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The Effect of Kebar Grass (*Biophytum Petersianum*) Extract to Histopathological Images of Lactating Mice' (*Mus Musculus*) Kidney Exposed to Carbofuran

Alfian Aulia Rakhman, Wiwik Misaco Yuniarti, Widjiati Widjiati, Lilik Maslachah, Rimayanti Rimayanti, Gracia Angelina Hendarti and Epy Muhammad Luqman*

Department of Veterinary Science, Faculty of Veterinary Medicine Universitas Airlangga Kampus C Unair Jalan Mulyorejo, Surabaya-60115 Indonesia

*Corresponding author details: Epy Muhammad Luqman; epy-m-l@fkh.unair.ac.id

ABSTRACT

This study aims to determine the effect of Kebar grass extract administration to reduce kidney damage in lactating mice exposed to carbofuran. Forty-two lactating mice were used in this study divided into seven groups, namely: K as a control group (treated with Aquadest), P1 was injected with carbofuran ¼ LD50 (0.0125mg/day), P2 was injected with carbofuran 1/8 LD50 (0.00625mg/day), P3 was injected with carbofuran 1/4 LD50 (0.0125mg/day) and Kebar grass extract (0.135mg/day), P4 was injected with carbofuran 1/8 LD50 (0.00625mg/day) and Kebar grass extract (0.135mg/day), P5 was injected with carbofuran 1/4 LD50 (0.0125mg/day) and vitamin C (0.2ml/day) and P6 was injected with carbofuran 1/8 LD50 (0.00625mg/day) and vitamin C (0.2ml/day) for 14 days. Then on the 15th day, the mice were sacrificed and the kidneys were collected, then histological preparations were made with HE (Hematoxylin-Eosin) staining. Data on tubular degeneration, necrosis, and inflammation were analyzed using the Kruskall Wallis test and after that the Mann Whitney test using the Statistical Product and Service Solutions (SPSS) application version 23 was conducted. The results showed that carbofuran caused a significant increase in tubular degeneration, necrosis, and inflammation ($p<0.05$). Administration of Kebar grass and vitamin C significantly reduced tubular degeneration, necrosis, and inflammation ($p<0.05$). It can be concluded that administration of Kebar grass extract was more effective to reduce kidney damage in lactating mice exposed to carbofuran compared to vitamin C ($p<0.05$).

Keywords: Kebar Grass extract; carbofuran; lactation; kidney; pesticide stress.

INTRODUCTION

Two million tonnes of pesticides are used yearly and the most common type of pesticides used in the world is insecticides[1]. Until now, there are 3,207 pesticide formulations used and registered for agriculture and plantations, including organophosphate and carbamate groups that are the most frequently used. Carbofuran is a type of carbamate insecticide which often causes poisoning in non-target organisms such as plants, animals and humans[2]. Carbofuran (2,3-dihydro-2,2dimethyl-7-7benzofuranyl methylcarbamate) is an insecticide from the carbamate group which has a broad spectrum. Carbofuran is usually used to increase plant productivity[3].

Carbamate insecticide poisoning has clinical symptoms, that is body weakness, headache, sweating, hypersalivation, vomiting and diarrhea[2]. In a study conducted by Purnomo et al (2019))[4], it showed that the damage to the kidneys of mice offsprings, such as tubular degeneration, tubular necrosis, and inflammatory cell infiltration, increased in line with the increased dose of carbofuran exposed to the lactating mice. Damage to the kidney tubules of the mice offprings was thought to be related to the reaction of carbofuran compounds in the mother's milk[5]. The chemical compounds in the mother's body entered the capillaries and then they entered the milk and were sucked by the offsprings[6].

Changes in the structure of the kidney are due to the process of reabsorption and excretion of these chemical compounds in the kidneys[7]. Cell damage due to exposure to carbofuran is caused by the formation of free radicals in the form of Reactive Oxygen Species (ROS) [3]. ROS in particular (OH-) can cause damage to DNA, lipids, and proteins. Excess ROS in tissues can cause oxidative stress because the presence of ROS is not balanced with antioxidant compounds[8]. In a study conducted by Luqman et al (2019) 9, it indicated that an increase in ROS was marked by an increase in malondialdehyde (MDA) in the brains of adult rats. Increased ROS can also reduce the activity of superoxide dismutase (SOD) and catalase (CAT) in the brain. Decreased catalase activity can reduce protection against free radicals in response to carbofuran[9]. Damage caused by excessive free radicals in the body requires additional antioxidants to neutralize free radicals that are formed. Antioxidants are chemical compounds that are able to inhibit damage caused by the oxidation process. Flavonoids are a group of phenolic compounds which are good antioxidants and can be found in fruits and vegetables. Phenolic compounds as antioxidants have mechanisms as reducing agents, free radical scavengers, metal binders, and preventing the formation of singlet oxygen[10].

One of the plants containing flavonoids is Kebar grass (*Biophytum petersianum*). Kebar grass is an herbal plant that wildly grows in Papua[11]. Other compounds contained in Kebar grass are vitamin A and vitamin E which function to destroy ROS compounds. Vitamin E is a fat-soluble antioxidant that can reduce lipid free radicals faster than oxygen[10]. The content of Kebar grass is expected to have an effect on preventing damage due to exposure to carbofuran.

METHODS

This research was conducted in an experimental animal laboratory and kidney histopathological preparations were made at the Department of Veterinary Pathology, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya Indonesia.

ETHICAL APPROVAL

All experimental procedures were performed according to the guidelines for the care and use of animals as established by the Animal Welfare and Experimentation Ethics Committee, Faculty of Veterinary Medicine, Universitas Airlangga, with register number: 1.KE.107.06.2019.

MATERIALS

The study used a completely randomized design (CRD) with seven groups and six repetitions. The experimental animals used were 42 lactating mice (*Mus musculus*). Materials used were: lactating mice (*Mus musculus*), kebar grass (*Biophytum Petersianum Klotzch*), CMC Na, Ethannol 70%, Carbofuran (2,3-Dihydro-2,2-dimethyl-7-benzofuranol N-methylcarbamate 98%) from Aldrich Chemistry with Bellstain Registry Number 1428746, Product of USA, Pellet Feed for mice, Aquadest as carbofuran solvent, Vitamin C, drinking water, husk as cage lining, ether, 10% formalin.

The tools used were plastic cages and wire mesh for experimental animal cages, sonde needles, drinking bowls, test tubes, and 3ml syringes. The equipments used for terminating mice were anesthetic jar, surgical scissors, scalpel, tweezers, and small potting bottles. The equipments for making kidney histology preparations were object glass, cover glass, automatic tissue processor, water bath, hot plate, microtome, and blade. Histological examination of the kidneys was conducted using a camera and an Olympus® CX-41microscope.

MATERIAL PREPARATION

This study used dry Kebar grass. The preparation of Kebar grass extract was carried out in the Pharmacognosy and Phytochemical Laboratory of the Faculty of Pharmacy, Universitas Airlangga, Surabaya Indonesia.

The dried kebar grass was then boiled in distilled water. 350 grams of Kebar grass simplicia which had been mashed, was macerated in a tube for approximately 3x24 hours with 70% ethanol solvent with a ratio of 1:10, next it was filtered and the dregs were macerated again twice with the same treatment. The existing macerate was evaporated with a rotary evaporator at a temperature of 30-40^{0}C to form thick extract. The extract was put in a bottle and was stored in the refrigerator.

DOSE DETERMINATION

Determination of the Kebar grass dose refers to study conducted by Labib (2018) [12]. Kebar grass dose which provides an effective antioxidant effect to prevent oxidative stress is 0.0135mg/g BW/day. The mice used had an average body weight of 25 grams, so the dose used was 0.0135 X 25 g = 3.375 mg/25g/day. The dose of carbofuran administered to mice used the LD_{50} fractions: ¼ LD_{50} (0.0125 mg/25g mice/day) and 1/8 LD_{50} (0.00625mg/25g mice/day) which did not cause death in the mother mice for 14 days [13].

TREATMENT

The lactating mice were treated on the first day of offspring's birth until the 14th day with kebar grass, vitamin C, and carbofuran orally using 1 ml of tuberculin. The treatments were described as follows: group K was treated with Aquadest (0.5 ml/mouse/day); group P1 was treated with carbofuran ¼ LD_{50} (0.0125mg/day); group P2 was treated with carbofuran 1/8 LD_{50} (0.00625mg/day); group P3 was treated with kebar grass extract 3.375 mg in 0.2 ml + given carbofuran ¼ LD_{50} (0.0125 mg/day); group P4 was treated with kebar grass extract 3.375 mg in 0.2 ml + carbofuran 1/8 LD_{50} (0.00625 mg/day); group P5 was treated with vitamin C 5mg in 0.2ml + carbofuran ¼ LD_{50} (0.0125mg/day); and group P6 was treated with 5 mg of vitamin C in 0.2 ml + carbofuran 1/8 LD_{50} (0.00625 mg/day). After being treated for 14 days, on day 15 the mice were sacrificed to collect the kidneys and the kidneys were placed into small pots containing 10% formalin buffer solution, then histopathological preparations were made using HE staining.

OBSERVATION ON THE RESULTS OF HISTOPATHOLOGICAL PREPARATIONS

Observation of kidney preparations using a microscope with a magnification of 100x and 400x was carried out at five different fields of view for each preparation. Furthermore, the evaluation was carried out by scoring the histological changes in the kidney including degeneration, necrosis of tubular cell, and inflammatory cell infiltration (Table 1).

TABLE 1: Scoring on Kidney Histopathology[14]

Score	Degeneration of kidney tubular cell
0	The degeneration of tubular cell does not occur
1	The degeneration of tubular cell occurs <25% from all view fields
3	The degeneration of tubular cell occurs 26-50% from all view fields
5	The degeneration of tubular cell occurs >50% from all view fields
Score	**Necrosis of kidney tubular cell**
0	The necrosis does not occur
1	The necrosis occurs <25% from all view fields
3	The necrosis occurs 26-50% from all view fields
5	The necrosis occurs >50% from all view fields
Score	**Inflammatory cell infiltration of tubule interstitial**
0	The inflammatory cell infiltration does not occur
1	The inflammatory cell infiltration occurs <25% from all view fields
3	The inflammatory cell infiltration occurs 26-50% from all view fields
5	The inflammatory cell infiltration occurs >50% from all view fields

DATA ANALYSIS

The scoring results of the changes made by kidney preparations were then analyzed using the Kruskall Wallis test and then, the Mann Whitney test using the Statistical Product and Service Solutions (SPSS) version 2 application was conducted.

RESULTS AND DISCUSSION

Table 1: Mean standard deviation values of degeneration, necrosis, and inflammatory cell infiltration in lactating mice exposed to carbofuran.

Treatment	Mean ± SD		
	Degeneration	Necrosis	inflammatory cell infiltration
K	0.00^{a} ±0.00	0.00^{a} ±0.00	0.00^{a} ±0.00
P1	4.6^{g} ± 0.00	4.2^{g} ± 0.00	4.6^{g} ± 0.40
P2	3.93^{f}± 0.23	3.93^{f}± 0.23	3.93^{f}± 0.46
P3	1.53^{c} ± 0.23	1.06^{c} ± 0.30	1.33^{c} ± 0.11
P4	0.86^{b} ±0.30	0.73^{b} ±0.11	0.80^{b} ±0.20
P5	2.73^{e}± 0.23	3.4^{e}± 0.69	3.13^{e}± 0.23
P6	1.93^{d}± 0.23	1.93^{d}± 0.23	2.06^{d}± 0.23

Notes: Different superscripts in the same coloumn show significant difference ($p<0.05$). K (aquadest), P1 (carbofuran 1/4 LD_{50} 0.0125 mg/day), P2 (carbofuran 1/8 LD_{50} 0.00625 mg/day), P3 (Kebar grass extract 3.375 mg (0.2 ml) + carbofuran 1/4 LD_{50}), P4 (Kebar grass extract 3.375 mg (0.2 ml) + carbofuran 1/8 LD_{50}), P5 (vitamin C 5 mg (0.2 ml) + carbofuran 1/4 LD_{50}), and P6 (vitamin C 5 mg (0.2 ml) + carbofuran 1/8 LD_{50}). x400.

DEGENERATION

Microscopic observation of degeneration in the kidney preparations of lactating mice was conducted by HE staining. Degeneration is characterized by the presence of swollen and turbid cytoplasm cell due to the presence of vacuoles. The results of statistical analysis based on table 1 showed that there were significant differences ($p<0.05$) between K and P1, P2, P3, P4, P5, P6.

There was a significant difference ($p<0.05$) between P1 and the P2, P3, P4, P5, and P6. There was a significant difference ($p<0.05$) between P2 and P3, P4, P5, and P6. There was a significant difference ($p<0.05$) between P3 and P4, P5, and P6. There was a significant difference ($p<0.05$) between P4 and P5 and P6. There was a significant difference ($p<0.05$) between P5 and P6.

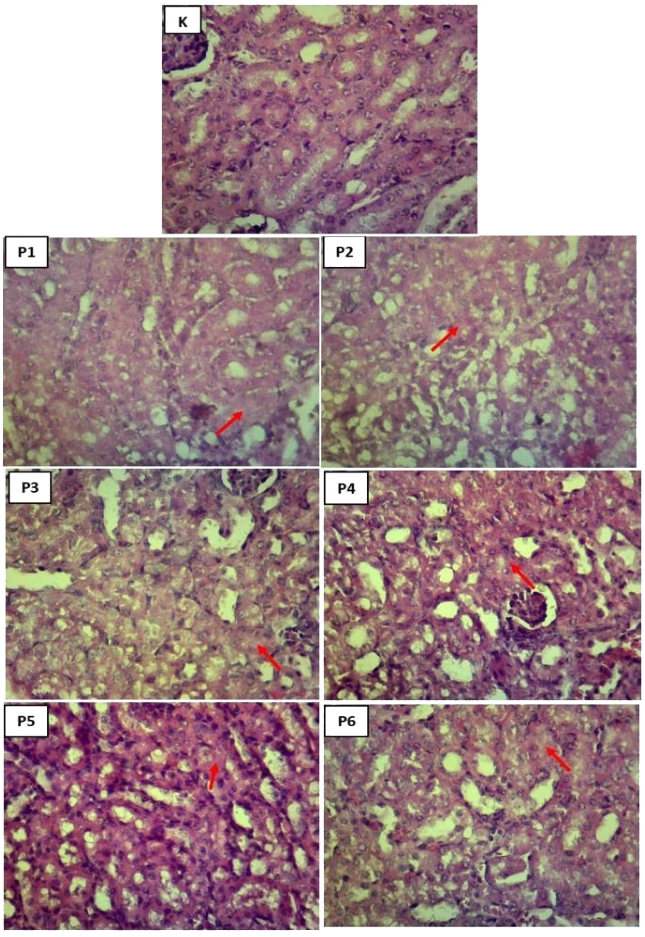

FIGURE 1: Comparison of the microscopic images of kidney degeneration in mice offspring with H&E staining, magnification 400x. Arrows indicate degeneration (→). K (aquadest), P1 (carbofuran 1/4 LD_{50} 0.0125 mg/day), P2 (carbofuran 1/8 LD_{50} 0.00625 mg/day), P3 (Kebar grass extract 3.375 mg (0.2 ml) + carbofuran 1/4 LD_{50}), P4 (Kebar grass extract 3.375 mg (0.2 ml) + carbofuran 1/8 LD_{50}), P5 (vitamin C 5 mg (0.2 ml) + carbofuran 1/4 LD_{50}), and P6 (vitamin C 5 mg (0.2 ml) + carbofuran 1/8 LD_{50}).

Necrosis

Evaluation on necrosis was carried out by microscopic observation of kidney preparations of mother mice (*Mus musculus*) which previously had been stained with HE staining. Observations were made using a microscope with a magnification of 400 times. The presence of necrosis is characterized by the presence of picnotic nuclei, karyorexis, and karyolysis. The results of the statistical analysis based on Table 1 showed that there was a significant difference ($p<0.05$) between group K and group P1, P2, P3, P4, P5, P6.

There was a significant difference ($p<0.05$) between group P1 and group P2, P3, P4, P5, and P6 treatment. There was a significant difference ($p<0.05$) between group P2 and group P3, P4, P5, and P6. There was a significant difference ($p<0.05$) between P3 and P4, P5, and P6. There was a significant difference ($p<0.05$) between P4 and P5 and P6. There was a significant difference ($p<0.05$) between P5 and P6.

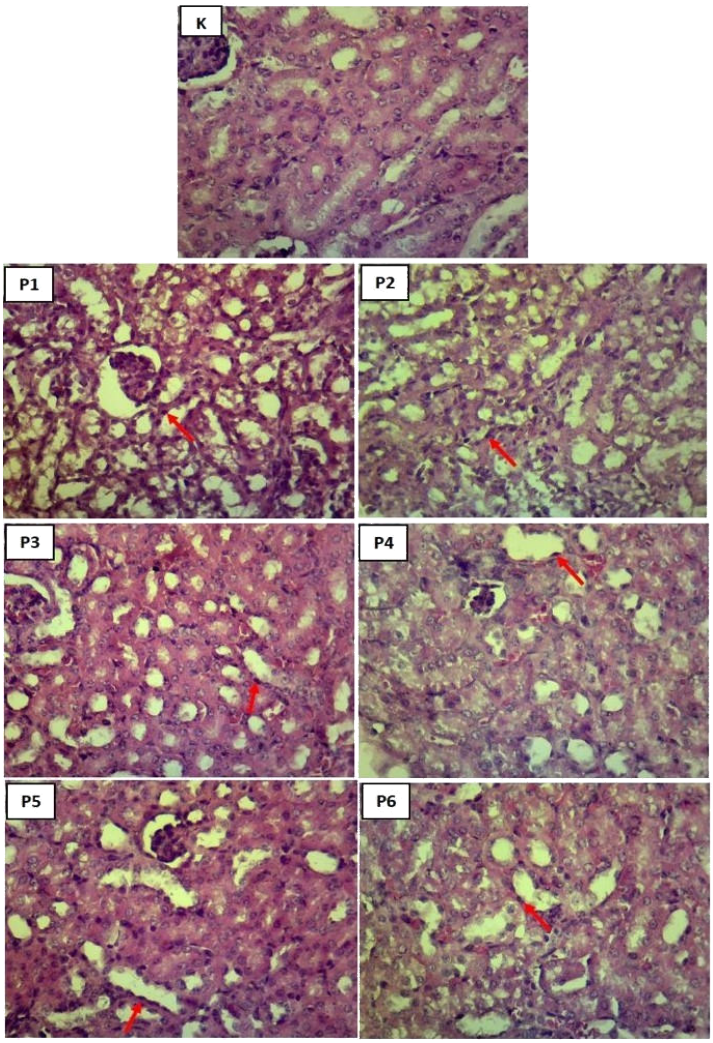

FIGURE 2: Comparison of the microscopic images on kidney necrose in mice offspring with H&E staining, magnification 400x. Arrows indicate necrose (→).K (aquadest), P1 (carbofuran 1/4 LD_{50} 0.0125 mg/day), P2 (carbofuran 1/8 LD_{50} 0.00625 mg/day), P3 (Kebar grass extract 3.375 mg (0.2 ml) + carbofuran 1/4 LD_{50}), P4 (Kebar grass extract 3.375 mg (0.2 ml) + carbofuran 1/8 LD_{50}), P5 (vitamin C 5 mg (0.2 ml) + carbofuran 1/4 LD_{50}), and P6 (vitamin C 5 mg (0.2 ml) + carbofuran 1/8 LD_{50}).

INFLAMMATORY CELL INFILTRATION

Evaluation on inflammatory cell infiltration was carried out by microscopic observation of kidney preparations of mother mice (Mus musculus) which previously had been stained with HE staining. Observations were made using a microscope with a magnification of 400 times. The presence of inflammatory cell infiltration can be seen in kidney tubule interstitial. The results of the statistical analysis based on table 1 showed that there was a significant difference ($p<0.05$) between group K and group P1, P2, P3, P4, P5, P6.

There was a significant difference ($p<0.05$) between group P1 and group P2, P3, P4, P5, and P6 treatment. There was a significant difference ($p<0.05$) between group P2 and group P3, P4, P5, and P6 s. There was a significant difference ($p<0.05$) between P3 and P4, P5, and P6. There was a significant difference ($p<0.05$) between P4 and P5 and P6. There was a significant difference ($p<0.05$) between P5 and P6.

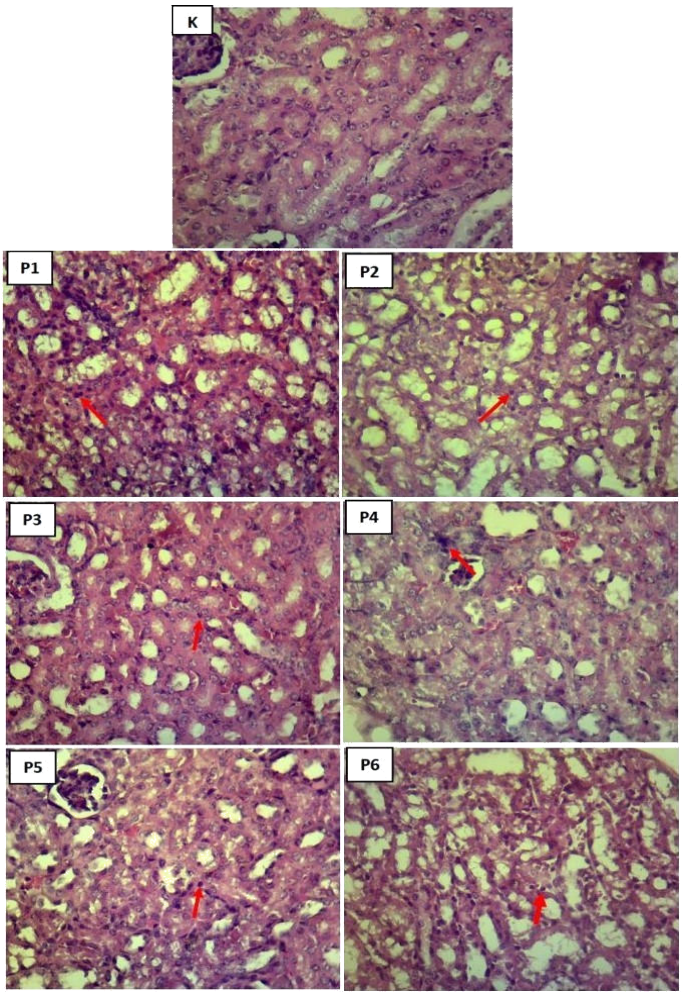

FIGURE 3: Comparison of the microscopic images on kidney inflammatory cell infiltration in mice offspring with H&E staining, magnification 400x. Arrows indicate inflammatory infiltration (→).K (aquadest), P1 (carbofuran 1/4 LD_{50} 0.0125 mg/day), P2 (carbofuran 1/8 LD_{50} 0.00625 mg/day), P3 (Kebar grass extract 3.375 mg (0.2 ml) + carbofuran 1/4 LD_{50}), P4 (Kebar grass extract 3.375 mg (0.2 ml) + carbofuran 1/8 LD_{50}), P5 (vitamin C 5 mg (0.2 ml) + carbofuran 1/4 LD_{50}), and P6 (vitamin C 5 mg (0.2 ml) + carbofuran 1/8 LD_{50}).

Toxic manifestations induced by metabolites of carbamate group can result in ROS which can then lead to the formation of free radicals[15]. Carbofuran is a carbamate insecticide which can cause residues in the environment that are easily absorbed by the body orally. Carbofuran is metabolized in the kidney by cytochrome p450 which results in 3-hydroxy carbofuran and two other metholyte products. 3-hydroxy carbofuran is a metabolite product that is systemic and is a carbofuran metabolite which can lead to increased manifestations of toxic activity[16].

The lipophilic nature of carbofuran will form lipid peroxidation in the kidney after binding to lipids and kidney cell membrane. Phospholipid, which is the main element of the plasma membrane, is subject to lipid peroxidation, increases the production of free radicals, and causes oxidative stress and damage to cell membranes[17]. Damage to cell membranes causes shifts in cell charge, changes in osmotic pressure, swelling and even cell death. The kidney is a critical target for xenobiotic compounds in the body, thus becoming a chemical excretion pathway in the body and enabling nephrotoxic effects[18].

Kebar grass as an antioxidant that contains flavonoids which are useful for preventing cell damage due to oxidative stress. Flavonoids have a working mechanism as antioxidants directly or indirectly. Flavonoids act as antioxidants directly by donating hydrogen ions and then neutralizing the toxic effects of free radicals. Meanwhile, flavonoids act as antioxidants indirectly by increasing the expression of endogenous antioxidant genes.

One of the increases in gene expression is the activation of nuclear factor erythroid related factor 2 (NrF2) which causes an increase in genes that function in the synthesis of endogenous antioxidant enzymes such as the SOD gene[19].

Vitamin C is an essential nutrient for several cells, one of which is immunity. Vitamin C is also an antioxidant and anti-inflammatory. There are high levels of free radicals in the body, so antioxidants are required to neutralize them so that the levels of free radicals decrease. The vitamin A and vitamin E contained in Kebar grass also functions as an active antioxidant. Vitamin A reacts with free radicals and stabilizes free radicals[10]. Vitamin E can inhibit oxidation reactions by binding to vitamin E radicals due to the process of breaking free radical reactions into free vitamin E which can re-function as antioxidants[20]

Kidney damage due to toxic compounds, one of which shows an image of degeneration of tubular cells. Degeneration is a condition in which a cell loses its normal structure due to influences from within or outside the cell. Degeneration is characterized by the presence of metabolic disorders[21]. Carbofuran can cause metabolic reactions to be disrupted so that ATP is reduced which is energy for cells and changes in cell membranes. This can affect cation pumps leading to increased permeability to water and ions[22].

At the level of the cell membrane, cells expend metabolic energy to pump sodium ions out of the cell to maintain a

stable internal environment. Toxic compounds that interfere with energy in cells or cell membranes result in cells being unable to pump sodium ions. Therefore, there is an increased concentration of sodium ions in the cell and the cytoplasm looks turbid[23]. This can be seen in this study that there was an increase in degeneration in line with the increase in the dose of carbofuran exposed. Group P1 and P2 showed significant differences ($p<0.05$). Cell swelling or degeneration is a reversible injury and the first manifestation that occurs due to the inability of cells to maintain ion homeostasis and fluid[24]. If the toxic compound is removed, cells can return to normal. These changes are mild disturbances from the normal state[23].

Group T3 and T4 which were treated with Kebar grass extract before the mice were exposed to carbofuran showed a decreased degeneration of tubular cell. The antioxidants such as flavonoids and Vitamin E contained in Kebar grass extract can reduce kidney damage because it can prevent and inhibit the toxic effects of carbofuran in the kidneys. These results are also in accordance with research conducted by Rabiah et al (2015) [25], which indicated that administration of vitamin E as an antioxidant is able to maintain the integrity of cell membranes and provide protection against cell damage in the kidneys. Vitamin E can be a hydrogen ion donor which is able to convert peroxyl radicals into less effective tocopherol radicals, so that the fatty acid chains cannot be damaged. Flavonoids can also enhance the regeneration process, provide competitive substrates for unsaturated lipids, and repair damaged cell membranes more quickly so that degeneration can decrease[26]. Vitamin C administered to group P5 and P6 was able to reduce kidney degeneration due to exposure to carbofuran. Vitamin C is an antioxidant that can reduce free radicals by inhibiting lipid peroxidation and preventing cell damage[27]. Necrosis is a continued degeneration that has passed the no return point and is irreversible damage due to cells failing to maintain their balance. Damaged cell membranes result in shifts in cell charge, changes in osmotic pressure, swelling and then cell death[24].

Toxic activity is caused by carbofuran as ROS can induce oxidative damage by increasing lipid peroxidation. Increased ROS is one of the free radicals that causes toxicity and cell death[28]. Antioxidant levels in the body that are not sufficient to compensate for free radicals will lead to cells to have necrosis[29].

High concentrations of toxic compounds in the body can cause reactions with all cell components, thereby suppressing cell function, resulting in cell death and organ damage. Damage to the cell due to toxic coagulates proteins in the protoplasm and nucleus. In the microscopic image, it can be seen as follows: there is a change in the nucleus which loses chromatin, it turns wrinkled, it is no longer vascular, it gets dense and dark, it is divided into fragments, and it is no longer colored[30]. Group P1 and P2 showed an increased necrosis due to carbofuran exposure with increasing doses. Necrosis that occurs in the tubules is caused by toxic carbofuran compounds in the bloodstream. Carbofuran which is absorbed by the glomerulus goes to the tubules. After it reaches the tubules, it will be metabolized to become more soluble in water and then it more easily enters the cells and affects mitochondrial metabolism[31]. The longer the kidney is exposed to toxic compounds, the greater kidney tissues have necrosis[32].

There was a significant difference ($p<0.05$) in group P3 and P4 treated with Kebar grass extract and it showed a decreased |necrosis in mice offspring's kidney. Decreased necrosis of P3 was lower compared to that of P4.

It is due to dose exposed to P4 is lower than that of P3. Necrosis in kidney decreases due to antioxidants contained in Kebar grass extract such as flavonoid, vitamin A dan vitamin E. Vitamin E as antioksidant is able to break chains in membranes which is able to prevent cell damage due to lipid peroxidation and hinder free radicals [20]. Flavonoid is also able to prevent lipid peroxidation at initiation stage with radical scavenger and propagation reaction is prevented with peroxy-radical scavenger. Vitamin C administration to P5 dan P6 showed that there was decreased necrosis in mice offspring's kidney and significant difference ($p>0.05$). Ascorbic acid as exogeneous antioksidant can reduce free radicals, then it hinders lipid peroxidation and prevent cell damage[27].

Inflammatory cells are a vascular reaction by dispatching fluids, solutes and cells from the blood circulation to the interstitial tissue in injured areas or necrosis[7]. Necrosis stimulates an inflammatory response by releasing inflammatory cytokines IL-6 to activate NFKB, p38 and MAPK. It can be seen that group P1 and P2 had an increase in inflammatory cell infiltration in the interstitial lumen of the kidney tubules of mother mice in proportion with carbofuran dose exposed to them. Inflammatory cells are a response of the body to tissue damage caused by pathogenic agents, dead cells, irritants, foreign bodies, physical injury, burns, radiation, or toxic compounds[24].

P3 and P4 had a significant difference ($p<0.05$) as well as reduced inflammatory cell infiltration in the interstitial lumen of the kidney tubules in mice offsprings. A decrease in inflammatory cell infiltration of P4 was greater compared to that of P3 because the dose of carbofuran administered to P4 was less than that of P3. The content of Kebar grass is also able to repair kidney cell damage so that inflammatory cells decrease. Antioxidants in Kebar grass such as flavonoids can bind Cu and Fe metal ions which can form ROS[33]. The binding of metal ions can reduce cell oxidative damage and prevent inflammatory cell infiltration. P5 and T6 which were treated with ascorbic acid had a decrease in inflammatory cell infiltration as well as a significant difference ($p<0.05$). Administering ascorbic acid as an antioxidant can neutralize and protect against the effects of free radicals, and can repair the functional structure of cells due to exposure to free radicals[34].

CONCLUSION

The results showed that carbofuran caused a significant increase in tubular degeneration, necrosis, and inflammation ($p<0.05$). Administration of Kebar grass and vitamin C significantly reduced tubular degeneration, necrosis, and inflammation ($p<0.05$). It can be concluded that administration of Kebar grass extract was more effective in reducing kidney damage in lactating mice exposed to carbofuran than vitamin C ($p<0.05$).

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