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# Plasma and mitochondrial membrane perturbation induced by aluminum in human peripheral blood lymphocytes

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ABSTRACT

Aluminum is a redox-inert element that could induce cell damage via activation of oxidative stress. In this work, the effect of aluminum on different cellular compartments of human peripheral blood lymphocytes was studied. The presence of aluminum induced a lipid peroxidation and physico-chemical modifications at the membrane level. A decrease in fluorescence anisotropy of TMA-DPH and in the polarity of the lipid bilayer with a concomitant shift toward a gel phase was observed, while the pyrene excimerization coefficient (Kex) increased.

Flow cytometry measurements, using JC-1, Rhodamine 123 and $H_2$-DCFDA as fluorescent probes, indicated that aluminum induces a slight mitochondrial membrane depolarization that was associated with a moderate increase in reactive oxygen species production. A significative influence on these parameters was measured only at high aluminum concentration.



## Introduction

In the last decades the concentration of metals in the environment has increased. Human exposure to metals can derive from natural erosion of metal-containing minerals as well as from anthropogenic activities. The highest exposure usually occurs at the work-place, even if significant exposure may take place due to its presence in certain foods (in particular in corn, yellow cheese, salt, spices, herbs) and beverages (such as tea) and drinking water [1], since aluminum (Al) absorption increases up to 4–5 fold in combination with the food matrix [2]. Metals play important roles in a wide variety of biological processes of living systems and therefore a tightly controlled homeostasis of metal ions is critical for life [3].

All metals can cause diseases through excessive exposure [4]. The toxic effects of metals depend on the amount of metal introduced in the organism, its solubility, chemical reactivity, tissue distribution and excretion rate (excretion may need years or decades). In the case of aluminum, the major route of excretion is represented by urine [5] even if Minshall et al. suggested that perspiration may also be a significant route for aluminum excretion [6].

Our interest in this field has focused on aluminum, a metal that despite having no useful biological function since its discovery, its accumulation may affect a variety of biological functions. Diseases such as nephropathies, dementia, encephalopathies, Alzheimer's, Parkinson's, osteomalacia and microcytic anemia have been associated with high Al(III) levels [7–9]. In spite of the numerous efforts, the accumulated evidence and progress in this research area, the mechanism(s) of Al toxicity are still not well defined.

Current research indicates that oxidative damage could play a role in aluminum toxicity [7,10–12] even if a direct generation of reactive oxygen species (ROS) by aluminum should be excluded, since this element is not a transition metal capable of undergoing valence changes. However aluminum pro-oxidant effect has been shown *in vitro* and *in vivo* as reviewed by Exley [12]. Aluminum, as a strong Lewis acid, is capable to producing oxidative stress and it is pro-oxidant on its one and sinergically with iron [7,12]. Formation of metal-superoxide complexes is also postulated by Kong et al. [13] and Meglio and Oteiza [14] in relation to the ability Al(III) and other non redox trivalent metals to bind to superoxide anion to

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form aluminum semireduced radical ion $AlO_2^{\bullet 2+}$. Pro-oxidant activity of aluminum on biological membranes has been well described but a clear definition of the molecular mechanisms involved is still lacking [10,15,16]. Also in this respect It has been proposed that aluminum is able to enhance iron-mediated peroxidation [17–21].

In light of the aluminum involvement in oxidative damage to membranes we designed the present study in order to investigate its pro-oxidant activity on mitochondria, that represent a major site of ROS production.

In a previous paper [22], we reported that Al affects both the status of the plasma membrane and the cytoplasmic antioxidant enzymes of human erythrocytes. Erythrocytes are routinely used as a model to study the toxicity of chemicals because numerous compounds persist in circulating blood before reaching target organs. However, information obtained by studying the toxicity of chemicals in erythrocytes is limited by the fact that these cells are structurally simple, devoid of nucleus and mitochondria. In fact, aluminum is also found in various tissues and inside the cells in different compartments, including mitochondria and the nucleus [23]. In this study we employed peripheral blood lymphocytes, presenting an active aerobic metabolism, and therefore suitable to study the influence of aluminum *in vitro* exposure on mitochondria.

In particular the aim of the study was to provide further information on the molecular basis of the toxicity of aluminum to biological membranes with particular attention to mitochondrial membranes and its implications on Al-induced mitochondrial dysfunction.

## Materials and methods

All reagents were of pure analytical grade. $AlCl_3 \cdot 6H_2O$, 1-(4-trimethylammoniumphenyl)-6-phenylhexa-1,3,5-triene (TMA-DPH) and Pyrene were obtained from Sigma (USA). 6-Dodecanoyl-N,N-dimethyl-2-naphthylamine (Laurdan), Carboxy-$H_2$-DCFDA,5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) and Rhodamine 123 were purchased from Molecular Probes (USA).

### *Samples and treatment*

The results reported in this paper were performed in two different laboratories located in Minsk (Belarus) and Ancona (Italy). The cells used in this study were thus obtained from the Department of Republican Theoretical and Practical Center of Hematology and Transfusiology (Minsk, Belarus) and from Italian donors prior to informed consent. The donor age ranged from 25 to 45 years. After dilution with Phosphate Buffered Saline (PBS) 1:1, whole blood was stratified on a lymphoprep solution and centrifuged at 1600 × *g* in a swinging bucket rotor at 4 °C for 20 min and deceleration was set without any brake. PBLs were collected and washed with PBS, pH 7.4. The different concentrations of $AlCl_3 \cdot 6H_2O$ were obtained dissolving the salt in Milli-Q water, 18.2 MΩ cm. Each experimental determination was conducted on separated aliquots from different donors.

### *Membrane lipid peroxides*

Membrane lipid peroxides were measured according to the method described by Strauss in 1981 [24] based on lipid peroxides reaction with thiobarbituric acid (TBA). 1 mL of lymphocyte suspension containing $1 \times 10^6$ cells obtained after 3 h of incubation at 37 °C in PBS at pH 7.4 for the control and in the presence of different amounts of $AlCl_3$ (10–25–50–75–100 μM), was combined with 2 mL of the TBA reagent and the mixture was placed in a boiling water-bath for 15 min. Debris were then removed by centrifugation at 1000 × *g* for 15 min. The clear supernatant was placed into clean tubes and read in a spectrophotometer at 535 nm. Six measurements were performed, each one from a different blood donor.

### *Fluorescence measurements on plasma membranes*

Regarding fluorescence experiments, lymphocytes previously incubated for 3 h at 37 °C with different amounts of $AlCl_3$ (from 10 to 100 μM) were used. Fluorescence measurements were performed using a luminescent spectrophotometer CM2203 ("Solar", Belarus). Three different fluorescent probes (TMA-DPH, Laurdan and Pyrene) were employed to investigate the physico-chemical state of the lymphocyte membranes and thus the influence of $AlCl_3$ on it. TMA-DPH steady-state fluorescence anisotropy measurements were performed at 37 °C and the excitation and emission wavelengths were respectively, 360 nm and 428 nm. Fluorescence anisotropy was calculated as previously described by Kuhry et al. [25] using the expression $I// - (I\perp \times g) + (2I\perp \times g)$ where *g* is an instrumental correction factor, I// and I⊥ are the emission intensities with polarizers parallel and perpendicular to the direction of the polarized light, respectively.

This amphiphilic probe anchors close to the bilayer surface because of its charged amino group [26]. In this assay lymphocytes ($1 \times 10^6$ cells/mL) were incubated with 1.5 μM of TMA-DPH.

Generalized polarization (GP) for laurdan, described by Parasassi et al. [27], was calculated by exciting laurdan at 340 nm (GP340) according to the following equation: GP = Ib − Ir/Ib + Ir where Ib an Ir are the emission intensities at the blue (440 nm) and red (490 nm) edges of the emission spectrum corresponding to the fluorescence emission maxima in the gel and liquid-crystalline phases, respectively, of the bilayer. This probe localizes in the hydrophobic-hydrophilic interface of the lipid bilayer at the level of the glycerol backbone [27]. Laurdan is sensitive to changes in the polarity of its microenvironment and modifications can be monitored by measuring the GP340. In this assay, lymphocytes ($1 \times 10^6$ cells/mL) were incubated for 15 min with 1 μM laurdan.

The fluorescent probe pyrene is an apolar molecule that buries completely within the hydrophobic region of the membrane and allows diffusion measurements in the interior part of the membrane [28]. Spectral properties of this fluorophore depend on its state. Pyrene monomers are located in the acyl chain region, and their spectrum is characterized by five vibrational bands which are highly sensitive to the environmental polarity. Excited pyrene molecules can interact with non-excited ones with the formation of excited state dimers called excimers. These pyrene species display a broad and red-shifted fluorescence spectrum compared to that of monomers. The extent of pyrene excimerization depends on the packing density of lipid molecules. The excimer formation in a fluid phospholipid membrane is a diffusion-controlled process that allows evaluation of the lateral mobility of aromatic molecules in the hydrophobic region of the bilayer.

The intensity of excimer and monomer forms of pyrene was measured at 473 nm (I473) and 393 nm (I393) as reported by Galla and Sackmann [28]; accordingly, λex = 330 nm and the coefficient of excimerization of pyrene was calculated as Kex = I473/I393. In this assay, lymphocytes ($1 \times 10^6$ cells/mL) were incubated with 5 μM pyrene. Control mean values were taken as 100% in laurdan and pyrene experiments. Five measurements were performed, each one from a different blood donor.

### *Flowcytometric determinations*

PBLs ($5 \times 10^5$ cells/mL) were incubated in the dark for 1 h at 37 °C in 250 μl of PBS alone (control) or with 25, 50 and 100 μM $AlCl_3$. At the end of incubation, to remove excess of salt, the samples were centrifuged at 500 × *g* for 5 min at 4 °C and resuspended in