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The effect of bleeding on peripheral blood and head kidney hematopoietic tissue in common carp (*Cyprinus carpio*)

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Abstract: Adverse environmental conditions (contamination of water with nitrite, pesticides, or other pollutants), nutritional deficiencies, bacterial and viral infections, and parasitic invasions may induce anemia in fish, in both natural and aquaculture conditions. Changes in hematological values and cellular composition of peripheral blood and head kidney hematopoietic tissue of bled common carp (Cyprinus carpio L.) were evaluated over a 4 week period post-bleeding (30% blood loss). The results showed that fish very quickly compensated for anemia. Erythrocyte count (RBC), hemoglobin concentration (Hb), and hematocrit (Ht) gradually increased to values significantly higher than they were before bleeding; this was accompanied by an increase in erythroblast frequency in blood and in the head kidney. In addition, WBC count increased, which was probably related to lymphocyte release from the spleen or other lymphoid sites; the percentage of blood lymphocytes and lymphoid lineage in the head kidney remained unchanged. Neutrophil frequency in the blood was also unchanged; however rejuvenation of their population occurred, accompanied by a decrease in total neutrophil abundance in the head kidney. The percentage of circulating monocytes, basophils, and eosinophils decreased, which indicates that their recovery was slow. The obtained results revealed very high hematopoietic (particularly erythropoietic) capacity in the carp head kidney. This was indicated by a significant increase in the frequency of early blast cells (precursors of various cell lineages) and RBC precursor cells at various stages of development. This indicates that carp are able to recover from anemia caused by various adverse environmental and/or nutritional factors.

Key words: Fish, anemia, hematopoiesis, erythropoiesis

Introduction

Anemia in fish may result from impaired erythropoiesis, an increased hemolysis rate, or from severe bleeding; it may also be induced by toxic chemical agents, viral and bacterial infections, parasitic invasions, malnutrition, or starvation. Therefore, fish may suffer from anemia both under natural and aquaculture conditions. Anemia results in a reduced oxygen supply to the tissues and thus, impairs the growth and health status of fish.

High nitrite concentrations may occur in intensive aquaculture systems as a consequence of high stocking densities. Intoxication with nitrite can result in anemia, which follows the development of methemoglobinemia (1,2). In addition, exposure to the cyanobacterial toxins (microcystins) often present in fish pond water may cause anemia in fish (3).

Various pesticides used in agriculture that enter surface waters with runoff from watersheds

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were reported as anemizing agents. Kawatsu (4) observed severe anemia in common carp as a result of intoxication with the herbicide Molinate, which caused body lesions and bleeding. Anemia in *Clarias albopunctatus* was observed as a result of intoxication with the pesticide Actellic (5). According to Anandkumar et al. (6), the insecticide Dimecron induced anemia in *Heteropneustes fossilis*.

The pollution of water with heavy metals may induce anemia in fish due to damage to circulating erythrocytes and/or impaired hematopoiesis. Kori-Siakpere et al. (7) and Kori-Siakpere and Ubogu (8) reported cadmium and zinc-induced anemia in *Heteroclarias*. According to Orun et al. (9), copper induced macrocytic anemia in *Oncorhynchus mykiss*. Acute mercury poisoning resulted in anemic response in *Channa gachua* (10).

Anemia was also reported to be a result of deficiencies in vitamin C (11), cyanocobalamin, and folacin (12) and starvation (13).

Various bacterial (14,15) and viral (16,17) infections often result in anemia in fish. Anemia is also induced by parasite infestations. According to Ruszczyk et al. (18) *Trypanosoma borrelli* induced anemia in common carp by digesting host hemoglobin by cysteine proteinase. According to Nakayasu et al. (19), the blood-feeding parasite *Neoheterobothrium hirame* induced anemia in cultured *Paralichthys olivaceus*. Wagner and McKinley (20) reported that sublethal infestation with blood-feeding sea lice may reduce swimming performance, due to anemia, in *Oncorhynchus mykiss*. Post-hemorrhagic anemia was observed in cage-cultured *Dicentrarchus labrax* as a result of attacks from the blood-feeding isopod *Ceratothoa oestroides*.

Anemia is usually followed by compensatory reactions: the release of erythrocytes from tissue reservoirs (spleen and head kidney) and activation of hematopoiesis. In most fish the head kidney is the main hematopoietic organ (21). However, little is known about changes in cellular composition of the hematopoietic tissue of anemic fish (22) or the hematopoietic capacity of the fish organism.

The aim of present study was a preliminary evaluation of the susceptibility of common carp to anemia and the rate of hematological renewal in fish subjected to experimental blood loss under good environmental and nutritional conditions.

Materials and methods

Common carp (*Cyprinus carpio* L.) were harvested from the rearing pond of the Inland Fisheries Institute at the age of 5 months and then reared for 1 year in the aerated flow-through laboratory tank in the Department of Animal Physiology, University of Podlasie, Siedlce at 18-20 °C under stable water quality conditions (dissolved oxygen saturation over 60%, NO_2^- under 0.5 mg/dm^3, and NH_4^+ below 1 mg/dm^3). The fish were fed Aller Classic 4 mm pellets (30% protein, 7% fat, 43% carbohydrate, 7% ash, 5% fiber) once a day at the rate of about 1% of body mass/day. Before the experiment, 20 fish with a body mass 60.7 ± 12.4 g were transferred to four 100 dm^3 aerated aquaria (5 fish in each), and left for 2 weeks to acclimate. They were fed in the same manner, water temperature was 20 ± 1 °C, and three-quarters of the water was renewed every day without removing or disturbing the fish. Oxygen saturation never dropped below 60%, NO_2^- concentration was below 0.3 mg/dm^3, and NH_4^+ level was below 1 mg/dm^3. Water quality parameters were measured daily before water renewal.

After the acclimation period, 1 cm^3 of blood was collected from each fish (about 30% of peripheral blood, assuming 5% blood content in carp body) with heparinized needles and placed into heparinized chilled plastic tubes. As a control sample 5 fish were sacrificed for head kidney analysis. After bleeding, the remaining 15 fish were returned to the aquaria. About 100 mm^3 of blood was collected from 5 fish for hematological analyses 1, 2, and 4 weeks after bleeding. Fish were then killed and the head kidneys isolated. Blood was analyzed for hematocrit (Ht), hemoglobin concentration (Hb), erythrocyte (RBC) and leukocyte (WBC) counts, and blood smears were analyzed for erythrograms and leukograms. Additionally, derived erythrocyte parameters—mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC)—were calculated.

Ht was evaluated by microhematocrit method in heparinized capillaries after centrifugation at

12,000 rpm. Hb concentration was measured by spectrophotometric cyanmethemoglobin method at a 540 nm wavelength in blood mixed 1:10 with Drabkin reagent. RBC and WBC counts were done by Burker hemocytometer in blood diluted 1:100 with Hayem's solution. The smears were stained with May-Grunwald and Giemsa solutions and viewed at 1000× magnification (using immersion) under a Nikon Eclipse E600 microscope. For the erythrograms, 300 erythrocytes were inspected, and percentage of erythroblasts (smaller, round, polychromatic cells) and abnormal and hemolyzed erythrocytes were calculated. For the leukograms 100 WBCs were viewed, and the percentage of lymphocytes, neutrophils (myelocytes, metamyelocytes; band and segmented), monocytes, basophils, and eosinophils were calculated. The derived RBC parameters were calculated according to the following formulas: MCV = Ht × 100/RBC, MCH = Hb/RBC, and MCHC = Hb × 100/Ht.

For hematopoietic tissue analysis fish were killed by rapid severing of the spinal cord with sharp scissors. Then, head kidneys were isolated and the fresh organ surface was blotted, smeared gently on degreased slides, left to dry for 24 h, and stained with May-Grunwald and Giemsa solutions. Slides were viewed at 1000× magnification. In each smear, 500 blood cells were identified and counted, according to Fijan (23); the results were expressed as percents. The identified cells were then grouped into main cell lineages (each included various developmental stages). Fields with crowded or damaged cells were excluded. Areas of kidney imprints with numerous mature erythrocytes (possibly blood vessel contents) were also ignored.

The obtained results were subjected to statistical analysis using the nonparametric Mann-Whitney test, assuming differences were significant at $P \leq 0.05$.

Results

Blood loss of about 30% resulted in some distinct alterations of hematological parameters and a picture of peripheral blood and hematopietic tissue. Fish exhibited the following, 1 week after bleeding: significantly reduced Ht and MCV values; increased RBC and MCHC, accompanied by an increase in erythroblast percentage; and elevated hemolysis rate; Hb level did not significantly change (Table 1). WBC also significantly increased, accompanied by a rejuvenation of neutrophil population increase in percentage of youngest cells (myelocytes and metamyelocytes) and decrease in mature segmented cells (Table 2). Ht returned to the initial (pre-bleeding) level 2 weeks after bleeding, MCV and MCH decreased, while RBC and Hb increased

Table 1. The effect of bleeding on red blood parameters in carp (mean ± SD; values within a row with different letter superscripts are significantly different; Mann-Whitney test, $P \leq 0.05$).

Parameters	Time after bleeding (weeks)			
	Before (n = 10)	1 (n = 5)	2 (n = 5)	4 (n = 5)
Ht (%)	27.0 ± 2.6 ^{a}	20.9 ± 4.1 ^{b}	26.6 ± 2.8 ^{ac}	31.6 ± 3.8 ^{c}
Hb (g/dm^3)	64.2 ± 12.9 ^{a}	68.8 ± 4.8 ^{a}	85.2 ± 5.2 ^{bc}	95.6 ± 16.1 ^{c}
RBC (10^6/mm^3)	1.17 ± 0.21 ^{a}	1.47 ± 0.37 ^{b}	2.55 ± 0.35 ^{c}	2.94 ± 0.37 ^{c}
MCV (fL)	236 ± 38 ^{a}	157 ± 84 ^{b}	106 ± 21 ^{b}	109 ± 23 ^{b}
MCH (pg)	56.9 ± 13.6 ^{a}	49.7 ± 15.4 ^{ac}	33.8 ± 4.1 ^{b}	33.0 ± 7.4 ^{bc}
MCHC (g/dm^3)	240 ± 42 ^{a}	336 ± 47 ^{b}	322 ± 24 ^{b}	302 ± 26 ^{b}
Erythroblasts (%)	7.3 ± 2.1 ^{a}	9.7 ± 1.3 ^{b}	21.6 ± 1.4 ^{c}	27.7 ± 3.3 ^{d}
Abnormal erythrocytes (%)	0.7 ± 0.5 ^{a}	0.4 ± 0.3 ^{a}	0.3 ± 0.3 ^{a}	0.5 ± 0.3 ^{a}
Hemolyzed erythrocytes (%)	3.0 ± 1.1 ^{a}	7.4 ± 1.5 ^{b}	2.1 ± 0.6 ^{a}	0.0 ± 0.0 ^{c}

Table 2. The effect of bleeding on white blood parameters in carp (mean ± SD; values within a row with different letter superscripts are significantly different; Mann-Whitney test, $P \leq 0.05$).

Parameters	Time after bleeding (weeks)			
	Before (n = 5)	1 (n = 5)	2 (n = 5)	4 (n = 5)
WBC (10^3/mm^3)	28.9 ± 10.3 [a]	59.7 ± 21.9 [b]	116.4 ± 17.6 [c]	157.0 ± 34.3 [d]
Lymphocytes (%)	72.9 ± 4.3 [a]	75.0 ± 1.0 [ab]	77.6 ± 3.9 [ab]	78.4 ± 4.9 [b]
Neutrophils (%)	19.4 ± 3.4 [a]	19.2 ± 3.6 [a]	18.4 ± 2.7 [a]	18.0 ± 3.4 [a]
Myelocytes (%)	3.3 ± 1.5 [a]	5.4 ± 1.1 [b]	2.0 ± 1.2 [ac]	1.2 ± 0.8 [c]
Metamyelocytes (%)	4.4 ± 1.8 [a]	6.8 ± 2.6 [b]	7.0 ± 2.3 [b]	4.0 ± 1.6 [a]
Band (%)	5.4 ± 1.9 [a]	3.6 ± 1.5 [a]	5.6 ± 1.5 [a]	10.4 ± 2.2 [b]
Segmented (%)	6.3 ± 1.5 [a]	3.4 ± 2.1 [b]	3.8 ± 1.8 [ab]	2.4 ± 0.9 [b]
Monocytes (%)	3.6 ± 2.0 [a]	3.2 ± 1.6 [ab]	2.8 ± 1.1 [ab]	2.4 ± 1.1 [b]
Basophils (%)	2.8 ± 1.3 [a]	1.8 ± 1.3 [ab]	0.8 ± 0.4 [b]	1.0 ± 0.7 [ab]
Eosinophils (%)	1.4 ± 0.8 [a]	0.8 ± 0.8 [ab]	0.4 ± 0.5 [b]	0.2 ± 0.4 [b]

significantly. Further increase in erythroblast levels was observed. WBC also increased significantly, and the percentage of myelocytes returned to the initial level; however the percentage of metamyelocytes was still elevated. The percentage of basophils and eosinophils dropped significantly as compared with the initial value. Further increase in Ht, Hb, and RBC took place 4 weeks post-bleeding, accompanied by ongoing reduced MCV and MCH and elevated MCHC. Percentage of erythroblasts increased compared to all previous values, and no hemolysis was observed. Further significant increases in WBC occurred, accompanied by a significant increase in lymphocyte percentage. The share of metamyelocytes dropped below the initial value, and the level of band neutrophils increased, while the percentage of segmented cells decreased again. This was accompanied by a significantly reduced level of monocytes and eosinophils.

The results of hematopoietic tissue analysis revealed a gradual increase in percentage of the earliest blast stages beginning from the second week post-bleeding (Table 3). A very sharp increase in the percentage of RBC precursors appeared 1 week post-bleeding, and was still significantly elevated after 4 weeks. The percentage of neutrophil lineage decreased and did not recover until the end of experiment. This was also the case with basophil and thrombocyte lineages. No significant differences occurred in percentage of lymphocyte, monocyte, of eosinophil cell lineages.

Discussion

The results of the present preliminary study revealed that a blood loss of 30% did not induce persistent anemia in carp, due to a very rapid compensatory reaction. An increase in RBC accompanied by a decrease in MCV and elevated erythroblast level in the peripheral blood—and a considerable percentage increase of RBC lineage in the head kidney—indicate very strong erythropoiesis activation. This is comprehensible in light of the fact that erythrocytes are the predominant cell species in circulating blood; thus, their loss due to bleeding was higher than other blood cells. As in mammals, fish erythropoiesis is triggered by erythropoietin release in response to tissue hypoxia (24). Montero et al. (25) reported very fast recovery in bled *Sparus aurata*; RBC count started to increase 2-4 days post-bleeding and returned to the basal value after 8 days. An increase in percentage of erythroblasts and young

Table 3. The effect of bleeding on composition of head kidney hematopoietic tissue in carp (mean ± SD; values within a row with different letter superscripts are significantly different; Mann-Whitney test, $P \leq 0.05$).

Cell lineages (%)	Time after bleeding (weeks)			
	Before (n = 5)	1 (n = 5)	2 (n = 5)	4 (n = 5)
Early unidentified blast cells	3.7 ± 0.6 [a]	3.3 ± 3.5 [a]	8.3 ± 6.1 [ab]	10.5 ± 5.0 [b]
Erythrocytes	3.1 ± 2.0 [a]	40.7 ± 11.3 [b]	34.0 ± 20.0 [bc]	14.9 ± 14.1 [c]
Lymphocytes	54.0 ± 5.0 [a]	44.4 ± 12.4 [a]	48.1 ± 10.7 [a]	58.5 ± 12.4 [a]
Neutrophils	15.2 ± 4.1 [a]	3.1 ± 2.0 [b]	1.9 ± 0.1 [b]	6.3 ± 8.5 [ab]
Monocytes	1.1 ± 0.6 [a]	1.2 ± 0.7 [a]	1.7 ± 0.7 [a]	1.0 ± 0.8 [a]
Basophils	2.5 ± 2.1 [a]	1.5 ± 0.6 [a]	0.8 ± 0.4 [ab]	0.3 ± 0.3 [b]
Eosinophils	0.0 ± 0.1 [a]	0.3 ± 0.3 [a]	0.2 ± 0.2 [a]	0.2 ± 0.2 [a]
Thrombocytes	20.4 ± 4.1 [a]	5.4 ± 2.5 [b]	5.4 ± 5.1 [b]	8.1 ± 1.6 [b]

erythrocytes in the peripheral blood of bled (20%-25% of blood loss) *Ictalurus punctatus* was observed by Fijan (23) 1 and 2 weeks post-bleeding; however, only a slight and insignificant increase in the contribution of erythroid populations occurred in the head kidney. According to Murad and Houston (26), maturation of erythrocytes in another cyprinid, *Carassius auratus*, required 16-20 days. An increase in hemolysis frequency 1 week post-bleeding may indicate that, with an increased erythropoietic rate, faster senescence and the destruction of older erythrocytes may take place and cell turnover rate is accelerated. On the other hand, no increase in the frequency of abnormal erythrocytes was observed, which shows that an increase in the erythropoietic rate did not involve the production of defective cells. Nakayasu et al. (20) reported that bled and fed *Paralichthys olivaceus* produced normal juvenile erythrocytes; in bled and starved fish numerous deformed, hypochromic, and vacuolated cells were observed.

Chudzik and Houston (27) reported that total recovery from anemia in *Carassius auratus* at 30 °C took 7 days, while at 7.5 °C no recovery was observed for 70 days. According to Rothmann et al. (28), activation of erythropoiesis in anemic *Cyprinus carpio* took place earlier at 25 °C than at 14 °C; however, no complete recovery was observed until 35 days post-bleeding. These data indicate that hematopoiesis in fish is a temperature-dependent process. Ability to recover from anemia obviously depends also on the nutritional status of the fish. According to Rios et al. (13), erythropoiesis in *Hoplias malabaricus* already decreased significantly during first 30 days of starvation. Nakayasu et al. (20) reported gradual recovery of fed *Paralichthys olivaceus* from severe anemia induced by repeated bleedings; unfed fish were not able to compensate for blood loss.

Sometimes fish are unable to compensate for anemia. Lecklin and Nikinmaa (29) observed no stimulation of erythropoiesis in anemic *Salvelinus alpinus* under good nutritional and thermal conditions.

The obtained results also showed a gradual significant increase in the WBC count post-bleeding and rejuvenation of the neutrophil pattern. A slight but significant decrease in the percentage of monocytes, basophils, eosinophils, and thrombocytes in hematopoietic tissue indicate that recovery of these cells was delayed compared to lymphocytes and neutrophils. The unchanged percentage of lymphocytes in the head kidney hematopoietic tissue indicates that their increase in the peripheral blood probably resulted from splenic or other lymphatic tissue supply. Depletion of neutrophils in the head kidney shows that this was the source of juvenile cells in the blood. An increase in percentage of early blast cells in the head kidney, up until the end of

experiment, indicates that hematopoietic activity was still elevated even at 4 weeks post-bleeding. No data were found in the literature on the changes in blood WBC count in fish subjected to anemia. Fijan (23) reported no change in percentage of lymphocytes, monocytes, or eosinophils and a slight decrease in neutrophil percentage in *Ictalurus punctatus* subjected to 20%-25% blood loss. He did not find any significant differences in the percentage of lineages in the head kidney; the frequency of thrombocyte lineage decreased but less significantly than in our study.

The observed increase in values of hematological parameters post-bleeding are difficult to explain, but it should be kept in mind that hematological values in fish are highly variable (30). According to Luskova (31), it is very difficult to establish normal hematological values in fish; these values are highly relative, and there is no sharp readily defined division between normal and abnormal.

The increase in frequency of early blast cells in the head kidney showed a general activation of hematopoietic function, and the increase in frequency of RBC precursors among hematopoietic cells indicates that the recovery of blood cells was proportional to their loss. The obtained results showed the very high potential of common carp head kidney hematopoietic tissue. These results indicate that under good environmental and nutritional conditions fish are able to compensate for anemia very quickly.

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