

ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Verapamil administration alleviates microcytosis and tissue accumulation after chronic aluminum exposure in rats

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SUMMARY

Introduction/Objective Research has demonstrated the toxicant potential of aluminum, but no therapeutic options have been suggested. The aim of the study was to investigate the extent of the aluminum-induced toxicity, evaluated by hematological/biochemical disarrangements, hepcidin concentration and tissue accumulation after chronic aluminum exposure and to determine possible protection with Ca²⁺-channel blockage, verapamil.

Methods Experimental animals (36 rats) were treated with different doses of AlCl₃ during eight weeks and after that their blood and tissues were analyzed.

Results The significant differences, regardless of the aluminum dose administered, were documented in all evaluated hematological ($p < 0.001$) and biochemical parameters ($p < 0.001$), as well as in aluminum tissue deposition in liver, kidneys and testicles ($p < 0.001$), respectively. After verapamil administration, a significant improvement in some hematological and biochemical parameters was demonstrated, $p < 0.001$, as well as the attenuation of aluminum deposits in liver and testes, $p < 0.001$. Evaluated parameters of inflammation and kidney deposition did not show significant change after verapamil application.

Conclusion The findings indicate that chronic AlCl₃ intoxication, regardless of the dose, results in the microcytic anemia associated with high hepcidin levels, numerous biochemical abnormalities and significant aluminum deposition in liver, kidney and testes and that these effects may be attenuated by verapamil administration. Overall, the results emphasize the significance of calcium homeostasis preservation in chronic aluminum exposure and propose possible therapeutic option.

Keywords: aluminum-induced toxicity; verapamil; chronic exposure; hepcidin; microcytosis

INTRODUCTION

Aluminum is one of the most widespread elements on earth; therefore, a progressive over-exposure of biota to biologically active aluminum has been reported, resulting in growing evidence of aluminum-related diseases [1]. The primary and physiological source of aluminum intake is food and drinking water, where an average ingestion stands for 5–40 mg of aluminum on a daily basis, while the accepted limit for aluminum intake is recognized as being 2 mg/kg/week [1].

In recent years, there has been abundant data on aluminum systemic toxicity, in order to clarify its role in the pathophysiology of many disorders: reproductive and breast diseases [2], bone impairment [3], lung disorders [4], impacts on the immune system [5], as well as neurological diseases [6], whereas inhibition of Ca²⁺ channels may represent potential therapeutic strategy for addressing aluminum-induced toxicity.

Therefore, we postulated the following aims of the study: to investigate the extent of the

obtained toxicity, evaluated by hematological and biochemical disarrangements and hepcidin plasma concentration and tissue accumulation after chronic aluminum exposure; and to determine a possible protection from aluminum-associated toxicity with Ca²⁺-channel blockage by verapamil.

METHODS

Animals

The experiment was performed on 36 male Sprague Dawley rats (Vivarium of the Research Center for Biomedicine of the Faculty of Medicine in Niš). The rats were two months old and weighed 130–160 g. They were given *ad libitum* access to food (Zemun Veterinary Institute standard rat chow pellets) and tap water. The ambient temperature was $20 \pm 2^\circ\text{C}$, the relative humidity was $55 \pm 10\%$, with a 12-hour light–dark cycle. The care and handling of the animals were in accordance with the guidelines and recommendations specified by the Federa-

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tion of European Laboratory Animal Science Associations (FELASA). Upon the request of the Ethical Commission for Experimental Animal Welfare of the Faculty of Medicine of Niš, the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia approved the experiment, decision number 323-07-01762/2019-05/10.

Experimental design

Rats were randomly divided into six groups ($n = 6$). The animals were treated daily during a period of eight weeks, as follows: 10 mg/kg of body weight (b.wt) AlCl_3 i.p. (group E1); 5 mg/kg (b.wt) verapamil by gavage, and after 60 minutes 10 mg/kg (b.wt) AlCl_3 i.p. (group E2); 20 mg/kg (b.wt) AlCl_3 i.p. (group E3); 5 mg/kg (b.wt) verapamil by gavage, and after 60 minutes 20 mg/kg (b.wt) AlCl_3 i.p. (group E4); 5 mg/kg (b.wt) verapamil by gavage (group V), and saline i.p. (control group C). The material used was as described: aluminum chloride, AlCl_3 (Honeywell Fluka, Fisher Scientific, Hampton, NH, USA) and verapamil hydrochloride (Verapamil, Alkaloid AD Skopje, Skopje, North Macedonia).

Blood analyses and tissue sampling

At the end of the study, the rats were euthanized with an overdose of Ketonal® (Richter Pharma AG, Wels, Austria). Hematological parameters: red blood cells (RBC), white blood cells, platelets, and relative white blood cell counts were determined by electrical impedance. The colorimetric method was used to determine hemoglobin (Hb) concentration. The hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and absolute white blood cell count were calculated. All hematological parameters were determined from whole blood, with EDTA K2 anticoagulant, and were determined on the hematology counter Celltac MEK 6510K (Nihon Kohden, Tokyo, Japan).

The biochemical analyzer AU 680 (Beckman Coulter, Inc., Brea, CA, USA) with original manufacturer reagents was used for biochemical parameters which were determined from serum. The concentration of ferritin, transferrin, and unsaturated iron binding capacity (UIBC) was determined by immunoturbidimetric method, and iron concentration by colorimetric method. Total iron binding capacity (TIBC) and transferrin saturation (TSAT) were then calculated. The activity of the following enzymes was determined by enzymatic colorimetric method: aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), amylase (AMY), lactate dehydrogenase (LDH), creatine kinase (CK), and γ -glutamyl transferase (γ GT).

The concentrations of hepcidin and C-reactive protein (CRP) were measured in plasma samples using commercially available ELISA kits, according to the manufacturer's instructions.

All animals were carefully examined macroscopically: body surfaces and orifices as well as all cranial, thoracic, and abdominal organs. The liver, kidney, and testes were

excised, frozen, and stored at -80°C prior to measuring the aluminum tissue concentration. The samples were prepared according to the method by Banni et al. [7], slightly modified. The samples were oven-dried (60°C) to a constant weight. The dried tissues (0.3 g from each sample) were then mixed with 3 mL of pure nitric acid at 90°C for 24–48 hours. The volume was then adjusted to 25 mL with 0.5% nitric acid.

Aluminum tissue concentration measurement

All analyses were carried out on an iCAP 6000 inductively coupled plasma optical emission spectrometer (Fisher Scientific, Hampton, NH, USA) which uses the echelle optical design and a charge injection device solid-state detector. The operating conditions for the inductively coupled plasma atomic emission spectrometers were the following: flush pump rate – 100 rpm, analysis pump rate – 50 rpm, RF power – 1150 W, nebulizer gas flow rate – 0.7 L min^{-1} , coolant gas flow rate – 12 L min^{-1} , auxiliary gas flow rate – 0.5 L min^{-1} , dual (axial/radial) viewed plasma mode and sample uptake delay – 30 s. The emission wavelength, the detection and quantification limits, and the correlation coefficient of the calibration curve for the aluminum are 308.215 nm, $0.3661 \mu\text{g g}^{-1}$, $1.2203 \mu\text{g g}^{-1}$, and 0.99998, respectively.

A TraceCERT® (Fluka Analytical, Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) ICP multi-element standard solution of about $40 \pm 0.1 \text{ mg L}^{-1}$ of aluminum was used as a stock solution for calibration. The plastic containers used for storing the samples were cleaned to avoid contamination of the samples with trace residues of any metals. Containers were treated with 20% nitric acid and then washed with ultra-pure water $0.05 \mu\text{S cm}^{-1}$ (MicroMed high purity water system, TKA Wasseraufbereitungssysteme GmbH, Niederelbert, Germany). The nitric acid (65%) was of analytical grade.

Statistical analysis

Data are presented as mean \pm standard deviation. One-way ANOVA was performed for comparing values among different animal groups. For non-normally distributed variables Kruskal–Wallis test was used, followed by Mann–Whitney test for comparing values between two groups. The null hypothesis was tested at the 0.05 level of significance. Statistical analysis was performed in R, version 4.0.3, 2020-10-10 (The R Project for Statistical Computing, the R Foundation).

RESULTS

Our study sample included 36 rats, whereas 12 of them were treated with saline and verapamil and were considered the control group, and 24 of them were treated with AlCl_3 in higher and lower doses, during eight weeks. We demonstrated that chronic aluminum exposure resulted in a significant change in almost all evaluated hematological

Table 1. The comparison of chronic aluminum chloride treatment effects on hematological parameters and the effects after verapamil administration

Hematological parameters	Control group	Verapamil group	AlCl ₃ 10 mg/kg b.wt	AlCl ₃ 10 mg/kg b.wt/verapamil	AlCl ₃ 20 mg/kg b.wt	AlCl ₃ 20 mg/kg b.wt/verapamil	p
RBC	8.33 ± 0.52	8.56 ± 0.42	7.86 ± 0.38 ^{a,b}	8.14 ± 0.38 ^{a,b,c}	7.14 ± 0.38 ^{a,b,c,d}	7.67 ± 0.52 ^{a,b,c,d,e}	< 0.001
Hemoglobin	155.17 ± 1.47	152.67 ± 2.07	138.71 ± 11.95 ^{a,b}	151.29 ± 1.38 ^{a,b,c}	126.57 ± 4.68 ^{a,b,c,d}	139.83 ± 2.79 ^{a,b,c,d,e}	< 0.001
Hematocrit	47 ± 0.89	46.17 ± 0.75	39.57 ± 0.98 ^{a,b}	44.14 ± 1.34 ^{a,b,c}	33 ± 2 ^{a,b,c,d}	37.5 ± 1.38 ^{a,b,c,d,e}	< 0.001
MCV	55.17 ± 1.17	55 ± 1.26	50.57 ± 1.9 ^{a,b}	53.71 ± 0.95 ^{a,b,c}	46.29 ± 1.6 ^{a,b,c,d}	49.83 ± 0.75 ^{a,b,c,d,e}	< 0.001
MCH	18.5 ± 0.55	18.33 ± 0.52	17.57 ± 1.51 ^{a,b}	18.29 ± 0.49 ^{a,b,c}	17.57 ± 0.79 ^{a,b,c,d}	18.5 ± 0.55 ^{a,b,c}	0.136
MCHC	329.67 ± 4.23	330.83 ± 0.75	352.14 ± 26.64 ^{a,b}	341.86 ± 8.76 ^{a,b}	381.29 ± 16.89 ^{a,b,d}	372.5 ± 10.88 ^{a,b,c,d,e}	< 0.001
Hepcidin	71 ± 5.4	72 ± 10.94	185 ± 64.54 ^{a,b}	132.14 ± 33.78 ^{a,b}	196.43 ± 45.25 ^{a,b,c}	160.33 ± 31.46 ^{a,b,c}	< 0.001
Ferritin	190.17 ± 48.35	187.33 ± 7.26	309.29 ± 19.33 ^{a,b}	282.71 ± 31.45 ^{a,b}	546.14 ± 85.1 ^{a,b,c}	456.5 ± 98.92 ^{a,b,c,d}	< 0.001
Transferrin	1.47 ± 0.18	1.53 ± 0.08	1.78 ± 0.07 ^{a,b}	1.67 ± 0.05 ^{a,b}	1.85 ± 0.11 ^{a,b,c,d}	1.68 ± 0.06 ^{a,b,c,d}	< 0.001
UIBC	52.17 ± 7.83	48.17 ± 11.58	78 ± 7.21 ^{a,b}	60.29 ± 9.71 ^{a,b}	80.29 ± 8.08 ^{a,b,c,d}	59.67 ± 7.34 ^{a,b,c,d}	< 0.001
TIBC	91.5 ± 8.41	92.33 ± 10.71	105.86 ± 6.56 ^{a,b}	88.14 ± 10.25 ^{a,b}	101.14 ± 10.14 ^{a,b,d}	83.17 ± 6.08 ^{a,b,c,d}	0.001
Fe	39.67 ± 1.86	38.17 ± 8.84	27.86 ± 2.26 ^{a,b}	28 ± 3.92 ^{a,b}	20.86 ± 3.08 ^{a,b,c,d}	21.83 ± 0.75 ^{a,b,c,d}	< 0.001
TSAT	0.43 ± 0.04	0.41 ± 0.1	0.26 ± 0.03 ^{a,b}	0.32 ± 0.05 ^{a,b}	0.2 ± 0.02 ^{a,b,c,d}	0.26 ± 0.02 ^{a,b,c,d}	< 0.001

Values are presented as mean ± S.D; p < 0.05;

RBC – red blood cells; MCV – mean corpuscular volume; MCH – mean corpuscular hemoglobin; MCHC – mean corpuscular hemoglobin concentration; UIBC – unsaturated iron-binding capacity; TIBC – total iron-binding capacity; TSAT – transferrin saturation;

^avs. control group;

^bvs. verapamil group;

^cvs. AlCl₃ 10 mg/kg b.wt;

^dvs. AlCl₃ 10 mg/kg b.wt/verapamil;

^evs. AlCl₃ 20 mg/kg b.wt

Table 2. The comparison of chronic aluminum chloride treatment effects on the platelets and white cells and the effects after verapamil administration

Parameters	Control group	Verapamil group	AlCl ₃ 10 mg/kg b.wt	AlCl ₃ 10 mg/kg b.wt/verapamil	AlCl ₃ 20 mg/kg b.wt	AlCl ₃ 20 mg/kg b.wt/verapamil	p
PLT	776.5 ± 63.46	760.17 ± 77.08	846.43 ± 90.65 ^{a,b}	820.71 ± 79.43 ^{a,b}	1133.17 ± 1.12 ^{a,b,c,d}	1017.17 ± 41.1 ^{a,b,c,d}	< 0.001
WBC	7.95 ± 0.83	7.67 ± 0.52	10 ± 1.83 ^{a,b}	8.71 ± 0.49 ^{a,b,c}	11.5 ± 0.53 ^{a,b,c}	9.33 ± 0.82 ^{a,b,c,d}	< 0.001
Ly %	53.2 ± 5.15	52.97 ± 1.72	57.16 ± 6.96 ^{a,b}	55.83 ± 9.37 ^{a,b}	50.1 ± 6.51 ^{a,b,c}	40.41 ± 9.82 ^{a,b,c,d}	0.004
Seg %	39.82 ± 7.13	40.725 ± 1.93	32.91 ± 6.51 ^{a,b}	34.81 ± 10.17 ^{a,b}	40.87 ± 7.93 ^{a,b,c}	51.65 ± 1.43 ^{a,b,c,d}	0.005
Mo %	6.98 ± 2.05	6.28 ± 0.75	9.93 ± 1.15 ^{a,b}	9.36 ± 1.11 ^{a,b}	9.03 ± 1.92 ^{a,b,c}	7.85 ± 74 ^{a,b,c,d}	0.001
Ly	4.2 ± 0.6	4.09 ± 0.24	5.78 ± 1.14 ^{a,b}	4.82 ± 1.01 ^{a,b}	5.7 ± 0.76 ^{a,b,c}	3.8 ± 0.97 ^{a,b,c,d}	< 0.001
Seg	3.07 ± 0.6	3.15 ± 0.3	3.33 ± 0.83 ^{a,b}	2.96 ± 0.77 ^{a,b}	4.66 ± 0.94 ^{a,b,c}	4.87 ± 1.25 ^{a,b,c,d}	< 0.001
Mo	0.54 ± 0.17	0.48 ± 0.06	1.02 ± 0.31 ^{a,b}	0.8 ± 0.11 ^{a,b}	1.03 ± 0.22 ^{a,b,c}	0.74 ± 0.17 ^{a,b,c,d}	< 0.001

PLT – platelets; WBC – white blood cells; Ly – lymphocytes; Seg – segmented leukocytes; Mo – monocytes;

^avs. control group;

^bvs. verapamil group;

^cvs. AlCl₃ 10 mg/kg b.wt;

^dvs. AlCl₃ 10 mg/kg b.wt/verapamil;

^evs. AlCl₃ 20 mg/kg b.wt

parameters (Table 1). The hematological parameters that were found to be significantly decreased after administration of 10 mg of AlCl₃ mg/kg b.wt. are as follows: the number of RBC (p < 0.001), Hg (p < 0.001), Hct (p < 0.001), MCV (p < 0.001), MCHC (p < 0.001), iron plasma concentration (p < 0.001), and TSAT (p < 0.001). Statistically significant increase was observed in plasma concentrations of hepcidin (p < 0.001), ferritin (p < 0.001), and transferrin, as well in values of UIBC (p < 0.001) and TIBC (p < 0.001). The identical result was obtained after 20 mg of AlCl₃ mg/kg b.wt. was administered, with p < 0.001 for all evaluated parameters (Table 1).

After verapamil administration, a statistically significant improvement in hematological parameters was demonstrated as follows: in the number of RBC (p < 0.001), Hg concentration (p < 0.001), Hct (p < 0.001), MCV (p < 0.001) and MCHC (p < 0.001). Parameters such as hepcidin, ferritin, transferrin, UIBC, TIBC, iron in plasma, and TSAT did not show statistically significant change after verapamil was applied, regardless of the AlCl₃ dose.

The results presented in Table 2 refer to the changes in parameters of WBC and platelets after AlCl₃ was administered. The lower dose of aluminum (10 mg/kg b.wt) presented with the significant increase in all evaluated parameters: the number of platelets (p < 0.001), WBC (p < 0.001), percentage of lymphocytes (p = 0.004), segmented leukocytes (p = 0.005) and monocytes (p = 0.001), as well as in absolute count of the same cells, number of lymphocytes (p < 0.001), segmented leukocytes (p < 0.001) and monocytes (p < 0.001). The same result was achieved with the higher dose of aluminum (20 mg/kg b.wt), for all analyzed parameters. However, no protective effects of verapamil application were observed, since no statistically significant differences were documented after verapamil was used, Table 2.

Table 3 presents comparison of chronic AlCl₃ treatment (10/20 mg/kg b.wt) on biochemical parameters and the changes after verapamil administration. In the cases of lower AlCl₃ dose, significant increase of evaluated parameters was observed in the following: AST (p = 0.001) and ALT (p < 0.001), and concentration of ALP (p = 0.002),

Table 3. The comparison of chronic aluminum chloride treatment effects on biochemical parameters and the effects after verapamil administration

Biochemical parameters	Control group	Verapamil group	AlCl ₃ 10 mg/kg b.wt	AlCl ₃ 10 mg/kg b.wt/verapamil	AlCl ₃ 20 mg/kg b.wt	AlCl ₃ 20 mg/kg b.wt/verapamil	p
AST	245.93 ± 17.56	246.17 ± 70.89	407.11 ± 143.95 ^{ab}	285.21 ± 41.11 ^{ab}	527.39 ± 227.91 ^{ab,c,d}	342.6 ± 58.89 ^{ab,c,d}	0.001
ALT	75.55 ± 5.91	75.70 ± 8.32	121.97 ± 48.33 ^{ab}	94.2 ± 10.09 ^{ab}	168.2 ± 32.09 ^{ab,c,d}	117.85 ± 19.5 ^{ab,c,d}	< 0.001
ALP	405.65 ± 16.51	405.18 ± 11.73	421.24 ± 28.04 ^{ab}	385.22 ± 34.22 ^{ab,c}	469.87 ± 63.59 ^{ab,c,d}	379 ± 42.78 ^{ab,c,d,e}	0.002
AMY	2356.12 ± 219.65	2364.1 ± 131.81	3362.58 ± 124.79 ^{ab}	2710.39 ± 499.39 ^{ab,c}	3451.43 ± 112.79 ^{ab,c,d}	2387.85 ± 569.49 ^{ab,c,d,e}	< 0.001
LDH	2837.30 ± 510.14	2789.9 ± 403.31	4516.35 ± 1166.46 ^{ab}	3058.11 ± 581.03 ^{ab,c}	7147.86 ± 1278.45 ^{ab,c,d}	4911.72 ± 2332.59 ^{ab,c,d,e}	< 0.001
CK	1302.63 ± 32.04	1299.77 ± 237.76	2719.81 ± 817.52 ^{ab}	2113.59 ± 687.64 ^{ab,c}	3446.76 ± 1492.33 ^{ab,c,d}	2320.35 ± 331.43 ^{ab,c,d,e}	< 0.001
γGT	0.1 ± 0.13	0.33 ± 0.2	1.51 ± 0.23 ^{ab}	0.91 ± 0.4 ^{ab,c}	2.09 ± 0.34 ^{ab,c,d}	0.67 ± 0.39 ^{ab,c,d,e}	< 0.001
CRP	2.1 ± 0.11	2.1 ± 0.16	15.5 ± 4.5 ^{ab}	14.6 ± 5.2 ^{ab}	22.6 ± 6.6 ^{ab,c,d}	20.5 ± 4.5 ^{ab,c,d}	0.004

AST – aspartate transaminase; ALT – alanine transaminase; ALP – alkaline phosphatase; AMY – amylase; CK – creatine kinase; γGT – glutamyl transferase; CRP – C-reactive protein;

^avs. control group;

^bvs. verapamil group;

^cvs. AlCl₃ 10 mg/kg b.wt;

^dvs. AlCl₃ 10 mg/kg b.wt/verapamil;

^evs. AlCl₃ 20 mg/kg b.wt

AMY ($p < 0.001$), lactate dehydrogenase ($p < 0.001$), CK ($p < 0.001$), γGT ($p < 0.001$), and CRP ($p = 0.004$). The same, significant results were obtained in higher (20 mg/kg b.wt) AlCl₃ doses for all the parameters. The protective effects of verapamil administration were observed in animals treated with aluminum 10 mg/kg b.wt as a significant decrease in plasma concentrations of ALP ($p < 0.001$), AMY ($p < 0.001$), LDH ($p < 0.001$), CK ($p < 0.001$), and γGT ($p < 0.001$). The identical effects of verapamil administration were documented in animals treated with higher doses of AlCl₃, seen also as significantly decreased values of ALP ($p < 0.001$), AMY ($p < 0.001$), LDH ($p < 0.001$), CK ($p < 0.001$), and γGT ($p < 0.001$) (Table 3). Plasma concentration of CRP and activity of AST and ALT did not change after verapamil administration.

Figure 1 presents results after comparison of chronic AlCl₃ treatment in tissue aluminum deposition and the effects after verapamil administration. Significant increase in aluminum tissue accumulation was observed in tissue measurements: the liver ($p < 0.001$), kidneys ($p < 0.001$), and testes ($p < 0.001$), regardless of the aluminum dose. The protective effect of verapamil was obtained in the liver ($p < 0.001$) and testes ($p < 0.001$), irrespective of the dose. In kidneys, however, no protective effect of verapamil was documented. The results are presented in Table 4.

DISCUSSION

In this study, it is documented that verapamil administration significantly mitigates aluminum-induced hematology intoxication and tissue accumulation, suggesting that it may be a protective agent against AlCl₃-induced toxicity. However, based on the results, the application of verapamil did not significantly decrease inflammatory parameters, implying that the inflammatory pathways in aluminum-associated toxicity are most likely independent of this mechanism of action and cannot be prevented by Ca²⁺ channel blocking.

It was evidenced that aluminum oral intake leads to its extensive accumulation in the liver, spleen, kidneys, and bones in higher rates compared to that of muscles, brain, lungs, and heart. After aluminum is deposited, its toxicity

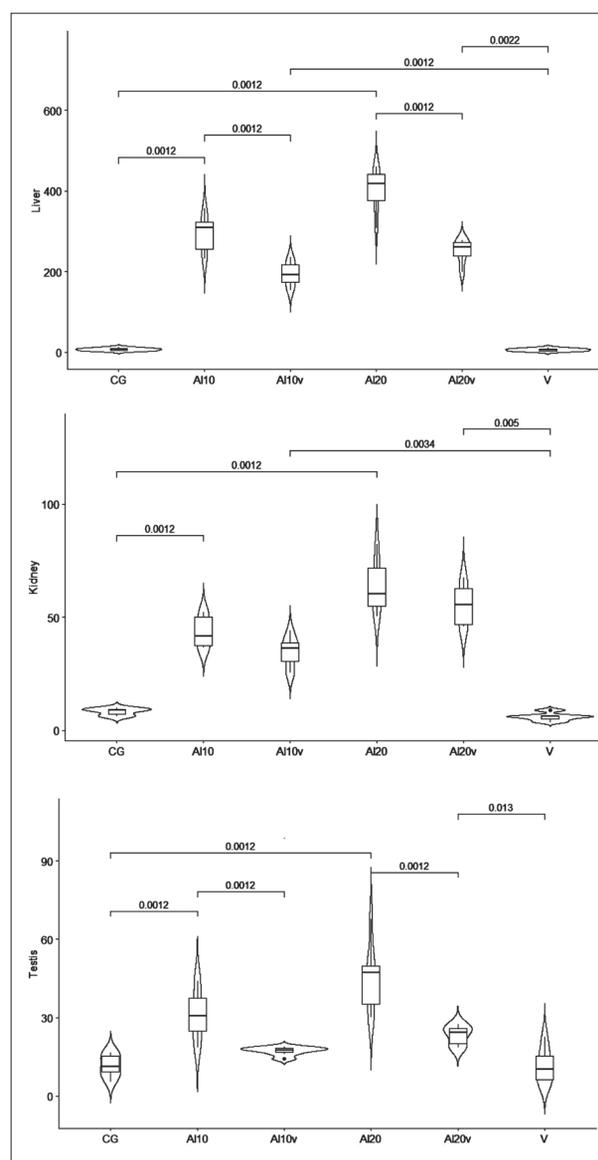


Figure 1. The comparison of chronic AlCl₃ treatment of tissue aluminum deposition and the effects after verapamil administration;

CG – control group; AI 10 – AlCl₃ 10 mg/kg b.wt; AI 10v – AlCl₃ 10 mg/kg b.wt/verapamil; AI 20 – AlCl₃ 20 mg/kg b.wt; AI 20v – AlCl₃ 20 mg/kg b.wt/verapamil; V – verapamil group

Table 4. The comparison of chronic aluminum chloride treatment effects on tissue aluminum deposition and the effects after verapamil administration

Tissue/groups	Control group	Verapamil group	AlCl ₃ 10 mg/kg b.wt	AlCl ₃ 10 mg/kg b.wt/verapamil	AlCl ₃ 20 mg mg/kg b.wt	AlCl ₃ 20 mg/kg b.wt/verapamil	p
Liver	7.18 ± 3.78	6.19 ± 3.70	293.24 ± 46.86 ^{ab}	194.44 ± 29.8 ^{abc}	403.29 ± 53.92 ^{abc,d}	251.17 ± 29.98 ^{abc,d,e}	< 0.001
Kidney	8.38 ± 1.56	6.05 ± 1.78	43.7 ± 9.96 ^{ab}	34.91 ± 6.8 ^{ab}	63.76 ± 11.85 ^{abc,d}	55.65 ± 9.6 ^{abc,d}	< 0.001
Testicle	11.63 ± 4.36	11.8 ± 6.79	31.14 ± 9.91 ^{ab}	17.27 ± 1.63 ^{abc}	45.01 ± 13.1 ^{abc,d}	23.35 ± 3.74 ^{abc,d,e}	< 0.001

^avs. control group;^bvs. verapamil group;^cvs. AlCl₃ 10 mg/kg b.wt;^dvs. AlCl₃ 10 mg/kg b.wt/verapamil;^evs. AlCl₃ 20 mg/kg b.wt

is achieved through many diverse pathways, the following being the most studied: oxidant/antioxidant/apoptosis [8], pro-inflammatory [9], immunosuppression [10], and interference with enzymes, proteins and metabolism [11]. In addition to these findings, free intracellular Ca²⁺ variations have been proposed as an index of aluminum-associated toxic injury [12]. Indeed, reports from many experimental models implicate that chronic aluminum exposure disrupts pathways of Ca²⁺-mediated homeostasis.

Nevertheless, Al³⁺ and Ca²⁺ share the same atomic radius; therefore, it is plausible that aluminum interacts with the binding sites of calcium, disrupting its homeostasis. In the milieu of disrupted Ca²⁺ homeostasis, mitochondria increase production of reactive oxygen species, resulting in oxidative damage, autophagy and apoptosis precipitation. Similarly, the function of calcium-ATPase enzymes may also be disrupted, resulting in cellular/organelles malfunction [13]. The inhibition of cytochrome oxidase, followed by the inhibition of respiratory chain, may lead to the depletion of ATP production via the aerobic pathway. In other words, the inhibition of cytochrome oxidase may enhance anaerobic metabolism, finally enabling the accumulation of Ca²⁺ [14]. All of this may provide a plausible pathophysiological link between Ca²⁺ disruption and oxidative damage.

Anemia was suggested as the first manifestation of aluminum toxicity, whereas much research has proved the manifestation of microcytic anemia without iron deficiency in cases of aluminum overload [15]. The results of the present study concur with these findings, since after chronic AlCl₃ exposure all experimental rats developed microcytic/hypochromic anemia. Importantly, a significant improvement of hematological parameters after verapamil administration was observed, regardless of the aluminum dose employed. It may, therefore, be postulated that verapamil mitigates aluminum-induced hematological toxicity.

The obtained results of a ~2.6-fold increase in hepcidin concentration suggest that the aluminum load indirectly reduces the possibilities of iron entering the erythrocyte precursors. Hepcidin concentrations are demonstrated to be significantly elevated in states of proinflammatory cytokines overproduction, leading to iron tissue sequestration, iron restriction of erythropoiesis, hypoferremia and low saturation of transferrin, which was demonstrated to be significantly decreased in this study [16, 17].

Hepcidin-related anemia is mostly presented as moderate normochromic-normocytic anemia, whereas significantly decreased values of MCV and MCHC were obtained here, so an additional pathophysiology explanation should

be provided for this aluminum-related microcytosis. The result of very low iron-transferrin saturation, is most likely due to increased hepcidin production, and this implicates the likeliness of a formation of a complex of aluminum-transferrin, delivering to the erythroid precursors aluminum, instead of iron. Finally, it is plausible that it is the elevation of hepcidin concentration that initially impairs iron metabolism and that therapeutic interference with hepcidin may improve plasma concentrations of iron and iron-associated proteins.

The most important finding, that MCV and MCHC values were significantly reversed after verapamil administration, may be explained by the hypothesis that AlCl₃ changes erythrocyte membrane permeability for ions, interrupting the function of the cation pumps, a phenomenon that, based on presented findings, may be alleviated by calcium channels' blockage [17].

Concerning the liver, after the application of verapamil, a statistically significant decrease in liver aluminum deposition was observed, but not in the biochemical markers of liver necrosis. It has been stated that tissue exposure to aluminum leads to necrosis and biochemical abnormalities, which is in accordance with the present findings [18, 19]. The necrosis of hepatocytes is presumed by the ability of aluminum to bind to microtubule-associated phosphoprotein, located in the cytoskeleton that is an integral part of the plasma membranes of the hepatic cells, resulting in liver cell necrosis. It may be suggested that it is more likely that necrosis of hepatocytes is achieved by impaired glycolysis, ATP production, Krebs cycles and lipids' and proteins' oxidation, whereas intracellular calcium deposition represents an additional mechanism, in a way to potentiate impaired ATP production via Krebs cycle. The blockage of Ca²⁺ channels by verapamil indisputably ameliorated aluminum liver deposition, regardless of the dosage, but did not significantly reverse the concentration of liver enzymes.

The reproductive system in experimental animals is known to be significantly affected by aluminum intoxication, resulting in decreased fertility. The key factors leading to infertility are reduced sperm production and count, decreased motility and production of aberrant sperm [20]. We demonstrated increased aluminum tissue concentrations, regardless of the dose, and more importantly that this effect may be alleviated by the verapamil administration. At odds with these findings, some authors did not prove aluminum accumulation in testes, but reported the impairment of testicular function, referred to as aberrant spermatogenesis and sperm malformation [20]. The

researchers postulated that aluminum exposure may cause inhibition of acid phosphatase, succinate dehydrogenase, and LDH isoenzyme. Aluminum presumably inhibits activity of Ca^{2+} -ATPase, resulting in the dysfunction of testicular membrane activity.

Finally, aluminum is proven to contribute to the pathogenesis of inflammation, by up-regulating proinflammatory cytokines and giving the signal for hepcidin synthesis. Within the study, inflammation was proven by increased CRP and ferritin concentration and, indirectly, by hepcidin evaluation. Based on the resulting outcomes, neither of the biomarkers was significantly decreased after verapamil was employed. It may even be speculated that these effects remain in consequence of the calcium generated from the intracellular store, a phenomenon that occurs in the presence of metals and may not be mitigated by verapamil [13].

CONCLUSION

The findings of the present study indicate that chronic AlCl_3 intoxication, regardless of the dose, results in the de-

velopment of microcytic anemia associated with high hepcidin levels, numerous biochemical disarrangements and significant aluminum deposition in the liver, kidney, and testes. In addition, a significant hemato-protective effect of verapamil was documented, as well as the attenuation of aluminum deposits in both the liver and the testes. Overall, the results of the research emphasize the significance of calcium homeostasis preservation in chronic aluminum exposure and propose a possible therapeutic option.

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Употреба верапамила ублажава микроцитозу и ткивну акумулацију после хроничне изложености алуминијуму

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САЖЕТАК

Увод/Циљ Циљ истраживања био је да се испита степен алуминијумске токсичности, процењен хематолошким и биохемијским параметрима, променом концентрације хепцидина и акумулацијом алуминијума у ткивима након хроничне изложености и да се утврди да ли постоји протективни ефекат верапамила, остварен блокадом канала Ca^{2+} .

Метод Експерименталне животиње (36 пацова) третиране су различитим дозама $AlCl_3$ током периода од осам недеља, а након тога анализирани су њихови крв и ткива.

Резултати Значајне разлике, без обзира на дозу алуминијума, постојале су у свим хематолошким ($p < 0,001$) и биохемијским параметрима ($p < 0,001$), као и у акумулацији алуминијума у јетри, бубрезима и тестисима. Након примене верапамила доказано је значајно побољшање вредности појединих хематолошких и биохемијских параметара, ($p < 0,001$), као и значајно смањење акумулације алуминијума

у ткиву јетре и тестиса ($p < 0,001$). Вредности анализираних параметара инфламације и акумулације алуминијума у ткиву бубрега нису биле значајно различите након примене верапамила.

Закључак Резултати овог истраживања показују да хронична интоксикација $AlCl_3$, без обзира на примењену дозу, доводи до развоја микроцитне анемије праћене високим концентрацијама хепцидина, биохемијским поремећајима и значајним таложењем алуминијума у ткиву јетре, бубрега и тестиса, као и да се неки од ових ефеката могу ублажити давањем верапамила. Добијени резултати сугеришу значајну патогенетску везу између хомеостазе калцијума и алуминијумске токсичности, на основу чега се може говорити о потенцијалној терапијској опцији током хроничне експозиције алуминијуму.

Кључне речи: токсичност изазвана алуминијумом; верапамил; хронична изложеност; хепцидин; микроцитоза