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Effects of Subchronic Exposure to LASSO MTX® (Alachlor 42% W/V) on Hematological Indices and Histology of the Common Carp, *Cyprinus carpio* L.

Premysl Mikula · Helena Modra · Danka Nemethova · Ladislav Groch · Zdenka Svobodova

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Abstract The aim of the study was to evaluate subchronic toxic effects of the preparation LASSO MTX (alachlor 42% W/V) on hematological indices and histology of the common carp (*Cyprinus carpio* L.). In carp exposed for 28 days to LASSO MTX in the concentration of 2,400 μg L^{-1}, significant differences ($p < 0.05$), were detected in all indices of the erythrocyte profiles tested except hematocrit (PCV) when compared to controls. At a lower exposure (240 μg L^{-1}) concentration of blood hemoglobin and mean corpuscular hemoglobin were significantly reduced. In contrast, no influence of the preparation on leucopoiesis was demonstrated, and only slight changes were observed in histopathological indices.

P. Mikula (✉) · H. Modra · Z. Svobodova
Department of Veterinary Public Health and Toxicology, University of Veterinary and Pharmaceutical Sciences, Faculty of Veterinary Hygiene and Ecology, Palackeho 1-3, 612 42 Brno, Czech Republic
e-mail: premysl.mikula@centrum.cz

D. Nemethova
Research Centre for Environmental Chemistry and Ecotoxicology, Masaryk University, Kamenice 126/3, 625 00 Brno, Czech Republic

D. Nemethova
Institute of Biostatistics and Analyses, Masaryk University, Kamenice 126/3, 625 00 Brno, Czech Republic

L. Groch
Institute of Pathological Morphology, University of Veterinary and Pharmaceutical Sciences, Faculty of Veterinary Medicine, Palackeho 1-3, 612 42 Brno, Czech Republic

Z. Svobodova
Research Institute of Fish Culture and Hydrobiology at Vodnany, Zatisi 728/II, 389 25 Vodnany, Czech Republic

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Chloroacetanilide herbicides are a group of chemicals widely used in agriculture. Alachlor-based herbicide preparations are used extensively in the Czech Republic. In 2006, 145.34 tonnes of alachlor were used in the production of rape, maize, and legumes. (The State Phytosanitary Administration of the Czech Republic 2007). Alachlor or its degradates are commonly detected in the aquatic environment. Some of the highest concentrations reported in surface water in the Czech Republic, Spain, and USA were 39.1 μg L^{-1} (Czech Hydrometeorological Institute 2007), 31.9 μg L^{-1} (Sanchez-Camazano et al. 2005) and 17.2 μg L^{-1} (Battaglin et al. 2000).

Adverse effects of alachlor have been demonstrated in vertebrates. In addition to disruption of thyroid hormone homeostasis in rats, which can lead to thyroid tumor formation (Wilson et al. 1996), alachlor has been related to genotoxicity (Chang et al. 2005), changes in serum levels of sex steroid hormones (Yi et al. 2007a), and altered activity of liver enzymes (Yi et al. 2007b; Peebua et al. 2007) in fish. The effects of alachlor on hematological indices have not been previously studied.

The aim of the present study was to evaluate through hematological and histological examination, the effects of alachlor resulting from subchronic exposure of common carp (*Cyprinus carpio* L.) to the herbicide LASSO MTX®.

Materials and Methods

The experiment was a semi-static assay conducted over a 28 day period. Seventy-eight 1-year-old common carp

(*Cyprinus carpio* L.) (mean body length 13.2 ± 0.8 cm, mean weight 61.2 ± 11.8 g) were allocated in duplicate, in groups of 13, to one of two experimental regimes or an untreated control group. Each of the six groups was held in a glass tank containing 100 L of water.

The physico-chemical parameters of the water (mean ± SD) used in the tanks were: $ANC_{4.5}$ (acid neutralization capacity) = 4.20 ± 0.18 mmol L^{-1}, COD_{Mn} (chemical oxygen demand) = 2.80 ± 0.11 mg L^{-1}, BOD_5 (biological oxygen demand) = 0.72 ± 0.06 mg L^{-1}, $NH_3 + NH_4$ = not detected, NO_3^- = 23.48 ± 3.80 mg L^{-1}, NO_2^- = not detected, Cl^- = 18.11 ± 2.19 mg L^{-1}. Water temperature varied from 20.0 to 21.8°C, pH from 7.9 to 8.1, and oxygen saturation from 53.2% to 70.0%.

After a 14 day adaptation period, experimental fish were exposed to the herbicide LASSO MTX added to the tank water in concentrations of either 240 or 2,400 μg L^{-1}. The manufacturer states that LASSO MTX contains c. 42% of the active substance alachlor, c. 41% water, and 17% other, unspecified, substances (Monsanto Europe S.A. 2007). Thus, expected concentrations of alachlor in the two baths were approximately 100 μg L^{-1} and 1,000 μg L^{-1}. Tanks for both the treated fish and the controls were replicated and fish were transferred to the replicate tank, containing freshly diluted LASSO MTX at the appropriate concentration or fresh water for the controls, every second day. Fish were fed about 1% of body weight per day in two feedings of commercial fish pellets.

After 28 days exposure of fish, 1 mL blood samples were taken by cardiac puncture and stabilized with sodium heparin (50 IU per mL blood). Erythrocyte count (RBC), hematocrit (PCV), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), leucocyte count (TLC), and differential leucocyte count (leucogram) were determined according to Svobodova et al. (1991). Hematological parameters were examined in six or seven fish from each tank and blood samples from the remaining fish were used for analysis of plasma biochemical parameters (unpublished data).

Samples of liver (hepatopancreas) from three fish in each tank, together with samples of both head and trunk kidney and gills, were removed for histology. All samples were fixed in 10% formalin before examination. Fixed tissue samples were drained, embedded into paraffin and stained with hematoxylin–eosin. The sections (5 μm) were examined by light microscopy.

The Shapiro–Wilk test was used to assess the normal distribution of hematological parameters (Zar 1999). Since non-normal distributions of parameters ($p < 0.05$) were identified, non-parametric tests were used. To compare values of hematological parameters among groups, the Kruskal–Wallis test was used. This test was followed by a multiple comparison when significant differences were found among groups (Zar 1999). Data analyses were performed using STATISTICA software (StatSoft Czech Republic, Prague).

Results and Discussion

The effects of LASSO MTX on the erythrocyte profile are shown in Table 1. The only parameter that did not differ among all groups was PCV. Significant ($p < 0.05$) differences compared to control fish were detected, not only in RBC, Hb, MCV, MCH, and MCHC of carp exposed to the preparation LASSO MTX at the concentration of 2,400 μg L^{-1}, but also in Hb and MCH values of carp exposed to LASSO MTX at the lower concentration tested (Fig. 1). Carp exposed for 28 days showed a dose-dependent decline of erythrocyte counts, hemoglobin concentration, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration. Conversely, mean corpuscular volume increased in carp exposed to the preparation LASSO MTX. Median value (range) of leucocyte counts in control group (n = 12) was 52 (42−68) G L^{-1} (i.e., 10^9 cells per litre). Leucocyte profile of control fish was as follows: lymphocytes 44 (34−58) G L^{-1}; monocytes 0.5 (0−3) G L^{-1}; neutrophile granulocytes 7 (5−14) G L^{-1}. Eosinophiles or basophiles were not detected. Neither leucocyte counts of fish exposed to LASSO MTX nor their leucocyte profile showed differences when compared to control carp.

Histological examination of tissues of exposed carp demonstrated only slight differences compared to control fish. In exposed carp, there were diffuse dystrophic processes in the liver parenchyma. In the group of carp exposed to 240 μg L^{-1} LASSO MTX these differences were very slight, however, in the group exposed to 2,400 μg L^{-1}, differences were more pronounced. Histological examination also demonstrated a higher incidence of melanomacrophage centers (MMCs) in head and trunk kidney. Such differences were observed mainly in carp from the group exposed to the higher concentration of LASSO MTX. Histopathological differences were also detected in gills of fish exposed to higher concentrations of LASSO MTX. These were characterized as hyperplasia of epithelial elements connected with chloride-cell activation. Neither of these pathological conditions was observed in control fish.

In our study, macrocytic hypochromic anemia was observed in fish following subchronic exposure to LASSO MTX. Anemia relating to the exposure of fish to a range of pesticides is commonly reported. Jenkins et al. (2003) reported decline in total erythrocyte counts, hemoglobin percentage, and hematocrit of common carp (*Cyprinus carpio* L.) exposed to endosulfan. Similar results were also published by Svoboda et al. (2001), who exposed common

Table 1 Effects of the preparation LASSO MTX on erythrocyte profiles in common carp (*Cyprinus carpio* L.)

Index	Control group			LASSO MTX 240 μg L^{-1}			LASSO MTX 2,400 μg L^{-1}		
	n	median	range	n	median	range	n	median	range
RBC [T L^{-1}]	12	1.52^{a}	(0.90–2.02)	13	1.42^{ab}	(1.09–1.58)	13	1.32^{b}	(1.22–1.40)
Hb [g L^{-1}]	11	67.27^{a}	(37.63–87.59)	13	44.96^{b}	(29.64–76.93)	13	40.30^{b}	(28.64–66.27)
PCV [L L^{-1}]	12	0.28^{a}	(0.22–0.39)	13	0.30^{a}	(0.27–0.39)	13	0.34^{a}	(0.26–0.41)
MCV [fL]	12	197.88^{a}	(133.66–248.41)	13	232.88^{ab}	(188.81–274.65)	13	250.00^{b}	(196.97–310.61)
MCH [pg]	11	44.55^{a}	(36.92–57.77)	13	31.47^{b}	(18.76–54.18)	13	28.79^{b}	(23.48–50.59)
MCHC [L L^{-1}]	11	0.23^{a}	(0.15–0.32)	13	0.16^{ab}	(0.09–0.20)	13	0.12^{b}	(0.09–0.17)

Groups with different alphabetical superscripts differed significantly at $p < 0.05$. Units of measured indices: T L^{-1}, 10^{12} cells per litre; g L^{-1}, grams per litre; L L^{-1}, litres per litre; fL, femtolitres; pg, picograms

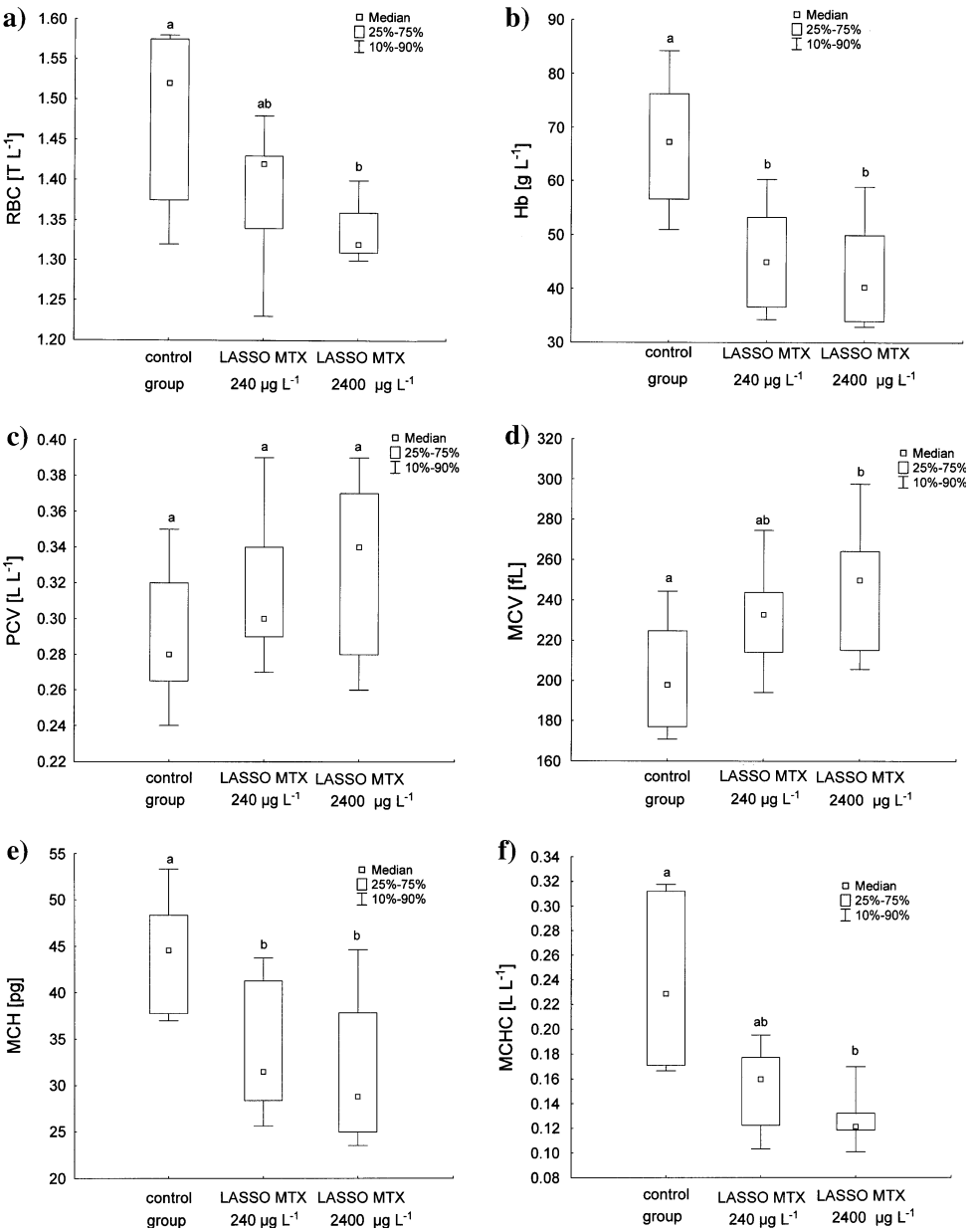

Fig. 1 Hematological indices of common carp (*Cyprinus carpio* L.) after subchronic exposure to LASSO MTX: (**a**) erythrocyte count (RBC); (**b**) hemoglobin concentration (Hb); (**c**) hematocrit (PCV); (**d**) mean corpuscular volume (MCV); (**e**) mean corpuscular hemoglobin (MCH) and, (**f**) mean corpuscular hemoglobin concentration (MCHC). Groups with different alphabetical superscripts differ significantly at $p < 0.05$

carp to diazinon, in the preparation BASUDIN 600 EW, for 96 h. Other authors have observed anemia in common carp exposed to the preparation DECIS FLOW 2.5, which contains deltamethrin as an active substance (Svobodova et al. 2003). Adhikari et al. (2004) investigated the effects of cypermethrin and carbofuran on certain hematological parameters in a freshwater teleost, *Labeo rohita* H. and estimated the rate of recovery of these parameters to pre-exposure levels during the recovery period. In fish exposed for 28 days to sublethal levels of either substance, a significant time and dose-dependent decrease in RBC, Hb, PCV, and oxygen-carrying capacity was observed. As with our study, increased MCV values were observed. During the recovery period following exposure of fish to tested pesticides, the parameters studied gradually returned to values determined at the beginning of the experiment (Adhikari et al. 2004). Anemia in fish exposed to the preparation LASSO MTX may be explained by both the higher degree of erythrocyte destruction and the reduced availability of substances important in erythropoiesis, such as iron and vitamins. Mild macrocytic hypochromic anemia has also been observed in rainbow trout (*Oncorhynchus mykiss* W.) not receiving essential ω-3 fatty acids (e.g., linoleic acid) in their diet (Castell et al. 1972).

Based on the results of erythrocyte profile investigation, detection of marked pathologies in hematopoietic tissues of exposed carp (i.e., liver and head kidney) had been expected. Surprisingly, only slight dystrophic changes in liver parenchyma and a more extensive occurrence of melanomacrophage centers in head and trunk kidney were observed in exposed fish when compared to controls. MMCs in hematopoietic tissues are formed by clusters of phagocytic cells containing erythrocyte fragments or certain pigments (e.g., hemosiderin, lipofuscin and melanin) (Herraez and Zapata 1986; Passantino et al. 2005). The occurrence of MMCs in teleost hematopoietic tissues is influenced by factors, such as vitamin deficiency, the disease process, and bleeding or starvation of fish (Herraez and Zapata 1986). However, a higher incidence of MMC occurrence in head and trunk kidney and in spleen was also reported by Capkin et al. (2006), who exposed rainbow trout to an organochlorine pesticide, endosulfan, for 96 h. Thus, the higher incidence of MMCs in kidney of exposed fish observed in our experiment could be related to their anemic condition. In addition to the above-mentioned histopathological changes, epithelial hyperplasia and chloride-cell activation was found in gills of fish in the group exposed to higher levels of LASSO MTX. A similar histopathological picture was described by Dobsikova et al. (2006) following short-term exposure of common carp to the pyrethroid cypermethrin.

In conclusion, an anemic condition was demonstrated in carp as a consequence of subchronic exposure to the preparation LASSO MTX containing alachlor as an active substance. Differences in certain parameters of the erythrocyte profile were detected, when compared to controls, not only in fish exposed at the higher alachlor concentration tested, but also at the lower exposure (approximately 100 $\mu g\ L^{-1}$), which can be considered to mimic environmentally relevant concentrations. Continuous long-term exposure of fish to high alachlor concentrations used here is unlikely in the aquatic environment. However, the possibility of synergistic effects of alachlor and other water pollutants on fish must be considered. Besides the adverse effects of LASSO MTX on the erythrocyte profile, slight histopathological changes, visible mainly in the group of carp exposed to the higher alachlor concentrations, were demonstrated. On the other hand, LASSO MTX did not affect cell-mediated immunity of fish on the basis of TLC and leucograms of exposed carp, neither of which differed from those of control fish.

Further research is necessary, not only into the effects of alachlor alone, but also of its effects in combination with other common pollutants in the aquatic environment.

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References

Adhikari S, Sarkar B, Chatterjee A, Mahapatra CT, Ayyappan S (2004) Effects of cypermethrin and carbofuran on certain hematological parameters and prediction of their recovery in a freshwater teleost, *Labeo rohita* (Hamilton). Ecotox Environ Safe 58:220–226. doi:10.1016/j.ecoenv.2003.12.003

Battaglin WA, Furlong ET, Burkhardt MR, Peter CJ (2000) Occurrence of sulfonylurea, sulfonamide, imidazolinone, and other herbicides in rivers, reservoirs and ground water in the Midwestern United States, 1998. Sci Total Environ 248:123–133. doi:10.1016/S0048-9697(99)00536-7

Capkin E, Altinok I, Karahan S (2006) Water quality and fish size affect toxicity of endosulfan, an organochlorine pesticide, to rainbow trout. Chemosphere 64:1793–1800. doi:10.1016/j.chemosphere.2005.12.050

Castell JD, Sinnhuber RO, Lee DJ, Wales JH (1972) Essential fatty acids in the diet of rainbow trout (*Salmo gairdneri*): physiological symptoms of EFA deficiency. J Nutr 102:87–92

Chang LW, Toth GP, Gordon DA, Graham DW, Meier JR, Knapp CW, de Noyelles FJ, Campbell S, Lattier DL (2005) Responses of molecular indicators of exposure in mesocosms: common carp (*Cyprinus carpio*) exposed to the herbicides alachlor and atrazine. Environ Toxicol Chem 24:190–197. doi:10.1897/03-509.1

Czech Hydrometeorological Institute (2007) On-line water quality database. http://hydro.chmi.cz/ojv/ (in Czech). (visited online 1.10. 2007)

Dobsikova R, Velisek J, Wlasow T, Gomulka P, Svobodova Z, Novotny L (2006) Effects of cypermethrin on some haematological, biochemical and histopathological parameters of common carp (*Cyprinus carpio* L.). Neuroendocrinol Lett 27(S2):91–95

Herraez MP, Zapata AG (1986) Structure and function of the melanomacrophage centers of the goldfish *Carassius auratus*. Vet Immunol Immunopathol 12:117–126. doi:10.1016/0165-2427(86)90116-9

Jenkins F, Smith J, Rajanna B, Shameem U, Umadevi K, Sandhya V, Madhavi R (2003) Effect of sub-lethal concentrations of endosulfan on hematological and serum biochemical parameters in the carp *Cyprinus carpio*. Bull Environ Contam Toxicol 70:993–997. doi:10.1007/s00128-003-0080-7

Monsanto Europe S.A. Safety data sheet of the preparation LASSO MTX. http://www.monsanto.cz/doc/cr_lasso_mtx.pdf (in Czech). (visited online 1.10. 2007)

Passantino L, Cianciotta A, Jirillo F, Carrassi M, Jirillo E, Passantino GF (2005) Lymphoreticular system in fish: erythrocyte-mediated immunomodulation of macrophages contributes to the formation of melanomacrophage center. Immunopharmacol Immunotoxicol 27:147–161. doi:10.1081/IPH-200051766

Peebua P, Kosiyachinda P, Pokethitiyook P, Kruatrachue M (2007) Evaluation of alachlor herbicide impacts on Nile tilapia (*Oreochromis niloticus*) using biochemical biomarkers. Bull Environ Contam Toxicol 78:128–131. doi:10.1007/s00128-007-9027-8

Sanchez-Camazano M, Lorenzo LF, Sanchez-Martin MJ (2005) Atrazine and alachlor inputs to surface and ground waters in irrigated corn cultivation areas of Castilla-Leon Region, Spain. Environ Monit Assess 105:11–24. doi:10.1007/s10661-005-2814-y

Svobodova Z, Pravda D, Palackova J (1991) Unified methods of haematological examination of fish. Method No. 20. Research Institute of Fish Culture and Hydrobiology, Vodnany, Czech Republic, 31 pp

Svoboda M, Luskova V, Drastichova J, Zlabek V (2001) The effect of diazinon on haematological indices of common carp (*Cyprinus carpio* L.). Acta Vet. Brno 70:457–465

Svobodova Z, Luskova V, Drastichova J, Svoboda M, Zlabek V (2003) Effect of deltamethrin on haematological indices of common carp (*Cyprinus carpio* L.). Acta Vet. Brno 72:79–85

The State Phytosanitary Administration of the Czech Republic (2007) Czech Republic—Usage of Actives 2006. www.srs.cz (visited online 1.8. 2007)

Wilson AGE, Thake DC, Heydens WE, Brewster DW, Hotz KJ (1996) Mode of action of thyroid tumor formation in the male Long–Evans rat administered high doses of alachlor. Fundam Appl Toxicol 33:16–23. doi:10.1006/faat.1996.0138

Yi XH, Liu HH, Lu YT, Tao J, Ding H, Zhang M, Jiang W (2007a) Altered serum levels of sex steroids and biotransformation enzyme activities by long-term alachlor exposure in crucian carp (*Carassius auratus*). Bull Environ Contam Toxicol 79:283–287. doi:10.1007/s00128-007-9245-0

Yi XH, Ding H, Lu YT, Liu HH, Zhang M, Jiang W (2007b) Effects of long-term alachlor exposure on hepatic antioxidant defense and detoxifying enzyme activities in crucian carp (*Carassius auratus*). Chemosphere 68:1576–1581. doi:10.1016/j.chemosphere.2007.02.035

Zar JH (1999) Biostatistical analysis, 4th edn. Prentice Hall, New Jersey, p 929