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Short communication

Immunotoxic and cytotoxic effects of atrazine, permethrin and piperonyl butoxide to rainbow trout following *in vitro* exposure

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ABSTRACT

For many current use pesticides, limited information exists on their cytotoxicity and immunotoxicity in non-target organisms such as fish. We examined the effects of atrazine, permethrin and piperonyl butoxide (PBO) exposure, *in vitro*, on rainbow trout (*Oncorhynchus mykiss*) lymphocyte viability and proliferation. Purified rainbow trout peripheral blood leukocytes (PBLs) were exposed *in vitro* to the test chemicals (0, 0.01, 0.1, 1 and 10 μM) for 96 h, with and without the mitogen lipopolysaccharide. All three chemicals caused a decrease in both lymphocyte viability and proliferation at 10 μM, while atrazine also suppressed proliferation of PBLs at 1 μM. The *in vitro* toxicity of these chemicals to this salmonid underscores the need for further investigation using *in vivo* studies and host resistance models.

1. Introduction

The immune system has been shown to be a sensitive target for toxic insults by xenobiotics, with impairment of the immune system associated with increased morbidity and mortality due to infectious diseases [1]. Immunotoxicity in fish has been demonstrated for a wide range of chemical classes including polycyclic aromatic hydrocarbons (PAHs) [2], polychlorinated biphenyls (PCBs) [3] and some pesticides [4]. However, for many pesticides, the potential for immunotoxicity in exposed fish has been largely unexplored or data is very limited.

Pesticides enter aquatic habitats as a result of direct use on adjacent lands, aerial drift, runoff following their applications, and/or accidental release. As a result, it is not surprising that many pesticides have frequently been detected in aquatic environments throughout North America [5,6]. In British Columbia, Canada, alone, more than 4 million kilograms of pesticides were sold in 2003 [7], while in the United States an estimated 450 million kilograms of pesticides are used each year [5]. The heavy use of pesticides, combined with their frequent detection in the aquatic environment, suggest that there may be significant exposures and health consequences in fish residing in waterways that are adjacent to application zones.

Atrazine, a triazine herbicide, has been subjected to intense scrutiny due to its heavy use patterns, frequent identification in environmental samples, and potential for causing detrimental effects in non-target organisms [8]. Atrazine concentrations close to 700 μg/L have been reported (reviewed in [9]), and Rohr and McCoy [10] suggest that concentrations up to 500 μg/L be considered ecologically-relevant. Recent reviews have highlighted the need for more information regarding the immunotoxicity of atrazine to fish in light of the lack of information in teleosts, but notable immunotoxic effects in other organisms [9,10].

Permethrin is a pyrethroid pesticide that is extensively used in agriculture, as well as in residential and commercial insect control [11]. As a result, permethrin is frequently identified in both sediment (up to 700 ng/g) and water samples (up to 66 ng/L) [12]. Permethrin exposure (50 ng/L) has been shown to decrease resistance to bacterial infections in medaka (*Oryzias latipes*), despite limited effects detected on cellular immune function [13].

Piperonyl butoxide (PBO) is a common synergist added to insecticide formulations, including those containing pyrethoids. This formulant (or 'inert ingredient') acts as an anti-oxidant and impairs the metabolism of xenobiotics, thereby increasing the effectiveness of the pesticide by allowing the active ingredient to persist in the target pest. Concentrations of PBO as high as 2–4 μg/L have been reported in aquatic environments following pyrethroid

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pesticide application for mosquito control [14]. While data from mammalian studies suggest that PBO affects the function of leukocytes [15], there exist no data for fish.

In the current study, the effects of atrazine, permethrin and PBO on B cell proliferation *in vitro* were assessed using purified peripheral blood leukocytes (PBLs) from rainbow trout (*Oncorhynchus mykiss*). Lymphocyte proliferation is one of the commonly used functional endpoints for the assessment of the immunotoxic potential of xenobiotics [16] and has been used as a biomarker of immunotoxicity in fish [13].

2. Materials and methods

All chemicals were obtained from Sigma (Oakville, ON, Canada) unless otherwise noted, with the pesticides being of analytical grade. Rainbow trout were obtained from Miracle Springs Trout Hatchery (Mission, BC, Canada) and acclimated at 12.7 °C ± 0.1 °C (mean ± SEM) in continuously flowing dechlorinated municipal water for at least two weeks prior to use. At the time of sampling, rainbow trout weighed 163 g ± 9 g (mean ± SEM). All work was conducted in compliance with an animal care permit issued by the Simon Fraser University Animal Care Committee and in accordance with Canadian Council on Animal Care (CCAC) guidelines.

Peripheral blood was aseptically collected from 12 rainbow trout by caudal venipuncture, following euthanization with 0.5 g/L bicarbonate-buffered tricaine methansulfonate (Argent Chemical Laboratories, Redmond, WA, USA). Blood was mixed 1:1 with sterile isolation medium consisting of Hank's Balanced Salt Solution (HBSS) with 15 mM 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 10% fetal bovine serum (FBS; Invitrogen, Portland, OR, USA), 10 U/mL heparin and 1% penicillin/streptomycin. Peripheral blood leukocytes were obtained using hypotonic lysis [17]. After washing twice, cells were assessed for viability using trypan blue exclusion. Cells from each fish were then resuspended in supplemented Roswell Park Memorial Institute medium (sRPMI) containing 15 mM HEPES, 10% FBS and 1% penicillin/streptomycin.

The cell suspension was adjusted to 5×10^6 viable cells/mL for each sample and 100 μL seeded into each of 20 wells of a 96 well round-bottomed plate. Cells in quadruplicate wells were cultured in the presence of the test chemical at concentrations of 0, 0.01, 0.1, 1, and 10 μM (control and 2.2 μg/L − 2.2 mg/L for atrazine; 3.9 μg/L − 3.9 mg/L for permethrin; 3.4 μg/L − 3.4 mg/L for PBO) for 96 h at 15 °C. The test chemical solvent (0.5% v/v ethanol) was shown to not affect viability or proliferation in a separate study [18]. Half of the cells also received 100 μg/mL lipopolysaccharide (LPS, *Escherichia coli* O111:B4) in sRPMI to stimulate B cell proliferation while the other half received an equivalent volume of sRPMI.

At the end of the incubation period, cells were transferred to glass test tubes and cellular viability was determined with propidium iodide exclusion using flow cytometry (Becton Dickenson FACSAria, Franklin Lakes, New Jersey, USA) [19]. Proliferation of these same cells was also assessed using flow cytometry based on changes in forward (FSC) and side scatter (SSC) properties as described elsewhere [18,19]. Gates were set up at the beginning of the experiment for resting cells (low FSC/low SSC) and proliferating cells (high FSC/high SSC), with these same gates used consistently throughout all experiments (see supplemental data, Figure S1, for representative plots). A proliferation index was calculated as the percentage of LPS-stimulated cells in the proliferating cell gate divided by the percentage of unstimulated cells in the proliferating cell gate.

Since cells from each fish received all possible treatments for a given test chemical and results are therefore not independent, the data were analyzed using a repeated-measures ANOVA with a post-hoc Dunnett's test when significant differences ($p < 0.05$) were detected.

3. Results and discussion

Prior to cell culture, PBL viability was 99.1% ± 0.2% (mean ± SEM). Following the 96 h incubation, cellular viability was significantly decreased at the highest exposure concentrations of the test chemicals (10 μM) for both unstimulated and LPS-stimulated PBLs when compared to the corresponding control cells, except for PBO where unstimulated cell viability was affected at both 1 and 10 μM (Table 1). Permethrin had the greatest impact on viability, while atrazine and PBO had more limited, yet still significant, effects.

Similarly, lymphocyte proliferation was significantly diminished by exposure to 10 μM of each pesticide, although atrazine also elicited effects on proliferation at 1 μM (Fig. 1). A reduction in the proliferation index may reflect either an increase in unstimulated cells or a decrease in LPS-stimulated cells in the proliferation gate. For atrazine, the effects on proliferation were evident at concentrations that may be considered ecologically-relevant [10], while for permethrin [12] and PBO [14], effects occurred at concentrations higher than those seen in the environment.

The decreased proliferation index following 10 μM permethrin or PBO exposure may be due to cytotoxicity, since viability was also affected at this concentration. Both permethrin and PBO have been found to disrupt the activity of the Ca^{2+}-ATPase present in rat leukocyte membranes at concentrations of 10 μM and 50 μM respectively, which may lead to ionic and cell signalling dysregulation, and contribute to cytotoxicity [20]. For these two treatments relative to the control group, the number of unstimulated cells in the proliferation gate was increased while the LPS-stimulated cells were unaffected (data not shown), suggesting that resting lymphocytes might be subject to preferential cytotoxicity. This is consistent with the findings of Battaglia et al. [15], who demonstrated that resting murine splenic lymphocytes are more sensitive to cytotoxicity following PBO exposure than the overall splenocyte population.

Table 1
The viability of both unstimulated and LPS-stimulated peripheral blood leukocytes from rainbow trout declined following *in vitro* exposure to atrazine, permethrin or piperonyl butoxide. Data is shown in percent as mean cell viability ± SEM, $n = 12$, and * indicates that the treatment group is significantly different from the corresponding control group (repeated-measures ANOVA with Dunnett's post-hoc test, $p < 0.05$).

Concentration of pesticide	Atrazine		Permethrin		Piperonyl butoxide	
	Unstimulated	LPS-stimulated	Unstimulated	LPS-stimulated	Unstimulated	LPS-stimulated
Control (0 nM)	72.9 ± 1.6	79.0 ± 0.9	65.8 ± 2.5	69.4 ± 1.5	64.6 ± 2.8	68.5 ± 1.7
10 nM	72.0 ± 1.8	78.9 ± 0.8	65.7 ± 2.5	68.8 ± 1.5	64.3 ± 3.0	67.5 ± 1.8
100 nM	71.6 ± 1.9	78.7 ± 0.8	65.9 ± 2.8	68.8 ± 1.4	63.6 ± 3.1	67.6 ± 1.8
1 μM	73.2 ± 1.3	78.3 ± 0.8	62.8 ± 2.7	66.2 ± 1.4	62.4 ± 3.1*	67.4 ± 2.0
10 μM	69.6 ± 1.2*	77.7 ± 0.9*	47.4 ± 2.7*	53.4 ± 1.7*	59.5 ± 3.1*	65.0 ± 2.3*