

## Brain Cells Death on Infant Mice (*Mus musculus*) Caused by Carbofuran Exposure During the Lactation Period

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### Abstract

An exposure to insecticide carbofuran has been reported able to generate a reactive oxygen species (ROS) in mice brains. This study was undertaken to evaluate the oxidative damage, biochemical and histopathological alterations by respectively examining malondialdehyde (MDA), cholinesterase (ChE) levels, necrosis and apoptosis in the suckling mice whose mothers were exposed to the carbofuran. The carbofuran was exposed via an oral route at the doses of 0.0208 mg/kg and 0.0417 mg/kg BW from the first until the fourth postnatal day after the delivery (n=27). The six-day-old pups were examined for its brain's MDA and ChE levels as well as the necrotic and apoptotic Purkinje cell were counted using the Tunel assay and hematoxylin-eosin (HE) staining. The mothers' exposure to carbofuran caused an increase in MDA levels, necrosis of Purkinje cells and a decrease in ChE, but there was no significant apoptosis in lactating pups. Carbofuran altered the level of the marker parameters related to the MDA, ChE and necrosis of Purkinje cells. Consistent changes were found in MDA, ChE and necrosis of Purkinje cells of the subjected pups, especially between the control and treatment groups, and there was no change between treatments. In conclusion, the transfer of carbofuran intoxication through the mother's milk resulted in the oxidative stress, biochemical and histopathological alterations in the suckling pups.

**Keywords:** Carbofuran, Purkinje cells, Infant mice brain, Lactation

## Laktasyon Döneminde Karbofurana Maruz Kalan Yavru Farelerde (*Mus musculus*) Beyin Hücreleri Ölümü

### Öz

İnsektisit karbofurana maruz kalmanın farelerin beyinlerinde reaktif oksijen türleri (ROS) ürettiği bildirilmiştir. Bu çalışma, anneleri karbofurana maruz bırakılan ve anne sütü emen farelerde malondialdehid (MDA), kolinesteraz (ChE), nekroz ve apoptozis düzeyleri incelenerek oluşan oksidatif hasar ile biyokimyasal ve histopatolojik değişiklikleri değerlendirmek amacıyla yapılmıştır. Fareler (n=27) karbofurana, oral yoldan, doğumdan sonraki 1. günden 4. güne kadar 0.0208 mg/kg ve 0.0417 mg/kg BW dozlarında maruz bırakıldı. Altı günlük yavruların beyin MDA ve ChE düzeyleri yanı sıra, Tunel testi ve hematoksilin-eozin (HE) boyaması kullanılarak nekrotik ve apoptotik Purkinje hücre sayısı incelendi. Annelerin karbofurana maruz kalması, MDA düzeylerinde artışa, Purkinje hücrelerinin nekrozuna ve ChE'de azalmaya neden oldu, ancak emen yavrularda belirgin bir apoptoz gözlenmedi. Özellikle kontrol ve tedaviye tabi tutulan yavruların MDA, ChE ve Purkinje hücrelerinin nekrozunda tutarlı değişiklikler bulunmuş, tedavi grupları arasında ise herhangi bir değişiklik olmadığı tespit edilmiştir. Sonuç olarak, anne sütü yoluyla karbofuran zehirlenmesi, emen yavrularda oksidatif stres, biyokimyasal ve histopatolojik değişiklikler ile sonuçlanmıştır.

**Anahtar sözcükler:** Karbofuran, Purkinje hücreleri, Yavru fare beyni, Laktasyon

## INTRODUCTION

Insecticide carbofuran residues in food may be harmful to organisms which are actually not the target of insecticide itself<sup>[1]</sup>. In a flower plantation which was contaminated by carbofuran in Ecuador in 2001, there were several cases of babies born with abnormalities, such as declining reflexes and motoric skill. At the child stage, there were

some brain function developmental abnormalities, such as the degeneration of memorizing and concentrating abilities<sup>[2]</sup>. In tested animals, carbofuran contamination causes an oxidative stress and weakens the motoric, memory, and cognitive functions<sup>[3]</sup>. Like organophosphate, carbofuran inductions result in a significant oxidative damage in the cerebral cortex, cerebellum, and brain-stem<sup>[4]</sup>. Carbofuran inductions in the cerebral cortex



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strongly correlate to the decline of cognitive and motoric functions [3].

An oral administration of carbofuran has been proven to strongly stimulate the reactive oxygen species (ROS) in mice brains and increase the levels of malondialdehyde (MDA) [3]. Intraperitoneal sub-acute administrations of carbofuran increase the brain's oxidative stress as the dose increases; and thus, improve MDA levels significantly. The increase of oxidative stress induces the activity of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase in the brain [5]. The presence of ROS could trigger the formation of hydroxyl radicals (OH\*) which breaks the DNA chains or changes the composition of nucleotides in DNA creating mutations and apoptosis [6]. Hydroxyl radicals (OH\*) as a result of an oxidative stress could also damage all membrane systems in the cell, such as creating leaks in the lysosome membrane which may cause the cell death (necrosis).

Uncontrolled increase in ROS causes the injury and death of neuron cells [7]. Cerebrum consists of 80% neuron cells and of 20% glial cells which are responsible for transmitting information to the spinal cord to control motoric functions [8]. During the embryonal stage of brain development, neuron cells develop earlier and reach their developmental peak at mid-pregnancy. Glial cells develop at mid-pregnancy until a few days before the fetus is born and reach the peak of development at the end of pregnancy [9]. The development of the cerebellum starts in the late period of pregnancy and develops at maximum (peak) at the beginning of birth (the beginning of lactation). The death of neuron cells, cerebral glial cells and Purkinje cells due to carbofuran exposure potentially degenerates reflexes and motoric functions.

A deeper understanding of the brain's cell death mechanism in mice pups due to carbofuran exposure during the lactation period is necessary to acquire the basic treatment and prevention during the lactation period. Besides, it is important to understand the brain's cell death mechanism in order to identify the most sensitive period as well as the type of cells affected by carbofuran exposure during the lactation period. If the mechanism and the type of dead cells are recognized, actions can be taken to prevent the degeneration of reflexes and motoric skills in infant mice.

The aim of this research was to determine the brain's cell death mechanism in mice pups whose mother were exposed to carbofuran during the lactation period by measuring the MDA levels as an indicator of ROS production, ChE levels as the indicator of a neural function response to carbofuran exposure, apoptotic and necrotic cerebral Purkinje cells. This study contributed in disclosing the prevention mechanism of brain's cell death in mice pups whose mother were exposed to carbofuran during the lactation period. In addition, this study also provided a scientific information of insecticide carbofuran exposure,

especially during the lactation period related to the attempts of inhibiting brain's development disorders.

## MATERIAL and METHODS

### Ethics Approval

The study was approved by the Faculty of Veterinary Medicine's Animal Ethics Committee. All variables had been considered in accordance with the Ethics Committee related to the animal handling to ensure no discomfort or pain caused to the animals during sampling (2011/111-KE).

### Laboratory Animals

The animals used in this study were 27 female mice (*Mus musculus*), 10 weeks old, with the weight range of 25-30 grams, and 12-weeks-old male mice. Environmental adaptation was done to female mice (*Mus musculus*) for 7 days. On the 8<sup>th</sup> day, pregnant mare serum gonadotropin (PMSG) with a dosage of 5 IU/mouse was injected into the female mice and followed by Human Chorionic Gonadotrophin (HCG) injections with a dosage of 5 IU/mouse which was performed on the 10<sup>th</sup> day. Afterwards, the female mice were mated with the 12-week-old male mice. On the 11<sup>th</sup> day, a gestation examination was carried out. The gestation of female mice was indicated by the visible mating plug covering the female mice vulva; and then the day was considered as the first day of gestation [10].

### Carbofuran Exposure

Carbofuran exposure was targeted at the suckling mice's brain and this study examined the Purkinje cells in the cerebellum. The female mice were exposed to carbofuran with a dosage of 0.0208 mg/kg (1/24 LD<sub>50</sub>) and 0.0417 mg/kg (1/12 LD<sub>50</sub>) [10] on the 1<sup>st</sup> to 4<sup>th</sup> day of the lactation period given orally using a sterile disposable syringe. The six-day-old mice pups were then tested and measured for MDA and Cholinesterase (ChE) levels and histopathologic preparations were made. A microscopic examination was conducted to estimate the number of cells experiencing necrosis and apoptosis by using HE staining and an Apoptag Apoptosis Detection Kit.

### Measuring Cholinesterase (ChE) Levels

Cholinesterase (ChE) was determined according to the manufacturer's instructions of Cholinesterase FS (DiaSys Diagnostic Systems, 11401) [11]. To measure the ChE levels on the six-day-old mice's cerebrospinal fluid, the following materials were used to create a substrate: S-Butyrylthiocholine iodide Phosphate buffer pH 7.7, 5,5 dithiobis-2-nitrobenzoate. The principle of ChE level measurement was that the process of S-Butyrylthiocholine iodide + H<sub>2</sub>O hydrolysis and with the help of ChE sample was converted to Thiocholine iodide + butyrate. Thiocholine iodide + 5,5-dithiobis-2-nitrobenzoate would transform into 5-Mercapto-2-nitrobenzoate - 5-Mercaptothiocholine. The

reaction solution is from Diluent 100 mL, 3 mL reagent mix (for 30 samples) with concentrations of Phosphate buffer pH 7.7 50 mmol/L, S-Butyrylthiocholine iodide 6 mmol/L, 5,5-dithiobis-2-nitrobenzoic 0.25 mmol/L. The standard wavelength of reaction solution was 405 nm and the length of translucent light was 1 cm measured at 25, 35 and 37°C. The 10 mL of sampled fluid was collected and mixed with 100 mL of reaction solution. The mixture was examined every minute for three minutes. To determine the sampled concentration, the enzyme activity of sample (U/L) =  $\Delta A/\text{min} \times 68500$  was measured.

### Measuring Malondialdehyde (MDA) Level

Malondialdehyde (MDA) was determined by the method of Conti et al.<sup>[12]</sup>. The measurement of malondialdehyde level on the six-day-old mice pups' brains was performed using the MDA/Thiobarbituric Acid Reactive Substance (TBARS). This method was performed by weighing 1 gram of the infant mice brain sample then putting it into a reaction tube, mixing it with 9 mL cold PBS and then crushing it with a spatula. The liquid was then centrifuged at 3000 rpm for 15 min. 4 mL of supernatant was collected and added to 1 mL of *trichloroacetic acid* (TCA) 15% solution. Then, 1 mL of 0.37% Thiobarbituric acid (TBA) solution was added into HCl 0.25 N and heated in a water bowl at 80°C for 15 min. After cooling the solution at room temperature for 60 min, the solution was centrifuged at 3000 rpm for 15 min. Finally, the value of the absorbance was read against the red lines which were formed using a spectrophotometer at  $\lambda = 532$  nm.

### Necrosis Examination Using HE Staining

Necrosis examination was performed using HE staining. The six-day-old mice's brains were fixated with 10% formalin buffer and brain tissues were processed in routine processing until they formed paraffin blocks. The paraffin blocks were cut using a microtome with a thickness of 5  $\mu\text{m}$  in a series and then glued to the object glass. The observed area was the cerebellum which was obtained through the coronal section at the position 11/1.56 mm from the edge of the posterior lobe<sup>[13]</sup>. After 24 h' fixation, the mice brain was washed in a 70% alcohol solution three times and stained using Haematoxylin Eosin (HE). Purkinje cells which experienced necrosis were characterized by cells undergoing pyknosis and karyorrhexis.

### Apoptosis Examination Using Tunel Assay

The examination of apoptotic cells was performed by Tunel assay. The paraffin blocks were cut using a microtome with a thickness of 5  $\mu\text{m}$  in a series and then glued to the object glass using polylysine. For counting the apoptotic cells, pieces of the tissue were processed with S7101 Apoptag Plus Peroxidase. Apoptotic cells were identified by the color absorbent (dark brown).

### Data Analysis

Data analysis and evaluation of statistical significance among different determined values was performed using one-way Analysis of variance (ANOVA) with post hoc analysis (Duncan test)<sup>[14]</sup>. The values were expressed as mean  $\pm$  SD and considered significant at P 0.05. The statistical analysis was performed using Statistical Product and Service Solutions (SPSS) version 17.0.

## RESULTS

### Malondialdehyde (MDA) Level

This study showed that there was an increase in MDA levels, as the results of the ANOVA test indicated a sign value of 0.000 between the control and treatment group, which was less than the significance value  $\alpha=0.05$ . It can be interpreted that there was a difference between the control and treatment groups. Thus, carbofuran administration in doses of either 0.0208 mg/kg or 0.0417 mg/kg might potentially produce the free radicals. The Duncan test indicated a sign value of 0.115 between 0.0208 mg/kg and 0.0417 mg/kg, which was more than the significance value  $\alpha=0.05$ . There was no significant difference in pups whose mothers were exposed to carbofuran of 0.0208 mg/kg and those with 0.0417 mg/kg. Although there was no significant difference, the increase in MDA levels was very high at 58.95% and 247.47% compared to that the control group, and the increase in MDA levels reached 118% between 0.0208 mg/kg and 0.0417 mg/kg (Table 1).

### Cholinesterase (ChE) Level

This study presented the decreasing ChE levels and the ANOVA test result indicated a sign value of 0.001 between

**Table 1.** Effect of carbofuran exposure in subacute doses for 4 days on the MDA, ChE levels, Purkinje necrotic cell and Purkinje apoptotic cell in mice pups' brains (n=27)

Parameters	Control Group (mean $\pm$ SD)	0.0208 mg/kg BW Carbofuran (mean $\pm$ SD)	0.0417 mg/kg BW Carbofuran (mean $\pm$ SD)
Malondialdehyde (nmol/mg)	25.53 $\pm$ 3.02	40.58 $\pm$ 5.77 <sup>a</sup>	88.7 $\pm$ 3.02 <sup>a</sup>
ChE levels (U/L)	801.75 $\pm$ 129.73	671.50 $\pm$ 50.53 <sup>a</sup>	606.75 $\pm$ 28.45 <sup>a</sup>
Purkinje necrotic cell	1.98 $\pm$ 1.92	6.24 $\pm$ 0.73 <sup>y</sup>	7.68 $\pm$ 1.01 <sup>y</sup>
Purkinje apoptotic cell	11.00 $\pm$ 1.92	13.00 $\pm$ 0.73	14.13 $\pm$ 1.01

Statistical difference from the control: <sup>a,y</sup> Significant at P $\leq$ 0.05

the control and treatment groups, which was less than the significance value  $\alpha=0.05$ . It can be concluded that there was a difference between control and treatment groups. Carbofuran exposure in the doses of 0.0208 mg/kg and 0.0417 mg/kg potentially lowered the ChE levels. The Duncan test analysis revealed a sign value of 0.707 between 0.0208 mg/kg and 0.0417 mg/kg, which was more than the significance value  $\alpha=0.05$ . There was no significant difference in pups whose mothers were exposed to carbofuran of 0.0208 mg/kg and 0.0417 mg/kg. Although there was no significant difference, the decrease in ChE levels reached 16.25% and 24.32% compared to the control group, and the decrease in ChE levels reached 9.64% between 0.0208 mg/kg and 0.0417 mg/kg (Table 1).

**Necrotic Cell**

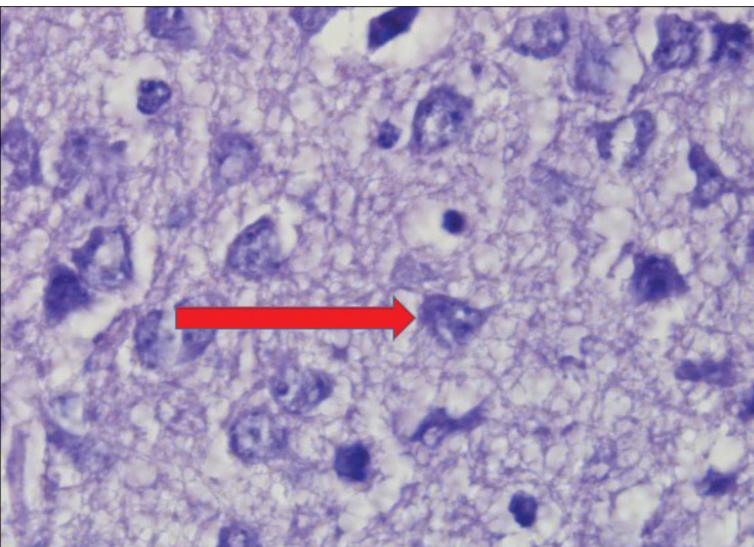
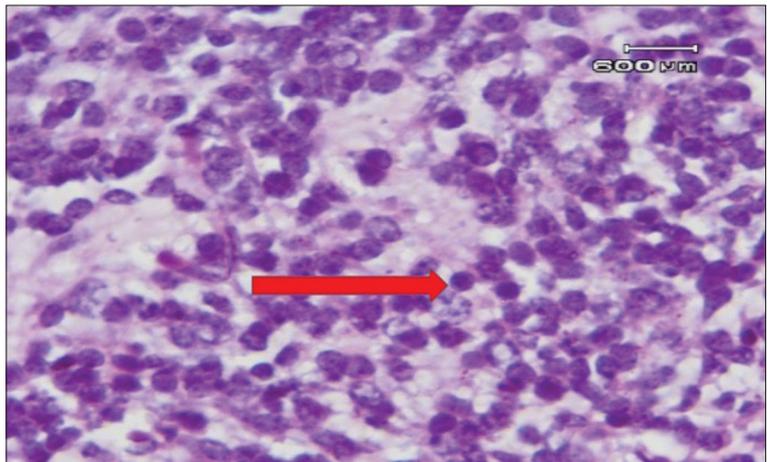
In light microscopic examinations, histopathological changes were observed in mice pups' brains of all exposed groups compared to the control groups. This study found an increasing number of Purkinje necrotic cell in the brains of six-day-old mice pups whose mothers were exposed to carbofuran on the first and the fourth day of the lactation

period. Carbofuran exposure in doses of 0.0208 mg/kg and 0.0417 mg/kg had enhanced Purkinje necrotic cell. There was no significant difference in pups whose mothers were exposed to 0.0208 mg/kg and 0.0417 mg/kg of carbofuran. Nevertheless, the increase in Purkinje necrotic cell was very high at 215.15% for a dosage of 0.0208 mg/kg and 287.87% for a dosage of 0.0417 mg/kg compared to the control group. Furthermore, the increase in Purkinje necrotic cell reached 23.07% between the dose administrations of 0.0208 mg/kg and 0.0417 mg/kg. An overview of staining results using HE showed necrosis of the Purkinje cells between the control and treatment groups (Fig. 1,2,3; Table 1).

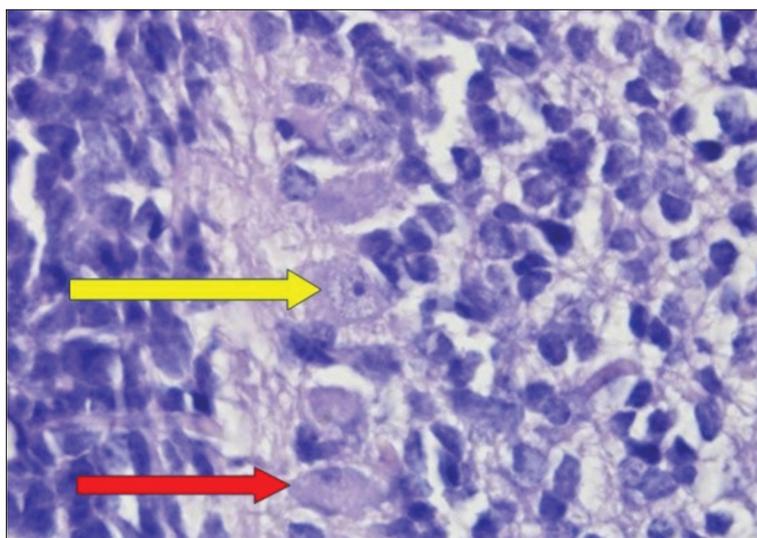
**Apoptotic Cell**

This study expressed that there was an increasing number of Purkinje apoptotic cell in the brains of six-day-old mice pups from mother mice which were exposed to the insecticide carbofuran from the 1<sup>st</sup> and 4<sup>th</sup> day of the lactation period. The results of the Kruskal-Wallis test indicated a significance level of  $0.549 > 0.05$ . There was no significant difference in Purkinje apoptotic cell in lactating

**Fig 1.** Histopathology of the cerebellum of mice pups (*Mus musculus*) from the control group. H.E Staining. 1000x



**Fig 2.** Histopathology of the cerebellum of mice pups (*Mus musculus*), red arrow indicates the Purkinje necrotic cells. H.E Staining. 1000x



**Fig 3.** Histopathology of the cerebellum of mice pups (*Mus musculus*), yellow arrow indicates the normal Purkinje cells, red arrow indicates the Purkinje necrotic cells. H.E Staining. 1000x

pups whose mothers received 0.0208 mg/kg and 0.0417 of carbofuran compared to the control groups (Table 1). The estimated increase of Purkinje apoptotic cell in the carbofuran groups was 18.18% and 28.45%, while the increase in Purkinje apoptotic cell reached 7.99% between the dose administrations of 0.0208 mg/kg and 0.0417 mg/kg (Table 1).

## DISCUSSION

The metabolism of carbofuran has been well studied in rats, mice, and lactating cows. Carbofuran is rapidly absorbed, metabolized, and eliminated, primarily via urine, in the species investigated<sup>[15]</sup>. Carbofuran was altered by oxidation of the number 3 carbon and of the N-methyl group, hydrolysis of the ester linkage, and conjugation of metabolites containing a hydroxyl group. Carbofuran metabolites in the milk were the 3-hydroxycarbofuran, 3-keto-carbofuran, and 3-hydroxy-N-hydroxy methyl derivatives of carbofuran, which were found both in free and conjugated forms. Conjugated 2,3-dihydro-2,2-dimethyl-3-keto-7-hydroxybenzofuran was the major hydrolytic product of carbofuran in the milk. The same metabolites were also found in the urine and feces. 3-Hydroxycarbofuran was one of the most rapidly formed metabolites and 3-ketocarbofuran phenol was one of the end products<sup>[16]</sup>.

3-hydroxycarbofuran and nitrosocarbofuran could induce the micronucleus formation, while 3-ketocarbofuran could not but caused a significant DNA migration in SCGE test. Moreover, 3-ketocarbofuran caused an obvious increase in damaged cells accompanied with a great decrease in undamaged cells which displayed a higher degree of cell damage than other three compounds, especially seriously damaged cells increased in number and suggested a more serious DNA damaging effects. There has been no report on the mechanisms of DNA damaging effects induced by carbofuran and its metabolites. But in the oxidation and the hydrolyze of its transformation, free radicals are a potential

outcome, which is widely known to reduce DNA damage. The presence of metabolic active system in organism could be degraded to less toxic phenols directly, while 3-hydroxycarbofuran was metabolized to 3-ketocarbofuran with a high direct toxicity to cells, thus, 3-hydroxycarbofuran revealed a stronger *in vivo* toxicity<sup>[17]</sup>.

Purkinje cell is a cell-specific marker of the cerebellar Purkinje cell and a suitable indicator for observing the postnatal development of the cerebellum after birth. The Purkinje cell levels in the mice's cerebellum during the critical postnatal (4 days after birth) is to determine the effect of external exposure on cerebellar growth in the offspring during lactation<sup>[18]</sup>.

Many pathophysiological conditions may have oxidative stress. In normal conditions, it is balanced by the antioxidant system and this balance is disturbed due to the increased oxidative stress. MDA is one of the fairly reactive metabolic products created from the effect of free oxygen radicals on tissues and from a series of reactions during lipid peroxidation. Malondialdehyde (MDA) is the best and a sensitive indicator of lipid peroxidation and so of oxidative stress<sup>[19,20]</sup>.

The final result of lipid peroxidation is MDA and a high MDA level indicates the damaged oxidation processes or cell membrane due to free radicals<sup>[21,22]</sup>. The results of this study showed that carbofuran exposure at subacute doses (0.0208 mg/kg and 0.0407 mg/kg) during a lactation period caused a significant enhancement in MDA levels of mice pups' brains. The data suggested that the significantly elevated MDA levels in the brain in turn produced a reactive oxygen species (ROS) which caused an oxidative stress in this organ. The increase of MDA levels results in cell death which is triggered by toxicants, depending on the dose. In this study, carbofuran dose increased MDA levels but there was no significant difference in pups whose mothers were exposed to carbofuran of 0.0208 mg/kg and 0.0417 mg/kg. Although there was no significant difference, the increase

in MDA levels reached 118% between 0.0208 mg/kg and 0.0417 mg/kg (Table 1). The increased doses could increase the significant MDA levels if exposed postnatally on the 1<sup>st</sup> until 20<sup>th</sup> day [23]. In another study, the increase in MDA levels due to an acute exposure of carbofuran may reach up to 175.04% for the dose administrations of 0.2 mg/kg which are compared to 0.4 mg/kg in mice [5]. Furthermore, oral sub-acute exposure of carbofuran for 28 days in male mice increases the MDA levels by 65% in other study [24].

This indicates that the increasing MDA levels due to carbofuran exposure depends highly on the dose, duration of exposure, and the type of affected organ. Vulnerability of brain development depends on the agent or the active metabolite which can be achieved during the development of nervous system and is associated with the exposure period. Exposure before or after the organ is fully developed makes the organ less vulnerable to inhibitions than if the exposure occurs during the organ development [25]. An adult mouse brain is protected by the blood-brain barrier (BBB) to chemicals, while such protection does not exist in mice fetus and 6 months old mice [26].

Neuronal membranes which are rich in polyunsaturated fatty acids are the source of lipid peroxidation reaction [27,28]. Lipid peroxidation causes destruction and damage to cell membranes and also changes the fluidity/membrane permeability [28,29]. Thus, the increase in MDA is caused by pesticides induction by forming ROS. Acute intraperitoneal carbofuran exposure may cause a significant increase in MDA levels of the brains and livers of adult mice. The increase in MDA levels is in line with the doses of carbofuran exposed. Carbofuran is proven to be efficiently absorbed and rapidly distributed to various organs of an organism. Lipophilic nature of carbamate also causes it to be able to interact with lipid serums and tissues [30]. The forming process of lipid peroxidation starts from hydrogen ions on the side chain of polyunsaturated fatty acids (PUFA), which construct the cell membranes by free radicals, form carbon radicals. Carbon radicals are oxidized to form peroxy radicals. Furthermore, peroxy radicals draw H<sup>+</sup> ion into the side chain of adjacent PUFA and form lipid peroxidation. This process is a chain reaction because the lipid peroxidation attracts more H<sup>+</sup> ions into the side chain of adjacent PUFA until the PUFA chain is finally split into other compounds, such as MDA, 9-hydroxy-nonanal, ethane and pentane [21,22].

Cholinesterase level measurements are often conducted to determine the exposure effect to insecticides. ChE used in this measurement was collected from tissues, plasma and red blood cells [31] and ChE collected from the brain was the best sample to be used as an indicator of the exposure to insecticides [32]. In this study, the insecticide carbofuran decreased the ChE levels between the control and the treatment groups. However, there was no significant difference in the ChE levels among lactating

pups whose mothers received carbofuran of 0.0208 mg/kg and the ones with 0.0417 mg/kg (Table 1). The increased doses could decrease the significant ChE levels if exposed postnatally on the 1<sup>st</sup> until the 20<sup>th</sup> day [23]. Almost all insecticide exposure resulted in the decrease of ChE levels, either during embryonic period, growth period, or adulthood. Generally, the response of the decreased ChE level is in accordance with the exposed dose. However, the decreased ChE levels due to the insecticide exposure in several phases of individual growth may induce varied responses.

There were correlations between the accumulation of acetylcholine and the extent of MDA. An increased oxidative stress by carbofuran might be a result of cholinergic hyperactivity or might be due to its direct effect on the production of reactive oxygen [33]. The peroxidation does not only alter lipid milieu, the structural and functional integrity of the cell membrane, but also affects the activities of various membrane-bound enzymes, including acetylcholinesterase (AChE) and different ATPases. The inhibition of ATPase activities may be a causative factor of neuronal/cellular dysfunction, due to an alteration in cationic transport across the membrane and a disturbance in uptake as well as a release of certain neurotransmitters [34].

The role of ChE is activated before the synaptogenesis during the formation of neural tube. The formation of ChE is in line with the axon growth [35]. The cholinergic system in early development acts as a regulatory growth and has morphogenetic functions [36] by controlling cell proliferation, motility, cell differentiation and genetic expression [37]. Thus, the cholinergic system has a very important role in the cell development and brain formation [38]. Although the brains of infant mice are extremely sensitive to carbofuran exposure during the lactation period and both treatments showed a decrease in ChE levels, all infant mice were still alive with symptoms of mild poisoning. The decrease in ChE levels indicates the response of brain or adult nervous system [35].

In this study, the insecticide carbofuran increased the necrotic death of Purkinje cells both in control and treatment group. However, there was no significant difference in necrotic cells among lactating pups whose mothers received carbofuran of 0.0208 mg/kg and the ones with 0.0417 mg/kg. In this study, we found an association between the increased MDA levels and the necrotic cells. The increased lipid peroxidation and lipid peroxidation products, such as MDA levels, contribute to neuronal loss in conditions associated with oxidative stress [39]. Increased MDA levels indicates a membrane damage and leads to a cellular necrosis. The attack of free radicals on a cell membrane makes it devoid of integrity and viability causing the cells to undergo necrosis [40]. Cell death caused by the swelling of cytoplasm, nucleus karyolysis and lysis are classified as necrosis [41,42].

The number of Purkinje necrotic cells was not as many as the number of cells which experienced apoptosis due to the carbofuran exposure. This was because during the neurogenesis period, the Purkinje cells had experienced more apoptosis physiologically through homeostasis efforts. However, when the number of Purkinje necrotic cells was compared to the control group, there was an increase in the number of Purkinje necrotic cells in the treatment group which was significantly higher than the number of Purkinje apoptotic cells (apoptosis increases 28.45% and necrosis increases up to 287.87%) (Table 1).

In this study, the insecticide carbofuran could increase the apoptosis of Purkinje cells between control and treatment groups. However, there was no significant difference in the number of Purkinje apoptotic cells among lactating pups whose mothers received carbofuran of 0.0208 mg/kg and the ones with 0.0417 mg/kg. Such different results from those of a study by Luqman<sup>[10]</sup> could be because the brain development phases during the embryonal period and lactation period have different critical time. In addition, the duration of carbofuran exposure during embryonal period was longer (10 days), while during the lactation period the exposure lasted only for 4 days. Although cerebellum is the most sensitive organ to oxidative-stress causing neurotoxins, longer exposure time is needed for the agent to reach the target of nuclear DNA and mitochondria to induce apoptosis<sup>[43]</sup>. The exposure of carbofuran insecticide can increase the activity on cerebral ROS during embryonal period and the expression of p53, caspase 3 and apoptosis. The increasing expression of p53, caspase 3 and apoptosis indicated that the insecticide carbofuran caused an apoptosis through an intrinsic pathway<sup>[10]</sup>.

In conclusion, this study revealed that the carbofuran had been distributed in pups' tissues through the milk of lactating mothers and had caused an oxidative damage of pups' brains. Carbofuran exposure indicated that mice pups' brains were particularly more vulnerable to oxidative stress, which may eventually lead to neurobehavioral disorders. In this study, we also found that the insecticide carbofuran dose in lactating mice of 0.0208 mg/kg BW had been able to increase ROS activity and Purkinje cell death. with the same dose, if converted to humans, according to dose conversion by Laurence and Bacharach (1964), it will be equal to 0.115 mg/kg BW. This result of dose conversion can be applied as a carbofuran potential standard in increasing the ROS activity and Purkinje cell death since the residual level found in cows' meat and milk is around 0.17 mg/kg BW and 0.349 mg/kg BW<sup>[44]</sup>.

The insecticide carbofuran exposure to the lactating mice made the mean of Purkinje apoptotic cell higher than the necrotic ones in all treatment doses. A high increase of apoptosis in Purkinje cells allowed an opportunity to prevent and manage the strategy to overcome Purkinje cell death due to carbofuran insecticide exposure during the lactation period, such as by providing antioxidant

variations. Some efforts to prevent the formation of ROS can be done to inhibit and countermeasure the neuronal development cell death due to the exposure to carbofuran. The administration of antioxidants, such as vitamin C, curcumin, and allopurinol during lactation is possible to reduce the oxidative stress through the inhibitor xanthine oxidase and scavenger effects of free radicals. Therefore, there are still opportunities to improve the environment of neonate's nerve by increasing the growth of axons, dendrites and synaptogenesis and myelination of axons<sup>[45,46]</sup>.

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