CLINICAL, HEMATOLOGICAL AND MORPHOLOGICAL STUDIES ON EX SITU INDUCED COPPER INTOXICATION IN CRUCIAN CARP (CARASSIUS GIBELIO)

КЛИНИЧНИ, ХЕМАТОЛОГИЧНИ И МОРФОЛОГИЧНИ ПРОУЧВАНИЯ НА EX SITU ПРЕДИЗВИКАНА МЕДНА ИНТОКСИКАЦИЯ ПРИ КАРАКУДА (CARASSIUS GIBELIO)

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ABSTRACT

The influence of increasing concentrations of copper sulphate ($CuSO_4.5H_2O$) on Crucian carp (Carassius gibelio) was investigated. The clinical signs, the morphologic changes in the spleen and gills, and the alterations in red blood cells were analyzed. The concentrations tested were respectively 0.1, 0.5, 1.0 and 2.0 $mg.l^{-1}$. Various clinical symptoms were observed resulting from damaged oxygen transport, hemorrhages being the most frequent. Also, different concentrations of copper caused three various types of anemia. Pathological alterations caused by compensatory reactions refer to the adaptation of Carassius gibelio to the hypoxia, caused by the pathologic changes in gills and the anemia that occurred.

Key words: Carassius gibelio, Copper, anemia, hematology, gills, spleen.

РЕЗЮМЕ

Проучено е влиянието на нарастващи концентрации меден сулфат ($CuSO_4.5H_2O$) върху външния вид, поведението, показателите на червената кръвна картина, както и върху хистоструктурата на хриле и слезка на каракуда (Carassius gibelio). Концентрацията на медния сулфат беше съответно 0.1, 0.5, 1.0 and 2.0 $mg.l^{-1}$. Наблюдавани бяха разнообразни клинични промени, обусловени от нарушения в транспорта на кислорода, като най-често срещани бяха кръвоизливите. Установено бе също, че различните медни концентрации предизвикват три различни типа анемия. Патологичните промени в изследваните органи са основно от компенсаторен тип, свързани с адаптацията на Carassius gibelio към хипоксията и анемията.

Ключови думи: Carassius gibelio, мед, анемия, хематология, хриле, слезка.

INTRODUCTION

Copper becomes toxic when its concentration is increased due to environmental industrial pollution. Nowadays, the amount of copper in water basins progressively grows due to some industrial and agricultural polluters. In comparison with other heavy metals, copper intoxication in freshwater fish hasn't been investigated as much. It is known that the copper causes oxidative stress [3, 5] and cytotoxic effect on some cell lines [12, 24, 31, 34, 35]. Also, the bioaccumulations of the copper and metallthionein induction have been investigated [4, 11, 16]. But for the needs of diagnostic and the biomonitoring, the investigation on clinical, histopatological and hematological alteration caused by copper appears quite interesting.

In many carp species, including Carassius gibelio, anemia was detected as an important clinical manifestation of intoxication with heavy metals and, in particular, with copper [15, 21, 26, 28, 34].

Accordingly, it is a matter of great interest to study the clinical and morphologic manifestations of anemia caused by copper intoxication. This has to be studied, due to the fact that erythrocyte's circulation flows mainly in the spleen (hemopoesis and destruction) and also in the girls (gas diffusion).

The aim of the present investigation; to reveal the clinical manifestations, the morphologic changes in the spleen and gills, and the changes in the characteristics of red blood cells of Carassius gibelio induced by copper.

MATERIAL AND METHODS

For the, experimental animals were used: gibelio carp (Carassius gibelio). These fish were taken from a clean water pond situated in a fish breeding farm. The specimens had no external pathological changes and they were of the same size and age group (length 9.5÷12 cm, weight 90÷120 g). The fish were not being fed during the experiment.

The stagnant tap water was put in aquariums with capacity of 25 liters. For the aim of the experiment a series of increasing concentrations of copper sulphate ($CuSO_4.5H_2O$) were used. Four experimental groups plus one control group were formed. Test concentrations were 0.1, 0.5, 1.0 and 2.0 $mg.l^{-1}$ of copper respectively. The initial concentration (0.1 $mg.l^{-1}$) is under the Threshold Limit Value of Concentration by the Bulgarian Standards for copper in waters. Stagnant tap water was used as a control sample. Each group possesses 30 specimens. Water for all experimental and control samples were maintained at the following parameters:

temperature – 20°C, pH – 7.0–7.5 and water hardness – 9.5 dH. The duration of the test for each concentration was 96 hours.

Clinical studies. The following characteristics were followed:

- behavior (motion, oxygen searching, time and type of reaction in conditions of irritation);
- status of the skin, eyes, scales and fins;
- mortality.

Red blood cell analysis. The blood test-samples were taken through heart puncture in monovet units with anticoagulant (EDTA). The following parameters were examined:

1. Total number of erythrocytes; to count the number of erythrocytes, we used a chamber method according to Angelov et al. [2], and used in fish laboratory practice in Bulgaria. Blood sample was taken with an erythrocytes pipette and diluted (1/200) with Hayem solution. One drop of hemolyzed blood was transferred onto Burker lamella and examined in a light microscope (Olympus CX21 model) with magnification of 400x. Normal and no tear cells were counted only.
2. Hemoglobin content; the amount of hemoglobin was determined according to cyanomethemoglobin procedure [2]. Non-clotted blood (20 µL) was diluted with Drabkin solution (1mL) and left stand for 10 min. The absorbance of the mixture was read at 540 nm (Specol 11, 1 cm kuvette) and the amount of hemoglobin was calculated from the Parelly Rumed hemoglobin standard.
3. Hematocrit; the microhematocrit method was used [2]. Non-clotted blood was transferred into microhematocrit pipette and centrofuged at 12.5 rpm for 5 min (microhematocrit centrifuge H-240 model). Then the ratio of shaped blood cells in plasma was determined.

These parameters served for calculation of erythrocyte indices – mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) [2].

Morphological analysis

Macroscopic examination. After taking the blood samples, the fishes were seceded .The type, size and colour of the spleen and gills were examined. The abdominal cavity was checked for any hemorrhages and other pathologic changes.

Microscopic examination. The gill lamellae and spleen from each experimental fish were collected. Tissue samples were fixed in 10% formaldehyde for 24 hours. These samples were processed in paraffin with a melting point of 54-56^{0}C. Paraffin sections (0.5 - 0.7 µm thick) were stained with hematoxylin and eosin (HE). These sections were examined by light microscopy (Olympus CX21 model), using as a reference Takashima and

Hybiya [30].

Statistical methods. The results were variably and statistically processed with methods described by Sepetliev [27]. A 95% confidence limit test was applied to compare the means whenever the data was significant ($p<0.05$).

RESULTS

Clinical appearance. In the lowest copper concentration: no external changes, compared to the control specimens, were found. No important alteration in their behaviour was noted. When copper sulphate concentrations got higher (0.5mg.l^{-1} and more), there were some progressive and developing clinical changes:

1. The fish gathered near the aerator and their motion activity got weaker. They moved slowly, uncoordinatedly and showed a very weak reaction to any external irritation. (Figure 1a).

2. The water got darker because of the mucus secreted. The water got distinctly darker in the higher copper concentrations. (Figure 1a).

3. Various pathologic changes were observed at the external inspection on the skin and visible mucous membranes. Hemorrhages were detected – spot-like hemorrhages appeared on the skin in submandibular areas, around both gill fins, the abdominal and hind fins. When the concentration got higher, spot-like and bigger hemorrhages in other skin regions were also found. In the highest concentration (1.0 mg.l^{-1} and 2.0 mg.l^{-1}) it could already see large regions of skin with scales that had fallen off. Also, the skin was with diffuse hyperemia. There were also distinctive hyperemia and erythrodiapedesis in the blood vessels of the back, gills, abdominal and hind fins (Figure 1c).

In some individuals, put under the effects of the highest copper concentration, massive hemorrhages over the eye sclera were found (Figure 1b). As well as a damaged wholeness of the back, anal and abdominal fins, which were seriously jagged.

The skin colour of the fish, affected by the highest copper concentration, was grey to dark grey.

The abdominal cavity of many of the fish specimens under observation was full of serous brownish liquid. In the fish, treated with 1.0 mg.l^{-1} copper sulphate and higher copper concentrations, we found hemorrhages on the inner surface of the abdominal cavity.

Mortality. In copper concentrations of 0.5 and 1.0 mg.l^{-1}, the death-rate was 30%. In concentration of 2.0 mg.l^{-1}, the death-rate got up to a sharply higher, 80%.

Red blood cell analysis.

Under the influence of low copper concentrations, the hematocrit value of the blood decreased, with minimum in concentration of 0.5 mg.l^{-1}. When the concentration got higher, the hematocrit value increased and was comparable to the control samples in concentration of 2 mg.l^{-1} (Table 1).

As a whole, in comparison with the control specimens, the volume of erythrocytes decreased throughout this experiment. However, with exception, there was the value of concentration 1.0 mg.l^{-1}. Where, a statistically significant difference was not found. It could be assumed that the possible reason for this is: an attempt made to compensate for the anemia that occurred in the foregoing concentrations. The value of MCH strongly changed depending on the copper concentration. It changed from critically low in 0.1 mg.l^{-1} and 2.0 mg.l^{-1} too. The "Mean hemoglobin concentration in erythrocytes" showed strong undulating alterations in dependence of the concentration used. In 0.5 mg.l^{-1} it even got higher due to the very low values of the hematocrit (Figure 1). Like MCH, MCHC found the lowest values of 2.0 mg.l^{-1} (Table 1).

Our findings concerning blood characteristics of gibelio carp, in the course of the experiment, definitely showed the presence of anemia. According to the values found, the type of anemia in the different copper concentrations was as follows:

In concentrations of 0.1 mg.l^{-1} and 2.0 mg.l^{-1} – it is microcytic, hypochromic, in concentration of 0.5 mg.l^{-1} – microcytic, normochromic, and in concentration of 1.0 mg.l^{-1} – normocytic, normochromic.

Morphological changes.

Table 1: Erythrocyte indices of *Carassius gibelio* after exposure to increasing copper concentrations

Experimental groups	Hematocrit (l/l)	MCV(fl)	MCH (pg)	MCHC (%)
Control	0.230±0.010	163.18±38.56	52.49±12.15	33.31±2.8
0. 1mg.l^{-1}	0.195±0.015**	101.32±14.29***	29.91±11.37***	29.98±7.02*
0.5 mg.l^{-1}	0.082±0.012***	125.97±50.05***	49.43±17.90	39.92±4.59***
1.0 mg.l^{-1}	0.105±0.037***	163.32±50.09	55.19±18.87	33.17±6.88
2.0 mg.l^{-1}	0.224±0.032	135.20±5.96***	16.19±1.56***	13.29±0.99***

*p≤0.05; **p≤0.01; ***p≤0.001

Gills. Macroscopically, hyperemia in the gills was found. In some fish specimens under lower copper concentrations (0.1, 0.5 and 1.0 $mg.l^{-1}$), hyperemia was combined with anemic or cyanotic regions. In the highest concentration, whitish polyps were found, as well as necrotic regions. Besides, the gills seemed severed and the wholeness of the lamellae was seriously damaged.

The histological analysis of gills showed degeneration in the lamellae and disorder of blood circulation in the lamellae – lamellar aneurysms, in all tested concentrations of copper sulphate. These results confirmed completely the observed macroscopic changes. In 0.5 $mg.l^{-1}$ copper sulphate and in the higher concentrations, besides degeneration in gills a fusion (sticking together of two and more contiguous filaments) was found and also hyperplasia of gill lamellae (increase in the number of erythrocytes in gill lamellae) (Figure 1d-g).

Spleen. Macroscopically, in the experimental specimens a distinctive anemia was observed. In contrast to fish from the control group, yet in the low copper concentration, thickening of spleen capsule was noted on tissue sections. No clumping of macrophages filled with hemosiderin was found. It can be assumed that the lack of hemosiderin is due to the intensive usage of the hemoglobin secreted by the destroyed erythrocytes for formation of new erythrocytes. In the higher concentrations, besides the thickening of the capsule, a strong congestion of the red pulp, as well as narrowing of the sinuses was found (Figure 1i-l).

DISCUSSION

As a result of the current study the following conclusions could be drawn:

Copper possesses manifested lethal effect on gibelio carp. However, this species is resilient than other teleost freshwater fish, by which the lethal doses of the copper are lower [18, 20, 33]. Only crucian carp (Carassius carassius) possesses a similar high resilience to copper toxicity [26]. Saulamtumur et al. [25] reported that the copper did not cause death in Oreochromis niloticus L., but by treatment with very low doses (from 0.1 to 5.0

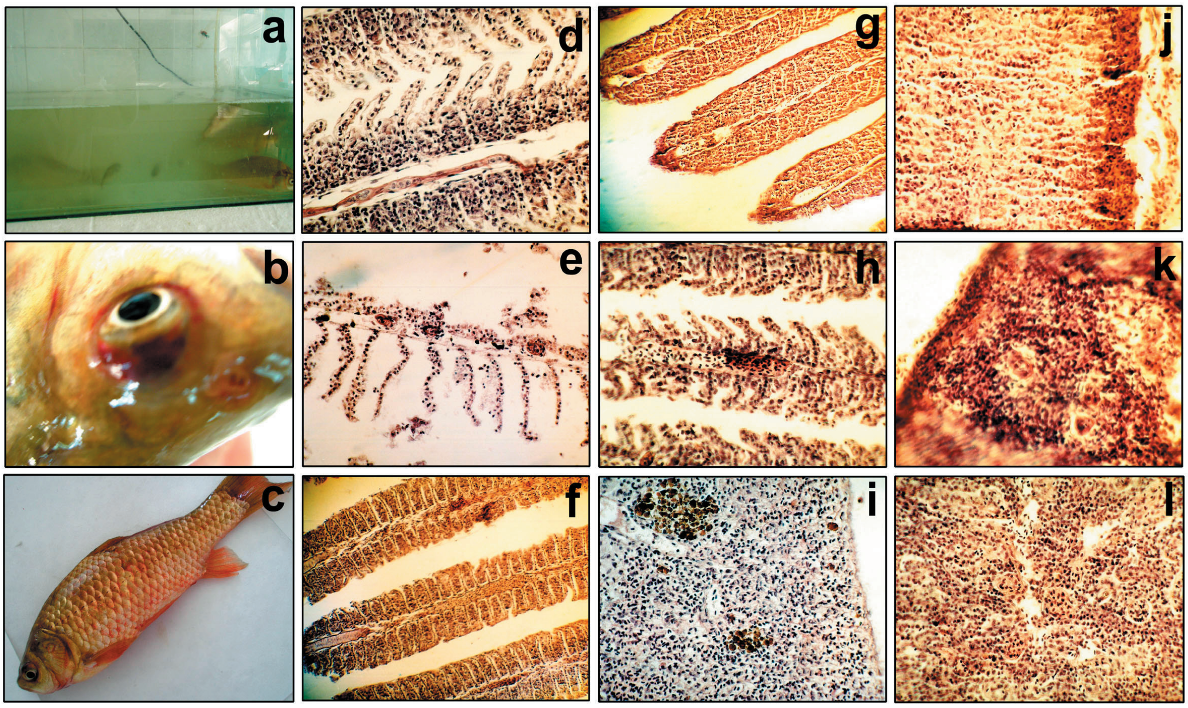

Figure 1. Morphological alterations in gills and spleen of Carassius gibelio induced by copper.
a. Heaping of the fish around the aerator and opaquing of the water; b. Massive hemorrhages in the sclera; c. Hyperemia and erythrodiapedesis in the back, gills, abdominal and hind fins; d. Control group, normal gills. HE, x 400; e. Degeneration of the gills. HE x 400; f. Fusion of lamellaes. HE x 200; g. Aneurysm of the central blood vessel of the lamellaes. HE x 200; h. Hyperplasia of the lamellaes. HE, x 400; i. Control group, normal spleen. HE, x 400; j. Thickness of the capsule and lack of hemosiderin. HE, x 400; k. Thickness of the red pulp. HE, x 400; l. Very thin sinuses HE, x 400.

ppm of copper). The toxic effect of Cu^{2+} on gibelio carp is evidenced by the pathological changes in the tissue and by the type of the induced anemia. Analyzing the toxic effect copper has on fish, however, the animals' size, nutrition, and water indexes should be considered - e.g. temperature, hardness, alkalinity and organic substances in it [8, 10, 17, 19].

The skin alterations found in our study are similar to these observed by Sola et al. [29] in trout fish Oncorhynchus mykis (skin edema, strong mucus production and cell desquamation). However, while in gibelio carp the haemorragic diathesis is more manifested, in Oncorhynchus mykis the skin edema is more visible.

Gelev et al. [9] also found massive hemorrhages in the skin and the abdominal cavity, as well as in the eye, but only in cases of hemorrhagic septicemia found in the rainbow trout.

The alternations in the gills observed in this investigation are similar to the pathological changes found by Muhvich et al. [23] in gills of goldfish Carassius auratus. However, there were different announcements for damages in gills under the effects of other heavy metals or in other species [1, 7, 13, 14, 21, 22, 25, 28]. Hence, gill damages are a common manifestation of heavy metal effects in some freshwater fish. However, in certain species they are not a specific effect of copper intoxication. It is established that by common and gibel carp (but not in rainbow trout) the sublethal concentrations cause respiratory stress [6].

The alterations in the spleen under the influence of copper are most likely a result of the hyperfunction of the organ, connected with the active erythropoiesis and destruction of abnormal erythrocytes. In our previous study, identical changes were registered in the spleen of Carassius gibelio under higher zinc concentrations [32]. Therefore, the histologic changes in the spleen, like the changes in gills, are not specific for a certain metal. However, they show a strong compensatory reaction of the organism connected with compensating the anemia caused by heavy metals.

As a result of the hematological investigation, it can be assumed that the occurring anemic changes provoke the development of a compensatory increase in the number of erythrocytes in the blood. However, the hemopoietic system is not able to provide normal hemoglobin filling in the increased number of the newly-formed erythrocytes. As a result of this, a considerably low hemoglobin concentration in erythrocytes was found. Probably, it is one of the general pathologic factors of the copper intoxication in Carassius gibelio.

Mazon et al. [23] studying the manifestation of acute copper poisoning in Prochilodus scrofa, reported a significant increase in the hematocrit and red blood cell values. The increase in red blood cells was associated with an increase in total blood hemoglobin, only in fish exposed to higer copper concentrations. An increase of the hematocrit value was also found by Schjolden et al. [26] in crucian carp (Carassius Carassius) under the effect of 300 μg/l Cu^{2+}.

The various compensatory reactions, which were detected in Carassius gibelio, probably refer to the adaptation of the organism to the hypoxia caused by the pathologic changes in gills, as well as the anemia that occurred. This observation might be confirmed by the fact that a lot of authors claim that bio-accumulation of heavy metals in fish occurs firstly in the gills [25].

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