The Effect of Lead Ions on Human Erythrocytes in Vitro

RALUCA CIUBAR1, CRISTINA CIOFRINGEANU1, VALENTINA MITRAN1, ANISOARA CIMPEAN1, AURELIAN STANESCU1, DANA IORDACHESCU1
1 University of Bucharest, Research Center for Biochemistry and Molecular Biology, 93 Splaiul Independenþei, 050095, Bucharest, Romania
2 Politehnica University of Bucharest, 313 Splaiul Independenþei, 060042, Bucharest, Romania

Despite the vast literature on lead toxicity, little is known about the biochemical mechanisms responsible for the toxicity of lead. The erythrocytes are an important target of lead toxicity Pb2+ that causes anemia owing to a decreased life span of circulating cells. The aim of this study was to estimate the influence of acute exposure (24 h) to lead ions (0.1 – 2 µM) on some antioxidant enzymes and malondialdehyde (MDA) levels of human erythrocytes in vitro correlated with exposure of phosphatidylserine at the cell surface. It was observed a decrease in superoxide dismutase (SOD) and catalase (CAT) activity and an increase in MDA levels in cells treated with higher Pb2+ doses (> 1 µM) compared to control and an oxidant-induced apoptosis as well.

Key words: lead, erythrocyte, antioxidant enzymes, apoptosis

Lead exposure is one of the greatest challenges of modern times to those responsible for public health and industrial hygiene [1]. In most of its chemical forms, lead can be toxic at the levels to which human beings are exposed in the workplace and in the general environment [2]. Analytical epidemiological studies suggest that some degree of exposure is almost universal [3]. The primary sources of environmental exposure to lead are leaded paint, auto emissions, and drinking water. Occupations related to house painting, welding, renovation and remodeling activities, smelters, firing ranges, the manufacture and disposal of car batteries, and the maintenance and repair of bridges and water towers, are particularly at risk for lead exposure [4]. Lead poisoning is usually caused by months or years of exposure to small amounts of lead in the home, work, or daycare environment. Acute exposure to high levels of lead is less common than chronic lead poisoning. During the last decade the risk of exposure to low levels of inorganic lead affecting the behaviour and intelligence of children have been a major issue. Fetal exposure can cause potentially adverse neurological effects during postnatal development since lead readily crosses the placenta [5].

The seriousness of lead damage depends on two factors: the amount of lead that gets into the body and the length of time it remains there. Lead can damage almost every organ system, with the most harm caused to the brain, nervous system, kidneys, and blood. Despite the vast literature on lead toxicity [6, 7], little is known about the biochemical mechanisms responsible for the toxicity of lead. Oxidative stress is proposed as a molecular mechanism in lead toxicity, which suggests that antioxidants might play a role in the treatment of lead poisoning [8].

The erythrocyte is an important target of lead toxicity that causes anemia owing to a decreased life span of circulating cells. A clear relationship between degradation of membrane phospholipids, osmotic shock, oxidative stress and exposure of phosphatidylserine (PS) at the erythrocyte surface was noticed in the case of cells incubated with lead [9, 10].

The aim of this work was to evaluate the influence of acute exposure to lead ions on the oxidative status of human erythrocytes in vitro and its possible relation with cell apoptosis. The development of specific software applications in relation health &environment and the evaluation of prognosis ways of environmental risk factors on health is supposed to be supported using in vitro experiments and the present paper is an example of such concept.

Experimental part

Erythrocyte cell culture

Blood from nine normal volunteers was collected in tubes containing citrate as the anticoagulant. Leukocytes and platelets were removed by the method of Beutler et al. [11]. After the blood was centrifuged, plasma was removed and the erythrocytes were used as hemolysates. Pb2+ ions were added to the medium at final concentrations varying from 0.1 to 2 µM lead nitrate, Merck. After 24 hours of treatment, erythrocytes were washed twice with PBS and then lysed by sonication, and different dilutions were used as hemolysates.

Phase-contrast microscopy

Morphological changes induced by treatment of erythrocytes with lead were examined with an inverted phase-contrast microscope Nikon Eclipse TS 100.

Phosphatidylserine detection

Control and treated erythrocytes were incubated with annexin V-FITC (Sigma Aldrich) for 15 min at room temperature in the dark. Then, cells were immediately analyzed under a fluorescence microscope.

Superoxide dismutase assay

SOD activity was measured in erythrocytes hemolysates following the method of Paoletti and Mocali [12]. SOD activity was expressed as U/g Hb.
Measurement was carried out the inhibition of the NADH oxidation by superoxide radicals generated chemically in the reaction mixture containing EDTA, MnCl₂ and mercaptoethanol.

**Measurement of catalase activity**
Immediately after obtaining erythrocytes hemolysates quantitative determination of catalase (CAT) was performed according to the method of Aebi [13]. Activity was expressed as U/g Hb.

**Estimation of MDA**
Malondialdehyde (MDA) content of erythrocytes was estimated as thiobarbituric acid reacting substances (TBARS) by spectrophotometric method as described by Jain et al. [14]. The MDA value was calculated from the MDA standard graph and expressed as nanomoles/g Hb. Hemoglobin concentrations were determined by a modification of the Drabkin method [15].

**Results and discussion**

**Effect of Pb²⁺ on erythrocyte morphology**
Severe alterations in red-cell morphology have been observed following a 24 h incubation with lead nitrate. As shown in figure 1a, control red cells incubated for 24 h at 37°C exhibited the biconcave appearance of normal discocytes. Echinocytic cells were observed on occasion, but represented less than 2% of the total cells. In contrast, more than 50% of the red cells exposed to doses over 0.5 µM Pb²⁺ for 24 h at 37°C had lost their discocytic morphology and exhibited moderate to severe degrees of echinocytosis (fig. 1d,e). The cells were characterized by several moderately-sized protuberances that were asymmetrically distributed on the cell surface. Exposure to higher doses of Pb²⁺ was accompanied by erythrocytes shrinkage.

**Studies of antioxidant enzymes in erythrocytes**
Oxidative stress can lead to oxidation of thiol groups of cytoskeletal proteins that oversee cell shape maintenance. So next, the enzyme activity of SOD and CAT was measured to evaluate Pb²⁺-induced changes in antioxidant defence system. As shown in figure 2 the *in vitro* treatment for 24 h of erythrocytes with Pb²⁺ alters the SOD activity in a dose dependent manner. As result of treatment with 0.1 µM lead, an increase in erythrocyte SOD activity by about 57% than in controls was noticed, suggesting an induction of the defense mechanism of cells against superoxide anion production. On the other hand at the highest concentration of lead tested (2 µM) the SOD activity was inhibited by 82% showing a decrease in antioxidant protection. Little or no change in SOD activity was noticed in the case of erythrocyte treatment with intermediary doses (2 µM > Pb²⁺ > 0.1 µM).

Catalase is an enzyme involved in H₂O₂ detoxification, that can act after SOD in a scavenger mechanism:

\[ \text{O}_2 \rightarrow \text{O}_2^- \rightarrow \text{H}_2\text{O}_2 \rightarrow \text{CAT} \rightarrow \text{H}_2\text{O} + \text{O}_2 \]

In response to treatment with the four doses of Pb²⁺ the erythrocyte CAT activity was modified to a lesser extent than SOD (fig. 3). Thus, for 0.1 µM dose of Pb²⁺, a 20% activation of CAT and for 2 µM Pb²⁺ a 21% inhibition of CAT was noticed, the results that suggest the higher sensitivity of SOD to lead than CAT. Therefore, in spite of increased SOD activity at low
doses of Pb²⁺, the CAT activity can not compensate the overproduction of H₂O₂ and a prooxidant state is induced. So, the imbalance of SOD/CAT ratio in erythrocytes could make the cells very susceptible to oxidative damage.

Investigation of the changes in MDA level in erythrocytes treated with different doses of Pb²⁺, for 24 h, serves as an index of extent of lipid peroxidation, as the first oxidative lesions that appear as result of oxidative stress (fig.4). These data show a good agreement with those obtained in enzyme activity assay and clearly illustrate that cytotoxicity of 5 µM dose of Pb²⁺ is owing to erythrocyte susceptibility to lead-induced oxidative stress.

Lead nitrate-induced phosphatidylserine externalization

It was demonstrated that oxidant-induced apoptosis yields rapid oxidation of different classes of phospholipids with substantial oxidation of PS [16]. This oxidation of PS preceds its externalization during apoptosis [17] and may be an integral part of the apoptotic program. Therefore, next we explored whether treatment with Pb ions triggers phosphatidylserine exposure using immunofluorescence microscopy. Incubation of erythrocytes in DMEM for 24 h resulted in very low PS exposure. Addition of lead nitrate at concentrations of 0.5–2 µM activated erythrocyte scramblase leading to PS exposure at cell membrane with subsequent annex binding (fig. 5). The effect of lead ions was paralleled by erythrocyte shrinkage, another typical feature of apoptotic cell death.

Conclusion

In conclusion, impaired oxidant-antioxidant balance in erythrocytes could be partially responsible for the adverse health effects of lead exposure. Treatment with lead nitrate leads to PS externalization and cell shrinkage, which favors premature elimination of circulating erythrocytes and the development of anemia after lead poisoning. The potential of oxidative stress parameters to be used as biomarkers of lead toxicity required further investigation.

References
1. GURER-ORHAN, H., SABIR, H.U., OZGUNES, H., Toxicology, 195, 2004, p.147

Manuscript received: 30.07.2007