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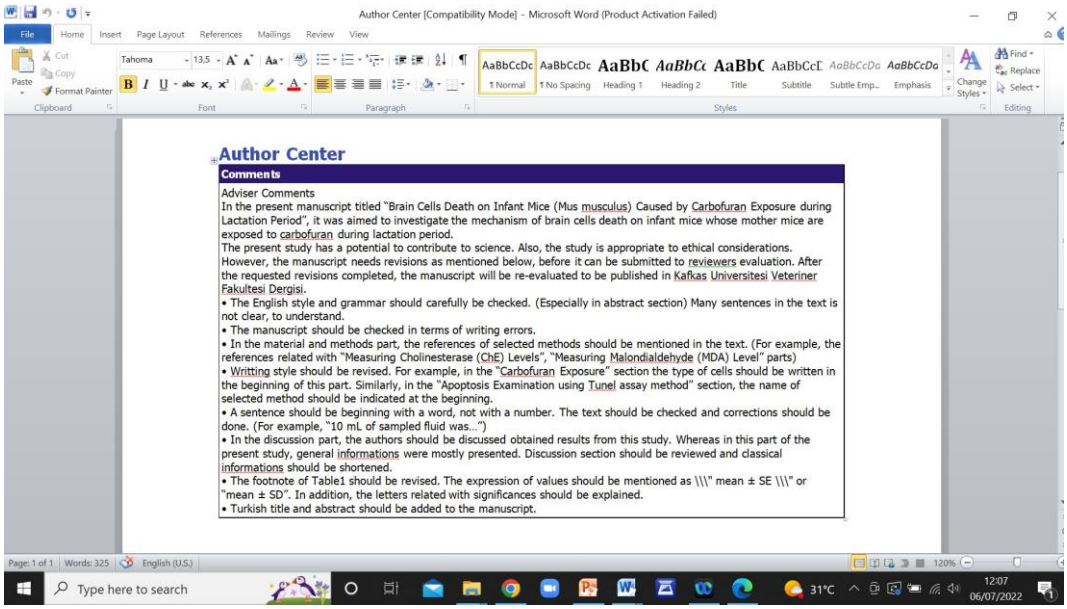

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Brain Cells Death on Infant Mice (*Mus musculus*) Caused by Carbofuran Exposure during Lactation Period

Abstract

Exposure to insecticide carbofuran has been reported to generate reactive oxygen species (ROS) in mice brains. The present study was undertaken to evaluate the oxidative damage and biochemical and histopathological alterations by respectively examining malondialdehyde (MDA), cholinesterase (ChE) levels, and necrosis and apoptosis in suckling

mice whose mothers were exposed to the carbofuran. The carbofuran was exposed via an oral route in doses of 0.0208 mg/kg and 0.0417 mg/kg BW from postnatal day 1 until day 4 after delivery (n=27). The six-day-old pups were examined for brain MDA and ChE levels as well as necrotic and apoptotic Purkinje cell count using Tunel assay and hematoxylin-eosin (HE) staining. Exposure of the mothers 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 control and treatment, and no change between treatments. In conclusion, the transfer of carbofuran intoxication through the mother's milk resulted in oxidative stress, and biochemical and histopathological alterations in the suckling pups.
Keyword*: Carbofuran, Purkinje cells, infant mice brain, lactation.*

Laktasyon Döneminde Carbofuran Maruziyetinin Neden Olduğu Bebek Farelerinde (Mus musculus) Beyin Hücreleri Ölümü

Öz

İnsektisit karbofurana maruz kalmanın farelerin beyinlerinde reaktif oksijen türleri (ROS) ürettiği bildirilmiştir. Bu çalışma, anneleri karbofurana maruz bırakılan anne sütü emen farelerde malondialdehid (MDA), kolinesteraz (ChE ve nekroz ve apoptozis düzeyleri incelenerek oksidatif hasar, biyokimyasal ve histopatolojik değişiklikleri değerlendirmek amacıyla yapılmıştır. Karbofurana, oral yoldan, doğumdan sonraki 1. günden 4. güne kadar 0.0208 mg/kg ve 0.0417 mg / kg BW dozlarında (n = 27) maruz bırakıldı. Altı günlük yavruların beyin MDA ve ChE düzeyleri yanı sıra, Tunel testi ve hematoksilen-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 bulunmuştur, tedavi grupları arasında arasında herhangi bir değişiklik olmadığı bulunmuştur. Sonuç olarak, anne sütü yoluyla karbofuran zehirlenmesi, emen yavrularda oksidatif stres, biyokimyasal ve histopatolojik değişiklikler ile sonuçlanmıştır.

Anahtar sözcükler **:** Carbofuran, Purkinje hücreleri, fare beyin çocuk, emzirme.

Introduction

Insecticide carbofuran residues in food may be harmful to organisms which are actually not the target of the insecticide itself [1]. In a flower plantation contaminated by carbofuran in Ecuador, there were several cases of baby born with abnormalities, such as declining reflexes and motoric skill in 2001. At the child stage, there were some brain function developmental abnormalities, such as the degeneration of memorizing and concentrating abilities[2]. In tested animals, carbofuran contamination causes oxidative stress and weakens motoric, memory, and cognitive functions[3]. Like organophosphate, carbofuran inductions result in significant oxidative damage in the cerebral cortex, cerebellum, and brainstem[4]. Carbofuran inductions in the cerebral cortex strongly correlates to the decline of cognitive and motoric functions[3].

Oral administration of carbofuran has been proven to strongly stimulate reactive oxygen species (ROS) in mice brains and increase the levels of malondialdehyde (MDA) [3]. Intraperitoneal sub-acute administrations of carbofuran increase brain 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 break the DNA chains or change the composition of nucleotides in DNA, resulting in mutations and apoptosis[6]. Hydroxyl radicals (OH*) resulting from oxidative stress could also damage all membrane systems in the cell, such as creating leaks in the lysozyme membrane which may result in cell death (necrosis).

Uncontrolled increase in ROS results in 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 mechanisms of brain cell death in mice pups due to carbofuran exposure during the lactation period is necessary to acquire the basic treatment and prevention of carbofuran exposure during the lactation period. Besides, it is important to understand the brain 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 mechanism of brain cell death in mice pups whose mother mice were exposed to carbofuran during the lactation period by measuring MDA levels as an indicator of ROS production, ChE levels as the indicator of a neural function response to carbofuran exposure, and apoptotic and necrotic cerebellum Purkinje cells. This study contributes to disclosing the prevention mechanism of brain cell death in mice pups whose mother mice were exposed to carbofuran during the lactation period. In addition, this study also provides scientific information of insecticide carbofuran exposure, especially during the lactation period, related to the attempts to inhibiting brain development disorders.

Material and Method

Ethics approval

The study was approved by the Faculty of Veterinary Medicine Animal Ethics Committee. All variables had been considered as in accordance with the Ethics Committee related to animal handling to ensure no discomfort or pain was 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, weighing 25-30 grams, and 12-week-old male mice. Environmental adaptation was done to female mice (Mus musculus) for 7 days. On day 8, Pregnant mare serum gonadotropin (PMSG) with a dosage of 5 IU/mouse was injected into the female mice, and Human Chorionic Gonadotrophin (HCG) injections with a dosage of 5 IU/mouse was performed on day 10. Afterwards, the female mice were mated with 12-week-old male mice. On day 11 a gestation examination was carried out. The gestation of the 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 brain and this study examined the Purkinje cells in the cerebellum. The female mice were exposed to carbofuran with a dosage of 00.0208 mg/kg (1/24 LD_{50}) and 0.0417 mg/kg (1/12 LD_{50}) [10] on days 1-4 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 Apopteg 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 cerebrospinal fluid of six-day-old mice, the following materials were used: 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_2O hydrolysis, 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: Diluent 100 ml, 3 ml reagent mix (for 30 samples) with concentrations: Phosphate buffer pH 7.7 50 mmol/l, S-Butyrylthio choline iodide 6 mmol/l, 5,5-dithiobis-2-nitrobenzoic 0.25 mmol / l. The standard wavelength of the reaction solution was 405 nm and the length of translucent light was 1 cm measured at 25, 35 and 37° C. The10 mL of sampled fluid was collected and mixed with 100 mL of the reaction solution. The mixture was examined every minute for three minutes. To determine the sampled concentration, the enzyme activity of sample (U/L) = ΔA/min x 68500 was measured.

Measuring Malondialdehyde (MDA) Level

Malondialdehyde (MDA) was determined by the method of Conti et al[12]. The measurement of malondialdehyde level on six-day-old mice pups' brains was performed using MDA/Thiobarbituric Acid Reactive Substance (TBARS). This method was performed by weighing 1 gram of the infant mice brain sample, putting it into a reaction tube and mixing it with 9 ml cold PBS and then crushing it with a spatula. The liquid was then centrifuged at 3000 rpm for 15 minutes. 4 ml of supernatant was collected and added to 1 ml of a *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 bath at a temperature of 80ºC for 15 minutes. After cooling the solution at room temperature for 60 minutes, the solution was centrifuged at 3000 rpm for 15 minutes. Finally, the value of the absorbance was read against the red lines formed using a spectrophotometer at λ = 532 nm.

Necrosis Examination using HE Staining

Necrosis examination was performed using HE staining. The six-day-old mice 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 µm in a series and then glued to the object glass. The observed area was the cerebellum obtained through the coronal section at the position 11/1.56 mm from the edge of the posterior lobe[13]. After 24 hours' fixation, the mice brain was washed in a 70% alcohol solution three times and stained using Haematoxylin Eosin (HE). Purkinje cells undergoing necrosis were signified by cells undergoing pyknosis and karyorrhexis.

Apoptosis Examination using Tunel assay

Examination of apoptotic cells was performed by Tunel assay. The paraffin blocks were cut using a microtome in a thickness of 5 µm 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)[14]. The values were expressed as mean ± 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 indicated 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 α = 0.05. It can be interpreted that there was a difference between control and treatment groups. Thus, carbofuran administration in doses of either 0.0208 mg/kg and 0.0417 mg/kg might potentially produce 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 α = 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 increase in MDA levels was very high at 58.95% and 247.47% compared to that of 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 indicated decreasing ChE levels and the results of the ANOVA test indicated a sign value of 0.001 between the control and treatment group, which was less than the significance value α = 0.05. It can be interpreted that there was a difference between control and treatment groups. Carbofuran exposure in doses of 0.0208 mg/kg and 0.0417 mg/kg potentially lowers 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 α = 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).

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±SD)	0.0208 mg/kg BW Carbofuran (mean±SD)	0.0417 mg/kg BW Carbofuran (mean±SD)
Malondialdehyde (nmol/mg)	25.53 ± 3.02	40.58[a] ± 5.77	88.7[a] ±3.02
ChE levels (U/L)	801.75±129.73	671.50[q]±50.53	606.75[q]±28.45
Purkinje necrotic cell	1.98±1.92	6.24[y]±0.73	7.68[y]±1.01
Purkinje apoptotic cell	11.00 ±1.92	13.00 ±0.73	14.13 ±1.01

Statistical difference from the control:

[a, q, y] Significant at $p \leq 0.05$.

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 days 1 to 4 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 with the control group, and the increase in Purkinje necrotic cell reached 23.07% between 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 (Figure 1-3 and Table 1).

Apoptotic Cell

This study showed that there was increasing number of Purkinje apoptotic cell in the brains of six-day-old mice pups from mother mice exposed to insecticide carbofuran from days 1 to 4 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 pups whose mothers received 0.0208 mg/kg and 0.0417 of carbofuran, compared to control groups (Table 1). The estimated increase in Purkinje apoptotic cell in carbofuran groups was 18.18% and 28.45%, the increase in Purkinje apoptotic cell reached 7.99% between 0.0208 mg/kg and 0.0417 mg/kg and (Table 1).

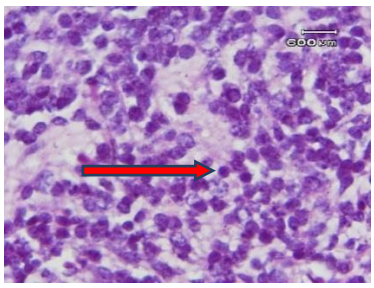

Figure 1. Histopathology of the cerebellum of mice pups (*Mus musculus*) from the control group. H.E Staining. 1000x

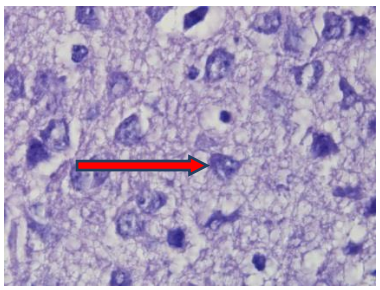

Figure 2. Histopathology of the cerebellum of mice pups (*Mus musculus*), red arrow indicates Purkinje necrotic cells. H.E Staining. 1000x

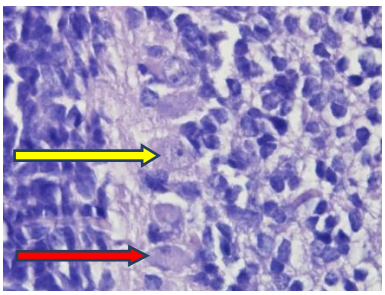

Figure 3. Histopathology of the cerebellum of mice pups (*Mus musculus*), yellow arrow indicates normal Purkinje cells, red arrow indicates Purkinje necrotic cells. H.E Staining. 1000x

Discussion

The metabolism of carbofuran has been well studied in rats, mice, and lactating cows. Carbofuran is rapidly absorbed, metabolized, and eliminated, primarily via the urine, in the species investigated[15]. 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-ketocarbofuran, and 3-hydroxy-N-hydroxy methyl derivatives of carbofuran, which were found in both the 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[16].

3-hydrocarbofuran and nitrosocarbofuran could induce micronucleus formation, 3-ketocarbofuran could not but caused significant DNA migration in SCGE test. 3-ketocarbofuran caused an obvious increase in damaged cells accompanied with a great decrease in undamaged cells, and it displayed a higher degree of cell damage than other three compounds, especially seriously damaged cells increased in number, which 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 hydrolyze of its transformation, free radicals are a potential outcome, which is widely known to reduce DNA damage. The presence of metabolic active system of organism, it could be degraded to less toxic phenols directly while 3-hydrocarbofuran was metabolized to 3-ketocarbofuran with a high direct toxicity to cells, so 3-hydrocarbofuran revealed a stronger *in vivo* toxicity[17].

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

Many pathophysiological conditions may have oxidative stress. In normal conditions, it is balanced by the antioxidant system and this balance is disturbed due to increased oxidative stress. MDA is one of the fairly reactive metabolic products resulting 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[19] [20].

The final result of the lipid peroxidation is MDA, and a high MDA level indicates damaged oxidation processes or cell membrane due to free radicals[21][22]. The results of the present study have shown that carbofuran exposure at subacute doses (0.0208 mg/kg dan 0.0407 mg/kg) for a lactation period caused a significant enhancement in malondialdehyde (MDA) levels in 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 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). Increased doses could increase significant MDA levels if exposed post-natally on days 1-20[23]. In another study, the increase in MDA levels due to acute exposure of carbofuran may reach up to 175.04% (in the dose of 0.2 mg/kg as compared to 0.4 mg/kg in mice)[24]. In the other study, oral sub-acute exposure of carbofuran for 28 days in male mice increases MDA levels by 65%[25].

This indicates that MDA levels increasing 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 the 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[26]. An adult mouse brain is protected by the blood-brain barrier (BBB) to chemicals, while such protection does not exist in mice fetoes and those aged 6 months[27].

Neuronal membranes rich in polyunsaturated fatty acids are the source of lipid peroxidation reaction[28][29]. Lipid peroxidation causes destruction and damage to cell membranes and also changes fluidity/membrane permeability[29][30]. 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 the carbamate also causes carbamate to be able to interact with lipid serums and tissues[31]. The forming process of lipid peroxidation starts from hydrogen ions on the side chain of polyunsaturated fatty acids (PUFA), that construct the cell membranes, by free radicals, forming carbon radicals. Carbon radicals are oxidized to form peroxyl radicals. Furthermore, peroxyl radicals draw H+ 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+ ions into the side chain of adjacent PUFA until the PUFA chain is finally split into other compounds, such as MDA, 9-hydroxy-nonenal, ethane and pentane[21][22].

ChE level measurements are often conducted to determine the effect of exposure to insecticides. ChE used in this measurement was collected from tissues, plasma and red blood cells[32] and ChE collected from the brain was the best sample to be used as an indicator of the impact of exposure to insecticides[33]. In this study, the insecticide carbofuran decreased ChE levels between the control and treatment groups. However, there was no significant difference in ChE levels among lactating pups whose mothers received carbofuran of 0.0208 mg/kg and 0.0417 mg/kg (Table 1). Increased doses could decrease significant ChE levels if exposed post-natally on days 1-20[23]. Almost all insecticide exposure results in the decrease of ChE levels, either during embryonic period, during growth period, or in adulthood. Generally, the response of the ChE level decrease is in accordance with the exposed dose. However, decreased ChE levels due to insecticide exposure in several phases of an individual's growth may induce varied responses.

There were correlations between the accumulation of acetylcholine and the extent of MDA. 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[34]. The peroxidation not only alters lipid milieu and 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 disturbance in uptake as well as release of certain neurotransmitters[35].

The role of ChE is activated before synaptogenesis during the formation of the neural tube. The formation of ChE is in line with the axon growth[36]. The cholinergic system in early development acts as a regulatory growth and has morphogenetic functions[37] by means of controlling cell proliferation, motility, cell differentiation and genetic expression[38]. Thus, the cholinergic system has a very important role in the development of cells and brain formation [39]. 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[36].

In this study, the insecticide carbofuran increased necrotic death of Purkinje cells 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 0.0417 mg/kg. In this study, we found an association between increased MDA levels and necrotic cells. Increased lipid peroxidation and lipid peroxidation products, such as MDA levels, contribute to neuronal loss in conditions associated with oxidative stress[40]. Increased MDA levels indicates membrane damage and leads to cellular necrosis. The attack of free radicals on a cell membrane makes it devoid of integrity and viability, causing the cells to undergo

necrosis[41]. Cell death caused by the swelling of cytoplasm, nucleus karyolysis and lysis are classified as necrosis[42][43].

The number of Purkinje necrotic cells was not as many as the number of cells which experienced apoptosis due to 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 increase of 28.45% and necrosis increase 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 0.0417 mg/kg. Such different results from those of a study by Luqman [10] could be because the brain development phase during the embryonal period and lactation period has 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[44]. Carbofuran insecticide exposure during embryonic period may increase the activity of embryonic cerebral ROS, increased p53 and of caspase 3 expression, and apoptosis. Increased expression p53 and caspase 3 and apoptosis indicated that carbofuran insecticide may cause apoptosis through the intrinsic pathway[10].

In conclusion, the present study revealed that carbofuran has been distributed in pups' tissues through the milk of lactating mothers and has caused oxidative damage of pups' brains. Carbofuran exposure indicates that mice pups' brains are particularly more vulnerable to oxidative stress, which may eventually lead to neurobehavioral disorders. In this study we also found that insecticide carbofuran dose in lactating mice of 0.0208 mg/kg BW has been able to increase ROS activity and Purkinje cell death. Such a dose, if being converted into human dose according to dose conversion by Laurence and Bacharach (1964), will be equal to 0.115 mg/kg BW. This result of dose conversion can be applied as a carbofuran potential standard in increasing ROS activity and Purkinje cell death since residual level in cows' meat and milk was found to be as much as 0.17 mg/kg BW and 0.349 mg/kg BW[45].

The exposure of lactating mice to the insecticide carbofuran has made the mean Purkinje apoptotic cell count higher than the necrotic ones in all treatment doses. A high increase of apoptosis in Purkinje cells allows an opportunity to prevent and manage the strategy to overcome Purkinje cell death due to carbofuran insecticide exposure during the lactation period, such as in providing antioxidant variations. Efforts to prevent the formation of ROS can be done to inhibit and countermeasure 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 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[46][47].

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Research Article

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*

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

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Abstract

Exposure to insecticide carbofuran has been reported to generate reactive oxygen species (ROS) in mice brains. The present study was undertaken to evaluate the oxidative damage, and biochemical and histopathological alterations by respectively examining malondialdehyde (MDA), choline esterase (ChE) levels, and necrosis and apoptosis in suckling mice whose mothers were exposed to the carbofuran. The carbofuran was exposed via an oral route in doses of 0.0208 and 0.0417 mg/kg BW from postnatal day 1 until day 4 after delivery (n=27). The six--days-old pups were examined for brain MDA and ChE levels as well as necrotic and apoptotic Purkinje cell count using Tunel assay and hematoxylin-eosin (HE) staining. Exposure of the mothers 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 control and treatment, and no change between treatments. In conclusion, the transfer of carbofuran intoxication through the mother's milk resulted in oxidative stress, and biochemical and histopathological alterations in the suckling pups.

Comment [A1]: plural

Comment [A2]: conjunction

Comment [A3]: determiners

Comment [A4]: plural

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Laktasyon Döneminde Carbofuran Maruziyetinin Neden Olduğu Bebek Farelerinde (Mus musculus) Beyin Hücreleri Ölümü

Öz

Reaktif oksijen türleri (ROS) fareler beyin üretmek için böcek ilacı carbofuran maruz bildirilmiştir. Bu araştırma oksidatif hasar (MDA), Biyokimya (ChE) ve histopatoloji değişiklikleri (apoptoz ve nekroz) büyük fareler anne maruz karbofuran emzirmek değerlendirmek için yapılmıştır. Carbofuran oral aracılığıyla varlık üst. Maruz kalma doz 0.0208 ve 0.0417 mg/kg vücut ağırlığı doğumdan sonra günde 1 gün 4 için (n = 27). Büyük fareler yaş altı gün için test MDA ve kolin Esteraz (ChE) düzeyde beyin ve nekrozu ve hücre ölümü apoptozis miktarı hesaplamak ölçü boyama tahlil ve o tünel kullanarak Purkinje hücreleri. MDA, Purkinje hücre nekroz ve ChE, bir düşüş, yüksek düzeyde karbofuran pozlama üst üzerinde neden olur ama önemli apoptosis fare çocuk emzirmek anlamına gelmez. Karbofuran değiştirme parametreleri: MDA, Purkinje hücre nekroz ve ChE. MDA üzerinde tutarlı değişiklikler bulundu ChE ve fare hücrelerinde nekroz Purkinje, denetim ve tedavi tedaviler arasında değişiklik arasında özellikle değişiklikleri hedef. Sonuçları gösterdi ki toksik transferini emzirmek genç farelerde oksidatif stres, Biyokimya ve histopatoloji değişiklikleri üst süt ile carbofuran sonuçlandı.

Anahtar sözcükler **:** Carbofuran, Purkinje hücreleri, fare beyin çocuk, emzirme.

Introduction

Insecticide carbofuran residues in food may be harmful to organisms which are actually ~~are~~ not the target of the insecticide itself [1]. In a flower plantation contaminated by carbofuran in Ecuador, there were several cases of baby born with abnormalities, such as declining reflexes and motoric skill in 2001. At the child stage~~ren's age~~, there were some brain function developmental abnormalities, such as the degeneration of memorizing and concentrating abilities.[2]. In tested animals, carbofuran contamination causes oxidative stress and weakens motoric, memory, and cognitive functions[3]. Like organophosphate, carbofuran inductions result in significant oxidative damage in the cerebral cortex, cerebellum, and brainstem[4]. Carbofuran inductions in the cerebral cortex strongly correlates to the decline of motoric functions[3].

Oral administrations of carbofuran ~~have~~ has been proven to strongly stimulate reactive oxygen species (ROS) in mice brains and increase the levels of malondialdehyde (MDA) [3]. Intraperitoneal sub-acute administrations of carbofuran increase brain oxidative stress as the dose increases; and thus, improve MDA levels significantly (12.50, 34.38 and 59.38%). 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 break the DNA chains or change the composition of nucleotides in DNA, resulting in mutations and apoptosis[6]. Hydroxyl radicals resultinged from oxidative stress could also damage all membrane systems in the cell, such as ~~a~~ creating leaks in the lysozyme membrane which may result in cell death (necrosis).

Uncontrolled increase in ROS results in the injury and death of neuron cells[7]. Cerebrum consists of 80% ~~of~~ neuron cells and of 20% ~~of~~ glial cells which are responsible ~~to~~ 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 ~~the~~ 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.

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A deeper understanding of the mechanisms of brain cell death in mice pups due to carbofuran exposure during the lactation period is necessary to acquire the basic treatment and prevention of carbofuran exposure during the lactation period. Besides, it is important to understand the brain 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, ~~prevention~~ actions can be ~~performed~~ taken to prevent the degeneration of reflexes and motoric skills in infant mice.

The aim of this research was to determine the mechanism of brain cell~~ls~~ death in mice pups whose mother mice were exposed to carbofuran during the lactation period by measuring MDA levels as ~~the~~ an indicator~~ion~~ of ROS production, ChE levels as the ~~indication~~ indicator of a neural function response to carbofuran exposure, and apoptotic and necrotic cerebellum Purkinje cells. This study contributes to disclosinge the prevention mechanism of brain cell death in mice pups whose mother mice were exposed to carbofuran during the lactation period. In addition, this study also provides scientific information of insecticide carbofuran exposure, especially during the lactation period, related to the attempts to inhibiting brain development disorders.

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Material and Method

Ethics approval

The study was approved by the Faculty of Veterinary Medicine Animal Ethics Committee. All variables had been considered as in accordance with the~~to~~ Ethics Committee related to animal handling to ensure no discomfort or pain was 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, weighinged 25-30 grams, and 12-week-~~s~~ old male mice. Environmental adaptation was done to female mice (Mus musculus) for 7 days. On ~~the~~ day 8, PMSG with a dosage of 5 IU/mouse was injected into the female mice, and HCG injections with a dosage of 5 IU/mouse was performed on day 10. Afterwards, the female mice were mated with 12-week-~~s~~ old male mice. On day 11 a gestation examination was carried out. The gestation of the 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.

Carbofuran Exposure

Carbofuran exposure was targeted at the suckling mice 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 and 0.0417 mg[10] on days 1-4 of the lactation period given per orally using a sterile disposable syringe. The six--days-old mice pups were then tested and measured for its MDA and Choline Esterase (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 Apopteg Apoptosis Detection Kit.

Measuring Cholinesterase (ChE) Levels

Cholinesterase (ChE) was determined according to the manufacturer's instructions of Cholinesterase FS[11]. To measure the ChE levels on the brains of six6 -day-s-old mice, the following materials were used: 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_2O hydrolysis, 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: Diluent 100 ml, 3 ml reagent mix (for 30 samples) with concentrations: Phosphate buffer pH 7.7 50 mmol/l, S-Butyrylthio choline iodide 6 mmol/l, 5,5-dithiobis-2-nitrobenzoic 0.25 mmol / l. The standard wavelength of the reaction solution was 405 nm and the length of translucent light was 1 cm measured at 25, 35 and 37° C. The10 mL of sampled fluid was collected and mixed with 100 mL of the reaction solution. The mixture was examined every one minute for three minutes. To determine the sampled concentration, the enzyme activity of sample [kU/I] = (ΔA/min) was measured.

Measuring Malondialdehyde (MDA) Level

Malondialdehyde (MDA) was determined by the method of Conti et al[12]. The measurement of malondialdehyde level on six-6-days--old mice pups' brains was performed using MDA / TBARS (Malondialdehyde / Thiobarbituric Acid Reactive Substance). This method was performed by weighing 1 gram of the infant mice brain sample, putting it into a reaction tube and adding mixing it with 9 ml cold PBS and then crushing it with a spatula. The liquid was then centrifuged at 3000 rpm for 15 minutes. Four 4 ml of supernatant was collected and added with to 1 ml of a TCA 15% solution. Then, 1 ml of 0.37% TBA solution was added into HCl 0.25 N and heated with in a water bath at a temperature of 80ºC for 15 minutes. After cooling the solution at a room temperature for 60 minutes, the solution was centrifuged at 3000 rpm for 15 minutes. Finally, the value of the absorbance was read against the red lines formed using a spectrophotometer at λ = 532 nm.

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Necrosis Examination using HE Staining

Necrosis examination was performed using HE staining. The six-~~6~~ day-old mice brains ~~was~~ were fixated with 10% formalin buffer and brain tissues were processed in ~~a~~ routine processing until they formed paraffin blocks. The paraffin blocks were cut using a microtome with a thickness of 5 µm in a series and then glued to the object glass. The observed area was the cerebellum obtained through the coronal section at the position 11/1.56 mm from the edge of the posterior lobe[13]. After 24 hours' fixation, the mice brain was washed in a 70% alcohol solution three times and stained using Haematoxylin Eosin (HE). Purkinje cells undergoing necrosis were signified by cells undergoing pyknosis and karyorrhexis.

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Apoptosis Examination using Tunel assay

Examination of apoptotic cells was performed by Tunel assay. The paraffin blocks were cut using a microtome in a thickness of 5 µm 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 ANOVA with post hoc analysis (Duncan test)[14]. The values were expressed as mean ± 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 indicated that there was an increase in MDA levels, as the results of the Duncan test indicated a sign value of 0.115 between the control and treatment group, which was less than the significance value α = 0.05. It can be interpreted that there was a difference between control and treatment groups. Thus, carbofuran administration in doses of either 0.0208 mg and 0.0417 mg might potentially produce free radicals. There was no significant difference in pups whose mothers were exposed to carbofuran of 0.0208 mg and 0.0417 mg. Although there was no significant difference, the increase in MDA levels was very high at 58.95% and 247.47% compared to that ~~i~~of the~~n~~ control group, and the increase in MDA levels reached 118% between 0.0208 mg and 0.0417 mg (Table 1).

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Cholinesterase (ChE) Level

This study indicated decreasing ChE levels and the results of the Duncan test analysis revealed a sign value of 0.115 between control and treatment groups which was less than the significance value α=0.05. It means that there was a difference between control and treatment groups. Carbofuran exposure in doses of 0.0208 mg and 0.0417 mg potentially lowers the ChE levels. There was no significant difference in pups whose mothers were exposed to carbofuran of 0.0208 mg and 0.0417 mg. Although there was no significant difference, the decrease in ChE levels reached ~~at~~ 16.25% and 24.32% compared to ~~that~~ the~~in~~ control group, and the decrease in ChE levels reached 9.64% between 0.0208 mg and 0.0417 mg (Table 1).

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Table 1: Effect of carbofuran exposure in subacute doses for 4 days on the MDA, ChE levels, Purkinje cell necrosis and apoptosis in mice pups' brains.

Parameters	Control Group (mean±SD)	0.0208 mg/kg BW Carbofuran (mean±SD)	0.0417 mg/kg BW Carbofuran (mean±SD)
Malondialdehyde (nm/ml)	25.53^{a} ± 3.02	40.58^{b} ± 5.77	88.71^{b} ±3.02
ChE levels (U/L).	801.75^{b}±129.73	671.50^{a}±50.53	606.75^{a}±28.45
Purkinje cell necrosis	1.98^{b}±1.92	6.24^{a}±0.73	7.68^{a}±1.01
Purkinje cell apoptosis	11.00^{a} ±1.92	13.00^{a} ±0.73	14.13^{a} ±1.01

Necrosis 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 cells necrosis in the brains of six~~6 -~~day-old mice pups whose mothers were exposed to carbofuran on days 1 to 4 of the lactation period. Carbofuran exposure in doses of 0.0208 mg and 0.0417 mg had enhanced Purkinje cell necrosis. There was no significant difference in pups whose mothers were exposed to 0.0208 mg and 0.0417 mg of carbofuran. Nevertheless, the increase in Purkinje cell necrosis was very high at 215.15% for a dosage of 0,0208 mg and 287,87% for a dosage of 0,0417 mg compared with the control group, and the increase in Purkinje cell necrosis reached 18.75% between 0.0208 mg and 0.0417 mg. An o~~O~~verview of staining results using HE showed necrosis of the Purkinje cells between the control and treatment groups (Figure 1-3 and Table 1).

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Apoptosis Cell

This study showed that there was increasing number of Purkinje cells apoptosis in the brains of ~~the~~ six~~6 -~~day~~-s~~-old mice pups from mother mice exposed to insecticide carbofuran from days 1 to 4 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 apoptosis cells in

lactating pups whose mothers received 0.0208 mg and 0.0417 of carbofuran, compared to control groups (Table 1). The eEstimated increase in Purkinje cell apoptosis in carbofuran groups was 18.18% and 28.45%, the increase in Purkinje cell apoptosis reached 7.99% between 0.0208 mg and 0.0417 mg and (Table 1).

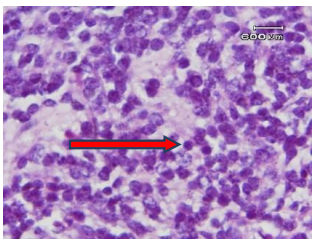

Figure 1. Histopathology of the cerebellum of mice pups (*Mus musculus*) from the control group. H.E Staining. 1000x

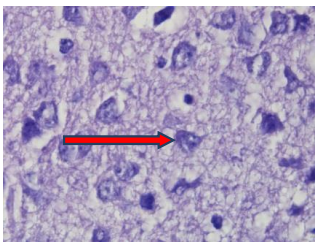

Figure 2. Histopathology of the cerebellum of mice pups (*Mus musculus*), red arrow indicates necrotic Purkinje cells. H.E Staining. 1000x

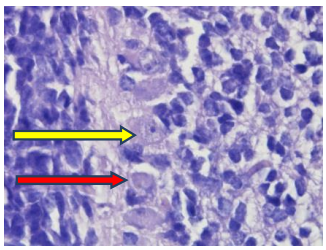

Figure 3. Histopathology of the cerebellum of mice pups (*Mus musculus*), blue arrow indicates normal Purkinje cells, red arrow indicates necrotic Purkinje cells. H.E Staining. 1000x

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Discussion

The final result of the lipid peroxidation is MDA, and a high MDA level indicates damaged oxidation processes or cell membrane due to free radicals[15][16]. The results of the present study have shown that carbofuran exposure at subacute doses (0.0208 mg dan 0.0407 mg) for a lactation period (4 days) caused a significant enhancement in malondialdehyde (MDA) levels in 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 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 and 0.0417 mg. Although there was no significant difference, the increase in MDA levels reached 118% between 0.0208 mg and 0.0417 mg (Table 1). Increased doses could increase significant MDA levels if exposed ~~to~~ post--natally on days 1-20[17]. In another study, the increase in MDA levels due to acute exposure of carbofuran may reach up to 175.04% (in the dose of 0.2 mg as compared to 0.4 mg in mice)[18]. In the other study, oral sub-acute exposure of carbofuran for 28 days in male mice increases MDA levels by 65%[19].

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This indicates that MDA levels increasinge 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 the 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[20]. An adult mouse brain is protected by the blood-brain barrier (BBB) to chemicals, while such protection does not exist in mice fetuiis and those aged 6 months[21].

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Neuronal membranes rich in polyunsaturated fatty acids are is the source of lipid peroxidation reaction[22][23]. Lipid peroxidation causes destruction and damage to cell membranes and also changes fluidity/membrane permeability[23][24]. 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 the carbamate also causes carbamate to be able to interact with lipid serums and tissues[25]. The forming process of lipid peroxidation starts from hydrogen ions on the side chain of polyunsaturated fatty acids (PUFA), that construct the cell membranes, by free radicals, forming carbon radicals. Carbon radicals are oxidized to form peroxyl radicals. Furthermore, peroxyl radicals draw H+ 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+ ions into the side chain of adjacent PUFA until the PUFA chain is finally split into other compounds, such as malondialdehyde (MDA), 9-hydroxy-nonenal, ethane and pentane[15][16].

ChE level measurements are often conducted to determine the effect of exposure to insecticides. ChE used in this measurement was collected from tissues, plasma and red blood cells[26] and ChE collected from the brain was the best sample to be used as an indicator of the impact of exposure to insecticides[27]. In this study, the insecticide carbofuran decreased ChE levels between the control and treatment groups. However, there was no significant difference in ChE levels among lactating pups whose mothers received carbofuran of 0.0208 mg and 0.0417 mg (Table 1). Increased doses could decrease significant ChE levels if exposed to post-natally on days 1-20[17].

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Almost all insecticide exposure results in the decrease of ChE levels, either during embryonic period, during growth period, or in adulthood. Generally, the response of the ChE level decrease is in accordance with the exposed dose. However, decreased ChE levels due to insecticide exposure in several phases of an individual's growth may induce varied responses.

The p~~P~~ostnatal lower dose chlorpyrifos exposure on days 1-21 resulted in decreased ChE levels on day 6, while decreased ChE levels that occurred along with the increase of the dose was found on day 14 postnatal. However, in the measurement on days 22, 30 and 40 insignificant decrease of ChE levels was found in medium and high doses[28].

There were correlations between the accumulation of acetylcholine and the extent of MDA. 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[29]. The peroxidation not only alters lipid milieu and 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 disturbance in uptake as well as release of certain neurotransmitters[30].

The role of ChE is activated before synaptogenesis during the formation of the neural tube. The formation of ChE is in line with the axon growth[28]. The c~~C~~holinergic system in ~~the~~ early development acts as a regulatory growth and has morphogenetic functions[31] by means of controlling cell proliferation, motility, cell differentiation and genetic expression[32]. Thus, the cholinergic system has a very important role in the development of cells and brain formation~~ing~~ [33]. Although the brains of infant mice are~~is~~ extremely sensitive to carbofuran exposure during the lactation period and both treatments showed a decrease in ChE levels, all o~~f~~ infant mice were still alive with symptoms of mild poisoning. The decrease in ChE levels indicates the response of brain or adult nervous system[28].

In this study, the insecticide carbofuran increased necrotic death of Purkinje cells 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 and 0.0417 mg. In this study, we found an association between increased MDA levels and necrotic cells. Increased lipid peroxidation and lipid peroxidation products, such as MDA levels, contribute to neuronal loss in conditions associated with oxidative stress[34]. Increased MDA levels indicates membrane damage and leads to cellular necrosis. The attack of free radicals on a cell membrane ~~has made~~makes ~~the membrane~~it ~~to~~ devoid of~~its~~ integrity and viability, causing the cells to undergo necrosis[35]. Cell death caused by the swelling of cytoplasm, nucleus karyolysis and lysis are classified as necrosis[36][37].

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The number of necrotic Purkinje cells was not as many as the number of cells which experienced apoptosis due to carbofuran exposure. ~~It~~ This was because during the

neurogenesis period, the Purkinje cells had experienced more apoptosis physiologically ~~as~~ through homeostasis efforts. However, when the number of necrotic Purkinje cells was compared to the control group, there was an increase in the number of necrotic Purkinje cells in the treatment group which was significantly higher than the number of apoptotic Purkinje cells (apoptosis increase of 28.45% and necrosis increase 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 apoptotic cells among lactating pups whose mothers received carbofuran of 0.0208 mg and 0.0417 mg. Such different results from those of a study by Luqman [10] could be because the brain development phase during the embryonal period and lactation period has 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 ~~time of~~ exposure time is needed for the agent to reach the target of nuclear DNA and mitochondria to induce apoptosis[38].

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In conclusion, the present study revealed that carbofuran has been distributed in pups' tissues through the milk of lactating mothers and has caused oxidative damage of pups' brains. Carbofuran exposure indicates that mice pups' brains ~~is~~ are particularly more vulnerable to oxidative stress, which may eventually lead to neurobehavioral disorders. In this study we also found that insecticide carbofuran dose in lactating mice of 0.0208 mg/kg BW has been able to increase ROS activity and Purkinje cell death. Such a dose, if being converted into human dose according to dose conversion by Laurence and Bacharach (1964), will be equal to 0.115 mg/kg BW. This result of dose conversion can be applied as a carbofuran potential standard in increasing ROS activity and Purkinje cell death since residual level in cow~~'~~s' meat and milk was found to be as much as 0.17 mg/kg BW and 0.349 mg/kg BW [39].

The exposure of lactating mice to the insecticide carbofuran ~~to lactating mice~~ has made the mean apoptotic Purkinje cell~~s~~ count higher than the necrotic ones in all treatment doses. A h~~H~~igh increase of apoptosis in Purkinje cells allows an opportunity to prevent and manage the strategy to overcome Purkinje cell death due to carbofuran insecticide exposure during the lactatio~~n~~g period, such as in~~by~~ providing antioxidant variations. Efforts to prevent the formation of ROS can be done to inhibit and countermeasure 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 oxidative stress through

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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[40][41].

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