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Low toxic herbicide Roundup induces mild oxidative stress in goldfish tissues

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ABSTRACT

The formulation of Roundup consists of the herbicide glyphosate as the active ingredient with polyethoxylene amine added as a surfactant. The acute toxicity of Roundup (particularly of glyphosate) to animals is considered to be low according to the World Health Organization, but the extensive use of Roundup may still cause environmental problems with negative impact on wildlife, particularly in an aquatic environment where chemicals may persist for a long time. Therefore, we studied the effects of Roundup on markers of oxidative stress and antioxidant defense in goldfish, *Carassius auratus*. The fish were given 96 h exposure to Roundup at concentrations of 2.5–20 mg L^{-1}. Exposure to Roundup did not affect levels of lipid peroxides (LOOH) in goldfish brain or liver, and in kidney only the 10 mg L^{-1} treatment elevated LOOH by 3.2-fold. Herbicide exposure also had no effect on the concentrations of protein thiols or low molecular mass thiols in kidney, but selective suppression of low molecular mass thiols by 26–29% occurred at some treatment levels in brain and liver. Roundup exposure generally suppressed the activities of superoxide dismutase (SOD), glutathione *S*-transferase (GST), glutathione reductase and glucose-6-phosphate dehydrogenase in fish tissues. For example, SOD activities were reduced by 51–68% in brain, 58–67% in liver and 33–53% in kidney of Roundup treated fish. GST activity decreased by 29–34% in liver. However, catalase activity increased in both liver and kidney of herbicide-exposed fish. To our knowledge this is the first study to demonstrate a systematic response by the antioxidant systems of fish to Roundup exposure.

1. Introduction

Herbicides are actively used in terrestrial and aquatic ecosystems to control unwanted weeds, and their use has generated serious concerns about the potential adverse effects of these chemicals on the environment and human health. In many European estuaries, pesticide concentrations are higher than European norms which have to be lower than 0.1 μg L^{-1} for individual pesticide concentrations and total pesticide concentrations should be below 0.5 μg L^{-1} (Marchand et al., 2006). Several studies found that total pesticide concentrations were particularly high in Germany in the Rhine (2 μg L^{-1}), the Elbe (0.9 μg L^{-1}), the Weser (3.2 μg L^{-1}), and the Ems (2.3 μg L^{-1}) and were also high in France, especially in the Vilaine estuary (0.1–2.5 μg L^{-1}) (cited after Marchand et al., 2006). In the United States of America 100% of all water surfaces, 33% of major aquifers, and 96% of all fish contained one or more pesticides at detectable levels (Nowell et al., 1999). Pesticides were identified as one of the leading cases of impairment for streams and one potential cause of declines and deformities among amphibians, pollinator species, and other beneficial insects. Several herbicides used including atrazine, diuron, isoproturon and the glyphosate-based herbicide, Roundup, are widely detected in European ecosystems (Quaghebeur et al., 2004).

The use of glyphosate (N-phosphoromethyl glycine) as a herbicide was first proposed by scientists at the Monsanto Company in 1970. It is a nonselective herbicide that inhibits plant growth through interference with the production of essential aromatic amino acids by inhibiting the enzyme enolpyruvylshikimate phosphate synthase. This enzyme is responsible for the biosynthesis of chorismate, an intermediate in phenylalanine, tyrosine, and tryptophan biosynthesis (Williams et al., 2000). Glyphosate expresses its herbicidal activity most efficiently through direct contact with leaves, followed by translocation to other organs. Absorption via roots is negligible. In ecological systems glyphosate is degraded mainly by bacteria, but plants do this to small extent (Karpouzas and Singh, 2006). The Roundup formulation was proposed in 1974 and contained glyphosate as the active ingredient with polyethoxylene amine (POEA), a non-ionic surfactant, added to increase the efficiency of the active ingredients by promoting the penetration of the herbicide through plant cuticle (Brausch and Smith, 2007).

The extensive use of Roundup on crops may cause environmental problems with a negative impact on wildlife. The acute toxicity of Roundup (particularly glyphosate) is considered to be low, according to data from the World Health Organization (WHO,

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1994). The toxicity and risk for humans, other mammals and birds was analyzed in detail by Williams and colleagues (2000) who concluded that "under present and expected conditions of use, Roundup herbicide does not pose a health risk for humans". However, the data appeared to show that aquatic organisms, particularly fish, could be more sensitive to glyphosate than mammals. For example, Grisolia (2002), using a micronucleus test, found that Roundup enhanced the frequency of appearance of micronuclei in erythrocytes of *Tilapia rendalii*, whereas the same dosages failed to affect mice. Aquatic ecosystems may be subjected to glyphosate as a consequence of weed control in terrestrial ecosystems and water bodies. For example, Olaleye and Akinyemiju (1996) reported that Roundup was efficiently used to control water hyacinth *Eichhornia crassipes* Mart. in Abaila creek, Delta State, Nigeria, which resulted in no fish mortality, and was expected to increase fish catch. In China, Roundup effects on whole ecosystems and specific components were extensively investigated and found to be not harmful (Tsui and Chu, 2003, 2008).

However, recent studies (mainly from 2000 onward) are showing potentially adverse effects of Roundup, and its components glyphosate and POEA, on fish. For example, Roundup affected energy metabolism, free radical processes, and acetylcholine esterase activity (Rendón-von Osten et al., 2005; Glusczak et al., 2006, 2007; Langiano and Martinez, 2008). The micronucleus test and, in some cases, the comet assay showed that Roundup (glyphosate) affected these parameters in *T. rendalii* (Grisolia, 2002), *Prochilodus lineatus* (Cavalcante et al., 2008) and *Carassius auratus* (Çavaş and Könen, 2007). Glyphosate also affected immune responses in *Tilapia nilotica* (el-Gendy et al., 1998), and produced histological changes in hepatocytes of *Oreochromis niloticus* (Jiraungkoorskul et al., 2003) and *Cyprinus carpio* (Szarek et al., 2000). Depending on its concentration, Roundup induced either preference or avoidance reactions in rainbow trout (Tierney et al., 2007). Nesković and colleagues (1996) investigated the biochemical and histopathological effects of glyphosate on carp, *C. carpio* L. and found that the LC_{50} value for 48 h exposure was 645 mg L^{-1}, and 620 mg L^{-1} (as active ingredient) after 96 h. In a subacute toxicity test, the effects of sublethal glyphosate concentrations (2.5–10.0 mg L^{-1}) increased alkaline phosphatase activity in liver and heart and affected the activities of glutamic-oxaloacetic and glutamic-pyruvic transaminases in fish tissues. The gills of fish exposed to glyphosate exhibited epithelial hyperplasia and subepithelial edema. Other morphological changes were also found in gills and liver, whereas kidney structure was not affected by the glyphosate up to 10 mg L^{-1} (Nesković et al., 1996).

Because Roundup usage has increased in recent years in Europe, including Ukraine, we studied its effects on the levels of markers of oxidative stress and antioxidant defense in a common freshwater species in Ukraine, the goldfish *C. auratus*. Since free radical metabolism in fish is affected by many factors (Martínez-Alvarez et al., 2005), we aimed to discover whether the components of Roundup, which do not directly enter redox processes, might still influence these markers.

2. Materials and methods

2.1. Reagents

Phenylmethylsulfonyl fluoride (PMSF), 1-chloro-2,4-dinitrobenzene (CDNB), oxidized glutathione (GSSG), reduced glutathione (GSH), glucose-6-phosphate (G6P), ethylenediamine-tetraacetic acid (EDTA), xylenol orange, cumene hydroperoxide, ferrous sulphate, 2,4-dinitrophenylhydrazine (DNPH), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), N,N,N′,N′-tetramethylethylenediamine (TEMED), Tris[hydroxymethyl]aminomethane and potassium phosphate monobasic were purchased from Sigma–Aldrich Corporation (USA). $NADP^+$ and NADPH were obtained from Reanal (Hungary) and guanidine-HCl was from Fluka (Germany). All other reagents were of analytical grade.

2.2. Animals and Roundup exposure

Goldfish (*C. auratus* L.) weighting 50–70 g were obtained commercially at a local market (Ivano-Frankivsk, Ukraine) in December 2007 and held for about 2 months in a 1000 L tank under natural photoperiod in aerated and dechlorinated tap water at 20 ± 2 °C and pH ~ 7.0. The experiments were carried out in 120 L glass aquaria, in a static mode, under the same conditions. Fish were not fed during pre-adaptation to laboratory conditions and experimentation.

For experiments, groups of six fish were placed in 120 L aquaria with different nominal Roundup concentrations (2.5, 5, 10 or 20 mg L^{-1}) and were exposed to these conditions for 96 h. Fish in the control group were treated in the same manner, but Roundup was omitted. Water was not changed during the course of the experiment in order to avoid stressing the animals. Fish were killed by transspinal dissection after a 96 h exposure time and brains, livers and kidneys were quickly dissected out, rinsed in cold 0.9% sodium chloride solution, placed in pre-chilled homogenization buffers and immediately processed.

2.3. Indices of oxidative stress

For measurement of free thiols tissue samples were homogenized (1:10 w/v) using a Potter–Elvehjem glass homogenizer in pre-chilled 50 mM potassium phosphate (KPi) buffer, pH 7.5, containing 0.5 mM EDTA; a few crystals of phenylmethylsulfonyl fluoride (PMSF) were added prior to homogenization. Samples were then centrifuged at 4 °C for 15 min at 16 000*g*. Supernatant was removed and total thiol content (sum of low and high molecular mass thiols) was measured spectrophotometrically with DTNB at 412 nm as described previously (Lushchak and Bagnyukova, 2006a). To determine low molecular mass thiol (L-SH) content, aliquots of supernatants were treated with 10% (final concentration) trichloroacetic acid (TCA) to precipitate proteins, centrifuged for 5 min at 16 000*g* and then thiol content was measured again in these supernatants. Thiol concentrations are expressed as micromoles of SH-groups per gram wet weight of tissue. The high molecular mass thiol (H-SH) content was calculated by subtracting the L-SH concentration from the total thiol level.

Lipid peroxide (LOOH) concentrations were assayed by the xylenol orange method (Hermes-Lima et al., 1995). For that, tissue samples were homogenized in five volumes of ice-cold 96% ethanol, centrifuged at 13 000*g* for 12 min at 4 °C, and then supernatants were used for assay as described previously (Lushchak and Bagnyukova, 2006a). LOOH concentrations are expressed as nanomoles of cumene hydroperoxide equivalents per gram wet weight (gww).

2.4. Assay of antioxidant and associated enzyme activities

Tissue homogenates were prepared as described above for the thiol assay and centrifuged at 4 °C for 15 min at 16 000*g* in an Eppendorf 5415R centrifuge (Germany). Supernatants were removed and used for enzyme activity assays using a Specord M-40 (Karl Zeiss Jena, Germany). The activities of primary antioxidant enzymes, SOD and catalase, as well as associated enzymes, GST, GR and G6PDH, were measured as described previously (Lushchak and Bagnyukova, 2006b). One unit of SOD activity is defined as the amount of enzyme (per milligram protein), that inhibits quercetin oxidation reaction by 50% of maximal inhibition. One unit of cata-