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Time-Effect Relationship of Toxicity Induced by Roundup® and Its Main Constituents in Liver of Carassius Auratus

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ABSTRACT

In order to evaluate the eco-toxicological effects of Roundup® on *Carassius auratus* (*C. auratus*), fish were exposed to 32 μg/L Roundup®, isopropylamine salt of glyphosate (G.I.S) and polyoxyethylene amine (POEA) over different periods (0.5, 1, 3, 7 and 14 d). Hydroxyl radical (·OH), malondialdehyde (MDA) and acetylcholinesterase (AChE) in liver were detected in this study. Results showed that the generation of ·OH increased before 7 d, but without significantly difference. ·OH was induced at 1 d for POEA group, 3 d for Roundup® group and 7 d for G.I.S group. At 14 d, ·OH generation returned to normal levels. MDA contents all increased significantly ($p < 0.01$) during 7 days and then reached a normal level at 14 d. AChE activity in all group tests revealed a significant inhibition ($p < 0.01$) after 7 days exposure and then rebounded a little, but remained below the control after 14 days exposure. The rate of AChE inhibition range from 13% - 42% in Roundup®, 6% - 40% in G.I.S, and 21% - 54% in POEA, suggesting that POEA was more toxic compared to Roundup® and G.I.S. 32 μg/L Roundup® exposure led to the change of physiological and biochemical indexes in *C. auratus*, which was a reversible process in the long run.

Keywords: *Carassius Auratus*; Roundup®; Hydroxyl Radical (·OH); Toxicity

1. Introduction

Roundup®, the main glyphosate formulations, is already mixtures of glyphosate and various adjuvants at different concentrations [1]. The original formulation of Roundup® contains isopropylamine salt of glyphosate (G.I.S) as the active ingredient and polyoxyethylene amine (POEA) as the surfactant agent [2]. Since Roundup® can easily reach the aquatic systems by runoff, drainage, leaching or inadvertent aerial overspray, the herbicide represents a dangerous and widely spread group of environmental contaminants [3]. However, the knowledge on the time–effect of toxicity induced by environmental concentration of Roundup® and its main constituents to fish is still limited.

Researches suggest that reactive oxygen species (ROS), can be induced in organisms exposed to some environmental contaminants [4-6]. Recent study suggested that Roundup® might induce oxidative stress in aquatic organisms through the increased levels of tissue lipid hydroperoxides [7]. Few direct evidences can prove ROS generation and oxidative stress in aquatic organisms exposed to Roundup® and its main constituents.

The measurement of acetylcholinesterase (AChE) activity in different fish tissues has also proved to be a sensitive method for detecting the presence of several herbicides [8,9]. The inhibition of AChE causes an accumulation of acetylcholine in the synapse, which therefore AChE cannot function in a normal way [10]. Glusczak et al. [11] reported that Rhamdia quelen showed significant reduction in AChE activity after exposed to Roundup®.

In this study, *Carassius auratus* (*C. auratus*), commonly found in China, is chosen as the testing aquatic organism. The aim of the study is to investigate the time-effect of environmental concentration glyphosate herbicide on oxidative stress and AChE activity of native freshwater fishes, and to compare the toxicity difference of Roundup® and its main constituents (G.I.S and POEA) to *C. auratus*.

2. Experimental Methods

2.1. Chemicals

Roundup® solution (41% purity, containing 41% G.I.S and 18% POEA), was obtained from Monsanto Company (St. Louis, MO, USA). G.I.S (41% purity) was purchased from Sigma Chemical (St. Louis, MO, USA). POEA was purchased from Haian petrochemical complex (Jiangsu, China). The other reagents used were analytically pure,

such as the spin-trapping agent, a-phenyl-N-tert-butylnitrone (PBN), 2-thiobarbituric acid (TBA), and bovine serum albumin (BSA), which were purchased from Sigma Chemical.

2.2. Experimental Fish and Pollutants Treatment

Gold fish (*C. auratus*) with 9.9 ± 0.10 cm body length and 20.2 ± 0.75 g body weight were purchased from Fuzimiao aquatic breeding base (aquaculture facility, Nanjing, China). All fish were acclimatized to water dechlorinated with activated carbon for two weeks before the experiment. The total mortality of fish was below 3%.

In the Canadian Water Quality Guideline, the safety exposure concentration of glyphosate was 65 μg/L considered protective of aquatic life [12]. So in this experiment, we set half of 65 μg/L as the sole concentration. After acclimatization, fish were randomly divided into sixteen groups and kept in glass aquaria. One group was designated for control and the other groups were employed as experimental groups that received concentrations 32 μg/L of Roundup® (containing 41% G.I.S and 18% POEA), G.I.S (41% purity), and 18% POEA (equal to the concentration in Roundup®), respectively, for 0.5, 1, 3, 7 and 14 d. During the experiment, 50% water was replaced daily by adding fresh Roundup®, G.I.S and POEA solution to minimize contamination from metabolic waste. Artificial dry food was provided once a day. Fish were sampled after exposure. Then the fish were dissected to obtain some fresh livers for the determination of hydroxyl radical. The rest of the livers were homogenized at 4˚C for other experiments. During the experiment, the water conditions were as follows: Keep the dissolved oxygen levels at 5 mg/L by continuous aeration, temperature at 20˚C ± 1˚C, pH 7.0 ± 0.3.

2.3. PBN Adduct Extraction and Electron Paramagnetic Resonance (EPR) Analysis

ROS production in livers of *C. auratus* were measured using PBN as the spin-trapping agent [13]. After being rinsed with ice-cold physiological salt water, 0.1g of an fish liver sample was removed and homogenized quickly in 1.0 mL 50 mmol/L PBN (dissolved in dimethylsulfoxide (DMSO)) using a Teflon pestle in a potter homogenizer. 0.1mL supernatants was transferred to a capillary tube with a diameter of 0.9 mm, and placed in liquid nitrogen for EPR meaurements. The whole operation was carried out in an incubation system with continuous N_2 puring. The EPR spectra were recorded on a Bruker EMX 10/12 X-band spectrometer (Bruker, Germany) at room temperature (25˚C), with the operation conditions of magnetic field center 3470 G, scan range 200 G, modulation frequency 100 kHz; modulation amplitude 0.5 G, microwave frequency 9.751 GHz, incident microwave power 20 mW, and sweep time 84 s for 5 scans.

2.4. Degree of Lipid Peroxidation Determined Using Malondialdehyde (MDA)

MDA content was measured by previous published thiobarbituric acid assay of Miller and Aust [14] with some modification. The reaction mixture containing 0.2 mL of tissue homogenate, 0.2 mL 8.1% sodium dodecyl sulfate (SDS), 1.5 mL 20% acetic acid buffer (pH 3.5), 1.5 mL 1% TBA, and 1 mL distilled water was heated at 90˚C for 90 min, then cooled at room temperature and centrifuged for 15 min at 3,000 rpm/min. The absorbance of the supernatant was determined at 532 nm using a UV-220 spectrophotometer (Shimadzu, Japan). The amount of MDA formed was calculated by measuring the absorbance at 532 nm using a molar extinction coefficient of $1.56 \times 105\ M^{-1}\cdot cm^{-1}$.

The total protein content from enzyme extraction was measured using BSA as a standard [15]. All the determination assays were performed in triplicate, at a minimum.

2.5. AChE Activity Assay

Fish liver samples were weighed and homogenized in normal saline. The homogenate was centrifuged for 10 min at 4˚C at 3,500 rpm and the supernatant was used as the enzyme source. AChE activity was determined with a AChE Detection kit from Nanjing Jian Cheng Biology Company (Nanjing, China).

2.6. Statistical Analysis

Statistical analyses were performed using SPSS statistical package version 16.0. Data were expressed as mean values±standard deviation (SD). The differences between experimental groups and the control were compared by a one-way analysis of variance (ANOVA), and significantly different treatments were identified by Dunnett's test. The level of statistical significance was set at significantly different from control $p < 0.05$ (*) and highly significantly different from control $p < 0.01$ (**).

3. Results and Discussion

3.1. Free Radical Production by Induction of Roundup®, G.I.S and POEA

Signals of PBN adducts of fish hepatic after Roundup®, G.I.S and POEA exposure could be detected using EPR (**Figure 1**). A six line spectrum of three groups with two hyperfine coupling splitting peaks was observed. According to previous literature [4,5,13,16], the trapped ROS was likely to be the hydroxyl radical (·OH) and the levels could be expressed with the second couplet intensity of the triplets in the EPR spectra.

Figure 2 showed the kinetics of ·OH generation over

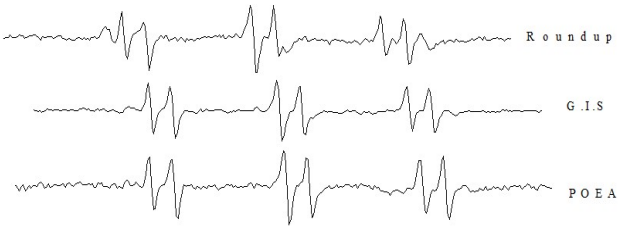

Figure 1. EPR was used to detect PBN-radical adducts in the liver of *C.auratus* with Roundup®, G.I.S and POEA.

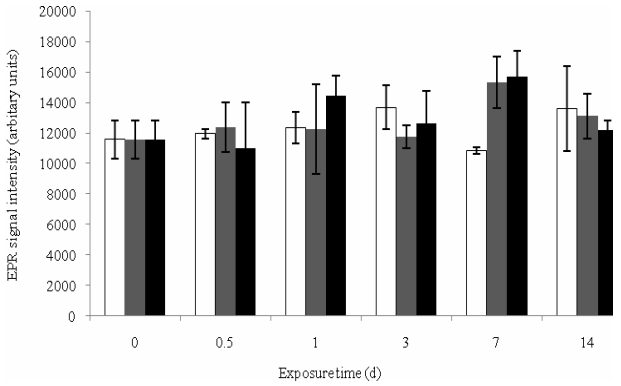

Figure 2. ·OH signal intensity in fish liver after exposure to 32 μg/L Roundup® (blank bars), G.I.S (grey bars) and POEA (black bars), for different experimental periods.

different periods (0.5, 1, 3, 7 and 14 d) of 32 μg/L Roundup®, G.I.S and POEA exposure. ·OH generation increased first and then decreased nearly with the control group, but without significantly difference. ·OH was induced at 1 d for POEA group, 3 d for Roundup® group and 7 d for G.I.S group. ·OH accumulation was earlier under POEA group conditions than under Roundup® group and G.I.S group, indicating that the speed of oxidant stress of POEA is faster than Roundup® and G.I.S in *C. auratus*. The maximum accumulation of ·OH generation was 118% (of the control) in Roundup®, 132% (of the control) in G.I.S and 135% (of the control) in POEA. Considering the speed and the maximum of ·OH accumulation, POEA is supposed to be more toxic than the other two substances. Howe et al. [17] found that for *Rana clamitans*, acute toxicity values in order of decreasing toxicity were POEA ＞ Roundup®.

It was reported that the ·OH can be significantly induced by phenanthrene, pentachlorophenol, pyrene and 2-Chlorophenol in fish liver [18-21]. In the present study, the ·OH generation induced by Roundup®, G.I.S and POEA, but without obviously accumulated. It might be explained that the activities of antioxidant enzymes were activated and induced to remove ·OH to protect organisms from oxidative stress. After 14 days, the ·OH signal intensity returned to normal levels. This could be explained as follows: First, the ·OH had been reduced by antioxidant defense systems, or metabolized to less harmful radicals. Second, the fish had adjusted itself to combat the oxidative stress. Third, glyphosate formulations does not accumulation in vivo [22] and protect the fish from many harmful effects.

3.2. Changes in MDA

One of the most damaging effects ROS and their products in cells is the peroxidation of membrane lipids, which can be indicated by MDA detection [16]. MDA contents in fish livers after exposure to Roundup®, G.I.S and POEA were illustrated in **Figure 3**. Roundup®, G.I.S and POEA group elevated ($p < 0.01$) the MDA contents during 7 days and returned to normal levels at 14 d. The MDA contents increased at 0.5 d after Roundup®, G.I.S and POEA exposure, and they kept increasing until a maximum level were reached at 3 d in Roundup® (148% of the control) and G.I.S (170% of the control), at 1 d in POEA (180% of the control). The speed of lipid peroxidation may be further confirmed that POEA was more toxic than the other two pollutants. Obviously, Roundup® and its main constituents exposure resulted in an accumulation of lipid peroxidation in *C. auratus* during 7 days. But at 14 d, MDA contents were the same as the control group, indicating that exposure of low concentration of Roundup® to fish is a reversible process. This maybe explained by an adaptive response takes place in the cells or might be due to the activities of various damage removal and repair enzymes, to minimize the concentration of ·OH to the basal level and blocked lipid peroxidation in the cell.

Detailed studies have provided evidence that some xenobiotics induced MDA contents following stress on many species [5,21,24]. In the present study, ·OH generation induced without significantly difference, which suggested the self-adjusting of fish by activating antioxidant capacity to combat the cellular excess ROS generation. However, the increased MDA contents during 7 days proved that the lipid peroxidation in fish liver was promoted and further revealed that the fish was already

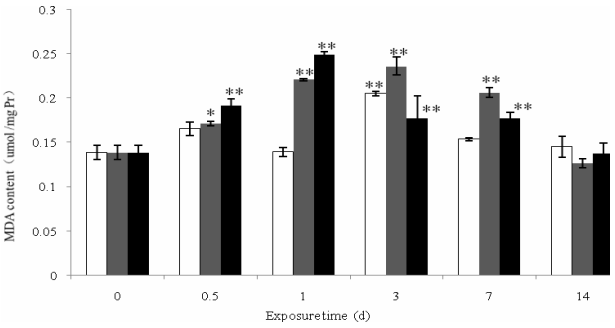

Figure 3. MDA content in fish liver after exposure to 32 μg/L Roundup® (blank bars), G.I.S (grey bars) and POEA (black bars), for different experimental periods.

in the status of oxidative stress although the results did not showed the accumulate of ·OH. According to Luo et al. [20], the higher MDA level by the end of the exposure to 2-chlorophenol in *C. auratus* suggested the oxidative damage occurred although hydroxyl radical returning to the normal level. In the study, the MDA content did not have a time-response pattern consistent with ·OH generation at all exposure time in Roundup®, G.I.S and POEA, which might be due to the effectiveness of the antioxidant system in providing protection. Sun et al. [21] found the similar change pattern of MDA in liver after different doses of pyrene were exposed to the fish (*C. auratus*).

3.3. Changes in AChE Activity

AChE activity in the liver of *C. auratus* exposed to Roundup®, G.I.S and POEA, for different experimental periods was displayed in **Figure 4**. All tests revealed a significant inhibition of AChE activity during 7 days exposure, then restored to the level of control group after 14 d. The inhibition percentages in Roundup®, G.I.S and POEA of the control fish was 13% - 42%, 6% - 40% and 21% - 54%, respectively. The degree of AChE reduction showed the toxicity order of the chemicals was: POEA＞ Roundup® ＞ G.I.S. On the basis of Giesy et al. [24], the LC_{50} values (mg/L) for rainbow trout were between 8.2 and 27 for Roundup®, between 0.65 and 7.4 for POEA.

Organophosphorus pesticides have several toxic properties, the most prominent effect of which is AChE inhibition. AChE activity is therefore widely used in biomonitoring studies as a biomarker of organopho- sphorus pesticide exposure [25]. In this study, the reduction of AChE activity is assumed to have been resulted from the direct action of Roundup®, G.I.S and POEA exposure on active site of this enzyme. Glusczak et al. [26] reported the inhibition of this enzyme in the brain of *Leporinus obtusidens* exposed to 3, 6, 10 and 20 mg/L glyphosate for 96 h. Modesto and Martinez [27] also reported a decreasing AChE activity in the brain of *Prochilodus*

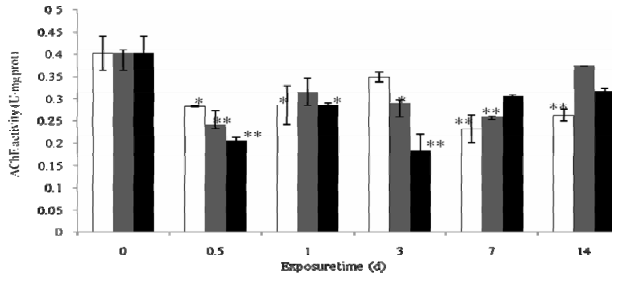

Figure 4. AChE activity in fish liver after exposure to 32 μg/L Roundup® (blank bars), G.I.S (grey bars) and POEA (black bars), for different experimental periods.

lineatus after exposure to 1 and 5 mg/L Roundup® for 96 h. The exposure of the three pollutants led to the maximum inhibition of AChE in POEA by 54%. The rate of AChE inhibition may not be considered a life-threatening situation since fish are capable of tolerating over 90% AChE inhibition [28]. After 14 days exposed to Roundup®, G.I.S and POEA, AChE activity rebounded a little, which were consistant with the alteration of ·OH generation and MDA contents, indicating that long-term exposure of Roundup®, G.I.S and POEA with low concentration in *C. auratus* is a reversible process.

4. Conclusions

The present study showed that Roundup®, G.I.S and POEA may cause changes in the metabolic and enzymatic parameters of fish during 7 days, such as ·OH generation addition, lipid peroxidation and AChE inhibition, implying that the fish was already in the status of oxidative stress. At 14 d, ·OH generation, MDA contents and AChE activity all returned to the normal level, indicating that the exposure of Roundup®, G.I.S and POEA with 32 μg/L in *C. auratus* is a reversible process in a long time. According to the toxic effects of Roundup®, G.I.S and POEA on *C. auratus*, POEA may be the most toxic pollutant. Roundup®, G.I.S and POEA should be distinguished when assessing the toxicity of this pesticide.

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