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Toxicological Responses of *Cyprinus carpio* Exposed to a Commercial Formulation Containing Glyphosate

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Abstract The effects of commercial glyphosate herbicide formulation on the activity of acetylcholinesterase (AChE) enzyme and oxidative stress were studied in *Cyprinus carpio* exposed for 96 h to 0.0, 0.5, 2.5, 5.0 and 10.0 mg/L and then allowed to equal recovery period in water without herbicide. The activity of AChE was inhibited in the brain and in the muscle after exposure. However, after recovery period brain and muscle AChE activity increased. Brain thiobarbituric acid reactive species (TBARS) were measured as an indicator of oxidative stress. Increased TBARS levels were observed with all concentrations tested of the glyphosate formulation, and remained increased after the recovery period. The results recorded clearly indicate lipid peroxidation and anti-AChE action induced by Roundup® exposure.

Keywords Glyphosate · Carp · AChE · TBARS

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In aquatic toxicology, laboratory experiments are normally used to estimate the potential hazard of chemicals and to establish "safe" levels of pollutants (Antón et al. 1994). Chemicals such as herbicides when present in aquatic system can contaminate fish and other animals or plants inducing a kind of effects including secondary effects sometimes due to indirect contamination (Sarikaya and Yilmaz 2003; Fonseca et al. 2008). Roundup® is a commercial herbicide formulation containing the active ingredient glyphosate, which is *N*-(phosphonomethyl) glycine (Fig. 1). It also contains the surfactant, polyoxyethyleneamine (POEA), which is known to be more toxic than glyphosate to fish (Folmar et al. 1979). The glyphosate is a broad spectrum, non selective post-emergence herbicide that inhibits shikimic acid pathway, and affects the aromatic amino acid biosynthesis phenylalanine, tyrosine, and tryptophan (Diaz-Sanchez et al. 2002; Lushchak et al. 2009). It is commonly used in agriculture and forestry for the control or destruction of herbaceous plants (Beuret et al. 2005). It is used in agriculture as a non-selective herbicide to control annual and perennial plants, grasses, and broad-leaved woody species (WHO 1994). The water solubility of glyphosate is 15,700 mg/L and its half-life in soil is 30–90 days, with a partition coefficient octanol/water of 3.2 at 25°C (Cox 1998). The half-life of glyphosate in aquatic environments is reported to range from 7 to 14 days (Giesy et al. 2000). The calculated glyphosate concentrations used in rice and soybean crops in Southern Brazil range from 0.36 to 2.16 mg/L (Rodrigues and Almeida 2005). Roundup® showed LC_{50}-96 h ranging from 2 to 55 mg/L, depending on the fish species, life stage, test conditions and herbicide formulation (Langiano and Martinez 2008).

Inhibition of acetylcholinesterase enzyme (AChE) is a common effect of carbamate-based and organophosphorated

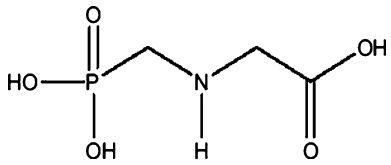

Fig. 1 Chemical structure of glyphosate

insecticides in fish. However, the determination of this enzyme in different fish species and tissues could be an important and sensitive method for detecting the presence of several herbicides including glyphosate (Miron et al. 2008; Modesto and Martinez 2010). Roundup® showed anti-AChE action inhibiting enzyme activity in brain of *Leporinus obtusidens* (Glusczak et al. 2006), *Rhamdia quelen* (Glusczak et al. 2007) and brain and muscle of *Prochilodus lineatus* (Modesto and Martinez 2010). Other herbicides class such as clomazone also inhibited AChE activity of *Rhamdia quelen* (Miron et al. 2005) and *Leporinus obtusidens* at different tissues (Miron et al. 2008). AChE is a key enzyme in the cholinergic transmission in the nervous system. The wide function of this enzyme is to catalyze the hydrolysis of acetylcholine into acetate and choline in the synaptic cleft. Inhibition of activity could affect the growth, survival, feeding and reproductive behavior of fish exposed to different pollutants (Dutta and Arends 2003). However, the effects of enzyme activation are still unclear. The activity of AChE is a parameter frequently used for environmental monitoring, usually in areas contaminated by pollutants. AChE is an enzyme that catalyzes the hydrolysis of acetylcholine (ACh) into choline and acetate in the synaptic cleft. When the inhibition of AChE activity occurs, the neurotransmitter acetylcholine is not hydrolyzed in the nerve synapses or neuromuscular junction, causing an abnormal amount of ACh to accumulate at these sites, leading to a disorder in cholinergic functions in tissues like brain and muscle. This may affect swimming or feeding behavior, for example. There are many hypothesis to explain changes caused by Roundup® in fish tissues especially due to surfactant POEA present in commercial formulation. Regarding brain and muscle AChE is possible that some aspect of chemical structure can interact with the active site of the enzyme or herbicide can reduce some important cofactor for AChE activity. Another issue is the oxidation of cysteine residues present in the structure of the enzyme which is equally important for its activity. Many pesticides can produce toxicity to fish tissues inducing oxidative stress, resulting in the production of reactive oxygen species (ROS). These reactive species are capable of causing damage to lipid, proteins, carbohydrates, and nucleic acids (Sevgiler et al. 2004; Üner et al. 2006). Lipid peroxidation may be the first step of cellular membrane damage and can be induced by environmental pollutants as herbicides (Wilhelm-Filho et al. 2001). ROS production associated with presence of pollutants such as herbicides and the establishment of oxidative stress has been imputed as a possible mechanism of toxicity in aquatic organisms exposed to pesticides (Oropesa et al. 2008). Fish is generally a good indicator of contamination by pollutants due to the fact that their biochemical responses are similar to those found in mammals. Fish frequently change metabolic state in response to pesticide contamination (Begum 2004; Fonseca et al. 2008).

The common carp, *Cyprinus carpio* is a fish species that is cultivated and consumed in the state of Rio Grande do Sul, Brazil. Studies linking the possible toxic effects of Roundup® in aquatic organisms are very important on the eco-physiological point of view. In fact, there is growing use of this product in Southern Brazil. At present more studies are necessary to explain the mechanisms of herbicide toxicity in fish and if Roundup exposure can induce lipid peroxidation and changes in AChE activity. In this context, the goal of this study was to evaluate Roundup® effects on the lipid peroxidation and cholinesterase enzyme after acute exposure to this herbicide. The final hypothesis is the possible enzyme alteration caused by lipid peroxides as a secondary effect of herbicide toxicity.

Materials and Methods

Carp (*Cyprinus carpio)* fingerlings (6.2 ± 0.5 g and 6.0 ± 0.4 cm) were obtained from a local fish culture and transported to the Biochemistry Laboratory at the Federal University of Santa Maria, in the state of Rio Grande do Sul. The fingerlings were placed in aquaria (250 L) at a stocking density of 50 fingerlings m^{-3} with aeration and temperature constant (25°C). After 10 days of laboratory acclimation, the fish were randomly redistributed in 40 L glass aquaria (five fish per aquarium), they were exposed to Roundup® (648 g/L of isopropylamine salt of Glyphosate, 480 g/L of acid equivalent of *N*-(phosphonomethyl) glycine (Ghyphosate) and 594 g/L of inert ingredients), at concentrations of 0 (without herbicide), 0.5, 2.5, 5.0 and 10.0 mg/L. For each treatment were made duplicates, so the "n" number represents five fish for each duplicate ($n = 10$). During acclimation, exposure and recovery experiment, fish were fed once a day with commercial fish pellets (42% crude protein, Supra, Brazil). Biological filters were used to maintain water quality. Glyphosate and AMPA were monitored according to Hidalgo et al. (2004) during 96 h and results are reported in Table 1. The analyses were made by a coupled-column liquid chromatography system with fluorescence detection (LC–LC–FD). This method was applied after water derivatization with fluorescent reagent 9-fluorenylmethylcloroformate (FMOC). A first short C18 column (3 cm) was used to perform

Table 1 Nominal concentrations and measured (mg/L) of glyphosate and AMPA (acid aminomethylphosphonic) at 96 h exposure to Roundup® quantified by HPLC

Nominal concentrations (mg/L)	Measured glyphosate (mg/L)	Measured AMPA (mg/L)
0.5	0.48 ± 0.01	0.22 ± 0.002
2.5	2.48 ± 0.02	1.05 ± 0.005
5.0	4.96 ± 0.01	2.35 ± 0.005
10.0	9.8 ± 0.05	4.55 ± 0.008

Values are expressed as mean ± SD (n = 5) in duplicate

large-volume injection (2 mL) and effect the efficient separation between the derivatized analytes and the excess of FMOC. It was coupled to a second amino analytical column (25 cm) for the anion-exchange separation of the derivatives. The limit of quantification (LOQ) was 0.1 µg/L (without pre-concentration) or 0.02 µg/L (after pre-concentration with 50 mL of water sample using anionic resin). The toxicity test was in 96 h to investigation of the glyphosate effects in *Cyprinus carpio* and the herbicide was applied only in the boxes at the beginning of the fish exposure without water changes. Previous studies in our laboratory were not able to obtain a lethal concentration (LC_{50}) for glyphosate at 96 h, because all fish survived even at the highest concentration tested (200 mg/L). Fish also showed normal swimming and feeding behavior. Thus, sub-lethal concentrations were choosing for this study. After exposure period, five animals per aquarium were sampled. The fish were killed by excision of spinal cord behind the operculum and the tissues (brain, liver, and muscle) were removed and quickly placed on ice and frozen at −70°C for analysis. The remaining fish were transferred to water without herbicide for the same period (96 h) to the recovery experiment. The work and experiments were approved by the board on experimentation on Animals of the Federal University of Santa Maria, reference number: 019/2008.

Water quality was analyzed daily and water pH was measured with a pH meter (Oakton). Water hardness was analyzed by the EDTA titrimetric method, total ammonia nitrogen ($NH_3 + NH_4^+$) was determined by the direct Nesslerization method and non-ionized ammonia nitrogen ($N–NH_3$) was calculated as described by Dutta and Arends (2003). Temperature and dissolved oxygen were determined with YSI oxygen meter (model Y5512). Alkalinity was estimated using a Tecnoquímica kit (Florianópolis, Brazil).

Tissues samples (brain and muscle) were homogenized in glass tubes under ice-bath with eight strokes of a motor driven Teflon pestle for 2 min. Homogenates were centrifuged for 10 min at 3,000*g* at 5°C in 150 mM NaCl, and the supernatant was used as the enzyme source. AChE activity was measured as described by Miron et al. (2005). Suitable amounts (50–100 µL) of homogenate were incubated at 25°C for 2 min with 0.8 mM acethylthiocholine (AcSCh) as substrate and 1.0 mM 5,5′dithio-2,2-nitrobenzoic acid (DTNB) as chromogen. The reaction was buffered with 0.1 M K-phosphate pH 7.5 for a final volume of 2.0 mL. The enzyme activity was followed at 412 nm. Enzyme activity was expressed as µmol AcSCh hydrolyzed/min/mg of protein.

Lipid peroxidation was estimated by a TBARS assay performed by a reaction of malondialdehyde (MDA) with 2-thiobarbituric acid (TBA), which was optically measured according to Buege and Aust (1978). Ten percent trichloroacetic (TCA) was added to brain homogenate (200 µL). Thiobarbituric acid (0.67%) was then added to a final volume of 1.0 mL. The reaction mixture was placed in a micro-centrifuge tube and incubated for 15 min at 95°C. After cooling, it was centrifuged at 5,000*g* for 15 min and optical density was measured by spectrophotometer at 532 nm. TBARS levels were expressed as nmols MDA/mg protein.

Tissue protein was determined by the Comassie blue method using bovine serum albumin as standard. Absorbance of samples was measured at 595 nm (Bradford 1976).

Enzymes parameters were analyzed by two-way ANOVA followed by Tukey–Kramer multiple range tests when appropriate. All data are expressed as mean ± standard deviation, with significance level $p \leq 0.05$.

Results and Discussion

The water quality temperature (23.0 ± 0.6°C), dissolved oxygen (7.6 ± 0.1 mg/L), pH (7.3 ± 0.2 units), total ammonia (0.8 ± 0.2 mg/L), total alkalinity (40 ± 0.5 mg/L $CaCO_3$), and hardness (33 ± 1.0 mg/L $CaCO_3$) of the acclimation period did not differ significantly between treatments and during all experimental periods. Glyphosate and the major metabolite, acid aminomethylphosphonic (AMPA), were monitored during the 96 h exposure according to the method described in Hidalgo et al. (2004). Results were showed in Table 1.

AChE activity in brain and muscle of fish exposed to glyphosate was lower than that in the control group (Figs. 2, 3). In this study, the brain and muscle AChE inhibition by glyphosate might lead to an accumulation of acetylcholine, causing the stimulation of the receptors. Thus, the inhibition of AChE can influence the process of cholinergic neurotransmission. The results of the present study concerning brain AChE activity are in agreement with those obtained in *Leporinus obtusidens* and *Rhamdia quelen* where exposure to glyphosate reduced brain AChE

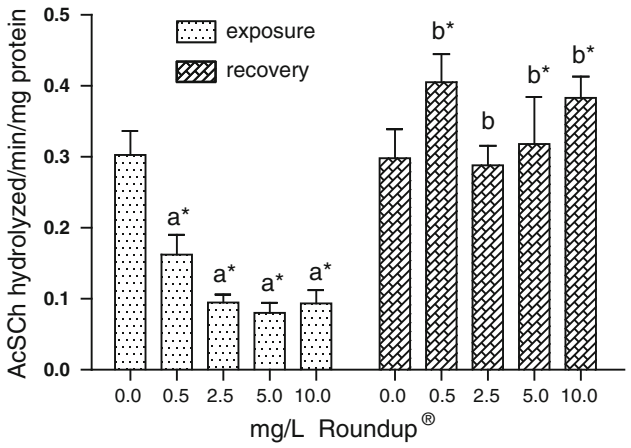

Fig. 2 Brain AChE activity (AcSCh/min/mg protein) of *Cyprinus carpio* exposed to Roundup® (96 h) and equal recovery period. Values are expressed as mean ± SD (n = 10). *Asterisk* indicates significant difference between groups and control group (0 mg/L) in the same period ($p \leq 0.05$). *Different letters* indicate significant difference between recovery and exposure periods ($p \leq 0.05$)

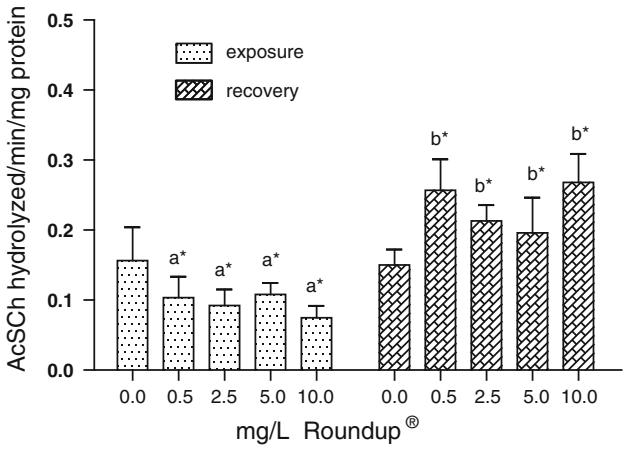

Fig. 3 Muscle AChE activity (AcSCh/min/mg protein) of *Cyprinus carpio* exposed to Roundup® (96 h) and equal recovery period. Values are expressed as mean ± SD (n = 10). *Asterisk* indicates significant difference between exposure groups and control group (0 mg/L) in the period ($p \leq 0.05$). *Different letters* indicate significant difference between recovery and exposure periods ($p \leq 0.05$)

activity (Glusczak et al. 2006). Recent study using tropical fish *Prochilodus lineatus* report for the first time AChE inhibition in muscle due to Roundup exposure (Modesto and Martinez 2010) and also brain inhibition like in this study. Miron et al. (2005) showed that clomazone herbicide exposure at concentrations above 5.0 mg/L inhibited both brain and muscle AChE activity. In addition clomazone caused erratic swimming and fish feeding alterations. However, no behavior change was observed when carp were exposed to glyphosate. A high AChE inhibition (93%) was also observed in *Oreochromis niloticus* exposed to the insecticide diazinon (1 and 2 mg/L) (Üner et al. 2006). Changes in AChE activity is frequently used as a biomarker of pesticide contamination in fish, particularly for OP and carbamate insecticides (Dembelé et al. 1999; Roex et al. 2003; Aguiar et al. 2004; Rodrigues et al. 2011). However, several studies in recent years have also shown that some herbicides inhibit AChE activity (Miron et al. 2005; Glusczak et al. 2006; Modesto and Martinez 2010; Moraes et al. 2011). The exposure of silver catfish to clomazone concentrations used in rice field significantly decrease muscle AChE activity (Crestani et al. 2007).

Regarding recovery of AChE activity after pesticide exposure some authors found that this hypothesis is true. Fernández-Vega et al. (2002) showed recovery of AChE activity to control values in muscle of ells exposed to thiobencarb for a period of 6 days in clean water. The time required to recovery for AChE activity varies with the type of pesticide and fish species tested (Fernández-Vega et al. 2002; Crestani et al. 2007). Dembelé et al. (1999) showed that brain AChE activity was almost completely recovered within one day after exposure to carbofuran (carbamate) and 15 days after exposure to chlorfenvinphos (organophosphate). In our study, AChE activity not only recovered to the level of the control, but exhibited a significant induction in activity level (Figs. 2, 3). The brain AChE activation observed in recovery period could represent an increase in the hydrolysis of the neurotransmitter acetylcholine, with consequent decrease activation of nicotinic and muscarinic receptors. In fact, animals could be compensating the metabolic stress enhancing brain AChE activity and this enhancement might influence cholinergic neurotransmission process. The AChE activation is a really unusual process, but some authors have shown similar results that those found in the present study regarding AChE increase activity in recovery period. Moraes et al. (2011) reported brain AChE increase in carps exposed to herbicide Only® (imzathapyr + imazapic) at field and laboratory study (7 days). Miron et al. (2005) showed similar results where brain AChE inhibition was observed in brain of *Rhamdia quelen* exposed to herbicide clomazone, but quinclorac and metsulfuron methyl cause AChE activation. Another hypothesis to consider is the possible interaction between lipid peroxidation and changes in AChE activity. Yang and Dettbarn (1996) in their study with diisopropylfluorophosphate suggested that AChE inhibitor-induced cholinergic hyperactivity initiates the accumulation of free radicals leading to lipid peroxidation, which may be the initiator of AChE inhibition. The results showed in this study also are in agreement with those observed by Miron et al. (2008) where TBARS increase in brain and muscle of *Leporinus obtusidens*. For the same tissues AChE inhibition was observed. This hypothesis is

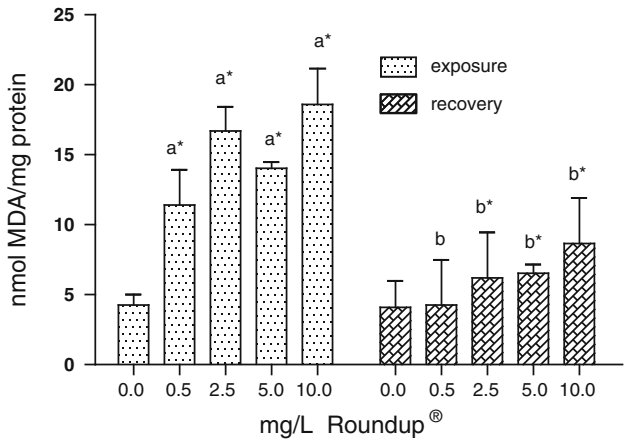

Fig. 4 TBARS levels (nmol MDA/mg protein) in brain of *Cyprinus carpio* exposed to Roundup® (96 h) and equal recovery period. Values are expressed as mean ± SD ($n = 10$). *Asterisk* indicates significant difference between groups and control group (0 mg/L) in the period ($p \leq 0.05$). *Different letters* indicate significant difference between recovery and exposure periods ($p \leq 0.05$)

feasible in the present study due to increase in brain TBARS observed in all exposure periods and consequent reduction of AChE activity recorded in the same period.

TBARS levels increased significantly at all concentrations tested, and remained high during the recovery period (Fig. 4), indicating that water contaminated with Roundup® at tested concentrations cause oxidative stress in carp. Lipid peroxidation results of the present study are in agreement with those obtained by Crestani et al. (2007), where increased brain TBARS levels were recorded in *R. quelen* after exposure to clomazone herbicide. Lipid peroxidation can be generated in various fish tissues after exposure to herbicides (Miron et al. 2008). Elevation of TBARS has also been described in liver of fish *Prochilodus lineatus* (Modesto and Martinez 2010) and muscle of the *Rhamdia quelen* (Glusczak et al. 2007) both exposed to Roundup®. In fact, Roundup® (RD) exposure may induce oxidative damage at different tissues leading to the generation of free radicals and lipid peroxidation could be a mechanism involved in pesticide toxicity. In addition to lipid peroxidation induced by RD exposure TBARS showed recovery levels only at concentration of 0.5 mg/L (Fig. 4). TBARS levels remained higher in most of herbicide treatment indicating that the fish continues to suffer toxicity even after returning to the water RD-free.

In summary, this study has shown that short-term exposure to concentrations of Roundup® herbicide between 0.48 and 9.8 mg/L were toxic to brain and muscle tissues of carp, causing cholinergic disruption and oxidative damage. Besides this, the Roundup® (particularly glyphosate) is considered low toxic to animals according to the World Health Organization. However, the extensive use of RD may still cause environmental problems with negative impact on wildlife, particularly in an aquatic environment where chemicals may persist for a long time (Lushchak et al. 2009). Regarding undesirable effects that could be caused by surfactant POEA and in line with this work more studies are necessary to discriminate what component of the formulated product, glyphosate or POEA could be responsible for ROS generation and AChE inhibition.

The results showed that the commercial formulation of glyphosate (Roundup®) affect the toxicological parameters of this species, indicating that the short-term exposure can affect their physiological conditions. After the recovery period in clean water, AChE activity was not recovered in the brain and muscle. In addition, the cerebral TBARS remained higher after the recovery period, a situation that confirms the hypothesis that cholinergic hyperactivity may increase lipid peroxidation.

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