Fish & Shellfish Immunology 30 (2011) 51–57

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Altered hematological and immunological parameters in silver catfish (*Rhamdia quelen*) following short term exposure to sublethal concentration of glyphosate

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ARTICLE INFO

Article history:
Received 3 August 2010
Received in revised form
17 September 2010
Accepted 18 September 2010
Available online 29 September 2010

Keywords:
Silver catfish
Glyphosate
Blood cells
Phagocytosis
Natural immunity

ABSTRACT

Using agrichemicals to control unwanted species has become a necessary and common worldwide practice to improve crop production. Although most currently used agrichemicals are considered relatively safe, continuous usage contributes for soil and water contamination and collateral toxic effects on aquatic species. Few studies correlated the presence of agrichemicals on fish blood cells and natural immune system. Thus, in this study, silver catfish (*Rhamdia quelen*) were exposed to sublethal concentrations (10% of the LC_{50-96h}) of a glyphosate based herbicide and hematological and natural immune system parameters were evaluated. Silver catfish fingerlings exposed to glyphosate for 96 h had a significant reduction on blood erythrocytes, thrombocytes, lymphocytes and total leukocytes in contrast to a significant increase in the number of immature circulating cells. The effect of glyphosate on natural immune system was evaluated after 24 h or 10 days exposure by measuring the phagocytic index of coelomic cells, and lysozyme, total peroxidase, bacteria agglutination, bactericidal activity and natural complement hemolytic activity in the serum of fingerlings. A significant reduction on phagocytic index, serum bacteria agglutination and total peroxidase was observed only after 24 h exposure to glyphosate. In contrast, fingerlings exposed to glyphosate for 10 days had a significant lower serum bacteria agglutination and lysozyme activity. Glyphosate had no effect on serum bactericidal and complement natural hemolytic activity after 24 h or 10 days exposure. Nonetheless, the information obtained in this study indicates that glyphosate contaminated water contributes to alter blood cells parameters and to reduce the activity of natural immune components important to mediate fish resistance to infecting microorganisms.

1. Introduction

Intensive crop production requires the use of agrichemicals with selective effect on unwanted invasive weeds. Glyphosate, a worldwide used herbicide, consist of an isopropylamine salt (IPA) mixed with a non-ionic polyethoxylated amine surfactant (POEA) and water, that has been used mostly in rice and glyphosate-resistant transgenic soybean culture [1]. Glyphosate-based herbicides used as expected and under field conditions is considered of low risk to humans, other mammals and birds [2]. Nonetheless, even though the active ingredient of glyphosate is readily dissipated in water, and soil micro flora contributes to its biodegradation, there is a potential for toxic effects mainly for aquatic organisms present in water ponds and springs found in agricultural areas, that are much more sensitive to glyphosate that other species [2,3]. Glyphosate and several other agrichemicals have been detected in soil and water in south America [4,5] and the adverse effect of commercial formulation of glyphosate on fish species has been a matter of increasing interest [6–10].

The toxicity of glyphosate to silver catfish (*Rhamdia quelen*), a South American teleostean fish from the Heptapteridae family, has been recently demonstrated [6]. Low concentrations of glyphosate, such as those used in rice and soybean fields, might cause changes in metabolic and enzymatic parameters of silver catfish [8] and other fish species [9,10] like inhibition of brain acetylcholinesterase (AChE), lipid peroxidation and protein catabolism. Sublethal concentration of glyphosate increases the level of silver catfish seric cortisol [11], but does not interfere with the ability to cope with an additional stress [12]. In addition, silver catfish females kept in earthen pound and exposed to sublethal concentrations of glyphosate had higher liver-somatic index (LSI), lower concentration of 17β-estradiol and decreased egg viability [7].

The effects of glyphosate in other fish species have also been investigated. In piava (*Leporinus obtusidens*) exposed to glyphosate,

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doi:10.1016/j.fsi.2010.09.012

a significant increase in hepatic glycogen and glucose was observed [9], concomitant with a reduction of hematological parameters and brain AChE. Following exposure of *Prochilodus lineatus* to glyphosate contaminated water, a significant increase in plasma glucose and catalase activity were reported [13]; in the same study, hepatocyte morphology and histological alterations were also detected. Hepatocyte histological changes were also reported in *Oreochromis niloticus* [14] after glyphosate exposure. In goldfish (*Carassius auratus*), glyphosate caused a significant reduction in superoxide dismutase, glutathione S-transferase, glutathione reductase and glucose-6-phosphatase dehydrogenase, and a substantial increase in liver catalase [10]. In the neotropical fish *Prochilodus lineatus* glyphosate induced oxidative stress and lipid peroxidation [15]. Thus, the data reported so far indicates that commercial formula of glyphosate causes a typical stress response on fish with a likely effect on immune response. Indeed, altered protein biosynthesis and immune function were observed in Tilapia (*Tilapia nilotica*) exposed to glyphosate [16].

Even though the adverse effect of commercial glyphosate on fish physiological and biochemical parameters have been reported, few studies aimed to investigate the natural immune response of aquatic organisms exposed to herbicide contaminated water [16,17]. Recently, we demonstrated that silver catfish exposed to sublethal concentrations of glyphosate were more susceptible to intracoelomic challenge with *Aeromonas hydrophila*; in addition, in these fish, macrophage collected from the coelomic cavity had reduced phagocytic activity [18]. Thus, because the use of commercial glyphosate has dramatically increased in recent years and many of the adverse effect are related to oxidative stress and a likely effect on immune response, we aimed to investigate whether hematological and natural immune parameter were compromised on silver catfish exposed to water containing non-lethal concentrations of glyphosate.

2. Materials and methods

2.1. Fish

Silver catfish fingerlings of both sexes, with an average weight of 18.0 ± 8 g were used to determine immunological parameters; juveniles catfish with an average weight of 80–100 g were used for hematological studies. All fish were transferred from the experimental farm to laboratory tanks containing aerated water and acclimated for 7 days (7–10 fish/tank; specific density < 1 g of fish/L). Water temperature was kept at 22.0 ± 2.0 °C, pH 7.4±0.6 units, dissolved oxygen 7.8±0.4 mg/L; total ammonia was lower than 0.01 mg l^{-1} and total hardness and alkalinity were 66 and 22 mg l^{-1} $CaCO_3$, respectively.

2.2. Experimental design

All experiments were carried out in triplicates with at least one control group. Following the adaptation period, commercial available glyphosate (N-phosphonomethyl glycine, 360 mg/L) was added to the water to a final concentration of 0.730 mg/L^{-1} which corresponded to 10% of the previously reported CL_{50-96h} [6] for silver catfish. The effects of glyphosate on hematological parameters were determined in fish kept in contaminated water for 96 h; immunological parameters were evaluated after 24 h or 10 days exposure to glyphosate contaminated water. Fish were fed twice daily with commercial fish pellets (42% crude protein, Supra, Brazil). Tanks were cleaned every two days and the water quality did not change during the experimental period.

2.3. Culture of pathogens

A. hydrophila [19] and *Micrococcus luteus* (ATCC 7468) were cultured in Brain Heart Infusion (BHI) for 18 h at 37 °C. The broth cultures were centrifuged ($600 \times g$ for 20 min), the supernatant discarded and the bacteria pellet was washed three times in PBS (pH 7.4). Final bacteria concentration was adjusted using a spectrophotometer: *A. hydrophila* was adjusted to 0.1 OD at 550 nm, and *M. luteus* to 0.5 OD at 450 nm. For both bacteria, the number of colony forming units (CFU)/ml^{-1} was determined using standard dilution methodology and plating on Tryptic Soy Agar (TSA) for 24 h at 37 °C. A local isolate of *Candida albicans* was grown on yeast extract (1%), peptone (2%) and glucose (2%) at 37 °C for 18 h, then washed three times with PBS (pH 7.4) and coupled to Fluorescein Isothyocyanate (FITC), according to manufactures instructions (Sigma, St Louis, USA). *A. hydrophila* was used for the bactericidal and hemagglutination assays and *M. luteus* was used to determine lysozyme activity. FITC-labeled *C. albicans* was used to determine the phagocytic index (PI).

2.4. Collection of blood and phagocytic cells

Following exposure to glyphosate all fish were captured and anesthetized with Eugenol (50 mg/L). For the hematological studies, blood samples were drawn from the caudal vein using sterile heparinized syringes. To determine immune parameter, blood was allowed to clot on ice-chilled water for 2 h, centrifuged ($600 \times g$, 4 °C, 10 min) and serum aliquots were stored (−18 °C) until use. To collect phagocytic cells, anesthetized fish were placed on ice-chilled water, transferred to the laboratory and the coelomic cavity was injected with 3 ml of ice-cold sterile phosphate buffered saline (PBS, pH 7.4). After 1 min, the PBS-containing phagocytic cells was collected from the coelomic cavity and the cells pelleted by centrifugation ($600 \times g$), counted and suspended in RPMI media containing 1% fetal bovine serum (FBS; Cultilab, Brazil) to a final concentration of 10^6/ml^{-1}.

2.5. Hematological parameters

Blood smear were prepared immediately after sampling, air-dried and submitted to Wright-Giemsa staining. Hematocrit, hemoglobin and erythrocyte counts were determined on whole blood within 2 h after sampling, as previously described [20].

2.6. Phagocytosis assay

The phagocytic activity of coelomic cells was determined, for each fish, using *C. albicans* coupled to fluorescein isothiocyanate (FITC). For the phagocytic assay, 10 μl of FITC-labeled *C. albicans* (10^9/ml) were thoroughly mixed with 200 μl of coelomic cells (10^6/ml^{-1}) in RPMI medium (1% FBS) and two aliquots of 100 μl each were layered over a circular (13 mm diameter) coverslip glass lamina placed inside the wells of a 24-wells tissue culture plates, and incubated at 22 °C for 15 min. Following that, the wells were washed three times with PBS to remove both non-adherent cells and non-phagocyted FITC-labeled *C. albicans*, the coverslip was removed, washed once more in PBS, quenched with Evans blue for 30 sec., fixed with standard histological solution and mounted over a microscopic lamina. To determine the phagocytosis index (PI), 100 cells were counted using an epifluorescent microscope, and the number of cells containing engulfed FITC-labeled *C. albicans* was registered, in duplicates, for each fish.