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Hematological Parameters and Ultrastructure of Hematopoietic Tissues in Common Carp (*Cyprinus carpio* L.) Exposed to Sublethal Concentration of Pendimethalin

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Original article

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The objective of this study was to evaluate the influence of the herbicide on juvenile common carp (*Cyprinus carpio*) hematological parameters, ultrastructure of hematopoietic tissues and plasma concentrations of catecholamines and cortisol. Fish were exposed to 2.5 µg/l of pendimethalin for 14 days and for the next 30 days subjected to purification. Pendimethalin slightly affected red blood cell values, while more pronounced and persistent changes in white blood cell parameters indicate inflammatory response. In hematopoietic tissues only minor alterations in precursor cells were observed followed by cleanup process. Pendimethalin exposure induced significant increase in concentrations of adrenaline, noradrenaline and cortisol which indicates stress response.

Key words: Fish, herbicide, blood parameters, hematopoietic tissues, stress.

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Pendimethalin is a dinitroaniline herbicide that inhibits plant cell division processes responsible for chromosome separation and cell wall formation. This active substance, alone or combined with other compounds, is frequently used in various formulations before crop emergence or planting (APPLEBY & VALVERDE 1988). Similarly as other herbicides, pendimethalin enters the surface waters mainly as runoff from newly sprayed areas, especially with heavy rain and surface flow, spray drift near water bodies (KLOPPEL *et al*. 1997; STRANDBERG & SCOTT-FORDSMAND 2004; SADOWSKI & KUCHARSKI 2007). Pendimethalin concentrations up to 0.1 µg/l or even up to 6 µg/l were recorded in freshwaters, especially after heavy rain (STRANDBERG & SCOTT-FORDSMAND 2004). KEESE *et al*. (1994) analysed the runoff from nurseries in a microplot experiment and found 2-134 µg/l of pendimethalin. RILEY *et al*. (1994) found 5 µg/l of pendimethalin in interconnected pond used for irrigation. HOFFMAN *et al*. (2000) found pendimethalin in 11% of tested US urban stream waters in which the maximum concentration of the herbicide was 0.32 µg/l. The results obtained by SADOWSKI & KUCHARSKI (2007) revealed up to 2.5 µg/l of pendimethalin in rural surface waters. According to SADOWSKI (1996), pendimethalin is highly persistent in soil and aquatic environments showing half-life over 50 days. In surface waters pendimethalin undergoes photodegradation but the rate of this process may be affected by adjuvants added to commercial formulas (SWARCEWICZ & OSUMEK 2004). The data concerning toxicity of pendimethalin to aquatic organisms are scarce. The results of the study of pendimethalin toxicity to three species of aquatic microorganisms: green microalgae (*Selenastrum capricornutum*), ciliate protozoa (*Tetrahymena thermophila*) and luminescent bacteria (*Vibrio fischeri*) revealed that green microalgae were the most sensitive to herbicide showing growth inhibition (BRAZENAITE & SAKALIENE 2006). FLIEDNER (1997) examined the influence of acute dietary pendimethalin exposure of *Daphnia magna* and found that 48h EC_{50} was 78 µg/l. Very few studies on toxicity of pendimethalin to fish are available. Acute exposure of *Channa punctata* to sublethal concentrations of pendimethalin changed various oxidative stress indicators, antioxidant defenses in the internal organs and caused alterations in brain epinephrine levels (TABASSUM *et al*. 2015). Chronic exposure of rainbow trout (*Oncorhynchus mykiss*) to low concentrations of pendimethalin did not alter activity of EROD (ethoxyresorufin-O-deethylase, phase I and phase II detoxification enzyme) but affected antioxidant defenses in dose- and organ-dependent manner and impaired immune system reducing antiviral resistance (DANION *et al*. 2014).

Hematological parameters provide extensive knowledge on physiological status of fish and their changes are sensitive indicators of stress and pathological processes caused by adverse environmental conditions (ROCHE & BOGE 1996; COFFIGNY *et al*. 2004; ZUTSHI *et al*. 2010; FAZIO *et al*. 2013; WITESKA 2013; FAGGIO *et al*. 2014 a,b). These parameters show very different changes in fish intoxicated with aquatic pollutants, including herbicides (HUSSEIN *et al*. 1996; GOMEZ *et al*. 1998; SANCHO *et al*. 2000; CRESTANI *et al*. 2006; GLUSCZAK *et al*. 2006; VELISEK *et al*. 2009; MODESTO & MARTINEZ 2010; VELISEK *et al*. 2010; KREUTZ *et al*. 2011; GHOLAMI-SEYEDKOLAEI *et al*. 2013; PEREIRA *et al*. 2013; BOJARSKI *et al*. 2015; LUTNICKA *et al*. 2018). The data obtained by these authors indicate that hematological changes in fish subjected to intoxication often include the alterations in hematocrit value, hemoglobin concentration and erythrocyte count as classical stress indicators (ROCHE & BOGE 1996). Some authors observed the reduction of the values of these parameters during exposure to butachlor (GHAFFAR *et al*. 2015), glyphosate based herbicides (GLUSCZAK *et al*. 2006; GHOLAMI-SEYEDKOLAEI *et al*. 2013), metribuzin (RBCc not altered) (VELISEK *et al*. 2009), clomazone (without RBCc) (PEREIRA *et al*. 2013), atrazine (HUSSEIN *et al.* 1996) or after recovery period post molinate exposure (SANCHO *et al*. 2000). Other authors noted the opposite changes of these parameters: the increase of RBCc after fish exposure to clomazone (PEREIRA *et al*. 2013) and terbutryn, while Hct and Hb values were not changed (VELISEK *et al*. 2010). KREUTZ *et al*. (2011) observed decrease of RBCc and unchanged Hct value as a result of fish exposure to glyphosate, while fish exposure to Roundup Transorb (MODESTO & MARTINEZ 2010) showed increase of Hct value and RBCc but Hb value was not altered. An increase of Hct and Hb values was reported by GOMEZ *et al*. (1998) during fish exposure to 2,4-D. The decrease of Hct, while RBCc and Hb were not altered during fish exposure to Gardoprim Plus 500 SC (terbuthyloazine and S-metolachlor) was noted by DOBSIKOVA *et al*. (2011). The exposure of fish to clomazone induced a decrease of Hct value, while Hb did not change (CRESTANI *et al*. 2006). A decrease or increase in the values of other red blood parameters: MCV, MCH and MCHC were observed as a result of fish exposure to metribuzin (VELISEK *et al*. 2009), glyphosate-based Roundup (GHOLAMI-SEYEDKOLAEI *et al*. 2013), terbutryn (VELISEK *et al*. 2010), atrazine (HUSSEIN *et al*. 1996) and clomazone (PEREIRA *et al*. 2013), ethofumesate and pendimethalin (BOJARSKI *et al*. 2015), and MCPA (LUTNICKA *et al*. 2018).

Exposure to herbicides may also affect white blood cell parameters. The decrease of white blood cell count (WBCc) was noted after fish exposure to molinate (SANCHO *et al*. 2000), metribuzin (VELISEK *et al*. 2009), Gardoprim Plus 500 SC (terbuthyloazine and S-metolachlor) (DOBSIKOVA *et al*. 2011) and pure glyphosate or glyphosate-based herbicide (KREUTZ *et al*. 2011; GHOLAMI-SEYEDKOLAEI *et al*. 2013). On the other hand, the increase in WBCc was found after fish exposure to Roundup Transorb (MODESTO & MARTINEZ 2010), butachlor (GHAFFAR *et al*. 2015) and MCPA (LUTNICKA *et al*. 2018). Pendimethalin and ethofumesate tested separately or in mixture (BOJARSKI *et al*. 2015), atrazine (RAMESH *et al*. 2009), terbutryn (VELISEK *et al*. 2010) and Roundup (GLUSCZAK *et al*. 2006) did not change WBCc. Alterations in differential leukocyte count were also reported. The decrease of lymphocyte frequency was observed as the effect of fish exposure to metribuzin (VELISEK *et al*. 2009), glyphosate (KREUTZ *et al*. 2011), ethofumesate or pendimethalin (BOJARSKI *et al*. 2015), while the increase – after MCPA (LUTNICKA *et al*. 2018) or Roundup Transorb (MODESTO & MARTINEZ 2010) exposures. The percentage of neutrophils decreased as a result of exposure to Roundup Transorb (MODESTO & MARTINEZ 2010), glyphosate (KREUTZ *et al*.

2011), MCPA (LUTNICKA *et al*. 2018) and during exposure to ethofumesate and pendimethalin (BOJARSKI *et al*. 2015), while increased after exposure to metribuzin (VELISEK *et al*. 2009) and terbutryn (VELISEK *et al*. 2010).

These data show that the values of hematological parameters may alter due to herbicide intoxication. Intoxication may also affect the structure and function of hematopoietic tissue (GOMEZ *et al*. 1998; VELISEK *et al*. 2010; KONDERA *et al*. 2018; LUTNICKA *et al*. 2018) but the data on hematopoietic effects of herbicides in fish are lacking.

Concentrations of stress hormones are widely used biomarkers of the effects of aquatic contamination on fish (WENDELAAR BONGA 1997). There are very little literature data concerning the effects of herbicides on catecholamine and cortisol levels in fish and no such data are available for pendimethalin. The data obtained by various authors indicate that herbicides may induce or inhibit stress reactions in fish, e.g. according to SOSO *et al.* (2007), cortisol level increased in *Rhamdia quelen* exposed to glyphosate, while CERICATO *et al.* (2008) reported a decrease in cortisol concentration in the same fish species after exposures to glyphosate and mixture of atrazine and simazine which indicates a disruption of the hypothalamus-pituitary-interrenal axis.

Therefore, the aim of present study was to determine the influence of sublethal and environmentally realistic pendimethalin concentration on hematological parameters, ultrastructure of hematopoietic tissues and plasma levels of catecholamines and cortisol in common carp, *Cyprinus carpio* Linnaeus, 1758.

Materials and Methods

Animals and experimental conditions

The study was approved by the I Local Ethic Commission in Kraków (permission No. 124/2010). The experiment was performed on clinically healthy common carp (*Cyprinus carpio*) of body mass 60±10 g obtained from the Department of Ichthyobiology and Aquaculture of Polish Academy of Sciences in Golysz. Before the experiment they were acclimated for 2 weeks to the laboratory conditions. During the experiment the fish were kept in 300 l aquaria, 10 fish in each. Water quality parameters were measured every 3 days and were in the range: temperature 17-18°C, pH 7.2-8.0, O_2 8.26-9.15 mg/l, hardness 16-18°n, NH_3 0.02-0.07 mg/l, NO_2^- 1-2 mg/l and NO_3^- 18-24 mg/l. Water was renewed every 3-4 days during exposure to maintain the nominal concentration of the tested herbicide and prevent the accumulation of fish nitrogen metabolites. Similarly, water was exchanged every 3-4 days during the purification period. The fish were fed once a day *ad libitum* with barley flakes and frozen chironomid larvae.

Experimental design

The fish (200 individuals) were divided into 2 equinumerous groups: control and pendimethalin-exposed. The animals were exposed to tested herbicide at nominal concentration of 2.5 µg/l for 14 days and then subjected to purification in clean water for another 30 days. Blood was sampled from 10 fish of each group after 1, 3, 7 and 14 days of exposure and after 7, 14 and 30 days of purification. Peripheral blood from each fish was taken only once, with a heparinized Pasteur pipette (sodium heparin 5000 IU/ml, Polfa, Poland) by cardiac puncture to heparinized Eppendorf tubes. Blood was subjected to standard hematological analysis. Erythrocyte and leukocyte counts (RBCc and WBCc) were counted in blood diluted 1:200 with Natt-Herrick solution in Burker hemocytometer. Hematocrit value (Hct) was measured using microhematocrit method. Hemoglobin concentration (Hb) was measured spectrophotometrically at 540 nm wave length after conversion of hemoglobin to cyanmethemoglobin with Drabkin solution. Mean cell volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated according to standard formulas. Blood smears were also made and stained using Hemacolor staining kit (Merck) to evaluate differential leukocyte count. Various types of leukocytes were identified per 100 cells at ×600 magnification. The following types of cells were identified: lymphocytes, monocytes, juvenile neutrophils, mature neutrophils and eosinophils.

For electron microscope study head and trunk kidney and spleen tissues were sampled from 5 control fish and from 5 fish from pendimethalin-exposed group twice: after the exposure period (14 days) and after the purification time (30 days). The tissue fragments were fixed using standard method (KARNOVSKY 1965) and embedded in epoxy resin (Epoxy Embedding Medium, Sigma Aldrich). Ultrathin sections were cut on Leica EM UC7 ultramicrotome with a diamond knife and contrasted with uranyl acetate and lead citrate. The preparations were subjected to transmission electron microscope analysis (TEM) using JOEL JEM-100 SX and Tecnai G2 Spirit (FEI Company, USA). For determination of catecholamine and cortisol levels blood was sampled after 3, 6 and 12 h, 1, 3 and 14 days of exposure, and after 30 days of purification. Measurements of total adrenaline and noradrenaline concentrations were done using 2-CAT RIA (LDN Labor Diagnostika Nord GmbH

& Co. KG) reagent kit. Total plasma cortisol concentration was measured using CORTISOL-RIA-CT reagent kit (DIAsource ImmunoAssays S.A., Belgium). Concentrations of the hormones were determined using RIA method, according to the manufacturer instruction, using gamma radiation detector Wallac 1470 Wizard (Perkin Elmer, Finland).

Chemical tested

Analytical grade pendimethalin of 99.3±0.2% purity obtained from the Institute of Organic Industry in Warsaw (Poland) was used in the experiment. Pendimethalin is highly persistent in aquatic environment with half-time equal to 64 days (SADOWSKI 1996). Fish were exposed to the environmentally-realistic concentration of 2.5 µg/l (SADOWSKI & KUCHARSKI 2007).

Statistical analysis of data

Normality of distribution was tested using Shapiro-Wilk's test and homogeneity of variance was determined using Levene's test. Then, data were analysed with ANOVA, followed by Tukey's post-hoc test to evaluate significance of differences. For the data that did not meet the assumptions of ANOVA (differential leukocyte count), a non-parametric U Mann-Whitney test was performed. The level of significance was set at α = 0.05. Data were presented as means ± SD. Results were analysed using STATISTICA 10.

Results

The fish showed no visible signs of intoxication or disease during the experiment and no mortality was observed. The fish showed normal feeding and behavior.

Hematological parameters

Pendimethalin exposure resulted in temporary significant changes in the values of red blood cell parameters (Table 1). After 7 days of exposure RBCc significantly increased, while Hct and MCV decreased. MCH significantly increased after 30 days of purification. Changes in Hb content and MCHC were not statistically significant.

White blood cell parameters also showed significant alterations in pendimethalin-exposed fish compared to the control (Table 2). The WBCc significantly increased after 7 days of exposure to the herbicide and after 7 and 14 days of purification

Table 1

The values of red blood parameters in common carp during exposure to pendimethalin (2.5 µg/l) and purification (n=10). P – pendimethalin; asterisks indicate the values significantly different from the control at the same time (Tukey's test, p<0.05)

Time of blood collecting / parameter tested		Exposure to pendimethalin (2.5 µg/l)				Purification		
		1 day	3 days	7 days	14 days	7 days	14 days	30 days
RBCc ($\times 10^6$/µl)	Control	0.81±0.16	1.06± 0.10	0.88±0.20	1.00±0.08	0.70±0.11	1.12±0.18	1.08±0.07
	P	0.75±0.12	0.88±0.22	1.12±0.20*	0.78±0.19	0.84±0.20	0.95±0.11	0.89±0.09
Hct (%)	Control	27.00±4.52	31.00±2.78	32.90±2.90	28.70±3.62	27.40±2.72	34.70±5.10	34.00±2.58
	P	29.02±2.62	27.90±3.14	26.30±3.30*	25.60±6.29	27.70±6.60	33.20±3.08	30.60±5.15
Hb (g/dl)	Control	6.08±1.42	8.33±1.37	5.88±0.86	7.93±1.37	6.85±1.77	7.79±0.94	4.80±0.57
	P	5.28±2.14	7.56±0.94	6.13±2.75	6.02±0.80	6.77±1.48	6.36±0.61	6.62±0.78
MCV (fl)	Control	342.07±75.93	302.57±48.30	394.50±117.69	290.29±49.31	399.44±75.65	319.65± 82.80	319.23±38.67
	P	397.93±77.57	341.68±120.84	240.99±46.37*	347.24±120.98	348.17±123.84	353.52±46.58	347.16±59.58
MCH (pg)	Control	78.10±26.06	78.93±14.58	69.14±13.79	79.70±13.00	97.48±21.65	71.02±12.51	44.51±5.05
	P	70.68±28.88	91.73±28.80	54.84±20.65	81.62±22.56	84.85±28.29	67.45±6.35	75.51±12.03*
MCHC (g/dl)	Control	22.53±4.62	26.20±3.77	18.21±3.38	28.00±5.80	25.08±6.70	22.66±2.63	14.04±1.63
	P	18.85±7.96	21.37±3.53	19.63±9.71	20.02±10.0	19.37±10.70	18.83±1.70	20.72±3.68

Table 2

The changes in the values of white blood cell parameters in common carp during exposure to pendimethalin (2.5 μg/l) and purification (n=10). P – pendimethalin; asterisks indicate the values significantly different from the control at the same time (Tukey's test for WBC and U-Mann Whitney test for differential leukocyte count, p<0.05)

Time of blood collecting / parameter tested		Exposure to pendimethalin (2.5 μg/l)				Purification		
		1 day	3 days	7 days	14 days	7 days	14 days	30 days
WBCc ($\times 10^3/\mu l$)	Control	35.00±5.90	42.60±5.41	34.20±5.29	33.80±9.82	23.40±4.72	24.20±3.33	45.80±9.36
	P	36.20±10.64	47.20±16.09	58.00±10.37*	40.00±11.00	45.20±15.9*	49.10±9.60*	48.60±10.29
Lymphocytes (%)	Control	90.50±2.58	93.60±1.74	96.80±1.60	95.60±1.91	92.10±4.06	88.70±2.03	85.00±6.36
	P	96.05±1.47	83.15±4.94*	90.45±4.54*	92.05±3.29	94.05±2.69	82.25±12.24	91.10±4.07*
Juvenile neutrophils (%)	Control	3.85±2.04	1.65±0.85	1.90±1.17	1.90±1.11	2.90±1.98	4.65±2.71	8.00±4.33
	P	1.45±1.09	7.90±3.59*	4.80±3.18	3.25±1.65	4.05±2.59	13.60±9.27*	5.35±3.97
Mature neutrophils (%)	Control	4.65±2.20	3.80±1.92	1.50±0.85	1.50±0.85	3.90±1.82	5.70±2.54	7.72±3.00
	P	1.45±1.09*	2.75±1.67	2.50±1.85	3.50±2.56	2.30±1.21	3.30±2.10	2.90±1.82*
Monocytes (%)	Control	1.60±1.07	1.40±1.18	0.95±0.55	0.75±0.68	1.40±1.22	0.50±0.53	0.60±0.22
	P	0.85±0.47	5.25±1.84*	1.85±1.13	1.05±0.69	0.35±0.41	0.65±0.75	0.15±0.34

period. After 3 and 7 days of fish exposure a significant decrease in lymphocyte percentage was observed, followed by an increase after 30 days of purification. The increase in percentage of immature neutrophils was noted during the exposure time (except after the first day) and purification but it was statistically significant only after 3 days of exposure and after 14 days of purification. After 1 day of exposure to the herbicide and after 30 days of purification a significant decrease in percentage of mature neutrophils occurred. Percentage of monocytes significantly increased after 3 days of pendimethalin exposure, in comparison to the control.

Ultrastructure of hematopoietic tissues

Electron microscope imaging of head kidney of fish from the control group (Fig. 1A) showed firm structure of the organ. In the hematopoietic tissue many immature neutrophils and lymphocytes, single eosinophils and erythrocytes were observed. Mitochondria, RER, free ribosomes, single vacuoles filled with different materials and showing different electron density were found in some cells. After 14 days of exposure (Fig. 1B and 1C) the structure of head kidney was still firm. Juvenile blood cells were observed: numerous neutrophils with abundant granules, single eosinophils and lymphocytes, numerous erythrocytes. They usually showed undisturbed ultrastructure, but in some cells altered structure of mitochondria, abundant vesicles with different electron density and different contents, and cell damage were observed. Some lymphocytes showed deformed nuclei. Single melanomacrophages were also observed. After purification (Fig. 1D) head kidney ultrastructure was similar as after herbicide exposure but vesicles of low electron density or showing myelin-like structures were more frequently observed.

No pathological changes were observed in trunk kidney hematopoietic tissue of fish from the control group (Fig. 2A). Firm structure and tightly packed cells were observed. Immature neutrophils and lymphocytes were the most frequently noted cells, accompanied by single eosinophils. After 14 days of exposure to pendimethalin structure of trunk kidney hematopoietic tissue (Fig. 2B and 2C) remained firm but juvenile neutrophils were less abundant and showed less granules compared to the control. Single erythrocytes and eosinophils were also observed. Some cells showed altered ultrastructure. Single melanomacrophages and myelin-like structures also appeared. After purification (Fig. 2D) trunk kidney hematopoietic tissue still showed firm structure with adjoining cells. Melanomacrophages were more abundant com-

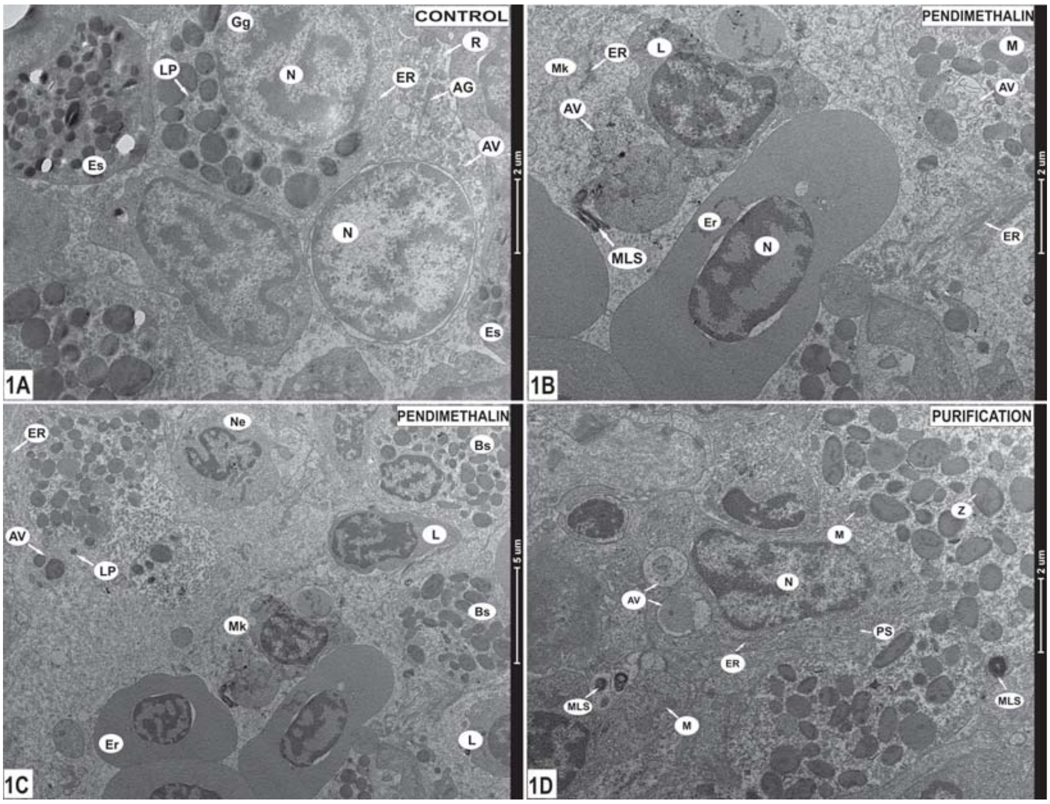

Fig. 1. Photomicrograph of the control head kidney cells (1A), after 14 days of pendimethalin exposure (1B, 1C) and 30 days of purification (1D).
AG-Golgi apparatus, AV- autophagic vacuoles, Bs-basophil, Er-erythrocyte, ER-endoplasmic reticulum, Es-eosinophil, Gg-promyelocyte, L-lymphocyte, LP-primary lysosome, M-mitochondria, Mk-macrophage, MLS-myelin structures, N-nucleus, Ne-neutrophil, PS-polysomes, R-ribosomes, Z-granulocyte granules.

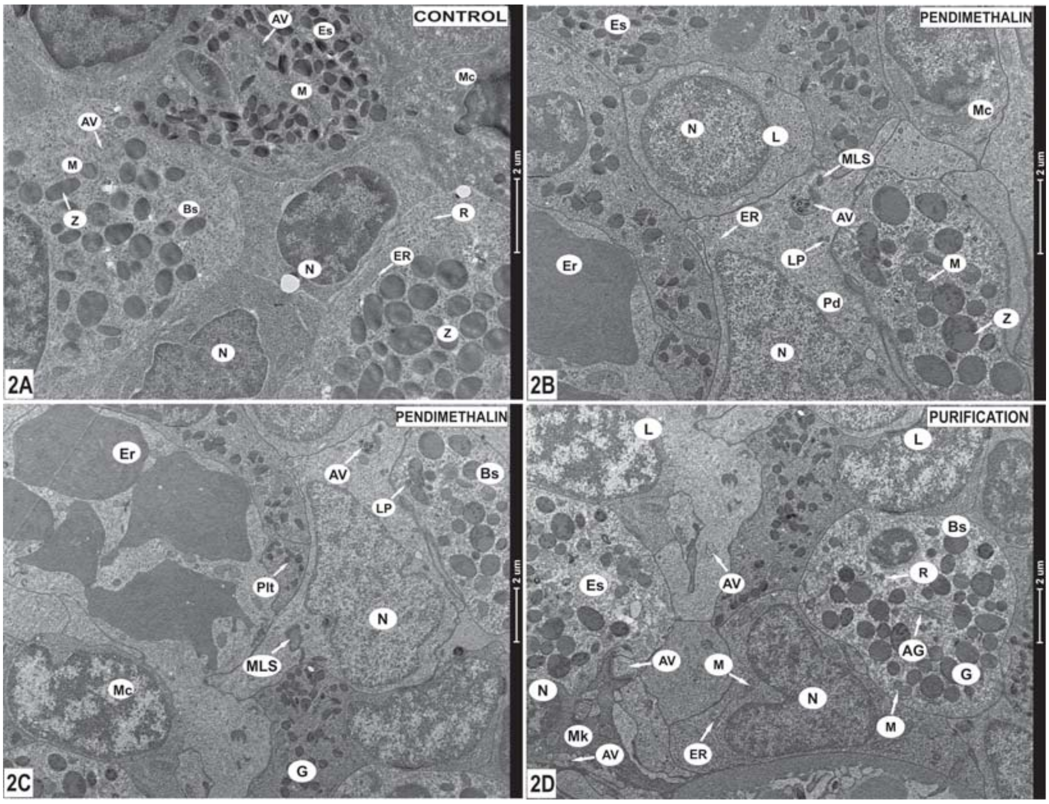

Fig. 2. Photomicrograph of the control trunk kidney cells (2A), after 14 days of pendimethalin exposure (2B, 2C) and 30 days of purification (2D).
AG-Golgi apparatus, AV-autophagic vacuoles, Bs-basophil, Es-eosinophil, Er-erythrocyte, ER-endoplasmic reticulum, L-lymphocyte, LP-primary lysosomes, M-mitochondria, Mc-monocyte, Mk-macrophage, MLS-myelin structures, N-nucleus, Pd-podocyte, Plt-platelets, R-ribosomes, Z-granulocyte granules.

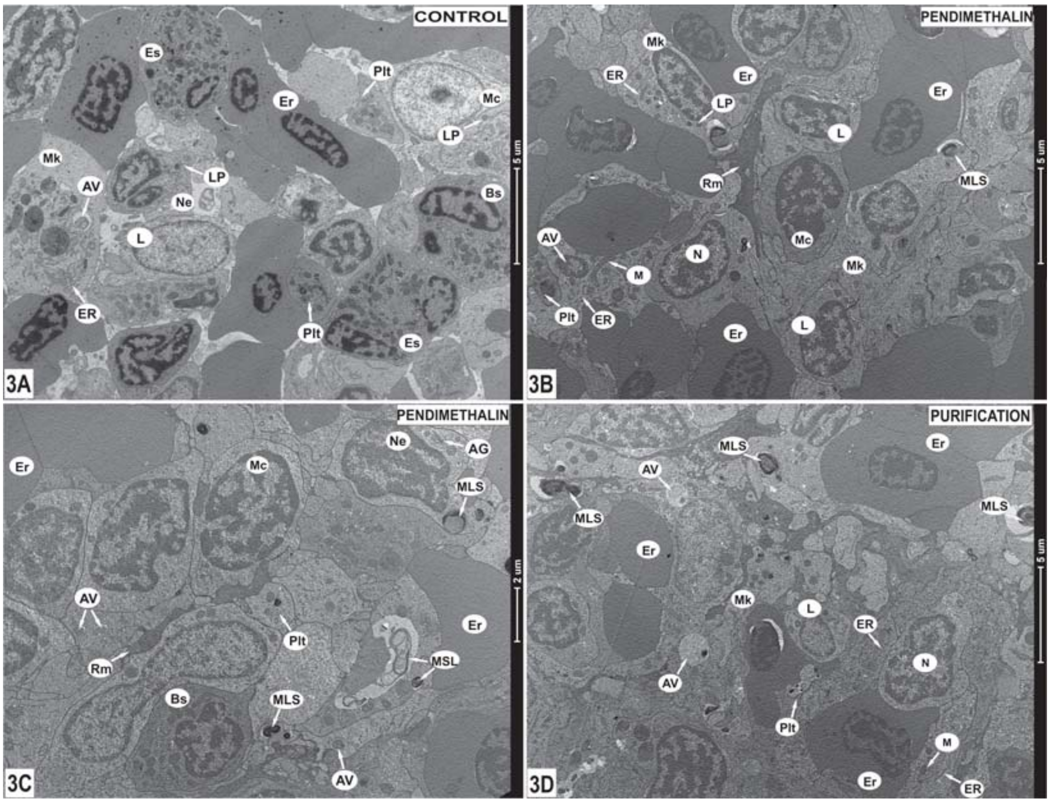

Fig. 3. Photomicrograph of the control spleen cells (3A), after 14 days of pendimethalin exposure (3B, 3C) and 30 days of purification (3D).
AV-autophagic vacuoles, Bs-basophil, Es-eosinophil, Er-erythrocytes, ER-endoplasmic reticulum, L-lymphocytes, LP-primary lysosomes, M-mitochondria, Mc-monocyte, Mk-macrophage, MLS-myelin structures, N-nucleus, Ne-neutrophil, Plt-platelets, Rm-reticular meshwork of the spleen.

pared to the samples taken immediately after the end of exposure.

The spleen of the control fish (Fig. 3A) showed firm structure with tightly adjoining hematopoietic cells. Lymphocytes and erythrocytes were the most abundant cells. Erythrocytes showed various stages of degradation and were present in aggregations of different size. Lymphocytes, single neutrophils and rare eosinophils were located among erythrocyte aggregations. After 14 days of herbicide exposure the structure of splenic hematopoietic tissue (Fig. 3B, 3C and 3D) was still firm but the structure of some cells was slightly changed. Numerous erythrocytes of different size showing symptoms of physiological destruction were observed. Lymphocytes were also abundant, usually normal, but sometimes deformed. Melanomacrophages and vesicles with single myelin-like structures were also frequently observed (Fig. 3B, 3C and 3D). After 30 days of purification (Fig. 3D) abundance of melanomacrophages and vesicles of different content and usually low electron density increased which indicates intensive cleanup process.

The levels of stress hormones

Pendimethalin caused significant increase in concentrations of all tested stress hormones in fish (Table 3). The concentrations of adrenaline and noradrenaline started to increase already after 6 hours of exposure and remained significantly elevated until the end of the experiment (30 days of purification). Cortisol level was significantly elevated compared to the control during exposure (1 and 14 days) and returned to the level similar to the control value after purification.

Discussion

Many authors reported that hematological parameters of fish may be affected by different stressors and water pollutants (COFFIGNY *et al*. 2004; ZUTSHI *et al*. 2010; WITESKA 2015), including herbicides (BOJARSKI *et al.* 2015; GHAFFAR *et al.* 2015; KONDERA *et al.* 2018). Thus, evaluation of fish blood parameters is a useful tool to understand the impact of agrochemicals on fish health (KREUTZ *et al*. 2011). In the present experiment we observed high individual variability of the hematological

Table 3

Concentrations of cortisol, adrenaline and noradrenaline in common carp plasma during pendimethalin (2.5 μg/l) exposure and after purification (PUR) (n=10). Asterisks indicate the values significantly different from the control at the same sampling time (Tukey's test, p<0.05)

Time of blood collecting / parameter		Adrenaline (ng/ml)		Noradrenaline (ng/ml)		Cortisol (ng/ml)	
		Control	Pendimethalin	Control	Pendimethalin	Control	Pendimethalin
Time of exposure	3 hours	0.52±0.15	1.24±0.31	0.72±0.10	1.18±0.27	146.3±32.2	207.95±35.52
	6 hours	0.63±0.12	2.17±0.60*	0.92±0.12	2.78±0.50*	152.3±41.2	116.94±22.50
	12 hours	0.54±0.10	1.95±0.44*	0.85±0.13	4.15±0.57*	132.3±10.0	115.84±23.51
	1 days	0.64±0.15	1.21±0.31	0.94±0.25	3.50±0.47*	156.3±52.7	225.61±72.78*
	3 days	1.72±0.35	2.41±0.58*	1.32±0.21	3.28±0.54*	160.1±29.8	210.14±61.04
	14 days	0.46±0.12	3.13±0.58*	0.66±0.08	2.30±0.44*	184.3±31.9	490.12±67.01*
PUR	30 days	1.30±0.29	3.34±0.85*	1.42±0.27	1.98±0.36*	149.2±24.9	165.04±51.48

values and thus little significant differences between the control and pendimethalin-exposed group occurred. This resulted from the experimental design – blood was sampled each time from different animals since after blood collecting the fish were killed for hematopoietic tissue analysis. High individual variability of some hematological parameters is typical for juvenile common carp (WITESKA *et al.* 2016). The obtained results showed that pendimethalin induced transient changes in red blood parameters, especially after 7 days of exposure, but after purification period (30 days) their values were similar as in the control. Transient increase of RBCc in this experiment was due to the release of new red blood cells into the bloodstream while in the spleen more pronounced destruction of erythrocytes was observed in relation to the control. More persistent alterations were observed in leukocytes. The earliest change noted was neutropenia (decreased percentage of immature and mature neutrophils) observed already after the first day of exposure. The most pronounced changes occurred after 3 days of exposure: lymphocytopenia and monocytosis accompanied by a significant increase in percentage of immature neutrophils. The increase of young neutrophils noted from 3 days of exposure to 14 days of purification probably indicates development of inflammatory response. The decrease of mature neutrophils and lymphocytosis were noted at the end of the experiment which indicates incomplete recovery and persistent alteration in fish immune system. The observed changes suggest migration of phagocytes to the tissues followed by enhanced recruitment of new cells which is typical for inflammatory response. This is confirmed by the structure of hematopoietic organs in which destruction of immature leukocytes was observed, accompanied by increased abundance of melanomacrophages and vesicles containing myelin-like structures. These symptoms indicate cleanup processes following herbicide-induced hematopoietic cell damage, particularly intensive after purification period. These changes show a weak cytotoxic effect of pendimethalin on common carp hematopoietic system. Other authors also observed changes in the values of hematological parameters during or after fish exposure to different herbicides. Short-term (96 h) exposure of *Leporinus obtusidens* to Roundup caused the alterations in red blood parameters indicating hemorrhagic anemia (GLUSCZAK *et al.* 2006). *Prochilodus lineatus* exposed to Roundup Transorb® for 6, 24 and 96 h showed a significant increase in Hct and RBCc as well as WBCc but only in group exposed to the higher concentration of the herbicide and only after 96 h of exposure (MODESTO & MARTINEZ 2010). Glyphosate caused a decrease of RBCc, WBCc and frequency of lymphocytes, accompanied by a significant increase in the number of immature circulating cells, and reduced the activity of natural immune components important for fish resistance to an infection (KREUTZ *et al.* 2011). VELISEK *et al.* (2009) reported a significant decrease in Hct, Hb and MCV in *Cyprinus carpio* exposed to metribuzin-based herbicide Sencor 70 WG for 96 h. Similarly, acute atrazine treatment of *Cyprinus carpio* caused a reduction of RBCc and Hb (RAMESH *et al.* 2009). HUSSEIN *et al.* (1996) tested the influence of atrazine exposure lasting

28 days on red blood cell parameters of *Oreochromis niloticus* and *Chrysichthys auratus*. They noted a significant decrease of RBCc, Hb, Hct, MCV, MCH and MCHC values A decrease in RBCc may result from a decrease in the erythropoietic activity of the kidney or from hemodilution caused by impaired osmoregulation across the gill epithelium (RAMESH *et al*. 2009). PEREIRA *et al*. (2013) observed hematological alterations in *Prochilodus lineatus* exposed to clomazone-based herbicide Gamit® 500 for 96 h: significant decrease in Hct, Hb, MCH and MCHC indicating anemic condition. According to the authors, anemia might have resulted from ROS-induced oxidative injury via oxidation of hemoglobin or other cellular components. In the same experiment RBCc significantly increased at the lowest herbicide concentration, possibly due to the release of new red blood cells into the bloodstream in response to splenic contraction, which was probably an adaptive response to the presence of toxic agent. *Cyprinus carpio* exposed to Gardoprim Plus Gold 500 SC (terbuthylazine and S-metolachlor) for 96 h (DOBSIKOVA *et al*. 2011) showed a decrease of Hct and the decline in WBC and lymphocyte counts indicating immunosuppression. Acute butachlor administration to *Labeo rohita* caused a decrease in the values of RBCc, Hct, MCH, Hb and lymphopenia while MCV, WBCc and the percentage of neutrophils and monocytes were significantly increased (GHAFFAR *et al*. 2015). The red blood parameters changes suggested macrocytic anemia, while leukocytosis and neutrophilia indicate the inflammatory reaction following injurious stimuli. Sublethal molinate administration (96 h) in *Anguilla anguilla* resulted in a decrease of Hct, RBc, Hb and WBCc. The observed effects may reflect a severe anemic state (hemorrhagic anemia) and immunosuppression (SANCHO *et al*. 2000). BOJARSKI *et al.* (2015) studied hematological alterations in *Cyprinus carpio* during one week exposure to ethofumesate and pendimethalin used separately or in mixture. The authors found that fish exposure to ethofumesate or pendimethalin caused fluctuations of red blood parameters: the increase and subsequent decrease in RBCc and Hct, and the decrease in Hb followed by an insignificant increase. Fish exposure to the mixture of the herbicides caused more pronounced and faster changes in the values of these parameters. At higher concentrations pendimethalin caused more pronounced and earlier alterations than ethofumesate. In the same experiment leukocyte profiles were also changed, especially after 3 days of exposure (the increase of the percentage of lymphocytes, while the frequency of neutrophils decreased in all tested groups). After 7 days of exposure, the opposite direction in the leukocyte profile changes occurred. Comparison of the data obtained in the present study with the results of the study by BOJARSKI *et al*. (2015) showed that the changes in hematological parameters in fish exposed to pendimethalin were different despite the same herbicide concentration used in both experiments (2.5 µg/l), similar environmental conditions and fish body mass. The differences might have resulted from different seasonal factors (late spring vs. autumn) of both experiments and probably from the different fish condition and thus their different sensitivity to the toxic agent, however the hematological changes were transient in both experiments. Common carp juveniles exposed for 7 days to Roundup showed alterations in peripheral blood and hematopoietic tissue cellular composition and activity (KONDERA *et al.* 2018). Hct and MCV increased, while Hb, MCH and MCHC decreased indicating a slight anemia. A reduction in WBCc and oxidative metabolic activity of phagocytes (NBT) were also noted. Analysis of head kidney hematopoietic tissue revealed that Roundup caused a significant increase in the rate of proliferation accompanied by an increase in frequency of early blast cells and the frequency of monocytoid, eosinophilic and basophilic lineage cells. These alterations suggest a slight immunosuppresive response although a considerable compensatory potential of carp hematopoietic system was observed. The exposure of silver catfish *Rhamdia quelen* to clomazone for 8 days with a recovery period of 8 days revealed a significant decrease of Hct but not Hb (CRESTANI *et al.* 2006). After purification the values of both parameters were similar as in the control. Hematological and hematopoietic effects of 12 days exposure of tench (*Tinca tinca*) to 2,4-D were studied by GOMEZ *et al.* (1998). They reported marked alteration of hematopoietic tissue, characterized by progressive swelling and moderate cell necrosis, activation of the phagocyte system, subsequent formation of myelin figures and auto- and heterophagic vacuoles. Transient Hct increase was attributed to hemoconcentration while gradual decrease in Hb was explained with binding of 2,4-D to erythrocyte membranes. *Cyprinus carpio* exposed to the herbicide MCPA for 14 days showed transient but statistically significant increase of Hb, MCH and MCHC values after 1 day of exposure that might have resulted from stress, followed by a decrease in Hb and MCHC after 14 days (LUTNICKA *et al*. 2018). RBCc increased after 7 days of exposure and decreased after 14 days of purification showing a transient anemic response to MCPA. After the purification period (30 days) red blood parameters recovered to the control levels. The leukocyte parameters were more variable in the same experiment (LUTNICKA *et al.* 2018). Despite transient leukocytosis, a persistent depletion of mature neutrophils accompanied by monocytosis was ob-

served. These alterations suggest a possible inflammatory response in fish exposed to MCPA and increasing migration of granulocytes to the affected tissues. Analysis of hematopoietic tissue revealed no major pathologic lesions but some minor ultrastructural anomalies. Hematopoietic precursor cells with blurred ultrastructure, vacuoles in cytoplasm showing different electron density and size, melanomacrophages and myelin-like structures were observed after exposure and particularly after purification. These changes indicate a weak cytotoxic effect of MCPA on carp hematopoietic system. ROCHE & BOGE (1996) did not find any major changes in Hct and Hb in sea bass (*Dicentrarchus labrax*) after 3 days post 15 days of atrazine exposure. Hematological response of *Cyprinus carpio* to sublethal Roundup® exposure (GHOLAMI-SEYEDKOLAEI *et al.* 2013) included significant decrease of RBCc, Hb, Hct and MCHC, while a significant time- and dose-dependent increase in MCH and MCV were observed. A decrease of Hb and Hct can be attributed to the disorders in hematopoietic processes and accelerated disintegration of erythrocyte membranes. Erythropoiesis inhibition and destruction of red cells are two possible causes of RBCc decrease. In the same experiment a significant dose- and time-dependent decrease in WBCc was found, indicating immunosuppression. Sub-chronic exposure of common carp to the terbutryn for 28 days led to a significant increase in RBCc, while MCV and MCH values were significantly reduced at higher doses. The lowest environmental concentration (0.02 mg/l) did not cause hematological perturbations (VELISEK *et al.* 2010). The increase of RBCc was probably the result of the release of immature erythrocytes from spleen to blood circulation and could be an immediate response to acute stress mediated by catecholamines.

The results of our study showed a singnificant increase of catecholamines and cortisol which indicated that pendimethalin at low concentration caused stress in fish. There are no literature data of the effects of this herbicide on the level of stress hormones in fish. According to SOSO *et al.* (2007), cortisol concentration increased in *Rhamdia quelen* after 20 and 40 days of exposure to 3.6 mg/l of glyphosate. On the other hand, CERICATO *et al.* (2008) reported a disruption of the hypothalamus-pituitary-interrenal axis in *Rhamdia quelen* exposed to sublethal concentration of glyphosate and mixture of atrazine and simazine – the fish showed lower cortisol level than in the control. DO CARMO LANGIANO & MARTINEZ (2008) reported that short-term (6, 24 or 96 h) exposures to 7.5 or 10.0 mg/l of Roundup resulted in no alteration of cortisol concentration in *Prochilodus lineatus*.

Hematological and biochemical parameters in fish can significantly change in response to chemical stressors; however, these alterations are nonspecific (SOSO *et al.* 2007; CERICATO *et al.* 2008; DO CARMO LANGIANO & MARTINEZ 2008; MODESTO & MARTINEZ 2010; WITESKA 2013, 2015). Usually, changes in red blood parameters are connected with anemia when a significant decrease of RBCc, Hb content and Hct value are observed (Witeska 2015). However, red blood parameters are relatively stable and show compensatory mechanisms (Witeska 2015). In the present experiment transient changes in red blood parameters were observed compensated by increased erythropoiesis in head kidney. Herbicide-induced alterations in leukocyte parameters were more persistent and therefore probably more dangerous to the fish. Immunosuppressive effect of chronic pendimethalin exposure was observed by DANION *et al.* (2012) in *Oncorhynchus mykiss* infected with VHS virus as the decrease of the Mean Time to Death (MTD).

Common carp exposure to pendimethalin at low (environmentally realistic) concentration caused stress reaction, inflammatory process and other minor hematological and hematopoietic changes.

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Author Contributions

Research concept and design: H.L.; Collection and/or assembly of data: H.L., B.B., W.T., E.T., A.K.-B., L.P.; Data analysis and interpretation: H.L., B.B., T.K., W.T., E.T., A.K.-B., M.W., L.P.; Writing the article: H.L., B.B., T.K., W.T., E.T., A.K.-B., M.W., K.P; Critical revision of the article: H.L., B.B., T.K., M.W., K.P; Final approval of article: H.L., B.B., M.W.

Conflict of Interest

The authors declare no conflict of interest.

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