Amphibian embryos as an alternative model to study the pharmaceutical toxicity of cyclophosphamide and ibuprofen

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Abstract

Pharmaceuticals are becoming potentially ubiquitous pollutants because of their extensive use by man. One of the most frequent groups of pharmaceuticals that have been identified as particularly concerning is that of nonsteroidal anti-inflammatory and chemotherapeutic drugs. In Albania, studies to determine the risk of pharmaceuticals in conjunction with their occurrence in water bodies and their adverse effects on living organisms, including humans, are scarce. The purpose of this study was to elucidate the possible toxic effects of ibuprofen (IBU) and cyclophosphamide (CP) on cellular physiology of frog tadpoles. For this purpose, individuals of *Pelophylax shqipericus* belonging to stage 21 Gosner were exposed to sub-lethal concentration (5 µg/L) of IBU and CP for 48 hours, and erythrocyte abnormalities and micronucleated cell frequency were evaluated as endpoints. Blood smears from tadpoles exposed to CP for 48 hours showed a pronounced decrease in the number of red blood cells and an increase in the percentage of micronucleated erythrocytes through chromatin fragmentation, while abnormalities like cellular and nuclear vacuolization, collapse and rupture of the cell membrane were caused by IBU toxicity. Understanding the biological effects of these drugs on frog tadpoles can help in using these animals as reliable bio-indicator organisms in monitoring aquatic environments health.

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Key words: Amphibian embryos; Tadpoles; Pharmaceuticals; Ibuprofen; Cyclophosphamide.

Conflict of interest: the authors declare no potential conflict of interest.

Funding: the work was supported by the University of Tirana, Albania.

Conference presentation: part of this paper was presented at the First symposium on experimental biology: sea and environment, Trapani, Italy, 24-25 May 2019.

Received for publication: 28 June 2019.
Revision received: 11 September 2019.
Accepted for publication: 12 September 2019.

Licensee PAGEPress, Italy
Journal of Biological Research 2019; 92:8370
doi:10.4081/jbr.2019.8370

Introduction

Nowadays, pharmaceuticals and personal care products (PPCP) that contaminate water sources are a worldwide problem. The widespread use of PPCP in hospitals, domestic residences, agricultural and industrial facilities has increased their effluent discharge into the surface waters and groundwater sediments,[1] rivers, estuaries and the sea.[2,3] Many studies conducted in freshwater environments[4-6] and in the marine environment,[7,8] have demonstrated that pharmaceuticals can cause adverse effects at concentrations typically found in the environment.

Decreases in amphibian populations have been observed on a global scale. In some cases, this phenomenon is associated with exposure to environmental pollutants such as pesticides and heavy metals.[9-11] Presence of pharmaceutical components in Albanian water bodies, is a new phenomenon. One of the most frequent groups of pharmaceuticals that have been identified as being of particular concern is the nonsteroidal anti-inflammatory drugs (NSAIDs) and chemotherapeutic drugs.[12-16]

In contrast to other pollutants in water, drugs are molecules with high biological activity on different organisms. Even though their concentrations in surface water are detected frequently in range from ng/L to tens of µg/L,[5,17] their ingrowing input into the water bodies and a long-term exposure may cause toxicity and adverse effects to aquatic organisms.[18] Kolpin *et al*. (2002) found ibuprofen (IBU) in 10% of stream water samples with maximal concentrations of 1 µg/L (median 0.2 µg/L).[19] In two stormwater canals levels of IBU were up to 674 ng/L and of naproxen up to 145 ng/L.[20] In Norway, IBU occurred in all sewage samples, and in seawater at concentrations of 0.1-20 µg/L (sum of IBU and metabolites).[21] In U.K. estuaries maximal concentration of 0.93 µg/L (median 0.05 µg/L) occurred.[22] Because of their aquatic embryonic and larval development as well as their sensitivity to a wide variety of toxic agents, amphibians are suitable in studies of environmental contamination[23,24] as well as for detection of genotoxic agents.[25-27]

Pelophylax shqipericus is a species of true frog (family

Ranidae) and is native in Albania and Montenegro. The Albanian water frog is an endangered species and known populations are currently in decline. Environmental pollution is one of the main causes of the decrease of amphibian population worldwide. In Albania, due to uncontrolled discharge of pharmaceuticals products and agricultural activity, freshwater bodies are polluted. This mostly affects the amphibian tadpoles whose life is closely related to water. To address the problem of *P. shqipericus* population decline, an important Albanian endemic amphibian species, assisted reproductive technology has been applied successfully.[28]

To detect possible effects of a contaminant in the environment, standardized short-term, sensitive, and low-cost methods are applied to estimate toxicity against organisms. Since the presence of pharmaceutical components in Albanian water bodies, is a new phenomenon never reported or measured before, the aim of the present study was to elucidate the possible toxic effects of sub-lethal and environmentally relevant concentrations of IBU, a new source of contamination in the aquatic environments, and also cyclophosphamide's (CP) effects on cellular physiology of amphibian tadpoles, with aiming of using tadpoles as good and reliable bio-indicator organisms for evaluating freshwater ecosystem's health.

Materials and Methods

Animals

All sexually mature Albanian water frog *P. shqipericus*, were obtained from a pond near Scadar Lake (42°10N 19°19E/42.167°N 19.317°E) in the north-western part, Albania, during the breeding season in April-May 2018. After acclimatization in the laboratory for 15 days, *in vitro* fertilization technique was applied, following the procedure described by Turani and Aliko (2018).[28] The eggs were evaluated as successful fertilized when they reached neural stage (stage 14, according to Gosner).[29] All experiments were carried out at a controlled room temperature of 20±0.58°C. In our bioassay, *P. shqipericus* tadpoles at Gosner stage 21, were used.

Chemicals

IBU (α-methyl-4-(isobutyl) phenyl-acetic acid) is a common NSAID, prescribed for the prevention and/or treatment of several human diseases and disorders. Doses of this drug were selected based on environmental concentrations reported in the studies carried out in surface waters, lakes and seawater worldwide. CP (CAS No 50-18-0, Endoxan, Asta), a well-known mutagen, was used as a positive control at a concentration of 5 ppm (mg/L). All test solutions were prepared immediately before each experiment.

Experimental design

The experiment was performed by dividing *P. shqipericus* tadpoles in three groups: a negative control group (n=10); a positive control (n=10) using 5 mg/L CP; and an experimental group (n=10) which was exposed to IBU added directly to water at a dose of 5µg/L for 48 hours. During the exposure period, the tadpoles were kept in 50 L aquaria, with aerated water at 21°C and no mortality was registered. The micronuclei frequency in each group was scored after 24h, 48h.

Blood smear preparation and analysis

The protocol is quick and simple: tadpoles were anesthetized for approximately 2 min in a 5% solution of benzocaine and the blood samples were obtained by cardiac puncture, under a magnifying glass. Two peripheral blood smears for each animal were immediately prepared on clean slides, fixed in absolute methanol for 3 min, and air dried. The slides were stained with Giemsa-Romanowsky for 20 min. For each tadpole, three slides were prepared and scored blind by a single observer, using a light microscope (Digital LCD microscope, DMC-653) linked directly with PC computer for image's processing. The micronuclei frequency was determined in 1,000 erythrocytes from each tadpole blood smear, using 1000× magnification. Coded and randomized slides were scored blind by a single observer. The frequency of micronucleated cells was expressed *per* 1000 cells.

Statistical analysis

Parametric analysis of variance (ANOVA) or the nonparametric analysis (Kruskal Wallis test) based on the data distribution (normality and homogeneity of variance) were used. When an indication of a significant difference ($P<0.01$) was observed, differences were analysed by the post-hoc Dunnett's test.

Results and Discussion

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 erythrocyte abnormalities and micronuclei detection, which can be readily counted in blood smears.[25,30] The frequencies of micronuclei after treatment are shown in Table 1 and the time-response curves at each dose level are shown in Figure 1.

P. shqipericus tadpoles exposed to 5 µg/L IBU showed no significant increase in the frequency of micronucleated erythrocytes compared to the negative control group. Meanwhile, tadpoles exposed to CP (CP positive control), showed a significant increase in micronucleated erythrocytes ($P<0.01$) after 24 and 48 hours of exposure. Statistical analysis was done with ANOVA (Table 2) and Dunn's test (Table 3).

Micronuclei are formed by the loss of whole chromosomes or portions of chromosomes from daughter nuclei at mitosis and exist separately from the main nucleus of the cell. Micronuclei result

Table 1. Frequency of micronucleated red blood cells (*per* 1000 cells) in *Pelophylax shqipericus* larvae exposed to different test compounds.

Treatment	Concentration (%)	No. of cells	No. of micronuclei	24h	48h
Control	-	10,000	3	0.33±0.04	1.0±0.11
Cyclophosphamide	5 mg/L	10,000	23	1.04±0.12*	2.2±0.44*
Ibuprofen	5 µg/L	10,000	18	0.67±0.09	0.63±0.12

*P<0.01.

either from chromosome breaks (clastogenic effects) or dysfunction of the spindle apparatus or centromere kinetochore complexes, with subsequent elimination of whole chromosomes (aneugenic effects).[25,31] Compared to other cytogenetic assays, the several advantages in quantifying micronuclei include the speed and ease of analysis, and the lack of requirement for metaphase cells.[32] Several authors have adapted the micronucleus test to assess the frequency of micronucleated cells in amphibians such as *Pleurodeles waltl*, *Ambystoma mexicanum* and *Xenopus laevis*[33] and tadpoles of the anurans *Rana catesbeiana* and *Caudiverbera caudiverbera*.[34,35]

Due to toxicity of IBU in *P. shqipericus* tadpoles exposed for 48 hours, erythrocyte abnormalities observed were cellular and nuclear vacuolisation, collapse and rupture of the cell membrane (Figure 2). In tadpoles exposed to CP, blood smears showed a pronounced decrease in the number of RBCs and an increase in the percentage of the micronucleated erythrocytes through chromatin fragmentation.

Our results demonstrated that the exposure to IBU caused lesser damage in chromatin level, but elevated the percentage of erythrocyte abnormalities. There is strong evidence that the mode of action of IBU is related to non-specific inhibition of prostanoids, *via* inhibition of the COX enzymes. Exposure to stressors can lead

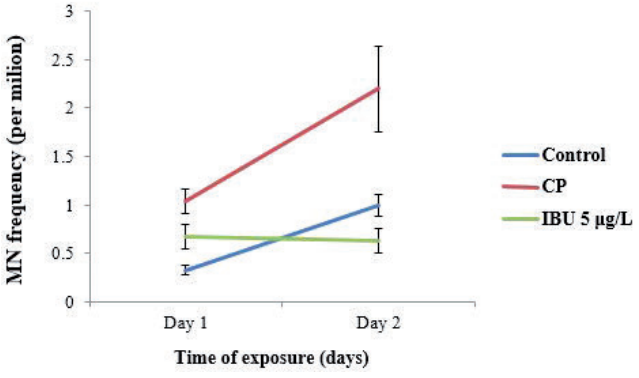

Figure 1. Variation in the micronuclei (MN) frequency with time in each treated group of *Pelophylax shqipericus* tadpoles. The graph shows control, cyclophosphamide (positive control) and the concentration of ibuprofen tested. Data are the mean ± standard error. CP, cyclophosphamide; IBU, ibuprofen.

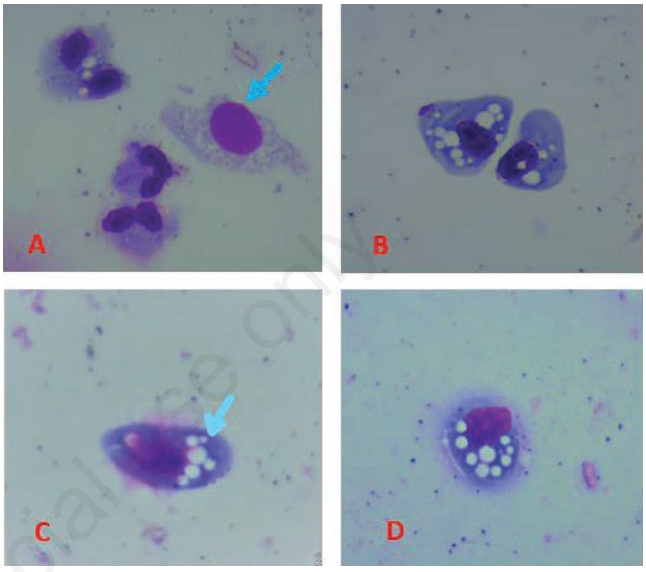

Figure 2. Erythrocyte abnormalities observed in *Pelophylax shqipericus* tadpole exposed to ibuprofen. Giemsa-stained blood smear 1000×. Membrane rupture (A), deformed and cytoplasm-vacuolated cells (B), vacuolated cell (C) and erupted nucleus and cytoplasm-vacuolated cell (D).

Table 2. Statistical analysis with analysis of variance.

Source of variation	SS	df	MS	F	P-value	F crit
Sample	12.35433	2	6.177167	14.28736	1.05E-05	3.168246
Columns	5.340167	1	5.340167	12.35144	0.0009	4.019541
Interaction	3.640333	2	1.820167	4.20992	0.019995	3.168246
Within	23.347	54	0.432352	-	-	-
Total	44.68183	59	-	-	-	-

SS, Sum of Squares; df, degrees of freedom; MS, Mean Square; F, F value; F crit, F critical value.

Table 3. Statistical analysis with Dunnett's test.

Dunnett t (2-sided)[b] (I) Treatment		Multiple Comparisons[a] Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
CP (day 1)	Control	1.2000*	.38893	.009	.2925	2.1075
IBU (day 1)	Control	-.3700	.38893	.542	-1.2775	.5375
CP (day 2)	Control	.7100*	.14722	.000	.3665	1.0535
IBU (day 2)	Control	.3400	.14722	.053	-.0035	.6835

Based on observed means. The error term is Mean Square(Error)=.756. I, Treatment; J, Control; CP, cyclophosphamide; IBU, ibuprofen. *The mean difference is significant at the .05 level. [a]Multiple comparisons; [b]Dunnett t-tests treat one group as a control, and compare all other groups against it.

to pain and inflammation, which in turn increase the proliferation of prostanoids, whose implication in several homeostatic functions in nonvertebrates, such as glucose metabolism and immunity regulation, are reported.[36]

The most frequent erythrocyte alterations following IBU exposure, were cytoplasmic vacuoles. It has been proven that IBU acts as a Ca^{2+} and PO_4^{2-} ions activator during the initiation of the process of opening of the channels found into the inner membrane of mitochondria.[28] There is also an interaction of IBU with lysosomal membrane lipid bilayer, modifying so its morphology. This could also explain the presence of deformed erythrocytes in blood smears. In this process, the alteration of ionic channels, receptors and enzymes found embedded into the membrane lipid layer, could also have been involved.[37,38]

It can be speculated that endoplasmatic reticulum (ER) vacuolization can be triggered by cellular osmotic stress probably induced by IBU toxicity. In this case, ER vacuolization proceeds probably due to mitochondrial dysfunction which lead to an imbalance K^+/Na^+ flux, can cause the increase of cell volume, which can lead to mitochondrial swelling.[39] However, given the incomplete data about the mechanisms of vacuolization, it remains possible that, in at least some cases, vacuole accumulation is an important initiating event, causing metabolic alterations or stress responses that lead to cell death, albeit indirectly.

Decrease in red blood cells in *P. shqipericus* tadpoles during 24 and 48 hours of exposure to IBU suggests anemic condition in the exposed animals. This may be happened due to the deleterious effect of IBU on the hematopoietic system, by inhibiting erythropoiesis *via* transferrin dysfunction.[40]

Our findings demonstrate the exposure to IBU causes several haematological damages, especially erythrocyte-related. It is very likely that IBU causes oxidative stress followed by eryptosis and animal health impairment. Thus, amphibian embryos represent a very useful bio-indicator model organism of *in vivo* studies of different pharmaceuticals effect on freshwater biota.

Conclusions

Tadpoles of *P. shqipericus* can be very good bio-indicators for *in vivo* monitoring of IBU pollution in aquatic environments. This study adds amphibian embryos as an alternative model to study the toxicity of pharmaceuticals.

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