent, ether solution, 10% formalin, alcohol, xylol, liquid paraffin, hematoxylin & eosin (HE).

Research Tools: The research tools that are used during the treatment includes: animal cages in the form of mouse cages, feed containers, drinking containers, sonde needles, Olympus® CX-41 microscopes, surgical equipment in the form of tweezers, scalpels, surgical scissors, glove, masks, cameras, glass objects glass cover, wire mesh, anesthetic jars, petri dishes, alcohol spray, analytical scales, opaque paper, ointment pots, label paper.

Preparation of Experimental Animals: Female mice Balb / C strains that were mated in pregnant state were divided into four groups namely K, P1, P2, P3. There are five repetitions for each group. Those which are exposed to carbofuran dose (P3) 1/4 LD50 as much as 1.25 mg / kg BW, (P2) 1/8 LD50 as much as 0.625 mg / kg BW, (P1) 1/16 LD50 as much as 0.3125 mg / kg BW (Luqman et al., 2018) during lactation days 1-9 were given orally using a single-use sterile syringe.

Method: In this study, female mice (*Mus musculus*) was mated with male mice. After being adapted in the pregnant state, it was randomly picked and then divided into 4 treatment groups namely K, P1, P2, P3. Mice who had given birth was exposed to carbofuran during the lactation period for 1-9 days orally. In the control group (K), carbofuran induction was replaced with 0.5 ml of aquadest / head / day, while the treatment group (P1) were given carbofuran dose 1/16 LD50, the treatment group (P2) were given carbofuran dose 1/8 LD50, and the treatment group (P3) were given carbofuran with a dose of 1/4 LD50. Each was given for 9 days with 5 repetitions. On the 10th day, the pups were extracted for the heart organs which then are prepared for the histopathological preparations and then observed using a microscope with 400x magnification.

Histopathological preparations: Microscopic observation of the pups' heart in histopathology

preparations using a microscope with a magnification of 400x followed by observations on five different field of view on each histopathological preparation of the pups' heart, and then an assessment was done to see the damage on the histopathological picture of the pups' heart using scoring. The damage that was observed includes: the presence of inflammatory cell infiltration, cell degeneration, cell necrosis, and congestion in the pups' heart organs. The scoring method used in this study is the Billingham method with modification and multi parametric total scoring (Table 1).

Data analysis: The data of analysis obtained was tabulated using the Kruskall Wallis test followed by the Mann Whitney test, using the IBM SPSS (*Statiscal Product for Service Solutions)* version 22.0.

RESULTS

The results of histopathological observations of microscopic heart of mice in children using scoring indicate the degree of damage to the heart organ. Observation of scoring results in heart organ damage in mice (*Mus musculus*) with HE staining. Observations were made using a microscope with a magnification of 400x optilab and observations can be seen in table 2.

The analysis results of table 2 shows that there are significant differences between the control and treatment groups. In the control group against groups P1, P2, and P3 also showed a significant difference. In groups P1, P2, and P3 shows an increase in the degree of heart damage. The scoring results of heart damage have a mean value of 0-6 which is normal and the control group produces a damage score of 0, for mild damage with a mean of 6-12 and the results obtained by the P1 group are 9.28, for moderate damage with a mean of 12-18 and the results obtained by P2 group is 12.56, for heavy damage with an average of 18-24 obtained by P3 group which is 18.04.

Table 1: Scoring Damage to Heart Organs

Form of Lesions	Score	Information
Inflammatory Cells (A)	0	If inflammation cells are found <5 cells in epicard/myocard/endocard
	1	If inflammation cells are found> 6-20 cells in epicard/myocard/endocard
	2	If inflammation cells are found> 21-50 cells in epicard/myocard/endocard
	3	If inflammation cells are found> 51-80 cells in epicard/myocard/endocard
	4	If inflammation cells are found> 80 cells in epicard/myocard/endocard
Cell Degeneration (B)	0	There is no degeneration in the form of vacuolization
	1	There is cell degeneration in the form of vacuolation <25% in the field of view
	2	There is cell degeneration in the form of vacuolization of 26-50% in the field of view
	3	There is cell degeneration in the form of vacuolization 51-75% in the field of view
	4	There is cell degeneration in the form of vacuolization> 76% in the field of view
Necrosis (C)	0	No histological changes found (normal)
	2	Not entirely normal, but there is no evidence of damage caused by the treatment
	4	Necrotic myocyte cell counts <5% of all myocytes observed
	6	Necrotic myocyte cell counts are 6-15% of all myocytes observed
	8	The number of necrotic myocyte cells is 16-25% of all myocytes observed, wherein myocyte cell necrosis occurs in one fasicle (cluster)
	10	Myocyte cell necrosis 26-35% of all myocytes were observed, some of which experienced vacuolization
	12	Myocyte cell necrosis> 35% wherein the cell necrosis occurs evenly in all parts (*diffuse)*
Congestion (D)	0	No congestion was found
	1	Congestion found <25% in the field of view
	2	Congestion was found 26-50% in the field of view
	3	Congestion was found 51-75% in the field of view
	4	Congestion was found> 76% in the field of view

Source: [17]

Table 2: Scoring results of heart organs in mice children due to carbofuran exposure in the parent lactation period

Treatment	Heart Damage Score (Mean ± SD)
Control	0.00 ± 0.00 [a]
P1 (1/16 LD_{50} = 0. 3125 mg/Kg BW)	9.28 ± 0.72 [b]
P2 (1/8 LD_{50} = 0.625 mg/Kg BW)	12.56 ± 0.71 [c]

P3 (1/4 LD_{50} = 1.25 mg/Kg BW)	18.04 ± 0.50 [d]

Note: Different superscripts show significant differences ($p < 0.05$).

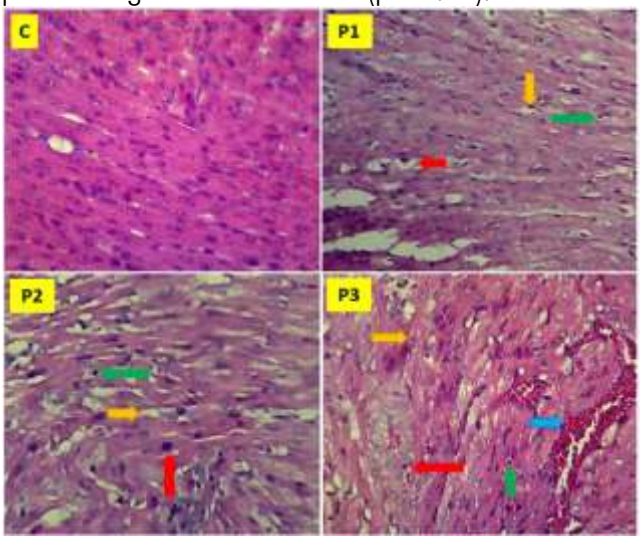

Fig.1: Damage to the heart organ of a mice, using HE staining, longitudinal cut with a magnification of 400x. (Yellow arrow = degeneration, red arrow = infiltration of inflammatory cells, blue arrow = congestion, green arrow = necrosis)

DISCUSSION

The control group (C) did not show any heart damage caused by treatment, the tissue and cells shape are still normal with the characteristics of the cell nucleus located in the middle, with an intercalary disc, and cell coir composition that is still regular. Figure P1, P2, and P3 shows that heart damage due to treatment have the following characteristics: Figure P1 contains inflammatory cell infiltration with core characteristics shaped like peanuts, has no granules (monocytes), cell degeneration in the form of vacuoles within or adjacent to cells in the cytoplasm, cell necrosis with a black cell nucleus and shrinking (picnosis). Figure P2 also contains inflammatory cell infiltration with no granular characteristics, cell nuclei appear to be almost filled with round and solid cells (lymphocytes), cell degeneration in the form of vacuoles within or adjacent to cells that have more numbers than the P1 group, vacuoles with spaces that is a hydropic degeneration with swollen cytoplasm, normal cell nucleus, cell necrosis with a nucleus at the edge, and shrinking by a greater number than the P1 group. Figure P3 contains infiltration of inflammatory cells with nuclear characteristics appear to almost fill in the cells, moon-shaped and dense, cells degeneration of vacuoles or adjacent to cells have larger numbers compared to groups of P1 and P2, cell necrosis with cell nuclei appearing to be blackened and shrinking and lysis with a larger number of groups P1 and P2, congestion in blood vessels is characterized by bleeding, dilated capillaries, full of erythrocytes and were found only in P3 group and spread evenly on each repetition.

In group P1, it can be seen that the degree of damage to the heart organ occurs at the smallest dose of 1/16 LD50 (0.3125 mg / kg). The results of the scoring calculations showed that the P1 group produced a multi-parametric total with a mean of 9.28 which is categorized as mild heart organ damage. In microscopic observations, infiltration of inflammatory cells was found with cell numbers range from> 6-20, the degree of inflammation based on infiltration of inflammatory cells with two important indicators namely distribution which covers on how widely the inflammatory cells spread on the inside of an organ or it is evenly distributed in the epicardium, myocardium, endocardium.

Cell degeneration which was found in the P1 group observations was vacuolation of <25% in the field of view. Necrosis cell in group P1 has shown the number of myocyte cells which experienced necrosis of 16 - 25% of all myocytes observed, whereas myocyte cell necrosis occurred in one facicle (a collection of several muscle fibers). In the P1 & P2 groups there was the same heart damage found with a higher difference in the amount of damage in the P2 group.

In the P2 group, it can be seen that the degree of cardiac damage occurs at a moderate dose of 1/8 LD50 (0.625 mg / kg). The results of the scoring calculations showed that the P2 group produced a multi-parametric total with a mean of 12.56 which was a heart organ damage in the moderate category. In microscopic observations, it was found that there is an infiltration of inflammatory cells with cell numbers range> 21 - 50, the degree of inflammation based on infiltration of

inflammatory cells with two important indicators namely distribution which covers how widely inflammatory cells spread on the inside of an organ or whether it is evenly distributed in the epicardium, myocardium endocardium.

Cell degeneration found in the P2 group observations was vacuolization of 51-75% in the field of view. Necrosis cell in P2 group, showed the number of myocyte cells experiencing necrosis within range 16 - 25% of all myocytes observed, whereas myocyte cell necrosis occurred in one fasicle (a collection of several muscle fibers). Inflammation and degeneration in the P2 group resulted in cell damage due to carbofuran exposure which could result in congestion occurring in the P3 group.

In the P3 group, it can be seen that the degree of cardiac damage occurs at high doses of 1/4 LD50 (1.25 mg / kg). The results of the scoring calculations has shown that the P3 group produced a multi-parametric total with an average of 18.04 which was categorized as severe damage to the cardiac. In microscopic observations, an infiltration of inflammatory cells was found with the cell numbers range> 51-80, the degree of inflammation based on infiltration of inflammatory cells with two important indicators namely distribution which covers how widely inflammatory cells are spread widely on the inside of an organ whether evenly distributed in the epicardium, myocardium, endocardium.

Cell degeneration that was found in the P3 group's observation with range of 51-75% vacuolation in the field of view. Necrosis cell in the P3 group has shown the number of myocyte cells experiencing necrosis 26-35% of all myocytes observed, which some of the rest experienced vacuolization. In group P3, a congestion ranging from 51 - 75% was found in the field of view in each repetition. This has resulted in the final of carbofuran causing congestive heart failure in pups due to exposure to carbofuran insecticide.

Carbofuran that are given orally has proven to be able to stimulate ROS which can cause oxidative stress [18, 19]. ROS formation can cause lipid, DNA and protein modification in various tissues. Molecular modification in various tissues will cause oxidative damage. Oxidative stress will lead to oxidative damage including tissue damage and cell death [20]. Antioxidants has the function of balancing free radical levels within the body. The body is able to produce endogenous antioxidants such as SOD (superoxide Dismutase), glutathione peroxidase, and catalase. The stability of ROS with disturbed antioxidants will result in oxidative stress resulting in organ damage [21].

Once the Kruskal Wallis test was done, it was found that there were significant differences between the control and treatment groups ($p < 0.05$). In the control group, no cardiac damage was found due to cell injury of carbofuran from environmental factors that were reversible. After further analysis using the Mann Whitney test, there was a noticeable difference between each treatment. Groups P1, P2, and P3 experienced an increase in organ damage that in line with the dose of carbofuran given to the parent during lactation. In this study, the highest degree of damage was experienced by the highest dose of carbofuran namely the P3 group (Table 2). This result was obtained due to the higher doses of the oxidative stress levels received by the body which result in a higher, so it is related to the body's maximum response to chemicals.

Carbofuran is absorbed by the digestive tract significantly and is distributed quickly to the body tissues. Carbofuran levels and the metabolites in the blood have shown to increase rapidly within two hours after treatment [22, 23]. Metabolites in milk can enter passively through the parent's blood plasma, whereas the level of concentration in milk depends on the solubility of the metabolite and the lipophilic nature of the metabolite. One of the main targets of ROS is membrane cells and endomembranes which will then trigger lipid peroxidation in unsaturated fatty acids or polyunsaturated fatty acids (PUFA). PUFA peroxidation can damage proteins, disrupt ion pumps, and reduce cell membrane fluidity [24].

Research conducted by [25] has shown that the exposure to carbofuran in mice for four days has resulted in the increased levels of MDA in breastfed mice. This theory supports this research that carbofuran exposure in mice causes free radicals in the mice which they eat. Carbofuran exposure is influenced by the duration of exposure and also the number of doses given in order to affect the body [26].

Inflammatory cells are one of the body's defense and defense systems against everything that attacks the body as well as in response to injury [27]. Inflammatory cell are activated as a result of the body being exposed to toxic antigens such as carbofuran, so that a non-specific immune response will work against this antigen where the damage occurs. The presence of inflammatory cells in the tissue is also a response to injury caused by carbofuran in the form of membrane damage and necrosis. Necrotic cells actively secrete cytokines that stimulate the inflammatory response (inflammatory cytokines). According to [28], cells that have active necrosis will release IL-6 which is an inflammatory cytokine to activate NKFB, p38 and MAPK, which then the existing inflammatory cells has the function to clear cells that experience necrosis.

Cardiotoxicity begins with vacuolization caused by mitochondrial dysfunction due to lipid peroxidation due to ROS. Mitochondrial dysfunction and DNA damage caused by the increase of free radicals or the decrease of endogenous antioxidants which result in ATP and NAD + depletion which will begin the process of cardiomyocyte death [24]. Mitochondria here acts as an important organ in forming ATP, when the mitochondrial membrane is damaged, there will be various disorders due to the decrease production of ATP which then result in cell death.

The mechanism of carbofuran in causing toxic effects that result in ROS has been widely studied [11, 25]. Excessive amounts of ROS can trigger the formation of hydroxyl radicals that are very reactive and harmful to the body [29]. Hydroxyl radicals disrupt the normal function of cells, in line with previous studies that shown these changes has caused cells to not be able to maintain ion and fluid homeostasis so that the exposure to carbofuran orally can cause degeneration of cells in a vacuola form [26].

Mitochondria acts as the main centers of intracellular production of ROS under normal conditions and is the central to cell damage when exposed to specific lesions, such as accumulation of insecticides that work systemically [30]. Injury can damage cells that cause necrosis which can occurs through several mechanisms. The mechanism of necrosis can be caused by lesions that damage the cytoskeletal cells which then cause damage to the membrane, and can directly damage the cell membrane by causing lipid peroxidation of the lysozyme membrane, plasma membrane, and mitochondrial membrane which causes the disruption of ATP formation, and directly disrupts the work of the mitochondria [31]. Cells that have undergone necrosis have a nucleus which tends to undergo autophagi and myocytolysis because the lysosome organism is destroyed and then releases the enzyme lysozyme. In addition, lipid peroxidation in the cytoskeleton will cause cells to lose structure and cause a faster lysis [24].

Congestion is a condition where there is excessive blood (an increase in the amount of blood) in blood vessels in certain areas. In the P3 group, there are congestion in several parts of the tissue that can cause congestive heart failure. Congestive heart failure begins with abnormalities of the heart muscle that cannot contract normally such as myocardial infarction, disturbances in hemodynamic pressure, excess volume, or hereditary cases such as cardiomyopathy. This condition causes a decrease in the cardiac pump capacity. This was caused by the body's compensation mechanism as the result of cardiac injury or an increase in myocardial contractions. When cardiac output decreases, baroreceptor stimulation in the left ventricle, carotid sinus and aortic arch, then provides afferent signals to the central nervous system at the center of the cardioregulator which will cause antidiuretic hormone (ADH) secretion from the posterior pituitary. ADH will increase the channel collector permeability so that water reabsorbance will increase [32].

This mechanism contributes to the functional and structural changes of the heart, fluid, and salt retention in congestive heart failure which further characterizes myocyte hypertrophy, myocyte contractile changes, decreased myocyte count due to necrosis, apoptosis, and autophagia cell death. The increase of oxidative stress and free radicals activate changes in heart structure due to decreased cardiac output, left ventricular dilatation, and hemodynamic overload. Both mentioned on the above contribute to the development of heart failure [32].

Free radicals stimulate intracellular stress which further stimulates changes in the endoplasmic reticulum and affects the permeability of lysosomes. Furthermore, pro-apoptosis is formed and activates Mitochondrial Permeability Translation so that the entry of protons occurs in the mitochondria. Mitochondria experiences failure in producing ATP which results in rupture of cell membranes. This triggers the release of cytochrome C and pro-apoptotic proteins resulting in cell death or apoptosis. One of the apoptosis tests is the Tunel test which is characterized by a brown discoloration [33]. Autophagi forms and break down cell components through lysosomes that play a role in cell homeostasis. The forming of membrane around the target cell area that separates its contents from other parts of the cytoplasm. Vesicles that are formed will then fuse with lysosomes and will break its contents [34].

CONCLUSION

Exposure to carbofuran insecticide in pups during breastfeeding can cause increased damage to its heart organs. Damage that occurs in the form of increase number of inflammatory cell infiltration, cell death, to the extent of bleeding in organs at a given dose are getting larger and larger, so that a further research is needed to determine heart damage due to the interactions of oxidative stress caused by carbofuran metabolites in breast milk.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this article.

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