EFFECTS OF ALUMINUM ON DELTA AMINOLEVULINIC ACID DEHYDRATASE *IN VIVO* AND *IN VITRO*

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Abstract. The present study examined the effect of aluminum on δ-aminolevulinic acid dehydratase (δ-ALAD) and hematocrit, and assessed the effects of zinc and selenium on activity of the enzyme affected by aluminum *in vivo* and *in vitro*. Experiments were done on white laboratory mice of (20-25) g body mass. To assess the effect of aluminum on δ-ALAD *in vivo*, mice injected i.p. with 0.5 LD_{50} aluminum chloride ($AlCl_3$) (25 mg Al^{3+}/kg body mass). To estimate the effect of zinc and selenium on activity of the enzyme affected by aluminum, twenty minutes before intoxication with 0.5 LD_{50} aluminum chloride mice were injected i.p. with 0.5 LD_{50} of sodium selenite (Na_2SeO_3) or with 1.56 mg/kg of zinc sulphate ($ZnSO_4$). Control animals received an injection of the same volume of saline.

Injection of mice with a single dose of aliuminium significantly increased concentration of metal in blood. However, δ-ALAD activity changed only slightly. Furthermore, addition of zinc before aluminum injection was related to significant increase of aluminum content and a little enhancement of δ-ALAD activity in blood. In blood of mice where selenium additives were used no changes in aluminum concentration or δ-ALAD activity was registered, and level of hematocrit decreased.

The *in vitro* effects of aluminum on δ-ALAD activity in blood of experimental mice were investigated. Concentration causing half-maximal inhibition (IC_{50}) of enzyme activity was used to assess the effects of Al^{3+} on δ-ALAD activity in blood.

The findings suggested that low concentrations of aluminum ions slightly decrease δ-ALAD activity *in vitro*, while high concentrations of aluminum ions inhibited the enzyme. Aluminum ions are medium whereas zinc ions are weak and cadmium ions are strong catalytic poison (IC_{50} Cd^{2+} < IC_{50} Al^{3+} < IC_{50} Zn^{2+}). Zinc ions also showed a weak protective effect on inhibition of δ-ALAD caused by aluminum ions, but do not remove it ((IC_{50} Al^{3+} < IC_{50} Al^{3+} + Zn^{2+}).

Key words: aluminium, zinc, selenium, δ-aminolevulinic acid dehydratase, *in vivo*, *in vitro*.

ALIUMINIO ĮTAKA DELTA AMINOLEVULINO RŪGŠTIES DEHIDRATAZEI *IN VIVO* IR *IN VITRO*

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Santrauka. Tirta aliuminio įtaka delta-aminolevulino rūgšties dehidratazei (δ-ALRD) ir hematokritui, įvertintas cinko ir seleno poveikis fermento, paveikto aliuminiu, aktyvumui *in vivo* ir *in vitro*. Eksperimentai atlikti su baltosiomis laboratorinėmis (20–25 g svorio) pelėmis. Tiriant aliuminio įtaką δ-ALRD aktyvumui *in vivo*, pelėms buvo įšvirkšta 0,5 LD_{50} aliuminio chlorido (25 mg Al^{3+}/kg kūno masės). Vertinant cinko ir seleno įtaką fermento, kuris bus apnuodytas aliuminiu, aktyvumui, 20 min. prieš intoksikaciją $AlCl_3$ pelėms buvo įšvirkšta 0,5 LD_{50} natrio selenito (Na_2SeO_3) arba 1,56 mg/kg cinko sulfato ($ZnSO_4$). Kontroliniams gyvūnams buvo įšvirkšta tiek pat fiziologinio tirpalo.

Mūsų duomenimis, didžiausia aliuminio koncentracija rasta tų pelių kraujyje, kurioms buvo įšvirkštas tik aliuminio chlorido tirpalas, tačiau δ-ALRD aktyvumas kito nežymiai. Cinko druskos įšvirkštimas prieš apnuodijant aliuminiu susijęs su ženkliu aliuminio kiekio ir mažu δ-ALRD aktyvumo padidėjimu pelių kraujyje. Pelių, kurioms buvo įšvirkšta natrio selenito, kraujyje aliuminio koncentracija ir δ-ALRD aktyvumas nepakito, tačiau sumažėjo hematokritas.

Buvo tirta aliuminio jonų įtaka δ-ALRD aktyvumui ekspermentinių pelių kraujyje *in vitro*. Apskaičiuota Al^{3+} koncentracija, sukelianti fermento inhibicijos pusės šuolio aktyvumą (IC_{50}). Pastebėta, kad maža aliuminio jonų koncentracija šiek tiek mažina δ-ALRD aktyvumą *in vitro*, o didelė – sukelia inhibiciją. Nustatyta, kad aliuminio jonai yra vidutinio stiprumo katalitinis nuodas palyginti su Zn^{2+} ir Cd^{2+} (IC_{50} Cd^{2+} < IC_{50} Al^{3+} < IC_{50} Zn^{2+}).

Įrodyta, kad cinko jonai pasižymi apsauginėmis savybėmis. Al^{3+} koncentracija, sukelianti δ-ALRD inhibicijos pusę šuolio, yra mažesnė už šio jono koncentraciją, kuri sukelia tokį patį efektą, kai į kraują pridėta cinko jonų (IC_{50} Al^{3+} < IC_{50} Al^{3+} + Zn^{2+}).

Raktažodžiai: aliuminis, cinkas, selenas, delta-aminolevulino rūgšties dehidratazė, *in vivo*, *in vitro*.

Introduction. Aluminum (Al) is the third most abundant element and the most common metal in the earth′s crust; however it has no known biological function. Aluminum enters the human body via air, water, food and drugs (Kim et al., 2001; Yokel, 2000). Although only a small portion of the metal is absorbed by the gastrointestinal tract, oral intake represents the route with greatest toxicological implications (Testolin et al., 1996).

Aluminum salts administered by different routes can produce toxic effects in animal models (Fiejka et al., 1996; Kaiser et al., 1985; Liu et al., 1996) some of which involve alterations in enzymatic activities (Schetinger et al., 1995; Zatta et al., 1995). It is presently accepted that aluminum intoxication may induce anaemia and encephalopathy in humans and animals (Wills et al., 1983). Some studies found that Al may cause microcytic anaemia by a direct effect on heme biosynthesis, emphasizing specifically alterations in the activity of δ-aminolevulinic acid dehydratase (δ-ALAD, EC 4.2.1.24) (Kaiser et al., 1985; Touam et al., 1983), i.e. enzyme that is inhibited by heavy metals and sulfhydryl reagents (Barbosa et al., 1998; Emanuelli et al 1998; Emanuelli et al., 1996; Oskarsson, 1989; Rocha et al., 1995) and seems to be the principal lead-binding protein in human erythrocytes (Bergdahl et al., 1997). The toxic effects of aluminum on δ-ALAD may involve protein synthesis, enzyme inhibition or enzyme activation (Zaman et al., 1993).

Zinc (Zn) is essential element for the function of more than 300 enzymes, including alkaline phosphatase, Cu,Zn-superoxide dismutase, δ-aminolevulinic acid dehydratase (Sandstead, 1994). Zn has three functions in these metalloenzymes: participation in catalytic functions, maintenance of structural stability, and regulatory functions (Walsh et al., 1994).

Selenium (Se) is a trace bio-element essential for the normal function of the body, being present at around 10 mg Se/60 kg body weight and toxic at higher concentration. Se is similar to sulfur in chemical property, and has to be discriminated biologically from abundant sulfur during its metabolism in the body. Animals can metabolize both inorganic and organic forms and convert non methylated Se to mono- or di- or tri- methylated forms, of which, mono-methylated forms are most toxic. Glutathione reductase converts selenoglutathione to H_2S in liver and erythrocytes and is ultimately excreted. Se affects the toxicities of xenobiotic agents, provides antagonistic effect to sulphur and co-administration with Zn increase Se retention in certain organs (Bedwal et al., 1993). Sufficient amount of zinc and selenium are related to normal function of many enzymes in an organism. Both Zn and Se are important to activity of δ-ALAD, enzyme of heme biosynthesis, and restore it when it's changed due to metal intoxication Bedwal et al., 1993; Walsh et al., 1994).

Our previous studies showed the *in vitro* and *in vivo* effects of lead, acetate, selenide and sulfide ions on δ-ALAD activity in blood (Ryselis et al., 2004a, 2006), the *in vitro* effects of lead, selenium, cadmium, zinc, chloride and sulphate ions on the enzyme activity (Ryselis et al., 2004b, 2007).

Inasmuch as the effect of aluminum on δ-ALAD and heme is not clear enough, the present study examined the effect of aluminum on δ-ALAD and hematocrit of mice *in vivo* and *in vitro*. We also tried to assess the effects of zinc and selenium on activity of the enzyme affected by aluminum.

Material and methods. Experiments were done on white laboratory mice of 20-25 g body mass. To assess the effect of aluminum on δ-ALAD *in vivo*, mice injected i.p. with a single 0.5 LD_{50} aluminum chloride ($AlCl_3$) (25 mg Al^{3+}/kg body mass) (1st group). To estimate the effect of zinc and selenium on activity of the enzyme effected by aluminum, twenty minutes before intoxication with a single 0.5 LD_{50} aluminum chloride mice were injected i.p. with a single 0.5 LD_{50} of sodium selenite (Na_2SeO_3) (2nd group) or 1.56 mg/kg of zinc sulphate ($ZnSO_4$) (3rd group). Control animals (4th group) received an injection of the same volume of saline. After 16 h mice were anaesthetized and terminated according to the rules defined by European convention for the protection of vertebrate animals used for experimental and other scientific purposes (License No 0028).

In vitro the samples of mice′s blood 0.8 cm^3 were mixed and heparin (0.4 % v/v) used as anticoagulant. Blood divided equally in 0.2 cm^3 to minisorption plastic tubes. The stock solution of $Al_2(SO_4)_3$ for Al^{3+} and $ZnSO_4$ for Zn^{2+} were prepared by weight method using deionized pure water, $Al_2(SO_4)_3 \cdot 18H_2O$ and $ZnSO_4 \cdot 7H_2O$. The solutions diluted and 0.04 cm^3 of different concentration solution added to each 0.2 cm^3 of the blood sample. At each stage of experiment multi-dot consecutive curves of δ-ALAD activity dependent on gradually increased concentrations of different ions (Al^{3+}, Zn^{2+}, Al^{3+} + Zn^{2+}) shed. In all cases concentration of any ions enhanced until maximal activity of δ-ALAD was followed by inhibition of the enzyme activity and graphically calculated IC_{50} for Al^{3+}, Zn^{2+} and Al^{3+} with addition of Zn^{2+} 20 μmol/l.

The activity of δ-ALAD was assayed by method of Berlin and Schaller modified by Semionova (Berlin et al., 1974; Semionova, 1985). After incubation at 37°C for 10 min, phosphate buffer pH 6.4 and 5-aminolevulinic acid were added to homogenized blood sample. Incubations were carried out for 1 h at 37°C. Reaction was terminated by addition of $HgCl_2$. The reaction product porphobilinogen was determined using modified Ehrlich′s reagent at 555 nm with a molar absorption coefficient of 6.1×10^4 per M for the Ehrlich-porphobilinogen salt.

δ–aminolevulinic acid were purchased from *Sigma (SIGMA-ALDRICH Chemie GmbH, Germany)*, mercuric chloride and 4-dimethylaminobenzaldehyde were purchased from *Fluka (SIGMA-ALDRICH Chemie GmbH, Germany)*. All other chemicals were purchased from Merck (Darmstadt, Germany).

The concentration of Al, Zn and Se in whole blood was determined by electro–thermal graphite furnace (ET HGA–600, AS–60) atomic absorption spectrophotometer Perkin–Elmer Zeeman 3030 (Schlemmer, 1989). Researches were accomplished in Institute for Biomedical Research of Kaunas University of Medicine.

The entire values of investigated indices are presented as median, a geometric mean, lower and upper quartiles. The Mann – Whitney U test was used to test differences between groups. Pearson correlation coefficients (r) of Al and activity of δ-ALAD as well as hematocrit were calculated in each group of mice. The level of significance was set at $p<0.05$.

Results. Our data showed the highest Al concentration in blood of mice that were injected aluminum or zinc and aluminum solutions (1st and 2nd groups) compare to that determined in mice got selenium and aluminum (3rd group) or control mice (4th group) (table 1). There was significant difference in zinc concentration between mice injected selenium and aluminum (3rd group) and control mice (4th group). We found rather high concentration of selenium i.e. 103.16 μg/dl in blood of mice that got selenium and aluminum (3rd group). Content of selenium in blood of mice from other groups were under detection limit.

Table 1. **Concentration of aluminum, zinc and selenium** (μg/dl) **in blood of mice *in vivo***

Group	Al			Zn			Se		
	Median	Geometric mean	Lower, upper quartiles	Median	Geometric mean	Lower, upper quartiles	Median	Geometric mean	Lower, upper quartiles
1st gr (Al) n=11	10.62	10.45**	7.63-14.32	487.63	501.48	431.17-600.81	-	<DL	-
2nd gr (Zn + Al) n=9	11.48	12.16*	11.43-12.90	480.78	477.72	470.01-509.05	-	<DL	-
3rd gr (Se + Al) n=11	5.42	5.43	4.09-6.48	636.04	650.74º	454.34-953.13	93.79	103.16	83.31-121.33
4th gr (control) n=7	2.16	2.70	1.43-7.31	429.38	452.12	409.47-509.26	-	<DL	-
5 gr (Zn) n=5	1.86**	2.42	1.33-5.29	518.52	526.57	507.43-535.92	-	<DL-	-
6 gr (Se) n=12	1.35	1.32	0.79-2.38	505.73	534.87	401.79-726.70	75.99	78.45	66.83-98.07

n – number of white mice in groups
DL= detection limit for Se in blood was 13.96 μg/dl
*$p<0.05$ between 2nd group (Zn + Al) and 3rd (Se + Al),
**$p<0.01$ between 1st group (Al), 3rd (Se + Al) and 4th (control) groups, 5 (Zn) groups, 6 (Se) groups
º$p<0.05$ between 3rd (Se + Al) and 4th (control) groups

Although the highest aluminum concentration was found in 1st and 2nd groups of mice, the activity of δ-ALAD did not differ significantly between the groups compared (table 2). Activity of δ-ALAD in mice that were injected selenium and aluminum and had the highest concentration of zinc (3rd group) did not differ from that in control mice as well as in other groups compared. However, hematocrit in blood of mice got selenium and aluminum (3rd group) was significantly lower than that in other groups.

Table 2. **Activity of δ-ALAD (nmol/l·s) and hematocrit** (%)

Group	δ-ALAD			Hematocrit		
	Median	Geometric mean	Lower, upper quartiles	Median	Geometric mean	Lower, upper quartiles
1st gr. (Al) n=11	237.53	237.74	176.57-358.91	55.00	51.92	44.10-58.00
2nd gr. (Zn + Al) n=9	208.11	216.55	153.75-320.96	56.00	56.16	54.10-58.00
3rd gr. (Se + Al) n=11	186.44	191.76	146.74-235.83	43.30	42.10*	38.80-44.40
4th gr. (control) n=7	204.26	197.80	176.89-231.66	55.50	53.62	47.30-57.00
5 gr. (Zn) n=5	167.69	170.15	144.31-204.68	53.00	59.24	52.70-64.00
6 gr. (Se) n=12	271.53	256.19*	204.40-348.53	42.60	42.34*	41.46-45.75

n – number of white mice in groups
*$p<0.05$ between 3rd (Se + Al) ,6 gr. (Se) and 1,2,4,5 groups

The coefficients of correlation of Al and δ-ALAD varied from -0.61 in control group to 0.48 in 3rd group (Se+Al), but relationships were not statistically significant; the coefficients of correlation of Al and hematocrit varied from 0.2 in 2nd group (Zn + Al) to -0.65 in 3rd group (Se + Al); the last one was statistically significant (p=0.032).

In vitro graphically estimated IC_{50} for δ-ALAD activity was: 8200 μmol/l for Al^{3+}, 52800 μmol/l for Zn^{2+} and 16900 μmol/l for Al^{3+} with 20 μmol/l Zn^{2+} addition.

Table 3. **Concentration causing half-maximal inhibition** (IC_{50}) **of δ-ALAD activity in blood of mice *in vitro*** (estimated graphically)

Ions	IC_{50} (μmol/l)
Al^{3+}	8200
Al^{3+} + add 20 μmol/l Zn^{2+}	16800
Zn^{2+}	52800
Cd^{2+} (Ryselis et al., 2007)	900

Discussion. Several lines of evidence have confirmed the fact that aluminum can induce anaemia (Mahieu et al., 2000). Parenteral exposure to aluminum has been recognized as a risk factor of microcytic anemia (Jaffe et al., 1995; Nasiadek et al., 1995). Other authors found that exposure to aluminum resulted in slightly hypochromic macrocytic anaemia with reticulocytosis (Drüeke et al., 1986). The mechanism by which an excess aluminum induces anemia remains to be clarified. It may be caused by aluminum overload resulting a reversible block in heme synthesis due either to a defect in porphyrin synthesis or impaired iron utilization (Nasiadek et al., 1995). Chronic exposure to relatively high doses of aluminum can change iron metabolism in different animal species (Nasiadek et al., 2001). δ-ALAD catalyzes the second step in the heme biosynthesis pathway, cyclizing two molecules of δ-aminolevulinic acid to form the heterocyclic monopyrrole porfobilinogen (Jaffe et al., 1995; Sassa, 1982).

Our data showed a slight, but not statistically significant increase in activity of δ-ALAD after injection of a single 0.5 LD_{50} aluminum chloride. Other authors have yielded conflicting results on aluminum and δ-ALAD activity. Vieira (Vieira et al., 2000) reported increased δ-ALAD activity *in vivo* after treatment with citrate and aluminum plus citrate and increase in enzyme activity was parallel to the increase in aluminum content in blood and plasma. *In vivo* studies with rodents have found that aluminum inhibits δ-ALAD activity and inhibition of this enzyme is associated with signs of anaemia (Zaman et al., 1993). It has been assumed that δ-ALAD inhibition was the main factor responsible for heme biosynthesis inhibition. Other studies have indicated that aluminum does not change the enzyme activity (Chmielnicka et al., 1996; Chmielnicka et al., 1994). However *in vitro* studies found that at relatively low concentrations aluminum activated and at high concentration it inhibited blood δ-ALAD (Vieira et al., 2000). Inactivation of δ-ALAD may lead to an accumulation of δ-aminolevulinic acid (substrate) that can cause an overproduction of reactive oxygen species which could explain the toxic effects of metals (Barbosa et al., 1998; Bechara, 1996). In view of the pro-oxidant effect of δ-aminolevulinic acid, a study of the inhibition of δ-ALAD by aluminum can contribute to a better understanding of the toxicology of this metal. These processes may contribute to oxidative stress in cells and may be related to degenerative cellular mechanisms.

We did not find significant changes in hematocrit after injection of a single 0.5 LD_{50} aluminum chloride. However hematocrit has been decreased in blood of mice where selenium was injected before aluminum chloride. We suppose that it could be due to intoxication with sodium selenite (0.5 LD_{50} Na_2SeO_3). Some authors reported that serum iron was lower in the aluminum group than in the control group. These results indicated that low doses of aluminum sulfate induced iron deficiency in rat with significant alterations in hematological parameters (hematocrit and hemoglobin). Since rats were exposed to aluminum sulfate by the intraperitoneal route, it was plausible to suppose that aluminum altered iron excretion. Accordingly, it has been reported that aluminum stimulates biliary iron elimination (Allain et al., 1988). Aluminum can interact with iron metabolism (Cannata et al., 1991; Nasiadek et al., 2001) and variations in iron status such as iron depletion may occur after aluminum intoxication (Rees et al., 1998; Skikne et al., 1990).

In vitro δ-ALAD activity was slightly decreased at Al^{3+} concentration to 402 μmol/l and do not caused inhibition of δ-ALAD, while high concentrations to 16000 μmol/l of these ions caused fast decrease in activity and inhibited the enzyme. Graphically measured Al^{3+} IC_{50} for δ-ALAD activity was 8200 μmol/l. Aluminum ions was medium catalytic poison in compare to Zn^{2+} (IC_{50} = 52800 μmol/l) and Cd^{2+} (IC_{50} = 900 μmol/l, Ryselis et al., 2007). Addition of zinc ions 20 μmol/l increased Al^{3+} IC_{50} from 8200 to 16200 μmol/l and shoved protective effect on inhibition of enzyme caused by Al^{3+}.

Conclusions

The data obtained show greater aluminum concentration in blood of mice injected with a single dose of the metal. However δ-ALAD activity has been changed slightly. Addition of zinc before aluminum injection is related to significant increase of aluminum content and a little enhancement of δ-ALAD activity in blood. In blood of mice where selenium additives are used no change of aluminum concentration or δ-ALAD activity is found, but hematocrit is decreased.

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