Giant Toads (*Rhinella marina*) From the Industrial Zones of Low Basin of the Coatzacoalcos River (Veracruz, MX) Presents Genotoxicity in Erythrocytes

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Abstract

The lower basin of Coatzacoalcos River is one of the most polluted regions of the southern Gulf of Mexico. Organochlorine compounds, polybrominated diphenyl ethers, polycyclic aromatic hydrocarbons, and heavy metals have been registered in this region. In the present study, genotoxicity was evaluated in the blood of giant toads (*Rhinella marina*) from Coatzacoalcos' rural and industrial zones, and compared with laboratory toads. Determination of the frequency of micronucleus and erythrocyte nuclear abnormalities by the light microscope and cell cycle and apoptosis by flow cytometry were used as biomarkers of genotoxicity. We found more variability in micronucleus and more nuclear buds in toads from industrial zones. Also, cell cycle alterations and an increase of apoptosis in erythrocytes were found in toads from rural and industrial zones. Multivariate statistics show that the toads from the industrial zone were more affected than toads from laboratory and rural zones.

Keywords Amphibians · DNA damage · Ecotoxicology · Flow cytometry · Non-destructive biomarkers

Throughout the years, thousands of pollutants have been emitted by industrial, agricultural, petrochemical, mining, or urban activities into different types of ecosystems. Some pollutants emitted into environmental matrices can have effects on living beings, including humans. Organochlorines, heavy metals, organophosphates, pyrethroids, pharmaceuticals, nanomaterials and microplastics have effects such as neurotoxicity, mutagenicity, carcinogenicity, teratogenicity, endocrine disruption, or genotoxicity (Sánchez-Bayo 2011). But, without a doubt, genotoxicity is one of the most

evaluated effects in organisms exposed to pollutants (Bolognesi and Cirillo 2014; Araldi et al. 2015).

Among the biomarkers most used in ecotoxicological studies to evaluate pollutants' genotoxicity are the comet assay, micronuclei (MNs), and nuclear abnormalities in some cells. MNs are small nuclei that appear in cells next to the central nucleus, a product of changes during mitosis in cells due to DNA damage (Frenzilli et al. 2009; Araldi et al. 2015). Nuclear abnormalities, mostly evaluated in erythrocytes (ENAs), are deformations in the nucleus due to exposure to genotoxic agents, although their origin has not yet been fully explained (Frenzilli et al. 2009). Both biomarkers can be determined non-destructively, only with a blood sample of the organism to be evaluated, without the need for their sacrifice.

Among other non-destructive techniques used in ecotoxicology, the use of flow cytometry stands out, a rapid technique that quantifies the structural and biochemical characteristics of cells. It is an easily adaptable technique for studies with various organisms, including small vertebrates. Likewise, it is suitable for non-destructive analysis (mainly in blood cells) in organisms with some protection or vulnerability status and has proven to be a powerful tool to identify and measure cytotoxic and genotoxic effects of

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environmental pollutants (Bihari et al. 2003; Pérez-Maldonado et al. 2004; Bihari 2017).

The giant toad (*R. marina*) is an anuran amphibian native to Mexico, Central America, and South America. As an amphibian, it has a highly permeable skin and a life cycle that develops in the aquatic and terrestrial environment (Solís et al. 2009). Additionally, it inhabits open, natural, or artificial areas, including habitats modified by humans, both in the rainy and dry seasons (Solís et al. 2009; Wilson and Johnson 2017). These characteristics make it an organism susceptible to the bioaccumulation of pollutants and their toxicological effects. Several studies have used this anuran to demonstrate the accumulation of pollutants, as well as the effects they produce on these organisms (Cruz-Esquivel et al. 2017; Sookoo et al. 2017; Triana Velásquez et al. 2017). In Mexico, *R. marina* has been used to demonstrate accumulation of persistent organic pollutants (POPs) and effects (mainly DNA Damage) in scenarios where there are complex mixtures of pollutants; for example, in the southern Gulf of Mexico (Gonzalez-Mille et al. 2013; Ilizaliturri-Hernández et al. 2013).

One of the most polluted regions in Mexico is the lower basin of the Coatzacoalcos River (Veracruz), located in the southern Gulf of Mexico. Because it has presented a rapid degradation of its ecosystems by urbanization, industrial and agricultural activities that take place there (Vázquez-Botello and Páez 1987; Bozada-Robles and Bejarano-González 2006). Studies in organisms and environmental matrices of this region's ecosystems have demonstrated the presence of POPs, heavy metals, and polycyclic aromatic hydrocarbons (PAHs) (González-Mille et al. 2010, 2013; Espinosa-Reyes et al. 2012; Ilizaliturri-Hernández et al. 2013).

In this context, the present study aimed to evaluate the genotoxicity in two areas of the lower basin of the Coatzacoalcos River, with MN and ENAs tests and flow cytometry in nuclear erythrocytes of giant toads (*R. marina*).

Materials and Methods

The study area is located in the Coatzacoalcos region, in the southern Gulf of Mexico (Fig. 1). The rural zone (RUR) sampling sites were located 35–40 km away from the

Fig. 1 Localization of low basin of Coatzacoalcos River and the sampling stations for the rural and industrial zones. A1 and A2 correspond to Rural Zone; B1 and B2 are the sampling stations of Industrial Zone. The map is based on INEGI (2010)

industrial area of Coatzacoalcos region, with slight urbanization and well-preserved ecosystems. The industrial zone (IND) sampling sites were in industrial and near petrochemical areas, with high urbanization and less-preserved ecosystems (González-Mille et al. 2013; Ilizaliturri-Hernández et al. 2013). Thirty adult giant toads (*R. marina*) (weighing an average of 183.8 ± 8.6 g and an average snout-vent length of 12.5 ± 0.1 cm) were collected in nocturnal transects at two sites in the lower basin of the Coatzacoalcos River. In RUR, 11 toads were captured, and 19 toads in IND. The male and female sex of toads were proportionally in each site, and the only criteria of exclusion were age (adults only).

A 1–2 mL of the blood sample was obtained from each toad by cardiac puncture using a heparinized syringe. The toads were stabilized and released at the sites where they were captured. For the determination of MNs and ENAs, blood smears were made on glass slides, which were fixed with methanol. For the analysis by flow cytometry, from another aliquot of blood, the erythrocyte package was obtained by centrifugation (3500 rpm, SOL-BAT J12), and this was treated with saline buffer (PBS, pH 7.4) for its fixation with formaldehyde at 3%, according to the method described by Gagné (2014). Both the smears and the erythrocyte packages were transported to San Luis Potosí for further analysis.

For comparison purposes, the same procedure was carried out with seven toads of the same species kept under laboratory conditions (LAB). These toads came from a non-industrialized site with little urbanization located in the state of San Luis Potosí. The collection of all organisms was carried out with a scientific collector's permit issued by the Secretaría de Medio Ambiente y Recursos Naturales de México (SEMARNAT). The capture and handling were carried out through adherence to international guidelines (Beaupre et al. 2004).

To determine MNs and ENAs frequency in toads' erythrocytes, the slides of blood smears were fixed and stained with the Wright-Giemsa stain method (Dunning and Safo 2011). A total of 2000 ENAs were examined per toad under the light microscope (Axio Imager A1, Carl Zeiss®) with $\times 100$ objective oil immersion. For identifying MNs and ENAs, we used the criteria of da Silva Souza and Fontanetti (2006) and Lajmanovich et al. (2014) applied to studies with fish and amphibian erythrocytes. MNs and ENAs frequency were standardized as MN/ENA per 1000 RBCs.

Through flow cytometry, the cell cycle and apoptosis in the toads' erythrocytes were determined using DNA content assay with some modifications of the method of Pérez-Maldonado et al. (2004). Cells were fixed with formaldehyde and were stained in a hypotonic staining solution (propidium iodide, sodium citrate, Tritón X-100, and RNase) at four Celsius grades for 30 min in the dark. Cells were analyzed in a FACSCalibur Flow cytometer (ex = 494 nm/

em = 521 nm, Becton Dickinson, San Jose, CA, USA), and a minimum of 10,000 cells per sample were registered. CellQuestPro® software (BD Heidelberg, Germany) was used to calculate the cells' percentages in each phase of the cell cycle and apoptotic cells. Results were expressed as the percentage of hypodiploid cells.

Differences in MNs, ENAs, cell cycle, and apoptosis in *R. marina* erythrocytes per zone were evaluated using the Kruskal-Wallis test, as they were non-normal data. We performed a permutational multivariate analysis of variance (PERMANOVA) to observe the differences with all biomarkers for the factor "Zone" (LAB, RUR, IND). The Similarity Percentage Subroutine (SIMPER) was used to identify which biomarkers contributed to differences between toads from the zones and LAB. A Principal Component Analysis was performed to observe the differences between zones and laboratory graphically.

Univariate statistics were performed by GraphPad Prism version 9.0.1 for Windows, GraphPad Software, San Diego, California, USA. And multivariate statistics by PRIMER 7 v.7.0.13 and PERMANOVA+ add on software (PRIMER-e Ltd, Anderson et al. 2008).

Results and Discussion

Figure 2 shows the presence of MNs and ENAs in the erythrocytes of the giant toads. Although the variability was more significant in the frequency of MNs in toads from the industrial zone, we only found statistically significant differences in the frequency of nuclear buds (BUDs). The frequency of BUDs increases in the ENAs of toads from the industrial zone (KW-H = 7.34, $p < 0.05$).

On the other hand, the results of the PCA analysis are shown in Fig. 3. The first two axes of the PCA explained 64.3% of the total variability. The biomarkers were different between LAB, RUR, and IND. These differences were statistically significant for the factor "Zone" (PERMANOVA test, pseudo F = 9.46, $p < 0.001$). To better understand the above, Table 1 shows the differences of the comparison by zones (pairwise PERMANOVA test) and the biomarkers that contributed to the differences in the factor "Zone", according to the SIMPER analysis (presented in order of importance showing at least one 60% dissimilarity). The biomarkers contributing to the differences between LAB vs. RUR and IND toads were the percentage of g2/m cells, phase s cells, and apoptosis. In contrast, the biomarkers that contributed to the differences between the RUR and IND toads were the percentage of g0/g1 cells, MNs, and BUDs.

In laboratory studies, the determination of MNs and ENAs in amphibian erythrocytes have been used as biomarkers of genotoxicity due to exposure to pesticides, heavy metals, and PAHs (Djomo et al. 1995; Lajmanovich et al. 2005;

Fig. 2 Normal, micronucleus, buds, and other nuclear abnormalities in erythrocytes of *R. marina*, the photographs were taken with CMOS C-Mount Amscope® Camera. **a** Micronucleus, **b** buds, **c** immature erythrocyte, **d** binucleated, **e** lobbed nucleus, **f** mitotic erythrocyte

Fig. 3 **a** Principal Component Analysis of biomarkers evaluated in erythrocytes of *R. marina* (PERMANOVA test, $p < 0.001$). **b** Percentage of cells in each phase of cell cycle and percentage of cells in apoptosis (different letters mean statistically significant differences, KW-H, $p < 0.001$)

Table 1 PERMANOVA pairwise and SIMPER analysis of biomarkers evaluated in *R. marina*

Zone comparison	t value (cumulative percent)
Laboratory vs. rural	3.20 (65.87%)* (% g2/m cells, % apoptosis, % phase s cells)
Laboratory vs. industrial	3.72 (59.82%)* (% g2/m cells, % apoptosis, % phase s cells)
Rural vs. industrial	2.45 (59.19%)* (% g0/g1 cells, MNs, BUDs)

t values are from PERMANOVA pairwise test

*Represents statistical differences ($p < 0.001$), cumulative percent (%) from SIMPER analysis represent the percentage of contribution and biomarkers that contributed to the dissimilarity

Mouchet et al. 2006, 2007). Furthermore, in urban-industrial sites or close to petrochemical complexes, MNs and ENAs have been found in erythrocytes of fish and amphibians (Gauthier et al. 2004; da Silva Souza and Fontanetti 2006; Huang et al. 2007; Hoshina et al. 2008). That is similar to what we found in our study because the industrial zone's evaluated sites are close to this type of activity. Moreover, concentrations of organochlorine pesticides and their metabolites (DDT, DDE, Lindane, HCB), polybrominated compounds, heavy metals, and PAHs have been recorded at some sites of Coatzacoalcos region (Table S1, Espinosa-Reyes et al. 2012; Ruiz-Fernández et al. 2016). Thus, we assume that the variability in MNs and a higher frequency of BUDs in the giant toads of the industrial zone evidence genotoxic effects due to the complex mixture of pollutants.

Although BUDs are considered nuclear abnormalities, their origin is known and is like that of MNs origin. Therefore, studies suggest that BUDs are precursors to MNs and that both can occur due to clastogenicity or aneugenicity from exposure to genotoxic compounds (Shimizu et al. 1998; Braham et al. 2017). Studies of our research group have shown DNA damage through the comet assay in erythrocytes of *R. marina*, fish, reptiles (turtles, crocodiles), and humans (children) from Coatzacoalcos, being related to the presence of POPs (Pelallo-Martínez et al. 2011; González-Mille et al. 2013, 2019). However, the comet assay only detects the breaking of the DNA double helix strands, that is, the first event that occurs after exposure to genotoxic agents, which is repairable. Whereas MNs, BUDs, and other ENAs result from DNA damage that could not be repaired in erythrocytes (Frenzilli et al. 2009). Thus, our results indicate that some pollutants found in Coatzacoalcos' industrial zone can produce clastogenic and aneugenic lesions in erythrocytes.

There is no study where the cell cycle phases are analyzed as an ecotoxicological biomarker in amphibians. The g1 phase (growth), s phase (DNA synthesis), and g2/m (mitosis) are the points of the cell cycle. They are critical for a cell cycle to be completed and another to start and prevent the formation of genetically abnormal cells (King and Cidlowski 1995; Pucci et al. 2000). Studies with other organisms in vivo or with cell lines (in vitro) have shown alterations in the cell cycle. Increases or decreases in the proportion of cells in some of the cell cycle phases in laboratory tests (Sánchez et al. 2000; Wickliffe et al. 2000; Xie and Shaikh 2006; Yu et al. 2019; Morozesk et al. 2020). Similar effects when organisms are found in contaminated sites (e.g., estuaries with industrial complexes) with genotoxic compounds (Castaño et al. 2000; Bihari et al. 2003; Batel et al. 2018; Gautam et al. 2020). This coincides with our results, where the percentage of cells contributed a large part to the differences between the biomarkers of the LAB vs. RUR and IND toads in the phases of the cell cycle, which were lower in the RUR and IND toads (Table 1, Fig. 3b).

Apoptosis or cell death is a natural process essential for all multicellular organisms' normal development and homeostasis. This process is critical to eliminate any damage, infection, or neoplastic proliferation (Sweet et al. 1999). Our results demonstrated an increase in the percentage of apoptosis in the erythrocytes of toads from the industrial zone followed by those belonging to the rural zone, where the pattern was from a lower to a higher percentage of apoptosis: LAB > RUR > IND (KW-H = 19.7, $p < 0.001$, Fig. 3b). Bihari et al. (2003) demonstrated that a decrease in the proportion of cells in g2/m and "s" phases could be due to the increase of apoptosis in organisms exposed to genotoxic compounds, which is also suggested by Telford et al. (1992). Studies with DDT metabolites have shown to induce apoptosis in cell lines due to an alteration of proteins that regulate it or the generation of antioxidant or pro-inflammatory responses (Pérez-Maldonado et al. 2005; Alegria-Torres et al. 2009; Yu et al. 2019). Therefore, the presence of a higher percentage of apoptosis in our study provides evidence of the alteration of the cell cycle of Coatzacoalcos toads and probably the effects produced by the pollutants found there.

As shown in the PCA (Fig. 3a), the presence of MNs and BUDs, the alteration of the cell cycle, and the increase in apoptosis showed that the most affected toads were those from the industrial zone. These effects could have consequences on amphibians' health since this means that they are under stress from pollutants causing damage to DNA or inducing apoptosis of cells, compromising their development, reproduction, or their immune system. Besides, as mentioned in Table S1, our research team has recorded many pollutants in environmental matrices in the lower basin of Coatzacoalcos region and has demonstrated their accumulation in *R. marina*. Determining all these pollutants and others of the emerging type in environmental monitoring can be difficult due to its costs and the sophisticated equipment. Therefore, a battery of genotoxicity biomarkers that included the evaluation of the phases of the cell cycle in red blood cells would be an excellent tool to differentiate between the most affected areas, as in the present study and as other studies suggest (Castaño et al. 2000; Batel et al. 2018; Gautam et al. 2020).

We conclude that the lower Basin of Coatzacoalcos River is contaminated with compounds that can produce clastogenic or aneugenic lesions, alterations in the cell cycle or induce apoptosis in organisms that live in the region, such as giant toads. Since these biomarkers are non-destructive, they could be applied to other species such as birds, fish, or reptiles, mainly because they have nucleated erythrocytes, which facilitates the determination of nuclear or cell cycle lesions. Therefore, they could be considered in future studies in this area or other contaminated scenarios in Mexico and other countries.

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