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# Effects of Acute Exposure to Metribuzin on Some Hematological, Biochemical and Histopathological Parameters of Common Carp (*Cyprinus carpio* L.)

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**Abstract** The aim of the study was to evaluate acute toxic effects of the preparation Sencor 70 WG (metribuzin 70% W/V) on hematological, biochemical indices and histology of the common carp (*Cyprinus carpio* L.). In carp exposed for 96 h to Sencor 70 WG in the concentration of 250.2 mg/L, showed significantly lower ($p < 0.01$) values of plasma total proteins, albumins, total globulins, triacylglycerols, lactate dehydrogenase, lactate, inorganic phosphate, hematocrit, hemoglobin concentration, mean erythrocyte volume, the leucocrite value, lymphocyte, and significantly higher ($p < 0.01$) values of glucose, ammonia, calcium, monocytes, neutrophile granulocytes, developmental forms myeloid sequence and basophiles compared to the control group. Histopathological examination revealed hyaline degeneration of the epithelial cells of renal tubules of the caudal kidney. This alteration of kidney resulted in hypoproteinemia, followed by generation of transudate in body cavity.

**Keywords** Triazine · Fish · Biochemical profile · Hematological profile · Histology

Metribuzin [4-amino-6-*tert*-butyl-3-(methythio)-1,2,4-triazin-5-one] is an asymmetrical triazine herbicide. It is distinct from the symmetrical triazines such as atrazine and simazine, in which the central ring structure has alternating carbon and nitrogen atoms, in that metribuzin possesses two nitrogen atoms and two adjacent carbon atoms. The extensive use of pesticides contributes to significant increases in crop yields and farm efficiency. Runoff of metribuzin, like other triazine and triazinone herbicides, is prone to contaminating surface waters due to its water solubility 1.220 mg/L; vapor pressure 1.3 mPa; and soil half-life 30 days (Pauli et al. 1990). Modeling has indicated that metribuzin can reach concentrations up to 390 g/L in surface water runoff (Pauli et al. 1990). Pesticide contamination of freshwater is a concern with respect to long-term and low-dose effects of pesticides on public health, as well as its impact on other non-target species. Thus, research on the fate of pesticides in the environment is needed (Guasch et al. 2007). The effect of metribuzin on hematological and biochemical profiles of rainbow trout (*Oncorhynchus mykiss* Walbaum) has been documented by Velisek et al. (2008). The assessment of the ecotoxicological risks of pesticides is based on toxicity data and the effects of pesticide preparations on non-target organisms (Guasch et al. 2007) such as fish. An important parametric finding following triazine exposure to fish is the presence of transudate in the body cavity (Velisek et al. 2008) We assume that the reason for this pathological change is interference with intermediate metabolism by the triazines. The aim of this study was to determine the effects of metribuzin (4-amino-6-*tert*-butyl-4,5-dihydro-3-methyl-thio-1,2,4-triazin-5-one) on the internal environment of 2-year-old common carp (*Cyprinus carpio* L.).

## Materials and Methods

The experiment was performed using 30 two-year-old common carp (M 72 polyhybrid strains of mirror carp),

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mean body weight 480.67 ± 128.37 g and mean standard body length 307 ± 31 mm. The fish were divided in six groups of five, each held in 200 L aquaria. Three groups were experimental fish, and were exposed to 250.2 mg/L of Sencor 70 WG, (Bayer CropScience GmbH, Germany) containing 175.1 mg/L of metribuzin, and three groups were untreated controls. This concentration of metribuzin is the median of the lethal concentration (96 h LC50) for carp, which was determined on the basis of previous acute toxicity tests with carp. The 96 h LC50 is the basic value in the acute toxicity test. Therefore this concentration was selected for determination of the effects of acute exposure of metribuzin on some hematological, biochemical and histopathological parameters of common carp. The tested substance was maintained at above 80% of the nominal concentration throughout the experiment. Determination of metribuzin concentration in water was measured using gas chromatography using the following method: the water was extracted with dichloromethane. The extract was then dried, redissolved and concentrating the extract in acetone. The extract was analyzed by gas chromatography with thermionic bead detector. The reporting limit of this method is 0.46 μg/L (Lyytikainen et al. 2003). The experiment was run as a semistatic 96 h acute toxicity test, with chemical renewal every 24 h, and the results compared with the non-treated control groups.

Physical and chemical indices of the test diluting water were as follows: pH 7.8–7.9; $ANC_{4.5}$ 1.11 mmol/L; chemical oxygen demand-$COD_{Mn}$ 1.3 mg/L; total ammonia 0.02 mg/L; $NO_3^-$ 10.0 mg/L; $NO_2^-$ 0.002 mg/L; $PO_4^{3-}$ 0.01 mg/L; Ca/Mg 13 mg/L. Water temperature and oxygen saturation were 20.0–20.2°C and 84–99%, respectively.

Blood samples, obtained by cardiac puncture, were stabilized with 50 IU sodium salt of heparin per 1 mL of blood. The hematological indices tested comprised erythrocyte count (RBC), hemoglobin concentration (Hb), hematocrit value (PCV), mean erythrocyte volume (MCV), mean erythrocyte hemoglobin (MCH), mean color concentration (MCHC), leucocrite value (Bc), leukocyte count (WBC), and differential leukocyte count.

Blood plasma was obtained by centrifuging chilled blood samples at $400 \times g$ for 15 min. Plasma biochemical indices included glucose (GLU), total proteins (TP), albumins (ALB), total globulins (GLOB), triacylglycerols (TAG), ammonia ($NH_3$), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatine kinase (CK), lactate (LACT), calcium ($Ca^{2+}$), and inorganic phosphate (PHOS). Plasma biochemical analysis was conducted with a VET-TEST 8008 analyzer (IDEXX Laboratories Inc., USA; Medisoft Co.). Analyzes were carried out on selective testing discs (Multi-layer film slides, Kodak) by laser reading of the bar codes. Detection limits of the methods were as follows: GLU (0.01 mmol/L), TP (1.0 g/L), ALB (1.0 g/L), GLOB (1.0 g/L), TAG (0.01 mmol/L), $NH_3$ (1.0 μmol/L), LDH (0.0167 μkat/L), AST (0.0835 μkat/L), ALT (0.0835 μkat/L), ALP (0.0167 μkat/L), CK (0.0167 μkat/L), LACT (0.01 mmol/L), $Ca^{2+}$ (0.01 mmol/L), and PHOS (0.01 mmol/L). QA/QC measures were consistently applied within the experiment according to validated standard operation procedures.

For histological studies, the gills, skin, liver, cranial and caudal kidney, and spleen were fixed in a solution containing ethanol, formalin, and acetic acid (ALFAC) and stored in 70% ethanol. The organs were embedded in paraffin, sectioned (5 μm), and the slides stained with hematoxylin and eosin (HE). The sections were examined by light microscopy, using as reference Takashima and Hibiya (1995), and photographed using a digital camera.

Statistical analysis was carried out using Statistica software 8.0 for Windows (StatSoft, Czech Republic). Data were first tested for normality (Kolmogorov–Smirnov test) and homoskedasticity of variance (Bartlett's test). If those conditions were satisfied, one-way analysis of variance (ANOVA) was employed to determine significant differences in measured variables among control and experimental groups. When a difference was detected ($p < 0.01$), Tukey's multiple comparison test was applied to identify which treatments were significantly different. If the conditions for ANOVA were not satisfied, the non-parametric Kruskal–Wallis test was used.

The study was conducted according to the principles of the Ethical Committee for the Protection of Animals in Research of the University of South Bohemia, Research Institute of Fish Culture and Hydrobiology Vodňany (based on the EU-harmonized animal welfare act of Czech Republic).

## Results and Discussion

During the course of metribuzin exposure all experimental carp experienced increased respiration and loss of movement and coordination. Fish lying on the bottom of the tank and moving in circles was a common observation, followed by a short excitation stage (convulsions). All fish survived the experimental exposure. Similar the behavioral symptoms were observed in *Carassius auratus* (Saglio and Trijasse 1998) following acute exposure with atrazine (5 μg/L).

Necropsy revealed increased watery mucus on body surfaces, black pigmentation of the skin, and abdominal distention with generalized edema. The body cavity contained transudate, and there was hyperemia of visceral organs and ascites. Svobodova and Pecena (1988) and Velisek et al. (2008) observed transudate in the body cavity of rainbow trout after acute exposure to triazine pesticide.

The results of hematological indices in experimental and control carp are given in Table 1. Exposure of carp to metribuzin at 175.1 mg/L caused a significant ($p < 0.01$) decrease in values of PCV, Hb, MCV, Bc, WBC and a significantly ($p < 0.01$) increased monocyte count, band, and segmented neutrophil granulocytes, developmental forms myeloid sequence, basophils. The remaining hematological indices, RBC, MCH, and MCHC, showed no significant differences between experimental and control groups.

The reduction in PCV and Hb of common carp in the present study can be interpreted as a compensatory response that reduces the oxygen carrying capacity to maintain gas transfer and indicates a change in the water–blood barrier for gas exchange in the gill lamellae (Jee et al. 2005). The hematological results indicated decreased nonspecific immunity. Similar changes in the hematocrit value, lymphocytes, monocyte count, and band and segmented neutrophil granulocytes were observed in carp following acute poisoning with atrazine (Svobodova and Pecena 1988).

The biochemical indices in experimental and control carp are given in Table 2. Exposure of carp to metribuzin at 175.1 mg/L caused a significant ($p < 0.01$) decrease in levels of TP, ALB, GLOB, TAG, LDH, LACT, PHOS but a significant ($p < 0.01$) increase in GLU, $NH_3$, and $Ca^{2+}$. The remaining biochemical indices, AST, ALT, ALP, and CK were not significantly different for experimental and control groups. Increase in blood $NH_3$ level indicated an increase in protein catabolism and/or disturbances in $NH_3$ removal. The increased blood GLU concentration demonstrated the response of exposed fish to metabolic stress. The decreased LDH level indicated metabolic changes, i.e., glycogen catabolism and glucose shift towards the formation of LACT in stressed fish, primarily in the muscle. These findings are in accord with those of Mekkawy et al. (1996), who observed a significant increase in GLU and significantly decreased TP levels in *Oreochromis niloticus* and *Chrysichthyes auratus* after acute exposure to 3 mg/L atrazine. Davies et al. (1994) observed a decrease in TP in rainbow trout after acute exposure to 50 µg/L atrazine. Prasad and Reddy (1994) observed increased serum $Ca^{2+}$ in *Tilapia mossambica* after exposure to atrazine.

After the acute toxicity test to metribuzin we performed the autopsy and observed transudate in body cavity. We believe the transudate in the body cavity resulted from escape of proteins due to damage to renal tubular epithelial cells. As a result, plasma showed marked hypoproteinemia, from 42.13 ± 4.53 g/L to 34.07 ± 5.05 g/L.

**Table 2** Derived biochemical indices of blood plasma in 2-year-old common carp after acute exposure to 175.1 mg/L metribuzin (250.2 mg/L Sencor 70 WG)

| Indices | Units | Control group | Experimental group |
|---|---|---|---|
| GLU | mmol/L | 4.87 ± 0.79 | 23.99 ± 5.59* |
| TP | g/L | 42.13 ± 4.53 | 34.07 ± 5.05* |
| ALB | g/L | 9.27 ± 2.43 | 7.01 ± 2.03* |
| GLOB | g/L | 32.67 ± 2.41 | 27.20 ± 3.82* |
| TAG | mmol/L | 0.52 ± 0.08 | 0.28 ± 0.15* |
| $NH_3$ | µmol/L | 292.20 ± 109.19 | 462.33 ± 85.55* |
| LDH | µkat/L | 6.14 ± 1.20 | 4.37 ± 1.38* |
| AST | µkat/L | 2.11 ± 1.02 | 1.51 ± 0.51 |
| ALT | µkat/L | 0.47 ± 0.13 | 0.40 ± 0.09 |
| ALP | µkat/L | 0.20 ± 0.05 | 0.22 ± 0.06 |
| CK | µkat/L | 14.35 ± 0.72 | 13.94 ± 0.58 |
| LACT | mmol/L | 0.97 ± 0.18 | 0.71 ± 0.18* |
| $Ca^{2+}$ | mmol/L | 2.26 ± 0.04 | 2.40 ± 0.15* |
| PHOS | mmol/L | 1.94 ± 0.33 | 1.01 ± 0.24* |

All values are mean ± SD, n = 15

* Statistically significant $p < 0.01$

**Table 1** Derived hematological parameters in 2-year-old common carp after acute exposure to 175.1 mg/L metribuzin (250.2 mg/L Sencor 70 WG)

| Indices | Units | Control group | Experimental group |
|---|---|---|---|
| RBC | T/L | 1.78 ± 0.36 | 1.64 ± 0.31 |
| Hb | g/L | 84.29 ± 14.73 | 70.81 ± 18.36* |
| PCV | l/L | 0.33 ± 0.04 | 0.27 ± 0.03* |
| MCV | fl | 189.52 ± 30.43 | 165.63 ± 25.76* |
| MCH | pg | 48.34 ± 8.81 | 43.92 ± 12.58 |
| MCHC | g/L | 255.94 ± 27.17 | 269.34 ± 67.12 |
| Bc | l/L | 0.028 ± 0.007 | 0.014 ± 0.005* |
| WBC | g/L | 104.01 ± 33.85 | 33.60 ± 18.20* |
| Lymphocytes | g/L | 97.70 ± 35.28 | 18.10 ± 11.79* |
| Monocytes | g/L | 1.66 ± 1.54 | 4.88 ± 4.36* |
| Neutrophile granulocytes segments | g/L | 0.45 ± 0.21 | 0.99 ± 0.31* |
| Neutrophile granulocytes bands | g/L | 2.56 ± 1.36 | 5.29 ± 2.87* |
| Developmental forms myeloid sequence | g/L | 0.01 ± 0.02 | 3.29 ± 5.08* |
| Basophils | g/L | 0.26 ± 0.53 | 2.08 ± 2.31* |

All values are mean ± SD, n = 15

* Statistically significant $p < 0.01$

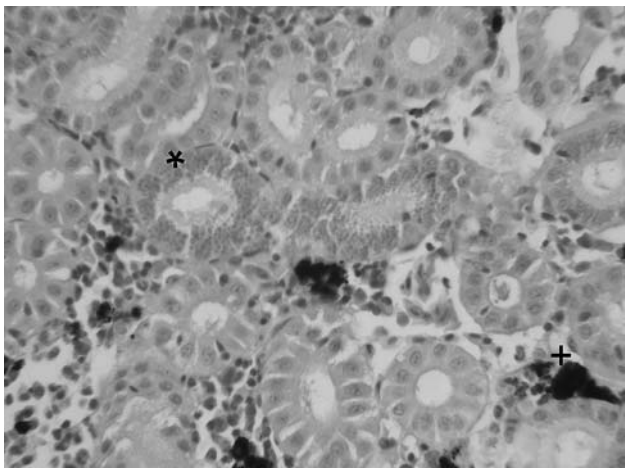

**Fig. 1** Section of caudal kidney. Hyaline degeneration of the tubular epithelial cells (*asterisk*). There is also intertubular hematopoietic tissue and melanomacrophages (*cross*). Hematoxylin and eosin ×400

We observed peritubular congestion of blood (teleangiectasiae) and the hyaline degeneration of the epithelial cells of renal tubules in the caudal kidney (Fig. 1). No histopathological changes were demonstrated in other tissues (skin, gill, spleen, liver, cranial kidney). Meyer and Hendricks (1985) observed gross morphological anomalies in the gill epithelium of yearling *Oncorhynchus kisutch* exposed to the herbicide atrazine (15 μg/L for 114 h), including necrosis, desquamation, hypertrophy and hyperplasia, and teleangiectasiae. Biagianti-Risbourg and Bastide (1995) reported that atrazine affects tissues in fish, particularly the liver, which shows a substantial increase in lipid inclusions followed by lipoid degeneration, as well as enlargement of the secondary lysosomes, mitochondrial malformation and vacuolization, and a reduction in glycogen content. Neskovic et al. (1993) reported hyperplasia of gill epithelial cells in *Cyprinus carpio* L. exposed to atrazine in concentration 1,500 μg/L.

Studies of toxicity of triazine pesticide indicate that it may induce morphological, biochemical, and physiological alterations in fish (Saglio and Trijasse 1998; Reddy et al. 1992; Davies et al.1994).

Acute exposure of 2-year-old common carp to 175.1 mg/L of metribuzin caused significant shifts in several hematological, biochemical and histopathological variables.

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