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Oxidative stress responses in zebrafish *Danio rerio* after subchronic exposure to atrazine

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A B S T R A C T

Atrazine is one of the most used pesticides all over the world and it is frequently detected in surface water. The aim of this study was to investigate if zebrafish exposure to atrazine could induce oxidative stress and changes in detoxifying system. Juvenile fish were exposed to sublethal concentrations of 0.3, 3, 30, or 90 $\mu g\ L^{-1}$ for 28 days. The level of oxidized lipids increased in experimental groups exposed to atrazine at 30 and 90 $\mu g\ L^{-1}$ compared to control. Activity of glutathione *S*-transferase decreased in group with the highest concentration compared to control. A significant decline was observed in catalase activity in all experimental groups compared to control. Activity of superoxide dismutase increased only in experimental group exposed to atrazine at 30 $\mu g\ L^{-1}$ compared to control. Activity of glutathione peroxidase and reductase (GR) increased in experimental groups exposed to atrazine at 0.3 (only for GR activity) and 90 $\mu g\ L^{-1}$ compared to control. Our results showed that atrazine exposure had profound influence on the oxidative stress markers and detoxifying enzyme of the exposed zebrafish. The changes in antioxidant enzyme activities could be an adaptive response to protect the fish from the atrazine-induced toxicity.

1. Introduction

The aquatic environment receives high amounts of pollutants that have the potential to induce oxidative stress in aquatic organisms through production of free radicals and reactive oxygen species (ROS). Exposure to chemical pollutants such as pesticides may induce an imbalance between intracellular ROS levels and antioxidant protection, and can subsequently cause changes in antioxidant defenses or direct oxidative damage in organism (Slaninova et al., 2009). Fish, like many other vertebrates, are endowed with defensive mechanisms to counteract the impact of ROS resulting from metabolism of various chemicals or xenobiotic (Nwani et al., 2010). The first line of defense consists of low-molecular-weight antioxidant compounds (e.g. glutathione, vitamin C and E), and the second defense mechanism comprises antioxidant enzymes (Valavanidis et al., 2006; Slaninova et al., 2009). Many studies have documented that pesticide exposure can induce oxidative stress in fish that may be linked to developmental alterations including embryotoxicity, teratogenicity, or reproductive effects (Paskova et al., 2011). Pesticides may induce oxidative stress via several mechanisms such as inactivation of antioxidants and associated enzymes leading to decreased antioxidant potential or by modification of core vital processes. Cellular metabolism of some pesticides may need consumption of endogenous antioxidants, including glutathione; this leads to depletion of reserves and may result in decreased antioxidant potential. Their interference with energy-providing processes may decrease supplement for metabolism and detoxification. Finally, pesticides are capable to enter redox cycles by accepting/donating electrons and consequently they may increase ROS level (Lushchak, 2011).

Atrazine (6-chloro-N-ethyl-N′-(1-methylethyl)-1,3,5-triazine-2,4-diamine), an s-triazine, is a selective systemic herbicide that acts as a photosynthesis inhibitor (inhibition of the Hill reaction). It is absorbed by roots and leaves, translocate acropetally in the

Abbreviations: CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione *S*-transferase; NBT, nitroblue tetrazolium; *r*, specific growth rate; ROS, reactive oxygen species; SEM, standard error of mean; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

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xylem, and accumulate in the apical meristems (Keith, 1998). It is widely used in agriculture for the production of corn, sorghum, sugarcane, and pineapple and used to some extent on landscape vegetation (Keith, 1998; Solomon et al., 2008).

The widespread use has resulted in the contamination of surface and ground water by atrazine and its metabolites. Among the latter, desethylatrazine is reported at levels similar to those of atrazine, while hydroxyatrazine and deisopropylatrazine are present at 25–50% of that level (Muller et al., 1997). The triazine ring of atrazine is resistant to degradation. Chemical degradation of atrazine may have greater environmental impact than biodegradation (Wexler, 2005; Solomon et al., 2008). Atrazine is listed as an environmental endocrine disruptor by the USA Environmental Protection Agency. It has low acute toxicity in mammals but is toxic to aquatic animals (Keith, 1998). Although its use has been banned by the European Commission (No. 2004/248/EC) in the Czech Republic since September 2005, atrazine and its degradation products are still detected in surface and ground waters. It still remains one of the most widely used herbicides in the world. According to the Czech Hydrometeorological Institute, high levels of atrazine residues are still found in Czech rivers. Water samples from Czech rivers in years 2005, 2006, 2007, and 2008 showed rates of occurrence of 88%, 86%, 46%, and 60%, respectively. The maximum concentrations in these years ranged from 0.3 to 1.0 $\mu g\ L^{-1}$ in these years.

Acute and chronic toxicity of atrazine in freshwater invertebrates and fish is well documented. Atrazine is not very acutely toxic to aquatic animals. Acute toxicity values for freshwater ranged from a 96hLC50 of 4.3 mg L^{-1} for the guppy (*Poecillia reticulata*) to 96hLC50 of >100 mg L^{-1} for the carp (*Carassius carassius*) (Solomon et al., 2008). But number of studies reported that chronic exposure to atrazine at low concentrations affected the behavior, physiology, immune function, reproductive system and metabolism of aquatic animals, especially of fish, which are among the most widely distributed aquatic organisms and are often used as bio-indicators of environmental pollution (Neškovič et al., 1993; Solomon et al., 2008; Ramesh et al., 2009; Nwani et al., 2010). Several toxicological studies have confirmed that exposure to atrazine can lead to enhanced ROS generation and induction of oxidative stress in fish (Elia et al., 2002; Nwani et al., 2010).

The aim of the present study was to investigate if zebrafish *Danio rerio* exposure to sublethal concentrations of atrazine could induce oxidative stress.

2. Materials and methods

2.1. Toxicity test

A toxicity test was performed on 30-day old zebrafish, according to OECD No. 215 Fish Juvenile Growth Test. Experimental fish were exposed to a range of sublethal concentrations of atrazine (Sigma–Aldrich, Czech Republic; chemical purity – 98.9%) in water: 0.3 $\mu g\ L^{-1}$, (i.e. the environmental concentration in Czech rivers), and 3.0, 30.0, and 90.0 $\mu g\ L^{-1}$ for 28 days. The fish were randomly distributed into 30 L glass aquaria, 40 specimens in aquarium. The experiment was conducted in a flow-through system, and the test solutions were replaced twice a day. The trial was duplicated. The fish were fed with dried *Artemia salina* without shells at 8% body weight per day. During the tests, conditions were checked at 24-h intervals and the number of dead fish was recorded for each concentration. The mean values of water quality were: temperature 25 ± 1 °C, oxygen saturation above 60% (ranged between 78% and 96%), pH 7.95–8.28. Gas chromatography with ion trap tandem mass spectrometry was used for the determination of atrazine, test substance concentrations were found above 80% of the measured initial concentrations. Experimental procedures were in compliance with national legislation (Act No. 246/1992 Coll., on the Protection of Animals Against Cruelty, as amended and Decree No. 207/2004 Coll., on the Protection, Breeding and Use of Experimental Animals, as amended).

2.2. Fish sampling and homogenization

At the end of the test, the fish were killed, immediately frozen, and stored at −85 °C until analyses. Whole body samples were weighed and homogenized (1:10 w/v) using phosphate buffer (pH = 7.2). The homogenate was divided into two portions, the first one for measuring thiobarbituric acid reactive substances (TBARS) and the second one was centrifuged (10.500*g*, 4 °C, 20 min) to obtain supernatant fraction for the determination of activities of glutathione *S*-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), and superoxide dismutase (SOD).

2.3. Measurement of detoxifying enzymes and oxidative stress parameters

The activity of GST was determined by measuring the conjugation of 1-chloro-2,4-dinitrobenzene with reduced glutathione at 340 nm (Habig et al., 1974). The specific activity was expressed as the nmol of the formed product per min per mg of protein. The activity of GR was determined spectrophotometrically by measuring NADPH oxidation at 340 nm (Carlberg and Mannervik, 1975). The activity of GPx was calculated from the rate of NADPH oxidation by the reaction with GR at 340 nm (Flohe and Gunzler, 1984). The specific activity of GR and GPx was expressed as the nmol of NADPH consumption per min per mg of protein. The activity of CAT was determined spectrophotometrically by measuring of H_2O_2 breakdown at 240 nm. The specific activity was expressed as the µmol of decomposed H_2O_2 per min per mg of protein (Aebi, 1984). Superoxide dismutase activity was determined spectrophotometrically by an indirect method using nitroblue tetrazolium (NBT). The specific activity was expressed in the units of SOD per mg of protein. One activity unit was defined as the amount of enzyme required for the inhibition of the initial rate of NBT reduction by 50% (Ewing and Janero, 1995). Protein concentrations were determined by a Bicinchoninic Acid Protein Essay Kit (Sigma–Aldrich, St. Louis, MO, USA) using bovine serum albumin as a standard Smith et al. (1985). To check lipid peroxidation, malondialdehyde was measured by the TBARS method at 535 nm as described by Lushchak et al. (2005). The concentration is expressed as nmol of TBARS per gram of tissue wet weight.

2.4. Statistical analysis

Oxidative stress markers were tested for normal distribution using the Shapiro–Wilk test. After testing for homogeneity of variance across groups (Levene test), an analysis of variance (one-way ANOVA) was used. The differences among test groups were assessed with the Tukey–HSD test with $p < 0.05$ chosen as the level of significance.

3. Results

3.1. Mortality and growth rate

Mortality did not exceed 5% during the 28-day trial period in both control and experimental groups. A lower specific growth factor (r) was observed in all test groups compared to the control ($r = 1.69$) but a significant difference ($p < 0.05$) was proved only at 90.0 $\mu g\ L^{-1}$ ($r = 1.41$).

3.2. Oxidative stress indices and detoxifying enzyme

Effects of chronic exposure to atrazine on selected oxidative stress indices and activity of detoxifying enzyme are presented in Table 1. An increase in GPx activity was found in fish exposed to atrazine at 0.3, 3, and 30 $\mu g\ L^{-1}$; but only in the group exposed to atrazine at 30 $\mu g\ L^{-1}$ the level reached significance ($p < 0.05$). The lowest value of GPx activity was observed in the group exposed to the highest concentration of atrazine, which was significantly different ($p < 0.05$) from all groups, with the exception of the control group. The activity of glutathione reductase was greater in groups treated with 0.3, 3, and 30 $\mu g\ L^{-1}$ compared to the control group; however, significant differences ($p < 0.05$) were obtained only between the control group and the 0.3 and 30 $\mu g\ L^{-1}$ groups. Significantly lower ($p < 0.05$) GR activity was found in fish exposed to 90 $\mu g\ L^{-1}$ compared to the experimental groups 0.3 and 30 $\mu g\ L^{-1}$, but no significant difference was found between the highest concentration and the control group. The activity of CAT was significantly lower ($p < 0.05$) in all experimental groups compared to the control group. No significance differences between control and experimental groups were obtained in SOD activity. The group exposed to 90 $\mu g\ L^{-1}$ was found to exhibit the lowest level of SOD activity. This result was significantly different ($p < 0.05$) only from the experimental group exposed to atrazine at 30 $\mu g\ L^{-1}$.