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Integrated biomarkers response confirm the antioxidant role of diphenyl diselenide against atrazine

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ABSTRACT

Atrazine (ATZ) is a herbicide worldwide used. That can cause oxidative damage in non-target organisms, such as fish. Furthermore, the threat of exposure to pesticides together with poor nutrition is hazardous to the normal development of fish, and supplementation of the fish diet with antioxidants compounds is an alternative approach to prevent the hazardous effects of pesticide exposure. Here we aimed to investigate the capacity of diphenyl diselenide $(PhSe)_2$ diet supplementation to improve the antioxidant defense of *Cyprinus carpio* (carp) exposed to environmental concentrations of ATZ. To prove the efficiency of $(PhSe)_2$, we used the Integrated Biomarkers Response (IBR) methodology. Therefore, carp were fed for 8 weeks diets either with or without $(PhSe)_2$ and exposed to 2 or 10 μg/L of ATZ for 96 h, euthanized, and their liver, gills, and muscle tissues were removed for biochemical assays. ATZ was able to cause oxidative damage from reactive species production in all tissues of carp, as observed by the increase of lipid peroxidation and protein damage. The activity of some antioxidant enzymes was inhibited in carp exposed to ATZ. However, $(PhSe)_2$ supplementation was able to prevent this ATZ-induced damage by improving the activities of antioxidant enzymes and through antioxidant competence of $(PhSe)_2$ *per se*. Furthermore, IBR was shown to be a useful tool to compare treatments, even at different concentrations, and identify the efficiently antioxidant behavior of the organoselenium compound

1. Introduction

Atrazine (ATZ; CAS Number 1912-24-9) is a selective herbicide (an inhibitor of photosynthesis) used worldwide in sugarcane crops, corn, and sorghum, as well as for some fruits and nuts. ATZ has a relative persistence in surface waters, possibly found in groundwater, thus increasing the risk to the aquatic environment (USEPA, 2007; Solomon et al., 2008). Recently, in Brazil, ATZ concentrations between 0.09 and 5.4 μg/L were registered in surface freshwater (Loro et al., 2015; Vieira et al., 2016). This pollutant might affect the abundance and diversity in aquatic ecosystems, promoting an imbalance in the food web by affecting the autotrophic organisms and consequently consumers (Graymore et al., 2001). Although ATZ acts selectively in plants, is found in the literature records of damage in fish. The potential teratogenic, genotoxic, and oxidative stress were reported in zebrafish embryos (*Danio rerio*) exposed to a mixture of arsenic and ATZ (Adeyemi et al., 2015). In liver of common carp (*Cyprinus carpio*) exposed to atrazine, chlorpyrifos alone or both in combination, were observed alterations in activity and transcription of glutathione S-transferase (GST), increase in ethoxyresorufin-*O*-deethylase (EROD) and pentoxyresorufin-*O*-deethylase (PROD) activity, rise in cytochrome P450 (CYP) content and accumulation of both agrochemicals (Xing et al., 2012, 2014). Common carp embryos exposed to environmental concentrations of ATZ (0.3 μg/L) showed alterations in antioxidant enzymes, indicating toxic effects even at low concentrations (Chromcová et al., 2013).

Besides the threat of exposure to pesticides, poor nutrition is hazardous to the normal development of fish and maintenance of aquatic biota. The adequate presence of micronutrients (*e.g.,* selenium) in the diet is essential for normal growth and health. In common carp,

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selenium intake improves the antioxidant system, protecting against chemical stressors when supplemented. On the other hand, carps fed a deficient diet showed pathologies, including hepatocytes degeneration and necrosis, lordosis, muscle atrophy, and oxidative stress (Elia et al., 2011; Wang et al., 2013; Ashouri et al., 2015). In this species, a diet supplemented for 60 days with 3 mg/kg of diphenyl diselenide $(PhSe)_2$, an organic compound of selenium, protected against oxidative damage induced by exposure to quinclorac for 192 h (Menezes et al., 2012). Similarly, silver catfish (*Rhamdia* sp.) fed a $(PhSe)_2$ supplemented diet were increased antioxidant defenses, protecting from clomazone exposure (Menezes et al., 2013). In mice, diet supplementation with $(PhSe)_2$ protects against the toxic effects induced by mercury chloride subcutaneous injection, increasing their antioxidant profile (Fiuza et al., 2015). Similarly, *Drosophila melanogaster* fed a $(PhSe)_2$ supplemented diet were protected against oxidative stress and mortality induced by chlorpyrifos (Adedara et al., 2015).

Fishes are frequently used as a bioindicator of environmental health. Carps have been effectively used in laboratory and field studies to assess the toxic effects of exposition to several types of pesticides (Clasen et al., 2014; Menezes et al., 2014a; Murussi et al., 2015a). This animal-model is also used in investigations of potential diet antioxidants (Menezes et al., 2012, 2013, 2014b), with the application of several biomarkers of oxidative damage. However, the use of multiple biomarkers could confuse the interpretation of the results. In this way, the Integrated Biomarker Response (IBR) is a useful tool for assessing the biological effects and health status in organisms submitted to several exposures, allow a comparison of the effects of different treatments (Zheng et al., 2013; Murussi et al., 2015b; Vieira et al., 2016; Hou et al., 2016).

Considering the harmful effects of ATZ and the antioxidant potential of $(PhSe)_2$ for fish species, Hence an attempt has been made to investigate the protective role of dietary supplementation with $(PhSe)_2$ by ameliorating the antioxidant system against concentrations environmentally relevant of atrazine in *Cyprinus carpio*. To demonstrate the efficiency of $(PhSe)_2$, we used the IBR methodology.

2. Materials and methods

2.1. Chemicals

Atrazine (ATZ) 99.4% was obtained from Dr. Ehrenstorfer (Germany). Diphenyl diselenide [$(PhSe)_2$], 98%, and all other reagents were purchased from Sigma-Aldrich (USA).

2.2. Animals

Thirty six juvenile carp of both sexes (mean weight 13.72 ± 0.37 g, body length 9.21 ± 0.09 cm) were obtained from a fish farm (Rio Grande do Sul, Brazil). The animals were acclimated for ten days to laboratory conditions, with natural photoperiod (12 h light/12 h dark). They were maintained in a static system in 250 L boxes of fiber glass, with continuously aerated tap water and physical and biological filters. Water quality parameters were verified daily (temperature 24.14 ± 0.96 °C, pH 7.13 ± 0.18, dissolved oxygen 7.98 ± 0.76 mg/L, non-ionized ammonia 0.18 ± 0.05 μg/mL, nitrite 1.09 ± 0.76 mg/mL). The fishes were fed twice a day with commercial feed to satiety during the acclimation period. This study was approved by the Ethics Committee on Animal Use of the Federal University of Santa Maria (protocol number 89/2014).

2.3. Experimental design

The two diets (control diet and $(PhSe)_2$ supplemented diet) were prepared in accordance with Menezes et al. (2014b). The ingredients were added to attend nutritional requirements of carp. For the supplemented diet, selenium compound $(PhSe)_2$ was added at the concentration of 3 mg/kg. Diets were stored at 4 °C until use. After acclimation, the carp were divided into two groups, (1) control group (n = 18) and (2) $(PhSe)_2$ supplemented group (n = 18). The animals were fed twice a day with 3% biomass per day of the diets with or without $(PhSe)_2$, in a period of 8 weeks, according to Menezes et al. (2014b). After feeding period, the fishes were divided in 6 treatments with 6 animals each treatment, as follows: (1) control diet + 0 μg/L ATZ, (2) control diet + 2 μg/L ATZ, (3) control diet + 10 μg/L ATZ, (4) $(PhSe)_2$ diet + 0 μg/L ATZ, (5) $(PhSe)_2$ diet + 2 μg/L ATZ, and (6) $(PhSe)_2$ diet + 10 μg/L ATZ. The fishes were exposed to atrazine for 96 h according to Mela et al. (2013), at 40 L aquariums, in a static system, without received food during the exposure period. The water quality parameters were the same of the acclimation period. Concentrations of ATZ in water were measured at the beginning and at the end of exposure time by gas chromatography-mass spectrometry according to Sabin et al. (2009). After the experimental period, animals were anesthetized using clove oil solution (concentration of 50 μL/L) according to da Cunha et al. (2010) and then euthanized by spinal cord section. After, liver, gills, and muscle were removed and stored at −80 °C for posterior analysis.

2.4. Biomarkers of oxidative damage

The lipid peroxidation was determined in accordance with Buege and Aust (1978), through the measure of thiobarbituric acid reactive substances (TBARS). Samples of liver and gills dilution of (1:20) and muscle dilution of (1:8) were homogenized with 20 mmol/L of potassium phosphate buffer (pH 7.5) in a Potter-Elvejhem glass/Teflon homogenizer. After that the samples were centrifuged at 10,000 × *g* for 10 min at 4 °C. For biochemical analyses were used homogenates of liver, gills (100 μL) and muscle (500 μL) and after added 100 μL of sodium dodecyl sulfate (SDS) 8.1%, 400 μL of acetic acid 2.5 M, 500 μL of 2-thiobarbituric acid (TBA) 0.67% and distilled water to adjust a final volume of 2.0 mL. A standard curve was performed using malondialdehyde (MDA) as a reference. The reaction mixture was incubated for 90 min at 95 °C. After cooling, samples and standard curve were read at 532 nm. The results were expressed as nmol TBARS/mg protein.

The content of protein carbonyl was measured in accordance with Yan et al. (1995), adapted to a microplate reader according to Müller et al. (2017). Liver, gills, and muscle tissues (1:18) were homogenized with Tris-HCl buffer 10 mmol/L (pH 7.4) in a Potter-Elvejhem glass/Teflon homogenizer. Samples were centrifuged at 10,000 × *g* for 10 min at 4 °C. An aliquot of homogenates (200 μL) was diluted with distilled water (800 μL) and then, 250 μL were reacted with 150 μL of 2,4-dinitrophenylhdrazine (DNPH) 10 mmol/L in 2 mol/L hydrochloric acid (HCl), incubated at room temperature in the dark vortex at 15 min of intervals during 60 min. Added 125 μL of denatured buffer (150 mmol/L of sodium phosphate buffer, pH 6.8, containing SDS 3%), 0.5 mL of heptane [99.5%], and 0.5 mL of ethanol [99.8%] sequentially, vortexes for 30 s, and centrifuged for 15 min. After, the protein isolated from the interface was washed twice by resuspension in ethanol and ethyl acetate (1:1) and suspended in 250 μL of denatured buffer. Then, 200 μL of mixture assay was put in 96 wells microplate and read at 370 nm. The assay was performed in duplicate, and two tubes blank incubated with 2 mol/L HCl without DNPH were included for each sample. Total carbonylation was calculated using a molar extinction coefficient of 22.000 M/cm and expressed as nmol carbonyl/mg protein.

2.5. Determination of non-enzymatic antioxidants

The content of non-protein thiols (NPSH) and ascorbic acid (AsA) was determined in liver, gills, and muscle, in accordance with Ellman (1959) and Roe (1954), respectively. The tissue preparation was the same in both analyses. Tissues were homogenized (1:10) with Tris-HCl