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Oxidative Stress and Inflammation Marker Profiles of White Rat Pup's Brain Endosulfan-induced Neurotoxicity in Pregnant Rat Model

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Abstract: Endosulfan is a forbidden insecticide that may cause some nerve related disorders. Some oxidative stress and inflammation markers may play a role in these neurotoxic events. This study aimed to analyze the effect of endosulfan exposure in white rat pup's brain in pregnant rat model. This study used pregnant white rats induced by endosulfan administered orally and divided to endosulfan treatment and control. After the female rats gave birth, endosulfan treatment was stopped. The pups were left to suckle on their mothers. When the pups had reached four weeks, 16 pups were terminated and brains were taken from each group were taken to examine levels of MDA, SOD, H_2O_2, AOPP, TNF-α, and Hsp70. The data collected were analyzed using Student's T-test or Mann-Whitney U test. The results suggested that MDA, H_2O_2, AOPP and TNF-α levels were higher in endosulfan group compared to control group ($p<0.05$), SOD levels decrease in endosulfan group ($p<0.05$) and no significant difference in Hsp70 levels between the group. This study concluded that endosulfan interfered the oxidative stress and inflammatory markers of pup's brain induced by endosulfan in the pregnant rat model.

1 INTRODUCTION

Endosulfan was used as an organochlorine insecticide in agriculture globally to improve farming production, fight against destructing pests and protection from vector-borne diseases and related epidemics for humankind. In several countries, endosulfan is banned permanently due to high toxicity profiles, but it is still largely used in some developing countries due to its low cost and high efficacy against pests (Menezes *et al.*, 2017; Patočka *et al.*, 2016).

Recent studies have shown that endosulfan can cause several diseases such as endocrine, reproduction, genotoxicity, teratogenicity and neurotoxicity disorders. Endosulfan may cause neurotoxic effects by overstimulating the central nervous system, affecting a number of targets on CNS and also crossing the barrier of the placenta (Pathak *et al.*, 2008; Silva and Gammon, 2009). Endosulfan is one of the factors that affect some neuropsychological disorders that occur in children and adults. Exposure to endosulfan during pregnancy and lactation in female rats can affect the neurotransmitter; γ-aminobutyric acid (GABA), glutamate, serotonin and dopamine (Lafuente and Pereiro, 2013; Wilson *et al.*, 2014). These findings suggested that neurotransmitter disorders may cause neurobehavior disorders caused by endosulfan exposure (Wilson, 2014). Endosulfan may also interfere with the ability of the zebrafish nervous system while swimming because it inhibits the activity of acetylcholinesterase (AChE) enzyme (Pereira *et al.*, 2012).

The neurotoxicity manifestation due to endosulfan exposure may be affected by oxidative stress disorder and an inflammatory reaction (Jang *et al.*, 2016; Lakroun *et al.*, 2015). Endosulfan exposure can cause oxidative stress in humans as well as rats (Koç *et al.*, 2009; Pathak,

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2008). Endosulfan had been shown to trigger systemic toxicity, reactive oxygen species (ROS) and lipid peroxidation, which can be seen with the increase of malondialdehyde (MDA) (Jang, 2016; Ullah *et al.*, 2016). The brain consists of phospholipids in the cell membranes so that if damage occurs, it can make some brain disorders (Kayhan, 2008; Zervos *et al.*, 2011). The rat liver cells induced by endosulfan exposure *in vitro* had reduced antioxidant enzyme activity such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) while elevated hydrogen peroxidase (H_2O_2) levels (El-Shenawy, 2010; Jang, 2016). Zebrafish induced by low concentration endosulfan may increase the activity of the enzyme SOD and catalase (CAT), whereas at high concentrations can lead to the production of reactive oxygen species (ROS) excess that causes SOD and CAT cannot handle it anymore (Shao *et al.*, 2012).

Endosulfan exposure may also increase the activity of inflammatory mediators such as tumor necrosis factor (TNF-a) in Mouse RAW 264.7 cells (ATCC) (Terry et al. 2018). Endosulfan exposure may also increase levels of advanced oxidation protein products (AOPP) in rabbits. AOPP is one of the inflammatory markers and oxidative stress in many diseases (Alagozlu *et al.*, 2013; Ozdem *et al.*, 2011). The 70 kilodalton heat shock proteins (Hsp70) are proteins that are formed to protect cells from oxidative stress and inflammation (Borges *et al.*, 2012). In endosulfan-induced zebrafish embryos Hsp70 increase significantly (Moon *et al.*, 2016).

Although endosulfan had been shown to have many toxic effects; especially neurotoxicity and prohibited already, many agricultural area use endosulfan as a weapon against pests legally or illegally (Patočka, 2016). This study aimed to determine whether the neurotoxic endosulfan can affect the levels of some markers of oxidative stress and inflammatory mediators in the brains of pups.

2 MATERIALS AND METHODS

This research had been received approval from the ethics committee of Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, Indonesia. This study was an experimental study with posttest-only with control group design.

Materials

The materials used in this study were white rats (*Rattus norvegicus*), pup's brain, distilled water, deionized water, standard rat feed (comfeed PARS 53% (12% water, 11% protein, 4% fat, 7% fiber, 8% ash, 1.1% calcium, 0.9% phosphorus, antibiotics, 53% coccidiostat), 23.5% wheat flour, 23.5% water), endosulfan, rat TNF-αelisa kit, rat HSP70 elisa kit, ether, phosphate buffer saline (PBS) pH 7, 200 µL 100% TCA, 100 µL 1%, sodium thiobarbiturate, 250 µL HCl 1 N, EDTA, dichromate, glacial acetate, H_2O_2, olive oil, adrenaline, sodium bicarbonate (Na_2CO_3) and potassium iodida (KI).

Animal Procedure

Acclimatization

Adult female and male rats were kept for 1 week before being treated to provide the same physical and psychological conditions. During maintenance, white rats were given the same distilled water and food sufficiently.

Endosulfan induction

After a one-week acclimatization period, female rats were injected by PMSG and HCG in accordance with estrous cycle. Female rats were mated with male rats from the same strain. 1 female was mated to 1 male. After mating, female rats were individually placed in a polypropylene cage. Female rats those had been positively pregnant were weighed and distributed randomly divided into 2 groups. The control group (K) without endosulfan induction while the treatment group (P) was induced by endosulfan with a dose of 1 mg/kg BW.

Endosulfan was given by dissolving it in olive oil and and administered orally during 21 days of pregnancy. After the female rats gave birth, endosulfan treatment was terminated. The pups were left to suckle on their mothers. When the pups had reached four weeks of age, 16 pups from each group were terminated and brain tissues were taken to measure levels of MDA, SOD, H_2O_2, AOPP, TNF-α, and Hsp70 of the brain.

SOD, H_2O_2, MDA and AOPP levels assay from brain homogenate

The brain was pounded with mortar at room temperature and added with 1 mL of PBS pH 7.4 until it became liquid. Then taken 5 mL and centrifuged at 8000 rpm for 20 minutes. The supernatant was then taken for measurement of H_2O_2, MDA, SOD, and AOPP levels.

Measurement of brain SOD levels

Incubation was performed on 3 ml of a solution containing 0.05 M Na_2CO_3, 0.1 M EDTA pH 10.2. Furthermore, in the solution was added 100 µL brain homogenate and 100 µL adrenaline with (3.10^{-4}) BM 189 M. Initial absorption measurements

(A_0) was performed with a spectrophotome at 480 nm wavelength. After that, the sample was incubated for 5 min at 30 °C and got the absorbance (A_1).

Measurement of brain H_2O_2 levels

Measurement of H_2O_2 was using a spectrophotometer. At first, making a standard curve. A total of 20 µmol H_2O_2 was added with ml of dichromate:glacial acetic acid (1:3) mixture. Then the mixture was heated in boiling water for 10 minutes. Then the cooled mixture was measured for absorbance at a wavelength of 570 nm. The same procedure was done for 40,60,80,100,120, 140,160 and 180 µmol H_2O_2. A graph was made between the absorbance on the Y axis with levels of H_2O_2 on the X axis to obtain a linear equation.

Preparation of test solution was made with a total of 1 ml of brain homogenate was added 5 ml of PBS pH 7.4. A mixture of 1 ml was taken and added to 2 ml of dichromate:acetate (1:3) mixture and then wrapped in aluminum foil for 30 minutes. The mixed solution was ated using a water bath for 10 minutes at 100 °C. The solution was cooled to room temperature. The solution was then transferred into the cuvette and measured its absorbance using UV-VIS at a wavelength of 570 nm.

Measurement of brain MDA levels

From the last procedure, 200 µL supernatant was taken for measurement of MDA levels. The first thing to do was making MDA standard curve. As many as 0.05 µM MDA standard added 1 mL of distilled water, then placed in Eppendorf tube. Thereafter, 100 µL of 100% TCA, 100 µL sodium thiobarbituric 1%, and 250 µL HCl 1 N were added respectively. Then heated at 100 °C for 20 minutes, and centrifuged 3500 rpm for 10 minutes. Subsequently, 450 µL supernatant was taken and the distilled water added to 3500 µL. Then read with the spectrophotometer with maximum wavelength 540 nm. The same thing was done to 0.025, 0.0125, 0.00625, 0.003125 and 1.56 x 10^{-5} µM MDA. Then making graphs for the relationship between absorbance on the Y-axis and MDA levels on the X-axis to obtain a linear equation.

Measurement of AOPP levels

The test solution was prepared by mixing 200 µL homogenate, 600 L PBS, and 100 L KI 1.16 M. Then the blank solution was prepared by mixing 800 L of PBS, 100 L KI 1.16 M. Test and bl solution were placed for 2 min and then add 200 µL of acetic acid. Absorbance was measured at = 340 nm. The concentration of AOPPs were expressed through A = ε b C with ε = 26 $mM^{-1}cm^{-1}$ and b = 1 cm.

TNF-α and Hsp assay in brain homogenate

Brain tissues were destroyed and homogenized with 1 mL of PBS pH 7.4. The measurement method refers to the rat TNF-α and HSP70 ELISA Kit (Novateinbio, USA). The materials and standard reference were placed at room temperature. As much as 100 µL standard reference, blank sta rd, samples were dissolved in dilution and placed into the well and then incubated for 3 hours at room temperature. The suspense washed with PBS and washed up to 4 times. The conjugates were filled into well as much as 200 µL. The suspensions were washe with washing buffer up to 4 times then 200 µL substrate solution was added to well and incubated 30 minutes at room temperatu The stop solution was added to every well and was read for 30 minutes at a 450 nm wavelength for TNF-α and HSP70.

Data analysis

The data were collected and tabulated. We used comparative analysis for this study. The data were tested using Shapiro W for normality test and Levene for homogeneity test. If the data were normally distributed and homogenous, Student's T-test would be performed with 95% confidence level. If the data were not distributed normally nor homogenous, the data would be analyzed by nonparametric statistic Mann-Whitney U test.

3 RESULTS

Effects of Endosulfan Exposure on Oxidative Stress and Inflammation Markers in The Brain of Pups

SOD levels

SOD plays an important role as an antioxidant in almost every cell exposed to oxygen. It is the first line of defense to fight harms superoxide radicals. Figure 1 showed the SOD levels in the experimental pup's brain.

The control grou had higher SOD levels than endosulfan exposure group. Mann-Whitney U test showed there was a significant difference between groups. It meant that endosulfan exposure reduced the quantity of SOD in the pup's brain.

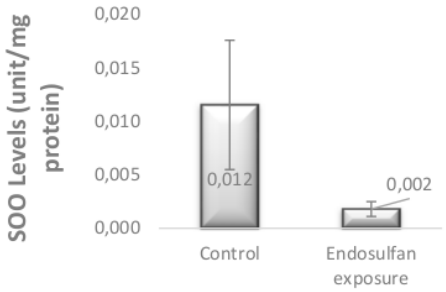

Figure 1. SOD levels of the experimental pup's brain

The control group had higher SOD levels than endosulfan exposure group. Mann-Whitney U test showed there was a significant difference between groups. It meant that endosulfan exposure reduced the quantity of SOD in the pup's brain.

H_2O_2 levels

Another marker of oxidative stress was H_2O_2 levels. H_2O_2 is a toxic substance to the brain. H_2O_2 levels were demonstrated in figure 2.

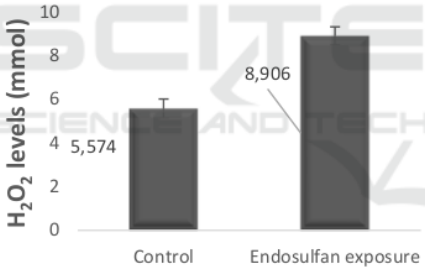

Figure 2. H_2O_2 levels of the experimental pup's brain

H_2O_2 levels in control group were 5.574 mmol and endosulfan exposure was 8.906 mmol. Endosulfan exposure had higher H_2O_2 levels with a statistically significant difference between group (Mann-Whitney U test, $p<0.001$).

MDA levels

MDA is one of the markers of oxidative stress, derived from polyunsaturated fatty acids peroxidation in the cells. MDA levels in the brain of pups were shown in figure 3.

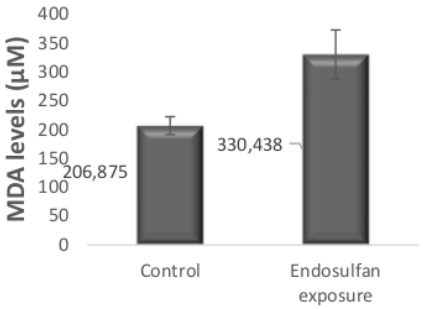

Figure 3. MDA levels of the experimental pup's brain

Figure 3 showed that pups with endosulfan exposure had higher brain MDA levels compared to control group. The statistical analysis with Mann-Whitney U test concluded that there was a significant difference between endosulfan exposure and control group ($p<0.001$). It suggested that endosulfan exposure may increase lipid peroxidation in the brain.

AOPP levels

AOPPs are defined as protein aggregates generated by disulfide bonds created as a result of oxidative stress. The modified proteins from oxidative modification are more stable than those of lipids and making AOPPs better marker for oxidative stress. AOPP levels of this study were presented in figure 4.

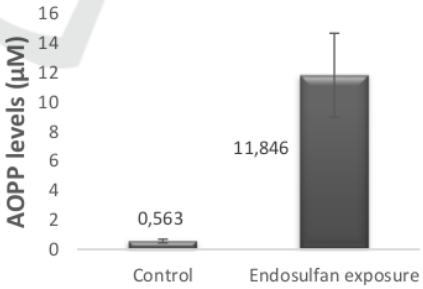

Figure 4. AOPP levels of the experimental pup's brain

Mann-Whitney U test showed a significant difference between groups ($p<0.001$) whereas endosulfan exposure group had worse AOPPs than the control group. It meant endosulfan may increase the damage in the brain of pups.

TNF-α levels

TNF-α is a cytokine that plays a role in systemic inflammation. TNFα levels (ng/L) can be seen in Figure 5.

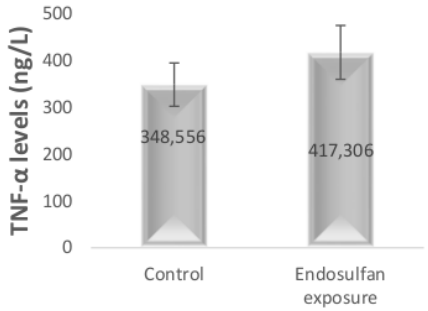

Figure 5. TNF-α levels of the experimental pup's brain

Figure 5 had shown us that endosulfan exposure had higher TNF-α levels compared to control group. Student's T-test statistical analysis showed significant difference among groups ($p<0.001$).

Hsp70 levels

Hsp70 has a significant role to bind the receptor in normal condition. Excessive Hsp70 can make some problems in the stress condition. Hsp70 levels in the experimental pup's brain were demonstrated in figure 6.

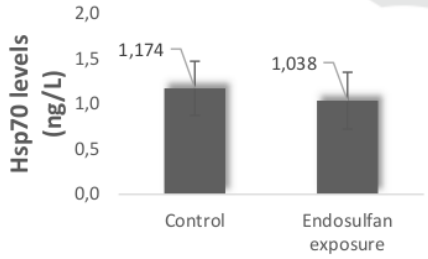

Figure 6. Hsp70 levels of the experimental pup's brain.

The endosulfan exposure gorup had lower Hsp70 levels than the control group, but statistical analysis with Student's T-test showed no significant difference between group ($p=0.218$).

4 DISCUSSION

This study used pup's brain with mothers induced by endosulfan during gestation. A study in India showed that endosulfan can be transferred from mother to fetus through human umbilical cord as much as 60-70%. This was very dangerous because it can disrupt the growth and development of the fetus (Pathak, 2008). Endosulfan was proven to increase the risk of teratogenicity such as cleft lip, limb malformation, eye deformity, hands and feet (Patočka, 2016).

Endosulfan is one of the most harmful pesticides responsible for environmental damage and cause disturbances to the nervous system (Kumar *et al.*, 2014; Wilson, 2014). The nervous system disorders can occur acutely such as hyperactivity, tremors, seizures, coordination disorders even breathing difficulties. A dose of 500 mg/kg can trigger permanent brain damage until death in humans. Farmers with chronic endosulfan exposure show rash and skin irritation (Patočka, 2016). Endosulfan also plays a role in several diseases such as autism spectrum disorder (ASD) and schizophrenia (Wilson, 2014).

The incidence of this neurotoxicity may occur due to impaired GABA function as a major inhibitory neurotransmitter (Patočka, 2016). Endosulfan inhibits the inhibition of [^{35}S]-t-butylbicyclophosphorothionate (TBPS) in the picrotoxinin-binding site of the GABA receptor in the rat brain synaptic membranes, which disrupts chloride flow through GABA-gated chloride channels ($GABA_A$) resulting in decreased neuronal excitability (Jang, 2016; Patočka, 2016). Some of the markers of oxidative stress, antioxidants and inflammation are thought to play some roles in nerve damage in endosulfan-induced rats (Jang, 2016; Lakroun, 2015; Moon, 2016).

SOD is metalloenzymes, which converts superoxide anion (O_2^-) become less reactive oxygen species, ie molecules (O_2) and hydrogen peroxide (H_2O_2). H_2O_2 is formed by SOD activity decomposed into H_2O and O_2 by CAT and/or GPx in the presence of reduced CAT and GSH (Bilodeau, 2014; Ighodaro and Akinloye, 2017). In this study, the pup's brain with endosulfan exposure had significantly lower SOD level than the control group. In contrast, H_2O_2 levels increased significantly in the endosulfan exposure group compared with control group. This indicated that existing SOD can not resolve the H_2O_2 excess in the brain. H_2O_2 is a reactive oxygen species (ROS). At low levels, H_2O_2 plays a role in normal cellular

metabolism, but at high levels, can trigger some diseases. Increased levels of endogenous H_2O_2 can potentiate $GABA_A$ which may trigger H_2O_2-induced brain dysfunction (Penna *et al.*, 2014).

Lipid peroxidation occurs when oxidants such as free radicals attack lipids in cells containing carbon-carbon double bonds, especially polyunsaturated fatty acids (PUFAs) (Ayala *et al.*, 2014). Damage to lipids due to oxidative stress can be seen through MDA; one of the biomarkers of oxidative stress in various diseases including neurobehavior (Khoubnasabjafari *et al.*, 2015). The pups which had received endosulfan exposure in this study had significantly higher MDA levels compared to control group. The increased MDA can lead to neurodegeneration and psychiatry disorders resulting from lipid peroxidation (Joshi and Pratico, 2014; Sultana *et al.*, 2013). A study of subjects exposed to endosulfan suggested that women with preterm delivery had higher levels of α-endosulfan and oxidative stress markers such as MDA compared to women with full-term delivery (Pathak *et al.*, 2010).

AOPP is a marker of oxidative injury and various inflammatory diseases (Alagozlu, 2013). AOPP is derived from oxidative stress (free radical) conditions in proteins and may act as a trigger of inflammatory mediators that will trigger neutrophils, monocytes and T lymphocytes to increase dendritic cell stimulation (Škvařilová *et al.*, 2005). There are many mechanisms for the induction of protein oxidation resulting in different types of protein modification. Detection of protein carbonyl groups is the most commonly used measure, AOPP is also a marker of protein oxidation. Most AOPPs are formed due to increased release of myeloperoxidase (MPO) from activated phagocytes (Hanasand *et al.*, 2012). This study showed that endosulfan exposure in pup's brain can increase AOPP levels up to 20 times greater than controls. High levels of AOPP may play a role in the incidence of neurodegenerative diseases such as parkinsonism and dementia (Demirbilek *et al.*, 2007; Miletić *et al.*, 2017).

The pup's brain exposed by endosulfan in this study had significantly higher levels of TNF-α than the control group. TNF-α is a cytokine that increases in the event of an inflammatory process. TNF-α can cause neurotoxicity by triggering the release of glutamate that can damage the nerves. TNF-α is thought to have a role in neurodegenerative diseases such as Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, and multiple sclerosis (Takeuchi *et al.*, 2006; Ye *et al.*, 2013). In the children with autism, there are elevated levels of TNF-α in lymphocytes, cerebrospinal fluid, and cerebrospinal fluid compared to the control group (Rose *et al.*, 2014).

In the case of brain injury, biomolecular process and pathological biochemistry occur that can cause cell damage, in the form of necrosis and apoptosis (Kayhan, 2008). This molecular damage results in the presence of symptoms of prolonged disability, such as cognitive impairment in the form of decreased attention, concentration, and memory (Demirbilek, 2007; Sultana, 2013). More and more severe the injury a person experiences, the greater the damage both neurons and glial cells as a support network. As a result, sequelae generated even more prolonged. Heat shock protein 70 (Hsp70) includes protein stress, which can be produced by neuron and glial cells that are under stress and inflammatory conditions (Borges, 2012). In this study, there was no significant difference between the groups with endosulfan exposure and control groups.

5 CONCLUSION

The study concluded that endosulfan exposure in pup's brain can trigger some oxidative stress and inflammation disorders. There were increases in MDA, SOD, H_2O_2, AOPP and TNF-α levels significantly accompanied by a decrease in SOD-free radical protection. This can play a role in some neurodegenerative related diseases; Alzheimer's, Parkinson's and psychiatry such as autism and schizophrenia. Endosulfan use should be restricted and prohibited to prevent the bad events.

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