

# Nanocurcumin Protective Effect on Lipid Peroxide of Lead Acetate Induced White Rats (*Rattus norvegicus*) Ovarian Granulosa Cells

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## Abstract

**Objective:** This study aims to investigate the protective effect of nanocurcumin on lipid peroxidation of rat ovarian granulosa cells induced by lead acetate (Pb).

**Methods :** A total of 45 mice were divided into a negative control group (C-) receiving corn oil, a positive control group (C+) receiving corn oil and lead acetate (Pb) 40 mg/kg bw/day, experimental group 1 (E1), experimental group 2 (E2), experimental group 3 (E3) receiving 50, 100 and 200 mg/kg/day nanocurcumin dissolved in corn oil and lead acetate (Pb) 40 mg/kg bw/day. The treatment was given orally and the treatment time was daily at 08.00 AM for 26 days. Ovaries were removed for immunohistochemical analysis of MDA expression.

**Result :** The positive control group had the highest MDA expression of ovarian granulosa cells ( $94.2 \pm 10.5$ ) and was significantly different ( $p < 0.05$ ) from negative control group ( $1.5 \pm 1.4$ ), experimental group 1 ( $17.4 \pm 10.5$ ), experimental group 2 ( $11.4 \pm 8.5$ ) and experimental group 3 ( $7.2 \pm 4.0$ ).

**Conclusion :** Nanocurcumin as a protective oxidative stress on ovarian granulosa cells due to exposure to lead acetate, by protecting lipid peroxidation.

**Key words :** Lead acetate, nanocurcumin, malondialdehyde (MDA).

## Introduction

Lead acetate (Pb) is a heavy metal compound that pollutes the environment, toxic and interfering various organ functions, including hematopoietic tissue, liver, kidney, brain, and testes<sup>1</sup> and interfering the function of digestive system, nervous system, respiratory system and reproductive system.<sup>2, 3</sup> Lead exposure has been

shown to cause toxicity in rat testes<sup>4, 5</sup> and alter normal histology and physiology of the ovaries and uterus of rats.<sup>6</sup> Exposure to lead acetate causes disruption to the body's organs through increased oxidative stress cell by destroying the balance between prooxidants and antioxidant systems, resulting in excessive production of reactive oxygen species (ROS) and depletion of cellular antioxidants.<sup>7</sup> Increased oxidative stress causes lipid peroxidation, the reaction between ROS and lipids in cell membrane system, especially the unsaturated lipids, and producing malondialdehyde (MDA), a cell-damaging toxic material.<sup>8</sup>

Malondialdehyde (MDA) is a secondary product of lipid peroxidation that can be used as a biomarker of

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toxicity in ovaries induced by lead acetate (Pb). Increased MDA level indicates a decrease in antioxidant activity to inhibit the formation of excessive free radicals.<sup>9</sup> The use of natural antioxidants is currently being a concern of researchers to reduce oxidative stress due to exposure to lead acetate.

Turmeric (*Curcuma longa*, Family *Zingiberaceae*) rhizome as a natural antioxidant contains 3 main components: curcumin, demethoxycurcumin and bisdemethoxy-curcuminoids in a ratio of 77: 17: 3. Curcumin (1,7-bis (4-hydroxy-3-methoxyphenyl) -1, 6 heptadiene-3, 5-dione), which is widely contained in the rhizome, has many benefits, especially as an antioxidant.<sup>10</sup> Although curcumin has an antioxidant effect, the bioavailability of curcumin is less understood. Nanotechnology is used to overcome problems related to the solubility, stability, and bioavailability of biological curcumin.<sup>11</sup> Nanocurcumin produced from curcumin nanotechnology has stronger potential compared to curcumin.<sup>12</sup> The aim of this study was to investigate the activity of nanocurcumin to decreasing lipid peroxidation in ovarian granulosa cells of white rats (*Rattus norvegicus*) exposed to lead acetate (Pb).

## Materials and Methods

### Chemicals

Curcumin (*Curcuma longa* (turmeric) powder, 65%, Product No. : CAS 458-37-7 molecular weight (MW):

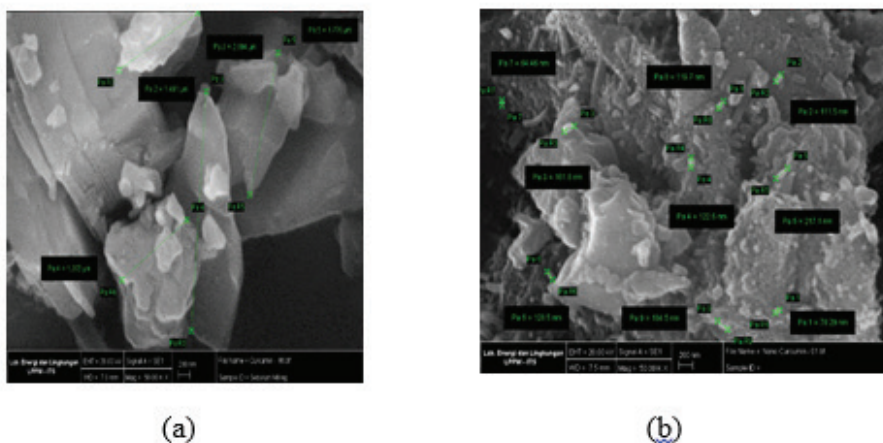
368.38 g/mol, product of China), and Lead acetate (Pb) (Product No: CAS 6080 -56-4, molecular weight (MW): 379.33 g/mol. Linear formula: Pb (CH<sub>3</sub>CO<sub>2</sub>) 2.3H<sub>2</sub>O), purchased from Sigma-Aldrich Co, USA.

### The making process of nanocurcumin

Nano Nanocurcumin was made at the Physics Laboratory, Universitas Airlangga, Surabaya, Indonesia. The method used was milling using the High Energy Miling (HEM) engine. Curcumin powder from curcuma longa (*turmeric*) rhizome was milled with collision by cubic zircornia balls, with a ratio between curcumin and cubic zirconia balls = 1 : 10. Curcumin and cubic zirconia balls were inserted into the tube and milled in a High Energy Milling (HEM) machine. The milling time was 20 minutes with a setting of 5 minutes milling and 5 minutes rest until the effective amount of processing time reaching 20 minutes outside the rest period.

### Nanocurcumin characteristics analysis

Analysis of nanocurcumin size characteristics was performed at the Robotics Laboratory, ITS, Surabaya, Indonesia, using Scanning Electron microscopy (SEM) method. Curcumin morphology before and after milling were compared. Curcumin morphology before milling appeared as irregular plates with a mean diameter of more than 1000 nm. Morphology of curcumin after milling had a more regular crystal shape with a mean diameter of less than 200 nm as shown in Figure 1.



**Figure 1** Morphology of nanocurcumin by Scanning Electron microscopy (SEM) examination. (a) Prior to milling with irregular plate, > 1000 nm in diameter. (b) After milling with a more regular crystal shape with a diameter <200 nm.

### The making of nanocurcumin solution

Nanocurcumin was dissolved with corn oil, because corn oil is the best carrier compared to butter, milk and water.<sup>13</sup> The solution was made by dissolving 2 grams of nanocurcumin with corn oil to 200 ml, so that 1 ml solution contained 10 mg of nano curcumin.

### Experimental animals

Female rats (*Rattus norvegicus*) with a mean body weight of 200-250 gr and an average age of 2.5-3 months were obtained from Institut Teknologi Bandung, Bandung, Indonesia, for experimental purposes. The rats were kept in plastic cages in air-conditioned rooms with temperatures of  $26 \pm 2^\circ$  degrees C and maintained with 12 hours of light and 12 hours of darkness. The rats were given with standard commercial food for rats and drinking water was given ad libitum.

### Experimental design

A total of 45 rats were divided into negative control group (C-) receiving corn oil and one hour later receiving distilled water, positive control group (C+) receiving corn oil and one hour later receiving lead acetate (Pb) of 40 mg/kg bw/day, experimental group 1 (E1) receiving 50 mg/kg/day nanocurcumin dissolved in corn oil and one hour later receiving lead acetate (Pb) of 40 mg/kg bw/day), experimental group 2 (E2) receiving nanocurcumin 100 mg/kg bw/day dissolved in corn oil and one hour later receiving lead acetate (Pb) of 40 mg/kg bw/day), and experimental group 3 (E3) receiving 200 mg/kg bw/day nanocurcumin dissolved in corn oil and one hour later receiving lead acetate (Pb) of 40 mg/kg bw/day. The treatment was given orally and the treatment time was every day at 08.00 AM for 26 days. On day 27 the rats were sacrificed by dislocation on the nape of the neck. Approximately 2 minutes after dislocation, the rats did not move, the eyes dimmed and the body did not move. Surgery was performed to remove the ovary. The ovaries were cleaned from

connective tissue, then washed with 0.9% physiological NaCl and put in Bouin's fixation solution (with saturated picric acid composition : pro-analysis formalin : glacial acetic acid = 15 : 5 : 1) for 24 hours. After the ovaries were fixed, the solution was replaced with 70% alcohol (stopping point) so that the tissue could be stored for a long time.

### Immunohistochemical analysis of MDA expression in rats ovarian granulosa cells

The removed ovarian tissues were embedded in paraffin blocks. Next, the slicing was carried out, and representative slices from the sample tissues were selected for immunostaining procedure. Each slice was stained using the streptavidin method by immunoperoxidase. The slicing of serial paraffin block in a thickness of 4 - 6  $\mu$ m was done. The best slices were chosen to calculate the MDA expression at 400X magnification. The presence of MDA was marked by the intensity of dark brown color. Observations were made quantitatively by counting the number of positive cells per visual field, counted up to 10 fields. The number of positive cells in each field was summed and divided by 10, and the final result was the average number of positive cells per visual field.

### Statistical Analysis

Data were presented with mean  $\pm$  standard deviation. Comparison test using Kruskal-Wallis Test was conducted to determine differences between groups, followed by Mann-Whitney test to determine differences between groups.

### Results

Kruskal-Wallis test showed MDA differences between groups (Kruskal-Wallis  $H = 33,697$ ;  $df = 4$ ;  $p = 0,000$ ). Then, we performed an analysis using Mann-Whitney test, with results as seen in Table 1.

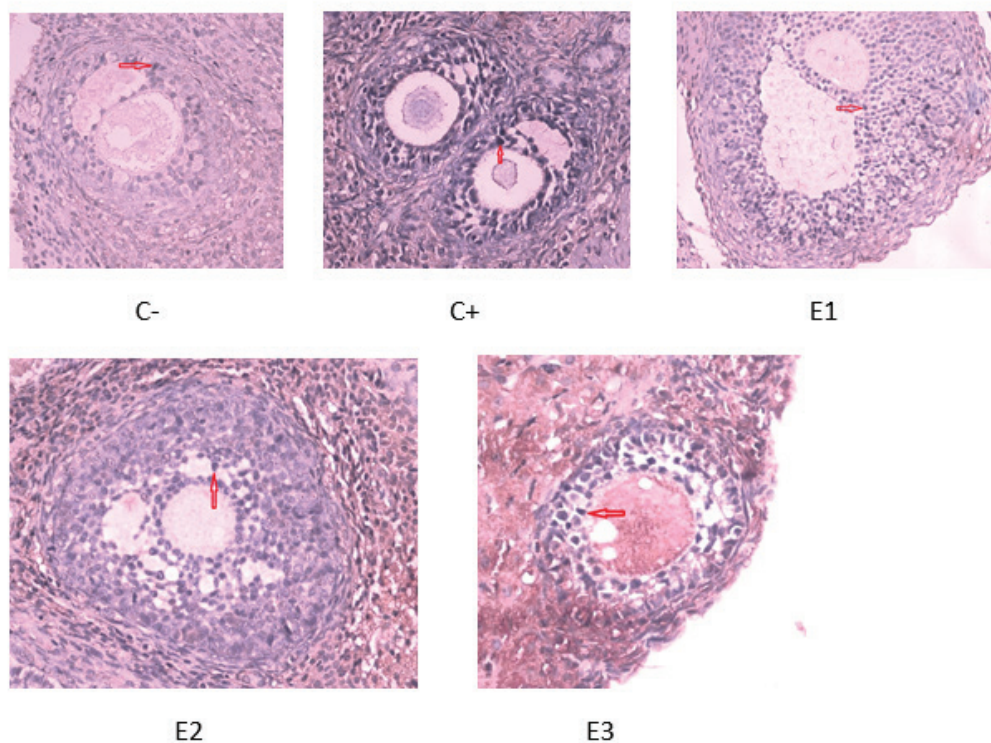
**Table 1. Effects of nanocurcumin on MDA expression of rat ovarian granulosa cells induced by lead acetate (Pb) (mean ± standard deviation)**

Groups	n	MDA	Minimum	Maximum
Negative control	9	1.5 ± 1.4a	0	4.4
Positive control	8	94.2 ± 10.5b	40.0	158.6
Experimental 1	9	17.4 ± 10.5c	8.0	37.2
Experimental 2	9	11.4 ± 8.5c	3.0	26.5
Experimental 3	8	7.2 ± 4.0d	2.2	13.0

a, b, c, d Different superscript within each column indicates significant difference between the means ( $p < 0.05$ ).

Table 1 shows that the positive control group had the highest MDA expression of ovarian granulosa cells and was significantly different ( $p < 0.05$ ) from negative control group, experimental groups 1, 2 and 3.

These results indicated that the administration of nanocurcumin of 200 mg/kg body weight of rats provided the greatest protection of white rats ovary cell lipid peroxide induced by lead acetate (Pb) of 40 mg/kg bw compared to the administration of 50 mg/kg bw nanocurcumin and 100 mg/kg bw. The administration of nanocurcumin of 50 mg/kg bw was the same as the administration of nanocurcumin of 100 mg/kg bw in lipid peroxide protection of white rat granulosa cells induced by lead acetate (Pb) of 40 mg/kg bw. The difference is presented in Figure 2.



**Figure 2. MDA expression of white rats (*Rattus norvegicus*) ovarian granulosa cells. C- (negative control group); C+ (positive control group); E1 (experimental group 1); E2 (experimental group 2); E3 (experimental group 3). Observation used light microscope with 400X magnification.**

Figure 2 shows the differences in MDA expression in various groups, MDA expression is in dark brown (examples are pointed with arrows) mostly in C+ group, and the least in C- group.

### Discussion

Lead acetate as one of heavy metals has a tendency to catalyze oxidative reactions and cause the formation of reactive oxygen species (ROS).<sup>14</sup> The results of this study indicated that lead acetate (Pb) exposure increased MDA expression of ovarian granulosa cells in white rats (*Rattus norvegicus*) compared to control group. However, the administration of nanocurcumin was significantly able to provide protection against lipid peroxide which was marked by a significant decrease in MDA expression.

If exposure to ROS cannot be offset by anti-oxidant enzymes or scavenger enzymes, where the ratio of ROS is higher than antioxidant enzymes, the cell experiences oxidative stress. Oxidative stress causes a reaction between ROS and lipids in cell membrane system, especially the unsaturated lipids (8). Oxidative stress due to increased ROS will cause lipid peroxidation, DNA damage and also a decrease in antioxidant defense system in the cells.<sup>14</sup>

Lipid peroxidation produces MDA which is a cell-destroying toxic substance.<sup>8</sup> MDA is the final product of lipid peroxidation in lead toxicity that induces oxidative stress.<sup>4</sup> High MDA concentration indicates oxidation process in cell membrane. MDA is also referred to as a highly reactive compound which is the final product of lipid peroxidation and is used as a biological biomarker of lipid peroxidation to assess oxidative stress.<sup>15</sup>

The inhibition of lipid peroxide can be induced by the nanocurcumin through: (1) the process of neutralizing free radicals involved in peroxidation by capturing hydroxy radicals (OH\*) produced by Fenton reaction and Haber Weiss reaction<sup>16</sup> and; (2) inhibition of hydroxyl radical (OH\*) formation. Lipid peroxidation can occur due to the increase in oxidative stress. Oxidative stress occurs because radical production exceeds the body's ability to neutralize it. This imbalance is present resulting from the decreased production of anti-oxidants or over-production of free radicals.<sup>17</sup>

Nanocurcumin has antioxidant activity because it has a phenolic hydroxyl group that functions as a free radical scavenger by inhibiting lipid peroxidase. Nanocurcumin has two aromatic rings that contain phenolic hydroxy, both of which are connected by short chains conjugated with  $\beta$ -ketones. Nanocurcumin also has a methoxy group in its aromatic ring which acts as an electron booster so that it makes it easier for the compound to capture hydroxy radicals. In nanocurcumin the dissociation energy of phenolic O-H is 5.04 kcal/mol lower than the dissociation energy of C-H binding in  $\beta$ -diketone group so that the tendency to donate H atoms from the phenolic group is higher.<sup>16</sup>

The inhibition of nanocurcumin in decreasing the expression of MDA by capturing hydroxy radicals (OH\*) is taking place because curcumin donates hydrogen atoms (H) from its phenolic hydroxyl (OH) groups when reacting with free radicals (R\*). This reaction will produce a curcumin phenoxyl radicals or flavonoid (KO\*/FO\*) which is less reactive because curcumin phenoxyl or flavonoid (KO\*/FO\*) can experience a change in resonance structure by redistributing unpaired electrons in the conjugated double bond structure in its aromatic ring. Phenoxyl curcumin or flavonoids (KO\*/FO\*) will react further to form unreactive compounds, which possibly takes place through the radical termination reactions.<sup>18</sup>

Another means of the inhibition of nanocurcumin in reducing MDA expression is by inhibiting the formation of hydroxyl radicals (OH\*). The inhibition of OH\* formation is to prevent Fenton reaction and Haber Weiss reaction by binding the transition metal (Fe<sup>++</sup> (Cu<sup>+</sup>), an OH\* catalytic.<sup>19</sup>

In conclusion, this study proved that the administration of nanocurcumin provided a protective effect of lipid peroxidation in ovarian granulosa cell induced by lead acetate (Pb) through free radical neutralization and the inhibition of hydroxyl radical formation. It is necessary to develop nanocurcumin for free radical protection due to lead exposure, so that it can be applied for supplementary therapy for women who have a risk of lead exposure.

**Conflict of Interest:** We declare that there is no conflict of interest.

**Source of Funding:** None

**Ethical Clearance:** Taken from Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya Indonesia.

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