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Effects of tebufenozide on some aspects of lake trout (*Salvelinus namaycush*) immune response ☆

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Abstract

Tebufenozide is a nonsteroid ecdysone agonist that causes premature and incomplete molting in Lepidoptera and is used on crops and in forest spray programs. Lake trout (*Salvelinus namaycush*) were exposed to one pulse of 0.25 ppm tebufenozide every 3 days (12 pulses in total). Cell ratios and respiratory burst responses of circulating white blood cells (WBCs) as well as head-kidney (HK) WBCs were investigated by using cell observation, Nitro-Blue Tetrazolium tests, and flow cytometry. Endpoints studied suggest a difference in sensitivity between HK and circulating blood cells to tebufenozide and show a stimulation of fish cell function and changes in percentages of cell types. Responses are not associated with a strong stress response as highlighted by the absence of effect on cortisol and blood protein levels. These results and tebufenozide persistence in water warrant further studies on pesticide impact on fish immunity when used on crops or in forest spray programs near lakes and streams.

Keywords: Tebufenozide; Pesticide; Lake trout; Immune response; White blood cells; Flow cytometry; Respiratory burst

1. Introduction

Tebufenozide, developed by Rohm and Haas Company (Philadelphia, PA, USA) for Lepidoptera control in agriculture and forestry, is one of the pest management alternatives designed to lessen the use of broad-spectrum synthetic pesticides. Tebufenozide is a nonsteroid ecdysone agonist that causes premature and incomplete molting in Lepidopteran larvae. Tebufenozide formulations are targeted for use in rice in Southeast Asia; vineyards and apple orchards in Europe; and agriculture, orchards and forestry in the United States, *etc*. In Canada, its use on fruits and peppers has been approved by the Food and Drugs Act and its application to forestry is being tested for the control of damage caused by the eastern spruce and jack pine budworms in coniferous forests. The formulation being tested, Mimic 240LV®, is composed of 25% tebufenozide and inert ingredients such as glycerol, canola oil and water.

Studies conducted so far on tebufenozide have shown that its acute toxicity on mammals, birds, nontarget terrestrial invertebrates and most aquatic organisms is low (Heller et al., 1992; Kreutzweiser et al., 1994; Addison, 1996; PMRA, 1996). Nontarget organisms, such as fish, inhabit ponds and lakes in areas that could be treated with Mimic. Moreover, long persistence of tebufenozide in both laboratory and field studies partly attributable to its low vapor pressure, low water solubility and high octanol/water coefficient (Sundaram, 1997) suggests that it is important to study fish exposure to this compound. Studies in mice, rats and dogs have shown that the most consistently observed effects of tebufenozide are related to the formation of methemoglobin which can lead to destruction of red blood cells (RBCs) (Clay, 1992; Hazelton and Quinn, 1995). To our knowledge, no information is available on tebufenozide effect on fish blood cells and in particular immune cells. The importance

☆Studies described in this research paper did not involve human subjects. Fish were used as experimental animals and all investigations were conducted in accordance with national and institutional guidelines for the protection of animal welfare. All protocols applied in this research study were reviewed by the committee for animal care and welfare of the Department of Fisheries and Oceans, Canada.

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of immune responses in fish disease susceptibility and its potential effect on population decline is now known and well documented (Gulland, 1995; Arkoosh et al., 1998).

In this study, the effect of tebufenozide on immune responses of lake trout was investigated by studying blood and head-kidney (HK) cells. Microscopic counts were used to evaluate lake trout blood cell numbers. Respiratory burst (RB) activity is the production of oxygen radicals released by phagocytic cells in an oxidative burst to destroy invaders (or when cells are stimulated). RB activity of lake trout white blood cells (WBCs) was measured using the nitroblue tetrazolium (NBT) reduction test and a flow cytometry (FCM) assay. These assays have been proven to be useful in investigating the effects of immunostimulant substances, environmental stress and vaccination on immune function (Anderson et al., 1992; Esteban et al., 2000). The aim of this study is to bring novel information on effects of tebufenozide on responses of lake trout immune cells by considering direct effects on cells *in vitro*, as well as by exposing fish to low levels of tebufenozide in the water.

2. Materials and methods

2.1. In vitro testing

Potential cytotoxic effects of tebufenozide on cells was tested by incubating circulating WBCs (separation protocol described below) with different concentrations of tebufenozide (0.001–0.14 ppm) and measuring cell viability with the trypan blue test. Moreover, effect on RB was investigated by preincubating WBCs with tebufenozide 1 h prior to the NBT test (protocol described below).

2.2. Fish exposure

Lake trout (*Salvelinus namaycush*) were grown in freshwater tanks from eggs and milt sampled from two lakes in Labrador, Canada. For this experiment, adult fish weighing 573 ± 164 g were kept in a flow-through system (2000 L tanks) with filtered water (8–9 °C) and fed dry food pellets every other day. Fish were divided into two groups of 12 fish. One group was exposed to one pulse of 0.25 ppm of tebufenozide (1 ppm of Mimic®) every 3 days and to a total of 12 pulses. Water flow in both control and exposure tanks was maintained at 3.75 L/min. Therefore, water dilution was such that the theoretical concentrations of tebufenozide in the exposure tank would be 0.15, 0.09, 0.05 and 0.03 ppm after 6, 12, 18 and 24 h, respectively. Water samples were also analyzed by an Ecology-accredited laboratory (ALS Laboratory Group, Edmonton, Alberta) using method number 8151 GC/MS modified. Fish were sacrificed 4 days after the last pulse. Blood and HK cells were sampled and processed the same day. Fish were sacrificed during two consecutive days (six control + six exposed per day) to allow immediate processing of blood and HK samples.

2.3. Cell separation

Blood was taken from fish with heparinized 10 mL syringes. Following a protocol described by Crippen et al. (2001), blood was diluted 1:12 in isolation buffer (MCHBSS + Alsever's buffer) and overlaid on an 8 mL cushion of Histopaque-1077. Gradients were centrifuged at $400 \times g$ for 40 min at 4 °C and cells at the interface were harvested.

Dissociated HK leukocytes were obtained by pressing through a nylon screen (50–60 μm) in the presence of isolation buffer (approximately 8 mL for 1 g of tissue). The cell suspension was layered on a cushion of 54% Percoll (4 mL on top of 10 mL of Percoll) (Funk et al., 2004) and centrifuged at 4 °C for 30 min at $650 \times g$. The cells at the interface were recovered and kept on ice or at 4 °C until use. The trypan blue test was performed on cells prior to any immune response testing to determine cell viability.

2.4. Cell counts

Cell densities were evaluated after WBC separation using a hemocytometer. Between 1.2×10^6 and 1.4×10^6 cells/mL of circulating leukocytes were obtained with blood and 1.9×10^6 to 2.0×10^6 cells/mL with HK (dilutions of initial samples were kept similar to minimize big variations in cell amounts). Blood smears (Wright–Giemsa) were also prepared and a first count was performed by counting WBCs and RBCs (250 cells). Percentages of both WBCs and RBCs were calculated. Counts were performed a second time on 400 WBCs to evaluate percentages of lymphocytes, thrombocytes and neutrophils.

2.5. Respiratory burst

2.5.1. NBT test

This test measures the reduction of NBT by superoxide anions (Secombes, 1990). This test was performed on both circulating leukocytes and HK cells. Between 0.25×10^6 and 0.7×10^6 cells (250 μL) were plated in each well and allowed to settle at room temperature for 90 min. Four replicate wells were used for every treatment: unstimulated, phorbol myristate acetate (PMA) or zymosan stimulated cells (12 wells with equal cell numbers per sample). After cell settling, the medium was removed by quickly inverting the plate. The replacement media contained 100 μL of NBT (1 mg/mL) + 100 μL of isolation buffer or 100 μL of NBT + 100 μL of PMA (1 μg/mL) or 100 μL NBT + 100 μL zymosan (2.5 mg/mL) (all products were dissolved for their final use in isolation buffer). The cells were incubated for 30 min at room temperature. The reaction was terminated by replacing the medium with 200 μL of absolute methanol followed by three washes with 70% methanol. The formazan was solubilized with 120 μL of 2.0 M KOH and 140 μL of DMSO. Absorbance values were determined at 630 nm using an ELISA plate reader. The level of stimulation was expressed as a stimulation index determined as the ratio of absorbance of PMA or zymosan stimulated cells to that of unstimulated cells.

2.5.2. Flow cytometry

Oxidative burst was also quantified using FCM as a measure of intracellular hydrogen peroxide production following activation with PMA (1 μg/mL, final concentration). Flow cytometric assessment of the RB was based on the technique described by Bass et al. (1983). The assay depends upon the cell incorporating 2′–7′ dichlorofluorescin diacetate (DCFH-DA), a stable nonfluorescent molecule which is hydrolyzed to DCFH by cytosolic enzymes, then by the action of H_2O_2 (produced in leukocytes stimulated with PMA), DCFH is oxidized to fluorescent DCF. Briefly, isolated circulating leukocytes (this test was not performed on HK cells) were incubated with DCFH-DA (5 μM) at room temperature and fluorescence measurements were done to acquire baseline fluorescence levels. PMA was added and fluorescence was measured immediately (time zero) and 12 min after cell stimulation. Adding PMA provoked only a two-fold increase in fluorescence (in all samples); "time zero" levels of fluorescence were considered when calculating stimulation index and standardized differences. Between 10 000 and 30 000 events were included in the analysis of every blood sample (12 controls, 12 exposed to tebufenozide). For each sample, a stimulation index was determined as the ratio of fluorescence of PMA stimulated cells (12 min) to that of cells at "time zero". Cellular debris were excluded from the analysis by raising the forward-scatter threshold only minimally. The stimulation ratio does not take into account the natural variability in fluorescence levels occurring among cell populations. Therefore, we also expressed our results as standardized differences. Cohen (1988) defined *d* as the difference between