Genotoxic effects of mercury chloride on the Neotropical fish *Andinoacara rivulatus* (Cichlidae: Cichlasomatini)

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Abstract: Understanding the effects of heavy metals in aquatic ecosystems is of significant importance due to their potential to bioaccumulate at various trophic levels and induce damage in DNA. Mercury is considered one of the most dangerous heavy metals, causing chromosomal breakage (clastogenic event) or spindle dysfunction (aneugenic event), that can lead to the formation of encapsulated chromatin into a separate smaller nucleus, generally referred to as a micronucleus. We evaluated the sensitivity of the micronucleus test in the neotropical cichlid *Andinoacara rivulatus* (Günther 1860). The fish were divided into four groups of 16 individuals, and each group was placed in separate aquaria (140 L) provided with filtered water and constant aeration. Fish were exposed to mercury chloride ($HgCl_2$) at doses 0.1, 0.25, and 0.50 mg/kg body weight, administered by intraperitoneal (IP) injection. Fish from the control group were injected with a physiologic solution. The following erythrocyte anomalies were identified: erythrocytes with micronuclei varying to some extent in size and position in the cytoplasm, blebbed nucleus, binucleated cell, nuclei showing a deep invagination toward the center (notched nuclei). Examination of blood smears demonstrated a higher level of micronucleus and notched erythrocytes in fish injected with $HgCl_2$ than in the controls. There were significant differences in the frequency of micronucleated and notched erythrocytes among the groups exposed to mercury. Linear regression analysis revealed a positive relationship between the frequency of micronucleated and notched erythrocytes (P< 0.0001), with a moderately strong correlation coefficient (R= 0.737). We propose that, in addition to the two so far known mechanisms of micronucleus formation (spindle apparatus damage and chromosomal ruptures), chromatin fragmentation in notched nuclei resulting from a combination of the cytotoxic effects of mercury and mechanical stress, may be a third mechanism of micronuclei genesis.

Key words: micronuclei; erythrocyte nuclear abnormalities; DNA damage; heavy metal; fish.

Heavy metals released into aquatic ecosystems from industrial, agricultural, and domestic sources, municipal sewage treatment plants, and from other anthropogenic activities have the potential to bioaccumulate at various trophic levels and can induce damage in DNA (Mitchelmore & Chipman, 1998; Ohe, Watanabe, & Wakabayashi; 2004, Bolognesi, Perrone, Roggieri, Pampanin, & Sciutto, 2006; Isani et al., 2009; Sharifuzzaman et al. 2016).

Thus, given the extreme toxicity of heavy metals, understanding their effects in aquatic ecosystems is of significant importance.

Mercury is one of the most dangerous heavy metals, causing deleterious effects on biota (W.H.O., 1990; Buhl, 1997; Rocha et al. 2011a; Anual, 2014; Nabi, 2014; Kandroo, Tripathi, & Sharma, 2015; Morcillo, Esteban, & Cuesta, 2017). This toxicant interferes with the formation of the mitotic spindle during cell division, causing the contraction of chromosomes, a delay in the division of the centromere, and a slower movement during anaphase, as well as chromosomal breakage (Skerfving, Hansson, & Lindsten, 1970; Catherine Ferens & United States, 1974; Thier, Bonacker, Stoiber, & Böhm, 2003). Chromatin fragments derived from chromosomal breakage (clastogenic event) or spindle dysfunction (aneugenic event), or even whole chromosomes in the case of chromosome lag, do not migrate to the poles during anaphase and are included in the daughter cells, becoming encapsulated into a separate smaller nucleus, generally referred to as a micronucleus (Miller,1973; Miller et al., 1998; Wirzinger, Weltje, Gercken, & Sordyl, 2007).

Fishes have a greater ability to metabolize xenobiotics, accumulate pollutants, and are capable of inhabiting practically all zones of the aquatic habitat. In addition, unlike mammals, fish erythrocytes are nucleated and have been shown to be more sensitive to the induction of DNA damages (Mir et al., 2014). These characteristics make them an excellent model for assessing the potential danger of chemicals introduced into the aquatic environment. Indeed it has been shown that after exposure to different pollutants under field and laboratory conditions the peripheral erythrocytes of fishes have a higher incidence of micronuclei formation (Al-Sabti & Hardig, 1990; Heddle et al., 1991; Vanparys, Deknudt, Vermeiren, Sysmans, & Marsboom, 1992; Torres de Lemos, Rödel, Terra, D'Avila de Oliveira, & Erdtmann, 2007; Ali, El-Shehawi, & Seehy, 2008; Yadav & Trivedi, 2009; Monteiro, Cavalcante, Viléla, Sofia, & Martinez, 2011; Özkan, Gündüz, Berköz, & Hunt, 2011; Bakar, Ashriya, Shuib,

& Razak, 2014; Fatima et al. 2014; Vignardi et al, 2015; Ivanova & Popovska-Percinic, 2016). Therefore, fishes are suitable organisms in which to evaluate the clastogenic and aneugenic effects of genotoxic compounds, with the assay being called the micronucleus test (MNT), a widely applied method due to its simplicity, reliability, sensitivity, and adaptive suitability (Carrasco, Tilbury, & Myers, 1990; Al-Sabti & Metcalfe, 1995; Udroiu, 2006; Junín, Rodríguez, Heras, & Braga, 2008). Indeed, research on clastogenic or mutagenic effects in neotropical fish has become increasingly common (Cestari et al., 2004; Rocha et al., 2011b; Zapata et al., 2016).

Some authors have observed, in addition to micronucleated erythrocytes (MNE), the occurrence of other nuclear abnormalities (ENAs), such as blebbed, lobed, binucleated, and notched nuclei, suggesting that these anomalies should be considered analogous to MNs, and, therefore, should be taken into consideration as potential indicators of cytotoxicity (Carrasco et al., 1990; Ayllon & Garcia-Vazquez, 2000, 2001; Çavaş & Ergene-Gözükara, 2003, 2005; da Silva Souza & Fontanetti, 2006; Matsumoto et al., 2006; Ergene et al., 2007; Jiraungkoorskul et al., 2007; Costa et al., 2008; Monteiro et al., 2011; Tasneem & Yasmeen, 2018). Our preliminary, unpublished observations, showed a significant proportion of erythrocytes with notched nuclei in fish exposed to mercury, leading us to investigate if this nuclear anomaly could be effectively employed as a genotoxic marker. Thus, in the present study, we assessed the proportion of MNE and notched erythrocytes (NE) after the administration of mercury chloride in *Andinoacara rivulatus* (Günther 1860), a freshwater teleost fish naturally distributed from the north of Ecuador to the north of Peru, and also spread throughout the world as an aquarium pet colloquially known as Vieja Azul or Green Terror.

MATERIALS AND METHODS

All specimens used in this study were acquired from a stock obtained via induced

reproduction that is carried out by the repopulation program of the species in La Tembladera Wetland, in El Oro Province of Ecuador (http://asogrotem.org/). Fish were transported in plastic bags to the laboratory and acclimated for 24 h.

Bioassay, blood sampling and smear preparation: Fish were divided into four groups of 16 individuals, and each group was placed in separate aquaria (140 L) provided with filtered water and constant aeration. Fish were not fed while the test was ongoing. Mercury chloride ($HgCl_2$) was administered by a single intraperitoneal (IP) injection (Al-Sabti & Metcalfe, 1995). Concentrations of 20, 50, and 100 μg/ml of mercury were used in a ratio of 0.5 ml of solution per 100 g of weight (0.1, 0.25, and 0.50 mg $HgCl_2$/Kg). Fish from the control group were injected with a physiologic solution. The mercury doses tested were chosen considering the mercury content (mg/kg) reported in some freshwater species of teleosts (*Cyprinus carpio* Linnaeus 1758*,* 0.1-0.5, max. 0.8; *Gambusia affinis* (Baird & Girard 1853), max. 0.5; *Lepomis macrochirus* Rafinesque 1819, 0.1-0.3, max. 0.41; *Micropterus salmoides* (Lacepède 1802) 0.35-0.85, max. 1.9) (Saiki, Jennings, & May, 1992). Four individuals from each group were randomly selected every 24 h until 96 h, and peripheral blood smears were prepared by draining blood via caudal vein puncture using a syringe without anticoagulant. All the fish were anesthetized with benzocaine before blood extraction. The fish of the control group were immediately released while the fish injected with mercury were euthanized with anesthetic overdose, incinerated and the ashes placed in plastic bags and buried in a landfill. Smears were air-dried, fixed in absolute ethanol for 20 min, and stained with 10 % Giemsa in phosphate buffer (pH 6.88) for 20 min. Three slides per fish were mounted using Canada balsam and archived until evaluation. The procedures were conducted in accordance with Institutional Authorization Research Project 396/2016, Universidad Técnica de Machala and Protocol number 1027 for experiments with animals of Universidade Estadual Paulista "Julio de Mesquita Filho".

Cell scoring: On each plate, a series of 15-20 photographs were taken at random with an Olympus BX53 photomicroscope (1 000 x magnification) to guarantee that no fewer than 1000 cells/slide were counted. Scoring was performed on the digital images by a single observer. The frequencies of MNE and/or NE were expressed per 1 000 counted cells.

Statistical analysis: Levene's and Kolmogorov-Smirnov tests were applied to check the homoscedasticity and normality, respectively. Since data obtained did not present a normal distribution and violated the homogenous variance principle for both MNE and NE frequencies, they were square root transformed to normalize and homogenize the variances. Then, data were analyzed by two-way ANOVA and Fisher's Least Significant Difference (LSD) post-hoc test ($P \leq 0.05$) for comparison of differences among groups. The relationship between MNE and NE frequencies was analyzed using linear regression analysis by the least squares method. Pearson's correlation coefficients were calculated between NE and MNE. The entire analysis was done with the statistical package STATGRAPHICS® Centurion XVI (version 16.1.18).

RESULTS

Fish exposed to 0.50 mg/kg of $HgCl_2$ died progressively during the period of the experiment. At 96 h only one fish was still alive in this group. Therefore, data from this group were excluded for statistical analysis. The analysis of peripheral blood smears of the studied fish showed that normal erythrocytes appeared with an oval shape and a centric ellipsoid nucleus with well-defined boundary (Fig. 1A). The following nuclear abnormalities were found: small, non-refractive circular/ovoid particles in the cytoplasm resembling a nucleus with respect to staining properties were considered an MNE varying to some extent in
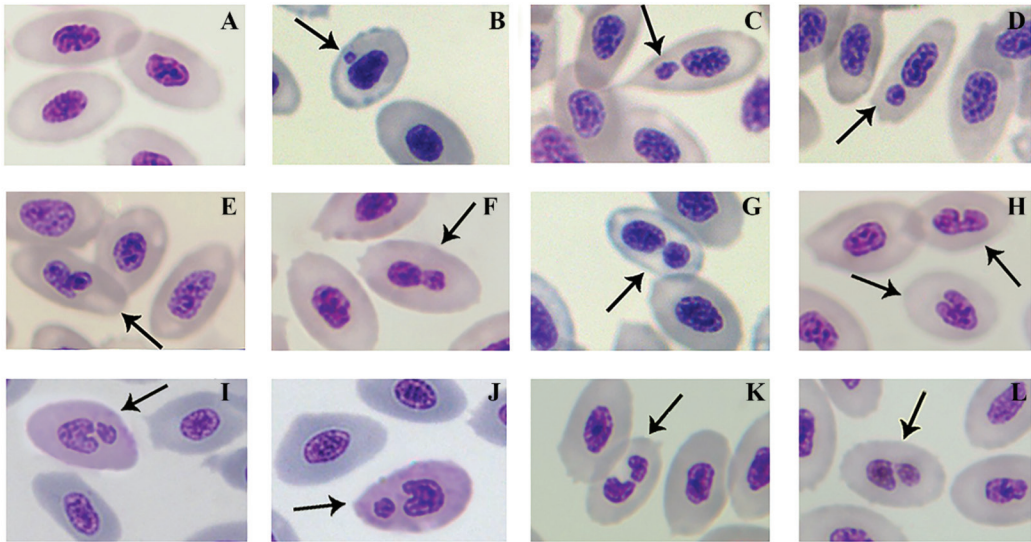
Fig. 1. Photomicrographs showing cells with normal nucleus **(A)** and nuclear abnormalities (arrows) in peripheral blood erythrocytes of *Andinoacara rivulatus* exposed to HgCl$_2$: micronuclei **(B, C, D)**, blebbed nucleus **(E, F)**, binucleated cell **(G)**, Notched nucleus **(H)**, putative fragmented notched nucleus **(L)**.

size and position in the cytoplasm (Fig. 1B, Fig, 1C, Fig. 1D); blebbed nucleus appeared as a small evagination of the nuclear envelope resembling a micronucleus (Fig. 1E, Fig. 1F); binucleated cell contains two nuclei that are not attached and relatively similar in size (Fig. 1G); nuclei showing a deep invagination toward the center were considered an NE (Fig. 1 H). Some fractured notched nuclei were also observed (Fig. 1I, Fig. 1J, Fig. 1K, Fig. 1L).

Compared with the control group, fish exposed to HgCl$_2$ showed an increase in the frequency of MNE and NE with a peak in MNE 24 h after dosing (Fig. 2). Two-way ANOVA with three levels of concentration of HgCl$_2$ (0, 0.1, 0.25 mg/kg) and four levels of exposure time (24, 48, 72, 96 h), followed by a post-hoc LSD test revealed significant differences in the MNE and NE among groups exposed to different concentrations of HgCl$_2$ (Table 1). Simple linear regression analysis between the frequency of MNE and NE (Fig. 3) revealed a moderately strong relationship (F = 54.84, P < 0.0001), with the linear model

$$\sqrt{MNE\,^0/_{00}} = -0,28801 + 0,2957\sqrt{NE\,^0/_{00}}$$

and a correlation coefficient R = 0.74. The adjusted model explains 54.38 % (R2) of the variability in $\sqrt{MNE\,^0/_{00}}$.

DISCUSSION

Fish exposed to HgCl$_2$ showed an increase in the frequency of MNE and NE, demonstrating that the species here studied is sensitive to mercury at the concentration used. Similar time-dependent effects in MNE frequency were also reported in *Oreochromis niloticus* (Linnaeus 1758), *Phoxinus phoxinus* (Linnaeus 1758), and *Poecilia latipinna* (Lesueur 1821*)* exposed to different heavy metals (Ayllon & Garcia-Vazquez, 2000; Jiraungkoorskul et al., 2007).

Differences in sensitivity to the induction of MNE and other erythrocyte nucleus anomalies among fish species have been reported after IP injection of mercurial compounds in *Salmo trutta* Linnaeus 1758*, P. phoxinus* (Sanchez-Galan et al., 1999), and *Channa punctata* (Bloch 1793) (Yadav & Trivedi 2009). Here, a low frequency of MNE and NE in the control group indicates that a percentage of these
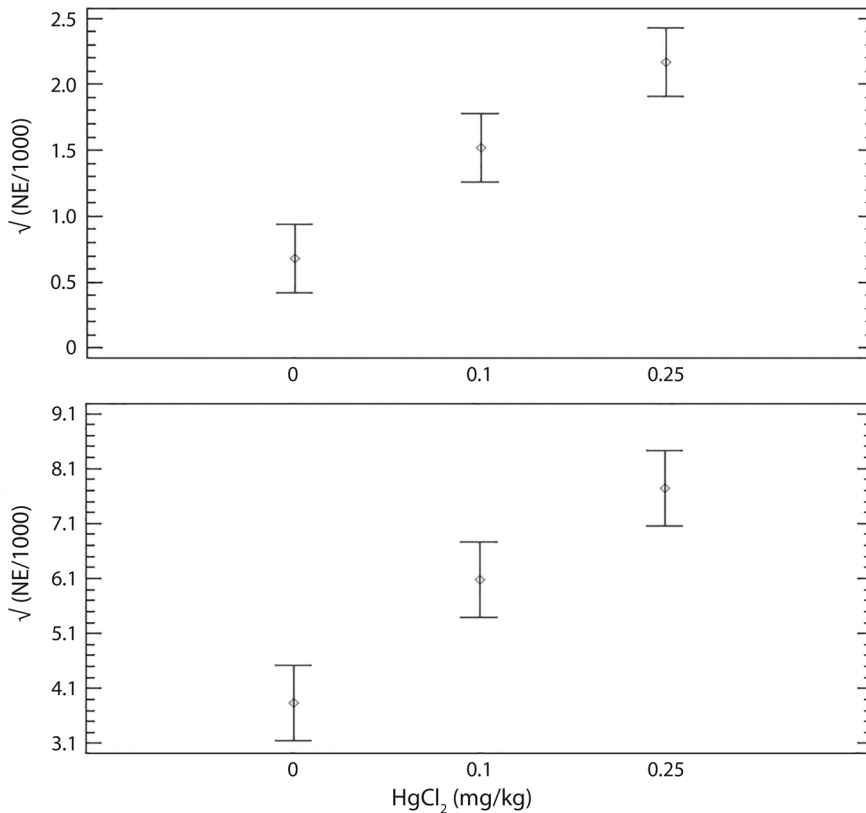
Fig. 2. Frequency of micronucleated erythrocytes (MNE) and erythrocytes with notched nucleus (NE) in *Andinoacara rivulatus* exposed to HgCl$_2$ (mg/kg). Bars represent 95 % Fisher's Least Significant Difference.

TABLE 1

Mean ± standard deviation of micronuclei (MN), notched nuclei (Notch), and MN+Notch per 1 000 erythrocytes in
Andinoacara rivulatus injected with two doses of HgCl$_2$ and subjected to 96 h of exposure

HgCl$_2$ (mg/g)	n	√MN	LSD	√Notch	LSD	√(MN+Notch)	LSD
0	16	0.68 ± 0.56	X	3.84 ± 1.75	X	3.93 ± 1.75	X
0.1	16	1.52 ± 0.94	X	6.08 ± 2.40	X	6.31 ± 2.46	X
0.25	16	2.15 ± 0.92	X	7.71 ± 1.76	X	8.05 ± 1.79	X
ANOVA		F= 16.96 *** p= 0.0002		F= 16.90 *** p= 0.000		F= 19.20*** p= 0.000	

Time (H)	n	√MN	LSD	√Notch	LSD	√(MN+Notch)	LSD
24	12	1.86 ± 1.09	X	5.29 ± 2.12	X	7.03 ± 2.29	X
48	12	1.35 ± 0.97	XX	5.33 ± 2.52	X	5.57 ± 2.54	X
72	12	0.94 ± 0.83	X	6.19 ± 2.54	X	5.41 ± 2.58	X
96	12	1.66 ± 1.03	X	6.75 ± 2.93	X	6.43 ± 3.05	X
ANOVA		F= 3.77 * p= 0.0189		F= 0.195 ns p= 0.1950		F= 1.92 ns p= 0.1433	
Interaction HgCl$_2$ x Time		F= 1.88ns p= 0.1116		F= 1.35ns p= 0.2591		F= 1.57ns p= 0.1853	

F-tests (F) and probability values (p) on Analysis of Variance (ANOVA) with results of post-hoc test (LSD) for establishing significantly different means are shown.
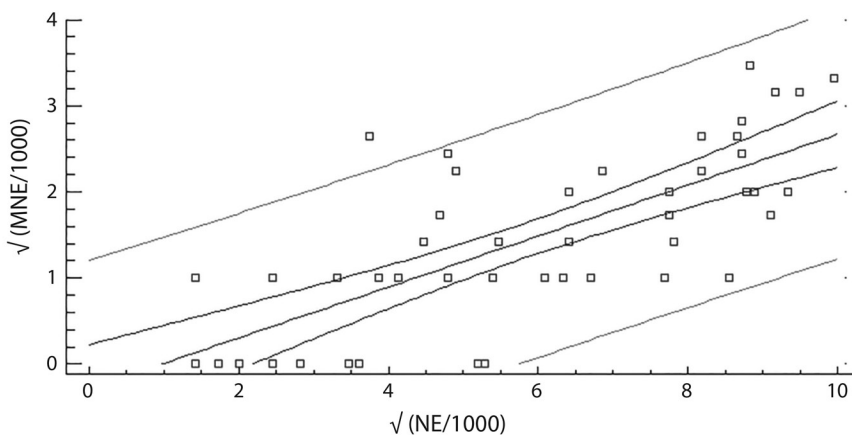
Rev. Biol. Trop. (Int. J. Trop. Biol. ISSN-0034-7744) Vol. 67(4): 745-754, September 2019

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Fig. 3. Regression between micronucleated erythrocytes (MNE/1 000) and cells with notched nuclei (NE/1 000). Data was square root transformed.

abnormalities were generated naturally. Indeed, spontaneous micronucleus frequencies (control) have been observed in several species of fish such as *Astyanax bimaculatus* (Linnaeus 1758) (Matsumoto & Cólus, 2000), *S. trutta, Anguilla anguilla* (Linnaeus 1758), *P. phoxinus* (Rodriguez-Cea, Ayllon, & Garcia-Vazquez, 2003), *Mullus barbatus* Linnaeus 1758 (Bolognesi et al., 2006), *Rhamdia quelen* (Quoy & Gaimard 1824), *C. carpio, Hypostomus punctatus* Valenciennes 1840, *O. niloticus* (Salvagni, Ternus, & Fuentefria, 2011), and *Colossoma macropomum* (Cuvier 1816) (Rocha et al*.,* 2011b), with analogous frequency to that found in this study.

Cell populations treated with a genotoxic agent must undergo mitosis for MNE to appear (Savage, 2000; Harding et al*.*, 2017). In fishes, erythrocytes arise from small lymphoid hemoblasts maintained by continuous mitotic divisions in the intertubular tissues of the kidney, although in some fishes like trout, the spleen is also active, while in others like perch, only the spleen is active (Catton, 1951). In fishes, a peak in micronucleated erythrocytes occurs 1-5 days after exposure to genotoxic substances, but in most species, it takes place after 2 or 3 days (Udroiu, 2006). Thus, the MNE peak here detected at 24 h suggests that mitotic spindle poisoning and/or the clastogenic effects of mercury (or their reactive metabolites) arose

from already active mitotic cells after dosing. By the contrary, the lower frequency of MN after 72 and 96 h of exposure could be a result of a weakening of the normal process of cell division (mitosis) in the hematopoietic tissues, probably produced by the toxic effect of mercury. Indeed, the decrease in the erythrocyte count in *Astronotus ocellatus* (Agassiz, 1831), *Anabas scandens* (Daldorff, 1797), *Salmo trutta caspius* Kessler, 1877*; Hypophthalmichthys molitrix* (Valenciennes, 1844), exposed to chronic doses of mercurial compounds has been attributed to the impairment of erythropoiesis due to direct effect of this heavy metal (Rodríguez et al., 2017).

The results of linear regression analysis revealed that an increase in the frequency of notched cells was accompanied by a proportional increase in micronuclei bearing cells, with a high significant correlation index (R = 0.74; P < 0.0001), suggesting a direct relationship between these nuclear anomalies. These data are congruent with previous results obtained in other fishes like *Clarias gariepinus* (Burchell, 1822), *Alburnus orontis* Sauvage, 1882*, Mugil cephalus* Linnaeus, 1758 (Ergene et al., 2007), *Dicentrarchus labrax* (Linnaeus, 1758), and *Mugil* spp. Linnaeus, 1758 (Strunjak-Perovic, Topic Popovic, Coz-Rakovac, & Jadan, 2009a). The cytotoxicity of mercury compounds causes damage in cell

function, resulting in the inability of cells to proliferate (Silva-Pereira et al., 2005). Studies in rats exposed to $HgCl_2$ have shown that this metal has a high affinity for chromatin in the cell nucleus (Rózalski & Wierzbicki, 1979) and studies on the effect of mercury on the nucleus of *Dictyostelium discoideum* Raper, 1935, a species of soil-living amoeba recognized as a model organism by the American National Institute of Health (http://www.nih.gov/science/model), revealed that 10 mM of $HgCl_2$ provokes an increase in MNE frequency, in addition to the carbonylation and quantitative changes in nuclear proteins (Boatti et al.*,* 2017). Other compounds, such as the herbicide sulfentrazone, have induced MNE and nuclear DNA fragmentation on *Allium cepa* L. cells (Bianchi et al., 2016).

Mechanisms of erythrocyte nuclei deformity have not yet been explained and there is no consensus about the causes of these changes (Strunjak-Perovic, Coz-Rakovac, Topic Popovic, & Jadan, 2009a, 2009b). Recently, it has been proposed that severe nuclear deformations can also originate from physical forces, such as the compression of the nucleus during migration through confined spaces that can lead to nuclear envelope rupture (Shah, Wolf, & Lammerding, 2017).

Although further studies are required to explain the mechanisms involved in the concomitant variation of MNE and NE in *A. rivulatus*, it is likely that mechanical stress might contribute to generation of chromatin fragmentation. In other words, in addition to the known cytotoxic effects of mercury on spindle apparatus damage and chromosomal ruptures, local defects in nuclear envelope might also results from a third mechanism linked to mechanical stress.

ACKNOWLEDGMENT

RESUMEN

Efectos genotóxicos del cloruro de mercurio en el pez neotropical *Andinoacara rivulatus* (Cichlidae: Cichlasomatini). Comprender los efectos de los metales pesados en los ecosistemas acuáticos es de gran importancia debido a su potencial de bioacumulación en los diversos niveles tróficos y de inducir daños en el ADN. El mercurio, considerado uno de los metales pesados más peligrosos, causa fracturas cromosómicas (evento clastogénico) o disfunción del huso mitótico (evento aneugénico), y puede conducir a la formación de fragmentos de cromatina encapsulada en un núcleo separado más pequeño, generalmente denominado micronúcleo. En este trabajo se evalúa la sensibilidad del test de micronúcleos en el cíclido neotropical *Andinoacara rivulatus* (Günther 1860). Los peces fueron divididos en cuatro grupos de 16 individuos, y cada grupo se colocó en acuarios separados (140 L) provistos de agua filtrada y aireación constante. Los peces fueron expuestos al cloruro de mercurio ($HgCl_2$) en dosis de 0.1, 0.25 y 0.50 mg/kg de peso corporal, administrada por inyección intraperitoneal (IP). Los peces del grupo control fueron inyectados con solución fisiológica. Se identificaron las siguientes anomalías en los eritrocitos: micronúcleos que varían en tamaño y posición en el citoplasma, núcleo con evaginaciones, células binucleadas núcleos con muesca (núcleos con una profunda invaginación hacia el centro). El examen de los frotis de sangre demostró un mayor nivel de eritrocitos micronucleados y con muesca en peces inyectados con $HgCl_2$ que en los controles. Fueron detectadas diferencias significativas en la frecuencia de eritrocitos micronucleados y células con núcleos con muescas entre los grupos expuestos al mercurio. El análisis de regresión lineal reveló una relación positiva entre la frecuencia de eritrocitos micronucleados y con muescas (P< 0.0001), con un coeficiente de correlación moderadamente fuerte (R= 0.737). Se propone que, además de los dos mecanismos hasta ahora conocidos de formación de micronúcleos (daño del huso mitótico y rupturas cromosómicas), la fragmentación de la cromatina en núcleos con muesca probablemente a causa de la combinación de los efectos citotóxicos del mercurio y el estrés mecánico, podría ser un tercer mecanismo de génesis de micronúcleos.

Palabras clave: micronúcleos; anomalías nucleares de eritrocitos; daño en el ADN; metal pesado; peces.

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