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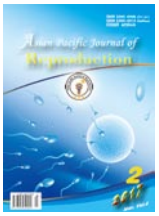

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The effect of oral lead acetate exposure on bax expression and apoptosis index granulose cells antral follicle in female wistar rat (*Rattus norvegicus*)

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ABSTRACT

Objective: To prove the effect of administered orally lead acetate exposure on Bax expression and the apoptosis index of granulosa cells on antral follicle female albino rats Wistar strain (*Rattus norvegicus*). **Methods:** Post-test only control group, using female albino rats Wistar strain aged 10-12 wk (reproductive age) weighing 100-200 g. Twenty-four animal samples were classified into one control group and three groups exposed to lead acetate in doses of 30, 100, and 300 ppm, respectively. Lead acetate was administered orally through a feeding tube over 30 d. Bax expression of granulosa cells on antral follicle was checked using immunohistochemistry, and apoptosis index were examined using Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). **Results:** Lead increases apoptosis index on doses of 300 ppm. The increase of the Bax expression granulosa cell on antral follicle was not statistically significant. **Conclusion:** The exposure of lead acetate administrated orally can increase apoptosis index of granulosa cell on antral follicle although it doesn't affect Bax expression.

1. Introduction

Environment pollution is a highly debated topic, one factor being the effect of heavy metals, for example, lead. Lead pollution is found in mining areas, industrial waste, vehicle emissions, pesticides, toddlers' toys, drinking water pipe installations, and cosmetics[1]. The pollution can spread through the air, water, and affecting mostly daily meals[2]. Previous research shows that the content of lead inside feather shells on Lekok beach, Pasuruan Indonesia, reached 2.031 ppm beyond the legal limit of lead by Indonesian National Standard(SNI) 7387: 2009, 1.5 mg/kg (ppm)[3].

Lead pollution can cause toxic effects on several body organ systems, such asthe central nervous system, the peripheral nervous system, the cardiovascular system, the endocrine system, immunity system, gastrointestinal system, hematology, kidneys, and male/female reproduction system[4]. Research about the effect of lead effect on women's reproduction systemis still limited.

Lead exposure binds to sulfhydryl proteins, alters calcium homeostasis and lowers the level of available sulfhydryl antioxidant reserves in the body. It increases the production of reactive oxidative species (ROS) causing an imbalance that will result in oxidative stress. Oxidative stress will damage DNA and trigger apoptosis through intrinsic pathways; involve Bax as a pro-apoptosis protein and Bcl-2 as an anti-apoptosis protein[5]. Previous research has examined the apoptosis caused by lead exposure inside several organs. Lead exposure can cause apoptosis inside the liver, kidneys, brain cells, and mesenchymal stem cells inside the spines[6–9].

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Lead passes through membrane easily and accumulates in softorgans, like the ovaries[5]. From the result of the research above, lead probably causes apoptosis in the granulosa cell on antral follicle ovarium. Therefore, the researcher is interested in proving the effect of lead acetate exposure,orally administered, on the apoptosis mechanism intrinsic pathway by looking at the protein expression involved, namely Bax.

2. Materials and methods

This research is a true experimental design using a randomized post-test only control group approach. Samples consist of 24 female albino Wistar rats classified into one control group and three groups exposed to lead acetate at doses of 30, 100, and 300 ppm, respectively[10]. This research has been stated to be properly conducted by the Ethics Commission for Health Research at Brawijaya University Malang.

2.1. Observation of rats

Rats were acclimatized a week before being treated. They were put into plastic basins, sized 45.0 cm × 35.5 cm × 14.5 cm, sealed by strong wires. The bottom of the cages were covered with (0.5-1.0) cm husk and changed once every 3 d. Each cage contained four rats. Indoor lights were designed to be 12 h light and 12 h dark with a room temperature of (27–28) ℃. Rats were given standardized food in the form of Merck Comfeed pellets (round) once a day in the afternoon. Drinking water was given using *ad libitum*. Weight measurement was conducted once a week.

2.2. Lead acetate and treatment

This research uses lead (II) acetate trihydrate (Merck) in the form of white powder. Lead acetate solution is made by combining 1 000 mg lead powder with 1 L Aqua Bidest using a magnetic stirrer. It is then divided into doses of 30, 100, and 300 ppm using the following formula:

$C_1 . V_1 = C_2 . V_2$

Lead acetate was administered orally by feeding tube 1 mL/d, for 30 d. Determination of the estrous cycle of the rats was conducted on the last treatment day to determine the proestrus phase.

2.3. Dissection of rats

Rats wereanesthetized by venereal injection 1% in their thighs with a dose of 0.2 mL. The ovaries were taken and put into a container of fixative buffered formalin solution (10%), soaked for 12-24 h to be formed into an immunohistochemistry preparation.

2.4. Bax expression by immunohistochemistry

A slidewas deparaffinized by washing it in xylol three times for 3 min each time. It was dehydrated by soaking it in absolute ethanol (2 × 10 min), ethanol 90% (1 × 5 min), ethanol 80% (1 × 5 min), ethanol 70% (1 × 5 min) and sterilized Aqua Dest (3 × 5 min). Antigen retrieval was conducted by heating up the slides inside a water bath in 95 ℃ for 20 min, then cooling them and washing them in phosphate-buffered saline (PBS) (3 × 5 min).

Immunostaining was performed after blocking 'endogenous peroxidase with H_2O_2 (3%) in methanol (20 min), incubated at room temperature (15 min), then washing in PBS for 5min, and two times for 2 min. Then unspecific proteins were blocked using background sniper, 15 min for incubation, PBS wash 1 × 5 min dan 2 × 2 min. Secondary antibody incubation for 60 min, PBS wash 1 × 5 min dan 2 × 2 min. Streptavidin-horseradish peroxidase (SA-HRP) incubation for 40 min, PBS wash 1 × 5 min dan 2 × 2 min. Rinsed using Aqua Dest 3-4 times, 3,3'-Diaminobenzidine (DAB) chromogen was applied for 3 min, then it was rinsed using Aqua Dest 3-4 mins. Afterward, counterstaining with Mayer's hematoxylinwas performed as follows: incubation 1 min, Aqua Bidest 3 drops, incubation again for 5 min, rinsed using Aqua Dest. Specimens were mounted then sealed using cover glass.

2.5. Apoptosis index by TUNEL assay

Rehydration was performed in xylol (2 × 5 min), absolute ethanol (2 × 5 min), ethanol 90% (3 min), ethanol 80% (3 min), ethanol 70% (3 min), Tris-buffered saline (TBS) wash (1 × 5 min). Then the specimens were permeabilized with 100 μL Proteinase K solution for 20 min, followed by TBS wash (1 × 5 min) to inactivate the endogenous peroxydation.

The inactivation of endogenous peroxydation step was followed by addition the 100 μL TdT equilibration buffer to specimens, incubated for 30 min. Then, simultaneously added again 40 μL TdT labeling reaction mix, and incubated 1.5 h.

Furthermore, 100 μL of buffer were added tothe specimen and incubated for 5 min, followed by TBS wash (1 × 5 min). Specimens were added with 100 μL of blocking buffer, incubated 10 min, added with 100 μL 1× conjugate liquid, incubated 30 min, and TBS wash (1 × 5 min). DAB solution was applied for 15 min, then the specimen was washed with water. Counterstaining was performed using Mayer's hematoxylin, incubated for (1-3) min. The specimen was mounted using Entellan (Merck) and covered with glass.

2.6. Data analysis

Bax expression was analyzed using one-way ANOVA. Apoptosis index was analyzed by Kruskal–Wallis and Mann–Whitney U-test (α=0.05). Stastistical analysis was conducted using SPSS 20.0 software for Windows.

3. Results

3.1. The effect of lead acetate exposure on Bax expression of granulosa cell on antral follicle

Bax expression is interpreted in Figure 1. According to Figure 2, P>0.05, which means that there is no significant difference in Bax expression of granulosa cell on antral follicle among observed groups. In other words, lead acetate exposure administered orally does not affect Bax expression of the granulosa cell antral follicle.

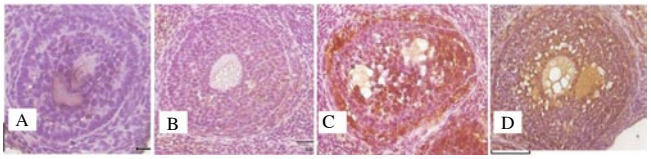

Figure 1. Bax expression on granulosa cells on antral follicle by imunohistochemistry (400 ×).
A. Granulosa cell on antral follicle control group has the least expression of Bax; B. Granulosa cell onantral follicle treatment group doses 30 ppm, expresses more Bax than control group does; C. Granulosa cell on antral follicle treatment group doses 100 ppm, expresses more Bax than treatment group doses 30 ppm and control group; D. Granulosa cell on antral follicle treatment group doses 300 ppm, expresses Bax the most among the other observed groups. Bax expression is marked with brown chromogen in granulosa cell.

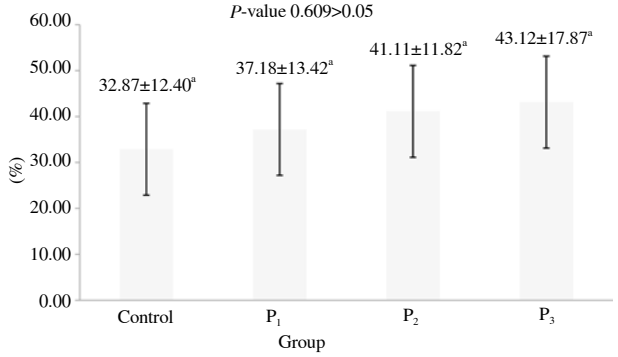

Figure 2. Bax expression of granulosa cell antral follicle.

3.2. The effect of lead acetate exposure administered orally on apoptosis index of granulosa cell on antral follicle

The apoptotic index is interpreted in Figure 3. Based on the Kruskal–Wallis test, P=0.027, less than 0.05, which means that there is a significant difference in apoptosis index of granulosa cell on antral follicle among the observed groups (Figure 4). In other words, lead acetate exposure administered orally has an effect on the apoptosis index granulosa cell antral follicle. Based on the Mann–Whitney U-test, there is a significant difference between the treatment group that received doses of 300 ppm.

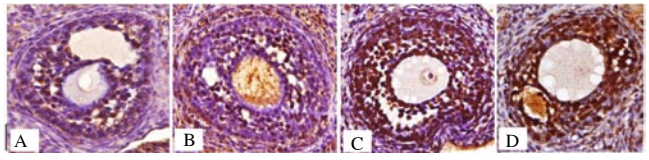

Figure 3. Apoptosis on granulosa cell antral follicle by tunel assay (400 ×).
A. Granulosa cell on antral follicle control group has the least apoptosis; B. Treatment group doses 30 ppm, has more apoptosis granulosa cell on antral follicle than control group does; C. Granulosa cell on antral follicle treatment group doses 100 ppm has more apoptosis than control group's and treatment group doses 30 ppm; D. Granulosa cell on antral follicle treatment group doses 300 ppm has the most apoptosis among all observed groups. Cells which get apoptosis appears brown in their nucleus.

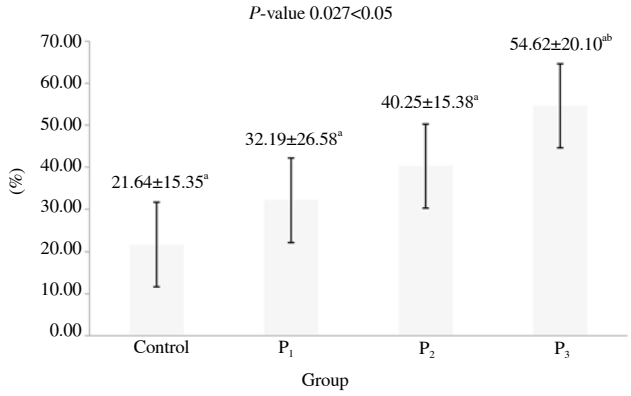

Figure 4. Bax expression of granulosa cell antral follicle.

4. Discussion

4.1. The effect of lead acetate exposure administered orally on Bax expression of granulosa cell on antral follicle

Bax is a protein belongs to Bcl-2 proteins, as pro-apoptosis protein that passing through intrinsic pathways. In normal conditions, apoptosis in ovarian follicles happens when the fetus becomes mature inside secondary granulosa cell and antral follicle, which express Bcl-2 and Bax. In this case, the granulosa cell that expresses Bax is the granulosa cell antral follicle. Based on this research, it is concluded that lead acetate exposure administered orally cannot increase Bax expression of the granulosa cell on antral follicle significantly. However, Bax expression increases as the doses increase[11,12].

The increase of Bax expression as a pro-apoptosis protein is based on a lead mechanism. Lead acetate enters the body orally and is then absorbed by the digestive system and metabolized in the blood. Afterward, lead will damage the hypothalamus, which automatically decreases the follicle stimulating hormone (FSH) level. The decrease of FSH will increase Bax expression and decrease Bcl-2 expression, then apoptosis occurs[11].

Another lead mechanism can be found in the increase in ROS. The lead within the blood binds the complex of sulfhydryl that could inactivate the work of glutathione, a body defense for filtering free radical agents[12]. Continuous lead exposure over a long time will make ROS and antioxidants within the body become imbalanced, thus leading to oxidative stress. Oxidative stress will destroy DNA, then stimulate apoptosis through intrinsic pathways. Intrinsic pathway apoptosis or the mitochondrial pathway is caused by a signal within the cells, involving the protein Bcl-2 family, like Bcl-2 and Bax.

Besides intrinsic pathways, apoptosis caused by lead could happen

through extrinsic pathways and directly affecting the mitochondria. It is supported by previous research that apoptosis happens in ovarium externally, stimulated by death receptor bound on the cell surface,such as Fas/FasL system, so that it can be recognized by a Fas-Associated protein with Death Domain (FADD). The FADD complex will change procaspase-8 to be active caspase-8. Caspase-8 changes procaspase-3, -6, and 7 to be active caspase-3, -6, and -7; thus, apoptosis occurs[13,14]. Apoptosis caused by lead could also happen through the direct pathway to the mitochondria. Flora *et al.*[15] stated that lead can resemble calcium increase intracellular metabolism which is able to stimulate mitochondrial depolarization, thus its pores will open. The opening of mitochondrial pores will release cytochrome C that soon binds with Apaf-1, activate caspase-9, and activate caspase-3 as an executor caspase, and finally, apoptosis occurs[15]. This is supported by another research, which proved that caspase-3 increases significantly on granulosa cell antral follicle as the effect of lead acetate exposure administered orally with the same doses and time as this research.

4.2. The effect of lead acetate exposure administered orally on apoptosis index granulosa cell on antral follicle

The result of statistical analysis shows that lead acetate exposure administered orally can increase apoptosis index of granulosa cell antral follicle significantly. The damage to DNA due to lead exposure can stimulate follicle apoptosis through an extrinsic pathway, such as apoptosis due to granulosa cell. In addition, there are other pathways for apoptosis caused by lead to occur, such as extrinsic pathways and direct pathways towardsthe mitochondria. Apoptosis in extrinsic or intrinsic pathways will end at caspase-3 as executor caspase.

The research on apoptosis as the effect of lead exposure has been frequently focused on organs other than the ovary and proven by TUNEL assay. Lead acetate can cause apoptosis in the mesenchyme salivary gland, liver, kidneys, brain, spine, epithelial endometrium, and ductus[6–9,16].

It is matched with the above theory that lead exposure can cause apoptosis within the ovary, as in the follicular granulosa cells. Bax expression as the first sign of apoptosis doesnot increase significantly. This proves that apoptosis probably happens through another pathway/other pathways, which is beyond the scope of this research. Lead acetate exposure administered orally increases apoptosis index granulosa cell on antral follicle at doses of 300 ppm, but cannot increase Bax expression of granulosa cell on antral follicle significantly.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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