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The Micronucleus Test in Erythrocytes of Amphibian Larvae as Tool for Xenobiotic Exposure Risk Assessment: A Brief Review and an Example Using *Lithobates catesbeianus* Exposed to Copper Sulphate

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Abstract: Micronucleus test is a useful tool on the evaluation short-term of environmental mutagenesis and it can estimate the genetic risk followed by xenobiotic exposures. In amphibians, this technique has been extensively used and based on erythrocytes, which in these organisms are nucleated. The aim of this study is to gather and evaluate published data on the use of amphibian MN assay in biomonitoring and genotoxicity assays. In addition, we show the results of this test in erythrocytes from *Lithobates catesbeianus* tadpoles, exposed to copper sulfate. The potential genotoxicity effect evaluated with Micronucleus test showed a reduced sensitivity of the animals to the assayed concentrations of the metal, exhibiting only a modest increase in the frequency of erythrocytes micronuclei, meanwhile larvae exposed to cyclophosphamide (positive control) showed significant increases.

Key words: Micronuclei · Bioassay · Biomonitoring

INTRODUCTION

The increased interest in environmental genotoxicity studies went ahead with the development of a great number of tests to evaluate genotoxic effects in aquatic environments. Considering this, Micronucleus test, one of the most popular and promising tests on ecotoxicology, represents a cytogenetic indicator of DNA damage for over 30 years [1].

Micronuclei (MN) are the result of chromosome acentric fragments (clastogenic effect) or whole chromosomes that, through incomplete migration, have been excluded from the main core (aneugenic effect). Thus, micronuclei represent a loss in chromatin as a result of damage to either chromosome structure (fragmentation) or dysfunction of the spindle apparatus or centromere kinetochore complexes [2-5].

When compared to other DNA damage detection techniques, Micronucleus test has some advantages: it can be performed rapidly, is not complex, presents low costs, its preparation and analysis are simpler and faster than chromosomal aberrations [6].

Micronucleus test has been applied extensively to test the genotoxicity of chemicals. The test has been successfully used with invertebrates, fish and amphibians, as a biological monitors of contaminated areas (*in situ* assay) [7, 8, 9] and in the screening of compounds to determine their genotoxicity, after direct or indirect exposure *in vivo* [6, 10, 11].

Many amphibian populations are declining in number throughout the world [12]. In some cases, this phenomenon is associated with pollution by pesticides and heavy metals. Although environmental pollution may interfere with amphibian growth and development, the induction of genetic damage after chronic exposure to low doses of chemicals is perhaps the most important biological effect [5].

Several works demonstrated that amphibians are sensitive organisms, suitable for detection of genotoxic agents [5, 11, 13].

Technical Aspects and Analysis: The original proceeding to Micronuclei assay was developed for Schmid and collaborators [14, 15, 16], using bone marrow erythrocytes

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of small mammalians. The erythroblasts, in mammalian bone marrow, undergo a final doubling of the chromosomes, after which they divide and differentiate in polychromatic erythrocytes [17].

The Micronucleus test has been widely used in erythrocytes because this cell type is easy handled once cellular dissociation is not required. Red blood cells (RBCs) in lower vertebrates such as amphibians are nucleated and undergo cell division in the circulation, especially during the larval stages. These cells are therefore suitable for micronuclei detection which can be readily counted in blood smears [5]. In most work with amphibians are used tadpoles. This stage was selected for the Micronucleus test because the larvae are large enough to allow cardiac puncture and provide a sufficient amount of blood for smears. In addition, this sampling interval corresponded to a period of intense hematopoiesis [18].

The protocol is quick and simple: blood samples are taken from each tadpole by cardiac puncture and one smear is prepared for each animal. Fixed in absolute methanol and stained with 5% of Giemsa (phosphate buffer pH 6.8), the smears are screened under a conventional microscope under 1000X magnification [19]. Micronuclei are circa 1/20 to 1/10 smaller than the main nucleus [17]. Only cells with well-preserved cytoplasm are considered.

Among current cytogenetic techniques, nuclear abnormalities such as binuclei and micronuclei are considered as well established indicators of cytotoxicity and genetic toxicology, respectively [20]. Moreover, the Micronucleus assay has been standardized on the amphibians *Xenopus leavis* and *Pleurodeles waltl* in France [21].

Although the significant differences between controls and exposed groups are usually reported using the non-parametric Kruskal-Wallis test [5, 22-25], other statistical analysis using the parametric ANOVA [11, 26] are valid and recommended according to the data distribution.

Ecological Biomonitoring and Genotoxicity Assays: The impact of toxic agents on the DNA integrity and function has been extensively investigated under environment conditions [4]. Because of their sensitivity to changes of their habitat and that their larvae live in the aquatic environment, amphibians have been regarded as bioindicators of aquatic and agricultural ecosystems [27]. Table 1 present some biomonitoring *in situ* studies using the MN assay in tadpoles.

Genotoxicity assays using amphibian larvae can be performed *in vitro* and/or *in vivo*. In the *in vivo* assays, the tested agents are usually added to water. In the last 12 years, the number of genotoxicity assays using the Micronucleus test in amphibian species has increased significantly. Some examples are presented in Table 2.

Since many heavy metals are well known to be genotoxic, mutagenic and even carcinogenic, several studies have evaluated its effects through bioassays.

The toxic potential of lead (Pb) in larvae of the toad *Xenopus laevis* after 12 days exposure in lab conditions was evaluated to Mouchet *et al*. [28]. The genotoxic effects were analyzed in the circulating blood from the levels of micronucleus induction according to the French standard micronucleus assay. Moreover, the toxic potential of lead, in aquatic media, was investigated in the presence of meat and bone meal combustion residues (MBMCR) known to be rich in phosphates and a potential

Table 1: Biomonitoring studies using the micronucleus assay in amphibian species

Organism	Cell Type	Localization	Contaminant	Reference	Publication year
Xenopus laevis	Erythrocytes	River Dadou (France)	Industrial effluents	Gauthier *et al*. [39].	2004
Rana ridibunda	Erythrocytes	Sumgayit (Azerbaijan)	Polycyclic aromatic hydrocarbons (PAHs), mercury, organochlorine pesticides and polychlorinated biphenyls	Matson *et al*. [25].	2005
Xenopus laevis	Erythrocytes	French channels (France)	Metals and hydrocarbons	Mouchet *et al*. [40].	2005
Lithobates catesbeianus	Erythrocytes	Paraíba do Sul River (Brazil)	Water at station used to treat water	Wirz *et al*. [9].	2005
Xenopus laevis	Erythrocytes	French Municipal Solid Waste Incineration Plant (France)	Polycyclic aromatic hydrocarbons, residues of solvents and metals	Mouchet *et al*. [41].	2006
Bufo raddei	Erythrocytes	Lanzhou Region (China)	Petrochemical (mainly oil and phenol)	Huang *et al*. [42].	2007
Scinax nasicus	Erythrocytes	Entre Ríos Province (Argentina)	Agricultural effluents	Peltzer *et al*. [43].	2008

Table 2: Genotoxicity assays using the micronucleus assay in amphibian species

Organism	Cell Type	Contaminant	Reference	Publication year
Pleurodeles waltl	Erythrocytes	Petrochemical Waste Waters	Djomo *et al*. [44].	2000
Lithobates catesbeianus	Erythrocytes	Pyrethroid insecticide	Campana *et al*. [5].	2003
Rana N. Hallowell	Erythrocytes	Imidacloprid and RH-5849 (Pesticides)	Feng *et al*. [19].	2004
Ambystoma sp.	Erythrocytes	Colchicine and cyclophosphamide	Zamora-Perez *et al*. [24].	2004
Hyla pulchella	Erythrocytes	Endosulfan (pesticide)	Lajmanovich *et al*. [23].	2005
Odontophrynus americanus	Erythrocytes	Pyrethroid insecticide	Cabagna *et al*. [26].	2006
Bufo bufo and *Rana arvalis*	Liver and bone marrow cells	N-nitroso-Nmethylcarbamide	Manskikh [45].	2006
Xenopus laevis	Erythrocytes	Lead	Mouchet *et al*. [28].	2007
Xenopus laevis	Erythrocytes	Double-walled carbon nanotubes	Mouchet *et al*. [46].	2008
Rana saharica	Erythrocytes	Artea 330EC	Bouhafs *et al*. [13]	2009
Rhinella arenarum	Erythrocytes	Pirimicarb (inseticide)	Candioti *et al*. [32].	2010
Lithobates catesbeianus	Erythrocytes	Potassium dichromate	Monteiro *et al*. [22].	2010
Lithobates catesbeianus	Erythrocytes	Copper sulphate	Ossana *et al*. [11].	2010

immobiliser of lead. The results obtained in this study demonstrated that lead is acutely toxic and genotoxic to amphibian larvae from 1 mg Pb/L and that lead in presence of MBMCR induced inhibition or reduction of the toxic and genotoxic potential of lead in water at concentrations that do not exceed the capacity of MBMCR of Pb-binding.

Ossana *et al*. [11] evaluated toxicity parameters of inorganic copper (Cu^{2+}) in *Lithobates catesbeianus* pre-metamorphic larvae. The potential genotoxicity effect evaluated with Micronucleus test showed a reduced sensitivity of the animals to the assayed concentrations of the metal, exhibiting only a modest increase in the frequency of erythrocytes micronucleated.

Monteiro *et al*. [22] exposed *Lithobates catesbeianus* tadpoles to potassium dichromate under different concentrations. Significantly increased frequencies of micronucleated erythrocytes, relative to control group, were observed in tadpoles treated.

Example Using *Lithobates catesbeianus* Exposed to Copper Sulphate

Amphibian Species: Bullfrog, *Lithobates catesbeianus* (Shaw, 1802) (Anura, Ranidae) originally distribute from Canada southwest, east and center south of United States up to Veracruz, in México, yet, due to its economic potential, was introduced in 41 countries [29]. In many cases, in the farming turned off, the animals were released into the natural environment where they have adapted to local climatic and ecological conditions [30].

Lithobates catesbeianus tadpoles been successfully used in genotoxicity tests of several xenobiotics, such as heavy metals [11, 22, 28], pesticides [5, 31, 32] and contaminants from paper mill and human sewage [9].

Chemicals: Copper (Cu) is a heavy metal commonly found in aquatic ecosystems due to its extensive use in agriculture and water treatments, in industrial activities, in aquaculture considering its algaecide properties as well as a therapeutic agent to control ectoparasitic infestations and bacterial diseases [11]. Copper, being a heavy metal, in trace amount is essential to life as it is toxic in excess. Its importance in health and disease is well documented. Also the clastogenicity of copper sulfate *in vivo* is being investigated for a long time in different test systems; however, results have been inconsistent [33].

Cyclophosphamide (CP; CAS 50-18-0, Genuxal®; Asta Medica, AG Frankfurt, Germany) was used as a positive control at a concentration of 5 $mg.L^{-1}$ (ppm). All test solutions were prepared immediately before each experiment.

Methods: In this study, the tadpoles used in the bioassay (1.66 ± 0.63 g and 61.98 ± 7.71 mm) were subsided from frog farming Rãmazon, in Belém (PA), Brazil. Acclimatization to laboratory conditions for fifteen days. Tests were run under a 14:10 h light–dark photoperiod regime was done using dechlorinated tap water with temperature = 26 ± 1.3°C and pH = 6.5 ± 1.3.

Larvae Were Divided into Four Groups: (1) a negative control, (2) positive control (using the well known mutagen ciclophosphamide, 5 $mg.L^{-1}$), (3) treated with CuSO4 0.2 $mg.L^{-1}$, (4) treated with CuSO4 0.4 $mg.L^{-1}$. The water containing the compound and the food was no changed during the bioassay. The micronuclei frequency in each group was scored after 24 and 48 hours of treatment.

Table 3: Micronuclei in *Lithobates catesbeianus* exposed to copper sulphate

			MN / 1,000 erythrocytes	
Treatment	Concentration	Cells Number (n x 1000)	24h	48h
Negative control	–	10,000		
μ ± SD			0.10 ± 0.31	–
Medians/Q1-Q3			0.00/0.00-0.00	–
Positive control	5 mg.L^{-1}		18,000	
(cyclophosphamide)				
μ ± SD			4.62 ± 2.06	2.30 ± 2.06
Medians/Q1-Q3			4.50/3.75-5.00***	1.50/1.00-3.00**
CuSO4	0.2 mg.L^{-1}	21,000		
μ ± SD			0.18 ± 0.40	0.40 ± 0.69
Medians/Q1-Q3			0.00/0.00-0.00	0.00/0.00-0.75
CuSO4	0.4 mg.L^{-1}	21,000		
μ ± SD			0.63 ± 0.50	0.20 ± 0.42
Medians/Q1-Q3			1.00/0.00-1.00*	0.00/0.00-0.00

MN: micronuclei; μ: mean; SD: standard derivation. *Significantly $p < 0.05$. **Significantly $p < 0.01$. Extremely Significantly $p < 0.0001$

The blood was spread onto a microscope slide accord to Feng *et al*. [19]. Upon exposure, blood was taken from each tadpole by cardiac puncture and one smear was prepared for each animal. Fixed in methanol and stained with 5% of Giemsa, the slides were observed for MN score in a transmission light microscope, using 1,000X magnification. The micronuclei frequency was determined in 1,000 erythrocytes from each tadpole.

BioEstat 5.0 software package [34] was used for statistical analyses. Data were tested for normality via the Shapiro-Wilk test and the Bartlett test before all statistical analysis, in order to check variance homogeneity. Data that presented normal distribution and homogeneous variances, whereupon a one way ANOVA was performed, followed by an *a posteriori* Tukey test. Several data demonstrating asymmetry and heteroscedasticity. Neverthless, a non-parametric Kruskal-Wallis test was done, followed SNK test. Differences between the negative control-group and individual dose-groups were analyzed at the 0.05 probability level.

Results are presented as the mean ± standard derivation and also in medians and quartiles (Q1-Q3) in Table 3. According to Ramsdorf *et al*. [35], when the data are not symmetrical, median is most useful that the mean like representing of the data. The median is at the middle of an ordered (ranked) data set and is a useful measure for ordinal variables. Strictly, the mean only makes sense for interval and ratio scales of measurement.

RESULTS AND DISCUSSION

Jaylet *et al*. [36] first adapted the Micronucleus test to amphibians. The bioassay has been extensively applied as an end-point for genotoxicity in amphibians [9, 25, 42, 43] to monitor contaminated areas (*in situ* assay) as well as for screening different mutagens and genotoxic compounds after direct or indirect exposure (*in vivo* assay) [5, 11, 22, 23, 28].

In our experiment, tadpoles exposed to cyclophosphamide (CP, positive control) showed a significant to extremely significantly increase in micronucleated erythrocytes. The Micronucleus test showed that there was not a significant difference between negative control and copper treatments groups, except in 0.4 mg $Cu^{2+}.L^{-1}$ / 24h ($p = 0.0378$).

The bioaccumulation of Cu is important as a bioindicator of environmental pollution at sublethal concentrations. Its toxicity to biota has been shown to be reduced by induction of the expression of metal binding proteins that may play a role in the tolerance of animals to the metal [37].

In this study, under the low copper concentrations used, response of *L. catesbeianus* tadpoles to copper sulphate differed from that to CP, exhibiting only a modest increase in the frequency of erythrocytes micronuclei in relation to negative control. According Bouhafs *et al*. [13], this also may due to the short time of exposition. In bioassay performed for Ossana *et al*. [11], the cells of the larvae only after 96 h exposure to the metal (2 mg $Cu^{2+}.L^{-1}$)

showed an increase in the frequency of MN with significant differences relative to negative control.

It is still interesting to consider the results reported in pre-metamorphic stages of *L. catesbeianus* by Krauter [38]. He found that the maximum number of MN occurred at two separate times, suggesting the presence of two erythrocyte population (coming from two hemopoietic sources, liver and kidneys).

GENERAL CONCLUSION

Environmental monitoring using living organisms has ranged from exploiting particular pollution responses observed in ecosystems as early warning signs, to attempting to use specific organisms as indicators, either in the field or the laboratory.

Genotoxicity studies using cytogenetic analyses in amphibian larvae have demonstrated the sensitivity of these organisms. The micronuclei frequencies may vary according to the season, the kind of pollution involved and the test specie. In laboratory assays involving tadpoles, several substances have been shown to have genotoxic potential. Micronuclei surveys in lower vertebrates such as amphibians have been generally performed on peripheral blood erythrocytes due to their easy use and because are nucleated.

Although the MN assay cannot give information about the type of chromosomal breakage, it is informative when the exposure causes aneugenic effects. Taken together, all the above-mentioned aspects render this methodology high applicability in the routine of mutagenesis studies. Research on environmental biomonitoring requires fast results and reproducibility. Exploration of the MN assay in amphibian species is welcome in order to standardize and improve the assessment of genotoxicity in aquatic and agricultural ecosystems.

We assessed the mutagenic potential of copper sulphate using samples of bullfrog tadpoles (*Lithobates catesbeianus*). This species showed a very low spontaneous frequency of micronuclei. The evaluation with Micronucleus test showed a reduced sensitivity of the larvae to the low assayed concentrations of the metal, exhibiting only a modest increase in the frequency of erythrocytes micronucleated, meanwhile larvae exposed to cyclophosphamide (positive control) showed significant increases.

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