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

Употреба верапамила ублажава микроцитозу и ткивну акумулацију након хроничне изложености алуминијуму

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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 Ca^{2+}-channel blockage, verapamil.

Methods Experimental animals (36 rats) were treated with different doses of $AlCl_3$ during 8 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 $AlCl_3$ 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

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+}.

Методе Експерименталне животиње (36 пацова) третиране су различитим дозама $AlCl_3$ током периода од 8 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 акумулација 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 алуминујуму.

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

INTRODUCTION

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

In recent years, there has been abundant data on Al 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 Ca^{2+} channels may represent potential therapeutic strategy for addressing Al-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 Ca^{2+}-channel blockage by verapamil.

METHODS

Animals

The experiment was performed on thirty-six male Sprague-Dawley rats (Vivarium of the Research Center for Biomedicine of the Faculty of Medicine in Nis). The rats were 2 months old and weighed 130-160 grams. They were given *ad libitum* access to food (standard rat chow pellets Veterinary Institute, Zemun) and tap water. The ambient temperature was 20±2 ºC, the relative humidity was 55±10 %, with 12-hour light-dark cycle. The care and handling of the animals were in accordance with the guidelines and recommendations specified by the Federation 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, number 323-07-01762/2019-05/10.

Experimental design

Rats were randomly divided into six groups (n = 6). Animals were treated daily during a period of 8 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) and verapamil hydrochloride (Verapamil, Alkaloid Skopje, North Macedonia).

Blood analyses and tissue sampling

At the end of the study, rats were euthanized with an overdose of Ketonal® (*Ketonal*®, 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 (Hg) 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, Brea, 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: AST, ALT, ALP, amylase (AMY), LDH, CK and γGT.

The concentrations of hepcidin and C-reactive protein 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 measurement of 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 gram from each sample) were then mixed with 3mL of pure nitric acid at 90 C for 24-48 h. 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 (Thermo Scientific, Cambridge, UK) which uses the Echelle optical design and a change injection device solid-state detector. The operating conditions for the ICP-AES instrument were: 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 (LOD) and quantification (LOQ) limits and the correlation coefficient of the calibration curve for the aluminum are: 308.215 nm, 0.3661 μg g^{-1}, 1.2203 μg g^{-1} and 0,99998.

A TraceCERT® (Fluka Analytical, Switzerland) ICP multi-element standard solution of about 40.00±0.10 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 traces residues of any metals. Containers were treated with 20 % nitric acid and then washed with ultra-pure water 0.05 μS cm^{-1} (MicroMed high purity water system, TKA Wasseraufbereitungssysteme GmbH). The nitric acid (65 %) was of analytical grade.

Statistical analysis

Data are presented as mean ± 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).

RESULTS

Our study sample included 36 rats, whereas 12 of them were treated with saline and verapamil and were considered as the control groups and 24 of them were treated with $AlCl_3$ in higher and lower doses, during 8 weeks. We demonstrated that chronic aluminum exposure resulted in a significant change in almost all evaluated hematological parameters, Table 1. The hematological parametres that were found to be significantly decreased after administration of 10 mg of $AlCl_3$ mg/kg b.wt. are as follows: the number of RBC ($p < 0.001$), hemoglobin ($p < 0.001$), hematocrit ($p < 0.001$), MCV ($p < 0.001$), MCHC ($p < 0.001$), iron plasma concentration ($p < 0.001$) and TSAT ($p < 0.001$). Statisticaly 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 $AlCl_3$ mg/kg b.wt. was administered, with $p < 0.001$ for all evaluated parameters (Table 1).

After verapamil administration, a statisticaly significant improvement in hematological parameters was demonstrated as follows: in the number of RBC ($p < 0.001$), hemoglobin concentration ($p < 0.001$), hematocrit ($p < 0.001$), 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 statisticaly significant change after verapamil was applicated, regardless of the $AlCl_3$ dose.

The results presented in Table 2 refer to the changes in parameters of WBC and platelets after $AlCl_3$ 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 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 analized parameters. However, no protective effects of verapamil application were observed, since no statisticaly significant differences were documented after verapamil was used, Table 2.

Table 3 presents comparison of chronic $AlCl_3$ treatment (10/20 mg/kg b.wt) on biochemical parameters and the changes after verapamil administration. In the cases of lower $AlCl_3$ dose, significant increase of evaluated parameters was observed in the following: AST ($p = 0.001$) and ALT ($p < 0.001$), and concentration of alkaline phosphatase ($p = 0.002$), amylase ($p < 0.001$), lactate dehidrogenase ($p < 0.001$), creatine kinase ($p < 0.001$), γGT ($p < 0.001$) and C-reactive protein ($p = 0.004$). The same, significant results was obtained in higher (20 mg/kg b.wt) $AlCl_3$ doses for all parameters respectively. 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 ($p < 0.001$), amylase ($p < 0.001$), 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 $AlCl_3$, seen also as significantly decreased values of ALP ($p < 0.001$), amylase ($p < 0.001$), 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 $AlCl_3$ treatment in tissue aluminum deposition and the effects after verapamil administration. Significant increase in aluminum tissue accumulation was observed in tissue measurements: 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 liver ($p < 0.001$) and testes ($p < 0.001$), irrespective of the dose. In kidneys, however, no protective effect of verapamil was documented.

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 $AlCl_3$-induced toxicity. However, based on the results, the application of verapamil did not significantly decrease inflammatory parameters, implying that the inflammatory pathways in Al-associated toxicity are most likely independent of this mechanism of action and cannot be prevented by Ca^{2+} channel blocking.

It was evidenced that Al 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 its deposition, aluminum-induced toxicity 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 Ca^{2+} 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 Ca^{2+}-mediated homeostasis.

Nevertheless, Al^{3+} and Ca^{2+} 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 Ca^{2+} 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* aerobic pathway. In other words, the inhibition of cytochrome oxidase may enhance anaerobic metabolism, finally enabling accumulation of Ca^{2+} [14]. All of this may provide a plausible pathophysiological link between Ca^{2+} 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 Al overload [15]. The results of the present study concur with these findings, since after chronic $AlCl_3$ exposure all experimental rats developed microcytic/hypochromic anemia. Importantly, a significant improvement of hematological parameters after verapamil administration was observed, regardless of the Al 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 Al 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].

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 $AlCl_3$ changes erythrocyte membrane permeability for ions, interrupting the function of the cation pumps [17], phenomenon that, based on presented findings, may be alleviated by calcium channels blockage.

Concerning 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 [18,19], that is in accordance with the present findings. 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 Ca^{2+} 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 lactate dehydrogenase 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-regulation of proinflammatory cytokines and giving the signal for hepcidin synthesis. Within the study, inflammation was proven by increased C-reactive protein 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 [12] and may not be mitigated by verapamil.

CONCLUSION

The findings of the present study indicate that chronic $AlCl_3$ intoxication, regardless of the dose, results in the development of microcytic anemia associated with high hepcidin levels, numerous biochemical disarrangements and significant aluminum deposition in liver, kidney and testicles. Besides, a significant hemato-protective effect of verapamil was documented, as well as the attenuation of aluminum deposits in both liver and 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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Conflict of interest: None declared.

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Table 1. The comparison of chronic aluminum chloride treatment 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	47.00 ± 0.89	46.17 ± 0.75	39.57 ± 0.98[a,b]	44.14 ± 1.34[a,b,c]	33.0 ± 2.00[a,b,c,d]	37.50 ± 1.38[a,b,c,d,e]	< **0.001**
MCV	55.17 ± 1.17	55.00 ± 1.26	50.57 ± 1.90[a,b]	53.71 ± 0.95[a,b,c]	46.29 ± 1.60[a,b,c,d]	49.83 ± 0.75[a,b,c,d,e]	< **0.001**
MCH	18.50 ± 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]	18.50 ± 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]	372.50 ± 10.88[a,b,c,d,e]	< **0.001**
Hepcidin	71.00 ± 5.40	72.00 ± 10.94	185.00 ± 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 ± 85.10[a,b,c]	456.50 ± 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	78.00 ± 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	91.50 ± 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]	28.00 ± 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 ± 0.10	0.26 ± 0.03[a,b]	0.32 ± 0.05[a,b]	0.20 ± 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;

[a]*vs*. control group;

[b]*vs*. verapamil group;

[c]*vs.* $AlCl_3$ 10 mg/kg b.wt;

[d]*vs*. $AlCl_3$ 10 mg/kg b.wt/verapamil;

[e]*vs*. $AlCl_3$ 20 mg mg/kg b.wt;

Table 2. The comparison of chronic aluminum chloride treatment 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	776.50 ± 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	10.00 ± 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 %	53.20 ± 5.15	52.97 ± 1.72	57.16 ± 6.96[a,b]	55.83 ± 9.37[a,b]	50.10 ± 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	4.20 ± 0.60	4.09 ± 0.24	5.78 ± 1.14[a,b]	4.82 ± 1.01[a,b]	5.70 ± 0.76[a,b,c]	3.80 ± 0.97[a,b,c,d]	**< 0.001**
Seg	3.07 ± 0.60	3.15 ± 0.30	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]	0.80 ± 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;

[a]*vs*. control group;

[b]*vs*. verapamil group;

[c]*vs*. $AlCl_3$ 10 mg/kg b.wt;

[d]*vs*. $AlCl_3$ 10 mg/kg b.wt/verapamil;

[e]*vs*. $AlCl_3$ 20 mg mg/kg b.wt

Table 3. The comparison of chronic aluminum chloride treatment 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[a,b]	285.21 ± 41.11[a,b]	527.39 ± 227.91[a,b,c,d]	342.60 ± 58.89 [a,b,c,d]	**0.001**
ALT	75.55 ± 5.91	75.70 ± 8.32	121.97 ± 48.33[a,b]	94.20 ± 10.09 [a,b]	168.20 ± 32.09[a,b,c,d]	117.85 ± 19.50 [a,b,c,d]	**< 0.001**
ALP	405.65 ± 16.51	405.18 ± 11.73	421.24 ± 28.04[a,b]	385.22 ± 34.22[a,b,c]	469.87 ± 63.59[a,b,c,d,]	379.00 ± 42.78 [a,b,c,d,e]	**0.002**
AMY	2356.12 ± 219.65	2364.10 ± 131.81	3362.58 ± 124.79[a,b]	2710.39 ± 499.39[a,b,c]	3451.43 ± 112.79[a,b,c,d]	2387.85 ± 569.49 [a,b,c,d,e]	**< 0.001**
LDH	2837.30 ± 510.14	2789.90 ± 403.31	4516.35 ± 1166.46[a,b]	3058.11 ± 581.03[a,b,c]	7147.86 ± 1278.45[a,b,c,d]	4911.72 ± 2332.59 [a,b,c,d,e]	**< 0.001**
CK	1302.63 ± 32.04	1299.77 ± 237.76	2719.81 ± 817.52[a,b]	2113.59 ± 687.64[a,b,c]	3446.76 ± 1492.33[a,b,c,d]	2320.35 ± 331.43[a,b,c,d,e]	**< 0.001**
γGT	0.10 ± 0.13	0.33 ± 0.20	1.51 ± 0.23[a,b]	0.91 ± 0.40[a,b,c]	2.09 ± 0.34[a,b,c,d]	0.67 ± 0.39 [a,b, c,d,e]	**< 0.001**
CRP	2.1 ± 0.11	2.1 ± 0.16	15.5 ± 4.5[a,b]	14.6 ± 5.2[a,b]	22.6 ± 6.6[a,b,c,d]	20.5 ± 4.5[a,b,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;

[a]*vs.* control group;

[b]*vs.* verapamil group;

[c]*vs.* $AlCl_3$ 10 mg/kg b.wt;

[d]*vs.* $AlCl_3$ 10 mg/kg b.wt/verapamil;

[e]*vs.* $AlCl_3$ 20 mg mg/kg b.wt

Table 4. The comparison of chronic aluminum chloride treatment of 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[a,b]	194.44 ± 29.80[a,b,c]	403.29 ± 53.92[a,b,c,d]	251.17 ± 29.98[a,b,c,d,e]	< **0.001**
Kidney	8.38 ± 1.56	6.05 ± 1.78	43.70 ± 9.96[a,b]	34.91 ± 6.80[a,b]	63.76 ± 11.85[a,b,c,d]	55.65 ± 9.60[a,b,c,d]	< **0.001**
Testicle	11.63 ± 4.36	11.80 ± 6.79	31.14 ± 9.91[a,b]	17.27 ± 1.63[a,b,c]	45.01 ± 13.10[a,b,c,d]	23.35 ± 3.74[a,b,c,d,e]	< **0.001**

[a]*vs.* control group;

[b]*vs.* verapamil group;

[c]*vs.* $AlCl_3$ 10 mg/kg b.wt;

[d]*vs.* $AlCl_3$ 10 mg/kg b.wt/verapamil;

[e]*vs.* $AlCl_3$ 20 mg mg/kg b.wt

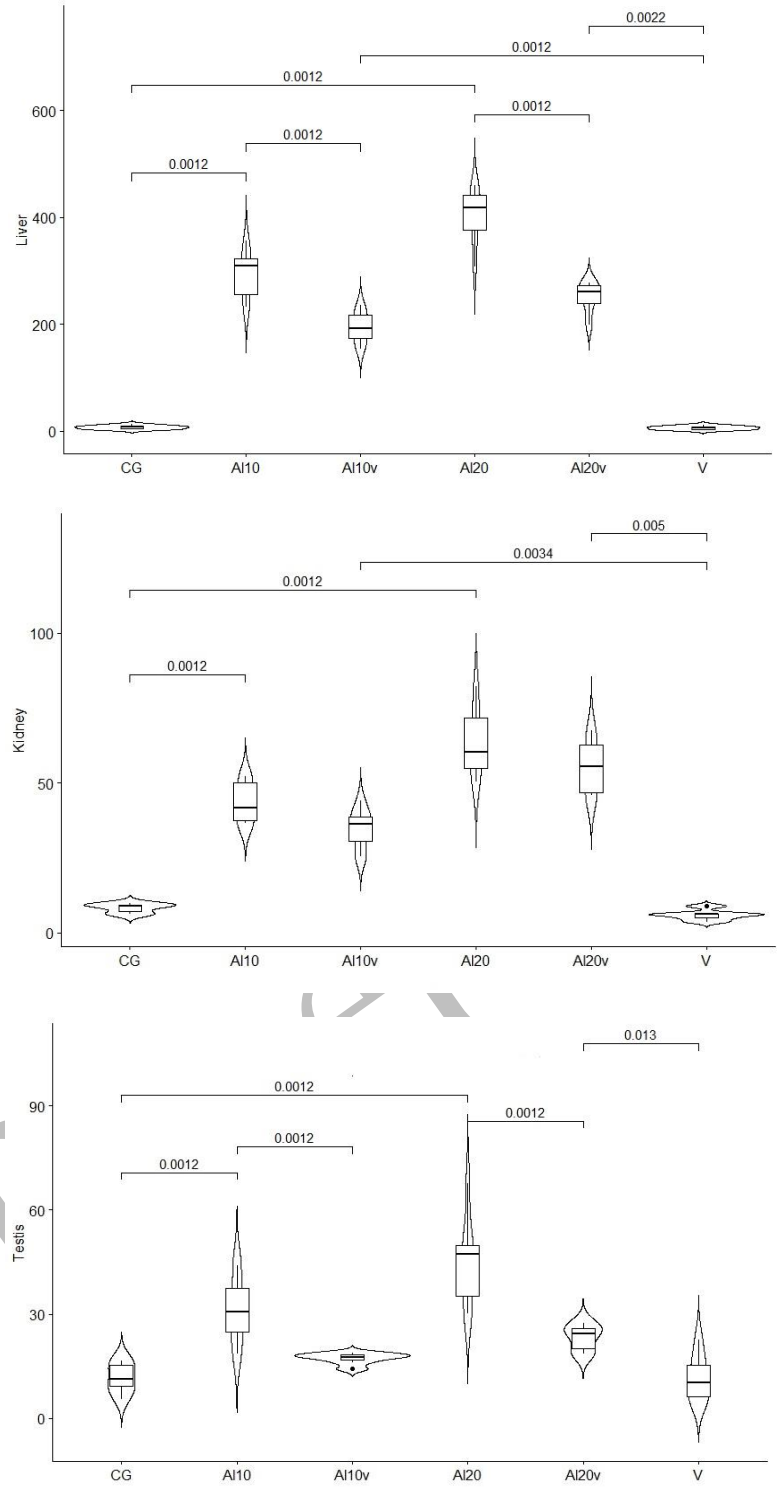

Figure 1. The comparison of chronic $AlCl_3$ treatment of tissue aluminum deposition and the effects after verapamil administration;

CG – control group; Al 10 – $AlCl_3$ 10 mg/kg b.wt; Al 10v – $AlCl_3$ 10 mg/kg b.wt/verapamil; Al 20 – $AlCl_3$ 20 mg mg/kg b.wt; Al 20v – $AlCl_3$ 10 mg/kg b.wt/verapamil; V – verapamil group

