

Zinc supplementation decreases basophilic stippling in rats exposed to lead

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ABSTRACT

BACKGROUND

Lead acetate inhibits pyrimidine-5'-nucleotidase resulting in ribonucleic acid and ribosome accumulation in erythrocytes (RBC), visible as basophilic stippling (BS). Lead exposure disrupts RBC membrane, shortens the RBC life span and decreases hematocrit. Zinc supplementation increases lead-binding proteins (metallothioneins). The study objective was to determine whether zinc supplementation prior to lead exposure decreases BS and increases the hematocrit in rats.

METHODS

The study was a randomized post-test only control-group trial, using 28 rats allocated to one control and 3 intervention groups (Zinc I, Zinc II, Zinc III) receiving 0.2 mg, 0.4 mg, and 0.8 mg of zinc by oral gavage daily for 3 weeks. All groups were then exposed to lead at 0.5 g/kg BW/day by gavage for 10 weeks. On the last day of the 10 weeks BS was determined from Giemsa-stained blood smears and hematocrit by hematology analyzer. Between-group differences were tested with one-way ANOVA, followed by Bonferroni's test.

RESULTS

Mean BS was significantly decreasing $7.93 \pm 0.99\%$ in controls, $6.91 \pm 1.04\%$, $4.66 \pm 0.79\%$, and $1.73 \pm 0.88\%$, respectively, in intervention groups ($p=0.000$). Mean hematocrit was significantly increasing $26.16 \pm 3.60\%$ in controls, and $30.33 \pm 6.12\%$, $36.83 \pm 3.31\%$, and $40.00 \pm 4.77\%$, respectively, in intervention groups ($p=0.000$). One-way Anova and Bonferroni's test showed significant differences in BS and hematocrit between controls and intervention groups receiving zinc supplementation of 0.4 and 0.8 mg ($p=0.000$).

CONCLUSION

Zinc supplementation before lead exposure significantly decreases basophilic stippling and increases hematocrit level in rats exposed to lead.

Keywords: Zinc, basophilic stippling, hematocrit, rats

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Suplementasi seng menurunkan jumlah basophilic stippling pada tikus yang terpajan Plumbum

ABSTRAK

LATAR BELAKANG

Plumbum (Pb) asetat menghambat enzim pirimidin-5'-nukleotidase yang menimbulkan akumulasi ribonucleic acid serta ribosom eritrosit ditandai adanya basophilic stippling (BS). Paparan Pb menyebabkan kerusakan membran eritrosit, umur eritrosit pendek dan penurunan hematokrit. Suplementasi seng meningkatkan protein metallothionein yang mengikat Pb. Tujuan penelitian ini adalah untuk membuktikan suplementasi seng sebelum paparan Pb menurunkan BS dan meningkatkan hematokrit pada tikus.

METODE

Randomized post test only control-group design digunakan pada 28 tikus yang dibagi menjadi 4 kelompok yaitu 1 kelompok kontrol dan 3 kelompok perlakuan yang disuplementasi seng 0,2; 0,4; 0,8 mg diberikan setiap hari melalui sonde sampai minggu ke-3. Kemudian semua kelompok diberi paparan Pb 0,5 gr/kg BB/hari melalui sonde sampai minggu ke-13. Hari terakhir minggu ke-13 diperiksa jumlah BS menggunakan pengecatan Giemsa dan kadar hematokrit menggunakan haematology analyzer. Perbedaan antar kelompok dilakukan menggunakan one way ANOVA, dilanjutkan dengan uji Bonferroni.

HASIL

Rata-rata BS menurun secara bermakna mulai kelompok kontrol sampai perlakuan ke-3 ($7,93 \pm 0,99\%$; $6,91 \pm 1,04$; $4,66 \pm 0,79\%$; $1,73 \pm 0,88\%$) ($p=0,000$). Rata-rata hematokrit meningkat secara bermakna mulai kelompok kontrol sampai perlakuan ke-3 ($26,16 \pm 3,60\%$; $30,33 \pm 6,12\%$; $36,83 \pm 3,31\%$; $40,00 \pm 4,77\%$) ($p=0,000$). Uji ANOVA dilanjutkan dengan uji Bonferroni menunjukkan BS dan hematokrit berbeda bermakna antara kelompok kontrol dengan kelompok yang disuplementasi seng 0,4 dan 0,8 mg ($p=0,000$).

KESIMPULAN

Suplementasi seng sebelum paparan Pb mampu menurunkan BS dan meningkatkan hematokrit secara signifikan pada tikus terpajan Pb.

Kata kunci: Seng, basophilic stippling, hematokrit, tikus

INTRODUCTION

One of the hazardous air pollutants is lead that may cause health problems and may be life-threatening to humans. The level of lead utilization in Indonesia is still considerable, although its use in gasoline has largely been abandoned. Several large cities such as Jakarta, Bandung, Semarang, Surabaya, Medan and others are at potential risk of lead poisoning.⁽¹⁾ In West and Central Java the use of phosphate fertilizers, pesticides, and

herbicides has increased, thus potentially increasing lead exposure. The main sources of lead poisoning are vegetables, batteries, paints, cosmetics, jewelry, toys, gasoline, and others. Exposure to lead in humans may occur by way of lead-contaminated air, foods, and drinks. The highest risk of lead poisoning is in children, pregnant women, and industrial workers.⁽²⁾ The main signs of lead poisoning are anemia,⁽²⁾ decreased hematocrit and the presence of inclusions in the red blood cell (RBC) called basophilic stippling (BS).^(3,4)

The lead toxicity threshold of 20-40 µg/dl is already capable of causing anemia in children.⁽⁵⁾ A previous study conducted in India on children found that 38% of the children had a blood lead content 10-19.9 µl/dl, and 9% had a lead level of ≥ 20 µl/dl. Children with a lead level of ≥ 10 µl/dl have a 1.7 times higher risk of anemia than those with a lead level of ≤ 10 µl/dl.⁽⁶⁾ The study conducted by Hariono⁽⁷⁾ showed that administration to rats of oral lead acetate at 0.5 g/kg/BW/day for 16 weeks caused anemia, weight loss, and increased numbers of reticulocytes since the 10th week, accompanied by increased activity of δ -aminolevulinic acid dehydratase (ALAD). Another study showed that blood lead levels of ≥ 7 µg/dl in children can inhibit the activity of enzymes involved in hemoglobin synthesis, resulting in subclinical effects characterized by elevated levels of δ -ALA and protoporphyrins.⁽⁸⁾

In heme biosynthesis, lead poisoning depresses enzyme ALAD activities at the starting point, at midpoint (coproporphyrinogen oxidase), and at the endpoint (ferrochelatase) of heme biosynthesis, thus leading to anemia. Approximately 90% of the lead introduced into the circulation will enter the RBC, where it acts as a prooxidant, causing oxidative stress that results in damage to the membranes of RBC and shortening of their lifespan.^(3,4) Membrane damage and shortened lifespan causes a decrease in RBC number and volume, as indicated by the hematocrit. Lead also causes glucose-6 phosphate dehydrogenase (G-6PD) deficiency and inhibition of pyrimidine-5'-nucleotidase. The latter results in accumulation of ribonucleic acid (RNA) and ribosomes in RBC, characterized by BS of RBC,^(3,4) which can be used as a marker of lead poisoning.

The management of lead poisoning by means of chelating agents has been studied previously. The chelating agents act to bind lead with the formation of polar (hydrophilic) complexes which are excreted by the kidneys. According to the study by Suaniti,⁽⁹⁾ the success rate of reducing lead toxicity by intravenous administration of the chelating agent EDTA is only 4.91%. The use of EDTA is only for therapeutic or curative purposes and thus not

maximal. Therefore preventive measures to combat lead toxicity are necessary.

Lead in the tissues is bound to the metal-binding proteins called metallothioneins at their sulfhydryl groups.⁽¹⁰⁾ Metallothioneins can be synthesized in the liver as well as the gastrointestinal wall through absorption of optimal amounts of zinc.⁽¹¹⁾ Zinc supplementation at stepwise increasing daily doses of 0.2 mg, 0.4 mg, and 0.8 mg to rats (*Rattus norvegicus*) may increase metallothionein levels.⁽¹²⁾ The administration of zinc supplements as an alternative preventive measure against lead poisoning needs to be subjected to further study, the results of which can be measured by the amount of BS and the hematocrit.

A meta-analysis of zinc supplementation in humans stated that doses of 1.5-50 mg zinc per day, by considering the influence of various food materials such as fibers, phosphates, and phytates, which are capable of inhibiting the level of zinc absorption.⁽¹³⁾ To observe the effectivity of certain doses in increasing metallothioneins in humans, stepwise increasing doses of zinc supplementation should be considered, e.g. 10 mg per day, 20 mg per day and 40 mg per day. Conversion of these doses to experimental animals yields the following doses of 0.2 mg, 0.4 mg, and 0.8 mg, respectively.⁽¹²⁾ Previous studies on lead exposure impacting on BS and hematocrit have yielded consistent results, i.e. considerable increases in BS and decreases in hematocrit. The present study differs from the previous ones, since it does not aim to merely observe the increase in BS nor the decrease in hematocrit after lead exposure. On the contrary, this study aims to determine whether zinc supplementation to rats before exposure to lead may decrease BS and increase the hematocrit.

METHODS

Research design

This study was of randomized post-test only control-group design. The experimental animals were kept and subjected to the intervention at the Integrated Research and Testing Laboratory, Gadjah Mada University, Yogyakarta. The animals

were kept for 100 days, starting from the selection period up to the end of the intervention period, from December 2012 to March 2013.

Experimental animals

The sample size was determined by means of the formula: $S = (t-1)(r-1) \geq 15$, where t = number of groups, r = number of animals per group.

This study uses 3 intervention groups and one control group, therefore $t=4$, $(4-1)(r-1) \geq 15$, yielding $r \geq 6$. The number of rats per group was 6, thus giving a sample size of 24 animals. To each group was added one animal in anticipation of death of the animals, so that the total number of animals used in this study was 28 male rats (*Rattus norvegicus*) aged 15 weeks.

Zinc supplementation

By simple random assignment, the rats were allocated to 4 groups, viz. one control group and 3 intervention groups. From the first until the third week, the intervention groups received zinc supplementation by oral gavage at stepwise increasing doses of 0.2, 0.4, 0.8 mg/day, respectively, whereas the control group did not receive zinc supplementation.

Lead exposure

From the third week until the 13th week all groups of animals were exposed to lead at 0.5 g/kg BW/day, in the form of oral lead acetate solution given by oral gavage (gastric tube).

Measurement of blood parameters

On the last day of the 13th week the control group and the intervention groups were examined for amount of BS and hematocrit level. Basophilic stippling was measured using methanol-fixed blood smears with Giemsa staining. The results were expressed as percentages (%), the normal values being 0-1%. The hematocrit was determined by means of a hematology analyzer, based on the principle of electrical impedance, using Lyse, Rinse, and Diluent solutions. The specimens used were whole blood samples of 3 ml with added EDTA as anticoagulant collected in vacutainer tubes.

Determination of blood parameters was done at the Health Analyst Clinical Pathology laboratory, Faculty of Medicine and Health Science, Muhammadiyah University, Semarang.

Data analysis

For testing of significant between-group differences in amounts of BS and hematocrit levels, one-way ANOVA test was used, followed by Bonferroni's test.

Ethical clearance

The present study obtained ethical clearance No.339/EC/FK/RSDK/2012 from the Ethics Commission, Faculty of Medicine, Diponegoro University/DR. Kariadi Hospital, Semarang. After the Head of the Laboratory for Integrated Research and Testing, Gadjah Mada University, Yogyakarta, was informed of the decision, the study was allowed to be conducted.

RESULTS

There was weight loss in the control animals between the third week until the 13th week after exposure to the lead acetate. In contrast, there was a continued increase in weight of the animals in all three intervention groups, in spite of exposure to the lead acetate at identical doses. In Table 1 is shown the weight of the animals of the control group and the groups receiving stepped doses of zinc, at baseline and at the end of the third and 14th weeks.

Basophilic stippling occurs from accumulation of ribonucleic acid (RNA) and ribosomes in RBC as a result of inhibition of pyrimidine-5'-nucleotidase upon lead exposure. As shown in Figure 1, the non-supplemented control animals had more BS than the animals in the Zinc III group receiving zinc supplementation of 0.8 mg.

The mean amount of BS in the control group differed from that in the intervention groups. The highest mean value ($7.93 \pm 0.99\%$) was in the control group receiving no zinc supplementation, while the lowest ($1.73 \pm 0.88\%$) was in the intervention group receiving zinc supplementation of 0.8 mg. According to the ANOVA test results,

Table 1. Mean weights of the animals

Group	Mean weight (gram)		
	Baseline	Before intervention (3 rd week)	After intervention (13 th week)
Control (n=7)	183.3 ± 7.44	257.8 ± 8.29	253.1 ± 46.35
Zinc I (n=7)	199.1 ± 11.85	269.9 ± 11.97	333.1 ± 54.73
Zinc II (n=7)	181.6 ± 3.22	253.3 ± 13.91	329.9 ± 30.54
Zinc III (n=7)	182.1 ± 3.62	264.2 ± 13.50	361.1 ± 26.87

there was a significant difference between control and intervention groups ($p=0.000$) (Table 2). On the basis of the Bonferroni test results (Table 3), the difference was between the control group versus the Zinc-II and Zinc-III intervention groups (both at $p=0.000$). There was a decrease in BS from the control group to the stepped-dose zinc supplementation groups.

The mean hematocrit values in the control and intervention groups are shown in Table 2. There was an increase in hematocrit values starting from the control group up to the third intervention group. The lowest mean was in the control group ($26.16 \pm 3.60\%$), and the highest in the third intervention group receiving zinc supplementation of 0.8 mg ($40.00 \pm 4.77\%$). The ANOVA results showed a significant difference between the control and intervention group ($p=0.000$). According to the Bonferroni test results (Table 3), the significant difference was between the control group versus the second and third intervention groups ($p=0.000$). Increased zinc supplementation was accompanied by an increase in hematocrit level in rats exposed to lead.

DISCUSSION

In this study there was a decrease in BS after zinc supplementation. The highest mean value for BS was in the control group who did not receive zinc supplementation, while the lowest mean was in the intervention group receiving zinc supplementation at the highest dose of 0.8 mg. Statistically, there was a significant difference in BS between the control group and all intervention groups, where the higher zinc supplementation dose resulted in the lowest amounts of BS in lead-exposed *Rattus norvegicus*.

Our findings are supported by those of Hariono,⁽⁷⁾ who found evidence that exposure of rats to lead at 0.5 g/kg/BW increased the amount of BS in the 10th week. Basophilic stippling is visible as blurred blue points in RBC and frequently occurs in cases of lead exposure. Lead inhibits pyrimidine-5'-nucleotidase and alters the functions of other nucleotides. Lead can interfere with heme synthesis, so as to alter the concentrations of enzymes and intermediates involved in heme synthesis or their derivatives.

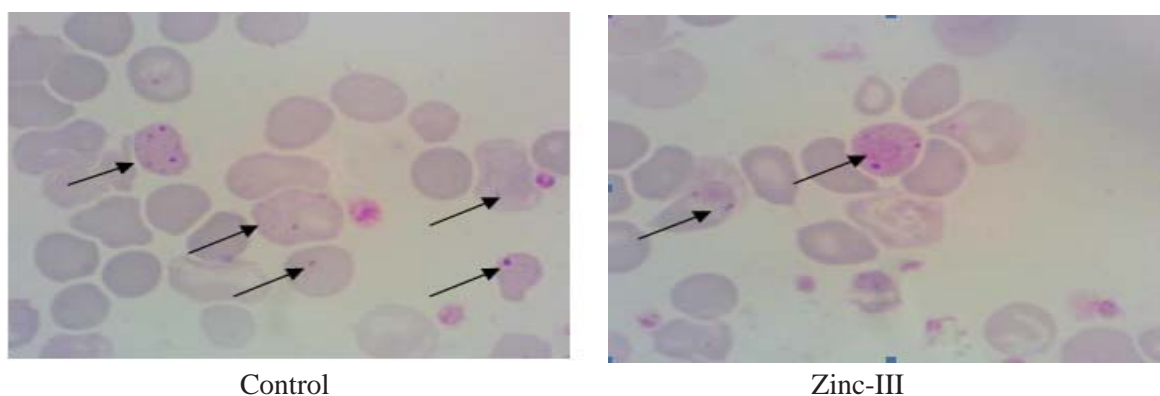


Figure 1. Basophilic stippling in control and zinc-III groups after intervention

Table 2. Mean basophilic stippling and mean hematocrit after the 13th week, by intervention group

Variable	Intervention				P value
	Control (n=7)	Zinc-I (n=7)	Zinc-II (n=7)	Zinc-III (n=7)	
Basophilic stippling (%)	7.93 ± 0.99	6.91 ± 1.04	4.66 ± 0.79	1.73 ± 0.88	0.000
Hematocrit (%)	26.16 ± 3.60	30.33 ± 6.12	36.83 ± 3.31	40.00 ± 4.77	0.000

Lead poisoning can also lead to increased amounts of BS in RBC.⁽¹⁴⁾

Lead exposure causes membrane damage in RBC, resulting in a shortened RBC lifespan and a decreased RBC count. The bone marrow as the site of erythropoiesis compensates for the decreased RBC numbers in the circulation by increased erythropoietic activity, leading to increased numbers of immature RBC in the circulation.^(4,15)

In the present study lead exposure without zinc supplementation results in high mean percentages of BS, whereas lead exposure with zinc supplementation results in reduction in mean percentages of BS. This shows that zinc supplementation is capable of decreasing the amount of BS in rats exposed to lead.

The hematocrit is the percentage volume of RBC in 100 ml of blood and is largely influenced by the RBC count. In our study, the hematocrit level of rats exposed to lead without zinc supplementation was lower than that of rats receiving preventive zinc supplementation. There was a significant difference in hematocrit level between the control group receiving no zinc supplementation and the intervention groups receiving stepped-dose supplementation.

Most of the lead in the blood is localized to the RBC, with 90% in the RBC cytoplasm and 10% in the RBC membrane, mainly bound to lipids and lipoproteins.⁽⁵⁾ The distribution of lead in RBC

cytoplasm and membrane is due to binding of lead to cytoplasmic elements, its transportation is through the RBC membrane, and its excretion via the calcium pump. The toxic effect of lead on RBC is caused by its ability to form complexes with negatively charged ligands, particularly sulfhydryl and carboxyl groups, and with imidazole enzymes and other proteins.⁽¹⁶⁾

Many investigators have reported that the most important mechanism of lead toxicity is the production of free radicals. Reactive oxygen species react with cellular macromolecules, namely DNA, proteins, and lipids.⁽¹⁷⁾ Red blood cells are very sensitive to induction of oxidative stress by high lead exposure.^(18,19)

Of the amounts of lead entering the circulation, around 90% goes to the RBC. In the RBC membrane, there are compounds or chemical reactions capable of producing potentially toxic oxygen species called pro-oxidants. Increased amounts of pro-oxidants may cause oxidative stress. Lead may also cause G-6PD deficiency that can inhibit RBC maturation in the bone marrow.^(3,4)

Gugliotta et al.⁽¹⁹⁾ in their investigations on the effect of lead on RBC membrane proteins, found a decrease in RBC count, in which membrane permeability plays an important role. This finding is supported by the study of Patit et al.⁽²⁰⁾ on lead exposure in battery manufacturing workers as compared to a normal group without lead exposure.

Table 3. Bonferroni test results for basophilic stippling and hematocrit after the 13th week, by intervention group

Variable	Intervention		
	Control vs Zinc-I (n=7)	Control vs Zinc-II (n=7)	Control vs Zinc-III (n=7)
Basophilic stippling (%)	1.01 (p=0.041)	3.26 (p=0.000)	6.20 (p=0.000)
Hematocrit (%)	-4.16 (p=0.782)	-10.66 (p=0.000)	-13.83 (p=0.000)


These investigators found a significant decrease in RBC count in the lead-exposed group as compared with the control group.

In addition, the findings of Gugliotta et al.⁽¹⁹⁾ was also supported by a laboratory experimental study on the addition of lead chloride to heparinized blood with a hematocrit concentration of 3%, which was incubated for 1 hour at 25°C in a medium containing stepped concentrations of lead chloride (0, 0.3, 0.5 and 1 µM). This procedure increased RBC permeability through cell damage and cell death, particularly at high lead concentrations, caused morphological and structural alterations in the RBC membrane, and decreased ATP concentrations in RBC.⁽¹⁹⁾

Exposure to lead causes reduced RBC counts, ultimately impacting on decreased hematocrit levels. In the present study the zinc supplementation prior to lead exposure was presumably capable of increasing the amounts of metallothioneins, which function to bind lead, so that the hematocrit levels increased in the zinc supplementation groups, and increased significantly at a doses of 0.4 mg and 0.8 mg.

One limitation of this study was that the hematology analyzer used was not capable of counting BS, which was therefore counted manually in Giemsa stained slides. The clinical implication of the study is that zinc supplementation is capable of decreasing BS in lead-exposed rats, indicating that zinc is capable of reducing the impact of lead exposure. Therefore further studies are needed to apply this finding to humans.

CONCLUSION

Zinc supplementation prior to lead exposure significantly decreases BS and increases hematocrit level in the blood of rats exposed to lead. 

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