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Effect of Molybdenum, Chrome and Cadmium Ions on Metamorphosis and Erythrocytes Morphology of the Marsh Frog *Pelophylax ridibundus* (Amphibia: Anura)

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ABSTRACT

The effect of high concentrations of molybdenum (Mo), chrome (Cr (VI)) and cadmium (Cd) ions on the metamorphosis of the Eurasian marsh frog *Pelophylax ridibundus* under laboratory conditions have been studied. The effects of these metals at concentrations exceeding Maximum Permissible Concentration (MPC) on the growth, survival and erythrocytes morphology of tadpoles and young frogs have been determined. Morphometry of mature animals and tadpoles and also their erythrocytes for assessment of this effect were used. The high levels of tadpole mortality during 1-20 days of metamorphosis were detected when animals were exposed to high cadmium (2.4 mg L^{-1}) and chrome (4.2 mg L^{-1}) concentrations. After 20 days of metamorphosis, both the survival of tadpoles and the growth of 30 and 60 day animals, exposed to the high concentration of chrome were decreased. Cadmium treatment slightly decreased the survival of tadpoles after 20 days. The morphology of erythrocytes of *Pelophylax ridibundus* was also changed in experimentally polluted water. In experimental environment containing molybdenum and chrome, the area and perimeter of young frog erythrocytes slightly differed from controls. The effects of long-time exposure to cadmium on the morphology of erythrocytes (area and perimeter) were substantially different than those of chrome and molybdenum. Thus, high concentrations of cadmium and chrome in the aquatic habitats are a danger for amphibians, especially, for the early stages of metamorphosis.

Key words: Frog, tadpole, toxic effect, survival, erythrocyte size

INTRODUCTION

With the development of industry, technogenic-pollutant migration has led to the contamination of water, soil and air systems. Industrial and power plant waste, as well as transport exhaust, contain toxic substances such as heavy metals, which accumulate in soil and water biotopes.

According to participation in biochemical processes, metals could be divided into two groups: (1) metals participating in processes of normal growth, development and reproduction, but having a toxic effect on living organisms in high concentrations (molybdenum, bismuth, manganese, cobalt, copper, zinc and chrome) and (2) metals not playing an important role in biochemical processes of living organisms and demonstrating a high toxicity with low concentrations. They can be accumulated in an organism during a long exposition (cadmium, lead and mercury) (Elinder and Piscator, 1978).

Cadmium and chrome are wide spread toxic contaminants. They enter water biotopes from many sources including natural (rocks, groundwater) and industrial and may accumulate in the organs of aquatic animals and plants. Toxic effects of cadmium and chrome (Cr (VI)) have been poorly studied in amphibian species (Selvi *et al*., 2003; Rollins-Smith *et al*., 2004; Snodgrass *et al*., 2005; Stacy *et al*., 2005; Sura *et al*., 2006; Mouchet *et al*., 2007; Sharma and Patino, 2008).

The study of toxic effects of the cadmium, chrome and molybdenum in aquatic organisms, particularly in amphibians, has great implications, since they are widely spread in the polluted biotopes. Amphibians are a key component of many ecosystems and their disappearance may complicate efforts to manage ecosystems on a sustainable basis (Corn, 1994). Amphibians are widely used as bioindicators to detect the presence of toxic agents in water, mainly because their high sensitivity makes them ideal for genotoxity monitoring of aquatic environments (Stacy *et al*., 2005; Sura *et al*., 2006; Edwards *et al*., 2006; Zhang *et al*., 2007; Chen *et al*., 2007). The study of the effects of toxic metals on the development of amphibians will help to identify both the degree of species survival and the causes of the decline in their populations.

Presently, the content of some heavy metals in the soil and water biotopes in the recreational sites on the territory of Armenia was increased up to 2 or 3 times from Maximum Permissible Concentration (MPC) (Avetisyan, 2007). There are many studies of accumulation in water and soil biotopes of Mo, Cr (VI) and Cd and their toxic effects on plants and human development that have been carried out in Armenia (Sagatelyan *et al*., 2003, 2007; Belyaeva, 2007; Mezhunts and Navasardian, 2007; Sahakyan and Amirkhanyan, 2007). However, there are no studies in Armenia regarding the effects of these metals on animals, in particular lower vertebrates such as amphibians. The Eurasian marsh frog *Pelophylax ridibundus* (Selvi *et al*., 2003). *Rana ridibunda* is a widespread species, which inhabits all humid and freshwater habitats in the territory of Armenia. This species, due to wide distribution, high sensitivity and short period of development is an ideal model to study the toxic effect of heavy metals.

The aim of this study is to detect the toxic effects of high concentration (exceeding Maximum Permissible Concentration, MPC) of cadmium, chrome and molybdenum ions on the development during metamorphosis and morphology of erythrocytes of *Pelophylax ridibundus*.

MATERIALS AND METHODS

Eggs of *Pelophylax ridibundus* (Selvi *et al*., 2003) were collected from a clean pond located near Vohchaberd village (Kotaik region) in April 2008 and incubated in glass containers with water from the natural habitat, up to a hatching of tadpoles. After hatching, the one-day tadpoles were placed into 40 L glass containers each with 30 animals. The tadpoles were kept in the laboratory under natural conditions of light-dark period (16:8 h), water temperature (25°C) and constant concentration of studied ions. Control group of tadpoles was incubated in dechlorinated tap water. One group of tadpoles was incubated in dechlorinated tap water+cadmium; second group of tadpoles was incubated in dechlorinated tap water+molybdenum and third group of tadpoles was incubated in dechlorinated tap water+chromium (VI). Experiments were done in two stages: exposing of animals in toxic solutions from one-day larvae to 10-days tadpoles and from 20-days tadpoles to young frogs (conditionally 60-days). Numbers of the survived animals were calculated up every 10 days at all stages of experiments. For modeling conditions with high content of heavy metals, toxicity tests were done using the following concentrations of heavy metals: Cd (2.4 mg L^{-1}), Mo (78.9 mg L^{-1}) and Cr (VI) (4.2 mg L^{-1}), chosen as exceeding Maximum Permissible Concentrations (MPC) for water fishery objects (State norms 2.1.5.690-98). Solutions of Cd, Mo and Cr (VI) were prepared from $CdCl_2$; Na_2MoO_3 and $K_2Cr_2O_7$ (Merck) correspondingly.

Measurements of the basic linear characteristics of the body (length of a body - L.b and length of a tail - L.cd) for 30 and 60-day animals were performed.

A blood films were made from a drop of blood according to the method of blood cells smear (Carmena, 1971). The smears were fixed with 96% methanol and stained with 4% Giemsa stain. Two smears on each individual have been prepared. Erythrocytes were estimated using to the geometric morphometrics. The blood smears were examined under a light microscope with digital chamber (Nikon Digital Camera COOLPIX 4500, USA) with the maximum optical zoom and objective x20. All erythrocytes images were 1024×768 pixels. The area and perimeter of erythrocytes were measured using Image J v.1.41 (Wayne Rasband, National Institute of Health, Maryland, USA, http//rsb.info.nih.govij). A polarization ratio was measured as Rp/Ra ratio according to Kuz'minykh and Petrov for a spreading of cultivated cells (Kuzminykh and Petrov, 2004; Petrov *et al*., 2007). At least 700 cells were analyzed per each specimen. A software package Origin 6.1 (OriginLab Corporation, USA) was used for obtain of curves of regression.

RESULTS

Metamorphosis of the marsh frog *Pelophylax ridibundus* under laboratory conditions proceeds on average for 60 days (control group). The dynamics of survival of tadpoles during the period of metamorphosis in tap water (control), as well as in molybdenum, cadmium and chrome solutions are shown in Fig. 1. All tadpoles incubated at studied concentrations of cadmium and chrome has died by tenth day of development. In contrast, molybdenum increased the survival of tadpoles.

For fitting of experimental data on survival rate the Fit Sigmoidal option from Origin 6.1 was used. The S-curves of control and molybdenum data are shown in Fig. 2. The initial parts of fitting

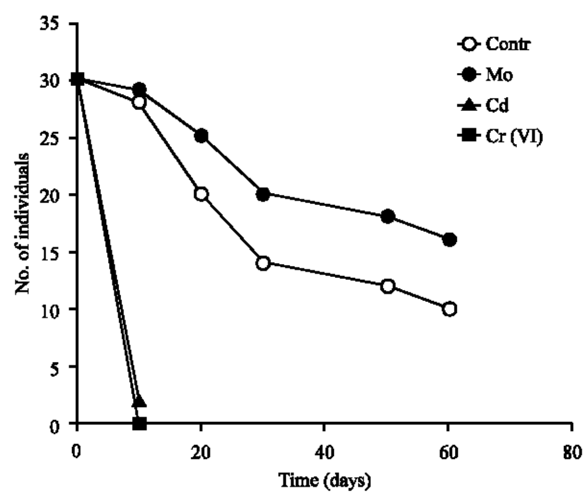

Fig. 1: Dynamics of the survival of *Pelophylax ridibundus*. Control experiments, Mo treatment, Cd treatment, Cr (VI) treatment

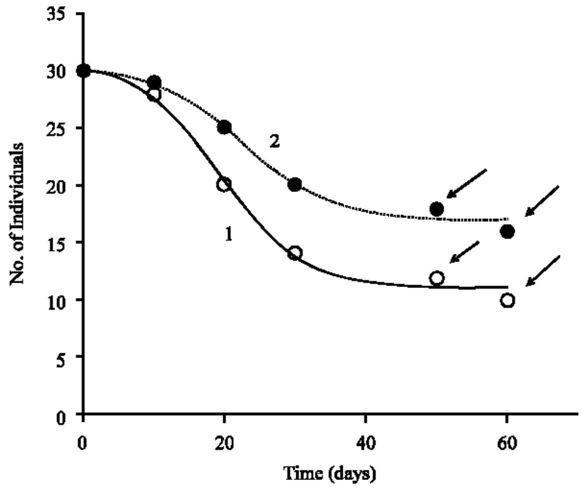

Fig. 2: Fitting of the experimental value by S-curve. (1) Control and (2) Mo treatment. Arrows indicate the experimental values with marked deviation from the theoretical ones

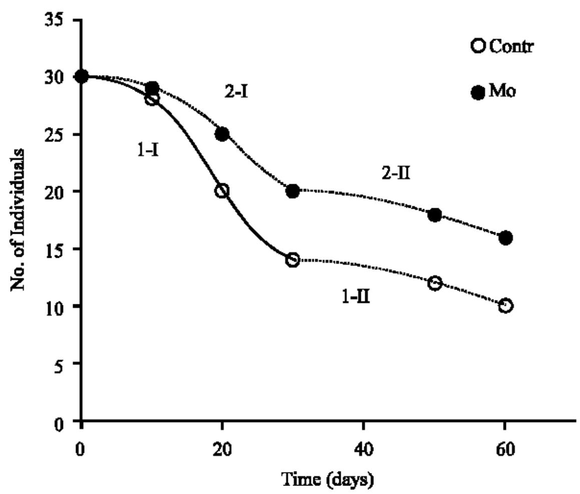

Fig. 3: Fitting of the experimental values of the survival of *Pelophylax ridibundus* by two S-curves (I and II). 1-control curve, 2-Mo-curve

curves coincide with experimental values. Further, despite obvious correlation between control and experimental groups, the experimental values (arrows) considerably differ from theoretical ones. This suggests that the processes described by S-curves have changed approximately after 30 days. The most successful fitting of experimental values in this part (later 30 day) can be with the use of different S-curve (Fig. 3). Thus, the experimental data can adequately be described by a combination of two S-curves-S-curve I (up to 30 days) and S-curve II (after 30 days).

The tadpoles, exposed in water environment with Cr (VI) and Cd during of ten days of metamorphosis have shown 90% mortality. Twenty-day tadpoles were exposed in water environment with Cr (VI), Cd and Mo and their number was taken as 100%. The results are shown in Fig. 4. The fitting curves are presented as S-curves. The Mo-and Cd-curves coincide and vary synchronously with the control. In the case of chrome treatment, the sharp decline of values and absence of synchronism with other variants (Mo, Cd) is observed.

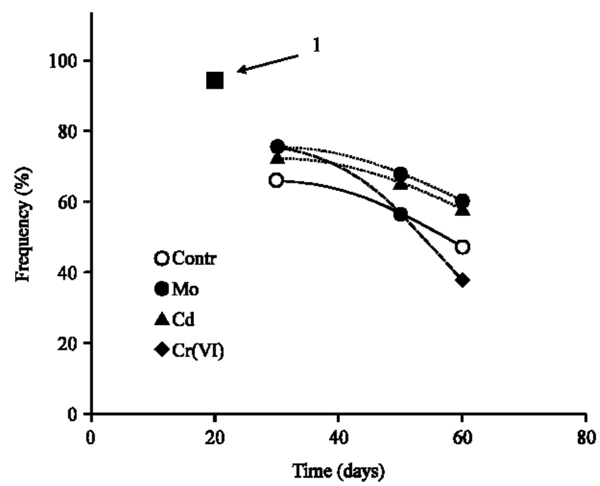

Fig. 4: Effect of Mo, Cr (VI) and Cd to the survival of the *Pelophylax ridibundus* after 20 days of the metamorphosis. The number of twenty days old tadpoles is 100% (1). Fitting was made with use of S-curves

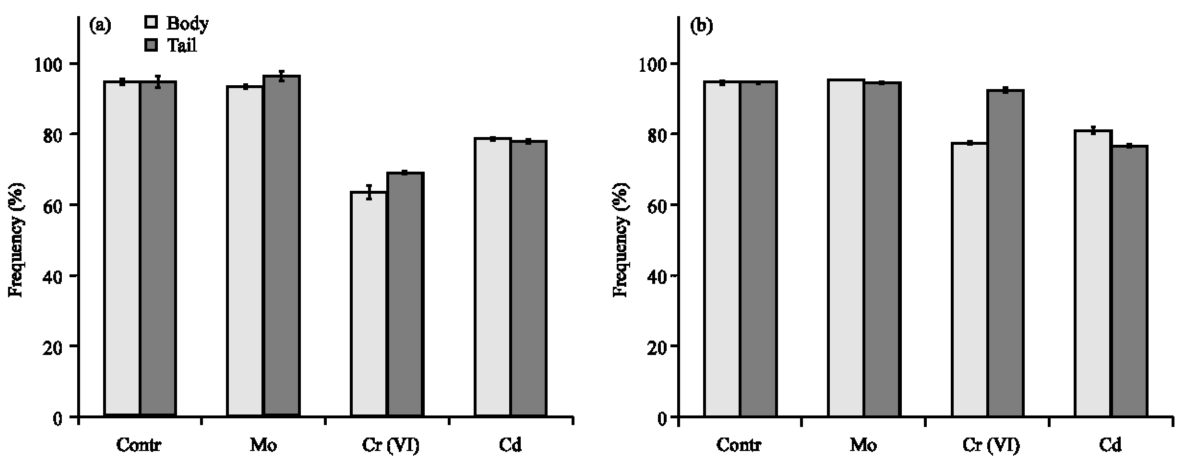

Fig. 5: Effect of Mo, Cr (VI) and Cd on the *Pelophylax ridibundus* body and tail growth. (a) Thirty day and (b) conditionally 60-day old animals. Control values are 100%. Values: Mean±Error

During metamorphosis, the size of the body and tail of tadpoles changes and legs develop. From 30-day, the size of body and tail of experimental tadpoles are changed. The body and tail of 30-day (tadpoles) and 60-day frogs (young) were measured to compare these important stages of metamorphosis. The results are presented in Fig. 5, where absolute values of control are shown as 100%. According to body and tail measurements, Cr (VI) causes a significant decrease in tadpoles body growth (30 day) and frog body growth (60 day), but does not affect the development of frog tail. Cadmium delayed the growth of 30 day tadpoles and young frogs (60 day). Molybdenum has not affected body and tail sizes in 30 day tadpoles as well as in 60 days frogs.

The morphological analysis of erythrocytes of *P. ridibundus* at the final stage of metamorphosis (60-day) was used to estimate the effect of Cd, Mo and Cr (VI) at the cell level. The effects of Mo, Cd and Cr (VI) on morphological cell parameters of erythrocytes (area and perimeter) are presented

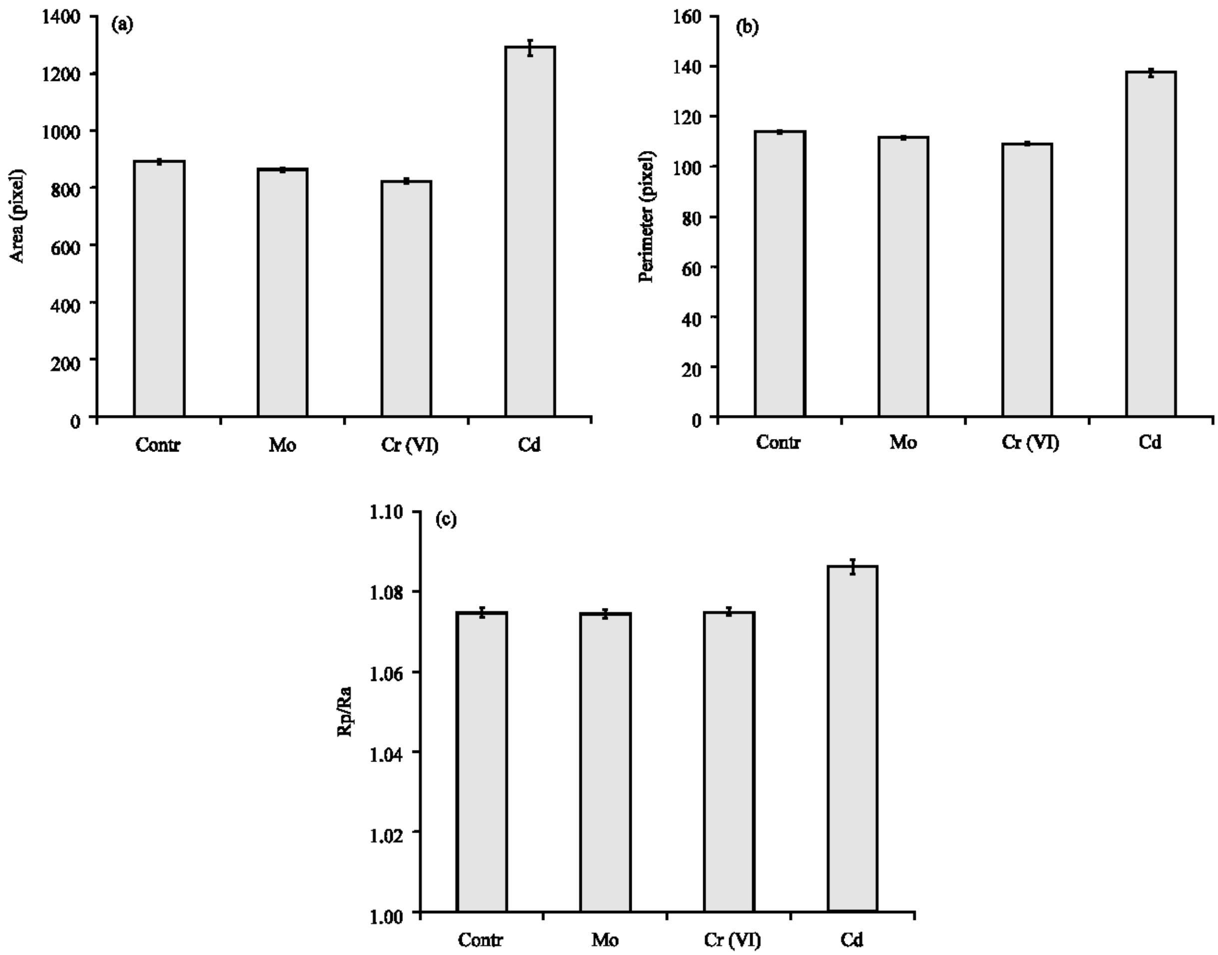

Fig. 6: Effect of Mo, Cr (VI) and Cd on erythrocytes morphology of conditionally 60-days old *Pelophylax ridibundus*. A-Area, B-Perimeter, C-Polarization coefficient (Rp/Ra). Values: Mean±Error

in Fig. 6a-c. There is a small decrease in the area and perimeter of erythrocytes of frogs incubated in water containing of Cr (VI) and Mo in contrast to control. However, no differences of Rp/Ra ratio between the erythrocytes of *P. ridibundus* incubated in tap water (control) and in mediums containing chrome and molybdenum were found. The significant differences in morphology of erythrocytes are observed after incubation of tadpoles in cadmium-containing medium. The area of these erythrocytes is increased approximately 1.5 times, while the perimeter is 1.2 times that of the control ones. Erythrocytes of frogs incubated with cadmium are polarized more strongly than in other cases.

DISCUSSION

Molybdenum and chromium are necessary microelements in small concentrations and are toxic in high doses. The metabolism of these metals in aquatic vertebrates, particularly in amphibians, is poorly studied under environmental pollution. The chemical analysis of soils and freshwater bodies from industrial and recreational territories of Armenia indicated the presence of compounds of Cd, Cr (VI) and Mo at concentrations 2 or 3 times higher than the maximum permissible concentrations (Avetisyan, 2007; Sagatelyan *et al.*, 2007; Mezhunts and Navasardian, 2007; Sahakyan and Amirkhanyan, 2007). In present study we have demonstrated that each of the mentioned metals has a specific effect on the survival and metamorphosis of the marsh frog *Pelophylax ridibundus*. At the studied concentrations exceeding MPC, molybdenum promotes the survival of tadpoles and young frogs. Ions of chromium and cadmium showed a strong toxic effect, increasing the animals' mortality from hatching and up to 10 days of development. However, tadpoles after 20 day of development became more resistant to toxic effect of cadmium and chromium (Fig. 1). After chromium treatment the viability of both tadpoles and young frogs (60 day) was decreased (Fig.1-4). The analysis of the theoretical curves of survival of frogs from hatching up to the young frog stages indicates a two-stage process. Both stages are described by the S-curve.

The study of the morphometric parameters of tadpoles and young individuals of *P. ridibundus* shows two polar processes during the metamorphosis. These processes are body size increase and synchronous tail reduction. This means that process of cells proliferation is accompanied by a local process of the programmed cell death. It is possible to assume that both processes should be relatively autonomous and synchronous. This assumption is in close accord with data received on Eurasian green toad Bufo viridis (Sahin and Balcan, 2006). Authors have shown programmed cell death of skeletal muscle tissues of the regressing tail of tadpole.

Ions of Cr (VI) inhibit the growth of tadpoles (Fig. 5a, b), but do not affect decrease in tail size (Fig. 5b). This indicates that the processes of proliferations in the bodies of animals are relatively independent from processes in the tails. The inhibition of cell proliferation in the body is increased during growth of tadpoles. Since, the processes of cell proliferation up to 60th day (young frogs) were decreased only in the body of animals, we assume that ions of chrome in the second stage do not influence the programmed cell death in their tail.

Present data show that Cd ions do not affect cell proliferation or cells death of 30 day tadpoles. However, Cd promotes a decrease of the size of the body and of the tail of 60 day animals (young frogs). Therefore, it could be assumed that Cd, in contrast to Cr (VI), enhances the processes of apoptosis in the tail at stage II.

Similar effects of Cd and Cr (VI) are known for others species of amphibians. The development of tadpoles of *Xenopus laevis* and *Rana pipiens* during metamorphosis, survival of adult individuals during hibernation, as well as oogenesis were inhibited by cadmium (Karasov *et al.*, 2005; Stacy *et al.*, 2005; Gross *et al.*, 2007; Sharma and Patino, 2008). The stage-dependent uptake features also related to toxic effects were reported for high concentration of chrome during developmental stages of Brazilian Hylidae frogs (Natale *et al.*, 2000). The above mentioned data indicate that cadmium and chrome exhibit high toxic effect during developmental stages of amphibians.

In spite of the numerous data regarding the toxic effect of Cr (VI), Cd and Mo (Natale *et al.*, 2000, 2006; Loumbourdis, 2005; Fenoglio *et al.*, 2006; Tejada-Jimenez *et al.*, 2007; there are insufficient data (Carmena, 1971; Vasilkov and Gricenko, 1989) about the effects of these metals on cell parameters.

Erythrocytes are very convenient object for studying of toxicity of investigated substances at cellular level (Witeska and Kosciuk, 2003; Velcheva *et al.*, 2006; Arnaudov *et al.*, 2008). First, they can be received easily without killing of animals and, secondly, they are true cells. According to the note, the obtained results with the high probability it is possible to extrapolate on any other cells of an organism. In the some known studies (Witeska and Kosciuk, 2003; Velcheva *et al.*, 2006;

Arnaudov *et al*., 2008) various pathological changes in the morphology of fishes and frogs' erythrocytes under the influence of increasing copper and zinc concentrations were found out.

The analysis of morphological parameters of erythrocytes of *Pelophylax ridibundus* has allowed us to estimate the effect of Mo, Cd and Cr (VI) at the cell level (Fig. 6). Molybdenum and chrome do not significant affect the form (Rp/Ra ratio), but they have effect on size (area and perimeter) of erythrocytes in comparison to control. This could indicate that the effects of chrome on processes of cells proliferation and apoptosis do not correlate with their direct influence on cells, if we consider the erythrocyte as a representative cell. Cr (VI) probably has a regulatory function at the level of the organism. On the contrary, the strong change of the size and the form of erythrocytes under cadmium treatment indicates that its effect on cell proliferation and apoptosis correlates with a direct influence on the structure of cells.

Cadmium is known to stimulate the deformation of cells and their damage, the increase in numbers of apoptotic bodies (Loumbourdis, 2005). Chrome induces the delay of growth and necrotic changes. Considering the known toxic effect of Cd and Cr (VI) and present results, we conclude that chrome and cadmium in high concentrations represent the greatest danger to amphibians in the water habitats, especially at early stages of development. The effect of some heavy metals on enzymes of human erythrocytes was shown (Al-Mustafa, 2006). However, the literature data about morphometry of erythrocytes of frogs during their long contact with heavy metals were not found.

The physiological role of molybdenum in life activity of animals is multiform (Tejada-Jimenez *et al*., 2007). Taking into account the important role of Mo and our results, we assume that for animals in the control experiments, the natural background of Mo was lower than physiologically optimal one.

So, examination of the toxic effects of the of high concentrations of molybdenum (Mo), chrome (Cr (VI)) and cadmium (Cd) ions on the metamorphosis and erythrocytes morphology of the eurasian marsh frog *Pelophylax ridibundus* under laboratory conditions has shown that the chrome ions was inhibit the growth of body and the survival of tadpoles. Cadmium treatment induces the slight decrease in both the survival of tadpoles after 20 days and in their growth, as well as affects erythrocytes morphology. Molybdenum treatment increases the survival of tadpoles and changes slightly erythrocytes morphology.

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