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Atmospheric Exposure to Cr III Powder Causes Genotoxicity in *Rattus Norvegicus.*

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Abstract: *Several chemical elements are responsible for altering the genetic integrity of living beings. The metal Cr stands out in this regard. It exists in two oxidation states, Cr VI and Cr III, and has been investigated as an important environmental and occupational contaminant. Although the former is considered carcinogenic, the latter is classified as safe, even for human use in food supplementation. However, most studies with Cr(III) have been carried out by different routes to how it is occupationally found – in the atmosphere. This study evaluated the genotoxicity of Cr(III) inhaled during 8 hours of exposure to the maximum concentration permitted by ATSDR. Fifteen male Rattus norvegicus were used in this study. There were 3 groups (n=5 per group); these were - group exposed to Cr (III) powder (S), the negative control group (NC) and the positive control group (PC). The animals were exposed to Cr aerosol particles at a flow rate of 9L/min and atmospheric concentration of 500µg/m3 for only 8 hours in this study. An increase in genotoxicity and mutagenicity in the group exposed to the metal powder was observed. These findings suggest that further studies should be carried out in order to establish safe levels of exposure to Cr III in work environments*

Keywords: *Inhalation mouse mode, Atmospheric exposure, genotoxicity, mutagenicity, micronucleus assay and comet assay*

I. Introduction

Several chemical elements can alter the genetic integrity of living beings. This change may affect the metabolism of DNA, disrupting its replication and transcription [1]. Mutations and chromosomal aberrations are some possible consequences. If tumor suppressor genes are involved, the onset of diseases is likely to occur [1; 2]. Chromium (Cr) is one of the metals of particular interest [3]. It exists in two oxidation states, Cr VI (hexavalent) and Cr III (trivalent), and it has been investigated as an important environmental and occupational contaminant [4; 5; 6]. Although the former is considered carcinogenic [7; 6], the latter is classified as safe, even for human use in food supplementation [8; 9; 10]. It is believed that Cr III is unable to cross the cell membrane, and cannot change the structure of DNA [4]. However, scientific evidence is challenging this position, and it may play a role in biological systems [11]. Recent findings indicate that Cr III interferes with protein transcription processes [12] and with the base stacking in yeasts [13]. Most *in vivo* toxicological studies assess the oral administration of chromium [14; 15], leaving a gap for investigations that address the effects caused by the exposure through inhalation. This scenario should be taken into consideration, since in tanneries, exposure to this metal occurs mainly through inhalation and some authors suspect it may lead to deaths and DNA damage [16; 17; 18; 19; 20;21; 5; 22). In order to contribute to this discussion, this study aimed to answer a simple question: What happens to the genetic material of *Rattus norvegicus* after approximately 8 hours of exposure to Cr III ?

II. Material And Methods

Chromium III

The Cr III used for this study was the sulfate form (Sigma Aldrich - co d.455954). This is the same form used in tanneries for the treatment of leather [23].

Animals

Fifteen male, 8 weeks of age, *Rattus norvegicus* were used in this study. Their weight was 200g ± 20%, and were supplied by the vivarium of the Faculty of Medical Sciences, State University of Piauí (FACIME/UESPI). During the experiment, each animal was kept in polypropylene cages under controlled conditions (temperature between 22 to 24° C, 40-60% humidity, with light and dark cycles of 12/12h) with *ad libitum* access to food and drink. The Ethics Committee of Animal Use CEUA / FACID approved this study under protocol n^0 24/2013, following the recommendations in the **Guide for Care and Use of Laboratory Animals** (The National Academies Press 2001).

Exposure chamber

To simulate an atmosphere rich in Cr III powder, two $5m^3$ inhalation chambers were used. The negative control chamber received filtered air (HEPA). The treatment group was coupled to a fluidised bed aerosol generator (Model 3400, TSI Inc., St. Paul, MN), which produced the Cr aerosol particles at a flow rate of 9L/min and atmospheric concentration of 500 $\mu g/m^3$ throughout the study. These parameters were based on the maximum daily exposure in work environments recommended by the *Agency for Toxic Substances & Disease Registry* (ATSDR): *http://www.atsdr.cdc.gov/csem/csem.asp?csem=10&po=8*. The two chambers were maintained at a temperature of 22^0C with 40% humidity during the exposure time.

Experimental groups

The animals were divided into 3 groups with 05 animals each, based on the protocol established by the OECD - guideline for the testing of chemicals in vivo mammalian alkaline comet assay: https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oecd/oecd-tg489-2014.pdf. The group exposed to Cr III powder (S), the negative control group (NC) with inhalation chamber as described above and the positive control group (PC), which received cyclophosphamide intraperitoneally at a concentration of 50 mg/kg and was not placed in an inhalation chamber due to the danger of handling the chemotherapeutic agent. The duration of exposure was 8 hours for each rat, which corresponds to more than the 24 hours of life of a human being [24] and to one working day, according to the labor laws in Brazil http: / /www.planalto.gov.br/ccivil_03/decreto-lei/Del5452.htm .

Ethical aspects

The study was approved by the Ethics Committee for Use of Animals of the State University of Piauí (CEUA / UESPI) under protocol number: 24/13.

Genotoxicity and clastogenicity studies

To assess the genetic integrity, genetic damage is measured by comet assay and the degree of calstogenicidade / mutagenicity by micronucleus test. The principle of the comet assay is based on the verification of the genotoxic effect by measuring the size of the comet's tail. This figure is derived after electrophoretic processing [25]. The tail size is proportional to the extent of genetic damage. These can be single strand breaks, double, crosslinks, excision repair sites and / or alkali-labile lesions. Such damage can be repaired (corrected) or not [26]. Therefore, measures the size of the tail 4 hours after the end of exposure to the test agent (acute damage verification) and 24 hours after the end of exposure to the test agent (chronic damage verification or repair) [27]. The degree of mutagenicity can be obtained by micronucleus count. The micronucleus test in vivo is a method designed primarily for screening chemicals that generate chromosome breakage (clastogenicity) [28]. The chromosomal fragment generated is surrounded by a nuclear membrane, similar to the primary nucleus and to have a smaller size is named micronucleus [29].

Comet assay

The comet assay was performed on peripheral blood taken from the tail of each animal. This was achieved after 8 hours of continuous exposure to the Cr III powder. The first sample was processed 4 hours after the end of exposure (acute effect) and the second collection occurred 24 hours after the end of the same exposure (chronic effect). At the end of each period 40 µL of blood was collected and transferred to microtubes containing 120 µL of low melting-point agarose (1.5%) at 37 ^{0}C. The mixture was homogenized and transferred to agarose-covered slides and received coverslips (in order to spread the content homogeneously) and stored at 4 ^{0}C for 30 minutes (da Silva et al. 2010). Then the coverslips were removed and immersed in vertical glass cuvettes containing lysing solution [NaCl (2.5M); EDTA (100 mM) and 1.2 g of TRIS (10mM)]. Upon use, 1% Triton X-100 and 10% of DMSO were added. The slides were placed in a vessel containing electrophoretic buffer, pH> 13.0 (300 mM NaOH and 1 mM EDTA, prepared from a stock solution of 10N NaOH and 200 mM EDTA, pH 10.0) and left to rest for 20 minutes. The electrophoretic "race" was carried out with 25 V and 300 mA at a temperature of 4 ^{0} C, for 15 minutes, in the dark. After electrophoresis, the slides were removed from

the vessel and immersed in neutralizing solution (0.4 M Tris, pH 7.5, for 5 min). This process was repeated 3 times. Finally, they were rinsed with distilled water and dried, flushing them with GelRed (2.0 Dilution: 10,000 µL for 10 minutes). All the slides were analyzed by immunofluorescence microscopy (X40 magnification), equipped with an excitation filter (420-490 nm) and barrier filter (520 nm). The images were captured using an Opton system (CCD digital camera, 5.0 mega pixel for immunofluorescence). DNA damage was evaluated by measuring the percentage of DNA in the tail (%DNA- measurement of the proportion of the total DNA present in the tail) and the momentum tail (TM- tail length times the percentage of DNA in the tail) (Kumaravel et al. 2009). These parameters were calculated on 100 nucleoids/sample (two slides per individual) using the software OpenComet [30].

Micronucleus test

After 24 hours exposure, with the last collection of peripheral blood for the comet assay, animals were sacrificed by cervical dislocation for the extraction of the medulla and subsequent counting of MN in polychromatic erythrocytes, according to Schmid (1975) [28] and Hayashi (1994) [31]. The medullary components were removed with using an insulin syringe containing foetal bovine serum. The syringe was introduced via the medullary canal pushing its contents toward the other extremity, where a 15 ml Falcon tube was placed. Then, the sample was re-suspended in foetal bovine serum and centrifuged for 5 minutes at 1,000 rpm. The supernatant was discarded and, using a Pasteur pipette, 02 drops of the material were smeared on the end of a matt slide. Three slides were prepared and colored with Giemsa for each animal. Finally, the slides were examined under an optical microscope with 100x magnification (immersion oil). For each animal, the number of micronuclei in 2,000 polychromatic erythrocytes was counted.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA). Analysis of variance (ANOVA) with *Tukey* post-test was carried out, at a 5% significance level.

III. Results

Genotoxic data is shown in **Figure 01**. From the significant DNA damage in the exposed group, it can be seen that the percentage of DNA in the tail is greater than the positive control and the negative control. Similar results were obtained in relation to tail length. Although observed differences between the comet 4h and 24h, the two results indicate that the repair was not effective even 24 hours after 8h exposure to Cr III and remained high content when compared to the negative control.

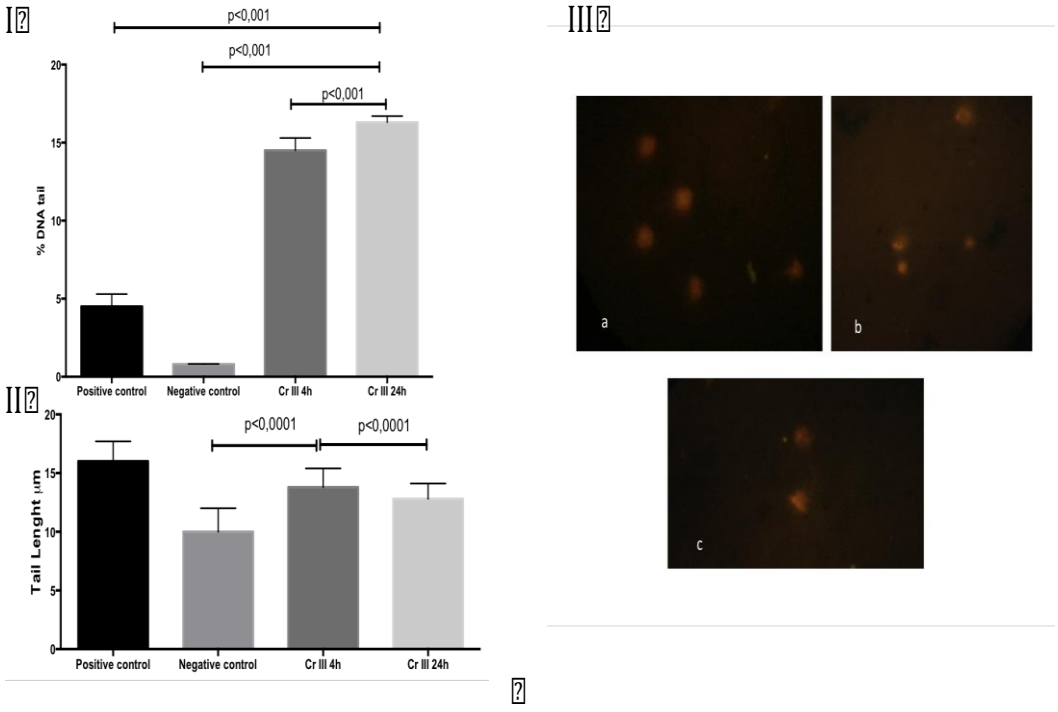

Figure 01. Results found after the comet assay. In (I) % of DNA found in tails and and (II) Tail length of the comets. In (III) patterns observed in the comets in Negative Control (a), Positive Control (b) and Exposed (c). 100 nucleoids were evaluated per animal exposed. ANOVA with *Tukey* post test.

Table 02 shows the results obtained by MN assay evaluation. Note that in E the number of MN is similar to PC and significantly higher than in NC ($P<0.05$).

Table 02. MN frequency in polychromatic erythrocytes in bone marrow of *Rattus norvegicus* exposed to 500 μg/ m^3 of Cr III powder.

Group	PCEMNs		Mean ± Standard deviation
	N^0	%	
NC	18	0,9	3.6 ± 0.89
PC	212	10,6	42.40 ± 11.93
E	178	8.9	35.60 ± 10.19 [a]

Captions: NC = Negative control; PC = positive control (cyclophosphamide 50 mg/kg); E = Exposed (Cr III); PCEMNs = micronuclei in polychromatic erythrocytes; N^0 = number of micronuclei and % = percentage of micronuclei Letter a indicates a statistically significant difference ($P <0.05$) when compared to the NC. There were no significant differences when compared to the positive control. ANOVA with *Tukey*.

IV. Discussion

Chromium III genotoxicity has already been discussed in the literature for some time [11; 12]. However, the existing data, to date, has classified it as non-mutagenic and non-carcinogenic http://monographs.iarc.fr/ENG/Monographs/vol49/mono49.pdf and http://www.atsdr.cdc.gov/toxprofiles/tp7.pdf. In our study, the findings indicated that the damage produced after 8 h exposure to Cr III powder in *Rattus norvegicus* remained high even 24 hours after the first measurement. This indicates that 24 was not enough time to repair the high damage caused by Cr. It is known that DNA repair is essential for homeostasis and, therefore, cell and tissue survival [31]. In most cases, repair restores the original molecule. However, when this does not occur, somatic mutations accumulate, deregulating transcriptional processes and produce tissue disorders with the development of diseases such as cancer [1]. Similar results were found by Fang (2014) [13] when a cell culture was exposed to varying concentrations of trivalent chromium. Cytogenetic evaluation corroborated the genotoxic data. The number of micronuclei found in the polychromatic erythrocytes in the bone marrow of *Rattus norvegicus*, points to high chromosomal damage. These findings reinforce the existing suspicions about the role that Cr III plays in the unhealthy environments of tanneries and related industries. Epidemiological studies have shown that in places like that, the exposed individuals have a high level of DNA and chromosomal damage [17; 19; 22]. It is worth stressing that the concentration used for Cr III powder was the same recommended by the ATSDR, as the maximum tolerable limit in work environments (http://www.atsdr.cdc.gov/csem/csem.asp? CSEM = 10 & po=8 .). Although these data are preliminary, requiring further *in vivo* investigations, the authors recommend further studies, in order to obtain the safe exposure values .

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