Effect of Chitosan on Histology of Reproductive Organs of Female Wistar Rats (Rattus norvegicus) Exposed to Acetate Lead

ABSTRACT

Lead is one of the pollutants widely spread in the environment because it is not easily decomposed. Lead can affect system functions such as the ovary and endometrium. Lead can trigger oxidative stress by reducing antioxidant enzymes and increasing Reactive Oxygen Species (ROS). Lead can also reduce Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) levels by disturbing the hypothalamus. Chitosan is an antioxidant compound that can reduce the toxic effects of lead. The purpose of this study was to study the effects of chitosan administration on the diameter of antral follicles, the number of endometrial arterioles, and the thickness of endometrial rats after lead acetate exposure. This study was an experimental laboratory using a posttest-only control group design approach applied on 25 female rats aged 8 weeks old, body weight 125-175 grams. Lead and chitosan were given orally with a sonde. There were 5 groups, namely, negative control group (without any treatment), positive control group (lead 175mg/kg/BW), treatment group 1 (lead 175mg/kg/BW + chitosan 16mg/kg/BW), treatment group 2 (lead 175mg/kg/BW + chitosan 32mg/kg/BW), and treatment group 3 (lead 175mg/kg/BW + chitosan 64mg/kg/BW) for 30 days. The rats were sacrificed at proestrus phase, which was proven from vaginal swab. Observations were carried out using the Hematoxylin Eosin (HE) staining method. The observations were analyzed using One Way ANOVA and followed by Least Significant Differences (LSD) test. The results showed significant results (p-value <0.05). Chitosan can increase the diameter of the antral follicle, increase the number of endometrial arterioles, and increase the thickness of endometrial rats exposed by lead acetate.

Keywords: Antral follicles, endometrial arterioles, chitosan, lead, thickness of endometrium
INTRODUCTION

Lead is one of the toxic pollutants that is widely spread in the environment because of its non-biodegradable nature (1). One of the main sources of lead exposure is through the digestive system that comes from lead-contaminated food and beverages. The US Centers for Disease Control and Prevention regulated that the limit of lead levels in the blood for adults is 10μg/dL (2). Symptoms of lead toxicity occur in continuous exposure in small amounts, so the effects of chronic lead toxicity are more common (1). Lead toxicity can affect the kidney function, the hematopoietic system, the central nervous system, and the reproductive system (3). Lead can induce oxidative stress (4).

Oxidative stress is an imbalance between antioxidants and oxidants, which are more dominant (5). Lead binds to the sulphydryl group and causes the decrease in the levels of superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) antioxidants (6,7). In addition, the sulphydryl group binding can deactivate several enzymes in heme biosynthesis, such as ALAD (Amino Laevulinic Acid Dehydratase) (8). Decreased ALAD results in an increase in Amino Laevulinic Acid (ALA) which can stimulate the production of ROS (9). Lead is able to pass through the Blood Brain Barrier (BBB) and affect to Ca2+ as a Second Messenger by binding to CaM, thus causing changes in cell function (10). This can affect the biosynthesis of sex hormones in the hypothalamus, the stimulus to GnRH is impaired so that the production of FSH and LH decreases (11). All of these mechanisms cause oxidative stress and a decrease in FSH and LH, which will damage reproductive organs such as the ovary and endometrium (7).

In the ovary, there are follicular developments on various stages, one of which is the antral follicle phase. Antral follicles are follicles that grow due to stimulation from FSH and LH (12). If the process of follicular growth is disrupted, it will affect hormone production, for example, the hormone estrogen produced by the antral follicle during the process of folliculogenesis (13). In the endometrium, a decrease in the hormone estrogen can result in the regulation of Vascular Endothelial Growth Factor (VEGF), which are the key compounds of angiogenesis (14). VEGF expression will increase during the proliferation phase, and the peak will occur in the middle phase of secretion in the menstrual cycle, which is responsible for ripening the spiral arteries during the implantation window. The spiral artery is a blood vessel branching from the arcuate artery which will form arterioles which supply 9mm from the outer shell of endometrial and arteriole number and endometrial thickness) in Wistar rats (Rattus norvegicus) exposed to lead acetate.

METHOD

Research design

This in vivo study was an experimental laboratory with posttest only control group design approach. The study used female rats (Rattus norvegicus) Wistar strain exposed to lead and given antioxidants in the form of chitosan.

Lead and Chitosan Solution Preparation

The study used lead (II) acetate trihydrate with catalog number 107375. The lead solution was made by dissolving lead acetate with distilled water according to the dosage set (24). Chitosan solution was made by dissolving chitosan and 2% acetic acid according to the dosage set.

Animal Treatments

The number of samples used in this study was 25 white female Wistar strain rats (Rattus norvegicus). The inclusion criteria in this study were Wistar strain rats (Rattus norvegicus), 8 weeks old, body weight 125-175 grams, female, healthy (clean white fur, active), not pregnant, and never been used in previous studies. The exclusion criteria in this study were rats that appeared sick before being treated. This research has approval from Ethics Commission of Faculty of Medicine, Brawijaya University No. 99/EC/KEPK-S2/03/2019.

The rats were divided into 5 groups, 2 control groups (negative and positive controls) and 3 treatment groups (given chitosan doses of 16mg/kg/BW, 32mg/kg/BW, 64mg/kg/BW). All groups were given lead at a dose of 175mg/kg/BW/day, except for the negative control group. Provision of lead acetate and chitosan solution was carried out orally with a sonde for 30 days.

After 30-days treatment, the rat was sacrificed due to the proestrus phase through vaginal smear. Before surgery, the rat was anesthetized by ketamine injection dose 0.2ml. The parts taken were the right ovary and uterus, and soaked in Buffer Neutral Formalin (BNF) solution and the histological preparations and HE staining were conducted on Anatomy Pathology Laboratory. The rest of the carcasses were buried together with a depth of 0.5 meters under the ground without being wrapped so as not to cause environmental pollution.

The procedure of Histopathological Preparations

The histopathological preparations consist of several processes, namely: tissue cutting, tissue blocking and cutting, deparaffinization, staining process (HE), immersion in multilevel alcohol, clearing, mounting, and closing with glass cover. Before reading the histological and endometrial histological slides, histological preparations were consulted to the Anatomical Pathologist. Histological
slide readings was using an Olympus microscope in 400 times magnification for parameters of antral follicle diameter and endometrial thickness, while 200 times magnification for the parameter of the number of endometrial arterioles.

**Measurement of Antral Follicle Diameter**

The first step in observing was to identify the type of follicle. Determination of antral follicles was based on the presence of antrum, an increase in the number of granulosa cells, the presence of Cumulus Oocyte Complex (COC), and theca cells surrounding the follicles. The antral follicle diameter was measured using Dot slide software with 2 intersecting lines drawn from the farthest distance of the follicle and the shortest distance of the follicles, which were then calculated and averaged.

**Calculation of the Number of Endometrial Arterioles**

Calculation of the number endometrial arterioles based on 10 visual fields using Olyvia software and cell counting. Determination of the number of endometrial arterioles was based on the characteristics of a thick round tissue that has intima, media, and adventitia, which are then calculated and averaged.

**Measurement of Endometrial Thickness**

The limit of measurement of endometrial thickness was from the epithelial lumen to the endometrial basal layer. Measurement of endometrial thickness was carried out at 12 points (which were described as the position of numbers on analog clocks) which reached 4 quadrants in the endometrium using Dot slide software, which was then calculated and averaged.

**Statistical Analysis**

The measurement results of antral follicle diameter, number of arterioles, and endometrial thickness of control and treatment rats were analyzed statistically using the One Way ANOVA test with a significance level of 0.05 (p=0.05) and a confidence level of 95% (α=0.05), and followed by Least Significant Differences (LSD) test.

**RESULTS**

**Effect of Chitosan Administration on Diameter of the Antral Follicle induced by Lead**

There are various types of follicles in the ovary, such as primordial follicle, primary follicle, secondary follicle, tertiary follicle, de Graaf follicle, and atresia follicle. The follicles are divided into 2, pre-antral follicles and antral follicles.

The One Way ANOVA test was used to compare the mean diameter of the antral follicles in the five sample groups. The Table 1 present One Way ANOVA test results on the antral follicle diameter.

One Way ANOVA test on antral follicle diameter obtained p-value 0.005 (<0.05) which showed significant differences in the diameter of the antral follicles in each group. Next, the Least Significant Differences (LSD) test was carried out. In the LSD test, the negative control group (370.34±86.25) compared to the positive control group (173.75±37.48) showed a significant difference in the diameter of the antral follicle. It showed that exposure of lead acetate in the female rat in a dose of 175mg/kg/BW could reduce the diameter of the antral follicle.

Table 1 also illustrated the comparison results of the negative control group (370.34±86.25) to group P1 (235.46±57.08), there was a significant difference in the mean diameter of the antral follicle. In other words, group P1, chitosan 16mg/kg/BW has not been able to increase the diameter of antral follicles in rats exposed to lead significantly. In addition, there were significant differences in the positive control group (173.75±37.48) and the P3 group (340.56±112.11). Lead + chitosan 64mg/kg/BW exposure in rats can significantly increase the diameter of the antral follicle.

**Table. 1 One Way ANOVA results of antral follicle diameter between research groups**

| Research Groups          | N  | Mean ± SD Antral Follicle Diameter | P-value |
|--------------------------|----|-----------------------------------|---------|
| Negative Control (without treatment) | 5  | 370.34±86.25                      |         |
| Positive Control (lead acetate 175mg/kg/BW) | 5  | 173.75±37.48                      | 0.005   |
| Treatment 1 (lead acetate 175mg/kg/BW+ chitosan 16mg/kg/BW) | 5  | 235.46±57.08                      |         |
| Treatment 2 (lead acetate 175mg/kg/BW+ chitosan 32mg/kg/BW) | 5  | 269.35±78.29                      |         |
| Treatment 3 (lead acetate 175mg/kg/BW+ chitosan 64mg/kg/BW) | 5  | 340.56±112.11                     |         |

**Note:** * One Way ANOVA & Least Significant Differences (LSD). The difference in notation indicates a significant difference (p-value <0.05). However, if the notation same it means there is no significant difference (p-value >0.05).

Therefore, it can be concluded that lead exposure can reduce the diameter of the antral follicles and chitosan can increase the diameter of the antral follicle. However, chitosan 16mg/kg/BW has not been able to increase the diameter of the antral follicle significantly. At a dose of 32mg/kg/BW, although there was an increase in the mean diameter of the antral follicle compared to lead-exposed rat, it was not sufficient to significantly increase the diameter of the antral follicle exposed to lead. The dose of chitosan 64mg/kg/BW was able to increase the diameter of the antral follicles in rats exposed to lead significantly.

**Effect of Chitosan Administration on the Number of Endometrial Arterioles Induced by Lead**

![Figure 1. Microscopic antral follicle measurement at 400 times magnification](Image)

**Note:** Identification of antral follicles, consisting of multiplying granulosa cells (blue arrow), presence of antrum cavities (green arrow), oocytes (red arrow), expansion of cumulus cells surrounding the oocytes (yellow arrow), and theca cells (orange arrow). Measuring the diameter using 2 auxiliary lines (black arrows) on all follicles.
The spiral arterioles were identified by recognizing their shapes, i.e., round, thick, and having intima, media, and adventitia tissue. The number of endometrial arterioles was calculated manually in 10 visual fields with 200 times magnification, and the results were averaged.

One Way ANOVA test on the number of endometrial arterioles obtained p-value 0.001 (<0.05) which showed significant differences in the number of endometrial arterioles in each group. Based on LSD test, the negative control group (8,228±4.3074) was significantly different compared with the positive control group (7.260±1.0065). This shows a significant difference in the number of endometrial arterioles. In other words, exposure to lead acetate in female rats at a dose of 175mg/kg/BW can reduce the number of endometrial arterioles.

Table 2 also showed the results of the positive control group (7.260±1.0065) compared to the P3 group (12.200±2.7359), indicating that there were significant differences in the mean number of arterioles. In other words, in the P3 group, chitosan 64mg/kg/BW was able to increase the number of arterioles in rats exposed to lead significantly.

Therefore, it can be concluded that lead exposure can increase the number of endometrial arterioles. At doses of 16mg/kg/BW and 32mg/kg/BW, although there was an increase in the number of endometrial arterioles compared to lead-exposed rats, it was not able to significantly increase the diameter of antral follicles exposed to lead. The dose of chitosan 64mg/kg/BW was able to significantly increase the number of endometrial arterioles in rats exposed to lead.

Effect of Chitosan Administration for the Endometrial Thickness Induced by Lead

Table 3. One Way ANOVA test results endometrial thickness between groups

| Research Groups | N  | Mean ± SD Endometrial Thickness | p-value |
|-----------------|----|---------------------------------|---------|
| Negative Control (without treatment) | 5  | 616.26±193.59b                 | 0.026   |
| Positive Control (lead acetate 175mg/kg/BW) | 5  | 626.64±107.10b                 | 0.001   |
| Treatment 1 (lead acetate 175mg/kg/BW+ chitosan 16mg/kg/BW) | 5  | 605.22±102.35a                 | 0.028   |
| Treatment 2 (lead acetate 175mg/kg/BW+ chitosan 32mg/kg/BW) | 5  | 616.26±193.59b                 | 0.026   |
| Treatment 3 (lead acetate 175mg/kg/BW+ chitosan 64mg/kg/BW) | 5  | 616.26±193.59b                 | 0.026   |

Note: * One Way Anova & Least Significant Differences (LSD). The difference in notation indicates a significant difference (p-value <0.05). However, if the notation same it means there is no significant difference (p-value>0.05).
Therefore, it can be concluded that lead exposure can reduce endometrial thickness. Administration of chitosan doses 16, 32, and 64mg/kg/BW can significantly increase endometrial thickness. However, the most effective dose of chitosan is a dose of 32mg/kg/BW.

**DISCUSSION**

*Effect of Chitosan on Antral Follicles Exposed to Acetate Lead*

Lead acetate exposure in the female rat at a dose of 175mg/kg/BW can reduce the diameter of the antral follicle. The results of this study are in line with the research conducted by Ahmed et al., that the administration of lead doses of 30mg/kg in experimental animals can increase follicular atresia, decrease the growth and maturity of follicles (25). According to the research of Dhir and Dhand, lead administration causes an increase in phosphoric acid in the ovary and shows the presence of phagocytosis (26). Lead that enters the bloodstream will flow into the soft tissue and can accumulate in the ovary. Ovaries have an important role in regulating the development, maturation, and ovulation of female gametes. Research shows that lead can cause direct damage to the ovary (11).

Infertility is associated with a decrease in the number and diameter of the antral follicle (27). The diameter of the antral follicle indicates that the antral follicle can receive a response from FSH and LH to develop so that it increases the number of granulosa cells to produce estradiol (28). The diameter of the follicle is used to increase follicular growth, oocyte growth, follicular fluid volume, and granulosa cell proliferation (12). The antral follicles grow because of FSH and LH stimulation. In the condition of lead toxicity, lead is able to pass through the BBB and affect to the role of Ca$^{2+}$ as a second messenger, thereby disrupting cellular processes or cell function (10). This disruption regulates the work of the hypothalamus and the function of the pituitary gland, which results in disruption of sex hormone biosynthesis. Impaired biosynthetic stimulation causes FSH and LH decrease (11).

The decrease in FSH and LH (the protective toxicities of lead), will reduce the diameter of the antral follicle. Oxidative stress caused by lead exposure can disrupt follicle growth by affecting the process of histological changes and cell components in the ovary, so that disrupting the growth of theca and granulosa cells (6). Lead exposure in female rats can reduce the diameter of the antral follicle. This is consistent with the research of Sharma et al., that lead exposed to female rats will affect the process of development and follicular growth (29).

The administration of chitosan in this study was able to increase the diameter of antral follicles of rats exposed to lead. This proves the administration of chitosan in lead-exposed rat can inhibit the effects of lead toxicity by increasing the diameter of the antral follicle. Chitosan has antioxidant compounds that can reduce damage due to lead exposure (23). This is consistent with the study of Niu et al., which showed that people with foods given chitosan extract had lower MDA than the control group (30). Chitosan has an antioxidant mechanism through its ability to stabilize free radicals (scavenging) and bind metal ions (chelathing). Scavenging, because hydroxyl groups and amino groups in chitosan will donate electrons so that free radicals can be controlled and inhibit the oxidative chain (20). Chelathing, derived from an amino group which is a cation, will bind to heavy metals, especially lead (31).

*Effect of Chitosan on the Number of Arterioles Exposed to Acetate Lead*

Lead is a metal that is very toxic to both animals and humans, even in a low dose (11). Chronic lead exposure can disrupt the reproductive system, such as menstruation and ovulation inhibition, decrease follicle count, damage the endometrium, myometrium, and perimetrium (32). In the reproductive organs, lead toxicity can interfere the activity of the hypothalamus and the function of the pituitary gland which will result in a decrease in the biosynthesis of the hormones FSH and LH (6). Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) are important hormones synthesized by the pituitary gland, which are important in regulating the reproductive system. Research conducted by Dumitrescu et al., stated that lead could disrupt FSH function, increase LH levels significantly, and significantly reduce estradiol and progesterone levels (33). Non-optimal secretion of FSH and LH can cause impaired maturation and development of follicle, resulting in a decrease in the progesterone and estrogen hormones which play an important role in the menstrual cycle, endometrial proliferation, and myometrium. The estrogen and progesterone hormones have an important role in forming a new layer of blood vessels in the endometrium of each menstrual cycle. New blood vessel tissues are formed through angiogenesis and vascular remodeling from pre-existing vessels in the endometrial basal zone during each menstrual cycle (34) under the control of estrogen and progesterone (35). This process is an important factor in each menstrual cycle. Apart from progesterone and estrogen, many factors play a role in angiogenesis, one of which is VEGF (Vascular Endothelial Growth Factor) (36). VEGF is a key compound in abnormal angiogenesis. Disrupted angiogenesis will disrupt blood vessel function such as abnormalities of bleeding during menstruation, amenorrhea, and failure of conception results.

The spiral arteriole is a uterine blood vessel that originates from the spiral arteries that supply 9mm from the endometrial layer (15). Spiral arteriole maintains stable blood flow to the endometrial functionalist stratum (37). The spiral arteriole in the uterus plays an important role in the menstrual cycle and becomes the center of placental formation after embryo implantation.

Chitosan as an antioxidant agent has a hydroxyl group (OH) and an amino group (NH2), which is a key group to prevent the occurrence of free radicals (21). In the study of Vedy, proved that chitosan have capability for binding heavy metals to the kidneys induced by lead acetate with renal histopathological images of rats. There is a reduction in damaged cells after chitosan administration compared to positive controls. The administration of chitosan in this study was able to increase the number of arterioles of rats exposed to lead (38). This proves that the administration of chitosan in lead-exposed rats can inhibit the effects of lead by increasing the number of endometrial arterioles.
Lead substitutes calcium (Ca2+) as a second messenger that binds to Calmodulin (CaM) so that protein kinase is inactive and cellular processes are impaired (2). This can disrupt the stimulation of hormone secretions caused by the damage on the hypothalamus and pituitary gland (6). Accumulation of exposure to lead acetate causes significant histological changes in the reproductive organs. This structural change is related to the level of exposure in the ovariates, uterus, and fallopian tubes. The reproductive organs experience edema and necrosis, denudation, and stages of abnormal follicle changes. The microscopic examination of the uterus and fallopian tube undergoes uterine gland necrosis at lead exposure of 0.150mg/L. This changed indicates the occurrence of infertility in female rats (11). However, the administration of oral lead acetate solution was not proven to cause a decrease in the thickness of the endometrial layer due to the influence of an increase in free radicals (39). In uterus exposed to 100 and 300ppm lead, cystic goblet cell degeneration and decay of the lining of endometrial epithelial cells occur. Degenerative and inflammatory changes in the uterus are in the form of a decrease in the height of columnar cells and areas of blood vessels, lymphatics, and connective tissue (40). The difference in changes in uterine histology in the treatment group depends on the dosage.

Besides that, lead acetate can cause lipid peroxidation and reduce SOD and GPx activity in rats' reproductive organs. Providing antioxidants can prevent oxidative stress conditions by increasing one of the antioxidant enzymes such as SOD (41). Chitosan is one of the antioxidant compounds which acts as an oxygen free radical scavenger that can inhibit the formation of free radicals, thus protecting the damage of testicular lead-induced rats (42). Chitosan acts as an ion exchanger and adsorbent to heavy metals including lead. An amino group is a cation capable of binding to lead heavy metals. The amino group as a chelating agent will bind lead heavy metal (31). If oxidative stress can be prevented, then the edema and necrosis of ovarian follicles and necrosis of the uterine gland will not occur. In this study, the endometrial cells observed were not necrosis and edema in the stroma which was described with no inter-cell spacing, no edema in the uterine gland, and no damage to epithelial cells. This results in an increase in endometrial thickness.

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