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Effect of Vitamin C on Xenobiotic Metabolism and Histopathology of Fetal Brain of Lead Exposed Mice.

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ABSTRACT

The objectives of this study are to investigate the effect of vitamin C on xenobiotic metabolism and histopathology of fetalbrain of mice after exposed to lead. The experiment used 27 pregnant mice and divided into three groups: the negative control group (NC), which wastreated with distilled water, the positive control group (PC) was exposed to 25 mg Pb/kg body weight (BW)/day, and the third group (T) was exposed to 25 mg Pb/kg BW/day during gestation day 7 to 16 and then administered with 64 mg vitamin C/kg BW/day started on the gestation day 9 to 16. Both lead and vitamin C were given to mice orally. The result showed that supplementationof vitaminCprovided protectionto thefetus. The histological structure of fetal brain administered to vitamin C (T) was betterthan PC. Theaverage concentration of lead inits fetal head (T) is the lowestamongthe othergroups.Supplementation of vitamin C can protect liver and fetuses, suggesting that vitamin C could bind the lead and excrete it via urine.

Keywords: lead, vitamin C, CYP1A1, GST, histopathology, liver, fetalbrain

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INTRODUCTION

Heavy metal pollution can occur through air and industrial pollution. Seventy percent of air pollution in Indonesia was caused by vehicle emissions. The number of vehicles increases gradually every year, consequently the fuel consumption also increase, while the gasoline produced has not been free of lead. One liter of gasolinepredicted containing 0.45 g of lead (1999 Annual Report, Directorate General of Oil & Natural Gas). Other sources of lead pollution arewaste of metal (iron and steel) industries, industry of batteries, canning industry, incineration, and cement production (Darmono, 2001). Research conducted by Soehendro et al (2005), showed that 84.4% of school children in 9 elementary schools in Sidoarjo district Indonesia has high blood lead levels (> 10 g/dL). Lead enters the body through the respiratory system. Lead in the blood will diffuse into the soft tissue (bone marrow, nervous system, kidneys, and liver) and hard tissue (bone, nails, hair) (Ardyanto, 2005). High lead levels will accumulate and adversely affect the cognitive function (Shih et al, 2006), causing impairment of neuropsychological function (Surkan et al, 2007), and is associated with abnormalities of pregnancy (Falcon et al, 2002), anemia, gastro enteritis, encephalopathy (Darmono, 2001), hyperactivity and other problems in children (Linder, 1992), and interfere with central nervous system. Lead bound to-SH groups of proteins at high concentrations causes the depletion of glutathione (GSH) (Halliwel, 1998), disrupt the immune system of children (Karmaus, 2005), lower kidney function (Weaver et al, 2005), inhibits the activity of enzymes and acids ferokelataseδ -amino levulinatdehydratase (Gibson, 2005). In *Rattusnovergicus* with blood lead levels of at least 1.39 g/ml, caused some histopathological alterations: degeneration, hyperplasia and edema of cerebral cortex (Hariono, 2005). Organic lead will affect the metabolism of xenobiotics in the liver and the enzyme in xenobiotics metabolism such as cytochrome P450.

Chronic lead exposurein mice will induce cytochromeP450 enzyme activity. Ifleadis addedto the incubation of microsomes, the activity of NADPH-cytochrome P450 reductase would be inhibited and an inhibitor of drug metabolism with a particular substrate. Leadal so inhibit certain enzymes in the synthesis of haem (Murray, 2006), affects the activity of cytochromeP4501A1(CYP1A1), which one form of cytochromeP450 is forms by Travenetal (2008) in vitro for pollutants in sediments from the sea, the used of enzymes CYP1A parameters and phaseII reactions, lead also affects the activity of the enzyme glutathione-S-transferase, sincethis enzymeis aglutathione substrat which has sulfhydryl groups, which are vulnerable to heavy metals (Murray, 2006).

Lead in the blood can be excreted through urine with vitamin supplementation is given to an officer of the General Fuel Filling Station (gas stations) in Abekuota, Nigeria(Onunkworetal, 2004). High calcium consumption is also reducing lead from the body (Linder, 1992;Ettinger etal, 2006). Lead can be reduced by administering chalet or such as British Anti Lewisite (BAL), Calcium Sodium Ethylene Diamine TetraAcetic (Ca Na-EDTA) and penicillamine. BAL binding of lead inserum, blood and cerebrospinal fluid but Ca Na-EDTA will be chelate lead from soft tissues, bones and excreted inurine (Darmono, 2001). VitaminC was chosenin this study because It can reduce lead in blood (Onunkworetal, 2004 (Christyaningsih,

2008) and it is non-toxic chelating agent that can be used to prevent the excessive effect of lead exposure. So far the effect of vitamin supplementation on the xenobiotic metabolism and fetal brain's histopathology in mice after exposed to lead has not been studied. Therefore, the objectives of this study are to investigate the effect of vitamin C on xenobiotic metabolism and histopathology of fetal brain of mice after exposed to lead.

MATERIALS AND METHODS

Animals

Adult female mice (*Musmusculus*) BALB/C strain, 10 weeks of age, approximately *25-30*g of body weight, physically healthy, and in 7 days pregnant condition were used in the experiment. The use of this animal has been authorized by the Research Ethics Committee of Veterinary Medicine Faculty of Airlangga University. Twenty seven pregnant mice are divided into 3groups, each containing nine mice

1. Negative control group (NC), as a place bowas given distillated water
2. Positive control group(PC) was exposed to neutral lead acetate at a dose of25 mg/kg BW/day, starting atgestationto7 to gestation to 16.
3. Treatment group(T) was exposed to neutral lead acetate at dose of25 mg/kg BW/day starting at gestation day7 to 16 and were given vitamin C at a dose of64mg/kg BW/day, starting ongestationalday9 to 16.

Isolation and purification of the enzyme glutathione-S-transferase cytosolic

The liver tissue wastransferred in phosphate buffered saline(PBS)solution with heparin (0.15 mg/mL) to separate the blood cells and clots. Tissue homogenized in glutatione S-transferase (GST) buffer (100 mg/0.5 mL) and centrifuged at 10,000g for 15 minutes at 4^{o}C. Supernatant were separated and analysedof GST activity. The sample is stable when stored at -80°C for 1-month (BioVision, Cat K263-100)

Preparation of microsomal, homogenates for CYP1A1 measurement

Liver was washed repeatedly in 0.15 M cold KCl solution, to clean it from blood fractions. Tissue stored in liquid nitrogen or stored directly in the temperature of -80°C to prevent the damage of CYP1A1 enzyme activity (Ikzus, 2006).

Measurement of cytochrome CYP1A1 enzyme activity (Ikzus, Cat. No. 4260-500K)

About 0.1 to 0.5 gram of tissue homogenized at 0-4^{o}C by adding 4 volumes of solution A (tissue extraction buffer) and B (protease inhibitors) from the kit. Homogenate was transferred on 2 pieces @ 2 ml microtube, and centrifuge at 9000 relative centrifugal force(RCF) at 4°C for 20 minutes. Two to five mL fractions used to analyse for total protein in 0.25 M sucrose by the method of Bradford reagent (Ikzus proteomics, Cat. No.1907-P). Afterwards transferring the

185 mL solution C (reaction buffer) at room temperature into the well. Solution E (substrate 1mm), added 4 mL, Ethoxyresorufin 1:10 into the well (with a final concentration of 2 µM). Supernatant was added to 10 mL in the well. The reaction begins when the addition of 10 mL solution of D (β NADPH) from the kit. Absorption spectrophotometer measurements performed with a wavelength of 573 nm at 10-20 minutes. Calculating Δ OD / minute from a standard curve. Spectrophotometer calibrated using a solution of H (standard resorufin) 1mL solution of resorufin: 2.0: 4.0: 6.0: 8.0 mL into solution C so that the concentration of resorufin 10; 20; 30; 40 nanomol / mL, absorbance read at 573 nm.

Measurement of glutathione S-transferase activity (BioVision, Cat K263-100)

Measurement of glutathione S-transferase activity using a spectrophotometer requires a reagent 1-chloro-2 ,4-dinitrobenzene (CDNB) as substrate. Mixed CDNB substrate, enzyme, 90 mL potassium phosphate buffer, 10 mL GSH is read at 340 nm 5 times every minute.

Fetal brain histopathology observations

Fetal brain was fixed with buffer formalin, embedded in paraffin and stained with Hematoxylin and Eosin. Brain histopathology observed is the thicknessof the cerebral cortex and corpus callosum.

RESULTS

Effect of vitamin C on the activity of the enzyme cytochrome P-450 1A1 (CYP1A1) in liver

Mean activities of CYP1A1 enzyme extracted from the liver of mice were presented in Fig. 1. Induction of CYP1A1 enzyme activity was higher at the positive control group (PC) (exposed to lead) than that of the negative control group (NC). Whereas, the CYP1A1 enzyme activity in the treated group (T) was lowest compared to the other groups.

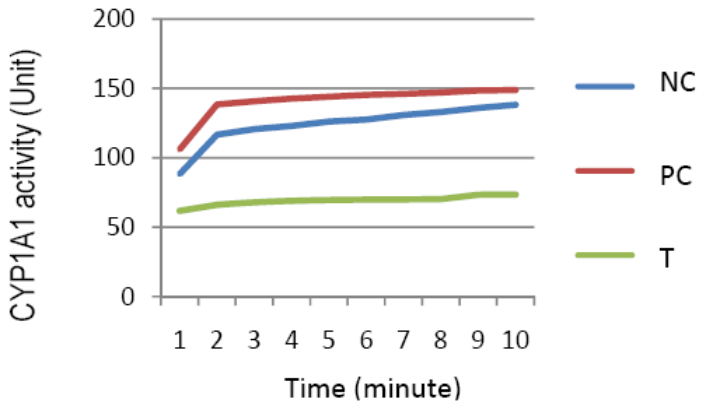

Figure 1: The mean values of hepatic CYP1A1 of negative control group (NC), positive control group (PC) and treatment group (T)

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Effect of vitamin C on the activity of the enzyme glutathione-S-transferase in liver

The mean values of GST enzyme activity of liver between groups of mice within 5 minutes were presented in Fig. 2. The PC(exposed to lead) shows higher value of GST enzyme activity than that of the NC. The lowest GST enzyme activity was noted at T.

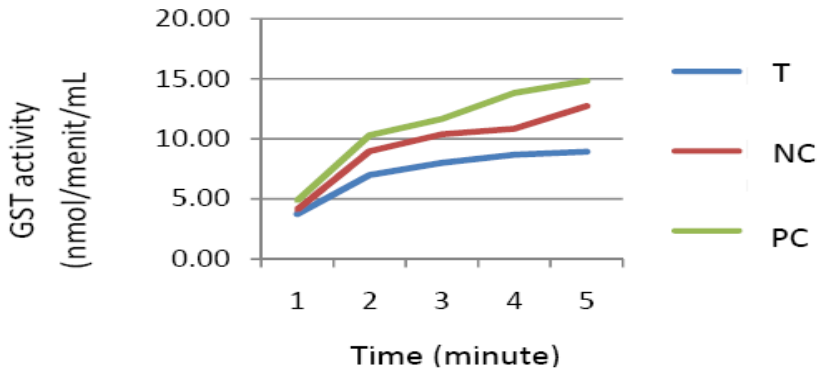

Figure 2:The meanvalues of glutathioneS-tranferase(GST) of liverthe negativecontrol group (NC), positive controlgroup (PC)andtreatment group (T)

Effect of vitaminCon the histopathology ofthe fetalbrain

The histopathologies of the fetal brains of mice are presented in Fig. 3. Thick cerebral cortex and corpus callosum were noted at NC and T. The thickness of carpus callosum of both NC and T was about 0.2 mm, or two-fold compared to that of the PC (0.1 mm).

There are the differences in the thickness of the cerebral cortex in the three groups of mice. The thickness of cerebral cortex in NCis about 0.2 mm, meanwhile the thickness of cerebral cortex of PC is 0.1 mm. The T has the highest thickness of cerebral cortex (0.3 mm).

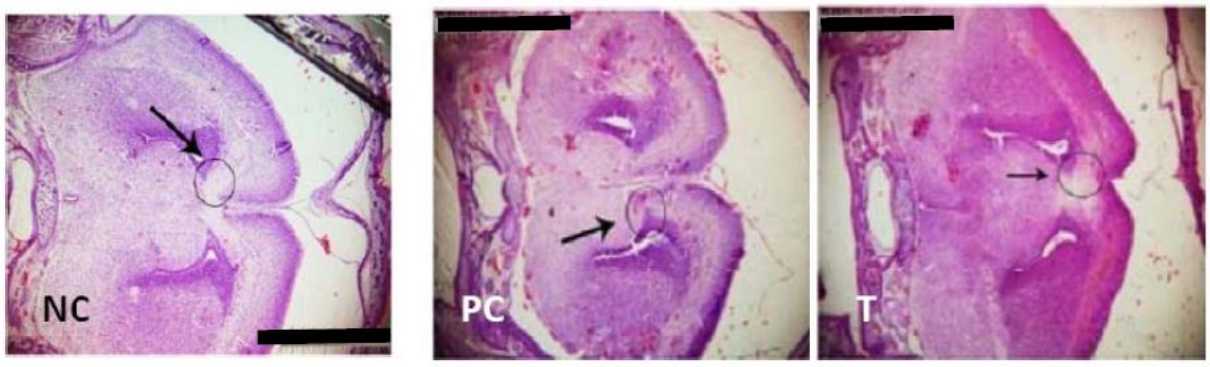

Figure 3: Histopathology of corpus callosum of fetal brain. Tissue is cut in the frontal or transverse cross section,and stained with Hematoxylin Eosin, with 40x magnification. NC is the negative control group, PC is the positive control group, T is the treatment group. Bar size = 1 mm

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DISCUSSION

The presence of lead in the body affect the hepatic drug metabolism, cholesterol metabolism, oxidative stress and causes hyperplasia in the liver, so it is also potentially damaging extrahepatic systems including the cardiovascular system(Mudipalli, 2007).

Effect of Vitamin C on the Activity of Liver CYP1A1

The results show that the highest CYP1A1 enzyme activity is achieved byPC (exposed to lead), and it's followed by NC and lowest CYP1A1 enzyme activity occurred in T (the group treated with vitamin C). CYP1A1 enzyme in the negative control group (placebo) had a highest activity when compared with the group treated with vitamin C, this is due to the negative control group received lead exposure from food which contained 98 µg Pb/kg according to the analytical laboratory measurement. Increasing CYP1A1 enzyme activity in the positive control group was consistent with the results of Traven et al (2008) which reported that the presence of heavy metals in marine sediment induced CYP1A1 enzyme of fish liver. Acute exposure to organic and inorganic lead caused a decrease in rat hepatic CYP450 activity and increase the excretion of δ-amino levulinic in the urine (Mudipalli, 2007). Methyl mercury was also reported affect the diminution of cytochrome P450 of rat (Alvares et al, 1972; Ryan et al, 1993). Acute exposure of copper was also reported inhibit cytochrome P450 enzymes and decrease the activity of the enzyme cytochrome C in liver microsomes (Joon-Sik Kim et al, 2002). Metals Hg^{2+}, Pb^{2+}, Cu^{2+} induced CYP1A1 mRNA expression. Korashy et al. (2008) reported that lead increased the activation of κBnuclear factor which played important role to modulate the induction of CYP1A1 mRNA. Exposure of lead nitrate in mice can induce TNF-α in the liver and affect the mRNA expression of CYP1A1 (Shinozuka et al, 1994). Supplementation of vitamin C can reduce the effect of lead on the activity of CYP1A1 enzyme in the liver. It's possibly that vitamin C play a prominent role to chelate lead before going to the liver. Consequently the levels of lead in the body becomelow, and the activity of CYP1A1 in the group treated with vitamin C (T) is lowest when compared with the NC (negative control group). CYP1A1 enzyme activity in the group treated with vitamin C (T) has the lowest activity, is consistent with previous studies conducted by Chang et al (2009) and Ueta et al (2003) which show that the supplementation of vitamin C affect the expression of genes that encode indirectly CYP1A1 at the level of transcription. Intake of vitamin C affects the gene expression of antioxidant enzymes and enzymes that catalyze the metabolism of xenobiotics by using a competitive reverse transcription-polymerase chain reaction method (competitive RT_PCR).

Supplementation of vitamin C provides protection to the liver, because vitamin C can chelate lead before transported into the liver and excreted via urine. In kidney most metals will be chelated by the protein containing a high thiol (called metalothionein). Exposure to toxic metalwill trigger the production of these proteins to bind metal, so it is not harmful to the body. Metals in sediments may increase the activity of CYP1A1 metalothionein and thus this enzyme is a potential indicator for metal exposure (Costa et al, 2009; De Favemey et al, 2009).

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Effect of Vitamin C on the Activity of Liver Glutathione S-Transferase

The results (Fig. 2) show that the highest activity of glutathione S-transferaseis noted in the positive control group (PC), followed by the negative control group (NC) and the lowest in the group treated with vitamin C (T). Columbano et al (1988) studied lead exposure in rats with a single injection can increase the activity of the enzyme glutathione S-transferase-placental in the liver, which did not occur in normal rat'sliver. Suzuki et al (1996) reported that there is a relationship between lead and activation of genes that encode glutathione S-transferase with glutathione S-transferase-placental enhancer I (GPEI) so that the presence of lead in the body can increase the activity of glutathione S-transferase enzymes, as occurs in positive control group (PC) exposed by the lead. Increased hydrophilicity of GSH conjugates facilitates further metabolism and elimination (Rouimi et al, 1996). GST enzyme activity is carried out by phase I enzymes CYP1A1, resulting in the same groove in the group treated withvitamin C (T), where the activity of this enzyme has the lowest activity among the other groups.

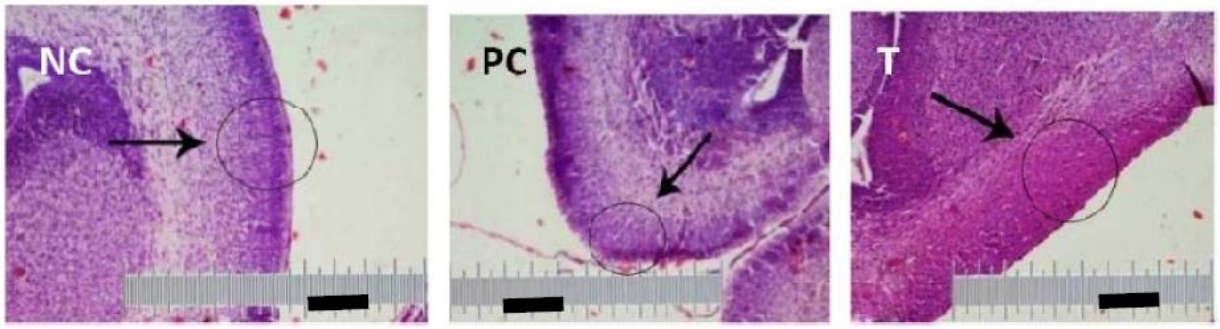

Figure 4.Histopathology of the cerebral cortex of fetal brain. Tissue is cut in the frontal or transverse cross section and stained with Hematoxylin Eosin,with 100x magnification. NC is the negative control group, PC is the positive control group, T is the treatment group. Bar size = 0.2 mm.

Effect of Vitamin C on Fetus's Brain Histopathology

The group treated with vitamin C (T) had the lowest lead levels at the fetus's head compared to the other groups; it's also indicated the existence of the corpus callosum (Fig. 3) and cerebral cortex (Fig. 4) which is thicker than the positive control group (PC). Exposure to lead can induce the expression of neurothropin and affect the nerve growth, brain-derived factor neurothropic neurothropin-3, and tyrosine kinase receptors and neurothropin receptor (P75NTR) (Nemoto et al, 2000). Rodrigues et al (1999) concluded that the lead will interact with G proteins and the catalytic subunit of the cerebral cortical activity to inhibit the enzyme adenylatecyclase so that an image acquired cerebral cortex is thinner than the control group. Wang et al (2002) examined the damage of cerebral cortex caused by the toxicity of lead. The increase of production of NOS activity, NO levels, enzyme activity superoxide dismutase (SOD) and malondialdehyde (MDA) was noted in the cerebral cortex. Wilson et al (1999) showed that exposure of 2 g Pb/ L to pregnant mice during 10 days result the decrease of brain weight and cortical thickness about 13.2% and 13.9% respectively. Lasky et al (2005) reported that lead reduced the volume of white matter, gray matter, cerebra spinal fluid, and the composition of

brain structures including the corpus callosum of monkey brains. Stewart et al (2006) reported that increase in tibia lead levels were significantly affected by the decrease in brain volume, the total and frontal gray and white matter volumes measured by Magnetic Resonance Imaging in 532 workers with a mean age of 56 years.

Supplementation of vitamin C provides protection to the fetus as indicated by normal structure of fetal brain. In general, supplementation of vitamin C can improve the mother's health and giving protection to the fetus.

Vitamin C has prominent role to reduce the lead toxicity through the binding of lead and then transfer to the compound which is more soluble in water, therefore it is more easily excreted from the body via urine, and not through increased metabolism of xenobiotics (CYP1A1 enzyme activity and GST). Vitamin C is a chelating agent that is safe and protectsliverand fetus from toxic effect caused by lead.

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