BalkanBio'2021 - 5th Balkan Scientific Conference on Biology – April 15-16, 2021, Plovdiv, Bulgaria

Chronic exposure to heavy metals induces nuclear abnormalities and micronuclei in erythrocytes of marsh frog (*Pelophylax ridibundus* Pallas, 1771)

Vesela Mitkovska[1], Hristo Dimitrov[1], Tsenka Chassovnikarova[1,2]

[1] Department of Zoology, Faculty of Biology, University of Plovdiv "Paisii Hilendarski", 24 Tzar Asen Str., Plovdiv 4000, Bulgaria

[2] Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 1 Tsar Osvoboditel Blvd., 1000 Sofia, Bulgaria

Introduction and Purpose

Amphibians have big potential as bioindicators based on their combined life cycle as aquatic and terrestrial form. They can play the role of prey or predator, making them a key element in toxic substances transfer between aquatic and terrestrial habitats. Due to their specific characteristics, amphibians can be used as bioindicators to detect toxic waste in water, soil, or bottom sediments. Most of their gas exchange takes place through the skin, which leads to easy absorption of substances that pollute their habitat. The nuclear abnormalities (NAs) in amphibians' erythrocytes in recent years have been used as a successful biomarker for anthropogenic pollution. The NAs including micronuclei in erythrocytes of the marsh frog (*P. ridibundus*) have been studied to assess the cytotoxic and genotoxic effect in heavy metal polluted area *in situ*. The marsh frog *P. ridibundus* is the anuran with the widest distribution in Bulgaria. Here we assess the cyto- and genotoxic potential of the polluted waters (Chaya River) close to the lead-zinc smelter near Plovdiv (Bulgaria) situated in an area that has been contaminated with heavy metals for 60 years. Frogs from Strandzha Natural Park (SNP) were used as a negative control.

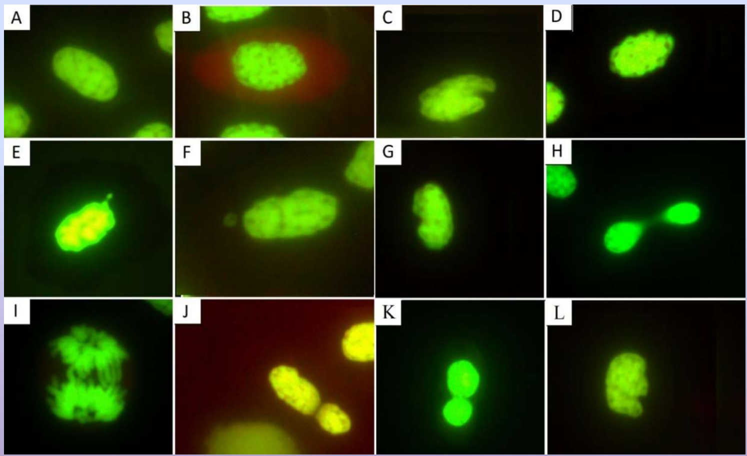

Fig. 1. Different type of NAs in erythrocytes of *P. ridibundus*: **A)** normal normochromatic erythrocyte without abnormalities; **B)** normal polychromatic erythrocyte; **C) notched nucleus** (NotchN) – with a substantial notch into the nucleus; **D) blebbed nucleus** (BlebN) – with relatively small evaginations of the nuclear membrane and contained euchromatin; **E) nuclear bud** (Nbud) – with relatively small formation connected to the nucleus by a stalk of nucleoplasmic material; **F) micronucleus** (MN) – in addition to the main nucleus, there is a less isolated nucleus with a round or oval shape, which is not larger than 1/5 of the main nucleus, focuses and fluoresces with the same color as the main nucleus; **G) kidney shape nucleus** (KN) – ; **H) nucleoplasmatic bridge** – NBr; **I) mitotic erythrocytes**; **J) binucleated erythrocyte** – BN; **K) "eight" shape nucleus** (EN) – represented a constriction resembling the shape of the number eight; **L) lobed nucleus** (LobeN) – with larger evaginations (lobe), including those with several lobes.

Material and Methods

Material: Total 79 specimens of *P. ridibundus* (40 from the heavy metal polluted area and 39 animals from the control region Strandzha NP) were investigated.

Sampling: The field research was conducted in the period May – June 2018. The animals were captured alive at night, blinded by artificial light, and then transported to a laboratory for analysis. All animals were identified based on secondary sexual characteristics: the presence of "marital corns" on the first finger and resonator bubbles in the corners of the mouth of the males. The animal handling and laboratory methodology was approved by the Ethics Board for Experimental Animals at the Faculty of Biology at the University of Plovdiv. In order to prepare thin blood smears, blood was isolated by cardiac ventricular puncture after anesthesia of the live frogs.

Observation for NAs including MN: Thin whole blood smears (fixed with absolute ethanol for 10 min) were stained with fluorochrome dye acridine orange (AO) according to Hayashi et al. (1983) immediately before evaluation with fluorescence microscopy (Leica DM 1000) equipped with appropriate for AO filter (I3). Photomicrographs taken with the Leica Application Suite are processed with the ImageJ program with the addition of the "Cell Counter" plugin. Analyses consisted of count and detection of erythrocytes with different NAs and MN (according to the criteria of Carrasco et al. (1990), Fenech (2000) and Furnus et al. (2014)). The average frequency of nuclear abnormalities per 2000 scored erythrocytes (polychromatic and normochromatic), expressed in per mille, was calculated for each individual with the following formula:

$$\textbf{NAs Frequency}\ ‰ = \frac{\textbf{Number of cells containing NAs}}{\textbf{Total number of cells scored}} \times \textbf{1000}$$

Statistical analysis: The data were first tested for both normal distribution (D'Agostino and Pearson omnibus normality test) and homogeneity of variance (Levene, F-test). The MN and NAs data was not normally distributed and therefore the non-parametric Mann-Whitney test was used. For all tests, the level of significance was set at $P \leq 0.05$. All calculations were performed with the software Prism, version 4.02 (GraphPad Software, San Diego, CA, USA).

Results

The fluorescent observation of blood smears indicated different types of nuclear abnormalities – notched nuclei (NotchN), blebbed nuclei (BlebN), nuclear buds (NBud), lobbed nuclei (LobeN), kidney shape nuclei (KN) and eight-shaped nuclei (EN), cells with MN, binuclear cells, mitotic erythrocytes (erythroblasts) (Fig. 1).

Using the non-parametric Mann-Whitney test, the absence of statistically significant differences between the sexes was observed in all scored NAs: BlebN (P = 0.3157; U = 1773), NotchN (P= 0.6956; U = 1896), NBud (P = 0.0553; U = 1584); Other NAs (P = 0.7973; U = 1924); MN (P = 0.5573; U = 1909) and Total NAs (P = 0.4574; U = 1824). Consequently, for the subsequent analyses, we combined males and females per site.

The frequency of the first three nuclear abnormalities in the impact site – NotchN (21.70±15.24‰), Nbud (15.32±11.06‰) and BlebN (8.30±4.91‰) is many times higher than the other abnormalities. Therefore, all other anomalies except the micronuclei were pooled in "Other NAs".

Significant differences in frequency of NochN, Nbud, BlebN, MN and Total NAs among impact and background sites were found (Fig. 2) with the lowest frequency recorded for SNP. No statistically significant differences were found only for "Other NAs" for the investigated sites (p = 0 .83).

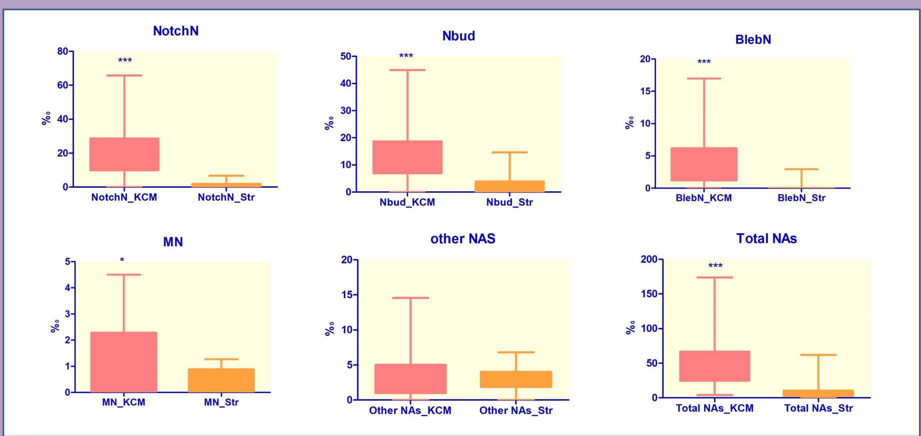

Fig. 2. Comparative analysis of frequencies of different types of NAs in polluted (KCM, Plovdiv) and background region (Strandzha Nature Park). Bottom and top of the box represent 25 and 75% percentile values, respectively. Error bars indicate minimum and maximum values. Significant difference at P ≤ 0.05 (*** P ≤ 0.0001; ** P ≤ 0.001; * P ≤ 0.05).

Conclusion

- The obtained results reveal the greater induction of NAs in erythrocytes of marsh frog *P. ridibundus* inhabiting polluted waters (Chaya river) close to the lead-zinc smelter near Plovdiv (Bulgaria).
- NAs of the following types: notched nuclei, nuclear buds and blebbed nuclei have shown the highest frequency.
- The significant differences (P≤0.0001) in the mean Total NAs (‰) and micronuclei (‰) in erythrocytes of marsh frogs from the polluted area compared to the Total NAs from the background region "Strandzha" NP, demonstrate the presence of *in vivo* active cytotoxic and genotoxic agents in the impacted area.
- The obtained results for NAs in erythrocytes of *P. ridibundus* are evidence for successful application of NAs as a biomarker in amphibians for the purpose of biomonitoring;

Acknowledgments

This study was supported by the MU19-BF-005 financed by the University of Plovdiv "P. Hilendarski", Bulgaria.