Molecular Study of Diazinon Toxicity Based on Inducible Nitric Oxide Synthase (iNOS) Expression in Hepatocytes and Kidney Histopathology of Rats (*Rattus norvegicus*)

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Abstract. Diazinon is an organophosphate insecticide that are widely used to eradicate pests but has a higher toxicity than other insecticides. Diazinon that accidentally consumed per orally has its impact in increasing reactive oxygen species (ROS) in the body. Reactive oxygen species (ROS) will activate proinflammatory cytokine which will induce the inducible Nitric Oxide Synthase (iNOS) at which iNOS will produce Nitric Oxide (NO) as free radical. High level of ROS will cause damage to the cells. This study was aimed to determine the toxicity effect of diazinon toward iNOS expression in liver and kidney histopathology of rats (*Rattus norvegicus*). The rats were divided into 4 groups which consist of negative control group and three experimental groups which were given diazinon 20 mg/kgBW (P1), 40 mg/ kgBW (P2), and 60 mg/ kgBW (P3). The parameters that was observed in this research are the expression of iNOS in liver using Immunohistochemistry staining then analyzed using one way ANOVA followed with Tukey test (α=0,05), and kidney histopathology that was observed using Hematoxylin-Eosin staining and analyzed qualitative descriptively. The results showed that administration of diazinon per orally was significantly increased iNOS expression (p<0,001) in liver and caused damage to the kidney’s cortex that was shown by inflammatory cell infiltration, necrotic of kidney tubule cells, and hemorrhage in inter-tubular space.

**Keywords:** Diazinon, Organophosphate, iNOS expression, kidney histopathology.

1. Introduction

The use of pesticides has been felt to increase production, especially as a preventive against pests and weeds (Kusriani, 2012). However, the frequently use of pesticides can cause these pesticides to be toxic to consumers, including animals both inhalation, oral and through the skin (Ameriana, 2006). One of pesticide that is commonly used is diazinon. Diazinon is an organophosphate pesticide that has a mechanism with inhibit the function of the enzyme cholinesterase. These pesticides also have less effect on non-target organisms and are more toxic
to vertebrate animals compared to organochlorines (Luthfanto, 2014). Normally pesticides will be neutralized in the liver by hepatocytes through detoxification, but these compounds also capable to form free radicals due to cell damage. Organophosphate exposure that occurs continuously can trigger liver damage as an organ that plays a role in the detoxification process (Ginting, 2014). Organophosphates that have been metabolized in the liver have the potency to inhibit the AChE enzyme. Inhibition of this enzyme directly results in an increase in acetylcholine levels in the nervous system and neuromuscular system. The increase in AChE can cause ions in cells became unstable and trigger the formation of Reactive Oxygen Species (ROS) (Uchendu, 2012). Increased ROS will trigger the production of proinflammatory cytokines. Naik and Vishva (2011) state that ROS affects the transcription of proinflammatory cytokines where high levels of ROS will be followed by high levels of proinflammatory cytokines as well as if ROS production is inhibited by inhibitors will affect the decrease in the production of proinflammatory cytokines such as IL-6 and TNF. Increased production of proinflammatory cytokines will induce iNOS and form NO in excessive amounts (Aktan, 2004).

Kidney as the main organ that maintain the balance of Extra Cellular Fluid (ECF) by carrying out its functions in excretion and metabolism (Curthoys, 2014). Excretion or elimination of xenobiotic metabolism result conducted in the kidney. The kidney damage can reduce the level of excretion of chemicals without increasing the toxicity of toxic substances (Gupta, 2012). The oxidative stress condition will initiate the lipid peroxidation process by taking hydrogen ions from the Poly Unsaturated Fatty Acid (PUFA) bond to form a more stable molecule. The bond breaking process on PUFA can cause cell or tissue necrosis (Sharma et al., 2003).

This study was conducted to determine the toxicity effect of diazinon against iNOS expression in the liver and renal histopathology of rats.

2. Materials and Methods

2.1. Animal Models and Experimental Design
Twenty rats (Rattus norvegicus) were divided into 4 groups: negative control group (healthy rats), P1, P2, and P3 groups were administrated with diazinon for 8 weeks orally using feeding tube with doses of 20 mg/kgBW, 40 mg/kgBW, and 60 mg/kgBW respectively. Diazinon 600EC mixed with distilled water until it contained 5 mg of diazonin in 1 mL (Baconi et al., 2013). The rats were acclimatized for a week before receiving treatment. All conditions and handling animal were conducted with the protocol approved by Ethical Clearances Committee of Brawijaya University (No: 732-KEP-UB).

2.2. Preparation and Observation of Kidney Histopathology
Preparation of kidney histopathology consists of the following stages: fixation, dehydration, clearing, embedding, sectioning, sticking to glass objects according to Muntiha (2001). Observations of kidney histopathology were carried out using a binocular light microscope with microscope magnification 100x followed up to 400x. The observation was done on renal glomerulus and renal tubules.

2.3. Analysis of iNOS Expression on Liver
iNOS expression on liver was analysed with immunohistochemistry methods, using anti iNOS2 as primary antibody and anti-rabbit IgG biotin labelled as secondary antibody. The measurement of iNOS expression on liver was conducted using software Immunoratio. The results of the iNOS expression percentage area were analyzed quantitatively using the one-way Analysis of Variance (ANOVA) test and followed with the Tukey test.
3. Results and Discussions

3.1. Analysis of iNOS expression on liver

To analysed the expression of iNOS on liver cells was done using immunohistochemistry method. The expression of iNOS can be shown as a brown color expression on the hepatocytes, that can be seen on Figure 1.

![Figure 1](image)

*Figure 1*. iNOS expression on liver. (1) Negative control group (K-). (2) P1 (Induced with diazinon 20 mg/kgBW). (3) P2 (induced with diazinon 40 mg/kgBW). (4) P3 (induced with diazinon 60 mg/kgBW).

White circle shown the area of iNOS expression.

The immunohistochemistry result was analysed qualitatively, and showed that there is an increasing of brown colour intensity (Figure 1). This result indicate that there is an increasing of iNOS expression levels on treatment group compared than control group.

To measure the level of iNOS expression on hepatocytes used software immunoratio. The results of quantitative analysis of the iNOS expression in the liver of rats can be seen in Table 1 and it can be concluded that there is a significant difference (p <0.001) between the treatment groups compared to the negative control group (K-). On negative control group that was the group without diazinon organophosphate induction, the average of percentage area of iNOS expression was 1.340 ± 0.092%. On treatment group 1 (P1) that was the group induced by diazinon organophosphate at a dose of 20 mg / kgBW, the average of percentage area of iNOS expression was 4.184 ± 1.041%. Treatment group 2 (P2) was a group induced by diazinon organophosphate at a dose of 40 mg / kgBW and the average of iNOS expression was 7.496 ± 0.997%. Treatment group 3 (P3) was a group of induced by diazinon organophosphate at a dose of 60 mg / kgBW and the average of iNOS expression was 11.604 ± 0.864.
### Table 1. The Average of Percentage Area of iNOS Expression on Hepatocytes

| Group                        | Average of Percentage Area of iNOS Expression ± SD (%) | Increasing to Negative Control (%) |
|------------------------------|--------------------------------------------------------|------------------------------------|
| Negative Control (K-)        | 1,340 ± 0.092<sup>a</sup>                               | -                                  |
| P1 (20 mg/KgBW)              | 4,184 ± 1,041<sup>b</sup>                               | 212                                |
| P2 (40 mg/KgBW)              | 7,496 ± 0.997<sup>c</sup>                               | 459                                |
| P3 (60 mg/KgBW)              | 11,604 ± 0.864<sup>d</sup>                              | 765                                |

<sup>**Different notations showed the significant differences (p<0.05).**</sup>

On negative control group showed iNOS expression in low intensity (1,340 ± 0.092%). McNaughton et al. (2002) found that iNOS was expressed in normal liver acini. The spread of iNOS in the normal liver shows the area around the portal with the strongest expression and intensity decreases along the liver acini. According to Kubes (2000), iNOS can provide therapeutic effects if it is regulated in the right amount and location, iNOS has a positive role including healing of the skin and intestinal mucosa, fighting certain bacteria, potential in regulation of T cell proliferation and differentiation, and regulating leukocyte recruitment.

The treatment group showed a significant increasing on percentage area of iNOS (p<0.001). Diazinon such as organophosphate in general will metabolized which mainly takes place in the liver. The metabolism that occurs is phase 1 metabolism which is an oxidation process, where organophosphate will be broken down into oxono-organophosphate metabolites and free sulfur ions with the help of cytochrome enzymes (CYP) (Gupta, 2009). Metabolites from phase 1 metabolism are free radicals, unstable substances that have no electron pairs. Free radicals will produce Reactive Oxygen Species (ROS) such as superoxide, hydroxyl radicals, and hydrogen peroxide through interactions with other molecules. Free radicals react with phospholipids from the plasma membrane, endoplasmic reticulum membrane, or mitochondrial membrane and cause cell death (Gupta, 2012). Organophosphate pesticides can also cause oxidative stress (Mishra, 2013). This oxidative stress condition is caused by diethyl as an organophosphate free radical (diazinon) that capable to produce large amounts of ROS. Oxidative stress is an impaired balance between prooxidants and antioxidants that causes potential damage (Uchendu et al., 2012).

The increasing of ROS will trigger the production of proinflammatory cytokines. Reactive Oxygen Species affect the transcription of proinflammatory cytokines where high levels of ROS will be followed by high levels of proinflammatory cytokines, likewise if ROS production is inhibited with inhibitors will also affect the decrease in production of proinflammatory cytokines such as IL-6 and TNF (Naik and Vishva, 2012). Dunlop et al. (2014) and Pahan et al. (2000) stated that TNF-α and IL-1 play a role in increasing the expression of iNOS in tissues through the activation of Nappar Kappa B (NF-kB) in which NF-kB enters the nucleus and binds to the promoter region of iNOS. Increased production of proinflammatory cytokines will induce iNOS and form excessive amounts of Nitric Oxide (NO) (Aktan, 2004).

### 3.2. Analysis of Kidney Histopathology

The results of this research on the effect of diazinon toxicity on the kidney histopathological features using the Hematoxylin-Eosin (HE) staining are presented in **Figure 2**. Histological picture of the rat’s renal cortex in the negative control group (K-) group was shown a normal condition and there is no abnormality was found. This is proved by the appearance of the renal corpus, the proximal and distal convulsive tubules with a layer of cuboid epithelial cells (**Figure 2 (1)**).
Figure 2. Kidney Histopathology of Rats using Hematoxylin–Eosin (HE) staining, 400x.

1: Negative Control Group (K-); B: P1; C: P2; and D: P3. G: Glomerulus; K: Proximal Convulatus Tubule; D: Distal Convulatus Tubule.

- ➡️: Hyperthrophy cell, ➡️: Inflammatory cells infiltration, ➡️: Karyolysis cell, ➡️: Haemorrhage

The histopathological picture of the renal cortex of rats in the P1 group showed abnormalities, that characterized by the presence of cells undergoing karyolysis and hypertrophy. The karyolysis cells were characterized by the disappear of cell nucleus, while epithelial cells undergoing hypertrophy have characteristics of enlarged cell size so that the structure of the renal tubules appears irregular. Erythrocytes in the inter-tubular space can be seen in this picture which indicates the presence of haemorrhage (Figure 2 (2)). The histopathologic picture in group P2 shown the abnormalities as similar in the group P1. Abnormalities that can be observed in the renal cortex of P2 rats include the appearance of cells undergoing karyolysis, cells hypertrophy and haemorrhage. In addition, the structure of the kidney tubules appears irregular, compared to the K- group (Figure 2 (3)).

Histopathological picture of the renal cortex of rats in the P3 group showed abnormalities as seen in the histopathology picture of the renal cortex in P1 and P2 rats, characterized by the appearance of cells hypertrophy, karyolysis, and erythrocytes in the inter-tubular space (haemorrhage). It also can be observed the presence of inflammatory cell infiltration in tissues (Figure 2 (4)).

Diazinon organophosphate that was given to the rats per orally will be digested by the digestive tract which will then be absorbed by the intestine, especially by the duodenum. Organophosphate metabolism mainly takes place in the liver, but to a lesser extent metabolism also occurs in the pulmo and intestine. Organophosphate will undergo phase 1 metabolism which is an oxidation process where organophosphate will be broken down into oxono-organophosphate metabolites and free sulfur ions with the help of cytochromes (CYP) enzymes (Gupta, 2009; Elersek and Metka, 2011). The oxono-organophosphate metabolite then enters the bloodstream and goes to the liver for
In the liver, oxono-organophosphate metabolites will be hydrolyzed with the help of the enzymes carboxylesterase (CE) and paraoxonase (PON), split into dialkylphosphate and leaving the group. Dialkylphosphatase will then be catalyzed by enzymes in phase 2 metabolism that will produce molecules that are hydrophilic and can be excreted in urine, while leaving groups (in the form of diethyl) can induce oxidative stress in the body. Diethyl is a very reactive molecule and can react with hydroxyl radical groups (-OH) to form new free radicals (Elersek and Metka, 2011). The high number of free radicals in the body cannot be neutralized by endogenous antioxidants, resulting in an imbalance between free radicals and antioxidants that causes oxidative damage (Caramori and Papi, 2004). This oxidative damage occurs because of diethyl-hydroxyl as a free radical from the organophosphate diazinon is able to produce large amounts of ROS (Uchendu et al., 2012). The increasing of ROS will then cause oxidative stress conditions and initiate the process of lipid peroxidation by taking hydrogen ions from Poly Unsaturated Fatty Acid (PUFA) to form more stable molecules. The bond breaking process of PUFA can cause cell or tissue necrosis (Sharma et al., 2003).

The abnormalities which occurred in the kidney tubular epithelium due to the direct contact of epithelium with the toxic substance of diazinon, and in the kidney tubule there is a process of reabsorption and augmentation in the process of urine formation (Mescher, 2010). The toxic substance of diazinon which is still in the blood vessels will be in direct contact with the endothelium, thus causing disruption of the vascular membrane permeability. Haemorrhage in intertubular space was caused by disruption of blood vessels membrane permeability (peritubular capillaries), so that erythrocytes can enter to the tissue (Djumadi et al., 2008). Injury to endothelial cells initiates inflammatory reactions, resulting in the accumulation of macrophages and platelets outside and inside the arteries. The appearance of inflammatory cell infiltration in damaged tissue indicates that metabolites from diazinon cause toxicity to the kidney (Khudair et al., 2017).

Based on the results of the histopathological appearance, the renal cortex in the treatment group experienced abnormalities compared to the K- group. The P1 group with the lowest dose of 20 mg/Kg BW was known to have shown the damage of kidney tubular cells compared to the K-group.

4. Conclusion

Administration of diazinon with the lowest dose of 20 mg/kg BW was shown to have a toxic effect in rats as indicated by a significant increase in iNOS expression (p <0.001) and showed the damage of the renal cortex in the form of renal tubular cell necrosis, haemorrhage, and renal tubular cell hypertrophy.

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