Original Research

The Effects of Cadmium on Common Carp Erythrocyte Morphology

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Received: 6 August 2010
Accepted: 29 December 2010

Abstract

The effect of short-term and long-term *in vivo* exposure to cadmium on the morphology of common carp erythrocytes was evaluated, and comparison of *in vitro* toxicity of various cadmium salts to these cells was also done. Juvenile carps were subjected to 3 h exposure at 6.5 mg/l or to a 4 week exposure at 0.65 mg/l of cadmium. Full blood was incubated for 2 h with cadmium chloride, sulfate, or nitrate at the concentration of 5 µl/ml of blood. All exposures resulted in a significant increase in frequency of abnormal erythrocytes. Anomalies included chromatin condensation at the nucleus border, nuclear malformation, cell body malformation, cytoplasm vacuolation, and swelling and hemolysis, with nuclear anomalies being the most frequent. In fish subjected to long-term cadmium exposure (and in some degree in fish after short-term exposure) an increase in erythroblast frequency occurred, which indicates hematological compensation for erythrocyte damage. A high percentage of abnormal erythrocytes observed under *in vitro* conditions (also in the control) indicates very high sensitivity of these cells to experimental factors. Cadmium nitrate induced more anomalies in carp erythrocytes (particularly nucleus malformations and cytoplasm vacuolation) than chloride and sulfate. The obtained results confirmed the genotoxic and cytotoxic properties of cadmium and showed that fish erythrocytes are good models for cytotoxicity studies.

Keywords: fish, heavy metal, genotoxicity, cytotoxicity, hemolysis

Introduction

Cadmium, a common aquatic pollutant, is a heavy metal that does not play any known physiological role. Very often cadmium-induced anemia is observed in fish [1-6] due to its adverse effect on iron uptake and metabolism, and direct damage to erythrocytes [7].

Fish erythrocyte morphology is more sensitive to various environmental agents than basic red blood parameters [8-10], and cellular anomalies are sometimes observed without distinct decrease in their values [2, 11, 12]. According to Sharma et al. [13], fish erythrocytes were more sensitive to water pollution compared to the other biological endpoints and thus should be included in the routine fish bioassay. Erythrocyte anomalies may result from various physiological disturbances. Evelyn and Traxler [14] and Eiras et al. [15] reported cytoplasmic inclusions and erythrocyte degeneration: irregular outlines, nuclear displacement, condensation and leakage of nuclear material, and cytoplasmic vacuolation as a result of infection with the viral erythrocyte necrosis virus. Buckley [16] observed Heinz bodies, poikilocytosis, clumped chromatin, ragged cell membranes, altered staining properties, and hemolysis in erythrocytes of *Oncorhynchus kisutch* from water contaminated with chlorinated sewage. Zeni et al. [9] reported erythrocyte echinocytosis in *Ictalurus melas* sublethally exposed to anionic detergent. Frequencies of nuclear anomalies such as irregular nucleus shape, vacuolation, binuclei

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and micronuclei that indicate genotoxic effects often increase in fish exposed to water pollution [17-19], but may also fluctuate seasonally [20]. Boge and Roche [21] observed cytotoxicity of various phenolic compounds to the erythrocytes of *Dicentrarchus labrax*. They reported loss of ATP and hemolysis. Erythrocyte anomalies were also reported in fish subjected to intoxication with heavy metals. Gill and Pant [22] observed erythrocyte swelling, poikilocytosis, vacuolation, amitosis, deformation, and deterioration of cell membranes in *Barbus conchonius* exposed to chromium, and nuclear aberrations such as chromatin condensation, nuclear puffs, and chromatin leakage in the same species subjected to cadmium intoxication [23]. Gwoździński [24] observed a decrease in membrane fluidity, change of internal viscosity, and internal protein conformation and hemolysis in erythrocytes of common carp subjected to copper and mercury, fish erythrocytes being more sensitive than those of humans. Witeska [10] observed various erythrocyte anomalies in common carp sublethally intoxicated with heavy metals. Toxicity of metals to carp erythrocytes ranged (according to the frequency of erythrocyte anomalies): Pb≥Zn>Cd>Cu, and the changes induced by various metals, were similar: nuclear malformation, chromatin condensation, cell swelling, and malformation. Karuppasamy et al. [6] reported increased fragility, rupture of erythrocyte membrane, and hemolysis in *Channa punctatus* sublethally exposed to cadmium.

The aim of the present study was an evaluation of the effect of a short-term and long-term *in vivo* exposure to cadmium on the morphology of common carp erythrocytes, and comparison of *in vitro* toxicity of various cadmium salts to these cells.

Materials and Methods

For the *in vivo* experiment 90 carp of 29.4±14.7 g body mass were brought from the Inland Fishery Institute rearing pond, and acclimated for a month to the laboratory conditions of a flow-through aerated tank at 18-20ºC. The fish were fed Aller Classic feed once a day. After acclimation the fish were transferred to 9 aerated aquaria of 100 l volume (10 fish in each). One group was in clean dechlorinated tap water (Control), four groups were subjected to 3 h exposure at 6.5 mg/l of Cd (as $CdCl_2 \cdot 2½H_2O$ solution), which was the 96 h LC50 value calculated using probit method from the results of earlier acute toxicity test and subsequently kept in clean water. Another four groups were continuously exposed to 0.65 mg/l of Cd (10% of 96hLC50 value). Water was changed daily by gentle siphoning out of about ¾ volume and replacing it with clean water or Cd solution, respectively. At the same time, feces were removed from the bottom using a plastic tube. Water temperature was 20ºC, DO saturation did not drop below 60%, ammonia and nitrite concentrations did not exceed 1 mg/l and 0.3 mg/l, respectively. Fish were fed Aller Classic feed once a day, 1-2 hours before water change. Blood was collected from the fish after 1, 2, 3, and 4 weeks post 3 h exposure at 6.5 mg/l of Cd (Cd_k1, Cd_k2, Cd_k3, Cd_k4), after 1, 2, 3, and 4 weeks of continuous exposure at 0.65 mg/l of Cd (Cd_d1, Cd_d2, Cd_d3, Cd_d4), and from the control group. From each fish blood was sampled only once.

For the *in vitro* test blood was collected from eight healthy live common carp of 70±15 g obtained from the Inland Fishery Institute rearing pond, and then reared for a year in the flow-through aerated tank at 18-20ºC. The fish were fed once a day Aller Classic feed. About 200 µl of blood was collected from each fish by heart puncture using heparinized needle into heparinized plastic tube. To evaluate the effects of various soluble cadmium compounds on erythrocyte morphology full blood from each fish was divided into 4 Eppendorf tubes (20 µl to each) and was incubated for 2 hours with $CdCl_2 \cdot 2½H_2O$, $3CdSO_4 \cdot 8H_2O$, or $Cd(NO_3)_2 \cdot 4H_2O$ solutions (2 µl of 50 mg/l solutions made in 0.6% NaCl), at final concentration of 5 µg Cd /ml of blood. Control samples were incubated the same way with buffered 0.6% NaCl solution.

In both experiments blood smears were made, stained with May Grunwald and Giemsa solutions, and fixed with Canada balsam and cover glass. The smears were inspected using a Nikon Eclipse E600 microscope (at 1000 × magnification) connected with Nikon Coolpix digital camera and computer equipped with a CoolView image analysis system. In each smear 300 erythrocytes were viewed and classified. Cellular and nuclear anomalies were registered and photographed, and their frequency was calculated. The obtained results were subjected to the U test ($p \leq 0.05$).

Results

The results of *in vivo* study revealed significant effect of both short-term and long-term cadmium exposure on carp erythrocyte morphology. Frequency of all anomalies was significantly elevated in all Cd-exposed groups compared to the control (Table 1). The most frequently observed abnormality was a nonuniform distribution of chromatin within the nucleus – it was condensed near the nuclear membrane forming a ring with poorly stained center (Fig. 1 A). After short-term exposure the maximum level of erythrocytes with marginal chromatin occurred in a week post exposure (Cd_k1), but remained elevated until the end of the experiment. In long-term exposure the frequency of such cells increased until the second week (Cd_d2), and then gradually decreased but also remained significantly elevated until the end of the experiment. Some erythrocytes showed deformed nuclei, but their percentage was low, and significantly elevated only in the Cd_d2 group. Cellular abnormalities included cell malformation (Fig. 1 B), vacuolation (Fig. 1 C), swelling (Fig. 1 D), and hemolysis (Fig. 1 E). The latter was the most frequently observed, and a gradual significant increase occurred during long-term exposure beginning from the second week, but great individual variability resulted in high standard deviations. Erythroblast level was significantly increased in Cd_k3, and in Cd2-Cd4 groups.

The results of the *in vitro* experiment showed high frequency of abnormal erythrocytes (also in the control), but

Table 1. Erythrocyte anomalies observed after *in vivo* short-term (3 h at 6.5 mg/l) and long-term (4 weeks at 0.65 mg/l) cadmium exposures.

Anomalies [%]	Control	Cd_k1	Cd_k2	Cd_k3	Cd_k4	Cd_d1	Cd_d2	Cd_d3	Cd_d4
All	1.9±0.6[a]	23.4±11.5[b]	7.1±2.2[cd]	11.8±3.4[c]	5.7±2.3[d]	5.6±1.2[b]	17.8±5.1[c]	14.7±11.7[c]	13.5±12.9[c]
Chromatin condensation	0.3±0.4[a]	17.3±13.3[b]	5.1±2.2[cd]	8.5±3.3[c]	4.2±2.7[d]	3.0±1.2[b]	12.9±5.0[c]	8.4±11.8[b]	4.7±4.2[b]
Deformed nucleus	0.5±0.2[a]	0.3±0.2[ab]	0.5±0.5[a]	0.8±0.2[ac]	0.4±0.4[ab]	0.5±0.5[a]	1.1±0.7[b]	0.5±0.3[ab]	0.4±0.4[a]
Deformed cell	0.2±0.3[a]	0.3±0.3[a]	0.4±0.6[ab]	0.9±0.2[b]	0.2±0.2[a]	0.9±0.5[b]	1.3±0.3[b]	0.2±0.3[a]	0.1±0.3[a]
Cytoplasm vacuolation	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.1[a]	0.0±0.1[a]	0.6±0.9[a]	0.3±0.9[a]
Cell swelling	0.5±0.3[a]	2.3±2.5[a]	0.7±0.4[a]	0.8±0.4[a]	0.4±0.5[a]	0.5±0.3[a]	0.9±0.4[b]	0.3±0.5[a]	0.3±0.3[a]
Hemolysis	0.5±0.3[a]	3.2±5.5[a]	0.6±0.4[a]	0.8±0.8[a]	0.5±0.7[a]	0.6±0.5[a]	1.5±0.6[b]	4.7±8.5[b]	7.6±13.6[b]
Erythroblasts	0.5±0.3[a]	0.9±0.5[a]	0.8±0.5[a]	1.3±0.3[b]	0.7±0.6	0.8±0.4[ab]	1.9±0.7[b]	4.7±5.1[b]	3.3±2.5[b]

Different letter superscripts indicate significant differences at p≤0.05 (U-test) in Control, Cd_k1-Cd_k4, and Control, Cd_d1-Cd_d4 pattern.

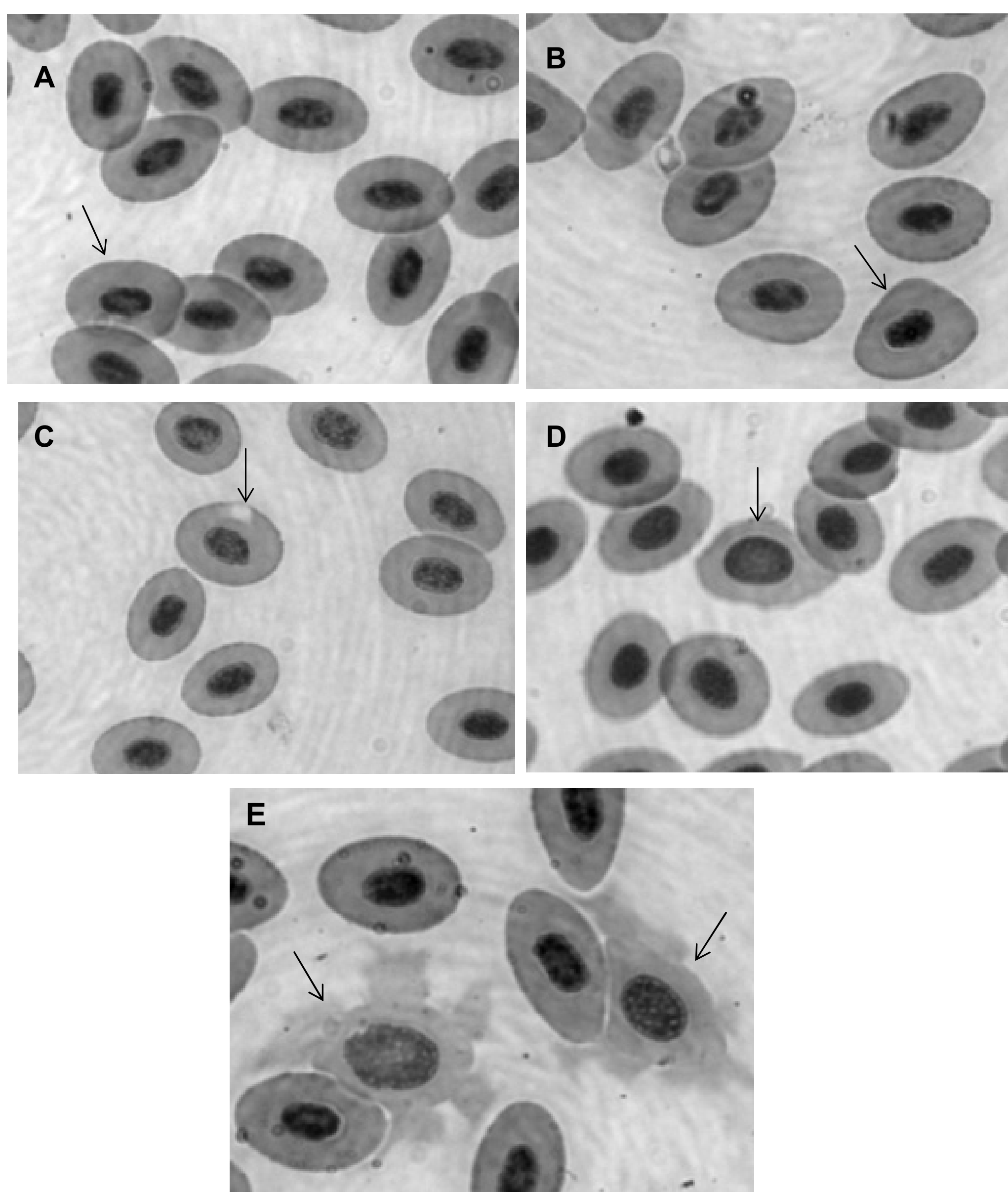

Fig. 1. Examples of cadmuim-induced erythrocyte anomalies in common carp subjected to an *in vivo* Cd exposure. A – chromatin condensation at the nucleus border, B – deformed cell, C – vacuolated cell, D – swollen cell, E – hemolyzed cells.

Table 2. Erythrocyte anomalies observed after *in vitro* exposure of full blood to cadmium (5 µg/ml).

Anomalies [%]	Control	$CdCl_2$	$CdSO_4$	$Cd(NO_3)_2$
All	46.2±7.1[a]	50.6±6.5[a]	56.7±12.1[a]	82.0±23.4[a]
Chromatin condensation	16.1±5.9[a]	18.5±9.3[a]	19.3±7.9[a]	25.7±13.0[a]
Deformed nucleus	3.9±1.0[a]	5.1±2.5[a]	5.0±1.9[ab]	8.6±3.3[b]
Deformed cell	13.0±6.6[a]	10.1±8.6[a]	7.1±2.5[a]	20.5±20.4[a]
Cytoplasm vacuolation	6.6±4.1[a]	8.3±4.0[a]	10.0±6.6[ab]	14.1±3.5[b]
Cell swelling	1.5±0.5[a]	2.8±2.0[ab]	4.6±4.0[b]	4.2±5.1[ab]
Hemolysis	5.1±2.1[a]	5.8±2.8[a]	10.6±10.2[a]	8.7±3.4a

Different letter superscripts indicate significant differences at p≤0.05 (U-test).

types of anomalies were similar as under *in vivo* conditions (Table 2). A significant increase of all anomalies occurred only in blood subjected to the cadmium nitrate. The percentage of cells with chromatin condensed at the nucleus border was slightly higher in cadmium-exposed blood, but the differences were insignificant. The rate of cells with deformed nuclei and with vacuolated cytoplasm was significantly higher in blood exposed to cadmium nitrate than in the control and blood subjected to cadmium chloride. The rate of deformed cells was high in all groups but did not show a relationship with cadmium exposure. Swollen and hemolyzed cells were more frequent in cadmium-exposed blood as compared to the control, but only the cell swelling rate was significantly elevated in cadmium sulfate-exposed samples.

Discussion

The results of both *in vivo* and *in vitro* studies revealed cadmium-induced fish erythrocyte anomalies, nuclear aberrances being the most frequent. However, a considerably higher percentage of erythrocytes with chromatin at the nucleus border and deformed cells in the control samples indicate that under *in vitro* conditions these anomalies cannot be attributed uniquely to cadmium action but also must have resulted from experimental procedures themselves, which suggests high cell sensitivity.

The results of the *in vivo* experiment revealed differences between the effect of short- and long-term exposure. After short-term Cd exposure fish showed the maximum level of erythrocyte anomalies in the first week post exposure, and later on the erythrocyte picture gradually recovered. On the other hand, during long-term exposure erythrocyte damage developed over time, reaching maximum in two weeks, and remained significantly elevated until the end of the experiment. This was probably related to elimination of cadmium from the organism after short-term exposure, and constant uptake and accumulation during long-term exposure.

Similar anomalies were observed in our earlier studies in another cyprinid fish, tench (*Tinca tinca*), subjected to short-term cadmium exposure (3 h at 4.5 mg/l) [25]. Erythrocytes of tench showed abnormal shape, vacuolation, swelling, chromatin disintegration, and nucleus indentation, and the frequency of these anomalies increased over 4 days post of exposure. Various nuclear and cellular anomalies were also observed in common carp subjected to acute exposure to cadmium, lead, and zinc [10]. According to Gill and Pant [23], the nucleus appears to be the primary target for the toxic action of cadmium to fish erythrocytes. They reported chromatin condensation at the periphery of the nucleus, followed by the formation of nuclear puffs and leakage of chromatin to cytoplasm. Abnormal chromatin distribution and condensation was also observed in *Carassius carpio* intoxicated for 14 days with 0.018-0.445 mg/l of cadmium [26]. Nuclear anomalies such as micronuclei, nuclear buds, irregular nucleus shape, binuclei, or vacuolated nuclei are commonly considered indicators of genotoxicity [19, 20]. Genotoxic action of cadmium upon rat erythrocytes was reported by Celik et al. [27, 28].

A concentration-related increase in the percentage of damaged erythrocytes (including mainly "ghost cells" with empty cytoplasm) in *Tilapia aurea* sublethally exposed to 6.8-52 µg/l of Cd for 16 weeks was reported by Papoutsoglou and Abel [29]. Similarly as in our study, the authors observed very high individual variability. Toxicity of cadmium to erythrocytes of other vertebrates was also reported. According to Romero et al. [30], cadmium induced apoptosis of mallard (*Anas platyrhynchos*) erythrocytes in a concentration-dependent way, and was more toxic than lead. Cadmium also increased fragility (susceptibility to hemolysis) of rat erythrocytes in both hypotonic and hypertonic solutions [31]. Mechanisms of cadmium cytotoxicity are not quite clear. It is known that cadmium shows a high affinity to membrane Ca^{2+} pump and competitively inhibits its activity, which may result in an increase in cytosolic Ca^{2+} concentration to toxic levels [32]. According to Suwalsky et al. [33], cadmium induced shape changes in human erythrocytes (echinocytosis) that resulted from disturbances in arrangements of the membrane lipid bilayer. Cadmium may disturb not only erythrocyte structure but also their function. According to Lionetto et al. [34], cadmium inhibited carbonic anhydrase (an enzyme that catalyses reversible hydratation of CO_2 to produce H^+ and HCO^-, essential for blood CO_2 transport), under both *in*

vivo and *in vitro* conditions. A dose-dependent inhibitory effect of cadmium on this enzyme in rainbow trout erythrocytes was also reported by Bektas et al. [35].

An increase in erythroblast frequency, especially in fish subjected to long-term cadmium exposure (accompanied by a high proportion of abnormal erythrocytes) indicate activation of erythropoiesis, probably to compensate for impaired function and elimination of damaged cells. However, Garofano and Hirshfield [36] reported that high Cd concentration (61 mg/l) caused complete elimination of hemoblasts and immature erythroblasts from the hemopoietic head kidney tissue of *Ictalurus nebulosus*. Celik et al. [28] observed a reduced ratio of erythroblasts in bone marrow but not in peripheral blood of cadmium-intoxicated rat. These data suggest that severe intoxication with this metal may disturb hemopoietic functions.

The obtained results, and the data provided by other authors, suggest that cadmium is both genotoxic and cytotoxic, and that nucleated erythrocytes are sensitive targets for its toxic action. It was also observed that time course of the anomalies was in accordance with the presence of metal in the water (transient disturbances after short-term exposure, and persistent anomalies during long-term exposure). The results of *in vitro* tests indicate that cadmium nitrate is probably more toxic to fish erythrocytes than cadmium chloride and sulfate.

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