Aluminum exposure affects transferrin-dependent and -independent iron uptake by K562 cells

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Abstract

Aluminum (Al) and iron (Fe) share several physicochemical characteristics and they both bind to transferrin (Tf), entering the cell via Tf receptors (TfR). Previously, we found similar values of affinity constant for the binding of TfR to Tf carrying either Al or Fe. The competitive interaction between both metals prevented normal Fe incorporation into K562 cells and triggered the upregulation of Fe transport. In the present work we demonstrated that Al modified Fe uptake without affecting the expression of Tf receptors. Both TfR and TfR2 mRNA levels, evaluated by RT-PCR, and TfR antigenic sites, analyzed by flow cytometry, were found unchanged after Al exposure. In turn, Al did induce upregulation of non-Tf bound Fe (NTBI) uptake. This modulation was not due to intracellular Fe decrease since NTBI transport proved not to be regulated by Fe depletion. Unlike its behavior in the presence of Tf, Al was unable to compete with NTBI uptake, suggesting that both metals do not share the same alternative transport pathway. We propose that Al interference with TfR-mediated Fe incorporation might trigger the upregulation of NTBI uptake, an adaptation aimed at incorporating the essential metal required for cellular metabolism without allowing the simultaneous access of a potentially toxic metal.

Keywords: Aluminum; Iron metabolism; Transferrin receptor; Transferrin-mediated iron uptake; Non-transferrin bound iron transport; K562 cell line

1. Introduction

Aluminum (Al) is an element which has no known biological role. However, the widespread use of products made of/or containing Al makes it unlikely that this metal is absent from any tissue in the body. Even though much controversy surrounds its role in human diseases, Al accumulation has been considered as an etiopathogenic factor affecting the erythropoietic [1] and nervous [2,3] systems.

Al and the essential metal iron (Fe) share several physicochemical characteristics (ionic radius, charge density, chelation by particular compounds) [4], and they both bind to transferrin (Tf), entering the cellular environment via specific Tf receptors (TfR) [5].

The same properties that make Fe an essential metal for basic biological processes also make it toxic. Fe is able to promote oxidative damage to vital biological structures, and thus its homeostasis should be tightly regulated balancing metal uptake with intracellular storage and utilization [6]. To guarantee this equilibrium, TfR expression is post-transcriptionally regulated by iron regulatory proteins (IRPs) that are sensitive to intracellular Fe concentration and interact with iron responsive elements (IREs) located in the untranslated region of TfR mRNA [7]. On the other hand, a recent study reported that the newly identified receptor TR2 is involved in another Tf-dependent Fe uptake pathway [8].

In addition to the well-described Tf-dependent pathways, many studies have demonstrated the existence of an uptake system involving non-transferrin bound iron (NTBI) [9–12].
Even though the function and regulation of this transport have not been completely elucidated yet, at least two mechanisms have been reported. Ferric ions are transported into cells via the β3-integrin–mobilferrin pathway [12], whereas the uptake of ferrous ions is mediated by the divalent metal transporter 1 (DMT1, Nramp2, DCT1) [13].

Unlike essential metals, no physiological role has been attributed to Al. Thus, its toxicity represents a major concern due to the growing bioavailability originated in its natural abundance and the expanding future of Al chemistry [14]. In previous studies on the relationship between Al and the development of anemia [1,15,16] we demonstrated the existence of significant Fe deposits in the bone marrow of animals chronically overloaded with Al, concurrently with erythroid progenitor cell growth depression and impairment of hemoglobin synthesis [16]. In this context, the presence of Al decreased Fe incorporation to the prosthetic group heme and inhibited erythroid differentiation of K562 cells [5,17]. These data agreed with impaired Fe utilization which could be a consequence of lower Fe uptake because of Al interference. In order to characterize the interaction of Al–Tf at the membrane TfR level, we performed kinetic studies. The affinity constant for Al–Tf binding, which was similar in magnitude to that of Fe–Tf, allowed us to report the first evidence on the behavior of Al–Tf as a non-physiological Fe–Tf competitor [17]. Therefore, the simultaneous presence of Al, Fe and Tf hindered Fe from being taken and incorporated to the hem group by K562 cells. On the other hand, the Al removal favored a rapid increase in cell Fe uptake by non-induced and hemin-stimulated cells [5], suggesting that the competitive interaction between Al–Tf and Fe–Tf at the cell surface would entail intracellular Fe depletion, which in turn might represent a stimulus to modulate Fe transport mechanisms. K562 cells induced to differentiate by sodium butyrate showed a quite different behavior after being exposed to Al. Even though adequate Fe uptake was achieved after Al withdrawal, the Fe incorporation to hem continued being inhibited. Therefore, Al might cause reversible or permanent effects on Fe metabolism depending on the cell’s physiological condition [5].

Taking into consideration our results as a whole, it was interesting to know whether the disruption of the different cellular metabolic pathways attributed to Al could be explained by its interference with Fe homeostasis. Therefore, the purpose of this work was to elucidate the contribution of Fe incorporation mechanisms to the cell adaptation in response to Al exposure under different conditions of erythroid maturation.

2. Materials and methods

2.1. Cell cultures

Human erythroleukemic K562 cells (American Type Culture Collection, Manassas, VA) were grown in HEPES-buffered RPMI 1640 medium, pH 7.0±0.3 (Sigma-Aldrich, St Louis, MO), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Bioser, Argentina) and 100 U/ml penicillin–100 μg/ml streptomycin (PAA Laboratories GmbH, Austria).

2.2. Induction of erythroid differentiation

In order to have models of K562 cells under different conditions of erythroid maturation, cell differentiation was induced by freshly prepared solutions of either sodium butyrate or hemin (Sigma-Aldrich), at final concentrations of 1.5 mM and 25 μM, respectively [5].

Cell viability, which was evaluated by the Trypan Blue (ICN Biomedicals) exclusion test, varied between 81% and 90% in the different assays, proving to be unaffected by either the erythroid differentiation induction or the Al treatment. In contrast, cell growth was 15% and 40% lower under the effect of hemin and butyrate than in non-induced cultures, respectively.

2.3. Al solutions and measurement of cellular Al content

On the day of the assay, Al citrate was freshly prepared in 0.1 M Tris–HCl buffer (pH 7.3) by mixing Al chloride and sodium citrate solutions (1:1.5 molar ratio). Al–Tf was prepared by adding Al citrate to human apoTf (Sigma-Aldrich) to yield a molar ratio Al:Tf of 4.5:1 as previously described [17].

Al content was determined in the cell lysates by atomic absorption spectrometry as previously reported [5].

2.4. Fe uptake assays

On completing culture periods, cells were washed, suspended in serum-free medium (RPMI-BSA) in which FBS was replaced by 1% bovine serum albumin (Sigma-Aldrich) and later subjected to either of the following protocols:

a) Tf-independent Fe uptake: Cells (5×10⁶ cells/ml) were incubated during 2 h, at 37 °C, in RPMI-BSA medium containing 0.5 μM ⁵⁹Fe citrate (Perkin Elmer Life Sciences Inc.).

b) Tf-mediated Fe uptake: a 4-h incubation of 2×10⁶ cells/ml was performed at 37 °C in RPMI-BSA medium with the addition of 1.0 μM ⁵⁹Fe–Tf. This radiolabeled compound was prepared from ⁵⁹Fe citrate and apotf, as previously reported [5]. During the ⁵⁹Fe pulse, Al–Tf was either absent or present at 1.0 μM final concentration.

In order to remove non-bound radioiron, cells were carefully washed (100 mM Tris buffer, pH 7.4, 25 mM NaHCO₃, 40 mM NaCl). The efficiency of this step was controlled by measuring the ⁵⁹Fe activity in supernatants.
Radioisotope incorporation was determined in cell pellets harvested by centrifugation (600×g, 4 °C, 10 min). The characteristic gamma radiation of 59Fe radionuclide (t1/2 44.6 days, β-, γ 1099, 1292 keV) was detected in a 3×3’ NaI(Tl) scintillation detector, coupled to a multichannel analyzer (Canberra series 35 Plus), calibrated by a 60Co standard source (CNEA, Argentina) [17].

2.5. Analysis of TfR expression by flow cytometry

The expression of TfR (CD71 antigen) was evaluated by an indirect immunofluorescence staining procedure. To examine surface TfR, cells were sequentially incubated with monoclonal anti-human CD71 (0.3 μg/10⁶ cells) (PharMingen, BD Biosciences) and goat FITC-anti-mouse IgG (5 μg/10⁶ cells) (Dako, CA). Incubations with primary and secondary antibodies in PBS containing 1% BSA were carried out for 30 min each, washing twice between steps. The cells were maintained on ice through the whole procedure. Analysis of membrane antigenic site density was performed by flow cytometry (Ortho Cytoron Absolute, Ortho Diagnostic System, Johnson and Johnson). Isotype-matched IgG2a antibody (Serotec) was employed as a non-specific binding control.

To determine total TfRs (membrane+cytoplasm), the cells were fixed with 1% paraformaldehyde in PBS for 20 min at room temperature. Then, the cells were incubated for 30 min with the primary antibody in PBS containing 10% BSA and 0.5% saponin to increase membrane permeability, and for additional 30 min with the secondary FITC-antibody, washing twice between steps. The cells were suspended in PBS-1% paraformaldehyde and stored at 4 °C in the dark for up to 24 h. Finally, they were washed and suspended in PBS-1% BSA to be analyzed by flow cytometry.

2.6. Analysis of TfR and TfR2 mRNA levels by reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted by means of Trizol Reagent (Gibco BRL). RNA integrity was verified by electrophoresis on 1% agarose gel and the concentration estimated by measuring the optical density at 260 nm [18]. Starting from a sample of total RNA (2.5 μg), cDNA was synthesized by reverse transcription using the Ready To Go T-Primed First-Strand Kit (Amersham Biosciences). An aliquot of cDNA was amplified by 28 PCR amplification cycles (94 °C for 30 s, 60 °C for 40 s, and 72 °C for 1 min) for TfR, and 33 PCR amplification cycles (94 °C for 30 s, 64 °C for 40 s, and 72 °C for 1 min) for TfR2. Specific primers (Invitrogen Life Technologies) were employed for TfR [19], TfR2 [8] and the internal standard glyceraldehyde 3-phosphate dehydrogenase, GAPDH [20]. The PCR products were examined by electrophoresis on 1.5% agarose gel containing ethidium bromide (19 V/cm, 25 min), using 90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.0 as running buffer. Gels were photographed and analyzed through the ArrayGauge (1.2 version) and ImageGauge (3.12 version) software.

3. Results

3.1. Transferrin receptor expression in cells exposed to Al

In order to investigate whether an intracellular Fe reduction originated by Al–Tf and Fe–Tf competition at the cell surface might have induced upregulation of TfR expression, TfR mRNA levels were evaluated by RT-PCR after Al exposure. Cell cultures were developed in an Al–Tf rich medium during 7 days since we had previously observed Fe uptake upregulation due to Al exposure for such a period [5]. However, no changes were detected in TfR mRNA levels of cells cultured under these conditions, regardless of the type of induction used (Fig. 1).

Since TfR2 expression has been recently reported in the K562 erythroleukemic cell line [21], TfR2 mRNA levels were also evaluated to analyze whether the increase in Fe uptake induced by Al pretreatment was related to the pathway mediated by this receptor. Fig. 1 shows that TfR2 mRNA levels remained unchanged in cells previously exposed to Al.

To assess if the expression of transferrin receptors (TfR and TfR2) of K562 cells was physiologically regulated by the intracellular Fe pool, cells were cultured in the presence of the Fe chelator desferrioxamine (DFO). As it was

![Fig. 1. RT-PCR analysis of TfR and TfR2 mRNA levels after Al exposure. K562 cells under different conditions of erythroid differentiation (NI: non-induced, H: hemin-induced, B: butyrate-induced) were cultured in the presence (+) or absence (−) of Al–Tf during 7 days. DFO-pretreated cells (DFO) were included in the assay. RT-PCR analysis was performed using specific primers for TfR, TfR2 and GAPDH. The PCR products were analyzed by electrophoresis and photographed. Results shown are representative of 5 separate experiments.](image-url)
expected, the upregulation of TfR mRNA under Fe depletion condition confirmed that the physiological regulatory mechanism was intact. In turn, TfR2 mRNA levels showed no response to DFO treatment (Fig. 1).

To further investigate whether Al effect was exerted at the post-translational level, TfR expression was analyzed by flow cytometry. Data described in Fig. 2 show no changes in TfR (CD71) surface antigenic sites due to Al exposure, despite the finding of significant differences related to the differentiation induction. Besides, total TfRs (membrane plus cytoplasm) were evaluated to investigate whether Al could induce rearrangements in TfR cellular distribution. As expected total TfR quantity was higher than membrane TfR number. However, no changes were observed due to Al cell exposure (data not shown).

3.2. Effect of Al exposure on NTBI uptake

The preceding experiments demonstrated that, under the conditions assayed, the Al effect on Fe uptake cannot be ascribed to a modified expression of Tf receptors. Therefore, the study was focused on the NTBI transport system. Cells induced to differentiation by hemin or sodium butyrate, as well as non-induced cells, were cultured in RPMI-FBS containing Al–Tf. Incubations were carried out for different periods (3 or 7 days), in which different behavior

### Table 1

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<th>Rate of 59Fe uptake (ng/10⁷ cells/h)</th>
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<td></td>
<td>Basal</td>
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<tr>
<td><strong>NI</strong></td>
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<tr>
<td>Tf free</td>
<td>0.76±0.16</td>
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<tr>
<td>Tf present</td>
<td>1.31±0.17</td>
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<td><strong>H</strong></td>
<td></td>
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<tr>
<td>Tf free</td>
<td>0.59±0.09</td>
</tr>
<tr>
<td>Tf present</td>
<td>1.00±0.19</td>
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Data of 59Fe activity measured in cell pellets, corresponding to experiences detailed in Fig. 3 (Panels A and B), were expressed as the mass of 59Fe incorporated to 10⁷ cells per hour. Cells induced to differentiate by hemin (H) and non-induced cells (NI) were exposed to Al for 3 and 7 days, respectively. Then, Fe incorporation was determined by incubation with either 56Fe citrate (Tf free) or 56Fe–Tf (Tf present). The differences of Fe uptake rate values between Al-pretreated cells and Al-unexposed cells (Basal) are displayed (Increment after Al exposure).

### Table 2

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<th>Rate of NTBI uptake (ng/10⁷ cells/h)</th>
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<tr>
<td><strong>Basal</strong></td>
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<tr>
<td><strong>Al excess</strong></td>
<td>0.41±0.03</td>
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<tr>
<td><strong>Fe excess</strong></td>
<td>1.14±0.26*</td>
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K562 cells suspended in RPMI-BSA medium were incubated with 0.5 μM 56Fe citrate for 2 h at 37°C (Basal). During the radioisotope pulse either 20 μM Al citrate (Al excess) or 20 μM FeCl₃ (Fe excess) was present. 56Fe activity associated to cell pellets was measured and the rate of Fe uptake was expressed as the mass of 56Fe incorporated to 10⁷ cells per hour (mean±S.E.).

* Statistically significant differences compared with Basal and Al excess (P<0.05, n=5, Kruskal–Wallis test).
had been observed between non-induced and induced cells [5]. Then, $^{59}\text{Fe}$ uptake was measured in a fresh medium free of Al and Tf (RPMI-BSA). Hemin-induced and non-stimulated cells showed significantly higher $^{59}\text{Fe}$ incorporation than did Al-unexposed cells after 3 and 7 days of Al exposure, respectively. On the contrary, Al failed to increase NTBI uptake in butyrate-induced cells (Fig. 3A).

These data were compared with the results obtained in parallel assays in which the $^{59}\text{Fe}$ uptake was measured in the presence of Tf (Fig. 3B). With this purpose, $^{59}\text{Fe}$ activity corresponding to experiences detailed in Fig. 3 (Panels A and B) was expressed in terms of mass of $^{59}\text{Fe}$ incorporated to $10^7$ cells per hour (Table 1). Through this comparison, the increase in $^{59}\text{Fe}$ uptake rates induced by Al was evident both in the presence or absence of Tf. As can be calculated from these data (increment of $^{59}\text{Fe}$ uptake rate in Tf free medium relative to the increment in a Tf-containing medium), 64–70% of the increase in Fe uptake rate observed in the presence of Tf after Al exposure can be ascribed to positive modulation of the alternative Fe transport pathway.

The next step was focused to elucidate whether Al might be able to compete with Fe for the sites involved in NTBI transport pathway. However, such effect was not observed in assays carried out with the simultaneous presence of both cations, even though Al concentration was 40 times higher than that of the essential metal. Conversely, the excess of unlabeled ferric ions during the $^{59}\text{Fe}$ pulse significantly increased the radioisotope uptake (Table 2). On the other hand, the Fe depletion caused by DFO did not induce significant modulation of the NTBI transport system. The $^{59}\text{Fe}$ incorporated into DFO-treated cells was 1780/1730–2250 cpm vs. 2110/1610–2830 cpm into control cells (median/range).

### 3.3. Al incorporation to cells

To analyze if Al is able to enter the cellular environment by mechanisms not related to Tf, cells were cultured in a Tf-free medium (RPMI-BSA) with the addition of Al citrate. The amounts of Al detected in the lysates were significantly higher than in control cells grown without Al (Fig. 4, Al vs. C). Although Al entered the cell by a Tf independent pathway, the contribution of this mechanism to total Al incorporation was of lesser magnitude than that of the Tf-mediated pathway (Fig. 4, Al vs. Al+Tf).

### 4. Discussion

This study provides information hitherto unknown on the action of the non-essential metal Al upon mechanisms of Fe homeostasis. Indeed, Al exposure proved to induce the modulation of the non-Tf bound Fe uptake rather than the classical Tf-dependent Fe transport.

Human K562 cells have been chosen for this investigation because they can mediate Tf-dependent [22] as well as Tf-independent [11,12] Fe uptake. In order to employ cellular models with different Fe requirements, cells were either non-induced or induced to differentiation by hemin or sodium butyrate, under which stimulus K562 cells showed different kinetic of erythroid maturation [5].

We have previously demonstrated the interference of Tf-bound Al with Fe uptake mediated by Tf receptors, which could be explained by the similar affinity for Fe–Tf and Al–Tf demonstrated for TfR in K562 cells [17]. This prevention of normal Fe uptake caused by Al proved to be reversible. Moreover, when Al was removed from the cell environment an Fe uptake stimulation was revealed [5]. However, the lack of changes in TfR mRNA levels despite previous Al exposure (Fig. 1) simultaneously with no variations in CD-71 antigenic sites (Fig. 2) strongly suggests that the intracellular Fe deprivation caused by the competitive interaction between Al–Tf and Fe–Tf was not enough to trigger the upregulation of TfR expression through the IRE–IRP interaction.

We then considered possible Al effects upon the expression of the TfR2 receptor, also expressed in K562 cells [8,21]. However, no changes were detected in TfR2 mRNA levels (Fig. 1).

The most outstanding fact herein demonstrated is that NTBI transport is positively modulated by Al exposure (Fig. 3A). The similar response exhibited by Al-treated cells irrespective of Tf availability (Fig. 3A and B) strongly suggests that, in the presence of Tf, Fe should have been simultaneously incorporated both through Tf-dependent and Tf-independent pathways. Whereas $^{59}\text{Fe}$–Tf is transported into cells via the TfR mediated route, low molecular weight $^{59}\text{Fe}$ complexes (e.g. ferric citrate) would be incorporated through alternative pathways. Therefore, NTBI uptake would be responsible for the increase in Fe acquisition. In terms of Fe uptake rate, 64–70% of the increment observed in the presence of Tf could be ascribed to the increase in Fe acquisition through Tf-independent routes (Table 1). Based on previous reports showing structural alterations of certain

![Fig. 4. Al incorporation to cells. K562 cells were cultured in a Tf-free medium (RPMI-BSA) in the presence of Al citrate (Al). At the end of a 4-day incubation period, the Al content was determined in cell lysates by atomic absorption spectrometry. Two controls were simultaneously assayed: cells grown without Al (C) and cells cultured in an Al–Tf containing medium (Al–Tf). Results are expressed as mean±S.E. *Statistically significant differences from both Al–Tf and C (P<0.05, n=4, Kruskal–Wallis test).](image-url)
proteins caused by Al [23], changes in TfR biological activity or in its related protein HFE [24] cannot be ruled out.

The positive regulation of NTBI incorporation due to Al exposure herein demonstrated agrees with a similar response found in primary cultures of fetal rat cerebral cortex cells pretreated with Al nitritotriacetate [25] and that reported in macrophages and other myeloid cell cultures developed in the presence of the metal [26]. Undoubtedly, the Al-induced modulation was not due to intracellular Fe decrease produced by the Al–Tf interference with Fe–Tf binding, since NTBI uptake proved not to be significantly regulated by Fe depletion induced by DFO. On the other hand, cell response to Al exposure is dependent on its metabolic condition (Fig. 3A and B). Even though similar disturbance of Fe uptake was observed when the three cell models were in contact with Al [5], butyrate-induced cells were unable to regulate mechanisms aimed to increase Fe incorporation. It has been reported that this short-chain fatty acid induces cell arrest [27] and our own results showed 40% cell growth depression under its effect. Therefore, it can be proposed that an active cell proliferative condition is required for the NTBI transport to be upregulated.

It is known that Al is incorporated to cells via the TfR route [5]. The detection of intracellular Al after Tf-free medium incubation supports the existence of a Tf-independent transport route, even though it would be of lesser magnitude than the Tf-mediated one (Fig. 4). Whether Fe and Al use different proteins to enter cells in the absence of Tf or Al is introduced through the disarrangement of cellular membrane remains to be elucidated. It is reasonable to assume that Al is unable to be transported by the two pathways of NTBI uptake described for K562 cells [12,28]. The divalent metal transporter DMT1 cannot be shared by ferrous and Al3+ ions since the latter cannot be reduced. Furthermore, it seemed unlikely that Al incorporation would be mediated by β3-integrin, the carrier of Fe3+. Since 59Fe3+ uptake was not inhibited by the simultaneous presence of the non-essential metal (Table 2).

The mechanisms that can be proposed to explain NTBI uptake modulation in response to Al exposure may involve the appearance of previously cryptic carriers [10] or the increase in the synthesis of transport proteins since Al proved to be able to translocate into the nucleus and interact with genetic material [29,30]. An alternative explanation is related to Al interference with the signaling pathway of calcium homeostasis [31], thus affecting NTBI transport since this is a calcium-dependent mechanism [9,10,25]. On the other hand, the disturbance of the dynamic regulation of essential events occurring at the cell membrane level, such as metal transport, could be explained by significant changes in cell morphology and structural protein organization caused by the presence of Al in the cellular environment [32–34].

In conclusion, the present contribution of hitherto unknown information allow us to describe the behavior of Al-exposed cells regarding the mechanisms of Fe uptake. The continuous presence of Al bound to Tf prevents normal Fe incorporation into K562 cells by interfering with Tf-mediated transport. Simultaneously, the upregulation of Tf-independent mechanisms of Fe uptake supports cell adaptation in response to Al exposure whereas an increase in TfR affinity cannot be discarded. It seems that through the regulation of NTBI transport rather than through Tf-mediated system, cells would be able to incorporate the essential Fe required for metabolism and growth without allowing the simultaneous access of a potentially toxic metal. Although no physiological role has been attributed to Al, or perhaps because of that, its accumulation in tissues is considered potentially harmful. In this concern, this study provides the basis for an understanding of the possible toxic role of Al in biological systems by disturbing Fe homeostasis, the unbalance of which is highly implicated in human diseases.

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