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Effects of zinc on morphology of erythrocytes and spleen in *Carassius gibelio*

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Abstract: *The influence of increased zinc concentrations (0.1, 0.5, 1.0, 1.5 and 2.0 mg^{-1} $ZnSO_4.7H_2O$) on the total number and the morphology of the erythrocytes, as well as the processes related to their formation and destruction in the spleen of Carassius gibelio were investigated ex situ. It was found that zinc concentrations caused pathological alterations in the erythrocytes that were not identical in the different concentrations – poikilocytosis; ruptures in cell membranes in the concentrations of 0.5 mg^{-1} and 1.5 mg^{-1}; cells with double nuclei (symplasts); in the concentration of 1.0 mg^{-1}; in the highest concentrations (1.5 mg^{-1} and 2.0 mg^{-1}) presence of erythrocytes at initial stage of atypical mitotic division. Against the background of those various alterations, the total number of the erythrocytes in the peripheral blood increased simultaneously with the increase of zinc concentrations ($p<0.001$). Morphological alterations in the spleen were also observed, indicating a compensational tendency against the toxic influence of zinc upon the fish erythrocytes – hyperplasia of the red pulp and lack of hemosiderin. These results show that the alterations in the total number and the morphology of the erythrocytes are connected with the relevant compensatory histopathological alterations in the spleen. The use of the ascertained alteration could be valuable in monitoring zinc-polluted waters.*

Key words: *Zinc, Toxic influence, Carassius gibelio, Erythrocytes, Spleen*
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Introduction

Fish is one of the most sensitive animals as bioindicators (Hybia, 1982; Hedreyarov and Paoopsu, 1983; Storelli and Macrotrigiano, 2001). Research of Brumbaut *et al.* (2005) point at the opportunity of using fish blood in biomonitoring.

There are studies examining the effects of sublethal chronic concentrations of heavy metals on fish and these studies aim at morphologic and biochemical variations in the organs of different species of fish (Davalli *et al*., 1990, Bieniarz *et al*., 1996, Lionetto *et al.,* 1998, Hollis *et al*., 1999; Wong and Wong, 2000; Zhou *et al*., 2001; Cavas *et al*., 2005; Tyagi and Srivastava, 2005; Loganathan *et al.,* 2006). The content, the allocation and the transfer of these elements are also a subject of research (Carpene *at al*, 1989; Marek, 1990; Radwan *et al*., 1990; Papagiannis *et al*., 2004; Velcheva, 2006; Satyaparameshwar *et al.,* 2006; Demirezen and Uruc, 2007; C'elik and Oehlenschlager, 2007; Vineeta *et al*., 2007; Ayas *et al.,* 2007).

Experiments that are done in the field of ecological toxicology (Vilella *et al*., 2000) are oriented towards studying the variations in the hematological fish indicators. According to Witeska and Kosciuk (2003), zinc causes increasing frequency of abnormal erythrocytes and compensatory reactions expressed by the appearance of immature erythrocytes in the blood flow. The morphologic characteristics of fish blood cells show the possibility of developing of compensatory adaptive processes under the noxious levels of content of heavy metals in waters.

Akahori *et al.* (1999) presume that zinc disrupts cell transport through erythrocyte membranes and that causes the confusion in the function of anti-oxidant protective system and changes in the function of these cell membranes. Palavi and Srivastava (2006) have found that sub lethal zinc concentrations lead to inconvertible histological changes in the kidneys of experimental fish specimens. Loganathan *et al*. (2006) report for histology changes in the brain, degeneration and hemorrhages in the liver of fish samples under the effects of zinc.

The processes of production and allocation of erythrocytes in fish organism are closely related with the function of the spleen. In this sense, there are interesting analyses on fish that have found the relation between the morphologic changes of erythrocytes and the histo-morphologic changes in the spleen under the effects of heavy metals, especially zinc. We could not find similar analyses, but they would enlighten the possible way of heavy metal effects in compensatory adaptive aspect in the above mentioned organs as well as the whole organism. In the present study we followed up the *ex situ* influence of increasing zinc concentrations (as zinc sulphate) on morpho-physiologic changes in erythrocytes of peripheral blood and these in the spleen in order to find the presence of dependent compensatory-adaptive changes in the organism of *Carassius gibelio.*

Materials and Methods

For the aim of the experiment. We used series of increasing concentrations of zinc sulphate solution ($ZnSO_4.7H_2O$), respectively - 0.1, 0.5, 1.0, 1.5 and 2.0 mg^{-1}. The duration of the test for each concentration was 96 hours. We put the control group of fish samples in aquaria with stagnant tap water. The quantity of water in these aquaria for the control samples and the test was 25 litres, the water

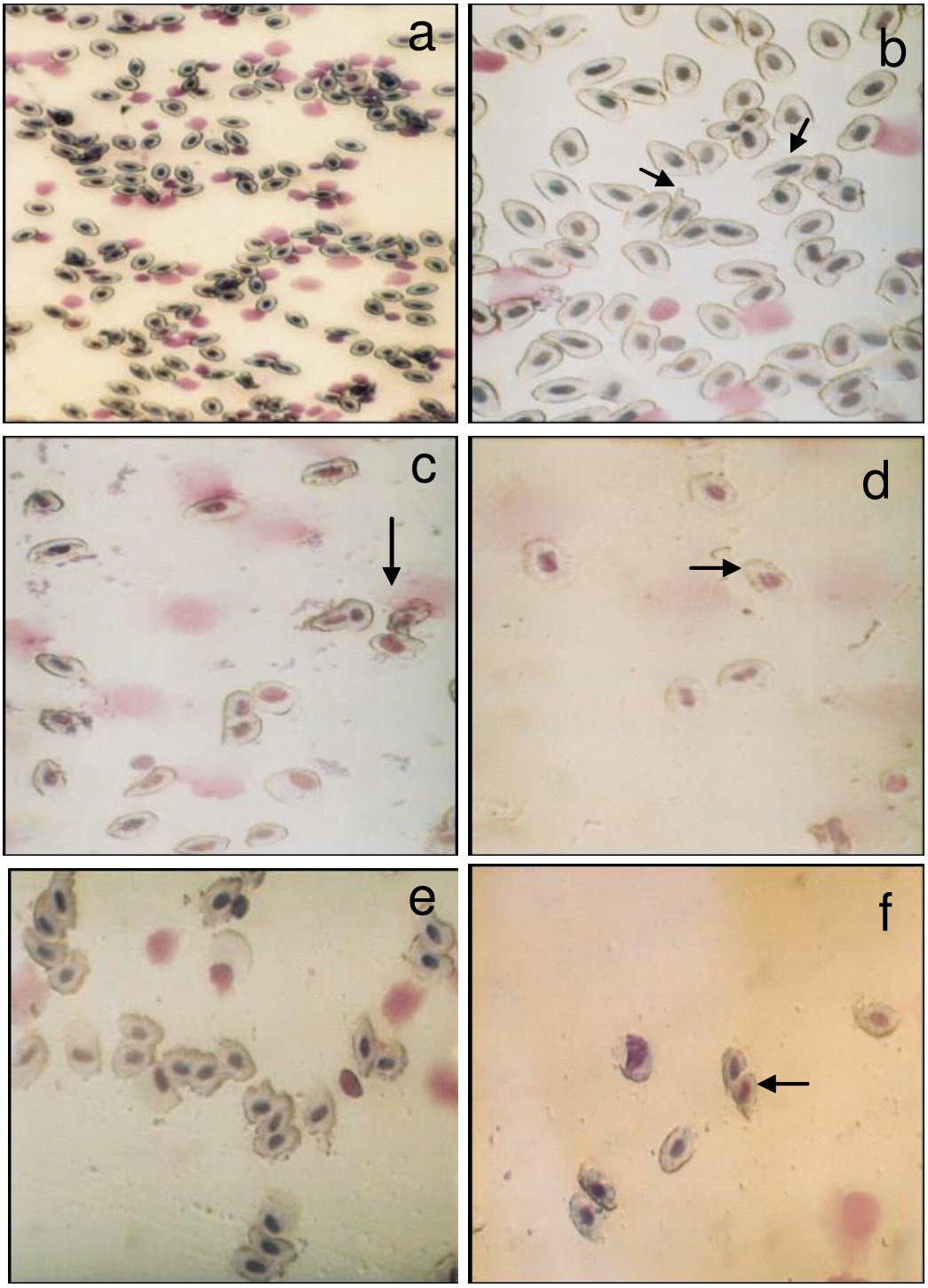

Fig. 1: Morphological alterations in erythrocytes of *Carassius gibelio* under the influence of zinc

a – control group, normal erythrocytes, H&E X 200
b – 0.1 mg l^{-1} $ZnSO_4$, alterations in the shape of some cells; lacerated membranes; a process of division, H&E X 400
c – 0.5 mg l^{-1} $ZnSO_4$, lacerated membranes and shape alterations (round cells; Inclusions in cytoplasm and cells merging) H&E X 400
d – 1.0 mg l^{-1} $ZnSO_4$, laceration of the membranes and granulation of the cytoplasm; alterations in shape towards round and angular cells as well as horseshoe-shaped cells; binucleate, H&E X 400
e – 1.5 mg l^{-1} $ZnSO_4$, alterations in the shape of some cells – misshapen, round as well as double cells (symplasts); lacerated membrane, H&E X 200
f – 2.0 mg l^{-1} $ZnSO_4$, alterations in the shape - mainly round cells; attempts at atypical mitotic division, H&E X 400

temperature was 20°C, pH from 7 to 7.5 and water hardness - 9.5° dH (German hardness).

We used 10 specimens of *Carassius gibelio* for the control for each of the tests. The experimental specimens were taken from a fish-breeding farm where the waters are not polluted with heavy metals (including zinc). The experimental specimens had no external pathological changes and they were of the same size (10-12 cm). The fish were acclimatized for ten days in aquarium with stagnant tapwater and they were not being fed.

The blood samples were taken through heart punctures. The samples were collected in monovet units with anticoagulant (EDTA).

The number of erythrocytes in the peripheral blood of each specimen was determined and the morphologic characteristics of cells were recorded (the shape, the pathologic changes in nuclei, the protoplasm and membranes) and they were registered. The morphologic variations were examined through light microscope (Karl Zeiss, Germany). The blood samples were treated with kits for instant colouring DKK Color-200 (VIVA-MT Bulgaria). The number of erythrocytes was counted in a Burker camera as $E10^{12}$ l^{-1}.

The spleen samples were fixed in 10% formalin solution for 12 hr. The samples for histology analysis were treated with increasing concentrations of ethyl alcohol (70%, 80%, 86%, 96%, 100%) and they were put in paraffin with a melting point of 54-56°C by Evgenieva's method (1983), 0.6 μm wide paraffin cuts were made and put for colouring in hematoxyline and eosin(H&E). The morphologic variations were examined through a light microscope (Karl Zeiss, Germany).

The results were mathematically processed through a variably-statistical analysis described by Sepetliev (1986) by defining t-criteria at a level of equivalence $p < 0.1$.

Results and Discussion

In the course of the present study we followed up the changes only in the number and morphology of red blood cells, as well as the morphologic changes in the spleen. The function of erythrocytes in gas exchange of fish is known and the processes of production and dissociation of these cells are directly connected with the spleen functions.

In our study we found that the number of erythrocytes increases in higher zinc concentrations ($p > 0.001$). It is clearly seen in the high levels -1.5 and 2.0 mg^{-1} zinc sulphate. In the concentration of 1.5 mg l^{-1} the average number of erythrocytes was 2.87 $E10^{12}$ l^{-1} which is twice bigger than that in the control and the preceding concentration (1.0 mg^{-1}). In the subsequent concentration of zinc sulphate the number of erythrocytes (3.86 E 10^{12} l^{-1}) is 2.5 times bigger than that of the control and the biggest in the whole study (Table 1).

Table - 1: Number of erythrocytes x10^{12} l^{-1} of *Carassius gibelio* depending of zinc concentrations

Experimental groups	$ZnSO_4$ concentration (mg l^{-1})	Number of erythrocytes (x10^{12} l^{-1})
Control	–	1.50±0.54
1	0.1	3.40±0.62*
2	0.5	1.30±0.26
3	1.0	1.29±0.06
4	1.5	2.87±0.34*
5	2.0	3.86±0.84*

Data are expressed as mean±standard deviation, *$p<0.001$

We think it is a possible compensatory release of immature erythrocytes as a result of a stress in the organism provoked by the effect of the toxicant. There are similar findings observed by Witeska and Kosciuk (2003).

Our findings show changes in the morphology of red blood cells. We found changes in the shape of cells and nuclei still in the first experimental concentration. The observed changes were not identical in the different concentrations and they reduce to the presence of erythrocytes with anomalous shapes (multinuclear, horseshoe-shaped, kidney-shaped, pointed cells). We found ruptures in cell membranes in the concentrations of 0.5 mg^{-1} and 1.5 mg^{-1} zinc sulphate. We observed cells with double nuclei (symplasts) in the concentration of 1.0 mg^{-1} and in the highest concentrations (1.5 mg^{-1} and 2.0 mg^{-1}) we found erythrocytes at initial stage of atypical mitotic division that is not a characteristic process in these cells. We suppose that it possibly concerns including an additional compensatory mechanism for increasing the number of erythrocytes in an environment of heightened zinc content. In 1.0 mg^{-1} concentration we also found changes in the cytoplasm expressed by granulation, and we observed necrosis in the nuclei in concentrations of over 1 mg^{-1} zinc sulphate (Fig. 1).

Some other researchers found changes similar to ours but they used different fish species. Akahori *et al.* (1999) report ruptures in erythrocytes membranes. Witeska (2004) prove nuclear malformations, cytoplasmic vacuolization and common changes in red blood cells. Koca *et al.* (2005) report micro-nuclei in erythrocytes as well.

The changes that we found in morphology of the spleen involve changes in the capsule, the parenchyma, sinuses and blood vessels which we found with a high level of expression in the experimental concentrations of zinc sulphate (Fig. 2).

The thickening of the capsule got stronger in increasing concentrations more than 0.5 mg^{-1}.

We found a progressive thickening of parenchyma in higher concentrations. Sinuses also become progressively narrower till

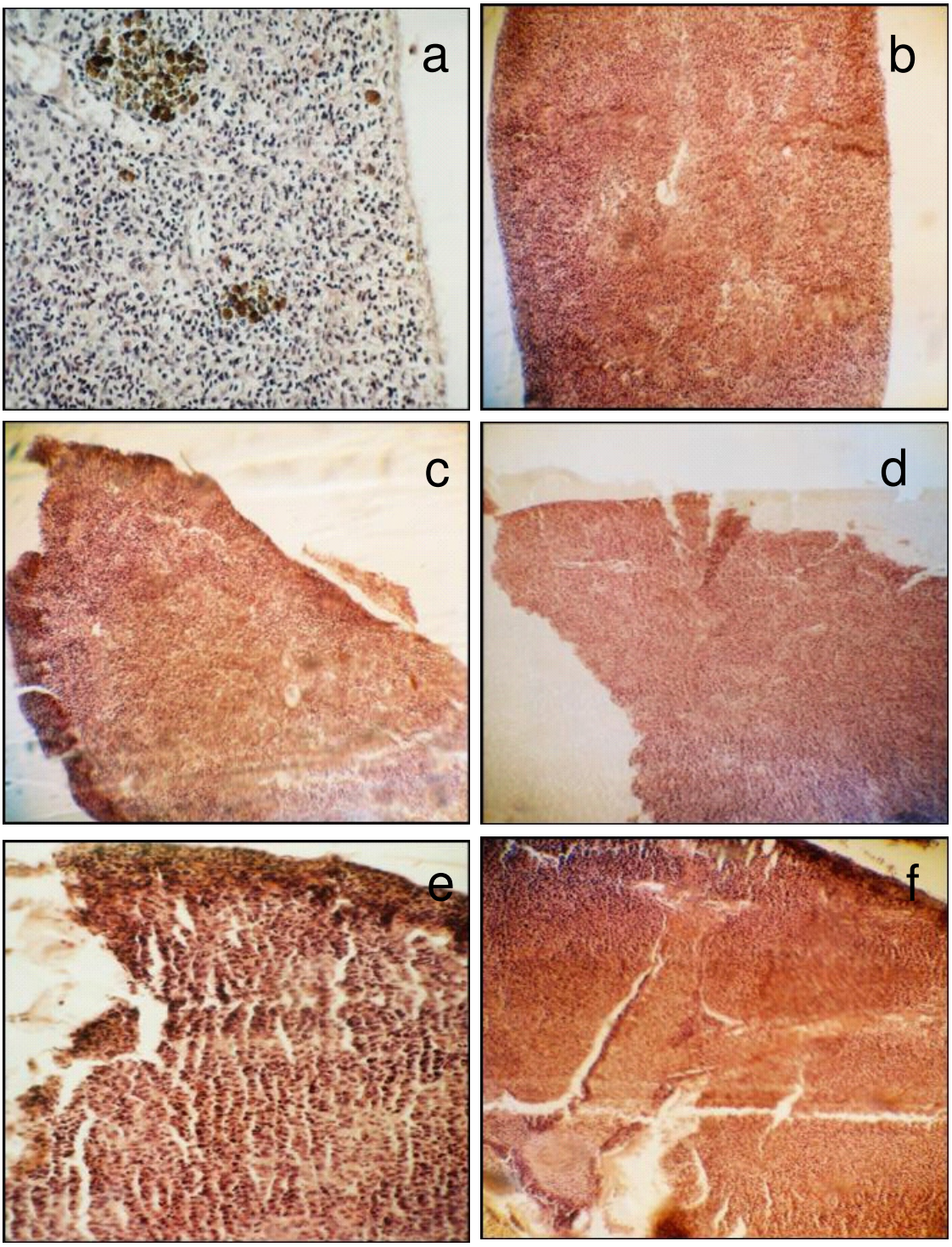

Fig. 2: Morphological alterations in spleen of *Carassius gibelio* under the influence of zinc, H&E X 200

a – control group, normal spleen
b – 0.1 mg l^{-1} $ZnSO_4$, lack of hemosiderin in the red pulp and thickening of the parenchyma; found no hemosiderin
c – 0.5 mg l^{-1} $ZnSO_4$, thickening of the capsule and heavy growth of parenchymal tissue; the sinuses - very thin; found no hemosiderin
d – 1.0 mg l^{-1} $ZnSO_4$, the capsule - highly thickened and the sinuses were hardly visible; thickening of the parenchyma; found no hemosiderin
e – 1.5 mg l^{-1} $ZnSO_4$, a highly thickened capsule; parenchyma – lacerated; found no hemosiderin
f – 2.0 mg l^{-1} $ZnSO_4$, highly thickened capsule and no sinuses; found no hemosiderin

their total disappearance in 2.0 mg l^{-1}. Still in the first concentration we found a disappearance of the hemosiderin from the pulp and we did not find it in the other test concentrations. In the highest content of zinc sulphate - 1.5 and 2.0 mg^{-1}, we observed spontaneous ruptures in the parenchyma and the blood vessels were full of big quantity of erythrocytes (hyperemia).

We think that the changes found in the spleen trace out two tendencies concerning the compensatory reactions of the organism towards the toxic effects of zinc on erythrocytes. The first tendency relates to the appearance of hyperplasia of parenchyma and a compensatory thickening of the capsule, and the second tendency – the loss of hemosiderin. We reckon that parenchyma hyperplasia is related to the intensive processes of destruction and formation of erythrocytes in the red pulp of the spleen. It is obvious that there is a very strong reaction towards the toxic zinc effects and these changes cause ruptures or even the formation of tumors like those found by Oliveira *et al.,* (2005), in the spleen of eel, *Anguilla anguilla*. Moreover, an additional compensatory thickening of the spleen capsule occured in order to impede the risk of spontaneous ruptures. Evidently, in the highest concentrations the processes of destruction and formation of new cells were quite intensive so the thickened capsule cannot with stand the hyperplasia of parenchyma which cause the spontaneous ruptures.

In contrast, Khan *et al.* (1994) report a hemosiderosis in the spleen of *Pleuronectes americanus* in waters with a chronic content of heavy metals. We think that the differences in the findings are due to the fact that zinc effects of the studied concentrations probably cause some operating mechanisms for conversely inclusion of the hemoglobin released from the destroyed cells in the intensively forming new ones. This idea is confirmed by our finding that the number of erythrocytes increases the peripheral blood of *Carassius gibelio* in the higher zinc concentrations.

It becomes clear from our results that zinc has expressively negative influence on morphology-physiologic characteristics of erythrocytes in the organism of *Carassius gibelio* which are related and dependent on the working compensatory processes in the spleen. This gives us good reason to recommend the changes and dependences found in our study to be used in monitoring zinc polluted waters in a way appropriate to the European Union directives for fish health.

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