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

Dian Aby Restanty1,2, Setyawati Soeharto2, I Wayan Agung Indrawan3,4

1State Health Polytechnic of Ministry Malang, Jl. Besar Ijen 77C, Malang, East Java, 65612, Indonesia
2Division of Pharmacology Science, Faculty of Medicine, Brawijaya University, Jl. Veteran Malang, East Java, 65145, Indonesia
3Division of Obstetrics Gynecology of Faculty of Medicine, Brawijaya University, Jl. Veteran Malang, East Java, 65145, Indonesia
4Dr. Saiful Anwar Hospital, Jl. Jaksa Agung Suprapto No.2, Malang, East Java, 65112, Indonesia

ARTICLE INFO

Article history
Received 3 January 2017
Revision 10 February 2017
Accepted 16 February 2017
Available online 1 March 2017

Keywords:
Lead acetate
Bax
Apoptosis

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 as the 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 systems 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].

Corresponding author: Dian Aby Restanty, State Health Polytechnic of Ministry Malang, Jl. Besar Ijen 77C, Malang, East Java, 65612, Indonesia.
Fax: (0331) 486613.
E-mail: dianaby87@yahoo.co.id
Lead passes through membrane easily and accumulates in soft organs, like the ovaries [5]. From the result of the research above, lead probably causes apoptosis in the granulosa cell on antral follicle ovum. 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 Committee 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) °C. Rats were given standardized food in the form of Merck Comfeed pellets (round) once a day in a room temperature of (27–28) °C. 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:

\[ C_1 \cdot V_1 = C_2 \cdot V_2 \]

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 were anesthetized 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 slide was 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 °C 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 H2O2 (3%) in methanol (20 min), incubated at room temperature (15 min), then washing in PBS for 5 min, and two times for 2 min. Then unspecified proteins were blocked using background snipper, 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 hematoxylin was 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 peroxidation.

The inactivation of endogenous peroxidation step was followed by addition the 100 µL TdT equilibrium 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 to the specimen and incubated for 5 min, followed by TBS wash (1 × 5 min). Then, specimens were added with 100 µL of blocking buffer, incubated 10 min, added with 100 µL 1x 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). Statistical 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.
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.

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 towards the 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 does not 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.

Acknowledgement

The authors thank to the Health Polytechnic of Malang and
Brawijaya University Malang for facilitating this research.

References

[1] Hartini E. Kadur plumbum (pb) dalam umbi bawang merah di kecamatan
karsa kabupaten brebes. J Visisk 2011; 10(1): 69–75.
[2] WHO. Air Quality Guidelines. 2nd ed. Copenhagen, Denmark: Regional
Office for Europe; 2001.
[3] Fitriyah. Pengaruh Bahan Peredam dalam Menurunkan Kadar Logam
Berat Timbal pada Kerang Bulu. Master thesis. Malang: Maulana Malik
Ibrahim University; 2007.
[4] Nordberg GF, Fowler BA, Nordberg M. Handbook the toxicology of
metals. USA: Elsevier; 2015.
[5] Gupta RC. Reproductive and developmental toxicology. USA: Elsevier; 2011.
[6] Niu Y, Zhang R, Cheng Y, Sun X, Tian J. Effect of lead acetate on the
apoptosis and the expression of Bel-2 and bax genes in rat brain cells.
Zhonghua Yu Fang Yi Xue Za Zhi 2002; 36(1): 30–33.
[7] Sharifi AM, Ghazanfari R, Tekiyehmaroo N. Investigating the effect of
lead acetate on rat bone marrow-derived mesenchymal stem cells
toxicity: Role of apoptosis. Toxicol Mech Methods 2011; 21(3): 225-230.
[8] Lu X, Jin C, Yang J, Liu Q, Wu S, Li D, et al. Prenatal and lactational
lead exposure enhanced oxidative stress and altered apoptosis status in
offspring rats’ hippocampus. Biol Trace Elem Res 2013; 151(1): 75-84.
[9] Sujatha K, Srilatha CH, Anjaneyulu Y, Rao C, Sreenivasulu D,
Amavartahi P. Expression of Bax and BCl-2 in lead induced apoptosis
in rat liver and kidney and its amelioration with ocimum sanctum: A leaf
extract. J Toxicol Health 2013; 103: 145-149.
[10] Nakade UP, Garg SK, Sharma A, Choudhury S, Singh RY, Gupta K,
et al. Lead-induced adverse effects on the reproductive system of rats
with particular reference to histopathological changes in uterus. Indian J
Pharmacol 2015; 47(1): 22-26.
[11] Yuan G, Dai S, Yin Z, Lu H, Jia R, Xu J, et al. Sub-chronic lead and
cadmium co-induce apoptosis protein expression in liver and kidney of
rats. Int J Clin Exp Pathol 2014; 7(6): 2905-2914.
[12] Gurer H, Ercal N. Can antioxidants be beneficial in the treatment of lead
poisoning? Free Radical Biol Med 2000; 29(10): 927–945.
[13] Abdollahi M, Salehnia M, Salehpour S, Ghorbanmehr N. Human
ovarian tissue vitrification/warming has minor effect on the expression of
apoptosis-related genes. Iranian Biomed J 2013; 17(4): 179-186.
[14] Osthoff KS. Chapter 1: How cells die: Apoptosis and other cell death
pathways. Apoptosis, Cytotoxicity and Cell Proliferation. 4th ed. Germany: Roche Diagnostics; 2008.
[15] Flora SJS, Saxena G, Mehta A. Reversal of lead-induced neuronal
apoptosis by chelation treatment in rats: Role of reactive oxygen species
and intracellular Ca²⁺. J Pharmacol Exp Ther 2007; 322(1): 108–116.
[16] Nandedkar TD, Dharma SJ. Expression of Bel(Xs) and C-Myc in atretic
follicles of mouse ovary. Reprod Biomed Online 2001; 3(3): 221–225.