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The Toxicity Effect of Organophosphate (Diazinon) towards Duodenum Histopathology and The Activity of Superoxide Dismutase (SOD) Serum in Rats (*Rattus norvegicus*)

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ABSTRACT

Diazinon is an organophosphate insecticide that can inhibit acetyl-cholinesterase competitively and increase the production of Reactive Oxygen Species (ROS). ROS affect the activity of antioxidant enzymes such as Superoxide Dismutase (SOD), and simultaneously ROS cause damage to the cells and duodenum tissue. The toxicity effect of diazinon was investigated by measuring the activity of SOD serum and histopathology of the duodenum in rats (*Rattus norvegicus*). The activities of SOD serum were measured by using spectrophotometry, and histopathology changes of duodenum were observed by using HE staining. Rats were divided into 4 groups, negative control group (K-), P1, P2, and P3 groups which were administrated with diazinon for 8 weeks orally with a dose of 20 mg/kgBW, 40 mg/kgBW, and 60 mg/kgBW respectively. The results showed that administration of diazinon orally decreased SOD activity significantly and caused damage of duodenal villi such as hyperplasia of epithelial cells, epithelial erosion, fatty degeneration, hyperplasia of Liberkuhn gland cells, and hemorrhage in the lamina propria.

Key word: Diazinon, organophosphate, SOD activity, duodenum histopathology

INTRODUCTION

Agriculture in Indonesia has experienced many improvements along with the increase of human's need of food. One of the examples is the use of pesticide to exterminate organism which causes diseases in plant and to control insects, weeds, and other pests in order to increase production. Many pesticides are used; one of the popular ones is organophosphate insecticides. Organophosphate insecticides are commonly used worldwide in agricultural and in pest control [1].

Diazinon is one of the toxic compounds in organophosphate to vertebrate animals such as fish, bird, lizard, and mammals. Diazinon in agriculture is usually used in soybean, fruit, and vegetable's spraying. In this case, the residue of pesticide sticks on leaves, fruits, seeds, and into the water which makes poisoning in animal and human, hence present a health hazard in long-term exposure even at low levels [2]. Some poisoning accidents of organophosphate pesticide on animal have been reported in Friesian Holstein (FH) cow in West Java, due to organophosphate presents in their feed [3].

Diazinon generally enters body after being absorbed through the skin, lung, and the digestive system. Since poison absorption begins with food consuming, digestive system is the most important place related to food poisoning. The food poison absorption in digestive

system occurs in stomach and small intestine. In the case of diazinon pesticide poisoning through digestive system, the damaging effect often takes place in small intestine, especially duodenum a place for the first time of diazinon metabolism [4].

Diazinon works in animal's body by blocking acetylcholinesterase (AChE) molecule competitively. AChE is the enzyme that is needed for the function of the nervous system [5]. Diazinon binds with AChE and inhibits the activity of the enzyme by irreversible phosphorylation. This result in elevated levels of acetylcholine thus stimulating the muscarinic and nicotinic receptors resulting in consequent toxicity [6]. Moreover, diazinon also causes oxidative stress condition [1]. Oxidative stress is an imbalance between pro-oxidant and antioxidant which causes potential cells damage [7]. Based on previous research, diazinon exposure of rats in laboratory tests has demonstrated that this insecticide can cause impaired hepatic and renal function. This is due to hepatocyte cell damage in the form of cell necrosis, inflammatory cell infiltration (mononuclear cells), hydropic degeneration and focal microvesicular steatosis in parenchymal tissue. In the kidneys, glomeruli show a mild widening of Bowman's space with glomerular atrophy and infiltration of interstitial inflammatory cells [8]. Diazinon exposure of pregnant animals can also cause a variety of reproductive problems, including damage to the developing nervous system, delays in sexual development, stillbirths, the death of newborn offspring, and birth defects [9]. However, there is a paucity of data regarding the effect of diazinon on duodenum. Therefore, the present study was conducted to investigate the toxicity effects of diazinon to Serum SOD activity and the duodenum histopathology of rats.

EXPERIMENT

Chemicals and Instrumentation

This study used female rats of *Rattus norvegicus* Wistar strain (10-12 weeks old) with weight 110 gram that purchased from Laboratory of Physiology, UIN Malang, Diazinon 600EC, distilled water, NaCl, Formaldehyde 10% (Sigma), Phosphate Buffered Saline (Sigma), hematoxylin-eosin (Sigma), Ethanol absolute (Sigma), xylol, paraffin block, SOD assay kit (Sigma-Aldrich19160: xanthine, xanthine oxidase, and Nitro Blue Tetrazolium). The equipment used in this study include feeding tube, a set of glasswares, micropipette, centrifuges, incubators, pipette, object glasses, cover glasses, a set of surgical equipment, microscope (Olympus BX51), freezer, spectrophotometer UV (Type Shimidzhu UV-Visible 1601), microtome rotary, tissue processor, tissue embedding, water bath, staining place, and paraffin cassette.

Animal 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. Diazonin 600EC mixed with distilled water until it contained 5 mg of diazonin in 1 mL [10].

The rats were acclimatized for a week before receiving their experimental. All conditions and handling animal were conducted with the protocol approved by Ethical Clearances Committee of Brawijaya University (No: 732-KEP-UB).

Data analysis was carried out quantitatively and qualitatively. Duodenum histopathology was analyzed qualitatively by comparing each group's duodenum histopathology. The activity of SOD serum was analyzed quantitatively using Microsoft Excel and SPSS for windows version 24 for Analysis of Variants (ANOVA) test and followed by Tukey test with α=0.05.

Observing Duodenum Histopathology and Measuring the Activity of SOD Serum

Duodenum histopathology was observed at its mucous membrane especially the villi and intestinal epithelium that have been colored with Hematoxylin-Eosin (HE). The observation was using Olympus BX51 microscope with 400x zooming.

Determination of SOD activity samples was performed using spectrophotometry method. 100 µL of rat's serum was put into the reaction tubes and added with 100 µL of xanthine, 100 µL of xanthine oxidase, 100 µL of NBT (Nitro Blue Tetrazolium), and 1 mL of NaCl solution. Further, the mixture was homogenized with the vortex. The mixture solution was incubated for 30 minutes at the temperature of 30 °C. Finally, the absorbance of the mixture solution was read using a spectrophotometer UV (Type Shimadzu UV-Visible 1601) with wavelength 580 nm.

RESULT AND DISCUSSION

The Toxicity Effect of Diazinon towards Duodenum Histopathology in Rats

Duodenum histopathology of rats in the negative control group (K (-)) is in the normal condition without any damages which were shown by simple columnar epithelium in duodenal villi. Goblet cells were found between those epithelial cells. Lamina propria of duodenal tunica mucosa was also seen in normal condition comprised of by loose connective tissue without any abnormality found (Figure 1 (A)). The duodenum histopathology was later used as a reference to observing the duodenum histopathology of experimented groups that were given diazinon.

The duodenum histopathology of rats in group 1 (P (1)) showed abnormality which compared to negative control group. It was shown by epithelial erosion (ruptured epithelial), hemorrhage appeared in lamina propria, and hyperplasia of the epithelial cell. Therefore, the duodenal epithelial should have neat and tight simple columnar with round nucleus which oriented to basal changed into disordered columnar epithelium with nucleus overlapping (Figure 1 (B)).

In group 2 (P (2)) also showed damages in duodenal tunica mucosa. The damages were much more severe in group 2 compared to group 1. Hyperplasia appeared in the duodenal epithelial cell indicated by duodenum disorder with the overlapping nucleus, shrinking goblet cell, and epithelial erosion. Many duodenal epithelial cells in group 2 had necrosis in pyknosis phase which was marked with the nucleus that shrinkage and turned darker as well as membrane cell that did not have distinct edge. Lamina propria in duodenal rat also underwent some damage which was the accumulated Liberkuhn gland cells caused hyperplasia, fatty degeneration, and hemorrhage in lamina propria (Figure 1 (C)).

Duodenum histopathology of rats in group 3 (P (3)) showed the most damages tunica mucosa among the other groups. Duodenal villi in group 3 did not have any neat and tight simple columnar epithelium. Most of group 3's epitheliums have been through hyperplasia leading to being an irregular shape with overlapping nucleus. It seems complex columnar epithelium and there was space at the edge of villi which showed epithelial erosion. Its duodenal lamina propria has also been damaged due to accumulated Liberkuhn gland cells lead to hyperplasia in much larger area than group 2's with followed fatty degeneration (Figure 1 (D)). Therefore, diazinon produces toxic effect to rat's duodenum.

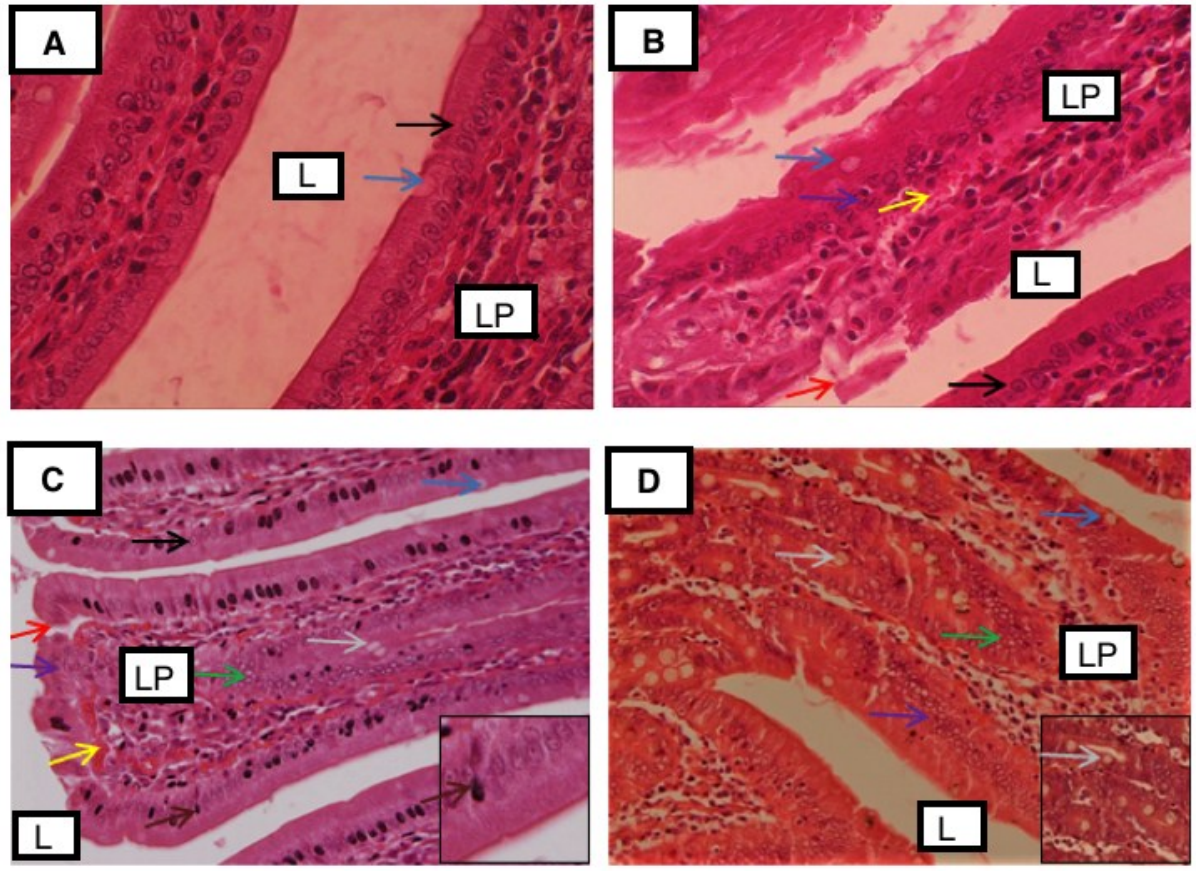

Figure 1. Duodenum Histopathology of Rats using Hematoxylin–Eosin (HE) coloring, 400x zoom. A: Control Negative Group; B: Group 1; C: Group 2; and D: Group 3. L is Lumen and LP is Lamina Propria. The arrow color represents:
(→): Simple Columnar Epithelium;
(→): Goblet Cell;
(→): Epithelial Erosion;
(→): Hemorrhage;
(→): Hyperplasia on Epithelial cells;
(→): Pyknosis on Epithelial cells;
(→): Hyperplasia on *Liberkuhn* cells;
(→): Fatty degeneration.

Diazinon (Figure 2) is an organophosphate insecticide, chemically related to other common insecticides like malathion and chlorpyrifos [9]. The diazinon that induced to rats orally, that was digested by the digestive system prior to absorption in the intestine, especially duodenum to perform metabolism [4]. Metabolism of diazinon mainly occurs in the liver. However, in lower level of metabolism, it also occurs in lung and intestine. Metabolism of diazinon in duodenum undergo phase I of metabolism through oxidation process to produce oxono-organophosphate metabolite and free Sulfur ion that catalyzed by cytochromes (CYP) enzyme [11]. Free sulfur ion as a by-product of diazinon metabolism in duodenum will accumulate in duodenal villi, especially at its epithelial cells. Duodenal mucosa layer tends to be acidic lead to free sulfur ion binds to hydrogen ion (proton) and form H_2S molecule. The H_2S will inhibit metalloprotease activity. The inhibition of

metalloprotease disturbs the normal physiological function of cell, particularly on the growth and maturation process of cell [12]. Consequently, that disturbs synthesis and degradation of protein bring to be hyperplasia on epithelial cells.

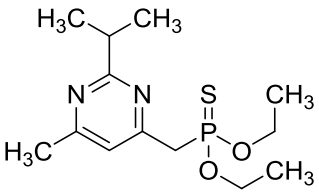

Figure 2. Molecule structure of diazinon (*O*,*O*-diethyl ((2-isopropyl-6-methylpyrimidin-4-yl)methyl)phosphonothioate)

Hyperplasia also occurred in lamina propria which is the intestinal gland cells or *Liberkuhn* crypts/gland. Type of hyperplasia that takes place in *Liberkuhn* is compensatory hyperplasia. It is because of formed H_2S in duodenal tunica mucosa, duodenum becomes very acidic. In order to neutralize the pH, the enteroendocrine cell in *Liberkuhn* produces in a large number of cholecystokinin hormones [11]. Cholecystokinin acts to stimulate the gallbladder to produce bile salt and secretion of pancreas enzyme. Bile salt will normalize duodenal pH and carry out the absorption and digest of food [13].

Oxono-organophosphate metabolite as the result of diazinon metabolism enters through blood circulation to the liver to be further metabolized. In liver, Oxono-organophosphate is hydrolyzed to dialkylphosphate and leaving group with by carboxylesterase (CE) and paraoxonase (PON). Dialkylphosphate is catalyzed by enzyme in phase II of metabolism to produce hydrophilic molecule and enable to be excreted in urine, meanwhile leaving group (in the form of diethyl) induces oxidative stress in the body. Diethyl is a really reactive molecule that react fast with the reactive hydroxyl radical group (-OH) to form new free radicals [4].

Free radicals in the body are scavenged with the endogenous antioxidant such as SOD. However, with the high amount of free radicals because of the continuous toxicity of diazinon decrease the activity of endogenous antioxidant. The imbalance of the number of free radicals and antioxidant lead to oxidative damage [14]. Since the diethyl-hydroxyl as free radicals from diazinon enable to produce a big amount of ROS [7]. The increase of ROS produce oxidative stress condition and initiate lipid peroxidation process by taking hydrogen atom off of Poly Unsaturated Fatty Acid (PUFA) to form more stable molecule. The process of breaking PUFA bonds cause necrosis in cells or tissue [15]. Those processes trigger erosion in epithelial, space on the edge of villi, and pyknosis from duodenal epithelial cells. The damages in the duodenal epithelium are caused by penetrating diazinon directly. The hemorrhage in lamina propria appears because of the intrusion of membrane permeability from blood vessel, as a result erythrocyte come into the tissue. In the subchronic and chronic toxicity of the diazinon, the diazinon cannot be metabolized due to the inhibition of CYP enzyme. Diazinon is fat soluble that accumulated together with fat lead to fatty degeneration in lamina propria [16]. In addition, in duodenum sulfur ion can bind haem ion of CYP in cysteine residues [17].

Based on the duodenum histopathology in rats, duodenum of experimented rats groups showed abnormality compared to the negative control group. The first group induced by the lowest dose of 20 mg/kgBW has displayed damages in tunica mucosa than the control group.

The Toxicity Effect of Diazinon towards Activity of SOD Serum in Rats

Measurement of the activity of SOD serum in rats was carried out to investigate the activity of endogenous antioxidants of rats induced diazinon. The activity of SOD is defined as the number of μmol anion superoxide radicals that converted to hydrogen peroxide (H_2O_2) and oxygen (O_2) by 1mL SOD isolated from rat's serum [18].

Table 1. Average Activity of Superoxide Dismutase (SOD) in Rats Serum

Experimented Group	Average activity of superoxide dismutase (SOD) (unit/mL)	Activity of superoxide dismutase	
		Increasing towards Negative Control	Decreasing towards Negative Control
Negative Control	5.989 ± 0.285^a	-	-
Group 1	4.756 ± 0.268^b	-	20.59 %
Group 2	4.256 ± 0.296^b	-	28.94 %
Group 3	3.178 ± 0.412^c	-	46.94 %

Note: Difference of notation a, b, c shows significance difference ($p<0.05$) between experimented groups.

As shown in Table 1, The SOD activity in the group receiving diazinon decreased significantly ($p<0.05$) in comparison with negative control (K (-)). SOD activity declined with the increasing dose of diazinon induction. It suggests that diazinon enable to produce free radicals. The decline of SOD activity related to the increase of lipid peroxidation in cell membrane that leads to the increase of cell membrane damage.

SOD is an antioxidant enzyme that catalyzes superoxide (O_2^-) free radicals into oxygen (O_2) and hydrogen peroxide (H_2O_2). Based on its action, this enzyme is grouped as the primary antioxidant that acts to decrease the forming of new free radicals by breaking the chain reaction and change it into a more stable product [19]. Decreasing activity of SOD in experimented groups indicates high oxidative stress in body. The oxidative stress is caused by the big amount of free radicals in body that cannot be scavenged by the endogenous antioxidant enzyme. Free radicals are derived from normal metabolism and diazinon detoxification in the body.

As described previously, diazinon mainly metabolized in the liver and lower level occurs in lung and instestine. The diazinon is metabolized through 2 phases; diazinon in phase I is oxidized to produce oxono-organophosphate metabolite and free Sulfur ion with the aid of CYP enzyme [11]. Oxono-organophosphate metabolite is then hydrolyzed by CE and PON into dialkylphosphate and leaving group (diethyl-hydroxyl). In phase 2, dialkylphosphate is converted to the hydrophilic molecule and enable to be excreted in urine, meanwhile leaving group (diethyl-hydroxyl) induce oxidative stress in the body [4]. Free sulfur ion as the by-product of diazinon metabolism in duodenum epithelial cells will bind proton to form H_2S molecule. The H_2S interact with metalloenzyme such as SOD and inhibit its activities lead to decline the activity of SOD [20-21].

Free radicals in body are scavenged with endogenous antioxidant such as SOD. However, with the high amount of free radicals as a result of diazinon toxicity, the activity of SOD decreases. The imbalance of the number of free radicals and antioxidant cause oxidative damage [14]. Oxidative damage occurs because diethyl-hydroxyl as free radicals from diazinon is able to produce significant amount of ROS [7]. The increase of ROS initiate lipid peroxidation process by taking hydrogen atom off of poly unsaturated fatty acid (PUFA) to form more stable molecule. The process of breaking PUFA bonds produce necrosis in cells resulting in the decrease of SOD activity [15].

CONCLUSION

Diazinon oral administration with varieties doses of (20; 40 and 60 mg/kgBW) showed the decreasing of SOD activity and the increase of damages on duodenal villi such as hyperplasia of epithelial cells, epithelial erosion, fatty degeneration, hyperplasia of *Liberkuhn* gland cells, and hemorrhage in the lamina propria.

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