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Flow cytometry evaluation of phagocytosis and respiratory burst in silver catfish (*Rhamdia quelen*) leukocytes exposed *in vitro* to atrazine

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Abstract: Water contamination by agrichemical is a matter of major concern to fish producers and researchers in that the presence of xenobiotics might affect fish immune system and their capacity to cope with water-borne pathogenic microorganisms. The purpose of this study was to evaluate flow cytometry as a tool to access the phagocytic and respiratory burst activity of silver catfish (*Rhamdia quelen*) leukocytes cultured in the presence or absence of atrazine. We found that phagocytosis and respiratory burst activity were reduced ($p < 0.05$) in leukocytes exposed to atrazine compared to non-exposed leukocytes. Our results further highlight the immunotoxic effect of atrazine and offers additional tools to monitor fish health in areas in which water contamination is a matter of concern.

Key words: fish, innate immunity, phagocytic assay, agrichemicals

Uso da citometria de fluxo para avaliar a fagocitose e explosão respiratória em leucócitos de jundiás (Rhamdia quelen) expostos in vitro ao atrazine. Resumo: A contaminação das águas por agrotóxicos é uma preocupação constante dos produtores e pesquisadores visto que a presença de xenobióticos na água pode afetar o sistema imune e a capacidade dos peixes em se defender de micro-organismos presentes na água. O objetivo deste estudo foi avaliar o uso da citometria de fluxo como uma ferramenta para a avaliação da fagocitose e explosão respiratória em leucócitos de jundiás (*Rhamdia quelen*), cultivados na presença ou ausência de atrazine. A fagocitose e a explosão respiratória foram significativamente reduzidas ($p < 0.05$) nos leucócitos cultivados na presença de atrazine em comparação com leucócitos não expostos. Nossos resultados ressaltam a imunotoxicidade do atrazine e oferecem novas ferramentas para monitorar a saúde dos peixes principalmente em regiões onde a contaminação das águas é preocupante.

Palavras-chave: peixes, imunidade inata, ensaio fagocítico, agrotóxicos.

Introduction

Phagocytosis is at the center of innate immune response and is carried out by different cell types (Uribe-Querol & Rosales 2020). Monocytic cells like macrophage and dendritic cells, for instance, are located mostly on tissues covering body entrances (Smith *et al*. 2019) and use specialized receptors to identify pathogen-associated molecular patterns (PAMP) which are considered a hallmark of pathogens. Upon recognition, these cells internalize the pathogens for destruction on specialized vesicles named phagolysosome by means of enzymatic

digestion; simultaneously, phagocytic cells increase the consumption of O_2 in a process known as respiratory burst, that triggers the production of reactive oxygen species (ROS) which are lethal to pathogens (Uribe-Querol & Rosales 2020). Furthermore, upon stimulation, macrophage and dendritic cells secrete a suit of cytokines that orchestrates the upcoming innate and adaptive immune response to better counterattack the invading pathogen (Secombes *et al*. 2011). Some cytokines like TNF-α and IL1-β drive circulating polymorphonuclear neutrophils to the site of infection and these cells provide additional support on pathogen phagocytosis and destruction. In addition, as demonstrated more recently in fish, the lymphocytic B cell is also capable of phagocytic and bactericidal activity through the formation of phagolysosomes (Smith *et al*. 2019; Wu *et al*. 2020). As such, phagocytosis is a vital early defense mechanism that plays a major role in linking innate and adaptive immune responses.

Fish encounter with water-borne microorganism is almost inevitable and impairment of phagocytosis and respiratory burst activity might compromise their capability to cope with infections. Several water contaminants have been shown to affect the innate and adaptive immune response of fish. Agrichemicals, for instance, are usually found on water bodies that run through or are located in the vicinity of crop field and their effect on fish health has been intensively investigated (van der Kraak *et al*. 2014). In previous work we found that glyphosate and atrazine, two of the most commonly found herbicides used in crop fields, have a major impact on innate immune response of silver catfish (*Rhamdia quelen*) (Kreutz *et al*. 2010), a bagrid fish species endemic in south American rivers and lakes (Schulz & Leuchtenberger 2006). Exposure of silver catfish to glyphosate or atrazine reduces the phagocytic index of cells collected from the intraperitoneal cavity (Kreutz *et al*. 2012) and, as a consequence, the resistance to *Aeromonas hydrophila* challenge. Interestingly, the *in vivo* effects of atrazine on phagocytosis are observed at most shortly after exposure (e.g. 24h) and return to basal levels up to 10 days after. Furthermore, monocytes exposed *in vitro* to sublethal concentration of atrazine had reduced expression of TNF-α and IL-1-β (Kirsten *et al*. 2017) which are central to optimal anti-bacteria resistance.

Phagocytosis and respiratory burst are evolutionary conserved across species (Uribe-Querol & Rosales 2020) and considered suitable markers to evaluate the immunotoxic effect of xenobiotics in indigenous animal species including fish. Traditionally, phagocytosis has been evaluated using particulate antigens and microscope-based techniques which are quite laborious. Recently, however, flow cytometric analysis have been applied to evaluate phagocytosis and respiratory burst in immune cells from several aquatic animal species like Lumpsucker (*Cyclopterus lumpus* L.), dolphins (*Tursiops truncatus*) and rainbow trout (*Oncorhynchus mykiss*) (Haugland *et al*. 2012; Keogh *et al*. 2011; Rehberger *et al*. 2021) but not yet in silver catfish. Thus, here we used flow cytometry to evaluate phagocytosis and respiratory burst in silver catfish leukocytes cultivated for 24h in the presence of atrazine.

Material and Methods

Fish: adult male silver catfish (300 g ± 50 gr) were used in this study. Before the experiment fish were acclimatized for 10 days into indoor tanks with a natural source of running water obtained from a water well (23/25ºC) free of atrazine. Water parameters were within accepted range for the species (Kirsten et al. 2017). A commercial pelleted feed (crude protein 30%; fiber 3%; fat 8%; and ash 14%; Supra, Brazil) was offered twice daily *at libitum*.

Leukocytes isolation from head kidney: silver catfish (n = 5) were profoundly anesthetized with Eugenol (50 mg/L^{-1}), immersed on ice and transported to the laboratory for aseptically removing of the head kidney. The head kidney was gently macerated and forced through a cell strainer (100µM) using a syringe plunger and ice cold RPMI containing 30 IU of heparin. The cells were collected by centrifugation (500x g; 10min at 4ºC) and then dispensed on top of a percoll (Sigma Aldrich, Brazil) layer of 1.02 g/ml overlaid on a ficcol (Sigma Aldrich, Brazil) layer of 1.077 g/ml. After centrifugation (25 min., 800 x g, 4ºC) the mononuclear leukocytes were collected from the top of the 1.077 g/ml layer and washed three times with ice-cold serum and antibiotic free RPMI medium. The cells were then distributed in at least 12 wells of a 24-well plates (1 x 10^6 cells/well) with serum and antibiotic free RPMI medium and incubated at 27ºC and 5% CO_2. Six wells of cells from each fish were exposed to atrazine (10 µg/ml; Sigma Aldrich, Brazil) as described in a previous study (Kirsten et al. 2017), during 24 h and the non-exposed cells from the remaining 6 wells were cultured under the same conditions and used as control. Cell viability

was assessed by trypan blue exclusion prior to plating and after 24h of incubation and was higher than 95%.

Bacteria labelling, phagocytosis and respiratory burst assays: the phagocytic assay was carried out using fluorescein isothiocyanate (FITC, Sigma Aldrich, Brazil)-labelled *Aeromonas hydrophila* (Canova et al. 2017). In brief, *A. hydrophila* was grown on LB media at 37ºC under constant agitation (200 rpm) until the optical density (OD_{600}) of the media reached 0.5. The bacteria were inactivated by adding formalin to a final concentration of 0.5% and kept under agitation for an additional 12h period at 37ºC. The inactivated bacteria were harvested by centrifugation (15 min, 800 x g), washed three times with phosphate buffered saline (PBS, pH 7,4) and counted by flow cytometry. Bacteria labelling was performed using 1µg of FITC per $1x10^9$ bacteria in the dark (30 min at 22ºC), under constant agitation (300 rpm). The labelled bacteria were extensively washed to remove the excess of FITC, diluted with PBS containing 1% albumin and stored in the dark at 4ºC.

The phagocytic assay was carried out in triplicates. Atrazine exposed and non-exposed cells were removed from the wells and mixed with $2x10^7$ FITC-labelled *A. hydrophila* (20 bacteria/leucocyte) and incubated at 37ºC for 15 min. Then, the samples were put on ice and analyzed by BD FacsVerse flow cytometry (BD life sciences, Brazil) with FSC and SCC linear amplification and biexponential fluorescence. Leucocytes were analyzed according to the size and complexity (FSC x SSC) and the phagocytic cells were identified within the population of leukocytes and evaluated regarding the fluorescence intensity and percentile of phagocytic cells (FL1 channel).

The respiratory burst assay was carried out similarly to the phagocytic assay using non-labelled bacteria and 2',7'- dichlorofluorescein diacetate (DCFH-DA, 500 µM, Sigma). DCFH-DA is a cell permeable probe that in the presence of ROS is oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) and, as such, has been widely used to access respiratory burst on immune cells. Atrazine exposed or non-exposed cells in triplicates were removed from the wells and mixed simultaneously with non-labelled *A. hydrophila* (20 bacteria/leukocyte) and 20µL of Diacetate de 2',7' – Dicloroflurescein (DCFH-DA, 500 µM, Sigma) and then incubated for 15 min at 37ºC. Samples were then ice-refrigerated and evaluated by flow cytometry as indicated above.

Statistical analysis: the data were evaluated by the Shapiro-Wilk's test and found to have normal distribution and were then analyzed by one-way Anova followed by Tukey's post-test. The data was plotted using GraphPad Prism Software v7 (GraphPad Software, Inc., USA). The results are expressed as the mean ± standard error of the mean (SEM) and p values of 0.05 or smaller were considered significant.

Results

Atrazine significantly ($p<0.05$) reduced the ingestion of FITC-labelled *A. hydrophila* (Fig. 1A) as deduced from the reduced mean fluorescent intensity (MFI) observed in the atrazine-exposed cells. In leukocytes exposed to atrazine, the MFI

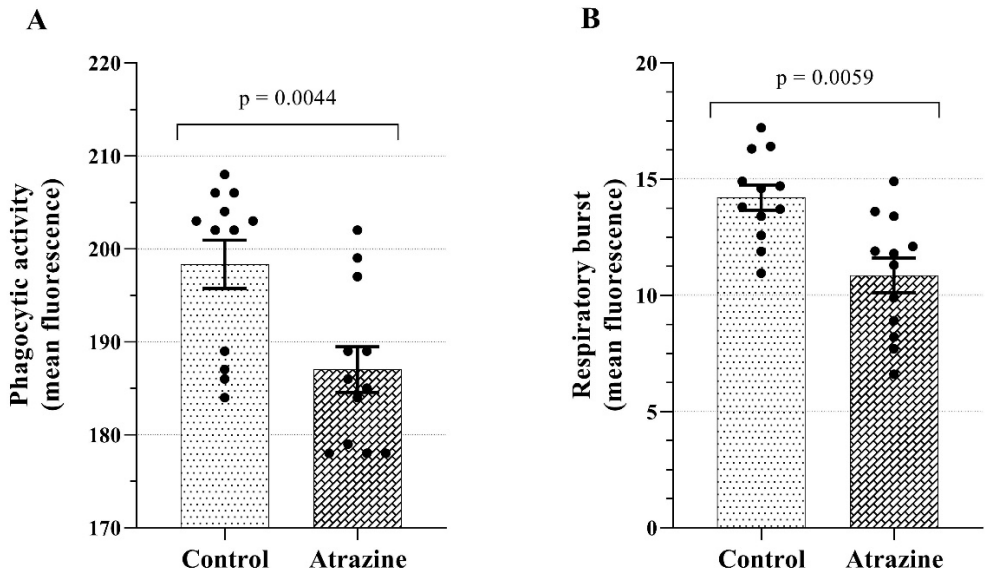

Figure 1. Phagocytosis and respiratory burst activity on silver catfish leukocytes. The cells were isolated from head kidney and exposed or not (control) to atrazine for 24h. A) Phagocytosis and B) respiratory burst activity were evaluated by flow cytometry and the data is expressed as the mean fluorescent intensity ± standard error of the mean. Significant differences (p value) between groups are indicated.

observed after addition of non-labelled bacteria and DCFH-DA was significantly lower ($p < 0.05$) compared to the MFI observed in cell cultivated in atrazine-free medium (Figure 1B).

Because fluorescent light is emitted only from DCF that is formed in the presence of ROS, the results indicate that atrazine reduces the bactericidal activity of cells mediated by respiratory burst.

Discussion

Here we used flow cytometry to evaluate phagocytosis and respiratory burst in silver catfish leukocytes exposed *in vitro* to atrazine and we provide further evidence on the deleterious effect of atrazine on fish immune system. In a previous study we found that most of the deleterious effect of atrazine on immune parameters (Kreutz *et al*. 2011) and expression of immune-related genes (Kirsten *et al*. 2017) peaked at 24h after exposure and, for this reason, here we only evaluated phagocytosis and respiratory burst at this time point. The subtypes of leukocytes were not identified here because specific reagent for discrimination of these cells are not yet available. However, the cells fraction on the top of the 1.077g/ml layer of ficcol contains mostly monocytes and lymphocytes (Pettersen *et al*. 2000) and both might have been affected by exposure to atrazine. Phagocytosis can be performed by several types of cells and consists of ingesting self and non-self-antigens larger than > 0.5µm (Uribe-Querol & Rosales 2020). Highly effective phagocytic activity towards invading microorganisms, however, is found mostly on professional phagocytes like the monocytic dendritic cells and macrophages, highly distributed on tissues, and blood circulating neutrophils. These cells are at the center of innate immune response and altogether with their secreted cytokines they coordinate subsequent events that counterattack the pathogens. Because of their key role on the immune response, impairment of phagocytosis might negatively impact on the capacity to cope with infections leading to disease outbreaks. Our results indicate that atrazine disrupt phagocytic function of the monocytic cells and, as such, has a major impact on the early defense mechanisms. In addition, the cell fraction retained by the 1.077 g/ml gradient contains B cells which in fish, beside producing antibodies, are also considered important professional phagocytic cells (Scapigliati *et al*. 2018) and might have, likewise, been affected by atrazine. Therefore, short term exposure to atrazine early during immune response might affect acquired humoral response as well.

During respiratory burst, myeloperoxidase and hydrogen peroxide (H_2O_2) are key elements on the production of ROS (Magnadóttir 2006) and their reduction in the presence of antigen is suggestive of improper immune function. Consistent with this, in a previous study we found that silver catfish exposed to atrazine had reduced serum myeloperoxidase activity and the expression of myeloperoxidase gene was reduced in fish monocytes exposed *in vitro to* atrazine (Kirsten *et al*. 2017; Kreutz et al. 2012). Thus, the data presented herein further strengthen our previous studies indicating that atrazine down regulates innate immune response.

In conclusion, the use of flow cytometry altogether with fluorochromes-labelled antigens and cell permeable probes activated during respiratory burst are sophisticated tools that allow unequivocal assessment of early cellular events (e.g. phagocytosis and respiratory burst) central to homeostasis and immune response. Thus, flow cytometry-based assays should provide further insights into fish immune cells and support ongoing research on this subject aiming to uncover the impact of atrazine and similar agrichemicals on fish immune system.

Data availability

The data presented in this study are available upon demand to the corresponding author.

Ethical statement

The study was approved by the Ethical and Animal Welfare Committee (University of Passo Fundo protocol number 017/2017).

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Authors' contribution

All authors contributed to the study. Conception, design and data analysis were performed by Paola Aparecida de Almeida, João Antônio Guizzo and Luiz Carlos Kreutz. The first

draft of the manuscript was written by Paola Aparecida de Almeida and all authors commented on previous versions of the manuscript. The article was reviewed by João Antônio Guizzo and Rafael Frandoloso and final writing was performed by Luiz Carlos Kreutz. All authors read and approved the final manuscript.

Declaration of competing interest

The authors have no interest to disclose.

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