The Influence of Orally Given Lead Acetate on the Expression of TNF-α and IL-6 of Fallopian Tube Epithel Cell of the Wistar Female Rat (Rattus Norvegicus)

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ABSTRACT

Objective: *This research aims at proving the influence of orally give lead acetate on TNF-a and IL-6 of fallopian tube epithel cell of The Wistar Female Rat (Rattus Norvegicus).*

Method: *Using true experimental post only control group method, there were 24 Wistar female rat (Rattus Norvegicus) with age of 10-12 weeks and body weight of 100-200 grams. The 24 rats were divided into 4 groups consisting of 1 control grup and 3 d groups with lead dosage of 30, 100, and 300 ppm. The lead was given with sonde for 30 days.The body weight and the fallopian tube weight of the rat were weighed, and expression of TNF-a and IL-6 with immune histochemistry.*

Result: *There were significant decrease of body weight difference of the treated groups compared with the control group, significant increase of TNF-a expression on the dosage of 30 ppm and 100 ppm and statistically insignificant increase of IL-6 with dosage of 300 ppm.*

Conclusion: *The lead given orally can increase the expression of TNF-a and IL-6 of the fallopian tube epithel.*

Key words*: lead actate, TNF- a and IL-6.*

1. INTRODUCTION

Lead (Pb) is heavy metal which can be found around us. It has naturally existed, but its level keeps increasing at this time due to the human activities. The high lead (Pb) level, both in atmosphere, water, and land is alerted because this metal heavy becomes toxic when it is in the body of an organism (Patrick, 2006). The main source of lead oxidation comes from gasoline and vegetables (from land or dash attached on the vegetables when consumed).

One of the mainly accumulated way of lead to human beings is through the digest system. The lead entering into the human body through the digestive system can pass through such consumed food and drink as meat, fruit, and vegetables

(Sudarwin 2008). The research result, by the Research and Development Centre of Agricultural Department, with analysis of vegetable samples such as cabbage, production centre in West Java and East Java, generally indicates that the lead (Pb) pollutant is over Residue Maximum Limit (Widowati, 2011). It is like the research result of Fitriyah (2007) which states that the lead (Pb) level of hairy shel (*Anadara Antiquata)* at Lekok Beach Waters in Pasuruan Regency is 2,031 ppm. This level has exceeded the threshold of lead (Pb) level allowed by Indonesian national standards 7378:2009 as amount as 1,5 mg/kg (ppm).

The lead exposure in society can cause such various negative effects in health as central nerve and peripheral nerves, cardiovascular system, hemato poetic system, kidney, digestion, reproduction system, and be carsinogenic (Darmono, 2008). In addition, the research result of Hemdan *et al.* (2005) indicates that he higher lead dosage can affect the immune modulator. All lead dosages significantly de All lead doses significantly reduce cell vitality and / or proliferation.

The accumulated lead causes the oxidative stress prompted by two mechanisms namely *reactive oxygen species (ROS)* formation and a decrease in the antioxidsidant system decrease in body (Patrick, 2006). The amount increase of ROS will cause the systematically damaged tissue which is signed by the increase of pro-inflammatory cytokines including N*uclear Factor Alpha* (TNF-α) *Tumor* (Agarwal *et al.,* 2007).

The research of Tarnate *et.al.* (1995) indicates that cytokine, prostaglandin, metabolite of lipid per oxidation and ROS in liquid sample of fallopian tube are found. Another research indicates that the fallopian tube weight of mature rat group with lead acetate decreases (Dumitrescu *et al*, 2008). However, the researcher wants to recognize the cause of weight decrease and structure change of the fallopian tube which can be due to the inflammation of fallopian tube because of ROS as a result of the reaction of cell membrane lipid per oxidation, activates inflammation cells that increase free radical in the body. Based on those researches, the researcher wants to recognize the influence of orally given lead acetate on the TNF-α, IL-6 of fallopian tube epithel cell of Wistar female rat (*Rattus Norvegicus).*

2. DESIGN, MATERIALS, METHOD, AND DATA ANALYSIS

This research was designed in *true experimental* with research approach of *post test only with control group design.* The samples consisted of 24 female rats divided into 1 control group and 3 treated gicroups given with dosage lead of 30, 100, and 300 ppm. Thin research was stated as feasible ethical by the Ethical Commission of Health Research of Malang Brawijaya University.

2.1 Rat Nourishment

The rat barn was made of platic material with wire cover of 45 cm x 35.5 cm x 14.5 cm completed with fodder place and water bottle. The husk was put into the barn and changed once three days or when in dirty condition it was changed soon. There were 6 in every barn. The barn room temperature was 26 – 27°C and the lamp did not need special specification for lighting. Aqua water was given using bottle with the pipe on the bottle's cap in adlibitum way. The lead acetate was given with sonde to put lead acetate solution into the experimental animals.

2.2 Lead Acetate Solution Making and Treatment

This research used *lead (II) acetate trihydrate* of Merck in the form of white powder. The lead acetate solution was made by mixing 1000 mg of lead powder into 1 L of aquabidest using *magnetic stirrer*. It was then divided into dosage of 30, 100, and 300 ppm with formula:

$$C1.V1 = C2.V2$$

The lead acetate was given orally through sonde, 1x/day, amount of 1 cc for 30 days. The lead acetate was given orally through sonde once a day in 1 cc for 30 days

2.2.1 Determination of Estrus Cycle of Experimental Animal

It was performed after the final day of exposure by the trained staff of reproduction laboratory of Veterinary Faculty of Malang Brawijaya University.

2.2.2 Experimental Animal Surgery

The surgery was performed by the skilled staff of Pharmacological Laboratory of Malang Brawijaya University. The rats were anaesthetized with ketamine injection of 1% on the rat's thigh with dosage of 0,2 ml. In this surgery the fallopian tube organ was taken and weighed using an analytical scale. In addition the fallopian tube was put into the container containing solution of fixative buffer formalin 10%, soaked for 12-24 hours to make the slide of immunohisto chemistry and Hema toxyin Eosin (HE).

2.3 HE Slide Making Procedure

The fallopian tube tissue was soaked in 10% formalin solution for one night and the tissue was cut at least 2-3 mm thickness, put into casset and labelled with the code in line with the researcher's code, put into 10%formalin, then into Tissue Text Processor for 90 minutes. The tissue was cut with Microtome thickness of 3-5 micron, and then put in oven for 30 minutes with temperature of 70-80°C, then soaked in xylol solution 2x, each for 20 minutes. It was soaked in alcohol 96%, 80%, 76% each for 3 minutes, then put into the flowing water for 15 minutes. After that it was given the maint paint of Harris Hematoxylin for 10-15 minutes, washed with the flowing water for 15 minutes, and dyed in acid alcohol of 1 % for 2-5x and ammonia water for 3-5x. In addition it was given Eosin paint 1% for 10-

15 minutes. Moreover dehydration in alcohol 70%, 80%, and 96% each for 3 minutes. Then soaked in xylol for 60 minutes. Mounted with engelan and coverglass. Let the slide dry at room temperature and ready to be observed.

2.4 Expression Examination Procedure of TNF-α and IL-6 with Immunohistochemistry Technique

The slide was deparaffinized by washing with xylol for 3 times each for 3 minutes. Then rehydrated by soaking into absolute etanol (2x10 minutes), etanol 90% (1x5 minutes), etanol 80% (1x5 minutes), etanol 70% (1x5 minutes), aquadest steril (3x5 minutes). In addition, performing *antigen retrieval* by heating the slide in waterbath of 95°C for 20 minutes. Then, it was freezed and washed using PBS (3x5 minutes). After that, *immunostaining* after blocking peroxidase endogen with H_2O_2 3% in methanol (20 mites), incubation for 15 minutes in room temperature. Wash the slide of fallopisn tube tissue 3 x 15 minutes with PBS. Incubation of fallopian tube tissue slide on the primary antibody in 4°C for 24 hours.

On the second day, wash the slide of fallopian tube tissue in PBS for 3 x 5 minutes. Incubate the slide of fallopian tube tissue with the secondary antibody for 60 minutes in room temperature. Wash with PBS for 3 times 5 minutes, rinse aquadest 3-4x. Drop with DAB (*Diaminobenzidine)* for 3 minutes or to the brownish colour. Wash the slide of fallopian tube tissue with aquadest for 3 x 5 minutes. After that, *counterstain* with Mayer's hematoxylin for 1 minute. Wash/drop with aquades 3 drops, incubate again for 5 minutes, rinse aquadest. Mount and cover with *coverglass.*

2.5 Data Analysis

The data of body weight difference, expression of TNF-α and IL-6 of fallopian tube epithel were analysed using *One Way Anova* test and LSD Post Hoc Test. The significance degree is 95%. Statistic analysis used *software* SPSS 22.0 *for windows.*

3. RESULT

3.1 General Characteristic

The Fallopian tube body weight was measured and analysed as written in the following table. Table 1 Mean of Rat's Fallopian Tube Weight after Lead Acetate Given with Various dosages

Treatment	Mean ± SD	P-Value
K	0,043 ±	
P1	0,125	
P2	0,041 ±	0,346
P3	0,131	
	0,039 ±	
	0,161	
	0,031 ±	

0,007

Based on the analysis result with Anova, it was found out that p-*value* was 0,346, larger than α= 0,05 (p<0,05). So from this test, it can be concluded that there is no difference of fallopian tube organ weight between the treated groups and the control group due to the orally given lead acetate.

3.2 The Influence of Orally Given Lead Acetate on the TNF-α Expression of Fallopian Tube Epithel Cell Sel Epitel Tuba Fallopi

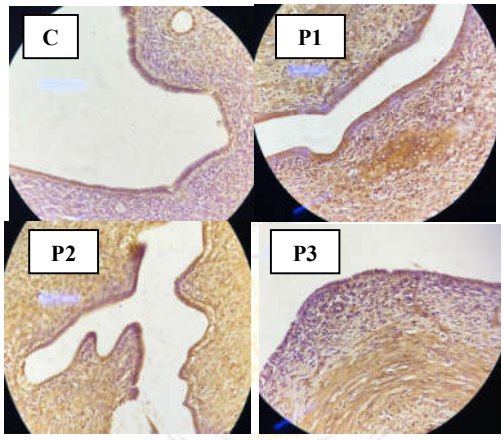

Figure 1 Description of esi TNF-α Expression of Epithel Cell through 5 Zoom View of Microscop 400x

Details: **C** indicates the control group, TNF-α expression looks little. **P1** (30 ppm), TNF-α expression looks more compared with the control group. **P2** (100pm), TNF-α expression looks more compared with P1. **P3** (300 ppm), TNF-α expression looks fewer on epithel cell compared with the treated groups of P1 and P2.

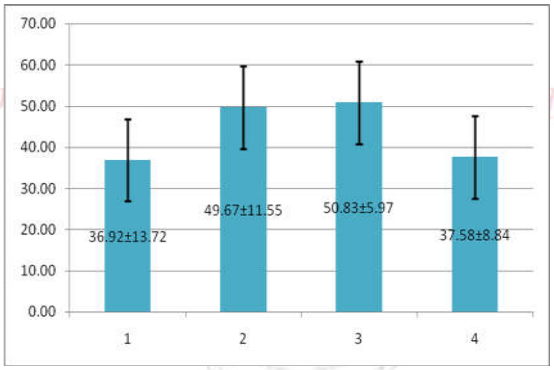

Figure 2 TNF-α Expression of Control Group and Treated Group

Details: TNF-α Expression increases on the acetate-given group at 30 ppm (P1) and 100 ppm (P2), and decreases on the lead acetate-given group 300 ppm (P3). Based on the analysis result with Anova, it was found out that p-*value* was 0,049, smaller than α = 0,05 (p<0,05), so that it can be concluded that there is a significant difference of TNF-α Expression between the treated group and the control group because of the given lead acetate.

Based on the test of Pos Hoc LSD, it was found out that TNF-α expression of P1 and P2 groups were larger than the control group. The TNF-α Expression of P3 group was larger than the control group, but it was not significant. This indicates that the significant increase of TNF-α expression was showed by the lead acetate given with dosage of 30 ppm (P1) and 100 ppm (P2) compared with the control group.

3.3 The Influence of Orally Given Lead Acetate on IL-6 Expression of Fallopian Tube Epithel Cell

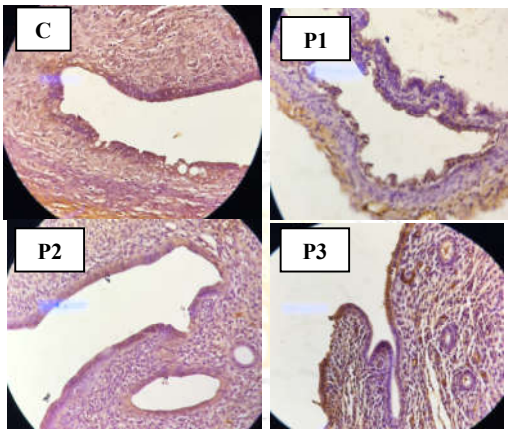

Figure 3 Description of IL-6 Expression of Epithel Cell through 5 Zoom View of Microscop 400x

Details: **C** is the control group, IL-6 expression looks little. **P1** (30 ppm), TNF-α expression looks more compared with the control group. **P2** (100 ppm), IL-6 expression looks more compared with **P1**. **P3** (300 ppm), IL-6 expression looks more compared with the treated groups of P1 and P2. Based on the analysis result with Anova, it was found out that p-*value* was 0,245, larger than α = 0,05 (p<0,05). For this, from this test it can be concluded that there is no difference of IL-6 expression between the treated group and the control group.

Based on the test result of LSD (appendix), IL-6 Expression of the treated group more and more increases but not significant. The mean difference of i IL-6 e following table.

Details: IL-6 expression more and more increases on the treated groups given with lead acetate. The highest mean of IL-6 expression is on the group given with acetate in dosage of 300 ppm (P3).

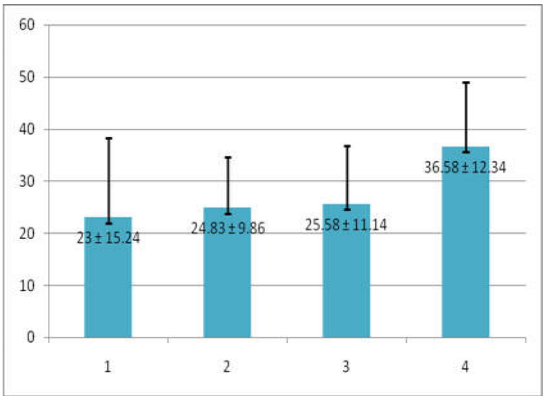

Figure 4 IL-6 Expression of Control Group and Treated Group

4. DISCUSSION

4.1 Overview Weight Loss and Weight Fallopian tubes Rat Betina

This study shows that the body weight of rats treated group were given exposure to lead acetate for 30 days did not appear to have weight gain as compared to the control group. However, statistically significant difference in final body weight and body weight early treatment group and the control group obtained significantly different. Trends decreased body weight of rats that is in line with the exposure dose of lead acetate is higher.

In accordance with research Seddik L. et al. (2010) which states that lead causes a decrease in body weight gain due to a decrease in appetite in mice. According to Shukla (1989) and Sharifi (2002) of lead can cause stunted growth and reduced consumption of food through mutual contacts with appetite receptors in the gastrointestinal tract.

The weight gain is a key indicator of the growth aspect. Normal weight gain in mice at 5-6 grams per day, or an average of 10% of their body weight per day (Suckow et al., 2005). In female mice will increase the weight quickly within 30 days and will continue to rise until age female mice reached 105 days. Mice that were contaminated by drugs or other contaminants will lead to weight gain minimum (Pahl, 1969). This is in line with previous research which states that exposure to lead acetate can reduce weight significantly in the treatment group compared to the control group (Hariono, 2006; Sharma et al., 2012).

Some heavy metals, including lead, known to cause the production of reactive oxygen species (ROS) is excessive. This generated ROS can affect the regulation of cell metabolism (Foyer CH. And Noctor G.,2002). This statement is supported by Hwang and Wang (2001) which states that the metabolism of the cells that cause weight gain as a result of lead acetate minimal associated with the presence of zinc metabolic imbalances in the body's cells. It is known that zinc is important for several metabolic processes of cells. Instead of pressing the toxicity of lead in the

cells (Taylor, 2010), Zinc is an important part in the regulation of nutrients in the body. Zinc deficiency associated with decreased appetite can be assessed by weight (Jing MY, et al., 2008).

4.2 Effect of Lead Acetate Against Expression of TNF - α

From this research it is known that a significant increase in the expression of TNF - α fallopian tube epithelial cells of female rats (Rattus norvegicus) group a dose of 30 ppm P1 and P2 group a dose of 100 ppm to the control group . But there was decreased expression of TNF - α in group P3 with a dose of 300 ppm . Lead (Pb), which enters the body will be distributed into the blood ± 95 % bound to red blood cells , and the rest is bound to plasma (Palar , 2008) . Once absorbed , 99 percent of lead in the blood is maintained for approximately 30-35 days and 4-6 weeks after the spread and accumulate in other tissues .

The relative distribution of lead in the soft tissue in the female organ is one of the ovaries (Barry , 1975; Gross et al. , 1975 in ATSDR , 2007) . Lead accumulates in the ovaries is also suspected to be accumulated in the fallopian tubes.

One of the mechanisms of toxicity of lead that can cause oxidative stress, an imbalance between oxidants and antioxidants (Flora, 2011). The onset of oxidative stress occurs along two pathways: first, emerging generation of ROS, such as hydrogen peroxide (H2O2) and second, antioxidant reserves become depleted (Patrick, 2006). ROS generated will activate NF-kB. TNF-α expression is under the control of NF-kB (Forman and Torres, 2001). Effect of lead exposure to TNF-α previously investigated by by Gillis et al. (2012). Levels of TNF-α were obtained in blood serum also increased due to lead exposure.

In figure 2 it can be seen that the expression of TNF-α tends to decrease closer to the control group at a dose of 300 ppm. These conditions indicate the existence of an alleged down regulation T-cell response (Karupiah et al., 1987) due to declining NO. Also according to Freeman et al. (1996) showed that lead acetate at doses greater and exposure time are getting longer, the level absorbsinya decreased so that researchers suspect that the dose limit for the maximum absorption of lead by the body that is decreasing the expression of TNF-α treatment group P3 significantly compared to group P2.

4.3 Effect of Lead Acetate Against Expression of IL-6

The results of this study showed that statistically expression of IL-6 both in the control group and the treatment group in various doses has no significant difference. However, seen from the histogram, tarjadi increased expression of IL-6 in the treatment group compared to the control group. Consistent with previous studies by Villanueva et al. (2000) also showed no difference in the levels of IL-6 in

the blood that are produced as a result of lead in the treatment group compared to the control group.

IL-6 is a pro-inflammatory cytokine that plays an important role in the immune response by stimulating the production of antibodies and as effector T cell development (Tanakal T. et al., 2014). Expression of IL-6 is a signal of tissue damage (Nishimoto et al., 1989). Another role of IL-6 is a rescue apoptosis of T cells through regulation of STAT3-dependent anti-apoptotic regulators such as Bcl-2 (Jones S.A. et al., 2005; Curnow S.J. et al., 2004).

In addition to its role as an anti-apoptotic T cells, IL-6 plays an important role in the differentiation of B and T cell IL-6 is an early sign as a factor that increases the production of antibodies from B cells (Hirano, T. et al., 1985). Expression of IL-6 is set in part by the transcription factor NF-kB. ROS increases will lead to an increase in NF-kB as an indicator that the cell inflammation (Olguin et al., 2015). The same is seen in the mean expression of IL-6 in the treatment group in this study are increasing with increasing doses of lead acetate given.

ROS due to increased lead through the bonding process sulphidryl cluster in the bloodstream that cause tissue damage (M. Ahamed and Siddiqui M.K., 2007). Several other important antioxidant enzymes become inactive because of lead that is super oxide dismutase (SOD) and catalase (CAT). Decreasing the concentration of SOD reduces the disposal of superoxide radicals, while the reduction of CAT disrupt binding of superoxide radical (O2 •).

Apart from targeting the sulfhydryl groups, lead can also replace the zinc ions that serve important for antioxidant enzymes and inactivate them (Flora et al., 2007). Lead also inhibits -ALAD, which results in increased substrate concentration of ALA in the blood and urine. ALA is a high-yield hydrogen peroxide and superoxide radicals and also interact with oxyhemoglobin, so that increased ROS (Patrick, 2006). The development of all the mechanisms mentioned above makes cells highly susceptible to oxidative stress and can lead to cell death. Lead can cause damage to the reproductive organs (Ahamed M. et al., 2005). ROS are generated can be associated with the expression of IL-6 (Aronis A. et al., 2003).

No significant effect of exposure to lead acetate on the expression of IL-6 fallopian tube epithelial cells suspected to be affected by factors related to the number of samples.

5. Conclusion

Exposure to lead acetate orally can inhibit weight gain , increased expression of TNF - α at a dose of 30 ppm and 100 ppm , lowering the fallopian tube epithelial

thickness at a dose of 300 ppm , but it cannot increase the expression of IL- 6 were significantly epithelial cells .

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