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Evaluation of Clastogenic/Aneugenic Damage Using the FISH Micronucleus Assay in Mice Exposed to Chromium (VI)

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Abstract. *Background/Aim: Exposure to chromium (VI) [Cr(VI)] has been postulated to be associated with the induction of cancer. In vivo studies utilizing biomarkers of genotoxic damage could aid in elucidating the mechanisms underlying the genotoxic effects of Cr(VI) and their relationship with carcinogenesis. In this study, the origin (clastogenic and/or aneugenic damage) and kinetics of micronuclei (MN) induced by Cr(VI) were investigated. Materials and Methods: Hsd:ICR female mice were divided into groups of five individuals each. MN kinetics were measured in groups treated with 20 or 25 mg/kg CrO_3 intraperitoneally using acridine orange-coated slides in peripheral blood obtained from the caudal vein 0, 12, 24, 36, 48, 60, and 72 h after treatment. Whereas identification of MN with centromeric DNA (MNK+) was measured at the dose of 20 mg/kg of CrO_3, using fluorescence in situ hybridization (FISH) with a centromere-specific probe in peripheral blood obtained at 0, 12, and 48 h after treatment. Control groups were administered vehicle only. Results: Total MN were quantified and the clastogenic/aneugenic effects of Cr(VI) were evaluated based on the proportion of MNK+ versus micronuclei without centromeric DNA (MNK–). There was a significant increase in MN frequencies beginning at 12 h in the Cr(VI)-treated groups demonstrating its genotoxicity. When calculating the MNK+ as a percentage of the total MN, the increase was significant beginning 12 h after treatment. Conclusion: The fact that the MNK+ and MNK– were observed at both evaluation times corroborates Cr(VI) as a genotoxic agent and demonstrates that both clastogenic and aneugenic damages are involved in the formation of MN.*

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Key Words: Hexavalent chromium, fluorescence *in situ* hybridization analysis, aneugenic, clastogenic, micronucleus test.

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The International Agency for Research on Cancer (IARC) has placed chromium (VI) [Cr(VI)] in Group 1, carcinogenic to humans (1). Human exposure to Cr(VI) is not rare. Currently, these compounds are used in several industries, including electroplating, welding, leather tanning, and pigment manufacturing. They can also be produced by combustion from automobiles and tobacco consumption (1, 2). Intracellular reduction of Cr(VI) can generate oxidative damage to DNA leading to DNA adduct formation, cross-linking (DNA-protein and DNA-DNA), abasic sites, and oxidized DNA bases (3, 4). These effects may be related to clastogenic damage leading to different health disorders such as cancer. Moreover, alterations such as the loss of chromosomes, originating from deficiencies in chromosome migration or nondisjunction may play a role in the onset of neoplasia. It is important to determine the origin of the damage (clastogenic and/or aneugenic) in order to propose prevention or treatment strategies against Cr(VI)-induced carcinogenic effects. Although studies are showing that exposure to Cr(VI) compounds has clastogenic and aneugenic effects (4-6), there are currently no studies *in vivo* that explore the mechanisms underlying the induction of micronuclei (MN) by Cr(VI) in the peripheral blood of mice, which is an important research gap.

This study initially focused on investigating the kinetics of micronucleus (MN) induction. Subsequently, the study aimed to determine the *in vivo* origin (clastogenic and/or aneugenic damage) of the MN induced by Cr(VI). The MN assay was used because it is a relevant biomarker of chromosomal/genomic damage to detect genotoxic agents

(7). MN kinetics were evaluated with acridine orange-coated slides and the origin of clastogenic and/or aneugenic damage in the formation of Cr(VI)-induced MN was identified using fluorescence *in situ* hybridization (FISH).

Materials and Methods

Chemicals. All the test chemicals and reagents were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Chromium trioxide [CrO_3, purity grade 99.9%, CAS 1333-82-0], acridine orange (AO) [CAS 10127-02-3].

Animals. Female Hsd:ICR mice were obtained from Harlan® ("Facultad de Química-UNAM", Mexico City, Mexico) and allowed to acclimate for two weeks prior to initiating the experiments. The mice (8-12 weeks old, 28-35 g) were randomly divided into groups of five individuals each. They were kept in plastic cages in a temperature-controlled room (22±2˚C) and with a 12-h light-dark cycle. They had free access to food (Purina®-Mexico, Mexico City, Mexico, small rodent chow) and water.

Experimental design. The Cr(VI) group was injected with CrO_3 intraperitoneally immediately after the solution was prepared. The control group was treated with the vehicle (distilled water) only. Although the intraperitoneal (*ip*) route is an artificial exposure route, it is useful for detecting genotoxic damage in short-term assays, such as MN, when testing compounds with potential clastogenic properties as it is more sensitive and direct (8). Thus, the *ip* route of administration was selected in this study since our aim was to examine the direct genotoxic damaging effects of CrO_3. The evaluation criteria and working conditions were set up according to the OECD guidelines (Test No. 474) for the testing of chemicals: mammalian erythrocyte MN test (7). Experimental protocols were approved by the Bioethics Committee of the "Facultad de Estudios Superiores-Zaragoza", UNAM (Code: FESZ-CE/21-118-13).

The doses of CrO_3 applied to the organisms to establish the kinetics of MN were based on the LD_{100} (30 mg/kg) using historical laboratory control data. The LD_{100} was reduced by 1/3 (20 mg/kg) and 1/6 (25 mg/kg). In accordance with OECD guidelines (Test No. 474) (7), the four CrO_3 doses were included in the MN kinetics graph: a) historical data from laboratory control (15 and 30 mg/kg) and b) experimental data from the present study (20 and 25 mg/kg).

Micronuclei assay. Genotoxic damage was assessed using the short-term MN protocol in peripheral blood. The evaluations were performed on sequential peripheral blood samples obtained from the same individuals. All groups' 0-h samples were also designated as negative control. Two slides were prepared for each mouse. The assessments of MN were performed based on 4,000 polychromatic erythrocytes (PCE) per mouse. In parallel, the relative proportion of PCE to normochromatic erythrocytes (NCE) was analyzed in 2,000 erythrocytes.

MN kinetics were evaluated using acridine orange-coated slides in three groups: (i) animals treated with vehicle only (vehicle water, distilled); (ii) animals injected with CrO_3 (20 mg/kg); and (iii) animals injected with CrO_3 (25 mg/kg). Moreover, the origin of MN (clastogenic *versus* aneugenic damage) was evaluated using FISH in four groups: (i) animals treated with vehicle only (vehicle water, distilled); and (ii) animals injected with the Maximum

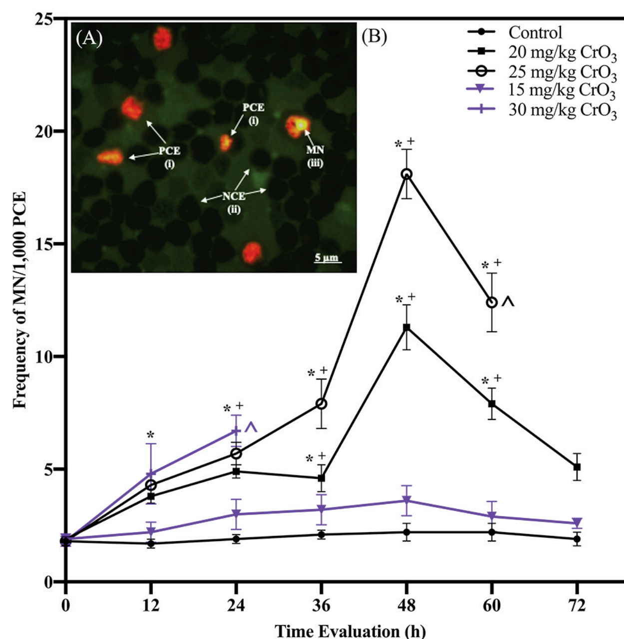

Figure 1. *Peripheral blood erythrocytes of mice treated with chromium trioxide. (A) Fluorescent microphotograph (1,000×) using the AO coating method. PCE stain fluorescent orange (i), NCE do not stain (dark shadow) (ii), and MN fluoresces yellow (iii). (B) Kinetics of the MN (mean±S.E./1,000 PCE) observed from 0 to 72 h with the 20 and 25 mg/kg CrO_3 treatments. A total of 4,000 PCE were evaluated in each mouse (n=5 mice/group). ^Three and five mice died at 60 and 72 h, respectively. ^Five mice died at 24 h. Statistical significance was determined using two-way repeated measures-ANOVA followed by Tukey's post-hoc test. Analysis by treatments: *$p<0.05$ vs. control; $^{+}p<0.05$ vs. 0 h. Control: Vehicle only; CrO_3: chromium trioxide; PCE: polychromatic erythrocytes; NCE: normochromatic erythrocytes; MN: micronuclei; AO: acridine orange; SE: standard error; ANOVA: analysis of variance.*

Tolerated Dose (MTD) of CrO_3 that showed the highest induction of MN based on the results of the kinetics. FISH studies were done in duplicate.

Acridine orange-coated slides. Sequential peripheral blood samples (5 ml) were obtained from the same individuals (0, 12, 24, 36, 48, 60, and 72 h) and placed directly onto slides previously treated with AO, as described by Hayashi *et al*. (9). The slides were stored in the dark at 4˚C for 24 h. The assessments were performed by identifying PCE, NCE, and MN in PCE using a fluorescence microscope (NikonTM OPTIPHOT-2; Tokyo, Japan) with blue excitation (480 nm) and a barrier filter emission (515-530 nm) at 100× magnification.

Fluorescence in situ hybridization analysis. Sequential peripheral blood samples (5 ml) were obtained from the same individuals (0, 12, and 48 h) and were spread directly on slides, allowed to dry at room temperature, and fixed with absolute methanol. The FISH analysis for the identification of centromeres in the MN (MNK+) was performed according to the manufacturer's instructions (Mouse Centromeric Probe Oncor Hybridization kit S1340, Gaithersburg, MD, USA). The

Table I. *Average number of MN, MNK+, and the ratio of polychromatic erythrocytes to normochromatic erythrocytes in the peripheral blood of mice treated with chromium trioxide.*

Treatment	Dose (mg/kg)	Time analysis (h)	n	PCE/NCE 1,000 cells (mean±S.E.)	MN /1,000 PCE (mean±S.E.)	MNK+ /1,000 PCE (mean±S.E.)
Control	0	0	10	35.0±2.6	1.9±0.5	0.1±0.1
		12		34.6±3.3	2.3±0.4	0.0±0.0
		48		36.0±3.0	2.7±0.4	0.2±0.1
CrO_3	20	0	10	35.7±2.8	1.7±0.4	0.1±0.1
		12		23.8±2.4	3.1±0.4	0.8±0.2d
		48		28.6±3.2	14.1±0.9[a,b,c]	3.8±0.3[a,b,c]

For MN, a total of 4,000 PCE, and PCE/NCE ratio, a total of 2,000 erythrocytes were evaluated in each mouse (n=5 mice/group). Control: Vehicle only; CrO_3: chromium trioxide; PCE: polychromatic erythrocytes; NCE: normochromatic erythrocytes; MN: micronuclei; MNK+: MN with centromeric DNA. Two-way repeated measures-ANOVA: [a]$p<0.0001$ *vs*. CrO3, 0 h; [b]$p<0.0001$ *vs*. CrO3, 12 h; [c]$p<0.0001$ *vs*. control, 48 h; [d]$p<0.0465$ *vs*. CrO_3, 0 h.

cell samples were incubated in 2xSSC at 37˚C, dehydrated with ethanol (70%, 85%, and 100%), denatured with formamide (70%, 70˚C), and dehydrated with cold ethanol. The centromeric probe was denatured at 70˚C and immediately placed on the slide with the target DNA (erythrocytes) and incubated for 24 h (37˚C) for hybridization to take place. Hybridized and washed sections were coverslipped with propidium iodide/antifade and were observed with a fluorescence microscope (DIA-ILL and TRIPLE D-F-T filters, Nikon™ OPTIPHOT-2; Tokyo, Japan) at 1,000× magnification.

Statistical analysis. The total MN and MNK+ averages and the PCE/NCE ratio are expressed as the mean±standard deviation (SE). Statistical significance for these parameters was determined using two-way repeated measures-ANOVA followed by Tukey's post hoc test. The percentages of MNK+ and MN without centromeric DNA (MNK–) were analyzed using Fisher's exact test. Data normality was verified with the Shapiro-Wilk test. GraphPad Prism 8.0® for macOS (Software 8.4.2, San Diego, CA, USA) was used for analyses, and tests with $p<0.05$ were considered significant.

Results

The genotoxic damage caused by Cr(VI) was evaluated using the MN assay in erythrocytes of peripheral blood. First, the kinetics of MN were determined using AO-coated slides. PCE exhibited orange fluorescence due to the presence of ribosomal RNA (Figure 1A), while NCE did not stain (Figure 1A). The AO also enabled the identification of MN, which exhibited yellow fluorescence due to their DNA content (Figure 1A). Figure 1B shows the kinetics of MN from 0 to 72 h when doses of 20 and 25 mg/kg of CrO_3 were administered. The doses of 15 and 30 mg/kg of CrO_3 correspond to historical control data. In these studies, although the 15 mg/kg treatment induced an increase in MN, it was not significant compared to the control group and against their own time 0. The treatment of 30 mg/kg induced the death of all organisms after 24 h. Based on these data, in the present study, the doses of 20 and 25 mg/kg of CrO_3 were selected to determine the time in which the greatest induction of MN occurs. The 20 and 25 mg/kg of CrO_3 treatments increased the MN beginning at 12 h after administration; significant results were obtained from 24 and up to 60 h compared to the control group and against their own hour 0. In the graph (Figure 1B), two peaks in the frequencies of MN are observed in the group treated with the 20 mg/kg dose. The first peak corresponds to an early induction between 12 and 24 h, and the second (48 h) corresponds to the greatest observed effect. Mice in the CrO_3 groups showed clinical signs of toxicity, including bristly hair, decreased motility, and loss of appetite. The 25 mg/kg dose induced the death of 3 mice at 60 h, and the death of the entire group at 72 h.

Table I shows the MN values (totals and MNK+), as well as the PCE/NCE ratio observed at 0, 12, and 48 h after Cr(VI) treatment in peripheral blood using FISH with the centromeric DNA probe. In the CrO_3 group, the PCE/NCE ratio decreased from 35.7 to 23.8 at 12 h, showing a slight increase to 28.6 at 48 h. The decrease was not significant against the control group and its own control (0 h). In this same group, an increase of about 12 MN was observed at 48 h, which was significant when compared to the MN observed before treatment administration (0 h) and to the control group at 48 h. In the control group, centromeric DNA was detected in the MN observed at 0 and 48 h. In the CrO_3 group, MN with centromeric DNA were detected from 0 h, and the difference relative was significant at 12 and 48 h (Table I). Centromeric DNA was identified in these MN using FISH with the centromeric DNA probe: (i) PCE stained pale red due to the presence of ribosomal RNA; (ii) NCE did not stain because the RNA had been degraded; (iii) MNK– exhibit yellow fluorescence due to their DNA content; and (iv) MNK+ show centromeric DNA as red dots

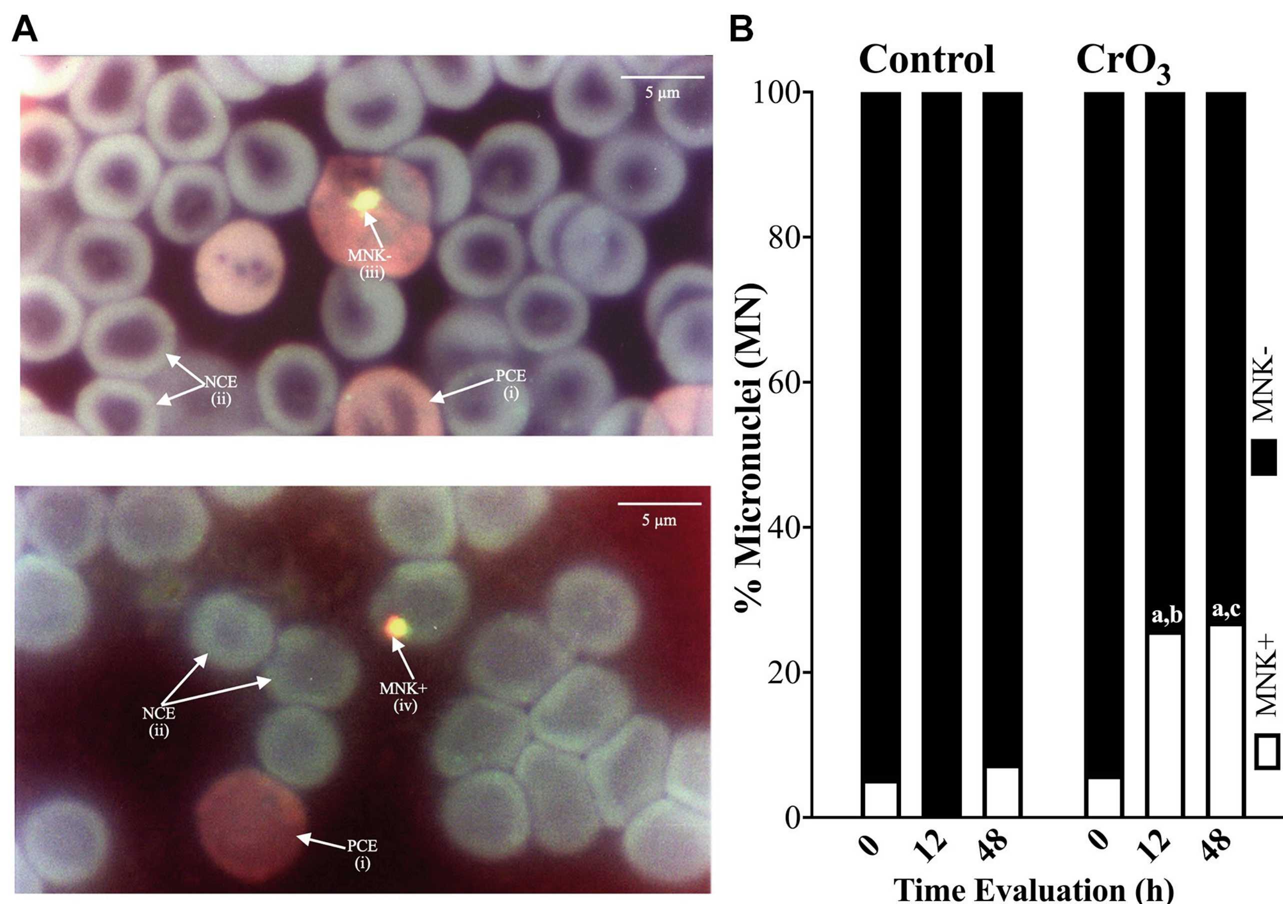

Figure 2. *Peripheral blood erythrocytes of mice treated with chromium trioxide. (A) Microphotograph (1,000×) using fluorescence in situ hybridization (FISH) with a centromeric DNA probe. PCE stain fluorescent pale red (i), NCE does not stain (white shadow) (ii), MNK– fluorescent yellow (iii), and MNK+ stain fluorescent yellow with a deep red dot (iv). (B) Data show the MN percentages (MNK+ or MNK– divided by the total MN) observed at 0, 12, and 24 h. A total of 4,000 PCE were evaluated in each mouse (n=5 mice/group). Fisher's exact test: [a]p<0.0002 vs. CrO_3, 0 h; [b]p<0.0001 vs. control 12 h; [c]p<0.0001 vs. control, 48 h. Control: Vehicle only; CrO_3: chromium trioxide; PCE: polychromatic erythrocytes; NCE: normochromatic erythrocytes; MN: micronuclei; MNK–: micronuclei without centromeric DNA; MNK+: micronuclei with centromeric DNA.*

in addition to yellow fluorescence (Figure 2A). Figure 2B shows the percentages of MNK+ and MNK– relative to the total MN observed in peripheral blood erythrocytes at 0, 12, and 48 h after Cr(VI) treatment. In the CrO_3 group, both MNK+ and MNK– were observed from 12 h. The increase in the percentages of MNK+ (25.8 and 27% at 12 and 48 h, respectively) was also significant when compared to the MNK+ observed before treatment administration (0 h) and to the control group.

Discussion

In this study, the genotoxic damage caused by Cr(VI) was assessed in the erythrocytes of the peripheral blood of mice using the short-term MN protocol with acridine orange-coated slides. These MN evaluations did not require the sacrifice of the animals; thus, we were able to explore MN kinetics by drawing sequential peripheral blood samples from the same individuals. A significant increase in MN frequencies (12-60 h after CrO_3 treatment) demonstrated Cr(VI)-induced genotoxicity. This is consistent with previous findings evaluating Cr(VI)-induced genotoxicity (10, 11), and particularly by CrO_3 (12, 13). The underlying genotoxic mechanism of the action of Cr(VI) has been linked to the intracellular reduction of Cr(VI) to Cr(III). During this chemical process ROS such as •OH are generated (14, 15). These species can induce cytogenetic damage which results in the formation of MN (11). Although direct oxidative damage to DNA is considered clear evidence of the clastogenic effect of Cr(VI) (4), supplementary MNK+ analysis may improve the sensitivity of the assay to detect both clastogenic effects and chromosomal instability. Hence, the MN assay with FISH

was used to identify MNK+ at 12 (early induction time) and 48 h (highest effect time). Applying FISH using centromeric DNA probes in the MN assay makes it possible to discriminate between chromosome breakage and chromosome loss, derived from either impairment in chromosome migration or non-disjunction (16). MNK– indicates that the MN contains only chromosome fragments (clastogenic damage), whereas the MNK+ indicate chromosome loss (aneugenic damage). Although the presence of MNK+ in the MN observed at 12 and 48 h confirms aneugenic effects in the group treated with CrO_3, the frequencies of MNK+ are low (25.8 and 27%, respectively), indicating that the main pathway in the MN induction is by DNA breaks. In *in vitro* studies performed on human lung cells, exposure to lead chromate was found to increase aneuploidies in a dose- and time-dependent manner. These findings suggest that centrosome dysfunction leads to the induction of aneuploidies that represent a possible mechanism in carcinogenesis (5). Eleftheriou *et al*. (6) reported that treatment with Cr(VI) interacted with microtubules in cells from the root tip of *Lens culinaris*. These observations could be related to the presence of MNK+ since Cr(VI) interacting with tubulin could induce the lag of chromosomes that were not included in the nuclei of the daughter cells during mitosis.

The toxic effects of the 25 and 30 mg/kg doses induced death at 60 and 24 h respectively, corroborating the toxic potential of exposure to this metal (1, 2). Although the cytotoxic effects of Cr(VI) have been documented in different study models (4), no marked changes in the PCE/NCE ratio were observed in the Cr(VI) group. Assessment of the PCE/NCE ratio is included in the MN assay as it may be an indicator of cytotoxicity (17). However, negative results must be interpreted with caution because, when toxicity occurs during erythropoiesis, cell division mechanisms are activated and may mask this effect (17, 18).

Conclusion

In summary, this study confirmed the genotoxicity of Cr(VI) and provided *in vivo* evidence that the formation of MN in mice's PCE involves both clastogenic and aneugenic damage. The identification of Cr(VI) as an aneugenic damage-inducing agent, in addition to its clastogenic properties, is an important endpoint for genotoxicity tests. Understanding the origin of MN can help elucidate the relationship between chromosomal/genomic damage and the initiation of carcinogenic processes resulting from exposure to metals like Cr(VI). However, further research is required to gain a deeper understanding of the underlying mechanisms involved in the formation of MN induced by metal exposure. This will aid in development of specific strategies to prevent or treat the harmful effects of these potentially carcinogenic metals.

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Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

MCG-R designed this experiment. MCG-R, YS-M, BM-A and MA-L conducted experiments. MCG-R, YS-M, BM-A and MA-L analyzed data. MCG-R and MA-L analyzed results. MCG-R prepared these figures. MCG-R wrote the manuscript. MCG-R and BM-A contributed to manuscript editing. MCG-R and BM-A revised the manuscript. All Authors participated in reading and reviewing of the manuscript.

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References

1 IARC Working Group on the Evaluation of Carcinogenic Risks to Humans: Arsenic, Metals, Fibres and Dusts. Lyon, France, International Agency for Research on Cancer, 2012.
2 U.S. Environmental Protection Agency (EPA) 2010: IRIS Toxicological review of hexavalent chromium: CAS No. 18540-29-9 (External Review Draft). Washington, DC, EPA/635/R-10/004A. Available at: https://cfpub.epa.gov/ncea/iris_drafts/recordisplay.cfm?deid=221433# [Last accessed on May 18, 2023]
3 DesMarais TL, Costa M: Mechanisms of chromium-induced toxicity. Curr Opin Toxicol 14: 1-7, 2019. DOI: 10.1016/j.cotox.2019.05.003
4 McCarroll N, Keshava N, Chen J, Akerman G, Kligerman A, Rinde E: An evaluation of the mode of action framework for mutagenic carcinogens case study II: Chromium (VI). Environmental and Molecular Mutagenesis 51(2): 89-111, 2010. DOI: 10.1002/em.20525
5 Holmes A, Wise S, Sandwick S, Lingle W, Negron V, Thompson W, Wise J: Chronic exposure to lead chromate causes centrosome abnormalities and aneuploidy in human lung cells. Cancer Research 66(8): 4041-4048, 2022. DOI: 10.1158/0008-5472.CAN-05-3312
6 Eleftheriou E, Adamakis I, Fatsiou M, Panteris E: Hexavalent chromium disrupts mitosis by stabilizing microtubules in *Lens culinaris* root tip cells. Physiologia Plantarum 147(2): 169-180, 2022. DOI: 10.1111/j.1399-3054.2012.01652.x
7 Organization for Economic Cooperation and Development (OECD): Test No. 474: Mammalian erythrocyte micronucleus test. OECD Guidelines for the testing of chemicals. Paris, France, OECD Publishing, Vol. 4, pp. 1-21, 2016.
8 Hayashi M, Sutou S, Shimada H, Sato S, Sasaki Y, Wakata A: Difference between intraperitoneal and oral gavage application

in the micronucleus test. Mutation Research/Genetic Toxicology 223(4): 329-344, 2019. DOI: 10.1016/0165-1218(89)90081-5

9 Hayashi M, Morita T, Kodama Y, Sofuni T, Ishidate M: The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange-coated slides. Mutation Research Letters 245(4): 245-249, 2019. DOI: 10.1016/0165-7992(90)90153-b

10 Sawicka E, Jurkowska K, Piwowar A: Chromium (III) and chromium (VI) as important players in the induction of genotoxicity - current view. Annals of Agricultural and Environmental Medicine 28(1):1-10, 2020. DOI: 10.26444/aaem/118228

11 Ventura C, Gomes B, Oberemm A, Louro H, Huuskonen P, Mustieles V, Fernández M, Ndaw S, Mengelers M, Luijten M, Gundacker C, Silva M: Biomarkers of effect as determined in human biomonitoring studies on hexavalent chromium and cadmium in the period 2008-2020. Environmental Research 197: 110998, 2022. DOI: 10.1016/j.envres.2021.110998

12 García-Rodríguez MC, López-Santiago V, Altamirano-Lozano M: Effect of chlorophyllin on chromium trioxide-induced micronuclei in polychromatic erythrocytes in mouse peripheral blood. Mut Res/Genet Toxicol Environ Mutagen 496(1-2): 145-151, 2001. DOI: 10.1016/s1383-5718(01)00225-x

13 Nicolás-Méndez T, Kacew S, Ortiz-Muñiz AR, Mendoza-Núñez VM, García-Rodríguez MC: Protective effect of resveratrol against hexavalent chromium-induced genotoxic damage in Hsd:ICR male mice. Molecules 27(13): 4028, 2022. DOI: 10.3390/molecules27134028

14 Shi X: Reduction of chromium (VI) and its relationship to carcinogenesis. Journal of Toxicology and Environmental Health, Part B 2(1): 87-104, 2016. DOI: 10.1080/109374099281241

15 Maeng S, Chung H, Yu I, Kim H, Lim C, Kim K, Kim S, Ootsuyama Y, Kasai H: Changes of 8-OH-dG levels in DNA and its base excision repair activity in rat lungs after inhalation exposure to hexavalent chromium. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 539(1-2): 109-116, 2021. DOI: 10.1016/s1383-5718(03)00154-2

16 Iarmarcovai G, Botta A, Orsière T: Number of centromeric signals in micronuclei and mechanisms of aneuploidy. Toxicol Lett 166(1): 1-10, 2006. DOI: 10.1016/j.toxlet.2006.05.015

17 Krishna G, Hayashi M: *In vivo* rodent micronucleus assay: protocol, conduct and data interpretation. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 455(1-2): 155-166, 2019. DOI: 10.1016/s0027-5107(00)00117-2

18 Tweats D, Blakey D, Heflich R, Jacobs A, Jacobsen S, Morita T, Nohmi T, O'Donovan M, Sasaki Y, Sofuni T, Tice R: Report of the IWGT working group on strategies and interpretation of regulatory *in vivo* tests. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 627(1): 78-91, 2019. DOI: 10.1016/j.mrgentox.2006.10.005

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