

In Vivo Effects of Lead on Erythrocytes Following Chronic Exposure through Drinking Water

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More than 95% of lead, an environmental heavy metal, entering into blood accumulates in erythrocytes suggesting erythrocytes as an important target of lead toxicity. Recent studies reported that erythrocytes could contribute to blood coagulation via phosphatidylserine (PS) exposure in erythrocytes. However, *in vivo* effects of chronic lead exposure especially by drinking water on procoagulant activity of erythrocytes have not been studied yet. In the present study, we investigated the effects of chronic exposure of lead by drinking water on erythrocytes in rats. Groups of 40 male rats were provided with drinking water containing various concentrations of lead for 4 weeks and complete blood cell count, procoagulant activities of erythrocytes and platelets were evaluated with basic inspections on body weight and food/water consumption. The administration of lead containing drinking water increased the blood lead level (BLL) in a dose-dependent manner up to 22.39 ± 2.26 $\mu\text{g/dL}$. Water consumption was significantly decreased while food consumption or body weight gain was not affected. In contrast to the previous findings with acute lead exposure, chronic lead exposure failed to increase PS exposure in erythrocytes with statistical significance although some trends of enhancement were observed. It implies that a certain adaptation might have happened in body during repeated exposure to lead, resulting in attenuation of PS exposure. With this study, we believe that a valuable information was provided for the study on the toxicological significance and the risk assessment of lead contaminated drinking water.

Key words: Lead, *In vivo* rat model, Drinking water, Erythrocytes, Blood lead level (BLL)

INTRODUCTION

Lead (Pb) is one of the most abundant heavy metals on earth. It has been widely used throughout human history, posing a serious health problem to susceptible populations, such as children or occupationally exposed people. The Center for Disease Control (CDC) defines lead poisoning as the blood lead level (BLL) exceeding 10 $\mu\text{g/dL}$, while the "normal" BLL is less than 5 $\mu\text{g/dL}$. This advisory BLL has been continually declining over the past few decades, from 60 $\mu\text{g/dL}$ (1960~1970), to 30 $\mu\text{g/dL}$ (1970~1985), to 25 $\mu\text{g/dL}$ (1985~1991), to 10 $\mu\text{g/dL}$ (1991), as the adverse health effects of lead poisoning become recognized widely (CDCP., 1997). The main source of lead in drinking water is from old lead piping and lead-containing solders. The use of lead solder and other lead-containing materials

in household plumbing or public water supplies was banned by EPA in June 1988. Many older structures, however, still have lead piping or lead-soldered internal plumbing, which may substantially increase the lead content of tap water.

Lead poisoning can directly cause peripheral neuropathy and chronic renal disease. The cardiovascular system, gastrointestinal tract, reproductive tissue and skeletal tissues can also be affected by lead exposure (Goyer, 1993; Goyer and Clarkson, 2001). Individuals with BLLs of 20 to 29 $\mu\text{g/dL}$ in 1976 to 1980 (15% of the US population at that time) experienced significantly increased circulatory, cardiovascular, and total mortality from 1976 through 1992 (Lustberg and Silbergeld, 2002). Some epidemiological studies reported the connection between BLL and an increased prevalence of peripheral arterial diseases (Navas-Acien *et al.*, 2004). The diversity of cardiovascular complications associated with chronic lead exposure raise the possibility that lead may induce the procoagulant activity of blood cells directly leading to thrombosis.

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In human blood, about 95% of lead is accumulated in erythrocytes (Goyer and Clarkson, 2001) suggesting erythrocytes could be an important target of lead toxicity in the cardiovascular system (Battistini *et al.*, 1971). In erythrocytes, lead could degenerate the lipid and protein component (Fukumoto *et al.*, 1983) and suppress hemoglobin synthesis (Monteiro *et al.*, 1989; Waldron, 1966). Most notably, it was very recently reported that lead-induced PS exposure in erythrocytes have an active role in procoagulant activation *via* phosphatidylserine (PS) exposure on their surfaces (Shin *et al.*, 2006). PS externalized to the outer membrane of erythrocytes could be recognized by macrophages (McEvoy, 1986) and as a result, PS exposed erythrocytes could be removed in blood circulation and anemia can be induced. More importantly, PS exposed on erythrocytes provides a site for an assembly of prothrombinase and tenase complex, leading to thrombin generation and clotting (Zwaal and Schroit, 1997; Zwaal, 2005). Furthermore, it was shown that lead induces thrombus formation in *in vivo* rat model (Shin *et al.*, 2006).

While lead toxicities have been well characterized in human *in vitro* studies and acute *in vivo* rat models, few studies have been focused on the effects of chronic lead exposure through drinking water on hematological alterations or procoagulant activation. We investigated whether PS exposure of erythrocytes could be induced by chronic lead exposure through drinking water in rat *in vivo* model in an effort to provide an insight into the toxicological impact of lead contaminated drinking water.

MATERIALS AND METHODS

Materials

Lead(II) acetate was purchased from Sigma Chemical Co. (St. Louis, MO). Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled annexin V (annexin V-FITC, annexin V-PE) and FITC-labelled antibody against rat glycoprotein IIIa (anti-rat GP IIIa-FITC) were obtained from Pharmingen (San Diego, CA). PE-labeled monoclonal antibody against human glycoprotein A (anti-glycoprotein-A-RPE) was from Dako Cytomation (Glostrup, Denmark). All other reagent used were of the highest purity available.

Drinking water study to rats

Male Sprague-Dawley rats were obtained from Dae Han BioLink Co. (Chungbuk, Korea) and were housed in groups of four or five, for at least 1 week prior to the experiment. The animals were fed a standard laboratory diet from Purina Korea and had access to food and water *ad libitum*.

Rats (105-150 g) were randomly grouped for control, 100, 250, 500 and 1000 ppm of lead(II) acetate. Lead contaminated water was supplied to each groups for 4

weeks. Body weight, water- and food-consumption were checked every two days.

Measurement of BLL

After 4 weeks, blood was collected from the abdominal aorta using 3.8% trisodium citrate as anticoagulant. BLL was measured by atomic absorption spectroscopy and We requested determination of haematological profiles to Neodin.

Whole blood flow cytometry for PS exposure

To investigate the PS exposure and microvesicle (MV) generation after *in vivo* exposure to lead (II) acetate, whole blood flow cytometry was conducted. After 4 weeks, blood was collected from the abdominal aorta using 3.8% trisodium citrate as anticoagulant. To prepare whole blood samples, blood was diluted with 200 fold of binding buffer (10 mM HEPES-Na, 136 mM NaCl, 2.7 mM KCl, 2.0 mM MgCl₂, 1.0 mM NaH₂PO₄, 5.0 mM dextrose, 5 mg/mL BSA, 2.5 mM CaCl₂, pH 7.4). And aliquots of samples were added to tubes containing antibodies and annexin V for 15 min in room temperature. Flow cytometric analysis was carried out in a FACScalibur (BD bioscience, San Jose, CA). Red blood cells RBCs were identified as being positive for glycoprotein A and within the RBC window defined by forward and side light scatter characteristics. To analyze platelets, platelets were identified as being positive for GP IIIa and within the platelet window defined by forward and side light scatter characteristics.

Preparation of human RBCs

With the approval from the Ethics Committee of Health Service Center at Seoul National University, human blood was obtained from healthy male donors (18-25 years old) using a vacutainer with acid citrate dextrose (ACD) and a 21 gauge needle (Becton Dickinson, U.S.A.) on the day of each experiment. Platelet rich plasma and buffy coat were removed by aspiration after centrifugation at 200 g for 15 min. Packed erythrocytes were washed 3 times with phosphate buffered saline (PBS: 1.06 mM KH₂PO₄, 154 mM NaCl, 2.96 mM Na₂HPO₄, pH 7.4) and once again with Tris buffered saline (TBS: 15 mM Tris-HCl, 150 mM NaCl, 5 mM KCl, 2 mM MgCl₂, pH 7.4). Washed erythrocytes were resuspended in TBS buffer to a cell concentration of 5×10^7 cells/ml and final CaCl₂ concentration was adjusted to 1 mM prior to use.

Measurement of Hemolysis

Pb was treated to human or rat RBC suspension at the concentration of 0, 1, 2, 5, 10 μ M for 4 h. And % Hemolysis of totally hemolyzed cells was calculated using absorbance at 540 nm of supernatant obtained after cell down.

Statistical analysis

Data are expressed as mean±SEM. Difference between two groups was evaluated using Student's t-test. When more than 3 groups were compared, ANOVA test was conducted, followed by Duncan's multiple range test. Significance was acknowledged when *p* value was less than 0.05.

RESULTS AND DISCUSSION

Chronic *in vivo* rat model was employed to investigate the effects of lead contaminated drinking water on erythrocytes. This model can demonstrate the toxicological impact of oral exposure of lead, the most important absorption route of lead in human. Rats were exposed to lead by drinking water at concentrations of 0, 100, 250, 500 and 1000 ppm for 4 weeks.

Body weight, water- and food-consumption were recorded on every two days during the administration of lead as indicators of general toxicities. While body weight gain or food consumption was not affected, there were statistically significant decreases of water-consumption at 500 and 1000 ppm group (Fig. 1). Previously, manganese containing drinking water was known to induce decreased water

and food consumption, possibly from neurological effect (Torrente *et al.*, 2005). It would be plausible, therefore, that the decrease of water consumption in the present study might be caused by neurological effect of chronic Pb^{2+} exposure although additional studies were necessary for the elucidation of mechanism.

After administration for 4 weeks, blood was collected from abdominal aorta and blood lead level (BLL) was measured using atomic absorption spectroscopy. As shown in Fig. 2, BLL was increased in a dose-dependent manner up to 22.39 ± 2.26 $\mu\text{g/dL}$. In addition, in complete blood cell count (CBC) analysis, the amount of hemoglobin was found to be slightly decreased at 500 ppm, reflecting the potential hematological effects of chronic lead exposure (Table I). It is well known that lead could block the synthesis of hemoglobin, but the clear dose response relationship was not found in this study. Hemolysis was not induced by lead either in rat blood or in human blood up to 10 μM (Fig. 3) excluding involvement of nonspecific cytotoxicities of lead in this observation.

It has been reported that treatment of lead on human erythrocytes or acute injection of lead in rat can increase phosphatidylserine (PS) exposure in erythrocytes (Shin *et al.*, 2006). Alterations in the erythrocyte membrane, such

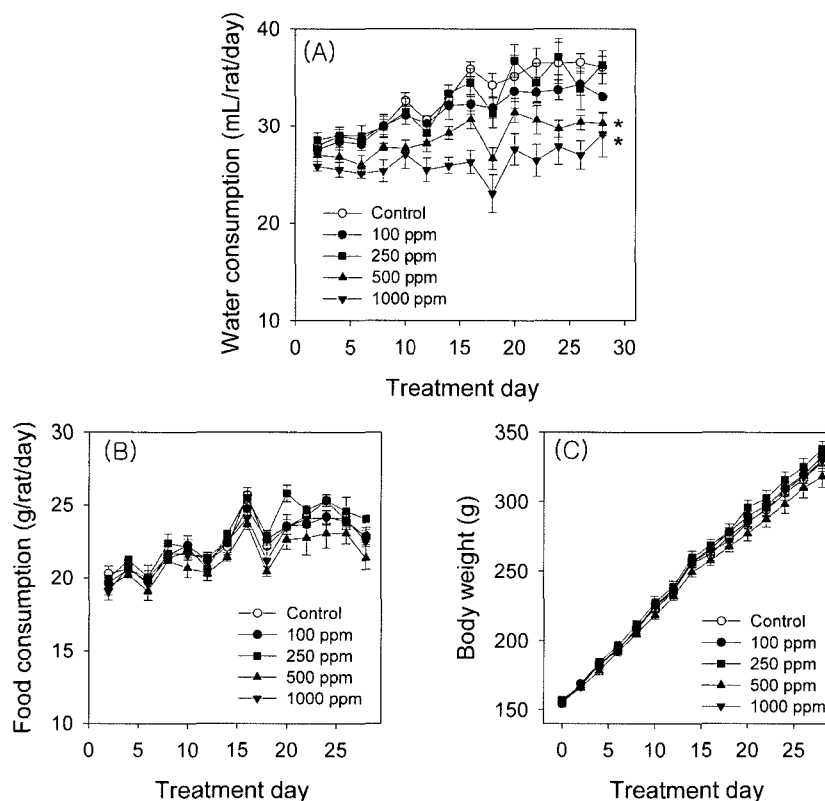


Fig. 1. Effects of Pb^{2+} exposure by drinking water on body weight and consumption in rat. Rats were exposed to Pb^{2+} in drinking water at DW(vehicle), 100, 250, 500, 1000 ppm *ad libitum* for 4 weeks. (A) water consumption, (B) food consumption and (C) body weight were measured every two days. Values are mean±SEM of 8-12 independent experiments. * represents significant difference from control ($p < 0.05$).

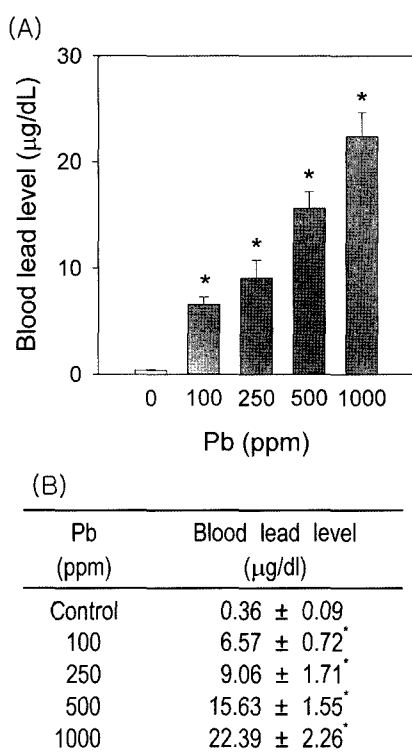


Fig. 2. Effect of Pb²⁺ exposure by drinking water on blood lead levels (BLL) in rat. Rats were exposed to Pb²⁺ in drinking water at DW (vehicle), 100, 250, 500, 1000 ppm *ad libitum* for 4 weeks. BLL was measured by atomic absorption spectroscopy. (A) and (B), The Values are mean±SEM of 8-12 independent experiments. * represents significant difference from control (p<0.05).

as PS externalization and PS-bearing microvesicle (MV) generation enable erythrocytes to participate in thrombus formation (Zwaal and Schroit, 1997). Therefore, measurement of PS exposure can give an important information on the cardiovascular effects of lead treatment.

PS exposure in erythrocytes was evaluated using flow cytometry from the whole blood of lead-administered rat by drinking water. As a result, there was a slight increase

of PS exposure in active dose groups, but all Pb²⁺ exposed groups failed to achieve statistical significance when compared to control group (Fig. 4). Moreover, there was no correlation between BLL and PS exposure on erythrocytes. Platelets which are another important blood cells in thrombus formation, did not show PS exposure and microparticle (MP) generation either, following lead exposure from drinking water (Fig. 5).

In the previous study, PS exposure on red blood cells was measured after intraperitoneal administration of Pb²⁺ and the treatment significantly enhanced PS exposure in a dose-dependent manner (Shin *et al.*, 2006). BLL of the blood sample was about 55.18 µg/dL (Table II), much higher than CDC advisory BLL, 10 µg/dL or that of the present investigation.

In the present study, the highest BLL achieved after 4 week administration, was around 22.39±2.26 µg/dL, well below 55 µg/dL, that from acute injection. Therefore, the BLL achieved by acute injection in the previous study seems to be high, unreachable even with 1000 ppm lead in drinking water and 4 week of repeated exposure. In view of toxicokinetics, it would need longer periods or higher concentrations of lead to signify the results of Shin *et al.* In addition, PS exposed to surfaces of cells is recognized and undergo a rapid phagocytosis by macrophage in a living body (McEvoy, 1986). Although there would be significant PS exposure on erythrocytes after 4-week administration of lead, it is possible that we could not detect any effects because of adaptive changes in body, such as attenuation of PS exposure or facilitation of lead excretion. If some methods to exclude the possible involvement of phagocytosis are available, it would be interesting to re-measure PS exposure again.

In conclusion, 4 week chronic administration of lead contaminated drinking water at 100 to 1000 ppm to rat can increase BLL up to 22.39±2.26 µg/dL in a dose dependent manner. Although a trend of increase in PS exposure was observed, statistical significance was not

Table I. Effects of Pb²⁺ exposure by drinking water on complete blood cell count (CBC) in rat whole blood

Complete blood cell count	Pb (ppm)				
	0	100	250	500	1000
RBC (M/µL)	8.4±0.1	8.4± 0.2	8.3± 0.2	8.5± 0.2	8.6± 0.2
Hb (g/dL)	13.9±0.2	14.0± 0.2	14.5± 0.3	12.2± 1.3	14.0± 0.2
HCT (%)	44.5±0.8	43.4± 0.7	44.3± 0.8	42.9± 0.9	43.2± 0.7
MCV (fL)	52.8±0.5	52.0± 0.3	53.2± 0.7	50.7± 0.8	50.2± 0.5
MCH (pg)	15.7±0.9	16.8± 0.5	17.5± 0.6	16.0± 0.5	16.3± 0.4
MCHC (g/dL)	31.4±0.2	32.2± 0.8	32.9± 0.8	31.6± 0.8	32.5± 0.6
PLT (K/µL)	155.7±5.6	268.1±96.3	253.0±96.8	270.4±97.7	253.6±86.3
WBC (K/µL)	9.5±0.4	9.9± 0.5	10.1± 0.6	9.7± 0.5	10.0± 0.6

Rats were exposed to Pb²⁺ in drinking water at DW (vehicle), 100, 250, 500, 1000 ppm *ad libitum* for 4 weeks. Complete blood cell count was measured as described in Materials and Methods. Values are mean±SEM of 8-12 independent experiments.

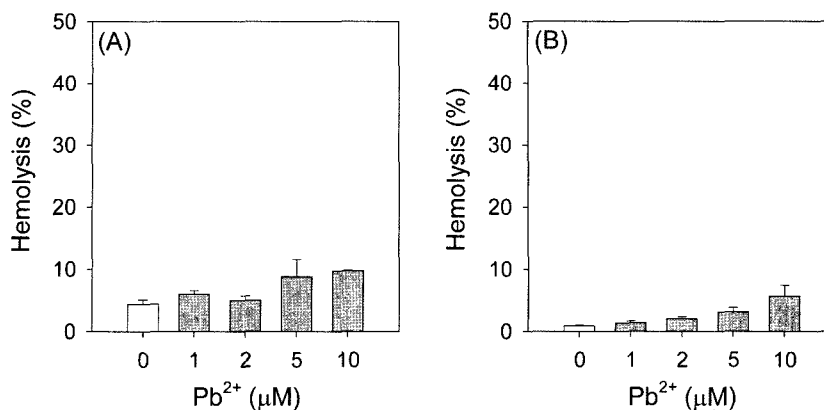


Fig. 3. *In vitro* effects of Pb²⁺ on hemolysis in red blood cells. After RBCs obtained from (A) rat and (B) human were treated with DW (vehicle) or 1, 2, 5, 10 μM lead(II) acetate for 4 h at 37°C, the extent of hemolysis was measured as described in Materials and Methods. Values are mean±SEM of three to four independent experiments.

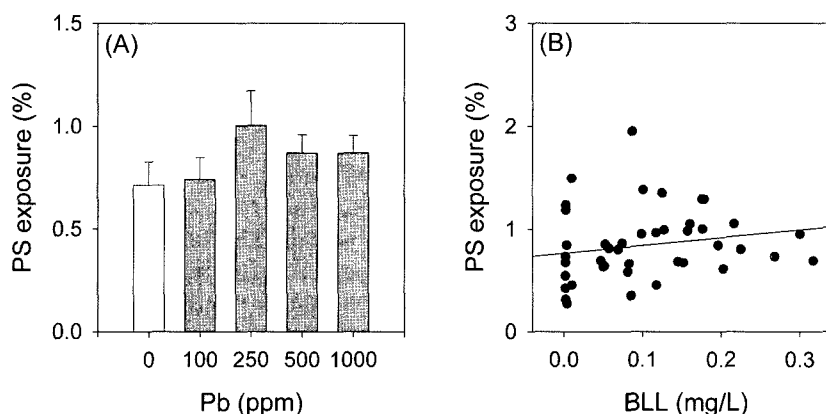


Fig. 4. *In vivo* effects of Pb²⁺ on PS exposure in rat red blood cells. Rats were exposed to Pb²⁺ in drinking water at DW (vehicle), 100, 250, 500, and 1000 ppm *ad libitum* for 4 weeks. (A) The extents of PS exposure on RBCs in collected whole blood were measured by flow cytometry. (B) The plot represents the correlation between blood lead level and PS exposure (r²=0.037).

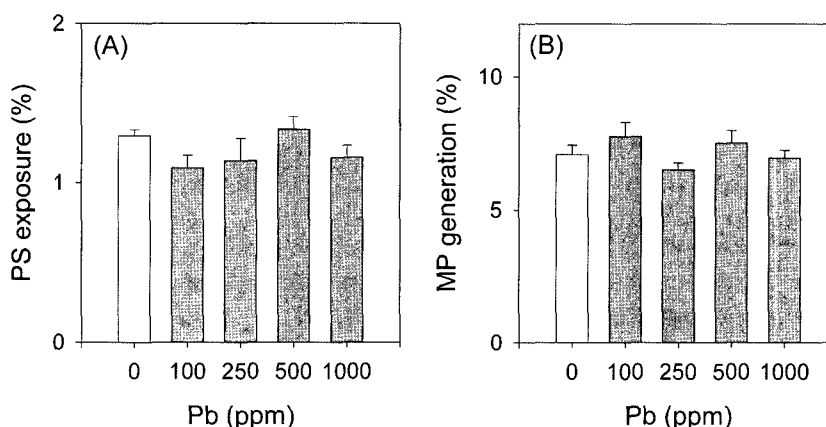


Fig. 5. *In vivo* effects of Pb²⁺ on PS exposure in rat platelets. Rats were exposed to Pb²⁺ in drinking water at DW (vehicle), 100, 250, 500, and 1000 ppm *ad libitum* for 4 weeks. The extents of (A) PS exposure and (B) microparticle (MP) generation on platelets in collected whole blood were measured by flow cytometry.

achieved, reflecting the potential existence of adaptive changes in body. With this study, we believe that a valuable information was provided for the study on the

toxicological significance and the risk assessment of lead contaminated drinking water.

Table II. Effect of intraperitoneally administered Pb²⁺ on PS exposure in rat red blood cells

Pb(II)Ac (mg/kg)	PS-exposing red blood cells (%)	Blood lead level (µg/dl)
Control	0.76±0.06	0.72±0.52
25	0.77±0.03	35.93±1.05*
50	0.99±0.07*	55.18±2.54*
100	1.36±0.09*	50.00±2.08*

Whole blood was collected from SD rats 4 h after *i.p.* administration of saline (vehicle), 25, 50, 100 mg/kg lead(II) acetate. Then, the extents of PS exposure on RBCs was measured by flow cytometry and Blood lead levels (BLL) was measured by atomic absorption spectroscopy. Values are mean±SEM of four independent experiments. * represents significant difference from control ($p < 0.05$).

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