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Subchronic Exposure To $AlCl_3$ Induces Hematological And Iron Metabolism Genes Expression Changes In Sprague Dawley Rats

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Abstract

Aluminum in the form of aluminum trichloride ($AlCl_3$) is a potentially toxic element depending on the entry route, concentration, and exposure time. This research aimed to evaluate a possible genotoxicity to iron metabolism genes profile expression and hematotoxicity effect as a consequence, due to subchronic exposure to $AlCl_3$ in Sprague Dawley (SD) rats. To this end, 14 female Sprague Dawley rats were aleatory selected forming two groups. Control group (A) was treated 90 days with physiological saline solution, whereas Experimental Group (B) was treated with $AlCl_3$ 40 mg/kg/day through intragastric route. Furthermore, rats were previously treated with halothane followed by blood drawing through intracardiac puncture. A complete blood count (CBC) and RNA extraction were done on whole blood samples, while serum was used to measure iron kinetic parameters using the micro-ELISA method. The CBC results point out that there are higher values of Hb and MCHC, PCT percentage, and white blood cells (WBC) predominantly due to neutrophils (NE), eosinophils (EO) and basophils (BA) in the experimental group compared to control ($\alpha = 0.05$). Moreover, experimental subjects showed lower ferritin values and higher total iron binding capacity (TIBC) in iron kinetics analytes than untreated individuals. In addition, hepcidin and transferrin gene expression appeared overexpressed, while the ferritin gene was observed slightly repressed in group B when analyzed in the heatmap. Significative changes in treated subjects were observed in hepcidin gene expression, while iron kinetics analysis showed a decrease in ferritin. In conclusion, $AlCl_3$ induces changes in blood cell count, iron kinetics parameters, and gene expression profile, which may be an oxidative stress consequence.

Keywords: *Genotoxicity, genes, iron metabolism, $AlCl_3$, Sprague Dawley rats.*

INTRODUCTION

Iron (Fe) is an essential element for the mammalian body and is required for the synthesis of oxygen carrier proteins and energy metabolism enzymes that use oxygen (O_2) to generate ATP [1,2]. Proteins such as cytochromes, myoglobin, hemoglobin (Hb), peroxidases, and others are required for the normal function of cells such as hepatocytes, myocytes, and erythrocytes. To perform these functions, proteins with Fe are usually synthesized with a heme to allow oxygen binding and transport. This is an important substrate for cellular respiration for electron acceptance [3,4]. These requirements make such elements available to keep metabolism and cell physiology in balance [5,6]. The disruption of this balance can be caused by Fe deficiency or excess, leading to serious damage to the organism.

The dietary contribution of elemental Fe and heme protein with Fe^{2+} is required to maintain iron homeostasis [7,8]. Absorption, transit, and storage of Fe are fundamental processes carried out by heme proteins including hepcidin, ferroportin, transferrin, and ferritin. Hepcidin is a central regulator of iron homeostasis, influencing Fe absorption in the gastrointestinal tract and release from stores in

macrophages and hepatocytes [9,10]. On the other hand, ferroportin is a membrane protein in enterocytes and macrophages that regulates the movement of Fe through the intestinal lumen into the plasma and from the macrophages into the erythrocytes as the final destination [11,12]. To do this, iron must be transported in carrier proteins called transferrin to erythrocyte precursors through the erythropoiesis process and regulate hepcidin expression [13,14]. Finally, ferritin is an iron store in muscle, liver, spleen, and bone marrow [15,16].

On the other hand, soluble forms of aluminum (mainly salts) may be biologically active and potentially hematotoxic [17], and they have been increasingly used in recent years [18]. It is well known that Al toxicity depends on solubility, route of entry, concentration, and exposure time [19]. All mechanisms potentially involved in aluminum toxicity can lead to hematological changes or hematotoxicity [20]. Hence, measuring iron metabolism parameters (iron, total iron binding capacity, transferrin saturation, and ferritin activity) may be a good predictor of hematological alterations or hematotoxicity. Recently, our research group found alterations in iron metabolism due to aluminum exposure [21]. The reasons are still unknown, so we consider it, is important to inquire whether such changes may be associated with changes in iron metabolism gene expression.

The biological activity of ferritin protein is related to iron cell storage capacity [22,23]. Also, changes in the concentration of this protein have been associated with iron overload or deficiency [24,25], and it is well known as an acute reactant since it is involved in infectious and inflammatory processes [26,27]. Finally, it is important to highlight that ferritin is related to the biological activity of many proteins involved with iron metabolism including hepcidin, transferrin, and ferroportin, among others [16,28-30]. Based on this, this work aimed to evaluate changes in blood cell parameters and iron metabolism, including gene expression profile and analytes, caused by subchronic exposure to $AlCl_3$ in Sprague Dawley rats.

MATERIALS AND METHODS

Bioethics.

This research protocol was evaluated and approved by the University of Sonora institutional review board with the code CEI-UNISON 13/2023. This work was performed according to international and national standards in the care and manipulation of animal experimental models to minimize animal suffering [31-34].

Experimental subjects

This experiment included 14 female Sprague Dawley (SD) rats with 50 days born and weight among 180 – 200 g provided by The University of Sonora Food Research and Postgraduate Department vivarium, that were randomized to form two experimental groups.

Experiment

Group A (Control) were 7 SD rats treated with intragastric 0.98 % saline solution, while Group B (Experimental) included an equal number of subjects treated with aluminum trichloride ($AlCl_3$) 40 mg/Kg/day intragastric during 90 days in 5 days a week cycles.

Animals conditions

Standard conditions were used, including 12 hrs dark/light cycles, 45 – 55% humidity and temperature between 24 – 26°C. Each subject was kept in individual cage, and were supplemented with food (Labdiet ® 5001) and water *ad libitum* [35].

Samples collection

After treatment (300 - 320 g weight), the animals were anesthetized in halothane chambers to obtain blood by intracardiac puncture. The rats were immediately euthanized by cervical dislocation. Whole blood samples were collected in heparinized tubes without additives. Whole blood samples were used for complete blood count (CBC) and evaluation of iron metabolism gene expression, while serum samples were used for evaluation of iron metabolism analytes.

Complete Blood Count

Blood cells were analyzed using 23 parameters Celltac ES MEK-7330K (Nihon Khoden; Tokyo, Japan) instrument.

Iron metabolism parameters

Serum samples were used to measure iron metabolism analytes using rat microELISA kits according to the manufacturer's instructions [36]. The parameters evaluated included serum iron, TIBC (Abcam 239715), transferrin saturation and ferritin activity (Abcam157732). MicroELISA was performed using an Eli Read RT-2100C (KONTROLab®) spectrophotometer. (Hamburg, Germany).

RNA extraction

A volume of 0.5 mL of whole blood was used to extract RNA using the TRIzol kit according to the fabricator's protocols [37]. Total RNA concentration and purity were measured using Nanodrop® 2000 (Thermo Fisher Scientific, Waltham, USA) [38]. Nucleic acid extraction and purification using the microvolume technique was previously validated and the expanded uncertainty was estimated by our research group [38,39].

RT-PCR gene expression determination

RNA isolated from experimental Sprague Dawley rats was used for a first RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction) reaction in a LightCycler96® (Roche Diagnostics; Basel, Switzerland). following the next program: RT 50 °C/30 min; Preincubation 95 °C/120 s; Denaturation 95°C /15 s and Annealing/Extension 55 °C/ 50 s for 45 cycles and Cooling 37 °C/ 30 s. Primers and probes sets were designed in-silico using the software BLAST-Primer and sequences deposited in GenBank database (National Center for Biotechnology Information; Rockville Pike, USA). Taqman probes used in these experiments were marked with FAM in 5' while BHQ1 quencher was included in 3'. All sets were synthesized in LGC Biosearch Technologies (Petaluma, CA, USA) in reaction vial containing: 25 µL of 2X reaction mix; 1 µL of sense and antisense primers (10 µM) each; 1 µL of probe (5 µM); 2 µL of SuperScript™ III RT/Platinum™ TaqMix; 10 µL of Template RNA and autoclaved distilled water up to a total volume of 50 µL. Then, master mix was made in a biosafety cabinet type II-A (Labconco™; Kansas City, MO, USA) by using a one-step qRT-PCR SuperScript III Platinum™ (Thermo Fisher Scientific, Waltham, USA) kit following the manufacturer instructions with slight modifications showed in Table 1.

In a second experiment, amplicons were recovered directly from reaction tubes and purified using GFX™ Columns (Cytiva Life Sciences; Marlborough, M.A., USA) following the kit instructions. cDNA eluted samples were quantified in a Nanodrop 2000 (Thermo Scientific; Waltham, M.A., USA), and then normalized to ~10 ng/µL. A calibration curve was done for each target by using cDNA 10-fold dilution from 1×10^{-2} to 1×10^{-6} ng/µL in five different points by triplicate to calculate PCR efficiency. The PCR program was carried out as previously mentioned avoiding RT cycle and adjusting reaction tube to 1 µL template. The calibration curves and PCR parameters were analyzed and obtained from the LightCycler Instrument Software.

Table 1.- Characteristics of the primers and probes used for target genes in the experiment.

Gene	Primer	Oligo (5' → 3')	Tm (°C)	5´ pos	Length (bp)
Ferritin (Fth1) NM_012848.2	Fw	ACCAGCGAGGTGGACGAATC	67.4	351	20
	Rv	AGCCCGCTCTCCCAGTCA	67.4	420	18
	Probe	TGCAGGATATAAAGAAACCTG ACCGTGA	69.4	375	28
Transferrin (Tf) NM_001013110.1	Fw	GCATGGGCTAAGGATCTGAA	65.4	410	20
	Rv	CTCGGTTACAGGCTTCTTGGT A	66.2	484	22
	Probe	AGGAAGACTTCCAGCTGCTGT GCC	69.2	432	24
Hepcidin (Hamp) NM_053469.2	Fw	GAGCAGCGGTGCCTATCTC	67.1	77	19
	Rv	CAGCGCACTGTCATCAGTCTT G	66.5	170	22
	Probe	CGGCAACAGACGAGACAGAC TACG	69.2	96	24
Ferroportin	Fw	AGCCCACGCTTGGAAAGGA	66.4	591	19

(Slc40a1) NM_133315.2	Rv	ACGTCGGCCCAAGTCAGTG	66.3	668	19
	Probe	CGGCAACAGACGAGACAGAC TACG	68.3	618	30
GAPDH (LOC134483923) XM_063279674.1	Fw	TCCGCTGTGGATCTGACA	65.0	482	18
	Rv	GCCTGCTTCACCACCTTCT	66.7	555	19
	Probe	TGCCGCCTGGAGAAACCTGA CAAG	70.4	500	24

Fw: forward; Rv: reverse; Tm: melting temperature; bp: base pair.

Data analysis

A two-tailed Student-t test was used to evaluate the effects caused by the experimental treatment. The analysis was performed using SPSS v.21.0 statistical software (IBM Corp., Armonk, NY, USA) at a 5% significance level ($\alpha = 0.05$). The resultant Ct data from real-time PCR main experiment was analyzed by $2^{-\Delta Ct}$ formula, using GAPDH as housekeeping gene as reference, to obtain the relative expression of each mRNA. The obtained data of expression of housekeeping and each target gene from control group was used as calibrator [40]. The results were used to build a heatmap in NG-CHM Builder web tool (MD Anderson Cancer Center. Houston, Tx. USA) [41] for displaying all relative expression data for each iron metabolism gene investigated in control and experimental groups. One-tail Student-t Test means comparison between groups were done for $2^{-\Delta Ct}$ [40] data for assessing significant differences ($p < 0.05$) by using MedCalc® v.19.3.1 (Ostend, Belgium).

RESULTS AND DISCUSSION

Complete Blood Count

The analysis showed significant differences between the experimental and control groups for some of the white blood cell (WBC) parameters. Treated subjects showed higher levels of WBC ($p = 0.008$) and neutrophils (NE) ($p = 0.005$) and low levels eosinophils (EO) ($p = 0.005$), and basophils (BA) ($p = 0.001$). These levels may be considered significantly high and, therefore, biologically significant for this species (Table 2) [21]. In addition, elevated levels of hemoglobin ($p = 0.047$) and mean corpuscular hemoglobin concentration (MCHC) ($p = 0.046$) (Table 2) were observed, and platelet count (PCT %) was also elevated ($p = 0.047$) (Table 2). In general, significant changes in hematological parameters were observed in the experimental group.

Previous studies by our research group reported that no hematological changes were observed at $AlCl_3$ 10 mg/kg/day for 90 days in SD rats [21]. Based on this work results, hematological changes may be induced with a subchronic dose of $AlCl_3$ 40 mg/kg/day. However, this dose is within the range of human consumption, estimated at 105 - 150 mg/day for a 70 kg adult individual [42-45], which is 1.5 a 2.1 mg de Al/Kg in people with these characteristics but 37.5 - 53 mg for a 0.250 Kg weight rat. This experiment was carried out at $AlCl_3$ 40 mg/kg/day, therefore, it is reasonable to consider that subchronic exposure to $AlCl_3$ might give rise to adverse effects on human health. Considering the hematological effects in SD rats as an experimental model under the conditions studied here, it is important to highlight that dose-response has much influence on the outcome of this study.

Iron metabolism parameters

Many researchers have previously studied the risk effects of $AlCl_3$ exposure in animal models. One of them has found that aluminum may cause damage to the bone marrow provoking erythropoiesis abnormalities that yield to microcytic-hypochromic anemia due to iron deficiency [46]. Bone marrow is one of the major aluminum deposits in the body, and some authors postulate that this element may alter the iron concentration, reducing its bioavailability for normal erythropoiesis [47]. Moreover, aluminum presence in bone marrow has been associated with micronuclei in erythroblasts, as well as the presence of chromatin aberrations in white blood cells [46-48]. On the other hand, Geyikoglu et al. (2012) [49], observed that $AlCl_3$ administration in SD rats resulted in hematological changes including a reduction of RBC, WBC, PLT, Hb, and HCT. A similar outcome has been observed by Asis and Zabut (2011) [50] when evaluating CBC, but not iron metabolism parameters. Nonetheless, Zhang et al. (2011) [51] reported that iron in serum levels stayed unchanged in SD rats when treated with 430 mg $AlCl_3$ at 30 and

90 day exposure. However, increasing iron in serum was observed after 150 - day exposure, similar to TIBC and transferrin levels.

In the past, our research group has found higher micronuclei count in polychromatic erythrocytes while increasing in the number of comets (Olive Tail Moment), produced because of $AlCl_3$ in SD rats, including in shorter exposure time of 15 days [35]. However, hematological changes were not observed at 10 mg $AlCl_3$/Kg/day [21,52]. Differing from our last experiment, in this work we evaluated iron metabolism analytes at a dose of $AlCl_3$ 40 mg/Kg/day.

A decrease of ferritin was observed in the experimental vs control group (463.9 vs 589.5 ng/mL, respectively; p = 0.043) with 21% less serum ferritin in treated subjects. On the contrary, the experimental group had 4.2 times higher TIBC than the control group (65.1 vs 15.5 µmol/L, respectively; p=0.035). These findings may be observed in Table 2. According to Osowata et al. (2020) [53] iron-deficient diets in SD rats may cause a decrease in hematocrit, serum iron, and ferritin, while TIBC is increased, which reflects a lack of iron effect on blood. Therefore, the results obtained in this work do not fit iron deficiency anemia.

Table 2.- Results obtained for the blood count and iron kinetic profile in Sprague Dawley rats from the control group and the experimental treatment.

HEMATOLOGICAL PARAMETER	**CONTROL**	**TREATMENTS**	**p VALOR**
WHITE SERIES			
WBC [10^3/µL]	2.13 ± 0.78	4.92 ± 2.21	0.008*
NE [%]	0.57 ± 0.39	2.41 ± 1.39	0.005*
LY [%]	1.39 ± 0.43	2.07 ± 1.10	0.152
MO [%]	0.11 ± 0.11	0.07 ± 0.07	0.441
EO [%]	0.04 ± 0.06	0.32 ± 0.21	0.005*
BA [%]	0.01 ± 0.01	0.07 ± 0.03	0.001*
RED SERIES			
RBC [10^6/uL]	8.61 ± 0.46	8.95 ± 0.21	0.114
HGB [g/dL]	16.22 ± 0.86	17.01 ± 0.35	0.047*
HCT [%]	45.90 ± 2.45	47.37 ± 1.38	0.190
MCV [fL]	53.41 ± 1.27	52.84 ± 1.20	0.405
MCH [pg]	18.84 ± 0.41	19.00 ± 0.24	0.390
MCHC [g/dL]	35.27 ± 0.44	35.94 ± 0.66	0.046*
RDW-CV [%]	12.17 ± 0.31	12.05 ± 0.43	0.583
RDW-SD [fL]	26.01 ± 0.73	25.47 ± 0.52	0.137
PLATELET SERIES			
PLT [10^3/uL]	631.85 ± 16.83	723.28 ± 23.20	0.098
PCT [%]	0.33 ± 0.04	0.39 ± 0.04	0.047*
MPV [fL]	5.57 ± 0.18	5.67 ± 0.31	0.486
PDW [%]	17.11 ± 0.63	16.91 ± 0.59	0.552
KINETIC PROFILE OF IRON			
Ferritin [ng/mL]	559.36 ± 75.58	479.04 ± 56.05	0.043*
TIBC [umol/L]	22.07 ± 17.24	71.42 ± 52.37	0.035*
Fe [umol/L]	39.39 ± 18.09	42.20 ± 20.02	0.787
STr [%]	2.48 ± 1.51	1.29 ± 1.56	0.173

± Standard deviation. * Significant differences.

Iron metabolism gene expression

The expression of relevant genes related to iron metabolism in mammals was studied here, comparing with concentrations of such analytes in the serum of the murine model Sprague Dawley rats under treatment with $AlCl_3$ 40 mg/kg/day. All relative expression results are displayed in Figure 1 and Table 3, where three main findings can be observed: overexpression of hepcidin and transferrin, while ferritin appears slightly repressed. Such observation in ferritin agrees with that in the iron kinetics analytes measurement (Figure 1), where subjects under treatment showed lower levels of serum ferritin compared to controls. Nonetheless, while differences in ferritin concentrations in serum were statistically significant, the mean difference of relative expression among groups was not, probably due to the small number of subjects.

Table 3.- Expression relative (in $2^{-\Delta Ct}$) [40].

GEN	CONTROL	EXPERIMENTAL	EXP/CTRL	p VALOR
Ferroportin	3.29E-01	2.83E-01	0.86	0.214
Hepcidin	2.26E-04	6.06E-04	2.68	0.027*
Ferritin	4.41E+01	3.62E+01	0.82	0.131
Transferrin	2.12E-01	3.60E-01	1.70	0.139

* = Significant differences.

Ferritin is a member of a family with three different subtypes of proteins structurally composed of 24 subunits of light (FtL) and heavy (FtH) chains forming a cage-complex harboring up to 4,500 iron atoms [54-56]. It is the main iron storage in mammals' bodies and is found in the liver, spleen, bone marrow, and many other tissues [57]. Also, this protein is a crucial component of iron homeostasis, and most disorders of this element kinetics including iron deficiency and hemochromatosis may show changes in serum ferritin, reflecting the nature of each disease [58]. However, this protein also has a role as an acute phase reactant [56], and patients with infectious diseases may exhibit increased levels of ferritin in serum.

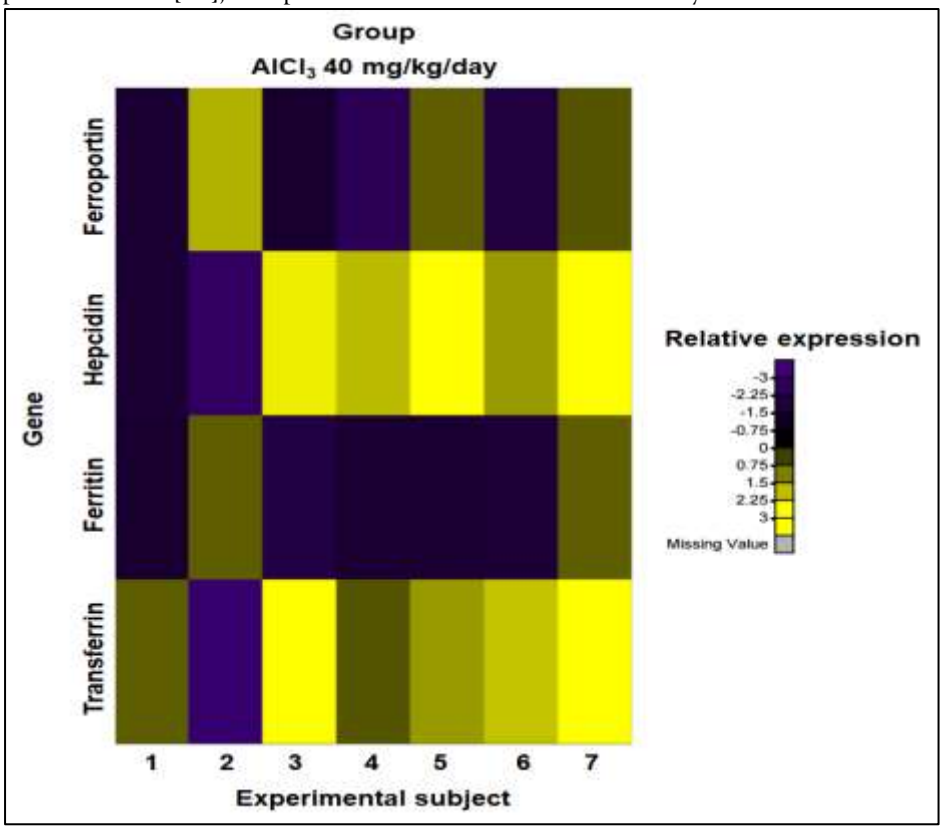

Figure 1.- Relative expression heatmap showing the tendency of the four genes of iron metabolism under investigation in the group of Sprague Dawley rats under treatment with $AlCl_3$ compared with the control group (basal condition). Yellow clusters represent overexpressed genes, while purple clusters are repressed genes. Slightly dark clusters show minor tendencies near to the lack of response. Gray clusters are missing values

In many pathological states involving iron metabolism, ferritin may be observed in abnormal concentrations. An uncommon and less-known cause of elevated ferritin levels is hereditary hemochromatosis, a type of iron overload [59]. In contrast, iron deficiency anemia is the most classic example where ferritin is usually in low concentrations, depending on the inflammatory status of the patient since it is an acute phase reactant [60]. Chronic inflammation is a frequent modifier of the ferritin status, but unlike hemochromatosis, the former is accompanied by an hepcidin increase since it is also an acute phase protein [61-63]. This is a physiological phenomenon followed by hypoferremia, and iron sequestration, which is thought to be related to the iron deprivation of microbes [64,65]. As a result, some patients develop chronic inflammatory anemia [66].

This behavior can be explained by the way the ferritin and hepcidin gene are regulated. It is well known that ferritin is strongly transcriptionally regulated by iron regulatory proteins (IRP) 1 and 2, which bind to iron regulatory elements (IRE) located in transferrin receptor 1 (TfR1) and ferritin genes [67], respectively, when iron concentration drops. The effect is that the TfR1 transcript becomes stable and may be expressed while ferritin and ferroportin remains repressed [68]. Inversely, when iron concentration rises, it binds to the IRPs, impairing interaction with IRE. As a result, TfR1 cannot be expressed, but ferritin is derepressed with a consequent upregulation [55]. The role of hepcidin expression in this mechanism of iron homeostasis is similarly complex and is related to iron and inflammation status. This topic has been extensively reviewed elsewhere [66], but it has been shown that hepcidin concentration correlates with ferritin in humans [69].

Differing from those abnormalities in diseases, ferritin is also known for its role as a protective element of the cell structures against Reactive Oxygen Species (ROS). This is possible by functioning as a protective shell and a detoxifying element of free iron by the H subunit ferroxidase activity [70,71]. Such action is important because free iron (Fe^{2+}) may yield ROS production by a Fenton reaction [64]. Now is clear that ferritin gene expression is also under the control of oxidative stress through the interaction of Nrf2/Small Maf protein with the antioxidant-responsive element (ARE) [55,72,73], located upstream of FtH and FtL genes [74,75]. In contrast, other research has demonstrated that H_2O_2 may enhance IRP1 interaction with IRE in the ferritin gene while stabilizing IRP2 [76-79], and consequently repressing ferritin while expressing TfR1.

On the other hand, iron concentration also induces BMP6 transcription, followed by the Nrf2 activation that subsequently interacts with AREs in BMP6 promoters. BMP6 and 2 induce hepcidin synthesis in the liver, after interacting with type I and II hepatocyte receptors, activating a mechanism that involves SMAD-Complex (Small Mothers Against Decapentaplegic) formation and signaling to the nucleus [64]. Altogether, the common denominator is the transcription factor Nrf2 (nuclear factor erythroid 2-related factor), which is considered a type of “cellular sensor” for oxidative stress status, and as previously mentioned has different effects in ferritin and hepcidin proteins [64,73]. Hence oxidative stress is a possible trigger of such a phenomenon.

In the present study, Sprague-Dawley rats under treatment with $AlCl_3$ 40 mg/kg/day had significantly less ferritin concentration in serum, while relative expression of the same protein was considerably lower than the control group. Control subjects were expected to have a certain level of ferritin expression since iron status in this group is adequate, but the lower level of the same protein in rats undergoing treatment may reflect increased oxidative stress in the organisms. The mechanism by which $AlCl_3$ exerts toxicity is still not completely elucidated, but the Fenton reaction has been proposed to be the way through which Aluminum (Al) reduces Fe^{3+} to Fe^{2+}, yielding to the increase of ROS [80,81] and possibly, a ferritin underexpression in the experimental group. This is supported by the observations of similar iron levels in both groups, while hepcidin expression is significantly overexpressed and ferritin remains slightly repressed. Therefore, it seems plausible that the ferritin drops in serum and changes in iron metabolism gene expression reported by this work could be caused by ROS increasing in animals as a consequence of the treatment.

Our research has important limitations, the most important is related to the small number of experimental and control animals, which may be the cause that the relative expression was not significantly different among groups. Other limitations may be the lack of TfR1 gene expression and serum hepcidin measurement. Still, this is a very good first approach to the mechanism by which $AlCl_3$ exerts its effect in mammals. Future research could increase the number of subjects and include the

aforementioned parameters. Also, antioxidant capacity, dose-response, and longitudinal analysis could be performed to confirm our findings. To the knowledge of the authors, this is the first study exploring the mechanisms of $AlCl_3$ toxicity in animal model involving iron metabolism markers, however, there is still much work to be done.

CONCLUSIONS

The results obtained in this work suggest that subchronic exposure to $AlCl_3$ at 40 mg/Kg/day in Sprague-Dawley rats may cause hematological changes leading to leukocytosis (mainly by neutrophils, eosinophils, and basophils), higher levels of Hb and CHCM, and increase in PCT. Effects on iron metabolism were also observed, including a decrease in ferritin and an increase in TIBC, while genotoxicity was demonstrated by overexpression of hepcidin and transferrin genes and repression of ferritin gene.

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Conflict of interest: The authors declare that there is no conflict of interest.

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