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Endosulfan Increases IL-1β, IL-6, IL-17, and TNF-α Production in Pregnant Rats and Causes Fetal Brain Cell Apoptosis

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Abstract

Endosulfan is a persistent organic pollutant commonly used as an insecticide in Indonesia. It has been reported to cause teratogenic effects, i.e., to decrease humoral activity, produce inflammation, and induce apoptosis in various type of cells. This study investigated the effect of endosulfan on the expression of IL-1β, IL-6, IL-17, and TNF-α in rats (*Rattus norvegicus*), as well as the incidence of fetal brain cell apoptosis. This experiment was carried out on pregnant rats divided into 4 groups: negative control (I), endosulfan: 1 mg/kg (II), 10 mg/kg (III), and 50 mg/kg (IV). The solution of endosulfan was given daily during the 20-day test period. Rat serum was collected for the measurement of IL-1β, IL-6, IL-17, and TNF-α using the ELISA kit. Fetal rat brains were taken and stained with Annexin V for apoptosis detection. The proinflammatory cytokine levels in Groups II, III, and IV were higher than in Group I, with significant increases of IL-1β ($p = 0.016$), IL-6 ($p = 0.009$), IL-17 ($p < 0.001$), and TNF-α ($p < 0.001$). The intensity of Annexin V in 4 groups of rats showed that the incidence of apoptosis increased with increasing endosulfan doses. In conclusion, the administration of endosulfan in pregnant rats increased the expression of IL-1β, IL-6, IL-17, and TNF-α and triggered apoptosis in fetal brain cells.

Keywords: Endosulfan, proinflammatory cytokine, brain cells, apoptosis, pregnant rats

Introduction

Endosulfan ($C_9H_6C_{l6}O_3S$), chemically referred as 6,7,8,9,10,10-Hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepine-3-oxide, is an organochlorine compound from the cyclodiene group, mainly used as insecticide [1]. In Indonesia, the use of endosulfan has been banned since 2001 by Agricultural Minister Regulation 434.1/KPTS/TP.270/7/2001; however, it is in fact still widely used by farmers, as it is an effective pesticide that is relatively cheap and easy to procure [2]. Prior studies reported that vegetables, milk, soil, and water contained high levels of endosulfan [3-5], which is persistent and causes bioaccumulation in the food chain. In Indonesia, a study in West Java detected endosulfan and other organochlorine pesticides residues in catfish [6]. Moreover, analysis of paddy fields in East Java found endosulfan residue in rice and paddy soil that exceeds Maximum Residue Limits by Alberta Environment [2].

Endosulfan enters the body through the skin, lungs, and intestinal tract. It has generally been shown to have high acute toxicity if swallowed or inhaled, and to be slightly toxic upon skin contact. In addition, it irritates the eyes, but does not cause skin sensitization [7-9]. In animals, endosulfan has a neurotoxic effect, resulting from excessive stimulation of the central nervous system [10,11]. The toxicity effects of endosulfan have previously been investigated in various organs such as the brain, kidney, liver, heart, and reproductive system [7,11-14]. It has also been found that pesticides can cause disturbances in the body's immune system through exposure of macrophages inducing the production of NO (nitric oxide), proinflammatory cytokines including IL-1, IL-6, IL-1β and TNF-α, and gene expression of NF-kβ, autoimmune deficiencies, decreases in CD4 cell counts, changes in the balance of T helper cell type 1 (Th1) and type 2 (Th2), decreases in T-lymphocyte responses to mitogens, decreased function of natural killers, and decreased levels of IgA humoral immunity [15-17].

Accumulation of endosulfan in the body is stored in fatty tissues, hence, women are at greater risk of endosulfan-induced toxicity because of their higher percentage of body fat [18]. Prior longitudinal studies mentioned that organochloride pesticide exposure, including endosulfan, was closely related to the incidence of fertility disorders, such as decreased fertility rates and spontaneous abortion [19]. Hematological and biochemical alterations, such as anemia, leukocytopenia, decreased serum protein and albumin values, increased serum urea, creatinine, and estradiol levels, were reported in endosulfan-exposed pregnant rats and rabbits [20,21]. Suppressed humoral and cell mediated immune responses of pregnant rats exposed to endosulfan, citrinin, or both, during gestation days 6 - 20 [22]. In endosulfan-exposed pregnant women, endosulfan metabolites are found in the placenta, umbilical cord blood, and breast milk [23-26]. Fetuses can be exposed through placental transmission during pregnancy, as well as breastfeeding. This indicates that the compound can affect the development of the fetus [25].

Endosulfan can cause apoptosis, necrosis, and a mixture of both. Necrosis increases the incidence of cytotoxicity, which is known for the insulation of thymosin in rats with elevated levels of endosulfan. Cytotoxicity caused by endosulfan is directly proportional to the amount and duration of endosulfan exposure [11,14]. Endosulfan also induces toxicity in cell cultures, namely sustainable human T cell leukemia, through apoptosis events [27]. Other study has reported that apoptosis events in liver, kidney, and spleen cells were seen in pregnant rats and their fetuses [28].

In modern life, people and animals are exposed to varying degrees to the chemicals found in air, water, food, or other consumer products. Humans are exposed to hundreds, or even thousands, of chemicals in food alone. The effects of chemicals on humans and the environment are, thus, worthy of special attention. In this study, we aimed to investigate the effect of various concentrations of endosulfan on the level of IL-1β, IL-6, IL-17, and TNF-α as the proinflammatory cytokines, as well as to detect the apoptosis levels in fetal rat brain cells caused by endosulfan neurotoxic activity.

Materials and methods

Research design

This study was a true experimental laboratory research with a randomized posttest-only control group design. The simple random sampling method was employed to obtain the study subjects.

Test animals

The test animals used as the study subjects were Wistar strain female white rats (*Rattus norvegicus*), 8 weeks old, pregnant, weighing 120 - 150 grams. After a one-week acclimatization period, the female rats mated with male rats of the same strain. After mating, each female rat was caged in an individual box and labeled according to the treatment group. The box was made from polypropylene sized 7×9.5×7 inches and covered with woven wire. Rats were given standard normal diet Comfeed PAR-S 20 gr/day and ad libitum access to water. A room with optimum temperature (22 - 24 °C), 50 - 60 % humidity, and 12:12 light-dark cycle was maintained regularly. All experimental procedures were performed in accordance to the guidelines of the Animal Ethics Committee of the Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia, and performed quadruplicate (4 times replication).

Provision of endosulfan in rats

Endosulfan insecticide 350 g/L (brand Akodan 35 EC, Saudara Tani Agrolestari Ltd, Indonesia) was obtained from local farmer's market. Twenty pregnant rats were weighed and randomly distributed into 4 groups, consisting of 5 rats each: Group I (negative control/without endosulfan administration), Group II (endosulfan 1 mg/kg), Group III (endosulfan 10 mg/kg), and Group IV (endosulfan 50 mg/kg). Solutions of endosulfan dissolved in olive oil were then given orally each day during the 20-day pregnancy period. After 20 days, the female rats were sacrificed. The blood of the adult female rats was then collected for the preparation of IL-1β, IL-6, IL-17, and TNF-α expression measurement. The fetal brains were collected and the apoptosis levels were observed.

Measurement of IL-1β levels

The IL-1β levels were determined using the ELISA kit (Rat IL-1β ELISA Kit, Koma Biotech, South Korea) with the procedure outlined in the manufacturer's instructions. Each microtiter well was filled with 300 µL of washing solution and placed on a tissue in an inverted position. 100 µL of IL-1β samples were put in the wells and incubated at ambient temperature for 2 h. The samples were then washed 4 times in washing solution and incubated with 100 µL of antibody solution (0.35 µL/mL) at ambient temperature for 2 h. Next, 100 µL of diluted color development enzyme (dilution 1:20) was added to the samples and incubated at ambient temperature for 30 min. The samples were again washed 4 times in washing solution and incubated with 100 µL of color development enzyme at ambient temperature. Approximately 22 - 32 min later, 100 µL stop solution (2N sulfuric acid) was added. ELISA measurement was conducted at 450 nm.

Measurement of IL-6 levels

Levels of IL-6 were determined using the ELISA kit (Rat IL-6 ELISA Kit, Koma Biotech, South Korea) method according to the manufacturer's manual procedure. A total of 100 µL of each sample was put into the microtiter plates and incubated at a temperature of 37 °C for 2 h. The samples were subsequently washed with 400 µL of washing buffer for 5 min. Following this, 100 µL of antibody solution was added to each well, except for the blank well. The microtiter plates were incubated at a temperature of 37 °C for one hour and then washed with 400 µL of washing buffer. Each well was stocked with 100 µL of conjugates. The microtiter plates were incubated at a temperature of 37 °C for 30 min. The samples were then washed again as in the previous procedure before the addition of 100 µL of tetramethylbenzidine (TMB) substrate solution and incubated at ambient temperature for 30 min. Then, 100 µL of blocking solution (HCl) was added to each well for 5 min. The reading of absorbance values was performed at OD 450 nm.

Measurement of IL-17 levels

IL-17 levels were measured using the ELISA kit (Rat IL-17 ELISA Kit, Koma Biotech, South Korea). A total of 100 µL of assay buffer in the blank well and the samples were dissolved in a solvent, placed in different tubes, and then incubated at ambient temperature for 3 h. The suspension was washed with phosphate buffer saline (PBS) 4 times. Then, 200 µL of conjugate was added to each tube. Serum samples were incubated at ambient temperature for 2 h. Next, the suspensions were washed with washing buffer 4 times; 200 µL of substrate solution was then added to each tube and incubated at ambient temperature for 30 min. Stop solution was added to each tube to stop the reaction. ELISA results were read at 450 nm after 30 min.

Measurement of serum TNF-α

The TNF-α levels were assessed using the ELISA kit (Rat TNF-alpha ELISA Kit, Koma Biotech, South Korea) according to the kit instructions. 100 µL buffer solution was put into the wells (including the well containing the blank solution), and then 100 µL TNF-α standard 1 - 7 was inserted into the other wells. Then, 100 µL of sample was put into the wells. The detection process was similar to that used for the measurement of IL-6 levels.

Apoptosis in fetal brain cells

The levels of apoptosis activity in the fetal rat brain cells were detected using Annexin V and a propidium iodide (PI) assay kit (KeyGen Biotech, China) to detect changes in the cell membranes. Annexin V binds to the phosphatidylserine on the cell surface undergoing apoptosis [29]. Samples were added to the slide preparations and washed using PBS pH 7.4. The samples were then incubated with 20 mg/mL of proteinase K at a temperature of 37 °C for 15 min. Annexin V dyeing solution and propidium iodide were added to the sample sand incubated at a temperature of 15 - 25 °C for 10 - 15 min. The apoptosis was observed using a fluorescent microscope (Bestscope, China) at 400x magnification. Apoptosis was indicated by green fluorescence of Annexin V with a corresponding color intensity of endosulfan.

Statistical data analysis

The data obtained from the test administration of endosulfan on IL-1β, IL-6, IL-17, and TNF-α levels in the pregnant rats as well as fetal brain cell apoptosis were statistically processed using SPSS for Windows. Data were checked for normality and the homogeneity of variance. The data were then analyzed correlatively using Pearson correlation test. Moreover, the comparation of different doses of endosulfan on the proinflammatory cytokines levels was analyzed using one-way ANOVA test, followed by a Tukey HSD post-hoc test. A simple linear regression analysis was performed to measure the effect size of endosulfan on proinflammatory cytokines levels. A significance level of 0.05 ($p = 0.05$) and a 95 % confidence interval ($\alpha = 0.05$) were considered statistically significant [30].

Results and discussion

Endosulfan association to increased serum levels of IL-1β, IL-6, IL-17, and TNF-α

This research used endosulfan in various doses (1, 10 and 50 mg/kg) in pregnant rats. The presence of endosulfan exposure has been known to improve several pro-inflammatory cytokines [31,32]. Therefore, we further investigated the effect of endosulfan on the proinflammatory cytokines IL-1β, IL-6, IL-17, and TNF-α.

Table 1 Mean concentrations of IL-1β, IL-6, IL-17, and TNF-α serum in pregnant rats exposed for 20 days to various amounts of endosulfan

Group	IL-1β (pg/mL)	IL-6 (pg/mL)	IL-17 (ng/mL)	TNF-α (pg/mL)
I	946.00±154.4^{a}	540.00±176.6^{a}	8.67±4^{a}	90.33±57.5^{a}
II	1102.00±315.4^{b}	556.00±454.3^{a}	18.35±7.6^{b}	138.33±56^{b}
III	872.00±216.3^{c}	996.00±393.7^{ab}	26.67±4.7^{c}	289.00±145.6^{c}
IV	604.00±131.6^{d}	1244.00±223.9^{b}	30.72±8.6^{d}	705.00±199.7^{d}

Group I: negative control; Group II: endosulfan 1 mg/kg BW; Group III: endosulfan 10 mg/kg BW; Group IV: endosulfan 50 mg/kg BW. The values were mean of 5 samples in each groups after 4 replication ±SD
^{a,b,c}Means with different superscripts differ significantly ($p < 0.05$)

As shown in **Table 1**, the average serum level of IL-1β in Group I (negative control) was 946.00±154.43 pg/mL. There was a tendency that, as the endosulfan dose increased, the concentrations of IL-1β decreased, as can be seen in Group III and IV. This was indicated by a negative correlation ($r = -0.473$; $p = 0.004$), meaning that the administration of endosulfan in larger doses lowered IL-1β levels. An ANOVA test on different doses showed a significant p-value ($p = 0.016$). Meanwhile, the regression test result indicated that endosulfan treatment impacts the production of IL-1β by 37.8 %.

A similar result was also seen in the measurements of IL-6 level. The concentration of IL-6 level was increased in Group II, III, and IV, compared to the negative control group (Group I), with the highest

value seen in Group IV, with 1244±223 pg/mL. Pearson test showed a positive correlation between endosulfan dose and IL-6 level ($r = 0.595$; $p = 0.006$). Based on the post-hoc test, Group IV showed a significant difference in IL-6 level to Group I ($p = 0.019$) or Group II ($p = 0.022$). Furthermore, the regression test result indicated that endosulfan administration increased IL-6 levels by 35.4 %.

The administration of endosulfan had different effects on the IL-17 levels of each group. Based on the post-hoc test, there were significant differences in the levels of IL-17 between Group I and Group II, and III and IV ($p = 0.018$, $p < 0.001$ and $p = 0.002$, respectively), as well as between Group II and Group III ($p = 0.018$). Meanwhile, the IL-17 levels were found to differ significantly between Group IV and Group I ($p < 0.05$). Increased dose of endosulfan was positively correlated with increased IL-17 level ($r = 0.771$; $p = 0.020$). In addition, the regression analysis showed that endosulfan increased IL-17 levels by 50.4 %.

Table 1 also shows the average serum levels of TNF-α. TNF-α level was lowest in Group I (90.33±57.51 pg/mL) and increased significantly with increasing doses of endosulfan ($r = 0.803$; $p < 0.001$). There was a difference between endosulfan doses ($p = 0.016$). The regression test result showed the effect of endosulfan on TNF-α levels to be 77.4 %.

In this study, we have shown that endosulfan has the potential to alter the humoral immune system of pregnant rats through increased production of proinflammatory cytokines, namely IL-1β, IL-6, IL-17, and TNF-α. This result was also in line with previous studies showing that endosulfan exposure could elevate IL-6 and IL-8 [32], IL-1β [33], and TNF-α [34,35] production in rats. In our study, the IL-1β level was at its peak by endosulfan exposure of 10 mg/kg BW, and tended to decline with higher doses. Similarly, a prior study showed that high endosulfan dose (20 μg/ml) also decreased cytokine production, which is LPS-induced TNF-α [35]. IL-1β production could be suppressed by IL-1β inhibitor, such as IL-1RA and sIL-1RII [36]. However, low levels of IL-1β can still induce inflammation [37]. Further research is needed to look at the effect of the inhibitor on the production of proinflammatory cytokines.

Endosulfan has been shown to suppress the cellular and humoral immune system in mice [15,16]. Furthermore, a comprehensive review concluded that pesticide exposure, including endosulfan, has an immunotoxic effect against animals and human beings [17]. Agents in HUVEC-C cell cultures led to increased levels of inflammatory factor (IL-6 and IL-8) secretions and mRNA expression, as well as causing endosteal dysfunction [32]. Increasing concentrations of inflammatory mediators in the body indicate that protective measures are being taken against the causative agent of the cell injury. In this process, the body defends itself by dissolving, destroying, or neutralizing the pathological agent [38]. A recent study suggested that a molecular mechanism, through the activation of nuclear factor-kappa β (NF-kβ) by intracellular redox status, external oxidants, and internal antioxidant systems, i.e., induced by endosulfan exposure, triggers production of inflammatory cytokines [39].

Endosulfan is a lipophilic pesticide compound that is stable in the environment and that blocks phagocytic metabolic activity in the peripheral blood [40,41]. This indicates that, even at low concentrations, endosulfan exposure can harm the immune system in the long term. It thus causes immunodeficiency in humans and animals exposed to endosulfan [41]. Endosulfan is classified as very toxic (Toxicity Category 1) by the EPA in the United States, so its use there is very limited when compared with that in emerging countries [42].

Fetal rat brain cell apoptosis index and endosulfan exposure

Apoptosis in fetal rat brain tissue was detected by means of Annexin V, to probe apoptosis, and propidium iodide, to probe cell necrosis. Annexin V is a phosphatidylserine (PS) binding protein; that is, it binds to the PS proteins, which flip from the inner to the outer membrane during apoptosis [29]. The observations, made by confocal microscopy, showed that endosulfan stimulates apoptosis in fetal rat brain cells.

Table 2 Apoptosis index in rats fed with various doses of endosulfan in each group

Group	Apoptosis Index (Mean ± SD)
I	1935.48 ± 343.63 [a]
II	2360.29 ± 458.72 [ab]
III	2388.61 ± 278.79 [ab]
IV	3697.92 ± 253.62 [c]

Group I: negative control; Group II: endosulfan 1 mg/kg BW; Group III: endosulfan 10 mg/kg BW; Group IV: endosulfan 50 mg/kg BW. The values were mean of 5 samples in each groups after 4 replication ± SD
[a,b,c]Means with different superscripts differ significantly ($p < 0.05$)

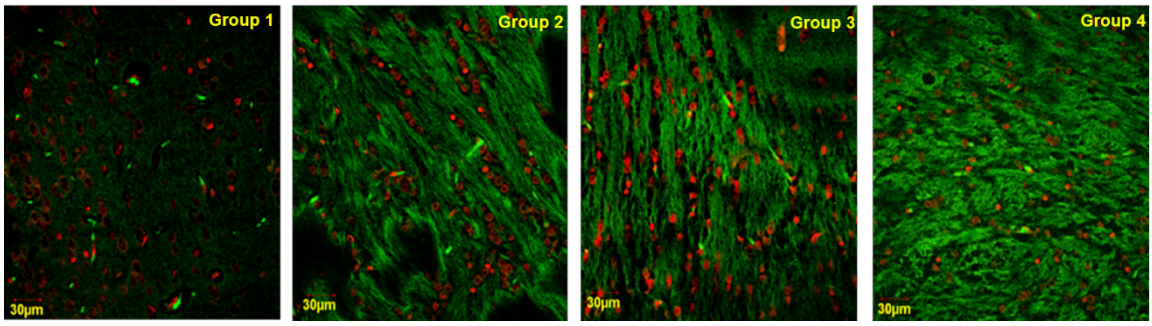

Figure 1 Quantification of fetal brain cell apoptosis by Annexin V for green fluorescent and propidium iodide for red staining; Group I: negative control; Group II: endosulfan 1 mg/kg BW; Group III: endosulfan 10 mg/kg BW; Group IV: endosulfan 50 mg/kg BW

According to **Table 2**, endosulfan significantly increased brain cell apoptosis in rat fetuses in Group IV compared to Group I, II, and III. This result, also depicted clearly in **Figure 1**, shows that the incidence of apoptosis increased with increasing endosulfan doses. The intensity of Annexin V seems weak in Group I, but begins to increase in Groups II (endosulfan 1 mg/kg) and III (endosulfan 10 mg/kg). The intensity of Annexin V is strongest in Group IV (endosulfan 50 mg/kg).

Apoptosis, or cell death, is an active and organized response to physiological stimuli or toxins from the environment. In our study, the higher the dose of endosulfan, the more apoptosis was detected in fetal rat brain cells. This result was in agreement with a prior study in rat endothelial cells, which suggested that an exposure of various concentrations of endosulfan had significantly induced apoptosis via a death receptor pathway, due to its ability to enhance oxidative stress [43]. Another study demonstrated increased levels of lipid peroxidation, decreased superoxide dismutase and catalase antioxidant, and apoptotic liver and kidney cells of pregnant rats resulting from endosulfan intoxicity [44].

Previous studies also reported the teratogenic effects of endosulfan and citrinin administration, given alone or in combination to pregnant rats [45,46]. Although it caused no deaths, the administration of endosulfan and citrinin led to weight loss in pregnant rats, increased numbers of fetal deaths, and increased numbers of fetal resorptions. The lengths and weights of the fetuses decreased, while visceral and skeletal anomalies increased significantly [46]. Furthermore, a study among pregnant women found that the average concentration of endosulfan in the placentas of women whose pregnancies were affected by neural tube defects (NTDs), such as anencephaly or spina bifida, was slightly higher than that in women who delivered healthy infants [47].

The above explanations reinforce the assumption that endosulfan is teratogenic, causes disabilities, and can inhibit the growth of the fetus via the process of apoptosis. Endosulfan, as a lipophilic compound,

tends to accumulate in fatty tissue and can be transferred to the fetus through the placenta [48-50]. Endosulfan can also compete with estradiol to bind to estrogen receptors; if estradiol cannot bind to its receptor, it cannot induce cell growth and development [51]. The evidence above demonstrates conclusively that endosulfan not only causes the death of fetal brain cells but also causes increased levels of proinflammatory cytokines that may affect nerve function and developmental stage nerve processes [52].

In recent years, more studies have emerged about the relationships between pro-inflammatory cytokines and fetal brain damage [53]. Meygeri *et al*. found that tumor necrosis factor (TNF)-α as one of the pro-inflammatory cytokines may influence brain and blood-brain barrier development [52] and that intrauterine infection and a pro-inflammatory cytokine response are involved in the pathogenesis of preterm brain white matter damage. Furthermore, pro-inflammatory cytokines are included in the pathogenesis of preterm brain white matter damage [55,56]. One of the mechanisms is through apoptosis inducing factor. A study by Cai *et al*. revealed that IL-1 injection caused neuronal death and delayed myelination in fetal rats [57]. Tumor-necrosis factor-α triggers apoptosis inducing factor and leads to cell death in mature oligodendrocytes. Tumor-necrosis factor-α is associated with reduced myelination through reduced staining for myelin basic protein, which is considered a hallmark of inflammation-associated diffuse white matter damage in neonatal rats [58]. However, more research is still required to reveal the details of the connection between inflammatory factors and cell death caused by exposure to endosulfan, particularly in pregnant women. Further work has the potential to form the basis for new scientific information regarding the dangers of xenobiotic agents for human life.

Conclusions

We conclude that endosulfan exposure during the gestation period of organogenesis in rats (*Rattus norvegicus*) causes increased serum levels of proinflammatory cytokines (IL-1β, IL-6, IL-17, and TNF-α) and triggers the apoptosis of fetal rat brain cells.

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