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PENELITIAN BERJUDUL	: Potensi Ekstrak Rumput Kebar (*Biophytum petersianum*) Dalam Mencegah Penurunan Fungsi Otak Anak Mencit (*Mus musculus*) Masa Laktasi yang Terpapar Karbofuran
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REVIEW

RESEARCH

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Research Article

The Carbofuran Exposure During Lactation Period in Reducing the Motoric Reflexes and Memory in Infant Mice *(Mus musculus)*

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ABSTRACT
This study aimed to determine the function of motoric reflexes and memory as a result of the necrosis of neuron cells in the cerebellum of infant mice *(Mus musculus)* from their carbofuran exposed mothers during lactation periods. The decreased motoric reflexes as an indicator of the reduced memory ability and concentration ability in infant mice. This experimental laboratory study used 36 mice and carbofuran injected using gavage method with the fraction of LD50 in mother mice in the dose of 5 mg/kg body weight (BW). The mother mice were exposed with carbofuran during lactation period from Day 0 to Day 9 and the 10-days-old infant mice were then terminated for microscopic examination by counting the necrosis of neuron cell by applying HE staining and the motoric-reflex function tests (neurobehavioral test) on the 10-days-old infant mice which included: surface righting reflexes, swimming and a memory test employing an Eight-arm radial maze at 20 days- old. The calculation results were analyzed using the Kruskal-Wallis test and the Mann Whitney test. The results of this study showed a significant increase in the necrosis of neuron cell, a fundamental decreased motoric reflex in infant mice in these activities; such as surface righting reflex and swimming ability (the head angle position and swimming direction) and eight-arm radial maze memory test. Conclusion: in sum, the exposure of carbofuran in the mother mice during lactation period causes the increase in necrosis of neuron cell, the decrease of motoric reflexes and the memory ability for infant mice.

Keywords: Carbofuran, neuron cells, lactation, motoric reflexes, memory

INTRODUCTION

The residues of carbofuran insecticide in food can harm non insecticide targeted organisms [1]. Carbofuran contamination provokes some cases of infants born with the impaired motoric reflexes, while at adolescent ages, there are abnormalities in the brain function development such as the decreased memory and concentration abilities [2]. Carbofuran contamination in animals leads to the oxidative stress and weakens the cognitive, memory and motoric functions. Carbofuran induction affects a significant oxidative damage to the cerebral cortex, cerebellum, and brain stem [3].

Furthermore, previous studies proof that any oral administration of carbofuran has been proven in stimulating the reactive oxygen species (ROS) in the mice's brain [4,5]. The sub-acute administration of carbofuran intraperitonially has been confirmed in increasing the brain oxidative stress along with the increasing doses. Consequently, an uncontrolled increase in ROS will in turn lead to injury and the death of neuron cell [6]. Meanwhile, in embryonal brain development, the neuron cells of cerebrum develop earlier and experience its development peaks in the middle of pregnancy, while the cerebellum develops in the middle of pregnancy until several days after the fetus is born [7]. The cerebellum is responsible for controlling movement, maintaining balance, adjusting position and coordinating body movements. Traditionally, the cerebellum is dedicated to motoric function, but its phylogenetic development and connectivity exhibit that the cerebellum also plays a role in cognitive processes in the human brain [8]. The necrosis of neuron cell from carbofuran exposure has the potential to decrease the motoric reflex function and memory capability. Meanwhile, the necrosis of neuron cells in cerebrum during embryonal exposure of carbofuran rises by 35.51% for low doses and 55.27% for high doses [5]. The understanding of the death mechanism in brain cells of infant mice due to the carbofuran exposure during the lactation period is very significant to obtain a fundamental information in handling and

preventing the carbofuran exposure during the lactation period. This is important because understanding the death mechanism will reveal the most sensitive periods and target cell types due to the carbofuran exposure during lactation. If the death mechanism is acknowledged, then the prevention efforts can be done, thus, the decline in the ability to memories and concentration in infant mice can be avoided by reducing their motoric reflexes during lactation periods.

This study was aimed to uncover the degree of necrosis in neuron cell and any changes of behavior between 10 days old and 20 days old infant mice with carbofuran exposed mothers during lactation period. The benefit of this study was to determine the impact of infant mice's abnormalities of brain function (neurobehavioral test) due to the carbofuran exposed during lactation period.

MATERIALS AND METHODS

This experimental laboratory research was conducted through these stages: the exploration of lethal dose 50% of responses (LD50), the exploration of carbofuran teratogenic doses, the synchronization of mice's estrous cycle using pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) hormones, the mice's pregnancy examination through vaginal observation using the pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) hormones, the mice's pregnancy examination through vaginal plug and gavage carbofuran administration for 9 days to the mice mothers during lactation period. On Day 10 of lactation period, the necrosis of neuron cells in the infant mice's brain was calculated and their behavior was tested which included: Surface righting reflex, swimming ability (swimming position and direction) and the eight-arm radial maze memory test for the 20-days-old infant mice.

Animal Models

The animal models used in this study were 10 weeks old female mice *(Mus musculus)* with the body weight around 25-35 grams and 12 weeks old male mice obtained from Veterinaria Farma, Surabaya, Indonesia.

Synchronizing mice's estrous cycle using PMSG and HCG hormones

Ten weeks old virgin Female mice (Mus musculus) with a body weight around 25-35 grams were environmentally adapted for 7 days. The injections of pregnant gonadotropin serum mare (PMSG) (Folligon ™, Intervet, Boxmeer, Holland) at a dose of 5 IU each in Day 8 and the injection of human chorionic gonadotropin (hCG) (Chorulon ™, Intervet, Boxmeer, Holland) at Day 10 with a dose of 5 IU each were conducted, then, they were mated with 12 weeks old male mice. After that, the mice were kept in a cage and fed ad-libitum [9].

Examination of mice's pregnancy

The pregnancy examination was performed on Day 11, if a vaginal plug was seen in the vulva, then that day was declared as Day 0 of pregnancy. Then, the pregnant mice were then grouped in cages of 5 mice each until giving birth.

Administration of carbofuran

Mice mothers which had given birth would be exposed to carbofuran which was 2,3-Dihydro-2,2-dimethyl-7-benzofuranol N-methylcarbamate 98% (Aldrich Chemistry USA-426008-5G) with the dose of ¼ LD50, 1/8 LD50, and 1/16 LD50 during the lactation period from Day 1 - by gavage administration using a 3 ml syringe (4). Next, the 10-days-old infant mice were terminated, and then histopathological preparation was made. Microscopic examination by calculating the necrosis of neuron cells was performed using hematoxylin and eosin staining (HE, Millicell®-HA, Merck, Germany). Each sample of three slices was observed and examined under a microscope (Olympus® CX-41). Furthermore, the motoric reflex function test of 10-days-old infant mice's brain was performed which included: body turn reflexes, swimming (head angle position and swimming direction), and a memory tests using eight-arm radial maze for 20 days old infant mice.

Motoric Reflex Test:

Body reverse reflex (Surface Righting Reflex). This test was carried out on the 10-days-old infant mice. The tested infant mice were placed in a flat position on the flat surface. The duration taken by the infant mice to change its position from the face up position to the face down position was recorded with a stopwatch [10].

Swimming Ability. Tests were performed on the 10-day-old mice. The infant mice were dropped into a vessel filled with warm water (27-30°C), then observed its movements, such as; **Head angle position**: Score 0: diving; Score 1: nose was above the water surface; Score 2: nose and upper head were on the surface/above the water surface; Score 3: the same position as score 2 with the eyes were above the water surface, ¼ earlobe was on the water surface; Score 4: the same position as in score 3 with all parts of earlobe were above the water surface. **Swimming direction**: Score 1: floating; Score 2: swimming in a circle; Score 3: swimming straight or approaching straight; Score 4: sinking [11].

Memory test using Eight-arm radial maze

In this test, the equipment consisted of 8 arms made of plastic, the length of each arm: 32 cm, width: 5 cm; and the middle area diameter: 20 cm. **Adaptation phase**, first, infant mice *(Mus musculus)* were adapted for 7 days and given with

daily pellet feed and water. **Initial phase**, on the last day of the adaptation phase (day 7), the radial maze of eight arms memory test were performed on the infant mice. The treatment was like in the test phase, but this phase was only completed for a day. The aim was to see the memory ability of infant mice between groups was not significantly different. **Training phase**, the infant mice were fasted for 12 hours before being trained on an eight-arm radial maze. Each end of the arm was placed a small bait (pellets) and made it invisible from the middle of the maze, thus, the infant mice did not know which arm contained with the bait. Then, infant mice were placed in the middle of the maze and allowed to explore the maze for 10 minutes. After all were completed, maze was cleaned using a cotton swab moistened with 70% alcohol to remove traces and odors from the infant mice. Every infant mouse was treated with the same thing once a day for 3 consecutive days. The results of this training phase were not included in the observation of infant mice's memory. **Test phase**, the test phase implemented in 1 day after the training phase was finished and done for 5 consecutive days. At this stage, the infant mice were treated the same as during the training phase, but the duration of the test was not limited to 10 minutes only. The test was declared complete when the infant mice entered all arms or 10 minutes had passed. Moreover, the results of observation which needed to be considered were (a) the number of errors in each session (entering the arm that was visited before and/or not entering one or more arms in each session was counted as an error) and (b) the number of correct choices in entering each arm in each session (entering an arm that had never been visited before in that session). To find out the memory score of each infant mouse, the memory score formula was applied [12]:

$$\text{Memory score} = \frac{(\text{Number of correct arms}) - (\text{number of incorrect arms})}{(\text{Number of correct arms}) + (\text{number of incorrect arms})}$$

The maximum memory score that could be obtained was 1. Memory scores stretched on a scale from -1 to 1, the closer score to 1, it meant that the better memory of the tested infant mice. On the contrary, a score of -1 indicated the opposite (= all arm entries are incorrect).

Data analysis: The average number of necrosis in neuron cell was characterized by neuron cells that were pyknosis and karyorrhexis. Data calculation on the necrosis number of neuron cells, motoric reflex ability and memory tests of eight-arm radial maze on infant mice were analyzed using the Kruskal-Wallis test, if the results were significantly different, then it was followed by the Mann Whitney test. Furthermore, to facilitate the statistical calculations, Statistical Product and Service Solution (SPSS) version 20.0 were applied.

RESULTS

In a preliminary study, it explored the lethal dose 50% of responses (LD50) of carbofuran through oral administration (gavage) to the mother mice with a dose fraction which could cause death by 50 percent of the tested population. Next, observations were made for 24 hours post carbofuran exposure and the live and dead percentage of mother mice was measured. LD50 of carbofuran was obtained at 5 mg/kg body weight (BW), then the exploration of teratogenic dose was performed by carbofuran exposing the mother mice during lactation period on Day 0 to Day 9 which did not cause death during carbofuran exposure. The tested doses were started with 2.5 mg/kg BW (½ LD50), 1.25 mg/kg BW (¼ LD50), 0.625 mg/kg BW (1/8 LD50) and 0.3125 mg/kg BW (1/16 LD50). During the carbofuran exposure to these doses, 100% of maternal mortality was obtained during 9 days of carbofuran exposure at a dose of 2.5 mg/kg BW (½ LD50). Meanwhile, the doses of 1.25 mg/kg BW (1/4 LD50), 0.625 mg/kg BW (1/8 LD50) and 0.3125 mg/kg BW (1/16 LD50) did not cause maternal death during 9 days of carbofuran exposure, thus, those three last doses were applied in the main study.

Table 1: The necrosis number of neuron cells, Surface Righting Reflex, head angle position and swimming direction on the 10-days-old infant mice and also 8-Arm Radial Maze test on 20-days-old mice with carbofuran exposed mothers

Variables	Control	Carbofuran 1.25 mg/kg BW	Carbofuran 0.625 mg/kg BW	Carbofuran 0.3125 mg/kg BW
Neuron Cell Necrosis (mean±SD)	29.28 ± 2.74^{a}	46.56 ± 7.21^{b}	46.16 ± 6.69^{b}	41.44 ± 6.11^{b}
Surface Righting Reflex (mean±SD)	0.68 ± 0.06^{a}	1.96 ± 0.10^{d}	1.60 ± 0.20^{c}	0.98 ± 0.01^{b}
Head Angle Position (mean±SD)	3.00 ± 0.01^{a}	2.14 ± 0.37^{b}	2.14 ± 0.37^{b}	2.28 ± 0.48^{b}
Swimming Direction (mean±SD)	3.00 ± 0.01^{a}	2.14 ± 0.37^{b}	2.25 ± 0.53^{a}	2.85 ± 0.37^{a}
8-Arm Radial Maze (mean±SD)	1.00 ± 0.07^{a}	0.85 ± 0.11^{c}	0.86 ± 0.07^{c}	0.92 ± 0.06^{b}

Note: * there was a significant difference result where (P<0.05)

In this study, carbofuran can increase the necrosis of neuron cells between control and treatment groups (p <0.05). The higher dose of carbofuran exposure, the higher number of neuron cells undergo necrosis, although there is no any significantly difference between treatment groups (see Table 1). The Surface Righting Reflex movement differs significantly between groups and it takes longer as the higher doses of carbofuran administration (p <0.05) (see Table 1). The swimming motion of head angle position also decreases significantly between control and treatment groups (p <0.05), although it does not differ between treatment groups (see Table 1). Moreover, the swimming motion related to swimming direction also drops significantly between control and treatment groups (p <0.05), especially at the highest dose (Carbofuran 1.25 mg/kg BW), while at low and moderate doses there are no any difference from control group (see Table 1). In the memory test using 8-Arm Radial Maze, it reveals a significant decrease between control and treatment groups. The decreased memory ability is affected as the increasing dose of carbofuran exposure; however, the highest dose does not differ from moderate dose (p <0.05) (see Table 1).

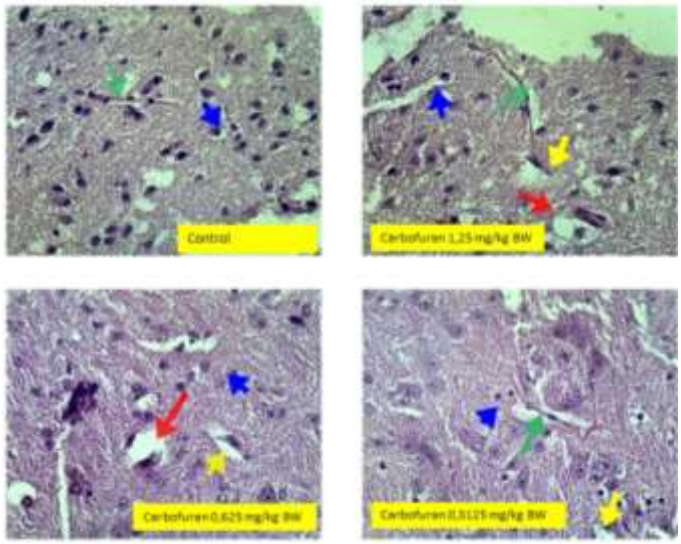

Fig.1: Histological figures of cerebellum in infant mice with carbofuran exposed mothers on Day 0 to Day 9 of lactation period (red arrows: astrocytes, yellow arrows: oligodendrocytes, green arrows: microglia, blue arrows: neurons - 400x magnification) [13].

DISCUSSION

This study analyzes the changes in motoric reflex activity and memory as the result of histological changes in infant mice with carbofuran exposed mothers during lactation periods. Carbofuran was administered orally (gavage) to the mothers and then the observation of cerebellum histology and brain function through motoric reflex movements and memory test were performed. The given carbofuran administration is a low dose that is still detected in foods of animal sources (meat and milk) [14], even though it is not the main source of contact for most individuals. It has been reported that carbofuran can cause cerebellar deficits [4]. Meanwhile, Purkinje cells and cerebellum granule cells are the most important targets of toxic substances [15]. Purkinje cells are one of the largest neurons in the cerebellum and are very sensitive to toxins. Granule cells are sensitive to intracellular glutathione loss. In this study, Purkinje cells are found in mice's brain on Day 14 to Day 16 pre-natal, whereas granule cells appear post-natal. Both cells are sensitive to excitotoxic chemicals and affect the mechanism of DNA repair [16].

Moreover, carbofuran is knows as one of the compounds that can induce cerebellar ataxia and is consistent with the findings in this study. The results of this study indicate the deficits of cortical cells and intracerebellar cells caused by carbofuran exposure. The necrosis number of neuron cells in this study increases to 41.53% at the lowest dose and 59.01% at the highest dose (see Table 1). Compared with the embryonal period, the necrosis number of neuron cell jumps to reach 580.17% at low doses and 662.64% at high doses when exposed to carbofuran at the peak of neurogenesis (Day 14-17 of pregnancy) [5]. Meanwhile, during the exposure of carbofuran at the peak of the lactation period (lactation Day 1-4), necrosis rises to 287.87% [4]. This shows that the response of neuron cell necrosis by carbofuran

exposure is highly dependent on the critical period of organ development. The necrosis of Neuron cell will be higher if it occurs in the peak phase of organ development (critical phase) [17]. In this study, perinatal carbofuran exposure to infant mice at the doses of 1.25 mg/kg BW, 0.625 mg/kg BW and 0.3125 mg/kg BW significantly increases necrosis. This is because carbofuran metabolites can be transferred in breast milk during breastfeeding. This finding is in accordance with the previous research, which reports that the effects of carbofuran on the autonomic nerve system can penetrate the blood brain barrier and affect the brain development and maturation [4].

Another objective of this study is to explore the impact of carbofuran exposure through lactating mothers on infant mice's behavior. A chronic exposure to carbofuran on the breastfed infant mice during lactation periods can cause the long-term behavioral changes characterized by the decreased motoric reflex activity and cognitive capacity. To determine the effect of carbofuran on neuromotor reflexes, the surface righting reflex test was applied. Carbofuran can significantly reduce the surface righting reflex with the increasing doses (see Table 1). This states that carbofuran exposure has a stimulating effect and reflex activity which is determined by the given doses. Generally, this test is performed during the embryonic period (exposure to pregnant mothers) to see the motoric reflex response when the infant mice are born. This is related to cerebrum development that occurs during the embryonic period [8]. While the function of motoric reflex is also determined by the function of the cerebellum which development occurs at the end of pregnancy until the beginning of the lactation period. Furthermore, this study also finds that there is the loss of most glia cells. These cells are responsible for maintaining homeostasis, myelin formation and providing the support and protection for neurons in the nerve system, including the cerebellum [18]. Therefore, glial cell loss caused by carbofuran exposure can be followed by neuron loss and dysfunction which appear as a deficit in motoric reflex performance.

Moreover, this study applies a swimming endurance method to determine the effect on breastfed infant mice with carbofuran exposed mothers. The swimming endurance test is a pharmacological screening method which is performed to determine the effects of toxic substances that work on the motion coordination, both the testing of decrease and increase in central nerve control. From the data presented in Table 1, it exhibits that all treatment groups reveal a decrease in control group even though the ability to swim related to swimming direction does not differ significantly at low doses. However, this is not found in moderate and high doses which are significantly different from control group. This indicates that the treatment can reduce swimming endurance or shorten the occurrence of fatigue. Furthermore, an excessive physical activity can progressively decrease the ability to produce muscle strength. Activity in the nerve system and muscles contributes to this fatigue. Besides the impaired motoric system function, fatigue and disruption of homeostasis can lead to performance degradation during the test. Thus, changes occur in all nerve systems including the brain, spinal cord, motor output, sensory input and autonomic function during the test [19].

Another interesting finding in this study is that carbofuran exposure during lactation period (perinatal) significantly decreases infant mice's learning patterns and memory, as shown by a decrease in latency time to reach food and time spent in the food arm. Memory function in this study is measured using 8-Arm Radial Maze in the form of mice error number in entering the 8-Arm Radial Maze arm can be seen in Table 1. The results of Kruskal test and Mann-Whitney test present a significant decrease indicating that the administration of carbofuran can reduce the infant mice's memory function. Moreover, this is consistent with previous research which states that carbofuran can cause neurochemical, neurophysiological and neurobehavioral deficits. Carbofuran exposure can lead to the rise in caspase 3 expression, an increase in the number of degenerative neurons and a very significant deficit in the learning process and memory [20]. Additionally, methyl mercury is a strong cytotoxic agent and any prenatal exposure can result in the extensive cortical changes, cerebellar changes characterized by reduced myelination, delayed migration and neuron cells loss. These morphological changes are accompanied by permanent changes in learning and memory [21]. Furthermore, observations in the 8-Arm Radial Maze are generally done for 12 days. The observations in this study are carried out for only 5 days, however they already present the decline of infant mice's memory function and the data obtained from the 8-Arm Radial Maze test can be used also to draw the same conclusions about the effects of carbofuran exposure on the infant mice's memory function. The prevention of decreased memory function is in line with the carbofuran working mechanism, thus, the use of antioxidants as neuroprotective and prevention of necrosis in neuron cells by free radicals is highly recommended [22].

The carbofuran dose used in this study is based on the LD50 fraction of 5 mg and given to mother mice on Day 0-9 of lactation period at the doses

of 1.25 mg/kg BW (1/4 LD50), 0.625 mg/kg BW (1/8 LD50) and 0.3125 mg/kg BW (1/16 LD50). The use of this dose is not different from the conversion of carbofuran content found in beef by 0.17 mg/kg BW and cow's milk by 0.349 mg/kg BW in Blora-Central Java Indonesia from the previous study [14]. Based on the administration of the lowest dose in this study with 0.3125 mg/kg BW, it is enough to cause an increase in the number of neuron cell necrosis, the decrease of body's motoric reflexes, the reduced ability to swim especially in head angle position and memory capacity. Thus, it is necessary to supervise for the use of carbofuran on agriculture and plantations that can provide adverse effects on the formation of residues in the source of livestock food products.

CONCLUSION

The conclusions of this study are a significant increase in neuron cell necrosis, fundamentally decreased motoric reflexes on the surface righting reflex and swimming ability related to the head angle position and swimming direction as well as an eight-arm radial maze memory test. The carbofuran exposure in mother mice during lactation period causes an increase in neuron cell necrosis as the result it can reduce reflexes, motoric skills and memory power. This study suggest that many opportunities open up to examine the provision of antioxidants to avoid the free radicals caused by carbofuran exposure. With various antioxidant variants that have a mechanism to prevent free radicals, breaking the chain of free radical reaction to the recovery of cell damage caused by free radicals is expected to increase the cell survival rate, thus, the increased necrosis of neuron cells, the decreased motoric reflex and memory ability can be avoided.

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