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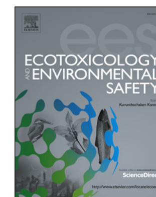

Assessing off-target cytotoxicity of the field lampricide 3-trifluoromethyl-4-nitrophenol using novel lake sturgeon cell lines☆

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ABSTRACT

Lampricides are currently being applied to streams and rivers to control the population of sea lamprey, an invasive species, in the Great Lakes. The most commonly used lampricide agent used in the field is 3-trifluoromethyl-4-nitrophenol (TFM), which targets larval sea lamprey in lamprey-infested rivers and streams. The specificity of TFM is due to the relative inability of sea lamprey to detoxify the agent relative to non-target fishes. There is increasing concern, however, about non-target effects on fishes, particularly threatened populations of juvenile lake sturgeon (LS; *Acipenser fulvescens*). There is therefore a need to develop models to better define lake sturgeon's response to TFM. Here we report the establishment of five LS cell lines derived from the liver, gill, skin and intestinal tract of juvenile LS and some of their cellular characteristics. All LS cell lines grew well at 25 °C in Leibovitz's (L) − 15 medium supplemented with 10% FBS. All cell lines demonstrated high senescence-associated β–galactosidase activity and varying levels of Periodic acid Schiff-positive polysaccharides, indicating substantial production of glycoproteins and mucosubstances by the cells. Comparative toxicity of TFM in the five LS cell lines was assessed by two fluorescent cell viability dyes, Alamar Blue and CFDA-AM, in conditions with and without serum and at 24 or 72 h exposure. Deduced EC_{50} values were compared between the cell lines and to the reported *in vivo* LC_{50}s. Tissues sensitive to the effects of TFM *in vivo* correlated with cell lines from the same tissues being most sensitive to TFM *in vitro*. EC_{50} values for the LSliver-e cells was significantly lower than the EC_{50} for the rainbow trout (RBT) liver cells RTL-W1, reaffirming the *in vivo* observation that LS was generally more TFM-sensitive than rainbow trout. Our data suggests that whole-fish sensitivity of LS to TFM is likely attributable to sensitivity at the cellular level. Thus, LS cell lines, as well as those of RBT, can be used to screen and evaluate the toxicity of the next generation of lampricides on non-target fish such as lake sturgeon.

1. Introduction

Lampricides are biocides intended to target and kill larval sea lamprey (ammocoetes) in lamprey-infested tributaries of the Great Lakes, prior to their metamorphosis into juvenile sea lamprey (*Petromyzon marinus*). Juvenile sea lampreys feed on the blood of teleosts, especially commercial and sport fishes, often causing death (Farmer 1980). Their predation/parasitism of lake trout (*Savelinus namaycush*), whitefish, and other fishes contributed significantly to the collapse of Great Lakes fisheries in the mid-twentieth century (Lawrie, 1970; Renaud, 1997; Smith and Tibbles, 1980). In response to the sea lamprey invasion, the Canadian and United States governments worked together to implement an integrated sea lamprey control program, consisting of barriers and dams on rivers to block the migration and reproduction of mature lamprey, and the use of lampricides which could specifically target multiple generations of larval sea lamprey in infested streams (McDonald and Kolar, 2007). After testing over 6000 chemical candidates for lampricidal potential in the 1950s and 1960s, one agent discovered to be a highly effective and lamprey-specific lampricide, was 3-trifluoromethyl-4-nitrophenol (TFM; Applegate et al., 1961; Howell et al., 1964). Current recommended field practices include the direct application of TFM alone, or in combination with

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niclosamide, into streams and rivers at concentrations that effectively eliminate larval sea lamprey while minimizing serious effects on other native species within the proximity of the treated areas (McDonald and Kolar, 2007). Applications of typically take place on 2–4 year cycles, last approximately 12 h, and result in the death of multiple generations of sea lamprey (Hubert, 2003; Siefkes, 2017).

Despite being used in the field for over 50 years, there is evidence that TFM can have adverse effects on non-target vertebrate organisms (Applegate and King, 1962; Marking and Olson, 1975; Bills and Marking, 1976; Johnson et al., 1999; Boogaard et al., 2003). While salmonids, bluegill, and sunfish are relatively TFM-tolerant, lake sturgeon (LS, *Acipenser fulvescens*) are among those aquatic animals that are highly sensitive to TFM toxicity, particularly in their early life stages (Boogaard et al., 2003; O'Connor et al., 2017). TFM targets the mitochondria of cells, uncoupling oxidative phosphorylation during cellular respiration, resulting in a lower yield of ATP for energy expenditure in both sea lampreys and non-target fishes (Birceanu et al., 2009, 2011). Sub-lethal concentrations of TFM can also decrease the olfactory reactions to biochemical and food cues in LS fingerlings and alter their behavior (Sakamoto et al., 2016). These effects are relevant because juvenile LS populations overlap with those of larval sea lamprey, and can therefore be episodically exposed to TFM when lamricides are applied (O'Connor et al., 2017). Moreover, LS is native to North America and recognized as a threatened/endangered species by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC, 2006).

Recently, there has been renewed interest in developing “next-generation” lampricides that are more target-specific, less harmful to non-target species, and able to avoid the development of lamprey resistance to TFM (Dunlop et al., 2017). A major challenge with developing such next-generation lampricides is that laboratory whole-animal testing is time-consuming, expensive, labor-intensive, space-demanding, and requires large numbers of lamprey and non-target fish. This challenge multiplies in magnitude with LS. First, it is a threatened/endangered species and requires protection. Second, this species has a long and complex life cycle with prolonged sexual maturation and sex-related differences and unseasonal changes in spawning cycle (COSEWIC, 2006). Thus, facility, labor, and cost to maintain them in the laboratory can be prohibitive.

Animal continuous cell cultures or cell lines circumvent many of these challenges and present a good alternative toxicological assessment tool to whole-animal testing (Lillicrap et al., 2016). For fishes, their continuous cell cultures or cell lines have been instrumental tools for the last several decades to evaluate toxicity of many classes of toxicants and environmental contaminants, and in many cases correlations between *in vitro* and *in vivo* data were met (Bols et al., 1985; Bols and Lee, 1991; Lee et al., 1993; Castano et al., 1996; Segner, 1998; Dayeh et al., 2013; Zeng et al., 2016a, b; Vo et al., 2017a, b). For LS, even though primary cell cultures could be established from gill and gonadal tissues (Clouthier et al., 2013), no continuous cell cultures or cell lines currently exist.

In this study, we report the development of continuous cell cultures from different organs of juvenile LS. We used these continuous cell cultures to demonstrate their suitability for toxicological testing, as well as comparing their sensitivity to that observed *in vivo* in response to TFM toxicity. We propose that these cell lines, not to mention those of other fishes, can serve as the initial screening tools to evaluate next generation lampricides to ensure their safety in non-target fishes.

2. Materials and methods

2.1. Fish husbandry

Eyed lake sturgeon (*Acipenser fulvescens*) were purchased from Sustainable Sturgeon Culture, Emo, Ontario and shipped to the Alma Research Station, University of Guelph, Alma Ontario. This population of sturgeon are from Rainy River, Northwestern Ontario near Emo and the North Dakota/Minnesota border. The eggs were subsequently hatched and reared at the Research Station, where they were initially fed brine shrimp on demand as larvae (hatchlings), before switching to bloodworms as fingerlings (2–3 months). When the fish were larger, with an average weight of 3.0 g in weight and 93 mm in length, they were transported to the aquatics facilities at the Centre for Cold Regions and Water Science, Wilfrid Laurier University. The fish at this stage of development were selected because they show higher sensitivity to TFM in vivo (Johnson et al., 1999; Boogaard et al., 2003) and it was our interest to determine if derived cells showed a similar response to the lampricide. The fish were held in a GHAB (PentAir, Aquatic Ecosystems, Sanford, NC) recirculating system (10% water replacement per day; Temperature = 13 °C) with UV filtration for at least 3–4 weeks. The animals were fed daily with bloodworms. Tissue samples from two fish were collected after the fish were euthanized with 0.1 g L^{-1} tricaine methanesulfonate buffered with 2 parts $NaHCO_3$, and then used for *in vitro* tissue cultures. All procedures followed the Canadian Council of Animal Care guidelines and were approved by the Wilfrid Laurier Animal Care Committee.

2.2. Initiation and subcultivation of primary cultures

After euthanizing two fish (above) gill, skin, liver, and intestinal tissues were removed in a level-2 Biosafety cabinet and placed in sterile Petri dishes containing Ca^{2+} and Mg^{2+}-free Dulbecco's Phosphate Buffered Saline (DPBS) solution (Corning) with 200 U/mL penicillin and 200 µg/mL streptomycin (2% P/S, Thermo Scientific; washing DPBS). Primary cell cultures were established by the explant outgrowth technique as demonstrated successfully for many fish species by Gignac et al. (2014), Vo et al. (2015a, b, c, d), Vo and Bols (2016), Bloch et al. (2016), Pham et al. (2017), and Semple et al. (2017). Briefly, removed tissues were minced into small finer pieces and washed three to five times with DPBS. Tissue fragments were then explanted into the 25-cm^2 tissue culture (TC) flasks (BD Falcon) containing 1 mL of Leibovitz's L-15 medium (Thermo Fisher) with 10% (v/v) fetal bovine serum (FBS, Thermo Fisher) and 2% P/S. Primary cultures were kept at 20–22 °C at atmospheric air and growth media with 10% FBS were changed daily during the first week and every 3–7 days afterwards. Adherent cells migrated out of the tissue explants and formed compact cell monolayers on the TC polystyrene surface. Cell cultures with high mitotic activities were further sub-cultivated by TrypLE (Invitrogen). Early cultures were maintained in growth media with 15–25% FBS to stimulate cell proliferation. Cultures that were successfully sub-cultured for multiple passages were then selected for further propagation to give rise to particular lineages of continuous lines.

2.3. Maintenance of five LS continuous cell cultures

The five lake sturgeon-derived cell lines that arose from the explant cultures were named: LSliver-e, LSskin-e, LSgut-f, LSgill-e and LSgill-f, with the suffix –e or –f delineating a more epithelial (-e) or fibroblastic (-f) morphology. LSgill-e and LSgill-f were derived from fish #1, LSskin-e, LSliver-e, and LSgut-f were derived from fish #2. Initially, for the first 12 months, 15 – 25% FBS was supplemented in the growth media and TryplE was used to sub-culture cells. The FBS concentration was reduced to 10% and 0.25% Trypsin/2.21 mM EDTA solution (in HBSS buffer free of $NaHCO_3$, Ca^{2+}, and Mg^{2+}, Corning) was used for cell passaging thereafter. All experiments were performed after the FBS percent for growth was switched to 10%.

2.4. Maintenance of rainbow trout cell lines

Two rainbow trout cell lines, RTG-2 (gonadal; Wolf and Quimby, 1962) and RTL-W1 (liver; Lee et al., 1993), were used for comparison purposes for specific assays. RTG-2 and RTL-W1 were grown in L-15

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