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Mutagenic and histopathological effects of hexavalent chromium in tadpoles of *Lithobates catesbeianus* (Shaw, 1802) (Anura, Ranidae)

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ABSTRACT

The potential mutagenic and histopathological effects of the hexavalent chromium were investigated in *Lithobates catesbeianus* tadpoles. These larvae (GS 25-31) were exposed to three nominal concentrations of potassium dichromate (4, 12, and 36 mg L^{-1}) and 5 mg L^{-1} of Cyclophosphamide as a positive control (PC), for 24 h. A negative control (NC) was also added to the experiment. Our results showed that, in general, the micronuclei (MN) were less frequent than the erythrocyte nuclear abnormalities (ENA); there was a significant difference in the frequency of MN between the NC and all treated groups ($p < 0.05$) in a concentration-dependent curve, in addition the PC did not differ from the chromium treatments. Also, only PC and the group treated with potassium dichromate at 36 mg L^{-1} showed significantly higher frequencies of ENA than NC ($p < 0.05$). Chromium treatments promoted cell retention in the Sub-G1 phase and a decrease of cells in the S and G2/M phases indicating inhibition of the cell cycle. All treatments with chromium led to liver and kidney histopathological lesions, especially with 36 mg L^{-1} (greater number of lesions). In conclusion, hexavalent chromium was mutagenic to *L. catesbeianus* tadpoles and its toxic effects also resulted in anti-mitotic activity, besides inducing histopathological alterations in liver and kidney. Amphibians have been proven to be useful bioindicators, and we suggest that tadpoles of different species can be used to represent the environmental impacts in aquatic ecosystems.

1. Introduction

Much of the waste produced, whether released into the atmosphere or soil, ends up having as final destination, through rain and runoff, the aquatic ecosystems, where they may interact with the biota, impairing the maintenance of aquatic life and the provision of good quality water (Oliveira-Filho et al., 2017; Corduk et al., 2018). Furthermore, environmental contamination through the bioaccumulation and biomagnification of compounds containing heavy metals, such as chromium, is a potential cause of damage to genetic material (Rocha et al., 2015; Siraj et al., 2018).

Chromium exists in nature in two main oxidation states, Cr3+ and Cr6+, which have different geochemical and biological activities. Although Cr6+ is more mobile, labile and toxic than Cr3+, the distribution of both is regulated by redox reactions in natural waters. Under acidic pH and reducing conditions, Cr3+ species will predominate in water, while Cr6+ species will prevail under alkaline pH and mildly oxidizing conditions. Cr3+ is an essential metal nutrient and Cr6+ is carcinogenic, so that hexavalent chromium compounds are classified by the International Agency for Research on Cancer (IARC) as

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group 1 agents, i.e. carcinogenic to humans (Ahmed et al., 2013; Rocha et al., 2015; IARC, 2018). Potassium dichromate ($K_2Cr_2O_7$) has several applications in the industry, such as cement components, chrome objects, antiseptics, fabric dyeing and leather tanning (Prieto et al., 2008).

A major problem in biomonitoring genotoxic pollutants is the choice of test organism. Misleading results can be produced by the uneven sensitivity among species, caused by differences in metabolic rates, physiological conditions and target organs used in the evaluation. Therefore, more than one species should be used to confirm the response to genotoxins under experimental conditions (Campana et al., 2003). Due to the universality of the genetic material, if an agent can cause DNA damage, it must also have genotoxic potential in any cell type (Poleto et al., 2011).

Analyses on potassium dichromate mutagenicity have already been conducted in aquatic organisms, such as the algae *Pseudokirchneriella subcapitata* (Labra et al., 2007), the crayfish *Procambarus clarkii* (De La Sienra et al., 2003), fish (Arunachalam et al., 2013; Nagpure et al., 2015) and amphibians (Wang et al., 2007; Fernando et al., 2016). Our group has also studied the mutagenic effects of potassium dichromate on fish (Rocha et al., 2011, 2015) and the effect of other metals on amphibians (Rocha, 2011; Rocha et al., 2012).

Cell cycle analysis by flow cytometry (FCM) allows the study of the expression of regulatory proteins and cellular kinetics, which provides, through quantification of cellular DNA, information regarding cell proliferation (Krueger and Wilson, 2011). Thus, it is possible to detect disturbances in certain phases of the cycle (changes in DNA content) and to identify clonal alterations (Darzynkiewicz et al., 2017). The use of FCM by toxicological genetics is on the rise due to rapid results, which makes the technique interesting and valuable, when applied to environmental studies, where the impacts promoted by xenobiotics must be clarified quickly, to ensure ecological balance and the health of organisms (Campagnaro et al., 2013). In this case, FCM is mainly focused on the evaluation of cytotoxicity and genotoxicity of chemicals.

Histopathological biomarkers use target organs of toxicity. Thus, the evaluation of the presence of lesions in these organs is used as an indicator of the acute or chronic exposure to toxic substances (xenobiotics) and its application is useful in the verification of the environmental quality and in biomonitoring the human interference (Triebskorn et al., 2008; Kaur et al., 2018).

To our knowledge, this is the first work of the literature that makes a concomitant analysis of the mutagenic, histopathological and cell cycle effects of chromium in the bullfrog model *Lithobates catesbeianus*. Our aim is to contribute to a better understanding of chromium intoxication. Therefore, we investigated the genotoxic and cytotoxic effects of hexavalent chromium in tadpoles of *L. catesbeianus*, by means of bioassays under controlled laboratory conditions. In addition, we analyzed the histopathological alterations in liver and kidney of the tadpoles.

2. Material and methods

2.1. Samples

Bullfrog tadpoles (n = 55), stages 25–31 (Gosner, 1960) (mean weight: 1.26 ± 0.25 g and mean length: 4.72 ± 0.45 cm) used in the experiments were commercially acquired, and acclimation was performed for one week in aired aquariums at 25 ± 1.41 ºC and pH 6.75 ± 0.35. The photoperiod was 12 h and water was renewed only once. All procedures used in this study were approved by the Ethics Committee on the Use of Animals of the Federal University of Pará - UFPA (Process CEUA 6500261017). Tadpoles were assigned to five groups (five aquariums, 15 L water), each with 11 specimens: negative control (NC), untreated; positive control (PC), subjected to 5 mg L^{-1} cyclophosphamide (Sigma-Aldrich, CAS Registry Number: 6055-19-2); three groups exposed to 4 mg L^{-1}, 12 mg L^{-1} and 36 mg L^{-1} $K_2Cr_2O_7$. There was no feeding of animals and no water renewal during 24 h of exposure. Specimens were anesthetized with ice, and peripheral blood was collected by cardiac puncture with heparinized syringes. A total of 0.7 mL blood sample was taken from each tadpole, for use in the micronucleus test and flow cytometry assay.

2.2. Micronucleus test

Genotoxicity was tested using micronuclei (MN) and erythrocyte nuclear abnormalities (ENA), carried out in mature peripheral erythrocytes according to the procedures of Fenech (2000) and Campana et al. (2003). Blood smears were fixed in methanol and stained with 5% Giemsa. The diameter of MN usually ranges from 1/16 and 1/3 in relation to the average diameter of the main nucleus; the MNs are not refractive and thus can be readily distinguished from artifacts, such as dye particles; the MN may touch, but should not overlap with the main nucleus; the micronuclear boundary must be distinguished from the nuclear boundary (Bosch et al., 2011). Three ENA alterations were considered: notched, lobed and blebbed. A single observer using a binocular microscope at 1000× magnification (Coleman – N107) examined one thousand erythrocytes per individual.

2.3. Flow cytometry assay

In this assay, the cell cycle distribution was evaluated following the procedure described by Nicoletti et al. (1991). Peripheral blood erythrocytes from the tadpoles were incubated at 37 °C for 3 h in a lysis solution containing 0.1% citrate, 0.1% triton X-100, and 50 µg/mL propidium iodide in the absence of light. Cellular fluorescence was determined using flow cytometry (EasyCyteTM Mini System cytometer Guava Technologies Incs., Hayward, CA, USA) and CytoSoft 4.1 software. Five thousand events per experiment were assessed, and cellular debris was omitted from the analysis.

2.4. Histopathology

After treatment, samples of liver and kidney were taken and preserved in phosphate buffered formaldehyde. For histopathological examinations, liver and renal tissues from five tadpoles per treatment group (i.e., control, low dose, medium dose, high dose and positive control) were embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin-eosin. Histological sections were analyzed and photomicrographed with a Zeiss Axiostar Plus photomicroscope.

For the determination of chromium in organs, liver and kidney were cut into small pieces, dried in an oven at 80 °C to constant weight and powdered with a pestle and mortar. The samples were digested in 10 mL HNO_3 over hot plate, at 130 °C under a reflux cap. Chromium quantities were estimated using an UV–Vis spectrophotometer. The chromium concentration tested in the organs was expressed in µg g^{-1} dry weight (Table 1).

2.5. Statistical analysis

Data set from different treatments were checked for normality (Lilliefors test) and homoscedasticity (Levene's test). Values from the flow cytometry assays met the assumptions of normal distribution and homogeneity of variances and were subjected to one-factor analysis of

Table 1
Chromium concentration (µg g^{-1}) in liver and kidney of the *L. catesbeianus* tadpoles.

Organs	Control	T1	T2	T3
Liver	0.27 ± 0.50	21.00 ± 5.10	35.00 ± 7.50	70.40 ± 6.67
Kidney	0.14 ± 0.03	11.50 ± 0.52	24.80 ± 1.95	42.40 ± 1.12

Three groups exposed to $K_2Cr_2O_7$: T1 = 4 mg L^{-1}; T2 = 12 mg L^{-1}; T3 = 36 mg L^{-1}.