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Effect of prochloraz fungicide on neopterin and biopterin concentrations in blood plasma of common carp

Petr Maršálek, Ivana Mikuliková, Helena Modrá, Zdeňka Svobodová

University of Veterinary and Pharmaceutical Sciences Brno, Faculty of Veterinary Hygiene and Ecology, Department of Veterinary Public Health, Animal Protection and Welfare, Brno, Czech Republic

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Abstract

Neopterin and biopterin are often used as markers of cell mediated immunity. The aim of this study was to evaluate the effect of prochloraz on neopterin and biopterin concentrations in blood plasma of common carp as possible indicators for monitoring perturbations of the immune system caused by xenobiotics like pesticides. Prochloraz is a widely used imidazole fungicide in horticulture and agriculture. A total of 60 juvenile common carp were divided into four groups of 15 fish and exposed to prochloraz at concentrations of 0, 50, 150 and 380 $\mu g \cdot l^{-1}$, respectively. The total length of exposure was 28 days. Neopterin and biopterin concentrations were determined by reverse phase high performance liquid chromatography with fluorescence detection. Our results indicated that concentrations of neopterin (25 ± 7.6 $nmol \cdot l^{-1}$) and biopterin (190 ± 29 $nmol \cdot l^{-1}$) in plasma of untreated common carp were comparable with those in mammals. Neopterin concentrations significantly ($P < 0.01$) increased after exposure to prochloraz in comparison to non-exposed fish, while biopterin concentrations were not influenced by exposure to prochloraz. This study contains new data on neopterin and biopterin concentrations in fish plasma and investigates neopterin and biopterin in their non-traditional role as markers of cell mediated immunity of fish associated with pesticide exposure.

Fish, Cyprinus carpio, imidazole, pterines

Neopterin and biopterin belong to a group of unconjugated pterins, derived from guanosine triphosphate (GTP) by guanosine triphosphate cyclohydrolase I and is synthesized mainly by activated monocytes/macrophages following stimulation by interferon-gamma cytokine (IFN-γ), which is released by NK cells and T-lymphocytes (Hoffmann et al. 2003). Because the neopterin concentration in body fluid reflects immune responses *in vivo*, neopterin is a useful biomarker of the activation of the cellular immune system (Murr et al. 2002; Hoffmann et al. 2003). Biopterin is produced by non-enzymatic oxidation of tetrahydrobiopterin. Synthesis of biopterin also takes place in T-cells, B-cells, the endothelium, smooth muscle cells and fibroblasts (Werner-Felmayer et al. 2002) and probably in the liver and kidney (Fujioka et al. 2008). The interest of researchers has focused mainly on the use of pterins in human medicine. Some authors used neopterin and biopterin in veterinary medicine; however, the concentrations in fish have not been investigated yet. A number of studies have found that environmental pollutants such as pesticides significantly affect fish immune systems (Monserrat et al. 2007; Dobsikova et al. 2011). Prochloraz (N-Propyl-N-(2,4,6-trichlorophenoxy)ethyl-imidazole-1-carboxamide) is an imidazole fungicide widely used in horticulture and agriculture. It has multiple endocrine activities (Kinnberg et al. 2007) and is also able to affect xenobiotic metabolizing enzymes, selected plasma indices and red blood cell count, and antioxidant activities, as well as to cause minor histological impairment of common carp (Haluzova et al. 2010) and to affect ovarian oestradiol production in fish (Hinfray

Address for correspondence:
Petr Maršálek
Department of Veterinary Public Health, Animal Protection and Welfare
Faculty of Veterinary Hygiene and Ecology
University of Veterinary and Pharmaceutical Sciences Brno
Palackého tř. 1/3, 612 42, Brno, Czech Republic

Phone: +420 541 562 792
E-mail: marsalekp@vfu.cz
http://actavet.vfu.cz/

et al. 2006). However, its ability to affect the immune system was previously unsuspected in fish or any other vertebrates.

The aim of this study was to establish concentrations of neopterin and biopterin in blood plasma of common carp (*Cyprinus carpio*) and to investigate the effect of subchronic exposure to prochloraz on blood plasma concentrations of neopterin and biopterin in common carp.

Materials and Methods

Experimental design

A total of 60 common carp (218.4 ± 64.5 g; 19.7 ± 2.0 cm) were obtained from a commercial fish farm. After a 2-week period of acclimation to laboratory conditions (water temperature 19−22 °C, photoperiod 12/12), the fish were exposed to Spartakus (prochloraz 450 $g \cdot l^{-1}$; BASF SE, Germany) for 28 days. Fish were randomly distributed into four 200 l glass aquaria, 15 fish per each. The aquaria were conducted in a flow-through system; the volume of test solutions containing prochloraz was exchanged twice a day. The pH of the solutions varied from 7.1 to 7.9, oxygen saturation was ≥ 60%. The fish were supplied with commercial feed twice a day at a total rate of 1.5% body weight. The 96 h LC_{50} of prochloraz was determined to be 2.2 $mg \cdot l^{-1}$ for bluegill sunfish (Gangolli 1999). Thus, proportional dosages of 1/45 (group 2), 1/15 (group 3) and 1/5 (group 4) of this value were chosen for the test. The control group (group 1) was subjected to dechlorinated tap water. After the exposure period (28 days), individual blood samples were taken from all fish by cardiac puncture, stabilized with an aqueous solution of heparin (50 IU per ml of blood), immediately centrifuged at 800 × *g* for 10 min at 4 °C, and stored at -85 °C. The experiment was approved by the Ethics Committee of the University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic, project no. 16/2009.

Determination of prochloraz

The concentrations of prochloraz in the glass aquaria were measured by gas chromatography with ion trap mass spectrometry (GC/MS) five times during the experiment. Sample preparation was based on simple liquid-liquid extraction into hexane. The separation, identification and quantification of prochloraz were based on the GC/MS method described by Charlton and Jones (2007). A Varian 450-GC gas chromatograph with a Varian 220-MS ion trap mass spectrometer and VF-5ms (30 m × 0.25 mm) column (Varian, Inc., Palo Alto, USA) were used for the separation of prochloraz. A certified standard of prochloraz was purchased from Dr. Ehrenstorfer GmbH (Germany). All solvents were GC/MS-grade purity (Chromservis, s.r.o., CZ). The detection limit (3σ) of prochloraz was 0.1 $\mu g \cdot l^{-1}$. The coefficient of variation was 3.5%. Prochloraz concentrations exceeded 80% of the nominal concentrations over the test period. Actual concentrations were 0.05 $mg \cdot l^{-1}$ (group 2), 0.15 $mg \cdot l^{-1}$ (group 3) and 0.38 $mg \cdot l^{-1}$ (group 4). These are further used to refer to the test groups.

Determination of neopterin and biopterin

The measurement of neopterin and biopterin was based on high performance liquid chromatography with fluorometric detection (Carru et al. 2004). All samples of blood plasma and standard pterin solutions were protected against light when handled using dark glass. For neopterin and biopterin analysis, 300 µl of trichloroacetic acid (5%) were added to 300 µl of standard or plasma. The samples were centrifuged at 800 × *g* for 10 min at 20°C. The supernatant was filtered through a 0.45 µm nylon filter (Millipore, Billerica, USA) and used for analysis. Elution was performed on a 150 × 4.6 mm, 5 µµm Zorbax Eclipse XBD-C18 column (Agilent Technologies, Santa Clara, USA). Isocratic elution was performed at a flow rate of 1 ml/min with water/acetonitrile 96/4 (v/v) at 35 °C. Fluorescence detection at 353 nm and 438 nm for excitation and emission, respectively, was used to selectively detect pterins. The chromatographic analysis was accomplished by means of an Alliance 2695 chromatographic system (Waters, Milford, USA) with an FD 2475 fluorescent detector (Waters, Milford, USA). Neopterin, biopterin and trichloroacetic acid were purchased from Sigma-Aldrich (St. Louis, USA). All solvents were HPLC-grade purity (Chromservis, s.r.o., Czech Republic). The detection limits for neopterin and biopterin were 0.23 $ng \cdot ml^{-1}$ and 0.41 $ng \cdot ml^{-1}$, respectively. The limits of quantification for neopterin and biopterin were 0.75 $ng \cdot ml^{-1}$ and 1.35 $ng \cdot ml^{-1}$, respectively. The coefficient of variation was 3.8%.

Statistical analysis

Statistical analysis was performed using Statistica 8.0 for Windows (StatSoft, Inc., Tulsa, USA). A normality check of all the data sets of results obtained for the properties investigated was performed with Kolmogorov-Smirnov test. One-way analysis of variance (ANOVA) was used to evaluate the results for neopterin and biopterin. When significant differences were found ($P < 0.05$), conservative Tukey's test was conducted as a *post hoc* test to determine differences between individual groups.

Results

Neopterin concentrations in plasma of carp are shown in Fig. 1. Analysis of variance revealed a treatment effect ($P < 0.001$). When using *post hoc* Tukey's test, concentrations

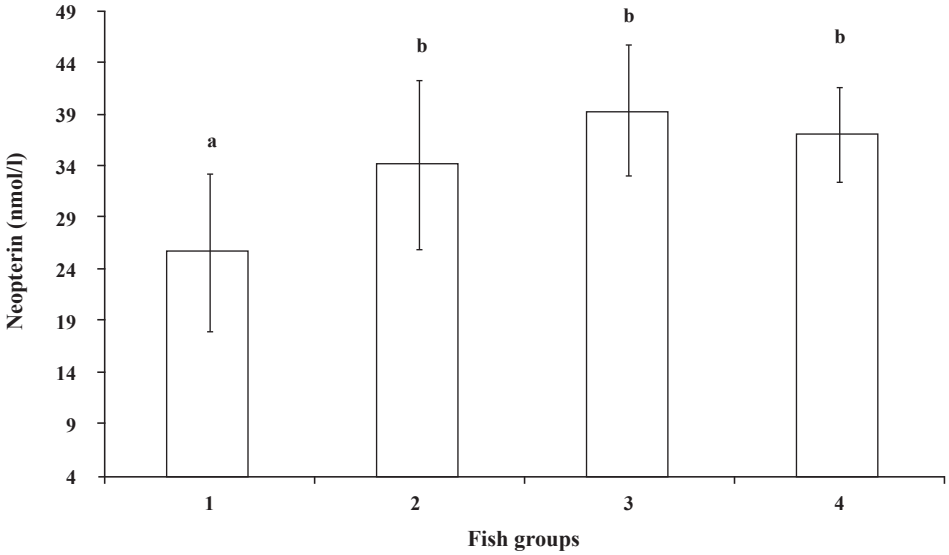

Fig. 1. Concentrations of neopterin in blood plasma from common carp after prochloraz treatment.

Data are expressed as mean ± SD. Fish groups: 1- concentration of prochloraz in test aquariums 0 mg·l^{-1}, 2–0.05 mg·l^{-1}, 3–0.15 mg·l^{-1}, 4–0.38 mg·l^{-1} (n = 15). ^{a,b} Significant differences ($P < 0.01$) are indicated by different superscripts.

of neopterin were higher in group 2 ($P < 0.01$) and groups 3 and 4 ($P < 0.001$) compared to the control group. Concentration of biopterin in plasma of fish (190 ± 29 nmol·l^{-1}) was not influenced by the treatment ($P > 0.05$).

Discussion

The pteridine pathway in fish has been studied in relation to the differentiation of neural crest precursors into neural elements, ectomesenchymal cells, and pigment cells (Ziegler 2003). The role of pteridine biosynthesis in the cell-mediated immunity of fish has not been investigated and current literature does not contain any data on neopterin and biopterin concentrations in fish plasma or serum. However, neopterin and biopterin concentrations in blood plasma of common carp found in our experiment are in agreement with the values of neopterin and biopterin concentrations in mammal serum or plasma (Stang and Koller 1998; Fujioka et al. 2008; Smutna et al. 2010).

The results of our study suggest that subchronic exposure to prochloraz increases plasma neopterin concentrations. Only studies in humans have investigated the influence of xenobiotics like pesticides on blood neopterin concentrations. Stiller-Winkler et al. (1999) reported that the exposure of humans to a mixture of pesticides such as organophosphates, carbamates, phenoxy herbicides and pyrethroids resulted in a significant increase in serum neopterin in comparison to a non-exposed group. Blood concentrations of pentachlorophenol also correlated positively with plasma concentrations of neopterin (Daniel et al. 2001a). In contrast, serum neopterin gave no indication of significant changes after pesticide control operation using pyrethroids (Hadnagy et al. 2003) and plasma neopterin showed no associations with blood concentrations of

polychlorinated biphenyls, hexachlorocyclohexane isomers and hexachlorobenzene (Daniel et al. 2001b).

Cytokine production is considered as a sensitive indicator for monitoring perturbations of the immune system caused by xenobiotics and could be a useful biomarker for environmental pollutant exposure (Hooghe et al. 2000; Jeon et al. 2001; Eder et al. 2004; Lee et al. 2010). Although published data show that IFN-γ (type 1 cytokine) is not necessary for neopterin production (Sghiri et al. 2005), it is the most potent inducer of neopterin production. Recent studies have shown that pesticide exposure could influence production of IFN- γ. After the exposure of common carp to various concentrations of atrazine, chlorpyrifos, and their mixture, Wang et al. (2011) identified different trends relating to the mRNA expression levels of IFN-γ. In comparison with cytokines, neopterin is biochemically inert and its half-life in the organism is only due to renal excretion. Thus, neopterin is a potentially superior marker of immune system perturbation. Biopterin is also released by monocytes/macrophages activated by cytokines. However, the origin of most plasma biopterin is thought to be in the liver, adrenals, or sympathetic nerves (Hashimoto et al. 2004) and despite the fact that biopterin could be used as a marker of immune system activation (Smutna et al. 2010), it is not used as often as neopterin.

In conclusion, the results show that concentrations of neopterin and biopterin in plasma of common carp are comparable with those in mammals. Prochloraz exposure influenced neopterin plasma concentration. Thus, neopterin is a possible marker of pesticide exposure. However, more research involving other xenobiotics and measurements of pro-inflammatory cytokines is needed to fully characterize the effect of xenobiotics on neopterin and biopterin concentrations in fish.

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