Nanocurcumin Protective Effect on Gpx Scavenger Enzyme Expression and Apoptosis of Lead Acetate-Induced Rats Ovarian Granulosa Cells

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Abstract

This study aims to prove the protective effect of nanocurcumin against the reduction of glutathione peroxidase (GPx) scavenger enzymeand the increase of apoptosis in lead acetate-induced rat ovarian granulosa cells.

A total of 45 female white rats were divided into 5 groups, the negative control group (receiving corn oil and one hour later receiving distilled water), positive control group (receiving corn oil and one hour later receiving lead acetate (Pb) of 40 mg/kg bw), and experimental groups 1, 2 and 3 (receiving nanocurcumin 50, 100 and 200 mg/kg, and one hour later receiving lead acetate (Pb) 40 mg/kg bw). All groups received treatments per oral once a day for 26 days. On day 27 the rats were sacrificed and then the expression of the GPx scavenger enzyme and apoptosis was performed by immunohistochemistry.

Lead acetate (Pb) decreased the expression of the GPx scavenger enzyme and increased apoptosis. The administration of nanocurcumin increased the expression of GPx scavenger enzyme and decreased the expression of apoptosis in lead acetate-induced rat ovarian granulosa cells.

Nanocurcumin functions as an antioxidant by providing protective effect against GPx scavenger enzyme and apoptosis in lead acetate (Pb)-induced rat granulosa cells.

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Introduction

Lead (Pb) is an easily found toxic heavy metal, one of environmental pollutants which can affect the function of organs such as testes, liver, brain, hematopoietic and kidneys[1]. Accumulation of lead in the body may cause toxicity and potentially affect the reproductive system [2]. Lead exposure can also cause abortion, preterm birth and fetal death [3] and has been shown to cause rat's nephron toxicity [4] and testicular toxicity[5] .

As a part of heavy metals, lead acetate has a tendency to catalyze oxidation reactions and lead to the formation of reactive oxygen species (ROS)[6,7]. If exposure to ROS is not able to be balanced by scavenger enzyme, where ROS ratio is higher than that of antioxidant enzymes, cells experience oxidative stress which causes lipid peroxidation and DNA damage which will end in apoptosis and decreased endogenous antioxidant defense systems in cells [8]. Endogenous antioxidants known as scavenger enzymes include the GPx enzyme[9]. GPx is the most important enzyme as an endogenous antioxidant that can scavenge free radicals. GPx is a first-line defense antioxidant and requires selenium to perform its function, so it is also called a selonoprotein antioxidant[10] . Glutathione peroxidase (GPx) is an important intracellular enzyme that breaks down hydrogen peroxide (H2O2) into water (H2O) and lipid peroxides into alcohol[11] .

Pb has effect on antioxidant enzyme GPx because Pb has a high affinity and reactivity for selenium, which is a component of the GPx enzyme, so that it can act as an antioxidant. The binding of selenium to Pb will cause glutathione

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peroxidase to lose its ability as an antioxidant[12].

Lead acetate exposure causes increased apoptosis of ovarian granulosa cells through oxidative stress mechanisms. Hydroxyl radicals (OH*) formed due to lead exposure can translocate into ovarian granulosa nuclei and stimulate the release of P53. P53 reacts with mitochondrial membrane and activates pro-apoptosis (Bax) and causes decreased anti-apoptosis (Bcl-2 and Bcl-x) which makes cytochrome c release to granulosa cell cytosol. In the cytosol, cytochrome c binds to Apaf-1 (apoptosis-activating factor 1) to form a caspase recruitment domain (CARD) which stimulates caspase 9 granulosa cells and caspase 9 will stimulate caspase-3, an effector that carries out granulosa cell apoptosis[10].

In protecting against the reduction of scavenger enzymes, including GPx, and the occurrence of apoptosis due to ROS, body's cells require exogenous antioxidants, such as curcumin rhizome (Curcuma longa). Curcumin has benefits as anti-bacterial, anti-inflammatory, chemopreventive, wound healing, anti-parasitic, and has a phenolic group that has great potential as an antioxidant substance[13].

Analysis of the antioxidant activity of curcumin also showed that curcumin had considerable antioxidant activity[14]. Several studies have shown that curcumin can reduce oxidative stress due to lead toxicity by inhibiting oxidative stress very effectively[15]. but its clinical application is still limited, both in vascular and oral administration.

The limitation of clinical application of curcumin is due to its poor solubility and absorption, leading to its low bioavailability[13] . Orally administered curcumin absorption undergoes presistemic elimination. Once absorbed, curcumin is conjugated by sulfates and glucuronates at various tissue sites. The poor absorption pattern makes it difficult to find high levels of curcumin in blood some time after administration, so the effect is less effective[16]. With innovations to improve bioavailability, longer circulation, and better permeability, curcumin is formulated in the form of nanoparticles. This study aims to investigate the protective effect of nanocurcumin against the reduction of the GPx scavenger enzyme and the increase in lead acetate-induced apoptosis of white rats' granulosa cells.

Materials and methods

Chemical materials

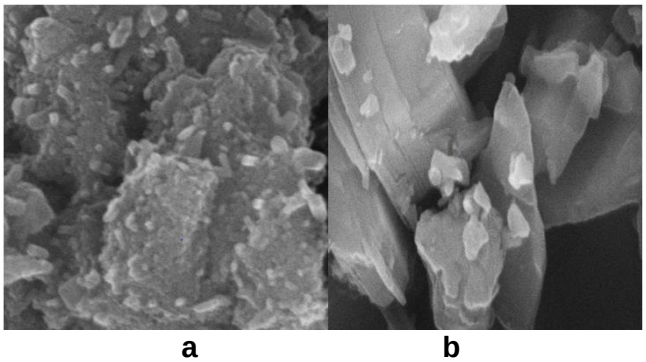
Lead acetate (Product No: CAS 6080-56-4, molecular weight (MW): 379.33 g/mol, Linear formula: Pb (CH3CO2) 2.3H2O), purchased from Sigma-Aldrich.co. USA and Curcumin (Curcuma longa (turmeric) powder, ≥65%, Product No: CAS 458-37-7 molecular weight (MW): 368.38 g/mol, product of China).

2.1. Making process of nanocurcumin

The method used for the making of nanocurcumin is by milling using a High Energy Milling (HEM) machine. Nanocurcumin was made at the Physics Laboratory, Universitas Airlangga, Surabaya, Indonesia. The milling time was 20 minutes with a setting of 5 minutes milling, 5 minutes resting until total effective processing time of 20 minutes outside the resting time. Curcumin powder from the rhizome of Curcuma longa (turmeric) was milled by pulverizing cubic zirconia balls, with the ratio of curcumin : cubic zirconia balls = 1 : 10. Curcumin and cubic zirconia balls were fed into the tubes and milled in a High Energy Miling (HEM) machine.

2.2. Analysis of nanocurcumin characteristics

Analysis of nanocurcumin size characteristics used the Scanning Electron Microscopy (SEM) method and was carried out at the Robotics Laboratory, ITS, Surabaya, Indonesia. The morphology of curcumin after milling was compared with that of before. The morphology of curcumin after milling showed a more regular crystal shape with an average diameter of less than 200 nm. The morphology of curcumin before milling appeared as irregular plates with a mean diameter of more than 1000 nm as shown in Figure 1.



a b

Figure 1. The morphology of nanocurcumin by scanning electron microscopy (SEM). (a) Curcumin after milling with a more regular crystal form with a diameter of <200 nm. (b) Curcumin before milling with irregular slab shape, diameter> 1000 nm.

2.3. Preparation of nanocurcumin solution

A solution was made by dissolving 2 grams of nanocurcumin with corn oil into 200 ml, so that 1 ml of the solution contained 10 mg of nanocurcumin. Nanocurcumin was diluted with corn oil, because corn oil was the best carrier compared to butter, milk and water [17].

Experimental Animals

Female wistar rats weighing about 180-200 g, aged 2.5 - 3 months were obtained from Institut Teknologi Bandung (ITB), Bandung, Indonesia, for experimental purposes. The rats were placed in cages in an air-conditioned room with temperatures maintained at 26°C - 2°C and 12 hours in a light and dark cycle. Rats were given with feed and mineral water ad libitum. This study has passed the ethical test by the Ethics Committee, Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia, and obtained ethical eligibility No: 2.KE.170.08.2019 on 29 August 2019.

Experimental design

A total of 45 female rats were divided into 5 groups, the negative control group (rats receiving corn oil, one hour later receiving distilled water), positive control group (rats receiving corn oil, one hour later receiving lead acetate of 40 mg/kg bw), and experimental groups 1, 2 and 3 (rats receiving 50 mg, 100 mg and 200 mg/kg bw of nanocurcumin orally, one hour later receiving lead acetate of 40 mg/kg). All groups received treatment orally once a day for 26 days. On day 27 the rats were sacrificed with neck dislocation, then the peritoneum was excised, the ovaries were taken to examine the expression of GPx scavenger enzyme and the apoptosis of granulosa cells by immunohistochemical examination.

Immunohistochemical examination

The removed ovaries were implanted in a paraffin block, then sliced, and a representative slice was selected from the tissue sample for immunostaining procedure. Each slice was stained with streptavidin method using immunoperoxidase. Serial cutting of paraffin blocks was carried out in a thickness of 4 - 6 uM.

The best slices were selected to examine the expression of GPx scavenger enzyme and apoptosis at 400X magnification. The presence of the GPx scavenger enzyme and apoptosis was characterized by dark brown color intensity. Observations were made quantitatively by counting the number of positive cells per visual field, counting up to 10 fields.

The numbers of positive cells for each visual field were summed up and divided by 10, and the final result was the mean number of positive cells per visual field.

Statistical analysis

Data were presented with mean ± standard deviation. The comparative test used Kruskall-Wallis Test to determine the differences between groups, followed by Mann-Whitney test to determine the differences between the groups.

Results

Nanocurcumin protection against lead acetate-induced reduction of GPx scavenger enzyme expression in rats ovarian granulosa cells

The results of Kruskal-Wallis test showed differences in the expression of GPx scavenger enzyme (Kruskal-Wallis H = 21,787; df = 4; p = .000). Then, to identify differences between groups, we used Mann-Whitney Test, as in Table 1.

Groups	n	GPx expression (%/micro)	Minimum	Maximum
Negative control	9	3.5 ± 0,5 [a]	2.9	4.5
Positive control	8	2.4 ± 0,3 [b]	1.7	2.8
Experimental 1	9	2.5 ± 0,2 [b]	2.3	2.9
Experimental 2	9	3.2 ± 1,0 [a]	2.1	5.4
Experimental 3	8	3.5 ± 0,6 [a]	2.6	4.3

Table 1. Effect of nanocurcumin on GPx scavenger enzyme expression of lead acetate-induced rat ovarian granulosa cells (mean ± standard deviation).

[a,b]Different superscript within each column indicates significant difference between the means ($p < .05$).

Table 1 shows that the mean expression of GPx scavenger enzyme of granulosa cells in rats' ovaries was the highest in the experimental group 3 (3.5 ± 0.6%/micro), the same (p = .481) with that of negative control group (3.5 ± 0.5%/micro) and also the same (p. = .139) with that of experimental group 2 (3.2 ± 1.0%/micro). The lowest value in control group was positive (2.4 ± 0.3%/micro) and the same (p = .263) with that of the experimental group 1 (2.5 ± 0.2%/micro).

These results indicated that the nanocurcumin at a dose of 100 mg/kg bw and 200 mg/kg bw increased the expression of GPx scavenger enzyme in lead acetate-induced rat

ovarian granulosa cells. This difference is also shown in Figure 2.

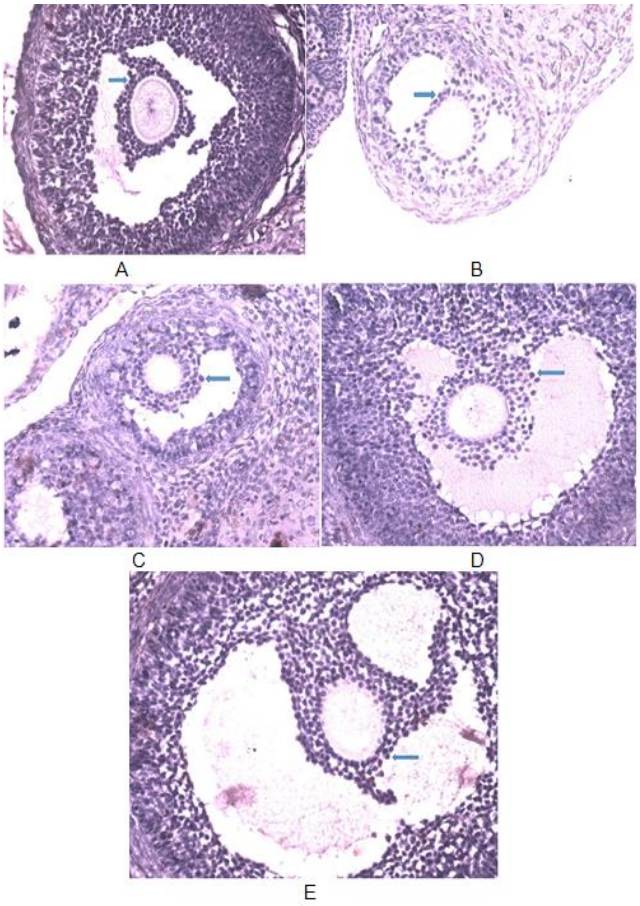


Figure 2. Comparison of GPx scavenger enzyme expression in rats' ovarian granulosa cells. (A) K- group; (B) K+ group; (C) P1 group; (D) P2 group; (E) P3 group. Observation used light microscope in a magnification of 400x. Arrow (⟹) shows an example of GPx scavenger enzyme expression.

Groups	n	Apoptosis expression (%/micro)	Minimum	Maximum
Negative control	9	2.2 ± 0.8 [a]	1.0	3.5
Positive control	8	4.3 ± 0.8 [b]	2.7	5.6
Experimental 1	9	3.4 ± 1.0 [c]	2.1	4.8
Experimental 2	9	2.4 ± 0.8 [a]	1.6	3.8
Experimental 3	8	2.0 ± 1.2 [a]	1.2	4.9

Table 2. Effect of nanocurcumin on the expression of apoptotic granulosa in ovaries of lead acetate-induced rats (mean ± standard deviation).
[a,b,c]Different superscript within each column indicates significant difference between the means ($p < .05$).

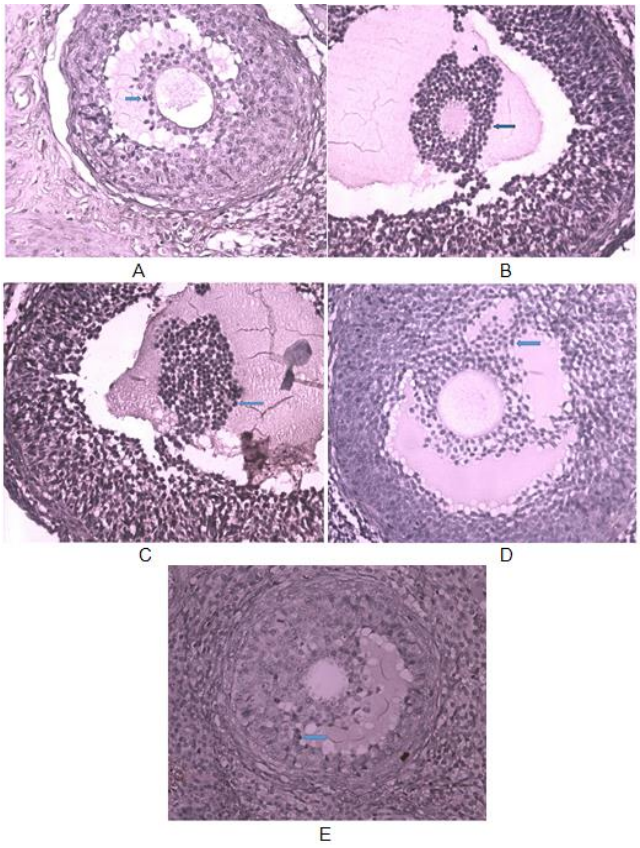


Figure 3. Comparison of the expression apoptotic of rats' ovarian granulosa cells with 400 x magnification. (A) K- group; (B) K+ group; (C) P1 group; (D) P2 group; (E) P3 group. Observation using a light microscope with a magnification of 400x. Arrow (⟹) shows one example of apoptotic expression.

Discussion

The results showed that exposure to lead acetate as much as 40 mg/kg bw reduced the expression of GPx scavenger enzyme in rats' ovarian granulosa cells. The decrease in the expression of GPx scavenger enzyme due to lead acetate induction was in line with previous studies. Rats receiving lead acetate injection of 20 mg/kg bw/day intraperitoneally for 5 days experienced a decrease in renal GPx compared to control group receiving distilled water injection [18]. Rats injected with lead acetate of 20 mg/kg bw for 11 days had a lower mean testicular GPx than control group injected with distilled water alone [5]. Rats that were given with normal saline (0.9% NaCl) of 3 ml/kg bw/day orally and one hour later injected intraperitoneally with lead acetate of 20 mg/kg bw/day for 7 days experienced a significant reduction in hepatic GPx compared to

that of control group with the same treatment and received a normal saline injection of 1 ml/kg bw/day [19].

As the first line defense besides SOD and caspase-3, GPx is the most important enzyme as an endogenous antioxidant capable of scavenging free radicals. As an antioxidant, GPx requires selenium to carry out its functions, so it is also called a selonoprotein antioxidant. GPx breaks down hydrogen peroxide (H2O2) as a result of ROS into water (H2O); and lipid peroxides into alcohol [10].

Pb has high affinity and reactivity to selenium, a component of GPx, so that it can act as an antioxidant. The binding of selenium to Pb causes GPx to lose its ability as an antioxidant. The increase in Pb in the body will cause the binding of Pb with selenium. As a result, the availability of selenium in the body decreases. The reduced availability of selenium causes GPx not to act as an antioxidant [20]. This was proved by the decrease in the expression of the enzyme GPx in rats ovarian granulosa cells induced with lead acetate of 40 mg/kg bw/day by sonde.

This study also proved that the administration of nanocurcumin increased GPx expression in rats granulosa cells induced with lead acetate of 40 mg/kg bw. This study found that mean GPx expression in P3 group was higher than that in P2 group and that mean GPx in P2 group was higher than in P1 group. This finding is in line with previous studies which reported that curcumin as an antioxidant can increase catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPx) [21].

Curcumin has a protective effect on reproductive organs, such as anti-inflammatory, anti-apoptotic and antioxidant in normal cells and acts as a pro apoptosis in malignant cells. Curcumin's effects depend on the dose and type of cells used for the trial[22] .

The protective mechanism of nanocurcumin against lead acetate-induced decreased GPx expression in rats' ovarian granulosa cells is through the inhibition of superoxide radical formation (O2*-) by suppressing the activity of cytochrome P450. P450 is an important isoenzyme for initial bioactivation of reactive oxygen species[23]. Inhibition of cytochrome P450 activity will inhibit oxidative phosphorylation, which is an important part of metabolism that produces reactive oxygen species, the superoxide (O2 -). The reduction of superoxide radicals (O2*-) will increase GPx expression[24] .

This study proved that lead acetate exposure of 40 mg/kg bw/day for 26 days in rats increased ovarian granulosa cell apoptosis compared to that of the control group. The results of this study were in line with previous studies, that the administration of intra-peritoneal injection of lead acetate of 20 mg kg bw for 7 days to rats increased pro-apoptosis markers (Bax and caspase-3 [25]. Researchers also reported that administering intraperitoneal injection of lead acetate of 20 mg/kg bw/day for 7 days to rats increased nephron apoptosis, as evidenced by the increase of pro-apoptotic nephron markers, the Bax and caspase-3 proteins, and, conversely, the decrease of anti-apoptotic marker, the Bcl-2 protein, as compared to control group receiving injection of 1 ml/kg bw/day normal saline[26] .

Lead acetate exposure causes increased H_2O_2 causes oxidative stress which can trigger apoptosis) [,27]. Hydroxyl radicals (OH*) formed as a result of lead exposure translocate to ovarian granulosa cell nucleus and stimulate the release of P53. P53 reacts with mitochondrial membrane and activates pro-apoptosis (Bax) and causes decreased anti-apoptosis (Bcl-2 and Bcl-x) leading to the release of cytochrome c into granulosa cell cytosol. In the cytosol, cytochrome c binds to Apaf-1 (apoptosis-activating factor 1), forming the caspase recruitment domain (CARD) which stimulates caspase-9 granulosa cells, and caspase-9 stimulates caspase-3 which is an effector that carries out apoptosis of granulosa cells[10] .

This study also proved that administering nanocurcumin reduced lead acetate-induced apoptosis of granulosa cells in rats ovaries at a dose of 40 mg/kg bw. This was evident in this study that the mean apoptosis expression in P3 rats was lower than in P2 group, the mean apoptosis in P2 group was lower than in P1 group, and that in P3, P2, and P1 groups were lower than in K+ group. This suggests that nanocurcumin provides a protective effect against ovarian granulosa cell apoptosis.

Curcumin isolated from Curcuma longa (turmeric) is very potential as an antioxidant which is thought to be caused by phenolic and 1,3-dicetone groups[14]. This natural polyphenolic antioxidant compound is multifunctional and can function as: (1) antidote to free radicals such as superoxide (O2*) and hydroxyl radicals (*OH), (2)

chelating metals such as iron (Fe), (3) inhibiting oxidative enzyme activity such as cytochrome P-450, and (4) reducing the formation of oxygen radicals[21]. The antioxidant activity of curcumin compound can occur because the formation of free radicals is inhibited by this compound by suppressing the activity of cytochrome p450[28].

As an antioxidant, nanocurcumin plays an important role in preventing apoptosis caused by oxidative stress due to lead toxicity by inhibiting the formation of hydroxyl radicals (OH*). The inhibition of hydroxyl radicals (OH*) formation is by preventing Haber Weiss reaction and Fenton reaction. The prevention is carried out by chelating the transition metals F ++ and C + which act as catalysts for OH* formation[29] . The prevention of OH* formation suppresses Bax, and Bax increases the release of Bcl-2 and Bcl-xl expression and suppresses cytochrome c expression out of mitochondrial membrane, resulting in the absence of binding between cytochrome c and Apaf-1, which is called apoptosome[30]. Thus, there is a decrease in stimulation to caspase-9 which results in the decrease of caspase-3 and ends with the decrease in apoptosis[31].

Conclusions

Nanocurcumin acts as protection against the reduction of GPx scavenger enzyme and the increase in apoptosis of lead acetate-induced rats ovarian granulosa cells.

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Declaration of Interest

Tidak ada konflik kepentingan dalam artikel ini.

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