EFFECTS OF LEAD CHLORIDE ON HUMAN ERYTHROCYTE MEMBRANES AND ON KINETIC ANION SULPHATE AND GLUTATHIONE CONCENTRATIONS

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Abstract: Our study concerns the effects of exposure to lead chloride on the morphology, K⁺ efflux, SO₄⁻² influx and GSH levels of the human erythrocyte. Blood was collected in heparinized tubes and washed three times. The cells were suspended at 3% hematocrit and incubated for 1 h at 25°C in a medium containing increasing concentrations of lead chloride (0, 0.3, 0.5 and 1 µM). After incubation, the suspensions were centrifuged and the erythrocyte pellets were divided into three aliquots for testing. The results show: an increase in the permeability of erythrocytes treated with lead chloride with consequent damage and cellular death, especially in the presence of high concentrations; an increase in potassium ion efflux; alterations in the morphology and membrane structure of the red blood cells; and a decrease in sulphate uptake, due either to the oxidative effect of this compound on the band 3 protein, which loses its biological valence as a carrier of sulphate ions, or to a decrease in the ATP erythrocyte concentration. In conclusion, the exposure of erythrocytes to Pb²⁺ ions leads to a reduction in the average lifetime of the erythrocytes and the
subsequent development of anemia. These data are discussed in terms of the possible effect of lead on the reduction-oxidation systems of the cell. Oxidant agents, such as lead, are known to cross-link integral membrane proteins, leading to K/Cl-cotransport. The increased K⁺ efflux affects the altered redox state.

**Key words:** Anion transport, Band 3 protein, Erythrocytes, Erythrocyte membrane, GSH, GSSG, Lead chloride, Scanning electron microscopy, Anemia, Adenosine 5’triphosphate, Sulphate influx

**INTRODUCTION**

Lead is the most abundant polluting agent in our environment and the most common toxic mineral found in the human body. It can be stored in the suprarenal glands, thyroid, bones, aorta, liver and other tissues, thus potentially causing hematological, gastrointestinal and neurological dysfunction, including peripheral neuropathy, due to myelin damage in the nerve fibers. Lead is a heavy metal that is found in nature as lead sulphide or lead chloride. It is sometimes used in dental prostheses and other metal objects. Major sources of lead exposure are dust, water, paint, cosmetics, folk remedies and food supplements [1]. Prolonged exposure to lead may also cause reproductive impairment, hypertension due to the involvement of endothelins [2] and nephropathy. Lead also slows interference with electrical nerve conduction, alters calcium homeostasis [3], inhibits various enzymes [4], inhibits ATP production and apoptosis factors [5], and induces the generation of reactive oxygen species (ROS) *in vivo*, with subsequent systematic mobilization, inhibition of nitric oxide and depletion of intrinsic antioxidant defenses.

When Pb²⁺ ions enter erythrocytes, they mainly bind to hemoglobin, with an estimated ratio in the cytosolic environment of bound to free lead of 6000:1. Binding with hemoglobin is not influenced by the oxygenation state [6]. Pb²⁺ is transported across the cell membrane via a number of mechanisms, but in erythrocytes Pb²⁺ transport is mediated by the anion exchanger Cl⁻/HCO₃⁻, band 3 protein, and the Ca-ATP pump.

Band 3 protein is the most abundant membrane protein in human erythrocytes, being present at approximately 1 million copies per cell [7]. It facilitates the electroneutral exchange of Cl⁻ and HCO₃⁻ across the membrane. Three anion exchanger (AE) isoforms are known, AE1, AE2 and AE3, each showing differing tissue expression. AE1 is found in erythrocytes and the kidneys, AE2 is found in a wide range of tissues, and AE3 is found in the brain, retina and heart. All members of the anion exchanger family have two domains: an N-terminal cytoplasmic domain that contains binding sites for glycolytic enzymes and hemoglobin; and a C-terminal membrane domain [8, 9]. The membrane domain is highly conserved. It spans the lipid bilayer 12 to 14 times and mediates anion transport. The cytoplasmic domain is anchored to the cytoskeleton and plays a structural role by connecting the cytoskeleton to the membrane [10, 11]. Band
3 protein is one of the main phosphorylated membrane proteins. It is generally phosphorylated at three sites in the cytoplasmic domain, the most important being tyrosine-8, followed by tyrosine-21 and tyrosine-46 [12]. Band 3 protein phosphorylation is accompanied by rigidification of the membrane skeleton, suggesting that the viscoelastic properties of human erythrocytes may be regulated by band 3 tyrosine phosphorylation [13].

Pb$^{2+}$ toxicity occurs due to its capacity to imitate Ca$^{2+}$ ions, allowing Pb$^{2+}$ ions to take the place of Ca$^{2+}$ ions in many important cellular processes. In addition to decreasing the capacity for calcium absorption, lead can accumulate in the bones, becoming a stable component that can be metabolized and returned to the blood, particularly in states of physiological stress (pregnancy, lactation or disease). This stable storage of lead in bones makes restoring blood lead levels to the norm slow, even after a complete removal of the toxic agent. Moreover, Pb$^{2+}$ ions adhere to erythrocyte cell membranes, decrease the erythrocyte ATP concentration, delay the decline in protoporphyrin concentration in mature erythrocytes and decrease 5'-nucleotidase activity.

We exposed human erythrocytes to different concentrations of PbCl$_2$ in order to study its effects on: 1) the mechanisms of anionic transport mediated by band 3 protein; 2) intracellular concentrations of GSH and GSSG; 3) K$^+$ efflux; and 4) the morphology of the erythrocytes.

**MATERIALS AND METHODS**

**Erythrocyte preparation**
Human blood was obtained with informed consent from 30 healthy volunteers. The blood was collected in heparinized tubes, washed in 150 mM NaCl and 25 mM HEPES (pH 7.4) and centrifuged three times for 5 min at 3000 rpm. At each step, theuffy coat and all leukocytes were carefully removed and the cells were suspended at 3% hematocrit and incubated for 1 h at 25°C in a medium containing 140 mM choline chloride, 10 mM NaCl, 25 mM HEPES and 10 mM glucose at pH 7.4 with increasing concentrations of lead chloride (0, 0.3, 0.5 and 1 µM). After incubation, the suspensions were centrifuged and the erythrocyte pellets were divided into three aliquots: one to study SO$_4^{2-}$ kinetics and to indirectly verify the oxidative effects of lead chloride on the -SH groups of the membrane proteins and on sulphate influx; one to examine the morphology using scanning electronic microscopy (SEM); and one to study the K$^+$ efflux. Whole blood was used for the measurement of GSH.

**Measurement of sulphate transport**
For kinetic studies, the erythrocytes were resuspended at 3% hematocrit and incubated at 25°C in a medium containing 115 mM Na$_2$SO$_4$, 5 mM KCl, 25 mM HEPES and 10 mM glucose with increasing concentrations of lead chloride (0, 0.3, 0.5 and 1 µM) at pH 7.4. At specified intervals, 5 ml samples of the suspension were removed, added to a test tube containing 5 µM 4,4'-diisothiocyanato-stilbene-2,2'-disulphonate (DIDS) stopping medium, and put on ice.
DIDS is a compound that binds irreversibly and specifically to band 3 protein and inhibits SO$_4^{2-}$ transport in both human and fish red blood cells. In some experiments, DIDS was added to the RBC suspension to give a final concentration of 5 µM. After withdrawal of the final sample, the erythrocytes were washed three times in a sulphate-free medium at 0ºC to remove extracellular sulphate, then lysed with distilled water and trichloroacetic acid. The membranes were removed by centrifugation and sulphate ions in the supernatant were precipitated by adding glycerol and distilled water (1:1), 4 mM NaCl and 37% HCl (12:1), and 124 mM BaCl$_2$ dihydrate to obtain a homogeneous barium sulphate precipitate. The intracellular SO$_4^{2-}$ concentration was measured by atomic absorption spectrophotometry at 425 nm. Using a standard curve obtained by precipitating known sulphate concentrations, we converted the absorption to mM of intracellular SO$_4^{2-}$ and calculated the rate constant in min$^{-1}$ by a non-linear least square curve-fitting procedure according to the equation:

\[ C(t) = C_\infty (1 - e^{-rt}) + C_0 \]

where $C_0$, $C_t$ and $C_\infty$ represent the intracellular sulphate concentrations measured at times 0, t and $\infty$, $e$ indicates the Euler’s number (2.7182818), and $r$ is a constant [14, 15].

**Measurement of K$^+$ efflux**

The blood collected in the heparinized tubes was washed and centrifuged three times for 5 min at 2000 rpm and 4ºC in an isotonic medium of 150 mM choline chloride, 20 mM HEPES, 15 mM glucose and 0.1 mM ouabain, (307 mOsm, pH 7.4), or 150 mM NaNO$_3$, 20 mM HEPES, 15 mM glucose and 0.1 mM ouabain (309 mOsm), pH 7.40. Erythrocytes at 3% hematocrit were incubated for 1 h at 25ºC in the same medium with 0.3, 0.5 or 1 µM PbCl$_2$. The suspensions were then centrifuged at 4ºC, 50 µl aliquots of the supernatants were placed in LiNO$_3$ solution, and the K$^+$ concentration was then measured by flame photometry [14]. The total K$^+$ content of the flux medium was corrected for initial cell volume and K$^+$ efflux was expressed in mEq/lRBC x h of the original cells.

**Measurement of GSH concentration**

The GSH concentration was measured in whole blood before and after treatment with 0.3, 0.5 and 1 µM of PbCl$_2$ using HPLC-UV [16] intended for the quantitative determination of glutathione in EDTA-blood. In this assay, the sample was treated with a diluted solution of PbCl$_2$ and divided into two aliquots. The reduced fraction (aliquot 1) was measured by adding 50 µl of the diluted sample, 100 µl reaction buffer and 100 µl derivatization solution. After incubation for 20 min at 60ºC, during which time GSH was converted to a fluorescent product, 100 µl of precipitation solution was added to remove higher molecular weight substances. The samples were precipitated for 10 min at 2 to 8ºC and centrifuged for 10 min at 6000 rpm, after which 200 µl of supernatant were added to 200 µl of the reaction buffer in autosampler vials. The
total glutathione (aliquot 2) was measured by adding 50 µl of the diluted sample, 20 µl of the reduction solution, 100 µl of the internal standard and 100 µl of the derivatization solution. The sample was then handled in the same way as the reduced fraction. After that, 20 µl of the supernatant was injected into the HPLC system. The HPLC separation followed an isocratic method at 30°C using a reversed-phase column in two runs. The chromatograms were scanned using a fluorescence detector and concentrations were calculated by integration of the peak height using the external standard method for the reduced fraction and the internal standard method for the total glutathione fraction. The amount of oxidized glutathione was calculated by:

½ GSSG = glutathione<sub>total</sub> - glutathione<sub>reduced</sub>

**Scanning electron microscopy and human erythrocyte morphology**

Sample preparation for electron microscopy involved fixation, dehydration, assembly, covering with gold, and observation. To analyze the effect of PbCl₂ on the morphology of human red blood cells, the samples were incubated in with increasing concentrations of lead at 25°C for 1 h, washed with physiological solution (166 mM NaCl), and fixed overnight at 5°C by adding one drop of each sample to plastic tubes containing 1 ml of 4% glutaraldehyde in Sorensen’s phosphate buffer (0.1 M, pH 7.4). The samples were washed three times in the same buffer for 30 min and then exposed to increasing concentrations (30, 50, 70, 90 and 95%) of ethanol for about 30 min each. Dehydration was carried out using liquid carbon dioxide until the critical point was reached. The samples were assembled on a particular type of glass using conductive silver paste and covered with a thin layer of gold (200-300 Å) via sputtering. The samples were then ready for observation by SEM.

**Statistical analysis**

Data are expressed as arithmetic means ± SD, and statistical analysis was performed using the paired t-test or ANOVA. P < 0.05 was considered statistically significant.

**RESULTS**

The experiments were carried out in order to highlight not only possible modifications of sulphate transport mediated by band 3 protein, but also variations in GSH levels, K⁺ efflux, human red blood cell membrane shape and -SH group oxidation. The data in Table 1 show K⁺ efflux in human erythrocytes in the presence of increasing concentrations of PbCl₂ (0, 0.3, 0.5 and 1 µM) after 1 h incubation at 25°C, in Cl⁻-containing and Cl⁻-free media. Lead ions at a concentration of 1 µM increased chloride-dependent K⁺ efflux relative to the control. A modest effect was seen after incubation with 0.3 µM lead chloride.
Table 1. K⁺ efflux in human erythrocytes in the presence of 0, 0.3, 0.5 and 1 µM PbCl₂, after 1 h incubation at 25°C and pH 7.40 in Cl⁻-containing and Cl⁻-free media. K⁺ efflux is expressed in mEq/l rbc×h. The data are presented as means ± SD of five experiments.

| Human red blood cells | Control | + 0.3 µM PbCl₂ | + 0.5 µM PbCl₂ | + 1 µM PbCl₂ |
|----------------------|---------|----------------|----------------|-------------|
| Cl⁻                  | 5.90 ± 0.1 | 7.69 ± 0.1     | 18.97 ± 0.1    | 38.8 ± 0.2  |
| NO₃⁻                 | 3.79 ± 0.1 | 5.67 ± 0.1     | 7.57 ± 0.1     | 11.3 ± 0.1  |

Fig. 1 clearly indicates that this was a process of facilitated diffusion mediated by band 3 protein. From the kinetic profiles and their rate constants shown in Table 2, it is clear that the sulphate influx was inhibited by 20.51% in erythrocytes treated with 0.3 µM compared to the influx measured for the control cells (0.039 ± 0.003 in min⁻¹).

Fig. 1 also shows that increasing PbCl₂ concentrations significantly increased the percentage of inhibition of sulphate influx into red blood cells: 41.02% inhibition occurred with 0.5 µM PbCl₂ and 64.10% with 1 µM PbCl₂, compared to the results for the control cells. The effect of PbCl₂ on the GSH concentrations in whole blood is shown in Tab. 3.

Fig. 1. Time course of sulphate uptake measured in human red blood cells at 25°C and pH 7.40 in the presence of 0 µM (●), 0.3 µM (□), 0.5 µM (○) and 1 µM (▲) PbCl₂. The bars represent means ± SD.
Table 2. \( \text{SO}_4^{2-} \) uptake rate constants in min\(^{-1}\), measured for human erythrocytes.

| Human erythrocytes | Rate constants in min\(^{-1}\) | % Inhibition |
|--------------------|-------------------------------|--------------|
| Control            | 0.039 ± 0.003                 |              |
| + 0.3 µM PbCl\(_2\) | 0.031 ± 0.002                 | 20.51%       |
| + 0.5 µM PbCl\(_2\) | 0.023 ± 0.001                 | 41.02%       |
| + 1 µM PbCl\(_2\)  | 0.014 ± 0.001                 | 64.10%       |

Table 3. Concentrations of reduced and oxidized glutathione expressed as µmol/l and the GSH:GSSG ratio measured in human whole blood after 60 min of incubation at 25ºC (pH 7.4) in the absence (control) and presence of 0.3 µM, 0.5 or 1 µM PbCl\(_2\).

|                | Control  | + 0.3 µM PbCl\(_2\) | + 0.5 µM PbCl\(_2\) | + 1 µM PbCl\(_2\) |
|----------------|----------|----------------------|----------------------|-------------------|
| GSH (µmol/l)   | 735 ± 145| 718 ± 103            | 625 ± 112            | 422 ± 130         |
| GSSG (µmol/l)  | 170 ± 36 | 267 ± 32             | 287 ± 112            | 328 ± 36          |
| GSH:GSSG ratio | 4.3 ± 1.5| 2.6 ± 1.3            | 2.2 ± 1.5            | 1.2 ± 1.8         |

The SEM observations confirmed that exposure to PbCl\(_2\) results in alterations to the red blood cell surface. Human red blood cells treated with increasing concentrations of PbCl\(_2\) (Figs 2B-D) appear deformed compared to normal red blood cells.

Fig. 2. Effects of lead chloride on the morphology of human erythrocytes. Scanning electron microscope image of (A) untreated erythrocytes, 1700 x; (B) erythrocytes incubated with 0.3 µM PbCl\(_2\), 15000 x; (C) erythrocytes incubated with 0.5 µM PbCl\(_2\), 15000 x; (D) erythrocytes incubated with 1 µM PbCl\(_2\), 15000 x.
DISCUSSION

The experiments revealed a novel effect of Pb\(^{2+}\) ions on erythrocytes. The trace element activated the erythrocyte scramblase, leading to phosphatidylserine exposure at the cell membrane. The concentrations needed to elicit this effect were well within the range of concentrations encountered in plasma [17-19], although most Pb\(^{2+}\) ions are bound to proteins such as serum albumin [20] and thus the published plasma concentrations do not reflect the actual free Pb\(^{2+}\) ion concentrations.

The active form of band 3 protein with intracytoplasmatic C- and N-terminals mediates the exchange of anions across the red blood cell membrane [21]. It is an integral membrane protein that crosses the bilayer lipid membrane many times. Because of its position, the protein is continuously damaged by chemical agents and drugs circulating in the blood. Previous studies reported that some metals enhance the degradation of band 3 protein after it has been treated with chemical substances likely to affect sulphate permeability and modulate anion influx through the erythrocyte membranes [22]. Oxidation, caused by high concentrations of lead chloride, changed the -SH groups into SS- groups, resulting in a conformational change in the protein structure. This led to a decline in the function of the band 3 transporter protein, with a 41% reduction in the rate constant of the anion influx compared to the control when oxidative stress was induced in the erythrocytes by treatment with 0.5 µM PbCl\(_2\). The percentage of inhibition of the rate constant of sulphate influx increased significantly (64%) compared to the control after treatment with 1 µM PbCl\(_2\). The decreased efficiency of anion transport may be either due to changes in the structural state of band 3 resulting from SH-group oxidation or due to cell shrinkage (increased K\(^+\) efflux), with a concomitant increase in band 3 tyrosine phosphorylation [23]. It is conceivable that K/Cl-cotransport activation induces a further decrease in SO\(_4^{2-}\) levels (Table 1).

Table 1 shows the mM/lRBC\(\times\)h of K\(^+\) efflux measured in human red blood cells after treatment with 0.3, 0.5 and 1 µM PbCl\(_2\). The oxidizing metal increased chloride-dependent K\(^+\) efflux with respect to the control. When Cl\(^-\) was replaced with NO\(_3^-\), the K\(^+\) efflux decreased, indicating that K\(^+\) efflux is Cl\(^-\)-dependent.

Pb\(^{2+}\) may activate K\(^+\) channels by increasing cytosolic Ca\(^{2+}\) activity. Increasing the extracellular K\(^+\) concentration and inhibitors of K\(^+\) channels not only reversed the Pb\(^{2+}\)-induced cell shrinkage but also significantly interfered with Pb\(^{2+}\)-induced phosphatidylserine exposure. Pb\(^{2+}\) may activate K\(^+\) channels by increasing cytosolic Ca\(^{2+}\) activity [24].

In addition, the interaction between hemoglobin-protein and band 3 protein plays an important role in modulating the speed of anion transport and, in general, in the control of structural and functional properties of red blood cells. This control seems to be influenced by several factors including the ionic complex, especially magnesium and 2.3 BPG [25, 26, 27], and ATP and hemoglobin [28].
The effect of lead on red blood cells includes interfering in the biosynthesis of heme and lipid peroxidation. Lead reduces the activity of many enzymes, including catalase, superoxide dismutase and glutathione peroxidase. The intracellular concentrations of GSH decreased significantly when human erythrocytes were treated with 0.5 and 1 µM of PbCl₂. These results indicate that lead exposure significantly reduces the cellular reserves of antioxidants. Lead also alters the organization of the erythrocyte membrane. SEM of erythrocytes treated with increasing concentrations of PbCl₂ revealed significant morphological differences compared with untreated erythrocytes. As seen in Fig. 2B, the erythrocytes exposed to lower concentrations of PbCl₂ (0.3 µM) seem slightly deformed in shape. At higher concentrations of PbCl₂ (0.5 µM), most of the erythrocytes showed significant morphological alterations. With 1 µM PbCl₂ the erythrocyte membranes were damaged and the cells appeared smaller, piled and more distorted.

Further mechanisms involved in the stimulation of erythrocyte apoptosis after Pb²⁺ ion exposure may involve a decrease in the cytosolic ATP concentration, because energy depletion was previously shown to trigger erythrocyte apoptosis and Pb²⁺ ion exposure is known to decrease erythrocyte ATP concentration [29, 30]. In conclusion, exposure to Pb²⁺ ions at concentrations that can be found in the environment activates K⁺ channels, leading to K⁺ loss and erythrocyte shrinkage and favoring phosphatidylserine exposure at the surface of the cell membrane. The affected erythrocytes are prone to being cleared from the circulating blood, which presumably contributes to the decreases in erythrocyte life span and in the number of erythrocytes in the circulating blood, and to the development of anemia after Pb²⁺ intoxication [31]. Increasing extracellular K⁺ concentration and inhibitors of K⁺ channels not only reversed the Pb²⁺-induced cell shrinkage but also significantly interfered with Pb²⁺-induced phosphatidylserine exposure. As reported recently [32-34], activation of the erythrocyte K⁺ channels and subsequent cell shrinkage participate in the triggering of scramblase activation. The sensitivity of erythrocytes to cellular K⁺ loss is similar to that observed in other cell types. Cellular loss of K⁺ has been demonstrated to run in parallel and support apoptosis in a variety of nucleated cells [35-41].

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