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Original Research

Hematological Changes in Common Carp (*Cyprinus carpio* L.) after Short-Term Lead (Pb) Exposure

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Abstract

The effect of short-term (3 h) exposure to 10 mg/l of Pb (96hLC50) on common carp hematological parameters was evaluated every 2 days for 16 days post exposure. Fluctuations of the values of red blood parameters were observed over the entire experimental period. Increase in frequency of morphological anomalies in erythrocytes also occurred and no complete recovery took place until the end of the experiment. Chromatin condensation at the nucleus border and nuclear malformation were the most common anomalies and seem the most pronounced hematological alterations induced by lead in carp.

In first days post exposure a decrease in immature neutrophil percentage, followed by a drop in mature cell frequency, accompanied by a decrease in phagocyte activity occurred. This was followed by a transient but significant decrease in leukocyte count 8 days after the end of exposure. A persistent decrease in basophil frequency was observed until 8 days post exposure. The observed changes suggest that lead disturbed non-specific immune responses in carp.

Keywords: blood, fish, heavy metal, erythrocytes, leukocytes

Introduction

Waterborne heavy metals exert various toxic effects upon fish [1, 2]. Lead is a common environmental pollutant used in many industries, and enters aquatic environments with effluents from mines, smelters, and chemical factories manufacturing pigments, paints, plastics, etc. [3, 4]. It does not play any known metabolic role in living organisms; it is a typical xenobiotic [5].

Lead has an extremely high affinity for erythrocytes [6, 7], and is a known inhibitor of dehydrogenase of delta aminolevulinic acid (ALA-D), an enzyme participating in heme synthesis [6-8]. Lead may cause deformities of fish erythrocyte [9], membrane disruption [10], and often induces anemia in fish [11-13].

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Despite some anatomical and physiological differences, the immune system of fish is similar to other vertebrates, and consists of various types of cells and chemical mediators. In fish both cellular and humoral, non-specific and specific immune mechanisms are present. Many data indicate that fish immune response may serve as an alternate or additional model for predicting the immunotoxicity of environmental contaminants [14].

Lead also is a known immunotoxicant [15, 16]. Immunotoxic effects of lead may result in immunosuppression, rendering an organism more susceptible to infectious diseases. On the other hand, lead may cause inappropriate enhancement of immune response, leading to allergies or autoimmune diseases [4]. An extensive review of the effects of lead on various immune functions in animals [4, 17] shows that this toxic metal affects almost all mechanisms of immune response: the function of B, T and NK lymphocytes,

chemotaxis, phagocytosis, and reactive oxygen intermediate production by phagocytes, cell-surface marker expression and antigen presentation, and cytokine profile. The results of various studies indicate different actions of various concentrations of lead upon immunocompetent cells. It was also proved that various leukocyte populations in various animal species show different sensitivity to the toxic action of lead. According to Markevieius and Dringeliene [18], dietary lead reduced the number of murine helper T cells but the increased the number of suppressors and did not affect NK cells. On the contrary, Qiao et al. [19] revealed a reduction of NK levels in women subjected to Pb intoxication, and no effect on memory Th cells. They also reported reduced activity of T, B, and NK cells. Sata et al. [20] observed that human NK cells were more sensitive to Pb than T lymphocytes. Steffensen et al. [21] reported similar sensitivity of lymphocytes T and B, and monocytes to cytotoxic action of metals, including Pb. Ferrer et al. [16] and Carey et al. [22] observed the stimulating effect of Pb on murine lymphocyte proliferation, while Mishra et al. [23] reported inhibited proliferation of human lymphocytes. According to Sarasua et al. [24], lead may cause an increase in IgA, IgG and IgM levels, while Sun et al. [25] reported a Pb-induced reduction in IgM and IgG titers, accompanied by an increase in IgE.

Data concerning *in vivo* toxic effects of lead on fish immune system are quite scarce. Johansson-Sjobeck, Larsson [26], Ghazaly [12], and Allen [27], observed no alterations in white blood cell absolute and differential count in fish exposed to waterborne Pb (at concentrations of 0.3-10 mg/l). Santos, Hall [28] reported lymphocytosis and neutropenia in eel *Anguilla anguilla* at 0.3 mg/l of Pb. Similarly, Olanike et al. [13] observed an increase in lymphocyte percentage, and a decrease in neutrophil contribution accompanied by a WBC decrease in African catfish *Clarias gariepinus* subjected to 25-250 mg/l of Pb for 4 days. On the contrary, Shah, Altindag [5], and Ates et al. [29] reported a WBC increase in lead-exposed fish. Our previous data showed that leukocyte counts changed in Pb-exposed carp over time post exposure; WBC increase and lymphocytosis were followed by a decrease in both parameters [30]. The data of *in vitro* studies obtained by Dunier and Siwicki [31] showed that 5.0-100 mg/l of Pb suppressed activity of common carp phagocytes while no effect was observed at 0.001-1.0 mg/l. Lymphocyte proliferation was enhanced at 6.2-100 mg/l, no effect occurred at 3.0 mg/l, while at 1.0 mg/l proliferation was completely suppressed. Witeska and Wakulska [32] observed an increase in mortality of lymphocytes subjected *in vitro* to 5-50 μM of Pb, but no effect on metabolic activity of phagocytes. O'Neill [33] reported a dose-dependent decrease in antiviral antibody level in *Salmo trutta* after intraperitoneal injection of Pb solution.

Natural waters usually contain low levels of lead due to precipitation and poor solubility of Pb compounds. However, low oxygen levels due to long ice cover, and pH reduction due to snow melt facilitate the release of metals from sediments, and their redistribution into water during spring mixing [34]. Therefore, waterborne concentrations of metals, including lead, may abruptly increase for a short time, particularly in early spring.

The aim of the present study was to evaluate the effects of short-term exposure of common carp to a high level of lead on the changes in peripheral blood leukocytes over two weeks after contact with metal.

Materials and Methods

The experiment was performed in autumn 2007 on healthy juvenile (six months old) common carp of body mass 31.0±11.2 g. The fish were obtained from the rearing pond of The Inland Fisheries Institute in Żabieniec, Poland, and acclimated for 4 weeks in the aerated flow-through tank, at 21°C and dissolved oxygen saturation 70-80%, supplied with dechlorinated tap water, at a density of 20 g/l. Water hardness was 178 mg/l as $CaCO_3$, and background Pb concentration was 0.0024 mg/l.

Fish were fed daily with carp starter at the rate of 2% of stock mass. Before the experiment, a 96 h acute toxicity test was performed, and 96 hLC_{50} value was calculated using the probit method (10 mg/l). Lead solutions were made of $Pb(NO_3)_2$.

The fish were exposed individually in 5 l aquaria to 10 mg/l of Pb for 3 hours, and then transferred to the metal-free water, and kept in groups of 12 fish in 30 l aquaria. Control group (C) was subjected to the same manipulations, except for Pb exposure. Blood was sampled using heparinized needles into plastic heparinized Eppendorf tubes by heart puncture from live fish immediately after the end of exposure (Pb0), and then every two days until day 16 post exposure (Pb2-Pb16). Some blood samples coagulated and were excluded from analyses. The number of samples were: C – n=10, Pb0 – n=10, Pb2 – n=11, Pb4 – n=10, Pb6 – n=12, Pb8 – n=12, Pb10 – n=10, Pb12 – n=8, Pb14 – n=12, and Pb16 – n=11. From each group, blood was sampled only once. The following parameters were evaluated: hematocrit (Ht), erythrocyte count (RBC), blood hemoglobin concentration (Hb), and differential erythrocyte count, leukocyte count (WBC), and metabolic activity of phagocytes (NBT). Additionally, mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC) were calculated. Ht was measured using the microhematocrit method, in heparinized capillaries after centrifuging at 12,000 rpm for 5 min. RBC and WBC were counted in Burker chamber, in blood diluted 100 times with Hayem solution. Hb was measured spectrophotometrically at wavelength of 540 nm using the cyanmeyhemoglobin method. Derived parameters were calculated according to the following formulas:

$$MCV=(Ht\times 10)/RBC$$
$$MCH=Hb/RBC$$
$$MCHC=(Hb\times 100)/Ht$$

Phagocyte activity was measured spectrophotometrically at 540 nm wavelength after 1 h incubation of blood with nitrotetrazolium blue solution at 28°C, as formasan concentration [35].

Table 1. Changes in the values in basic hematological parameters (means±S.D.) in common carp over 16 days after 3-hour exposure to Pb (10 mg/l).

Parameter	Experimental groups									
	C	Pb0	Pb2	Pb4	Pb6	Pb8	Pb10	Pb12	Pb14	Pb16
Ht [%]	27.7±2.9	29.7±2.3	26.4±2.5	22.3±3.0*	27.4±2.3	25.8±2.7	25.9±2.9	29.8±3.0	31.1±5.0	24.7±3.3
Hb [g/l]	99.0±27.2	90.4±18.3	119.9±11.3*	64.4±15.2*	106.4±9.0*	84.1±13.0	98.7±11.6	85.1±11.6	107.2±16.3	69.2±15.4*
RBC [10^6/µl]	1.91±0.22	2.18±0.34	2.58±0.58*	1.35±0.22*	2.15±0.42	1.86±0.56	1.88±0.35	1.63±0.24*	2.32±0.35*	1.46±0.33*
MCV [fl]	147±25	139±25	106±21*	168±32	131±26	150±49	142±32	186±36*	135±22	174±35
MCH [pg]	52.3±14.1	42.0±9.3	48.4±10.5	49.0±14.6	50.7±8.3	48.6±14.6	54.3±12.5	54.0±15.7	47.1±9.7	48.3±11.0
MCHC [g/l]	356±84	306±68	457±51*	288±47	390±36	330±68	383±29	286±30*	347±29	279±50*
WBC [10^3/µl]	64.5±25.3	74.6±33.6	75.5±28.9	63.0±19.6	52.1±14.1	40.1±16.9*	74.9±22.6	64.2±21.8	60.7±20.2	63.0±25.1
NBT [g/l]	0.90±0.34	0.51±0.18*	0.81±0.32	0.44±0.14*	1.03±0.3	0.78±0.43	0.97±0.38	1.13±0.27	1.32±0.38*	0.84±0.31

* – values significantly different from the control (Mann-Whitney U test, $p<0.05$).

Table 2. Changes in erythrocyte morphology (means±S.D.) in common carp over 16 days after 3-hour exposure to Pb (10 mg/l).

Erythrocytes [%]	Experimental groups									
	C	Pb0	Pb2	Pb4	Pb6	Pb8	Pb10	Pb12	Pb14	Pb16
Normal mature	98.5±0.8	91.6±3.6*	65.8±5.7*	64.7±9.2*	69.1±7.0*	72.4±12.1*	67.9±11.0*	89.8±4.6*	77.9±11.8*	92.5±6.9*
Erythroblasts	0.1±0.2	0.3±0.4	0.2±0.2	0.6±0.6*	0.4±0.4	1.3±1.2*	0.3±0.4	0.3±0.4	0.9±1.0*	0.1±0.2
Abnormal	1.4±0.8	7.7±3.7*	33.9±6.2*	34.7±9.5*	33.3±8.4*	26.1±12.6*	28.6±12.4*	9.8±4.7*	21.2±12.0*	7.5±6.6*
Nuclear anomalies	0.1±0.2	4.3±3.8*	24.9±10.8*	22.4±8.8*	29.6±8.4*	22.0±13.8*	24.5±11.7*	5.8±4.2*	15.7±12.2*	4.3±5.2*
Cell body anomalies	1.3±0.6	3.4±3.0*	8.8±6.6*	12.1±4.8*	3.6±1.8*	4.0±3.3*	3.8±2.6*	4.0±4.7	5.1±2.3*	3.1±2.1*
Amitotic	0.0±0.0	0.0±0.0	0.2±0.2	0.2±0.3	0.1±0.2	0.2±0.3	0.3±0.5	0.1±0.2	0.5±0.5*	0.1±0.2

* – values significantly different from the control (Mann-Whitney U test, $p<0.05$).

Differential erythrocyte and leukocyte counts were done at 1000 × magnification, using a Nikon Eclipse 300 microscope, on blood smears stained with May-Grunwald and Giemsa solution, and shown in percent (300 erythrocytes were viewed in each smear and 100 leukocytes). Photographs of erythrocyte anomalies were taken using a Nikon Coolpix digital camera connected to the microscope, and the computer image analysis system CoolView. The obtained results were subjected to statistical analysis using the non-parametric Mann-Whitney U-test. *P* values <0.05 were considered statistically significant and all data are presented as means±S.D.

Results

Red blood parameters fluctuated throughout the entire experimental period. Two days after the end of Pb exposure (Pb2) RBC and Hb increased, and MCV decreased, while on day 4 (Pb4) RBC, Ht and Hb decreased significantly (Table 1). Fluctuations of these parameters were accompanied by a persistent increase in the percentage of cellular anomalies, including mainly chromatin condensation at the border of the nucleus and nuclear malformation (Table 2, Fig. 1). Erythroblast level was low but significantly increased in Pb4, Pb8, and Pb14, which suggests increased erythropoiesis.

The leukocyte count in Pb-exposed fish gradually decreased from 4-day post exposure (Table 1), and on day 8 (Pb8) reached the minimum that was significantly lower when compared to the control. Then, WBC recovered and until the end of the experiment remained at a level similar to the control.

A differential leukocyte count showed some significant changes after Pb exposure (Table 3). Immediately after the end of exposure (Pb0) the level of lymphocytes (particularly small resting lymphocytes) significantly increased, while the percentage of neutrophils dropped. Another significant increase in percentage of lymphocytes took place in Pb8 and Pb12. In Pb4 the level of large lymphocytes significantly increased.

The level of metamyelocytes decreased in Pb0, while the percentage of mature segmented neutrophils was significantly reduced in Pb2 and Pb4, which suggests a transient disturbance in neutrophil production and maturation. A decrease in neutrophil percentage was accompanied by a significant and deep reduction of phagocyte activity in

Table 3. Changes in percentage of various leukocyte populations (means±S.D.) in common carp over 16 days after 3-hour exposure to Pb (10 mg/l).

Leukocytes [%]	Experimental groups									
	C	Pb0	Pb2	Pb4	Pb6	Pb8	Pb10	Pb12	Pb14	Pb16
All lymphocytes	93.4±1.8	97.6±2.5*	94.3±3.5	94.4±3.5	93.8±2.4	95.2±3.2*	93.5±2.8	95.9±2.1*	93.4±3.3	92.3±7.4
Small lymphocytes	91.4±2.4	95.3±2.9*	91.9±3.9	86.0±10.6	90.8±4.1	92.6±4.2	89.9±4.8	93.6±3.5	90.4±4.9	89.7±7.6
Large lymphocytes	2.0±2.2	2.3±1.9	2.4±2.4	8.4±8.0*	3.0±2.5	2.6±2.9	3.6±3.6	2.3±1.8	3.0±3.1	2.6±3.3
All neutrophils	3.6±1.7	1.4±1.1*	2.2±1.5	2.1±1.5	2.8±1.9	3.1±2.3	4.1±1.9	2.6±1.3	4.8±2.3	4.5±5.0
Myelocytes	0.8±0.8	0.5±1.0	0.7±0.7	0.6±0.8	1.1±0.7	1.2±1.3	1.0±0.7	0.8±0.9	0.8±0.9	1.3±1.8
Metamyelocytes	0.8±0.8	0.0±0.0*	1.1±1.0	0.6±0.7	0.5±0.7	0.9±0.7	0.9±0.6	0.5±0.5	0.8±0.9	0.8±0.9
Band neutrophils	0.6±1.0	0.4±0.7	0.3±0.6	0.6±0.8	0.3±0.7	0.3±0.8	1.1±1.2	0.1±0.4	1.3±1.0	0.5±0.8
Segmented neutrophils	1.4±1.4	0.5±0.9	0.1±0.3*	0.3±0.7*	0.9±1.2	0.7±0.8	1.1±1.3	1.3±1.0	1.9±1.3	1.9±2.3
Monocytes	1.0±1.3	0.0±0.0	1.6±1.8	1.6±1.9	2.0±1.7	0.7±0.8	0.3±0.7	0.6±0.7	1.0±1.2	0.8±0.4
Basophils	0.7±0.5	0.0±0.0*	0.0±0.0*	0.1±0.3*	0.1±0.3*	0.2±0.4*	0.2±0.4	0.4±0.5	0.1±0.3*	0.5±1.2
Eosinophils	0.2±0.4	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.2±0.4	0.1±0.3	0.3±0.5	0.2±0.4	1.0±1.0
Blasts	1.0±0.8	1.0±1.9	1.9±1.9	2.0±1.7	1.3±0.9	0.8±0.9	1.6±1.3	0.3±0.5	0.5±0.8	0.8±0.8

* – values significantly different from the control (Mann-Whitney U test, $p<0.05$).

Pb0 and Pb4, while from the 10-day post exposure phagocyte activity gradually increased and in Pb14 reached a value significantly higher as compared with the control. Another significant alteration was a decrease in basophil share that was statistically significant in Pb0-Pb8, and Pb14.

Discussion

In the present study a decrease in red blood cell parameters was temporary, but abnormal erythrocytes were observed over the entire experimental period. According to Nakagawa et al. [6] and Alves, Wood [7], lead shows a high affinity for erythrocytes. Reduction of Hb and RBC accompanied by a compensatory response (increased hematopoietic rate) in lead-intoxicated rainbow trout was reported by Johansson-Sjobeck and Larsson [26]. A severe microcytic anemic state was reported by Tewari et al. [11] in *Barbus conchonius* exposed to 47.4 µg/l of lead (decrease in Ht, RBC, Hb and MCV). Ghazaly [12] reported a decrease in RBC and Hb, but not Ht in *Tilapia zillii* exposed to 8.3 mg/l of Pb, and Allen [27] – a decrease in RBC, Hb and Ht in *Oreochromis aureus* treated with 10 mg/l of Pb. Olanike et al. [13] observed a non- dose dependent decrease in Ht, Hb, and RBC in *Clarias gariepinus* exposed to 25-200 mg/l of lead. Another effect of lead upon fish erythrocytes is structural damage. Membrane disruption and increased frequency of amitoses were reported in *Salvelinus alpinus* from a lead-polluted Alpine lake [10]. The increased rate of hemolysis, and particularly high rate of nuclear anomalies in lead-treated common carp, was also observed in our earlier studies [36]. Area increase and changes in erythrocyte shape were observed by Oliveira Ribeiro et al. [9] in *Hoplias malabaricus* fed lead-containing feed. According to Cavas [37], lead was toxic to the newly developing erythrocytes of *Carassius auratus*, and also showed a genotoxic effect. Romero et al. [38] reported an increased rate of apoptosis of mallard (*Anas platyrhynchos*) erythrocytes incubated *in vitro* with Pb solutions. The obtained results showed that erythrocyte morphology was more sensitive to lead intoxication than basic red blood parameters, and that the erythrocyte nucleus was the main target of Pb action.

In the present study a transient leukopenia, neutropenia and reduced phagocyte activity were observed, accompanied by a persistent decrease in basophil count. A decrease in leukocyte count in Pb-intoxicated common carp was also observed by Słomińska et al. [39] during prolonged exposure, and by Witeska [30] on the days 1 and 2 post short-term exposure. A slight decrease in WBC was also reported in African catfish subjected to very high Pb levels [13]. Other authors did not observe any changes or even noted a WBC increase in fish subjected to Pb exposures. According to Santos and Hall [28], Pb-induced tissue damage might have activated an immune response in fish. Ghazaly [12] observed an increase in Pb level in blood and other tissues, including liver and kidneys, which indicates that not only circulating blood leukocytes could be affected by lead but also peripheral population, as well as new cells developing in hematopoietic organs.

A similar shift in leukocyte composition (lymphocytosis and neutropenia) was reported in Pb-intoxicated eel [28], and in African catfish [13].

Phagocytosis in fish is the most important and potent immune mechanism [31]. Siwicki et al. (1994) reported a suppresory effect of high Pb levels (5.0-100.0 mg/l) on the activity of carp phagocytes *in vitro,* while Witeska and

Wakulska [32] observed no effect of 1-10 mg/l. Bussolaro et al. [40] observed a decrease in murine phagocyte activity, while Baykov et al. [41] – an increase in pagocytosis and hydrogen peroxide production by phagocytes. Valentino et al. [42] and Governa et al. [43] noted that lead inhibited chemotactic activity of human phagocytes *in vitro*. The data obtained by Burchiel et al. [44] indicate that Pb may affect not only circulating blood cells but also hematopietic tissue. They observed a shift into immature cells in murine bone marrow, and suppression of primary humoral response of splenocytes. According to Suzuki [45], blood basophil levels decreased in puffer fish during experimental acute inflammation, and increased at the same time in the inflammatory exudate.

The results of the present study showed that short-term exposure of common carp to Pb resulted in moderate and transient alterations in white blood cell systems, indicating a temporary immune disturbance.

Comparison of the effects of lead and other heavy metals show relatively little immunotoxicity of lead. Witeska and Jezierska [46] found that 5 and 10 mg/l of Cd caused a WBC decrease in common carp, while 12 mg/l of lead did

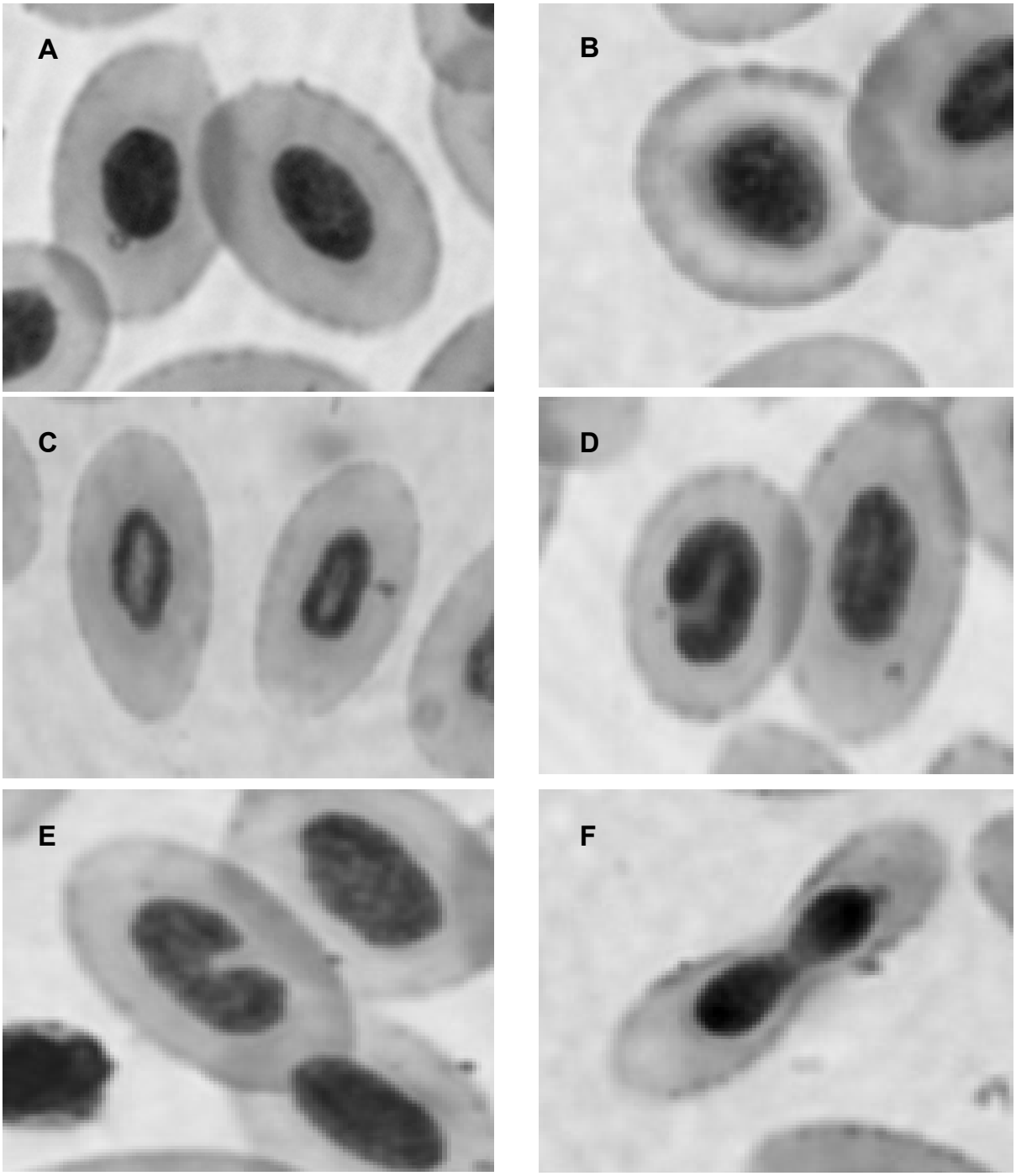

Fig. 1. Changes in erythrocyte morphology in common carp over 16 days after 3-hour exposure to Pb (10 mg dm^{-3}): A – normal mature erythrocyte, B – polychromatophilous erythroblast, C, D – condensation of chromatin at the nucleus border, E – nuclear malformation, F – amitosis.

not cause any change. The results obtained by Witeska et al. [47] under the same conditions as in the present study showed a pronounced and prolonged reduction in WBC in carp exposed to Cd and Cu, which indicates higher toxicity of these metals compared to Pb. Shah and Altindag [5] reported that exposure to lethal concentrations of Hg and Cd resulted in a significant WBC reduction, while exposure to Pb caused a slight increase showing toxicity of metals to this parameter: Hg>Cd>Pb. Dunier and Siwicki [31] observed that Cu, Zn and Mg inhibited carp lymphocyte proliferation *in vitro* at the concentrations beginning from 0.1 mg/l, while Pb over 1.0 mg/l. According to Camara Pellisso et al. [48], toxicity of metals to bottlenose dolphin leukocytes *in vitro* followed the ranking: Hg>Cd>Pb. The only effect of Pb was a slight reduction of lymphocyte proliferation ability, no effect on viability, apoptosis/necrosis, and phagocyte activity was observed. Krocova et al. [49] observed increased mortality of murine macrophages and lymphocytes at 1, 10 and 100 µg/µl of Pb, and at 10 and 100 µg/µl of Cd, which indicates higher toxicity of Pb. Similarly, Witeska and Wakulska [32] observed high toxicity of Pb to lymphocytes *in vitro* (Pb>Cd≈Zn>Cu), while phagocytes were less sensitive, and Pb did not affect their metabolic activity (Cd>Zn>Cu≈Pb).

According to Steffensen et al. [21], the sensitivity of human T and B lymphocytes and monocytes to heavy metals was similar, and metal toxicity was: Hg≈Ag>Cd≈Cu>Pb≈Zn. De la Fuente et al. [50] observed that Cd and As (but not Pb) induced apoptosis in human peripheral blood mononuclear cells *in vitro* (even at 500 µM). According to Borella et al. [51], inhibitory effect of metals on PHA-induced blastogenesis and proliferation was: Cd>Cr≈Ni>Pb (no effect of the latter was observed). Labedzka et al. [52] reported high toxicity of Cd, As and V to rabbit alveolar macrophages, moderate toxicity of Cu, Hg, Zn and Sb, and low toxicity of Sn, Ni, and Pb. The results obtained in the present study, and the data reported by other authors, show relatively low toxicity of lead to the immune system of animals, including fish.

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