Comparative Biochemistry and Physiology, Part C 157 (2013) 259–265

Contents lists available at SciVerse ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Goldfish can recover after short-term exposure to 2,4-dichlorophenoxyacetate: Use of blood parameters as vital biomarkers

Olga I. Kubrak [a], Tetiana M. Atamaniuk [a], Kenneth B. Storey [b], Volodymyr I. Lushchak [a,*]

[a] Department of Biochemistry and Biotechnology, Precarpathian National University named after Vassyl Stefanyk, 57 Shevchenko Str., Ivano-Frankivsk, 76025, Ukraine
[b] Institute of Biochemistry, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario, Canada K1S 5B6

ARTICLE INFO

Article history:
Received 14 July 2012
Received in revised form 27 December 2012
Accepted 27 December 2012
Available online 4 January 2013

Keywords:
Biomarkers
Blood parameters
2,4-Dichlorophenoxyacetic acid (2,4-D)
Goldfish
Recovery

ABSTRACT

This study investigated the effects of 2,4-dichlorophenoxyacetic acid (2,4-D), a widely used herbicide, on the metabolism of goldfish, *Carassius auratus*, using only vital (non-lethal) approaches. After 96 h exposure to 1, 10 or 100 mg/L of 2,4-D selected hematological (total hemoglobin and hematocrit) and biochemical (glucose content, aspartate transaminase and acetylcholinesterase activities) parameters were unchanged in blood of exposed fish. At 100 mg/L of 2,4-D lymphocyte numbers decreased by 8%, whereas promyelocyte and metamyelocyte numbers increased by 7- and 2-fold, respectively. Exposure to 100 mg/L of 2,4-D also elevated carbonyl protein levels (by 2-fold), triglyceride content (by 43%) and alanine transaminase activity (by 46%) in goldfish plasma. All of these hematological and biochemical parameters reverted to control values after a 96 h recovery period. These data indicate that 2,4-D has toxicological effects on goldfish that can be monitored with multiple diagnostic tests using non-lethal blood testing.

1. Introduction

Pesticides are used world wide in agriculture (Sancho et al., 2000; Oruç et al., 2004; de Menezes et al., 2011). Considerable amounts enter runoff to become major pollutants in aquatic ecosystems causing disturbances of the delicate balance of aquatic ecosystems and affecting the health status of non-target aquatic organisms, such as fish (Oruç and Üner, 1999; Bretaudt et al., 2000; de Menezes et al., 2011).

Fish are highly vulnerable to the presence of contaminants and are considered to be important indicators of environmental pollution (Prusty et al., 2011). The response of fish to pollutants can be studied through assessment of hematological, biochemical and physiological parameters (Gimeno et al., 1995; Sancho et al., 2000). Hematological analysis and biochemical parameters of blood are particularly useful for monitoring health of aquatic organisms (Pimpão et al., 2007; Li et al., 2011a). Blood often exhibits pathological changes before fish exhibit any external symptoms of toxicity and blood can frequently be sampled without causing substantial disturbances or death to the animal. Differential blood cell counts and plasma enzymes are frequently used as effective indicators of environmental stress and provide a general overview of the integrity of the immune system (Cole et al., 2001) and the state of the internal environment of the organism (Li et al., 2011a). Many studies have documented changes in blood parameters resulting from exposures to certain environmental conditions and/or the presence of contaminants (Borges et al., 2007; Vasylkiv et al., 2010, 2011; Li et al., 2010, 2011b). Hematological variables such as hemoglobin (Hb), hematocrit (Ht), red blood cell (RBC) count, white blood cell (WBC) count and others together with biochemical parameters like plasma glucose and protein are widely used to assess stress induced by environmental pollutants (Saravanan et al., 2011). Variations in types, numbers, and appearance of leucocytes are the most important factors limiting accurate hematological analysis in different fish species (Barton and Iwama, 1991; Kreutz et al., 2011). Furthermore, the evaluation of hematological and biochemical characteristics in fish blood has become an important means of understanding possible mechanisms of toxicological impacts (Borges et al., 2007; Sudova et al., 2009). The activities of serum/plasma enzymes have also been used as sensitive indicators of stress in fish exposed to diverse water pollutants (Kavitha et al., 2010). Among these, transaminases such as alanine transaminase (ALT, EC 2.6.1.2) and aspartate transaminase (AST, EC 2.6.1.1), as well as lactate dehydrogenase (LDH, EC 1.1.1.27) are commonly used as indicators of tissue damage and cell rupture (Saravanan et al., 2011). Thus, leucocyte profiles and selected biochemical indices of fish blood can be valuable parameters for non-lethal diagnostics of fish intoxication with pollutants, particularly pesticides (Svoboda et al., 2001; Svobodová et al., 2003; Vasylkiv et al., 2010; Li et al., 2011b).

Among the different pesticides that contaminate aquatic ecosystems, the phenoxyacetic herbicides attract special attention because they constitute one of the largest groups of herbicides used around the world. In particular, 2,4-dichlorophenoxyacetic acid (2,4-D) has been the most widely used pesticide for over 60 years and is present in more than 1500 formulations of 2,4-D-based products currently on the market (Tayeb et al., 2011). 2,4-D is a selective systemic herbicide (Tayeb et al., 2011) with an auxin-like capacity to alter normal

* Corresponding author. Tel./fax: +380 342 59 61 71.
E-mail address: lushchak@pu.if.ua (V.I. Lushchak).

1532-0456/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cbpc.2012.12.005

protein synthesis and cell division in plant meristems and leaves (Stevens and Breckenridge, 2001). The toxicity of 2,4-D to non-target organisms has been a topic of extensive research and it is known that 2,4-D disturbs metabolism in rat hepatocytes (Palmeira et al., 1995) and exhibits immunosuppressive (Pistl et al., 2003), neurotoxic (Bortolozzi et al., 2004), genotoxic (Abul Farah et al., 2003) and hepatotoxic effects (Tayeb et al., 2010).

However, to our knowledge, information concerning 2,4-D toxicity in fish is scarce. The present study evaluates toxicological impacts of this herbicide on goldfish, *Carassius auratus*, and does so using only vital diagnostic data obtained from blood sampling in order to determine if blood analysis could provide a potential diagnostic of 2,4-D intoxication. Furthermore, taking into account the fact that various fish species have exhibited a potential to recover after chemical exposure (de Menezes et al., 2011), depending on pesticide type, time of exposure and fish species (Crestani et al., 2006, 2007), we also aimed to assess the capability of a known stress-resistant freshwater species, the goldfish, to recover from metabolic disturbances after short term exposure to 2,4-D in sublethal concentrations.

2. Materials and methods

2.1. Reagents

Ethylenediaminetetraacetic acid (EDTA), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), KH_2PO_4, Tris(hydroxymethylaminomethane), β-nicotinamide adenine dinucleotide reduced (NADH) and lactic acid were purchased from Sigma-Aldrich Corporation (USA). Acetylcholine iodide was from Roth (Germany) and 2,4-dichlorophenoxyacetic acid (2,4-D) of greater than 98% purity was obtained from Shanghai Synnad Fine Chemical Co., Ltd. (China). All other reagents were obtained from local suppliers (Ukraine) and were of analytical grade.

2.2. Animals and experimental conditions

Goldfish (*C. auratus* L.) with body masses of 35–45 g were obtained from a local fish farm (Rohatyn district, Ivano Frankivsk region) in October 2011. Fish were acclimated to laboratory conditions for 3 weeks in a 1000 L tank under natural photoperiod in aerated and dechlorinated tap water. Water parameters were 19–20 °C, 8.5–9.2 mg/L O_2, pH 7.4–7.5 and hardness (determined as Ca^{2+} concentration) 42–44 mg/L. Fish were fed *ad libitum* once a day with commercial Cyprinid CarpCo Excellent (Koi Growner, The Netherlands) pellets, containing 36% protein, 7% fat, 3.6% cellulose, 8.7% ash, 1% phosphorus and vitamins C, E, D_3 and A. Fish were fed during the acclimation period (3 weeks), but were fasted for 1 day prior and during experimentation.

Experiments were carried out in 120 L glass aquaria (containing 100 L of water) in a static mode under the same conditions with the addition of 2,4-dichlorophenoxyacetic acid (2,4-D) (free acid formulation from Shanghai Synnad Fine Chemical Co., Ltd., China) in concentrations of 1, 10 and 100 mg/L. To reverse aquarium water acidification by 2,4-D, the pH values in all aquaria with 2,4-D were adjusted with NaOH to the same value as the pH in the control aquarium before fish was introduced. Groups of 6 fish were placed in aquaria with the above mentioned nominal concentrations of 2,4-D and exposed to these conditions for 96 h (no mortality occurred during exposure in any group). Fish in the control group were maintained in the same manner, but without addition of 2,4-D to the water. Fish in an additional aquarium were exposed for 96 h to the highest concentration (100 mg/L) of 2,4-D and after that were transferred to herbicide-free water for an additional 96 h to recover. Experimental 2,4-D exposure levels were selected to represent an environmentally permissible concentration (1 mg/L), a moderately toxic concentration (10 mg/L) (reviewed in Borges et al., 2004) and a toxic concentration (100 mg/L) as determined from LD_{50}^{96} values for goldfish (187 mg/L) (Birge et al., 1979). Experiments were carried in two independent runs with six fish placed in each aquarium, but total number of repeats for each parameters consisted of 6 (6 animals per a group, if not stated otherwise). Aquarium water was not changed over the 96 h course in order to avoid stressing the animals. Levels of dissolved oxygen, temperature and pH were monitored every 24 h. After fish exposure, blood was quickly taken from caudal vessels using 50 mM EDTA as an anticoagulant. Fish were sacrificed after blood sampling. All experiments were conducted in strict accordance with the institutional animal ethics guidelines of Precarpathian National University and were approved by the Animal Experimental Committee of Precarpathian National University.

2.3. Evaluation of hematological parameters and leucocyte formula

2.3.1. Estimation of total hemoglobin concentration and hematocrit value

Total hemoglobin concentration was determined after erythrocyte hemolysis in Drabkin's solution using a commercial kit (Genesis Co, Ltd., Ukraine) following the manufacturer's instructions.

Hematocrit was determined following the procedure of Ptashynski et al. (2002). Immediately after blood sampling, a small amount of whole blood was transferred into microcapillary tubes, which were then sealed at both sides and centrifuged (2000 *g*, 20 min, 4 °C) using an OPN-8 centrifuge (USSR). Hematocrit values were calculated as the percentage of red blood cell pellet in the total blood column.

2.3.2. Examination of leucocyte content

For microscopic examination of leucocyte content, small drops of whole blood were directly smeared on slides ($n=2$ per fish) and air-dried. Smears were fixed and stained with azure-eosine water solution as described previously (Vasylkiv et al., 2010). Cytological analysis was conducted by scoring at a 1600× magnification using a Leitz microscope (Leitz Wetzlar GmbH, Germany). Different types of leucocytes were identified according to the Fish Blood Cell Atlas (Ivanova, 1983). A total of 200 leucocyte cells were counted per smear and assigned to different leucocyte categories. Data are shown as numbers of different leucocytes among 200 counted.

2.4. Plasma biochemical parameters

2.4.1. Protein carbonyls in plasma

Blood samples were centrifuged (1500 *g*, 20 min, 4 °C) using a CV 1500 centrifuge (Lithuania) and then plasma was removed. Aliquots (100 μL) of plasma were mixed 1:1 v:v with 40% w:v trichloroacetic acid and then centrifuged (5000 *g*, 5 min, 4 °C). Carbonyl protein (CP) levels were measured in the resulting pellets by reaction with 10 mM 2,4-dinitrophenylhydrazine (DNPH), leading to the formation of dinitrophenylhydrazones. The amount of CP was evaluated spectrophotometrically at 370 nm using a molar extinction coefficient of 22×10^3 M^{-1} cm^{-1} (Lenz et al., 1989). Data are expressed as nanomoles CP per milligram protein (nmol/mg protein).

2.4.2. Total protein concentration in plasma

Protein concentration was measured by the Coomassie brilliant blue G250 method (Bradford, 1976) using bovine serum albumin as a standard. To fit within the calibration standard curve plasma samples were diluted with 0.9% NaCl before use. Data are expressed as milligram of total protein per milliliter of plasma (mg/mL).

2.4.3. Triglyceride content in plasma

Aliquots of plasma were mixed 1:1 v:v with phosphate-buffered saline buffer containing 0.05% Triton X100 and incubated in a boiling water bath for 15 min in tightly covered centrifuged tubes. After centrifugation (16,000 *g*, 15 min, 21 °C) deproteinized plasma samples were collected and triglyceride (TAG) contents were determined using the Liquick Cor-TG (Cormay, Poland) commercial kit. Data are expressed as microgram TAG per milliliter of plasma (μg/mL).