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Original Article

Evaluation of Subchronic Exposure to Aluminium Chloride and N-Nitroso-N-Methylurea on the Hematopoietic System and the Bioavailability of Iron, Copper, and Zinc in Sprague Dawley Rats

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Abstract *- Aluminum (Al) in its soluble form as $AlCl_3$ and N-Nitroso-N-Methylurea (NMU) are considered potentially hemotoxic. For this reason, the objective of this research was to determine whether the administration of Al, alone or in combination with NMU, affects hematological parameters and/or the bioavailability of essential metals. Respecting the postulates of the three R's, 12 Sprague Dawley rats were treated with 10 mg Al/kg body weight/day and administered intragastrically for 15 days. NMU was administered a single dose of 50 mg NMU/kg body weight intraperitoneally at 50 days of age. Blood biometry was performed using a Coulter HmX Hematology Analyzer, whereas peripheral blood was digested with mineral acids using a microwave oven. The concentration of iron (Fe), copper (Cu), and zinc (Zn) was determined using flame atomic absorption spectroscopy. In contrast, Al levels were analyzed using the graphite furnace technique using a Perkin Elmer AAnalyst 400. Statistical analysis was performed using the IBM SPSS statistics package, version 21 and randomization tests in Mathlab version 2014. The results obtained indicate that the administration of $AlCl_3$, alone or in combination with NMU, does not affect blood parameters (hemoglobin, hematocrit, red blood cells, and white blood cells, among others) or the bioavailability of Fe, Cu, and Zn obtained from the diet, under the proposed experimental conditions. The micronucleus analysis was the exception, as for treatment C (+2000 Al/−NMU), an increase in the number of micronuclei resulting in 17.5, 26.2, and 30 micronuclei was observed when increasing the exposure time of 5, 10, and 15 days, respectively, with respect to the other experimental treatments.*

Keywords *- Aluminium, Hematological alterations, Metal bioavailability, Rats, Toxicity.*

1. Introduction

Aluminium (Al) in its soluble forms has been the subject of studies to determine its toxic potential in laboratory animals and humans [1,2]. Human consumption of Al has been estimated to range between 100 and 150 mg/kg body weight of Al per day [3], with the tolerable weekly intake set by the European Food Safety Authority at 1 mg Al/kg body weight [4].

Some investigations have reported that there is an association between soluble forms of Al and iron deficiency anemia and microcytic anemia [5]. Moreover, there is a possibility that Al may interfere with iron metabolism [6,7], and this is directly associated with anemia [8] or with morphological alterations in the erythrocyte membrane [9-11]. Exposure to soluble forms of Al causes a decrease in peripheral blood and/or blood serum and in the levels of essential metals, such as Fe, Cu, Zn, Ca, and Mg, among others [12]. On the other hand, N-Nitroso-N-Methylurea (NMU) is a carcinogenic agent associated with bone marrow alterations and the induction of acute promyelocytic leukemia in Sprague Dawley rats [13,14].

All the mechanisms by which Al can interact with the organism produce morphological or physiological alterations

either in erythrocyte, leukocyte, and platelet counts, in addition to changes in hemoglobin, hematocrit, mean corpuscular volume, and mean platelet volume, among others [15-17]. In most of the afore mentioned studies, there is no information on options related to the applications of the three R's rules in the management of experimental animals. For this reason, in this exploratory pilot study, we intend to evaluate subchronic exposure to Al ($AlCl_3$) and determine whether the administration of Al ($AlCl_3$) intragastrically, alone or combined with NMU, causes hematological alterations in Sprague–Dawley rats, as well as determine the possible impact on the bioavailability of essential metals, such as iron (Fe), copper (Cu), and zinc (Zn). We aimed to conduct the study in accordance with the three R's postulates, which propose to reduce the number of experimental animals, as well as to design or use analytical options with mathematical models or software that allow obtaining reliable exploratory results [18-22]. Results obtained in the present investigation are intended to contribute to the generation of relevant information on the risk posed by exposure to soluble Al salts ($AlCl_3$) in animal models.

2. Materials and Methods

2.1. Bioethical Considerations

The present study was approved by the Research Ethics Committee of the University of Sonora with official letter No. CEI-UNISON 10/2018.

2.2. Experimental Model

This prospective study did not require many experimental animals because it aimed to determine whether subacute exposure to Al causes alterations at the hematological level without reaching chronic exposure levels, which is what has normally been done in similar hematotoxicity experiments. For this reason, in accordance with international regulations on the three R's, which promote reducing the number of experimental animals used in research, among other things [23,24], twelve female Sprague Dawley rats, weighing between 180 and 220 g. and 50 days of age, were selected from the biotherium of the Department of Food Research and Postgraduate Studies of the Universidad de Sonora. For the administration of aluminium trichloride ($AlCl_3$) and NMU, four groups of three rats were assigned completely at random to each of the following experimental treatments: treatment A in which rats were neither administered Al nor NMU (Negative control, +Al/+NMU), treatment B in which rats were administered Al at 2000 mg/L and NMU (+2000 Al/+NMU), treatment C in which rats were administered Al at 2000 mg/L but were not NMU (+2000 Al/-NMU), and treatment D in which rats were not administered Al but were administered NMU (positive control, −Al/+NMU). A dose of +2000 Al is equivalent to a dose of 10 mg $AlCl_3$/kg body weight of the rats/day. Rats that received treatment A were treated with 0.98 % (w/v) physiological saline to equalize animal handling conditions. Subsequently, the rats were administered 1 mL of the Al solution (as $AlCl_3$) intragastrically (gavage) five days a week for 15 days (three weeks). The route of NMU administration was intraperitoneal, at 50 days of age, beginning on the first day of initiation of the experimental treatments [23,24].

The rats were treated with NMU, a genotoxic agent that produces alterations in lymphohematopoietic tissue, among other affected tissues or organs [25,26], at a dose of 50 mg NMU/kg body weight (N-Nitroso-N-Methylurea, N1517-1G Sigma-Aldrich, St. Louis, MO, USA).

2.3. Biotherium Conditions

In terms of the biotherium and diet conditions, the rats were provided with an environment that had 12 hr light/dark cycles, humidity between 40% and 70%, and temperatures between 18ºC and 22ºC, whereas water and food was ad libitum [27] and a basal diet of pellet-type feed was provided [28].

2.4. Blood Sampling

Peripheral blood samples were collected by intracardiac puncture from rats anesthetized in a halothane chamber and subsequently euthanized by cervical dislocation, according to the recommendations of international standards to minimize animal suffering [29,30] (FDA, 2014; EMEA, 2009). Samples were collected in vacutainer tubes with heparin and stored under refrigeration (4ºC) for subsequent laboratory analysis.

2.5. Determination of Complete Hematic Cytometry (CHC) and Hematologic Alterations

These parameters were analyzed using a Coulter HmX Hematology Analyzer (Beckman brand). Subsequently, blood smears were prepared by extension on slides, and Wright staining was performed to look for alterations in the red and white series using light microscopy [31].

2.6. Micronucleus Analysis

Additionally, micronucleus (MN) analysis was performed, and for this purpose, 2000 erythrocytes were counted per experimental treatment and in triplicate, using a LEICA model DM 300 optical microscope.

2.7. Determination of Metals (Al, Cu, Fe, and Zn) in Peripheral Whole Blood

A total of 0.4 ± 0.02 g of peripheral blood was weighed in Teflon tubes, following which 7 mL of concentrated HNO_3 (Baker brand) was added, and the resulting solute was digested in a TITAN MPS microwave oven [32] at 200ºC, 35 bar, and 1600 watts for 47 minutes [33]. Deionized water was added to the resulting acid extract to increase its volume to 100 mL. The metals (Cu, Fe, Zn) in it were determined using flame atomic absorption spectroscopy using a Perkin Elmer AAnalyst 400 model. For Al, a graphite furnace (EAAGF) was used [34] as the amount of sample available for analysis was very low (1.0 g).

2.8. Statistical Analysis

The Shapiro-Wilk normality test was used to determine if the blood cytometry data presented a Gaussian distribution. [35]. This test was performed on the analyses of samples smaller than 50 [36]. Since the data obtained in the study showed a non-normal distribution, the Kruskal–Wallis nonparametric test was performed [37]. $p<0.05$ indicated statistical significance. All analyses were performed with the IBM SPSS statistics package, version 21. However, it should be noted that we only had three observations per treatment group; therefore, the sample size in each group was so small that the application of a conventional statistical technique to compare the groups would be invalid. Therefore, to perform statistical analysis, it was necessary to simulate a randomization test to indicate whether the observed measurements were due to the treatments or whether the observed measurements were attributed to chance. The randomization test involved creating a random combination/permutation of the twelve actual observations from the experiment and assigning them to each group in the resulting random order. Each combination represented the simulation of a complete laboratory experiment. For example, the 12 measurements for the number of red blood cells in peripheral blood were taken and the randomization algorithm was run using the scientific computing software Mathlab version 2014. The same was done for the rest of the hematological parameters evaluated, as well as for the metals Al, Fe, Cu and Zn, with a significance level of 5%.

2.9. Quality Control

To ensure the quality of the analytical data, two certified reference materials (oyster tissue, NIST 1566, and sediment, CRM-S-B) were analyzed to validate the precision and accuracy of the measurements of the metals Al, Fe, Cu and Zn. Calibration curves were performed in triplicate for five different concentrations for each metal for analysis using the atomic absorption technique, obtaining regression coefficients (R^2) and Pearson correlation coefficients (R) that fluctuated in the range of 0.9987 to 0.9998 (Figure 1), which were considered acceptable [38,39]. The recovery percentages obtained for Al, Fe, Cu, and Zn fluctuated between the range of 97.02% and 100.51% in the NIST-1566 certified reference material and between 95.00% and 100.71% in the CRM-S-B certified reference material (Table 1). Results obtained for the recovery percentages for the four metals are acceptable as international standards establish that there should be 100±5% recoveries [40].

3. Results and Discussion

In previous studies, our team had demonstrated that Al ($AlCl_3$) and NMU cause early alterations in hematopoietic tissue of peripheral blood of Sprague Dawley rats, given that increases in the number of micronuclei in erythrocytes have been found along with an increase in the number of comets and clouds of white blood cells determined using comet assay in peripheral blood of Sprague Dawley rats following 5 to 15 days of exposure to $AlCl_3$ [41]. These results justified the conduction of this study. However, the study is novel in that it adhered to the postulates of the three R's in relation to a low number of laboratory animals and used randomization tests using Mathlab software version 2014.

3.1. Determination of Complete Hematic Citometry (CHC) and hematologic alterations

Figure 2 shows the results of blood cytometry of blood smears, indicating no morphological changes between the experimental treatments. Meanwhile, the values obtained for the red series (Hb, Hct, number of red blood cells) show no significant differences ($p\geq0.05$) for these parameters in each of the experimental treatments (Table 2).

This behavior is similar to that occurring in the white series (basophils, lymphocytes, monocytes, among others), as no significant differences were observed ($p\geq0.05$) between the experimental treatments for each of the parameters evaluated (Table 3).

On the other hand, Geyikoglu et al. (2012) [42] reported that chronic exposure to $AlCl_3$ causes a decrease in hemoglobin concentration, hematocrit, number of red blood cells, platelets, and white blood cell count, when Al is administered at a dose of 5 mg Al (as $AlCl_3$) per kg body weight of Sprague Dawley rats, for 10 weeks (70 days).

Farina et al. (2005) [43] reported a behavior similar to that observed by Geyikoglu et al. (2012) [42], but with chronic exposure to Al sulfate in Wistar rats, unlike the values obtained in the present investigation, with subacute exposure to doses of 10 mg of Al (as $AlCl_3$) per kg body weight of rats for 15 days.

Mahieu et al. (2000) [44] reported a behavior similar to that observed by Geyikoglu et al. (2012) [42], but with chronic exposure to Al sulfate in Wistar rats, unlike the values obtained in the present investigation, with subacute exposure to doses of 10 mg of Al (as $AlCl_3$) per kg body weight of rats for 15 days. In this regard, it has been documented that one of the toxic effects of Al is its binding to the erythrocyte membrane with the consequent alteration of the cell membrane [45].

3.2. Micronucleus Analysis

Regarding the results of the micronucleus (NM) analysis, it was found that treatment C (+2000 Al/−NMU) presented a greater genotoxic effect with respect to the other experimental treatments, with significant differences ($p\leq0.05$) as the amount of MN in treatment C is greater at 5, 10, and 15 days of exposure to Al with a micronuclei count of 17.5, 26.2, and 30.0, respectively (Figure 3). It is important to note that although there was no significant effect of exposure to Al ($AlCl_3$) alone or with NMU in the values obtained for the red and white series or in the concentrations of Fe, Cu, and Zn, in

a similar study [41] a genotoxic effect could be proved when evaluating exposure to Al using micronucleus analysis (MA) and corroborated by comet assay (CA). This means that it is likely that the genotoxic effect of subacute Al exposure cannot yet be appreciated at this level of study; therefore, it is possible to observe it at a higher resolution level (MA or CA analysis), or perhaps it is only a matter of time before the effects begin to manifest at this macroscopic (cellular) level. In other words, it is highly likely that the first genotoxic and/or hematotoxic effects can be detected using a micronucleus assay. However, other types of morphological or physiological hematological alterations require more exposure time to manifest themselves from bone marrow to peripheral blood [46].

3.3. Determination of Metals (Al, Fe, Cu, Zn) in Peripheral Whole Blood

With respect to the results obtained, Al alone or combined with NMU does not affect the bioavailability of essential metals (Fe, Cu, and Zn) in the peripheral blood of Sprague–Dawley rats. Table 4 shows that there are no significant differences ($p \geq 0.05$) between the experimental treatments with respect to the concentration of Fe, Cu, and Zn obtained via diet. That is to say, the administration of Al and/or NMU as genotoxic agents do not affect the bioavailability of these essential elements. Similar results are reported in the scientific literature [18,21,47]. However, it should be noted that the administration of Al was performed for 15 days and the same could not be asserted if the administration of Al were for longer or chronic exposure periods.

Finally and in summary, results similar to those previously described were obtained with the Mathlab software version 2014, where it was shown that there are no significant differences between the experimental treatments, except for the micronucleus analysis.

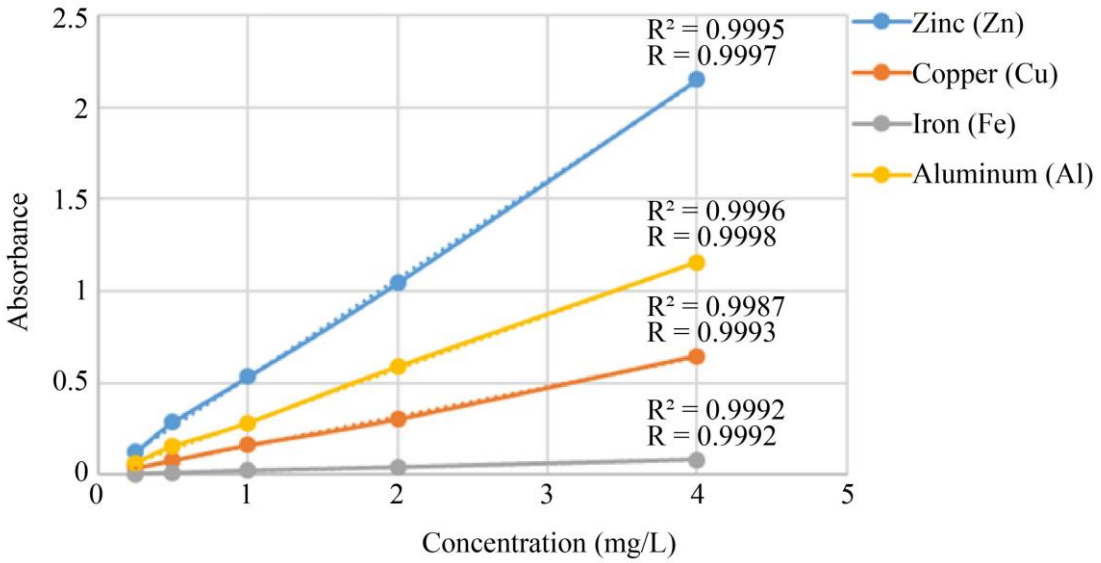

Fig. 1 Calibration curves for aluminum, iron, copper and zinc.

Table 1. Recovery percentages for Al, Fe, Cu and Zn were obtained from two certified reference materials (CRM)

CRM	Aluminium (Al)	Iron (Fe)	Copper (Cu)	Zinc (Zn)
NIST1566b	100.51	98.29	97.02	97.16
CRM-S-B	97.75	100.71	95.00	100.71

CRM NIST1566b is oyster tissue, while CRM-S-B is soil sediment in an aqueous state.

Table 2. Haematological parameters of the red series to determine whether or not there is iron deficiency anemia due to exposure to aluminum

TREATMENTS	Hemoglobin (Hb)	Haematocrit (HTO)	Number of RBC^{+}
GROUP A (-Al/-NMU)	12.93	48.3	7393333
GROUP B (+2000Al/+NMU)	13.19	48.6	7396666
GROUP C (+2000Al/-NMU)	13.47	48.3	7410000
GROUP D (-Al/+NMU)	12.24	48.3	7413333

The value of each determination represents the average of three replications obtained for each of the experimental treatments. ($p \geq 0.05$). * Number of red blood cells.

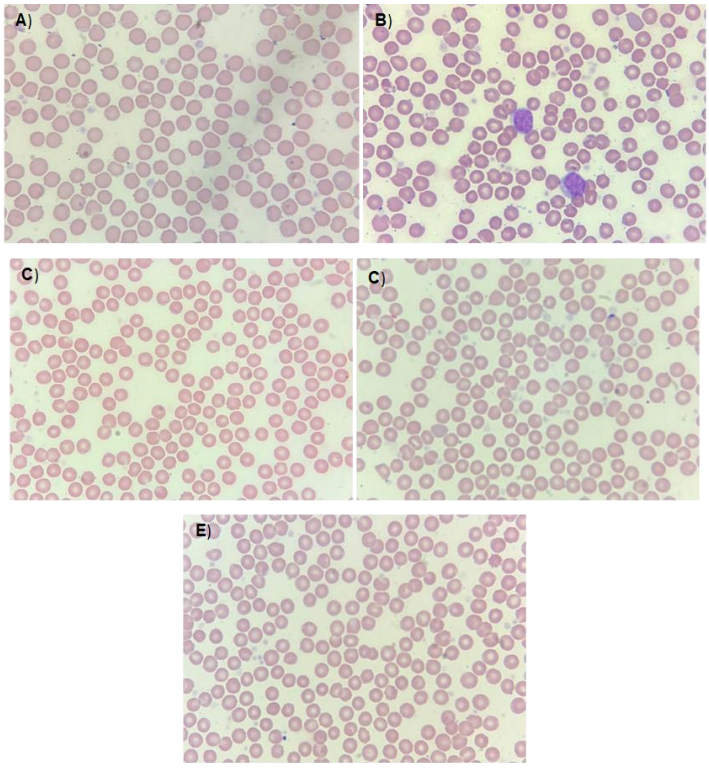

Fig. 2 Blood smears from Sprague Dawley rats stained with wright's stain

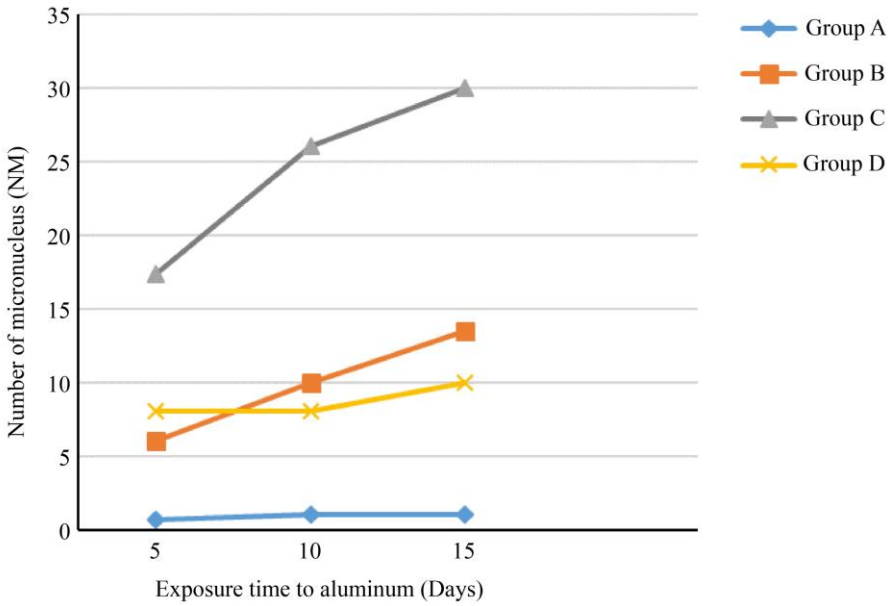

Fig. 3 Number of micronuclei (NM) with respect to the exposure time of aluminum as ALCL, and/or NMU, in days.

Table 3. Determination of the white series count in peripheral blood of sprague dawley rats

TREATMENTS	No. WBC*	Baso	Eosino	Mielo	Juvenil	Band	Lympho	Mono
GROUP A (-Al/-NMU)	6658	0	0	0	0	2	86	3
GROUP B (+2000Al/+NMU)	6650	0	0	0	0	2	90	5
GROUP C (+2000Al/-NMU)	6600	0	0	0	0	2	87	3
GROUP D (-Al/+NMU)	6343	0	0	0	0	2	89	3

The value of each determination represents the average of three replications obtained for each of the experimental treatments. * No. WBC is the number of white blood cells. ($p \geq 0.05$). Baso = Basohyls, Eosino = Eosinophyls, Mielo = Mielocytes, Lympho = Lymphocytes, Mono = Monocytes.

Table 4. Al, Fe, Cu and Zn concentration in peripheral blood of Sprague Dawley rats, determined by the atomic absorption technique by flame (Fe, Cu and Zn) or graphite furnace (Al).

TREATMENTS	Al (µg/L)	Fe (mg/L)	Cu (mg/L)	Zn (mg/L)
GROUP A (-Al/-NMU)	0.401	2.443	0.000	0.078
GROUP B (+2000Al/+NMU)	376.362	2.331	0.003	0.081
GROUP C (+2000Al/-NMU)	372.564	2.223	0.005	0.097
GROUP D (-Al/+NMU)	0.443	2.302	0.007	0.099

The concentration of each element represents the average of three replications obtained for each of the experimental treatments. ($p \geq 0.05$).

4. Conclusion

The results obtained in the present investigation indicate that there is no objective evidence that Al and/or NMU cause toxic effects on subchronic exposure at the hematopoietic level or on the bioavailability of Fe, Cu, and Zn.

Only the analysis of micronuclei indicates evident genotoxic effects in treatment C (+2000 Al/−NMU, equivalent to 10 mg of Al/kg body weight of the rat), as the number of micronuclei increased to 17.5, 26.2, and 30 with increasing exposure time of 5, 10, and 15 days, respectively, with respect to the other experimental treatments. The results obtained in this work contribute to the generation of relevant information regarding the exposure of metals in animal models.

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