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Acute Exposure to Glyphosate Herbicide Affects Oxidative Parameters in Piava (*Leporinus obtusidens*)

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Abstract In recent years, commercial glyphosate herbicide formulations have been widely used in agriculture to control aquatic weeds. These pesticides may result in disruption of ecological balance, causing damage to nontarget organisms including fish. Teleostean fish (*Leporinus obtusidens*) were exposed to commercial glyphosate herbicide formulation at 0 (control), 3, 6, 10 or 20 mg L^{-1} for 96 h. The effects of herbicide on plasmatic metabolic parameters, thiobarbituric acid reactive substances (TBARS), catalase activity, protein carbonyl, and mucus layer parameters were studied. Plasmatic glucose and lactate levels increased but protein levels showed reduction after herbicide exposure. TBARS levels in brain showed a reduction at all tested concentrations. However, liver demonstrated increased TBARS levels at all tested concentrations, whereas in white muscle TBARS production did not change after exposure to herbicide. Fish exposed to all concentrations of glyphosate showed increase in liver catalase activity and protein carbonyl. Herbicide exposure increased protein and carbohydrate levels of the mucus layer at all tested concentrations. The present results showed that, in 96 h, glyphosate changed toxicological parameters analyzed in piava. Parameters measured in this study may be useful in environmental biomonitoring.

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The increased use of herbicides in agriculture can affect aquatic ecosystems and cause ecological imbalance. In aquatic toxicology studies, fish are important indicators of the effects of toxic compounds. The commercial glyphosate formulation, which is the acid equivalent of the isopropylamine salt of glyphosate, contains in its formulation polyethoxylated tallow amine (POEA) as its predominant surfactant (Jiraungkoorskul et al. 2002), which is more acutely toxic to aquatic organisms than the active ingredient itself (Giesy et al. 2000). Glyphosate herbicide is nonselective and has been used for controlling aquatic weeds (Abdullah et al. 1995; Jiraungkoorskul et al. 2002). Glyphosate does not bioaccumulate in terrestrial or aquatic animals and presents low toxicity. The glyphosate formulation rapidly dissipates in surface waters, and soil microflora biodegrade it into aminomethylphosphonic acid (AMPA) and CO_2. It is widely used worldwide due to its high efficiency and cost effectiveness (Giesy et al. 2000; Tomlin 2000).

There are various studies considering herbicide toxicity in fish, especially concerning metabolic, oxidative, and hematological parameters in response to Roundup® exposure (Fonseca et al. 2008; Cavalcante et al. 2008; Salbego et al. 2010). Modesto and Martinez (2010), studying the effects of Roundup® on biochemical biomarkers in the Neotropical fish *Prochilodus lineatus*, showed that the herbicide interferes with antioxidant defense, leading to occurrence of lipid peroxidation. Long-term exposure to Roundup® causes metabolic disruption affecting brain acetylcholinesterase activity as well as metabolic and hematologic parameters of *Leporinus obtusidens* (Salbego et al. 2010).

The response of some aquatic organisms to environmental contaminants has been studied through measurement of general physiological concentrations such as glucose, lactate, and protein (Fonseca et al. 2008; Gimeno et al. 1995; Sancho et al. 2000).

It is known that contaminants such as pesticides may induce formation of reactive oxygen species (ROS), resulting in imbalance between pro-oxidant and antioxidant defense mechanisms. Lipid peroxidation (LPO) induced by pollutants such as pesticides has been observed in fish species (Schlenk et al. 1997; Üner et al. 2005, 2006). Reactive oxygen species (ROS) produced in biological systems are detoxified by antioxidant defenses. Variations in the activities of antioxidant enzymes such as catalase have been proposed as indicators of pollutant-mediated oxidative stress (Ahmad et al. 2000; Sayeed et al. 2003; Üner et al. 2005). It has been established in mammals that protein damage or chemical modification of its amino acids during the oxidative stress process can produce high levels of protein carbonyls (Parvez and Raisuddin 2005). Many studies have suggested that protein carbonyl content may be used as a complementary biomarker of oxidative stress in humans and other vertebrates (Pey et al. 2003). However, few such studies have been reported in fish (Almroth et al. 2005; Parvez and Raisuddin 2005). The mucus layer that covers exposed surfaces of fish is important not only for its effective role as a protective barrier but also as a hydrodynamic lubricant, as well as an active antiparasitic and antibacterial agent (Sabóia-Moraes et al. 1996; Tromeur et al. 1992).

Considering that there is no information available about changes in plasmatic metabolism, oxidative stress, and mucus layer parameters in piava exposed to herbicides, the present study aimed to investigate the effects of commercial glyphosate formulation on oxidative stress parameters in piava (*Leporinus obtusidens*) as a complementary study concerning the toxicity of this herbicide.

Materials and Methods

Fish

The piava (*Leporinus obtusidens*) species was chosen for this study because it is a native freshwater fish of Southern Brazil with good acceptance in the consumer market (Andrian et al. 1994; Baldisserotto and Gomes 2005). Piava of both sexes were obtained from the Santa Maria Federal University (UFSM) fish farm (Rio Grande do Sul, Brazil). Fish (weight, 12.0 ± 1.0 g; length, 8.0 ± 1.0 cm) were acclimated to laboratory conditions for 10 days. They were kept in continuously aerated tanks (250 L) with a static system and a natural photoperiod (12 h light/12 h dark). Throughout the experimental period, water quality was as follows: temperature 23 ± 0.5°C, pH 7.5 ± 0.05, and dissolved oxygen 7.1 ± 0.2 mg L^{-1}. Fish were fed once a day with commercial fish pellets (42% crude protein; Supra, Brazil), and feces and pellet residues were removed daily by suction only in the acclimation period.

Experimental Design

Acute toxicity assays were carried out in static fashion for 96 h, according to Antón et al. (1994). Previous experiments carried out in our laboratory were not able to obtain a lethal concentration (LC_{50}) of glyphosate at 96 h, because all fish survived even at the highest concentration tested (100 mg L^{-1}) and showed normal swimming and feeding behavior. Therefore, experimental glyphosate concentrations were chosen considering nominal sublethal concentrations. This study was approved by the Ethics and Animal Welfare Committee of the Federal University of Santa Maria (no. 23081.016049/2005-40). After acclimation, groups of eight fish were placed in 45-L continuously aerated glass tanks and exposed for 96 h to 0 (control), 3, 6, 10 or 20 mg L^{-1} commercial glyphosate formulation (480 g L^{-1} acid equivalent, 692 g L^{-1} inert ingredients; Monsanto Company, St. Louis, MO, USA). Before experiments, stock solution (500 mg L^{-1}) was prepared by diluting commercial glyphosate formulation (480,000 mg L^{-1}), then diluted to the required concentrations. All tests were carried out in triplicate. Herbicide was added to the water only at the beginning of the experiment. Water quality did not change throughout the experimental period, and water was not replaced.

Analytical Procedures

At the end of the exposure period (96 h), mucus was carefully scraped from dorsal body surface (total area 6 cm^2) using a cotton-tipped swab. After scraping, the cotton was immersed in 2 mL distilled water, and the sample was used to determine soluble sugar (Duboie et al. 1956) and protein (Lowry et al. 1951). All fish were sampled, and blood was collected from the caudal vein with a 1-mL heparinized syringe. One blood aliquot was centrifuged at 1,500×*g* for 10 min, and plasma was separated. Plasma glucose was measured by the glucose oxidase method with Bioclin test kit. Plasma was dissolved in 10% trichloroacetic acid (1:20 dilution), and lactate was estimated according to Harrower and Brown (1972). Plasma total protein levels were measured according to Lowry et al. (1951) using bovine serum albumin (Sigma) as standard. Brain, white muscle, and liver samples were quickly removed, washed with 150 mM saline solution, packed in Teflon tubes, and kept at −20°C for posterior analyses.

Lipid peroxides produced from oxidative stress were quantified by TBARS assay, performed using the malondialdehyde (MDA) reaction with 2-thiobarbituric acid (TBA), measured optically. Tissues samples (brain, white

muscle, and liver) were homogenized in a Potter–Elvejhem glass/Teflon homogenizer with 20 mM potassium phosphate buffer, pH 7.4 (with 0.1% Triton X100 and 150 mM NaCl) (1:20 dilution), centrifuged at 10,000×*g* for 10 min at 4°C. Brain, white muscle, and liver homogenates (100–400 μL) were added to 8.1% sodium dodecyl sulfate (SDS), 2.5 M acetic acid (pH 3.4), 0.8% thiobarbituric acid were added to adjust to final volume of 2.0 mL. The reaction mixture was placed in a microcentrifuge tube and incubated for 90 min at 95°C. After cooling, it was centrifuged at 5,000×*g* for 10 min, and the optical density at 532 nm was determined. TBARS levels are expressed in units of nmol MDA mg protein^{-1} according to Ohkawa et al. (1979).

Catalase (EC 1.11.1.6) activity was assayed by ultraviolet spectrophotometry (Nelson and Kiesov 1972). Liver samples were prepared as reported for TBARS assay. Briefly, the assay mixture consisted of 2.0 mL potassium phosphate buffer (50 mM, pH 7.0), 0.05 mL H_2O_2 (0.3 M), and 0.05 mL homogenate. Catalase (CAT) activity was determined by following the H_2O_2 decrease using absorbance at 240 nm. Enzyme activity was expressed as micromoles of H_2O_2 reduced per milligram of protein per minute (μmol mg protein^{-1} min^{-1}).

Liver tissue was homogenized in 10 volumes (w/v) of 10 mM Tris–HCl buffer, pH 7.4 using a glass homogenizer. Protein carbonyl content was determined by the method described by Yan et al. (1995), with some modifications. Briefly, homogenates were diluted to 0.7–0.8 mg mL^{-1} of protein in each sample, and 1-mL aliquots were mixed with 0.2 mL 10 mM 2,4-dinitrophenylhidrazine (DNPH) or 0.2 mL 2 M HCl. After incubation at room temperature for 1 h in dark, 0.5 mL denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, containing SDS 3%), 2.0 mL heptane (99.5%), and 2.0 mL ethanol (99.8%) were added sequentially, followed by vortexed agitation for 40 s and centrifugation for 15 min. Next, the protein isolated from the interface was washed two times with 1 mL ethyl acetate:ethanol 1:1 (v/v) and suspended in 1 mL denaturing buffer. Each DNPH sample was read at 370 nm using a Femto Scan spectrophotometer against the corresponding sample (blank), and total carbonylation was calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1}.

Protein levels for oxidative stress parameters were estimated spectrophotometrically by the method of Bradford (1976), using bovine serum albumin as standard.

Statistical Procedures

One-way analysis of variance (ANOVA) and Duncan's multiple-range tests were used. Data ($n = 3$), representing the mean of each triplicate (8), were expressed as mean ± standard deviation (SD), and mean differences were considered significant at $P < 0.05$.

Results and Discussion

Plasma glucose and lactate levels increased after exposure to glyphosate (Table 2). The hyperglycemia present in fish exposed to the herbicide may partially be a consequence of brain cholinesterase inhibition shown in this fish species (Glusczak et al. 2006). Blockage of the neuroeffector in the adrenal medulla favors glycogen breakdown and glucose export due to hypersecretion of adrenaline (Aguiar et al. 2004; Crestani et al. 2006). In addition, Roundup® causes cholinesterase inhibition in brain and muscle of *Prochilodus lineatus* (Modesto and Martinez 2010), and brain of *Leporinus obtusidens* after 90 days exposure (Salbego et al. 2010). The changes in lactate levels also indicate metabolic disorders. Lactate has been widely used as a measure of anaerobic metabolism, and this increase has been demonstrated to be a rapid and clear response to energy depletion caused by lack of oxygen (Gimeno et al. 1995). Lactate levels also increase in liver and muscle of the same fish *Leporinus obtusidens*. However, increase in lactate levels was recorded after 90 days of exposure (Salbego et al. 2010). The results observed after long-term exposure are in agreement with those obtained in the present study, where plasma lactate increase indicates anaerobic preference. The decreased plasma protein in *L. obtusidens* at all tested concentrations may indicate use of plasmatic protein to supply energy metabolism disrupting of any tissue. In addition, these results may indicate physiological adaptability of fish to compensate for oxidative damage caused by this herbicide. *Leporinus obtusidens* exposed to environmentally relevant concentrations of Roundup® showed similar results with plasma protein reduction after exposure to 1 or 5 mg L^{-1} for 90 days (Salbego et al. 2010). The results presented herein, when compared with those obtained for long-term exposure, could indicate a compensatory mechanism due to increased demand for energy expenditure for herbicide biotransformation. Hypoproteinemia is usually associated with fish exposure to pesticide and has been correlated with disturbance in osmoregulation (Begum 2004; Salbego et al. 2010; Sancho et al. 2000).

Measurement of lipid peroxidation through TBARS quantification has been used as an indicator of oxidative stress in fish. In this work, TBARS levels were altered after exposure of fish to glyphosate. TBARS levels in brain tissue showed a reduction at all concentrations tested when compared with the control group ($P < 0.05$). Similar results were observed in our laboratory concerning brain TBARS levels, where *L. obtusidens* exposed to clomazone

(0.5 mg L^{-1}) or propanil herbicides (3.6 mg L^{-1}) showed a decrease in TBARS levels in this tissue (Moraes et al. 2009). Hepatic tissues showed an increase in TBARS levels at all tested concentrations. In the liver, elevation in TBARS suggests participation of free-radical-induced oxidative cell injury caused by glyphosate toxicity. In muscle tissues, TBARS production did not change at any tested concentration (Fig. 1). Apparently, glyphosate caused lipid peroxidation only in liver tissues, and changes in TBARS varied depending on the tissue considered. Recently, Roundup® exposure was reported to cause lipid peroxidation and also to impair oxidative defenses of *Prochilodus lineatus* (Modesto and Martinez 2010).

As in this study, *L. obtusidens* exposed to clomazone (0.5 mg L^{-1}) or propanil herbicide (3.6 mg L^{-1}) exhibited a TBARS increase in the liver (Moraes et al. 2007). The level of lipid peroxidation may differ among the fish species and tissues considered; for example, TBARS levels in *L. obtusidens* poisoned with herbicides at rice field conditions showed reduced TBARS levels in white muscle after exposure to quinclorac (0.375 mg L^{-1}), propanil (3.6 mg L^{-1}), and metsulfuron methyl herbicide (0.002 mg L^{-1}). However, another fish species, *Rhamdia quelen*, exposed to clomazone (0.5 or 1.0 mg L^{-1}) showed an increase in TBARS levels, particularly in the liver, after 12, 24, 48, 96 or 192 h exposure (Crestani et al. 2007). Li et al. (2003) also observed elevated TBARS levels in the liver of *Carassius auratus* after exposure to 3,4-dichloroaniline. The differences in peroxide levels have also been attributed to the variation in antioxidant mechanisms of fish species (Radi et al. 1985; Ahmad et al. 2000).

The antioxidant enzyme catalase showed an increase in activity in the liver with increasing herbicide concentration (Fig. 2). This enzyme seems to be important as an antioxidant defense against possible lipid damage generated by glyphosate. Catalase activity showed an increase in hepatic tissue after exposure to herbicide (Fig. 2). According to previous experiments (Moraes et al. 2007), elevation in catalase activity was observed in the liver of *Leporinus obtusidens* exposed to commercial formulations of clomazone (0.5 mg L^{-1}) or propanil herbicide (3.6 mg L^{-1}). However, Crestani et al. (2006) showed a reduction in catalase activity in hepatic tissue of silver catfish exposed to clomazone (0.5 or 1.0 mg L^{-1}) after 12, 24, and 96 h. Sayeed et al. (2003) also observed a 45% decrease in hepatic catalase activity as well as high levels of TBARS in freshwater fish (*Channa punctatus*) exposed to the insecticide deltamethrin. Thus, oxidative stress generated by water containing glyphosate-based herbicides may suppress the antioxidant defense represented by catalase, leading to a loss of this compensatory mechanism.

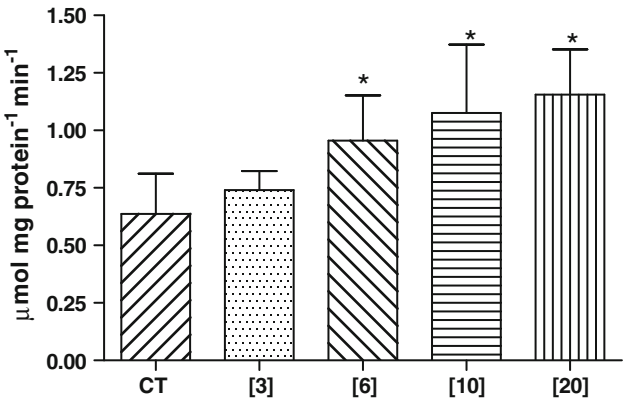

Fig. 2 Catalase activity (μmol mg protein^{-1} min^{-1}) in liver of *Leporinus obtusidens* exposed to glyphosate (mg L^{-1}) for 96 h. Data are reported as mean ± SD ($n = 3$). *Significant difference from control ($P < 0.05$)

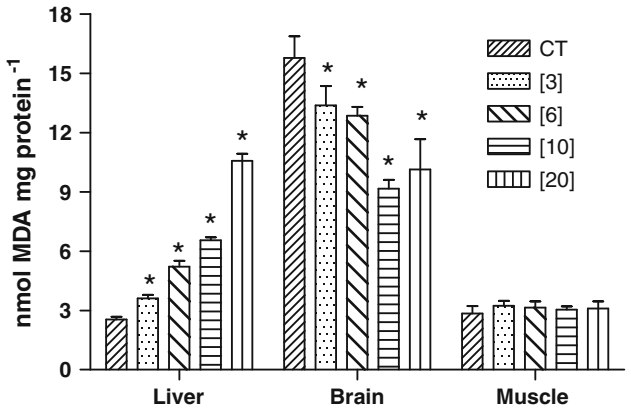

Fig. 1 TBARS levels (nmol MDA mg protein^{-1}) in brain, liver, and white muscle of *Leporinus obtusidens* exposed to glyphosate (mg L^{-1}) for 96 h. Data represent mean ± SD ($n = 3$). *Significant difference between groups and control values ($P < 0.05$)

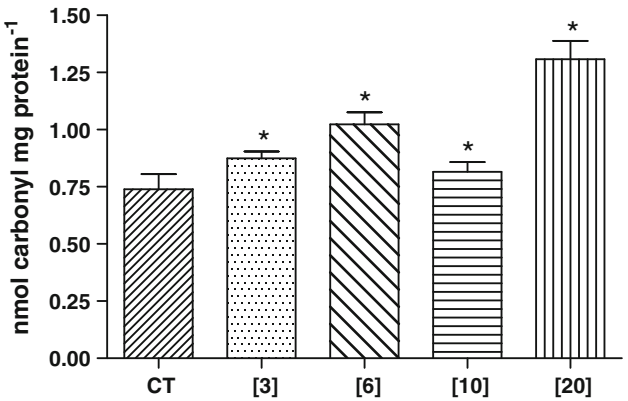

Fig. 3 Protein carbonyl (nmol carbonyl mg protein^{-1}) in liver of *Leporinus obtusidens* exposed to glyphosate (mg L^{-1}) for 96 h. Data are reported as mean ± SD ($n = 3$). *Significant difference from control ($P < 0.05$)

Table 1 Protein and glucose levels of the mucus layer of *Leporinus obtusidens* after glyphosate exposure (96 h)

Glyphosate (mg L^{-1})					
Mucus layer	Control	3	6	10	20
Protein (μg protein cm^2)					
	0.018 ± 0.002	0.025 ± 0.001*	0.046 ± 0.001*	0.051 ± 0.001*	0.053 ± 0.001*
Glucose (μg sugar cm^2)					
	0.011 ± 0.0006	0.020 ± 0.0005*	0.023 ± 0.0008*	0.022 ± 0.0007*	0.033 ± 0.0001*

Data represent mean ± SD ($n = 3$)

* Significant difference from control ($P < 0.05$)

Table 2 Plasma metabolic parameters in *Leporinus obtusidens* after glyphosate exposure (96 h)

Glyphosate (mg L^{-1})					
Plasma	Control	3	6	10	20
Protein (mg mL^{-1})	26.3 ± 2.02	23.6 ± 0.50*	24.0 ± 0.44*	23.9 ± 0.53*	24.3 ± 0.64*
Glucose (mg dL^{-1})	32.0 ± 5.90	45.5 ± 0.54*	40.5 ± 1.36*	41.3 ± 1.36*	39.3 ± 1.36*
Lactate (μmol mL^{-1})	2.67 ± 0.07	3.05 ± 0.14*	3.09 ± 0.23*	3.24 ± 0.14*	3.55 ± 0.34*

Data represent mean ± SD ($n = 3$)

* Significant difference from control ($P < 0.05$)

Fish exposed to glyphosate concentrations showed an increase in protein carbonyls in liver tissue (Fig. 3). Parvez and Raisuddin (2005) also observed an increase in protein carbonyls after 48 h of exposure to deltamethrin, endosulfan or paraquat. Few studies have been carried out using protein carbonyl formation in teleost fish. The presence of carbonyl groups in protein has been used as a marker of ROS-mediated protein oxidation (Madhusudhanan et al. 2004; Parvez and Raisuddin 2005). The reduction of TBARS formation shown in our study may affect protein oxidation. In addition, the increase in protein carbonyl levels would indicate that normal protein metabolism is disrupted, resulting in accumulation of damaged molecules. The relationship between TBARS, catalase activity, and protein carbonylation in this study may indicate a response of the fish to survive after herbicide toxicity.

In this study, *L. obtusidens* exposed to glyphosate showed an increase in protein and carbohydrate levels of the mucus layer when compared with controls (Table 1). Glycoproteins represent the major component of the mucus coating of fish skin. The changes in carbohydrate and protein content in the fish surface observed in *L. obtusidens* could be a mechanism to protect against external agents and microbial development. The suggested functional significance of the fish epidermal mucus includes osmoregulation, protection from abrasions, entanglement of particulate materials, defense against pathogens and parasites, reduction of swimming drag or friction, and protection against environmental contaminants (Tromeur et al. 1992; Hinton et al. 2001). In this context, our results can represent a protective mechanism against glyphosate toxicity.

In summary, the present work demonstrates that the concentrations of glyphosate-based herbicide used in agricultural fields cause changes in oxidative stress parameters in piava (*L. obtusidens*). It is evident that, from an ecophysiological point of view, use of this herbicide formulation in agriculture and aquaculture must be carefully evaluated. We conclude that the health of this fish species may be affected by the presence of glyphosate in water. However, more studies are necessary to discriminate which compound of the commercial formulation could be responsible for the oxidative liver damage found in the present study. In this context, TBARS, protein carbonyl, CAT, and mucous layer analyses can be used as biomarkers of fish poisoning by glyphosate. The observed alterations indicate also the potential use of native fish species as toxicity bioindicators in biomonitoring studies considering the environment risk of contamination by pesticides of rivers near areas of rice or soy cultivation in Southern Brazil.

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