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Effects of pulp and paper mill effluent extracts on liver anaerobic and aerobic metabolic enzymes in rainbow trout

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ABSTRACT

This study investigates whether pulse exposure to Chilean pulp and paper mill effluent solid phase extracted (SPE) extracts via intraperitoneal injection (IP), would result in changes in the activities of the respiratory metabolic enzymes citrate synthase (CS) and lactate dehydrogenase (LDH) in rainbow trout livers. It also investigated if an alteration in liver metabolic capacity influenced the liver detoxification processes and estrogenic effects previously reported. Besides, a comparison of those enzymatic activities with fish IP injected with SPE extracts of two model effluents coming from industries that process 100% different type of feedstock (softwood, SW and hardwood, HW) was also evaluated. An initial induction of the anaerobic metabolism (increase in LDH enzymatic activity) was detected in all Chilean pulp mill effluent extracts evaluated, contrary to the initial unaltered aerobic metabolism (CS enzymatic activity) observed. A compensatory relationship in energy metabolism (Pasteur effect) was observed when comparing both enzymatic activities of fish exposed to those effluent extracts. LDH and CS activities observed in fish injected with Chilean extracts seem to be related to the effects observed in fish injected with SW extracts. This study showed that intraperitoneal injection of pulp and paper mill effluent extracts affected the anaerobic and aerobic metabolic capacities in rainbow trout livers, but this metabolic alteration did not affect detoxification capability or estrogenic effect previously reported.

1. Introduction

Numerous studies have demonstrated that pulp and paper mill effluents affect fish, especially at the reproductive level (Hewitt et al., 2008). However, effluent quality can vary substantially between mills using different manufacturing processes, mill furnish, operation conditions and effluent biotreatment, influencing its ability to affect fish. Specifically, Chilean pulp mill industries have been implementing standard procedures such as the kraft pulping process and elementary chlorine free (ECF) bleaching technologies (bleached kraft mill effluents, BKME). However, a high variability in feedstock (softwood, SW and hardwood, HW) and effluent biotreatment is still being observed (Orrego et al., 2009).

Chilean pulp mill effluents have been shown to induce liver mixed-function oxygenase (MFO) enzymes and exert consistent estrogenic effects (e.g. increased plasma yolk-precursor protein, vitellogenin, VTG) in fish under both laboratory and field conditions (Orrego et al., 2005, 2006, 2009). Although induction of MFO enzymes has been extensively used to assess fish responses to different pulp and paper mill effluents (Munkittrick et al., 2002; Parrot et al., 2006), their mechanistic link to other indicators of liver impairment or damage due to such exposures in fish have rarely been considered. Our experience indicates that compounds such as resin acids (principally derived from SW) consistently found in Chilean pulp and paper mills in spite of the effluent secondary treatment, have the potential to cause a liver impairment, suggesting an indirect anti-estrogenic effect by reducing E2-induced VTG production (Orrego et al., 2010b). However, the final effects of Chilean pulp mill effluents are estrogenic and potentially related to compounds that act as estrogen receptor agonists or compounds that can induce changes leading to increased endogenous estrogens and up-regulated gene expression for enzymes such as CYP19 aromatase, involved in androgen to estrogen conversion in the final steps of the steroid biosynthetic pathway (Orrego et al., 2010a).

Metabolic enzymes such as citrate synthase (CS) and lactate dehydrogenase (LDH) are part of the respiratory enzymatic system, which can be affected independently from the detoxification enzymatic system (CYP450 1A1) under stress conditions in fish (Pollino and Holdway, 2002). However, disturbances in their catalytic properties as a result of xenobiotic interactions can cause impairment in cellular homeostasis affecting other enzymatic activities, which can lead to adverse effects at higher levels of biological organization such as tissues, organs or individuals (Konradt and Braunbeck, 2001).

LDH is an enzyme that catalyzes the last step in anaerobic glycolysis; the reversible oxidation of lactate to pyruvate. LDH is a soluble enzyme located in the cytoplasm of all cells in the body; it is

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consequently used as a quantitative marker enzyme for intact cells, providing information on cellular glycolytic capacity (Ozmen et al., 2007; Tugiyono and Gagnon, 2002). Glycolysis is an anaerobic process that generates much less energy than the citric acid cycle. However, it has been found that unlike the healthy mammalian heart, it is common for fish ventricles to be hypotoxic due to low environmental oxygen levels and as a result, fish may depend more on carbohydrates for energy than mammals (Brattiprolu et al., 2006). Consequently LDH may play a greater role in fish energy metabolism than it does in mammals, particularly in conditions of chemical stress when high levels of energy may be required in a short period of time (Cohen et al., 2001).

Alterations of normal LDH activity pattern have already been reported in several fish species in situ exposed to pollution (Castro et al., 2004; Monteiro et al., 2007), exposed to crude oil (Gagnon and Holdway, 1999), PCB's (Tugiyono and Gagnon, 2002), naphtoflavone (Mathilakath et al., 1997), heavy metals and organochlorines (Ozmen et al., 2007), or under hypoxia conditions (Cooper et al., 2002). However, under field conditions no alteration in LDH enzymatic activity was found in caged rainbow trout in areas of pulp and paper mill discharged (Soimasuo et al., 1995).

CS is the first pace-maker enzyme of the citric acid cycle (Krebs cycle), located within the cytoplasm of the mitochondria (Dickson et al., 1993). Its role is to catalyze the conversion of oxaloacetate to citrate. CS is used as a quantitative marker enzyme for the content of intact mitochondria aerobic capacity and energy production (Konradt and Braunbeck, 2001; Tugiyono and Gagnon, 2002). Increased levels of CS activity have been demonstrated in male rainbow trout myocardium during sexual maturation (Clark and Rodnick, 1998). No changes were detected in livers of fish IP injected with PCB126 (Tugiyono and Gagnon, 2002) and it was recently demonstrated that there were no effects on liver CS gene expression in rainbow trout fed a lipid-rich diet (Kolditz et al., 2008), contrary to the observed reduction of gene expression of lipogenic enzymes.

Fish livers with cellular injuries related to xenobiotic exposure are less capable of MFO induction than non-injured liver (Holdway et al., 1994). Consequently, hepatocellular injury can represent a confounding factor when interpreting detoxification enzymes and endocrine disruption effects, especially when assessing toxicity and estrogenicity of complex matrices such as pulp and paper mill effluents.

The aim of this research was to investigate whether pulse exposure via IP injection to solid phase extracted Chilean pulp and paper mill effluent extracts (untreated, primary and secondary treated effluents) results in changes in the activity of the respiratory metabolic enzymes LDH and CS in livers of rainbow trout. These results were simultaneously compared to responses in fish injected with several laboratory standards including phytosterols (β-sitosterol, stigmasterol and stigmastanol) and steroids (androstenedione, testosterone and 17β-estradiol). We also investigated if livers with altered metabolic capacity due to pulp mill effluent extract IP injections, influenced the detoxification process (increased liver 7-ethoxyresorufin-O-deethylase, EROD activity) and the estrogenic effects (increased endogenous plasma estradiol, testosterone and vitellogenin levels and up-regulated gene expression of CYP19a) previously reported (Orrego et al., 2009). A secondary objective was to evaluate the relationship of changes in LDH and CS activities observed following pulp mill extract injection, with respective changes following injection with SPE extracts obtained from two different pure feedstock lines (100% SW and 100% HW).

2. Materials and methods

2.1. Effluent samples

Untreated, primary (primary clarifier) and secondary treated Chilean pulp mill effluent samples (aerated extended lagoon and activated sludge basin) were obtained from ECF/BKME mills. Untreated and primary treated extracts correspond to samples of the same industrial effluent obtained before and after its primary treatment. This mill processes equal amounts of pine and eucalyptus (SW–HW, 50–50%), with an annual white cellulose production of 550,000 tons. The mill with secondary treatment processes 60% SW and 40% HW, and has an annual production of 850,000 tons.

Additionally, both samples assessed in the pure lines injection experiments (100% SW and 100% HW) correspond to non-Chilean mills with ECF/BKME and secondary treatment.

2.2. Effluent solid phase extractions (SPE)

Effluent samples were extracted using reverse phase C-18 (non polar) cartridges as previously described for the analysis of organic compounds derived from treated pulp mill effluents (Burnison et al., 1999). Briefly, 3 L samples (24 h composite) of each effluent were filtered through a Whatman Binder-free Glass microfiber filter (type GF/C: 4.7 cm in diameter and 0.3 μm particle retention) and extracted by solid phase extraction (SPE) using ACCUBOND ODS C-18 reverse phase cartridges previously conditioned with two volumes of Milli-Q water and one volume of methanol. Extractions were performed under 4 ml $\times$ min^{-1} of vacuum (2 cartridges were prepared for each sample).

The effluent compositions for Chilean extracts were analyzed and previously reported from one of the cartridges previously eluted with two ethyl acetate volumes, two of methanol and derivatized with diazomethane (Orrego et al., 2009). The extracts were reconstituted in methanol and analyzed by gas chromatography mass spectrometry (GC/MS) in a Hewlett-Packard 5890 II with HP 5972 detector series (Avondale, PA, USA) and a HP 5MS column (0.25 mm diameter and 0.25 μm thickness), using an injector temperature of 250 °C and a mass detector temperature of 300 °C.

The second cartridge of the Chilean and pure line extracts were eluted using a sequence of hexane, ethyl acetate, methanol and gaseous nitrogen reduced. Finally the ethyl acetate and methanol elutriates were re-suspended together in acetone and dissolved in corn oil (carrier) for subsequent injection to the fish. The acetone was evaporated prior to intraperitoneal injection.

2.3. Dose calculation and intraperitoneal injection

Non-lethal intraperitoneal doses of the steroid standards androstenedione (ADD), testosterone (T) and 17β-estradiol (E2), the phytosterols β-sitosterol (BS), stigmasterol (S1) and stigmastanol (S2), Chilean pulp mill effluents untreated (ME1), primary (ME2) and secondary (ME3) treated and two pure line effluents softwood (SW 100%) and hardwood (HW 100%), were derived from preliminary 96 h acute toxicity tests. The doses (μl/g of body weight) of extracts and standards were calculated immediately before injection based on individual fish weight (Rottmann et al., 2001); fish were injected with a desired injection volume of 200 μl.

In a first experiment, a total of 375 female hatchery-reared (Linwood Acres Trout Farm, ON, Canada) rainbow trout *Oncorhynchus mykiss* (129 $\pm$ 35 g), were used in a 28 day pulse exposure toxicity experiment including injection of 25 ppm of three Chilean effluent extracts (ME1, ME2 and ME3), 5 ppm of ADD and E2; 1 ppm of T, and 5 ppm the phytosterols standards (BS, S1 and S2). In this experiment four sets of controls were included: time zero controls (no treatments, TZ), carrier controls (intraperitoneal injection of corn oil and tagged fish, CO), experimental controls (tagged fish, EC) and stress controls (tagged fish held in a glass aquaria supplied with 25 °C and 25 kg/m^3 of density constantly maintained during the whole experiment, ST). The ST control was used to evaluate the influence of high stress treatment on the variability of the molecular biomarkers analyzed (respiratory metabolic and detoxification enzymatic activities along with estrogenic hormonal and genetic effects). In a second experiment a total of 50 fish were injected with 25 ppm of SW 100% and HW 100% effluents extracts, while 25 fish were injected with corn oil only (CO) as their respective controls.

All the fish in both experiments were acclimated for two weeks to 12 $\pm$ 1 °C flowing water in 1000 L tanks (7.5 kg/m^3 of density and 3 L/min tank flow) and fed once a day (5PT pellets, Martin Mills Inc. Elmira, ON, Canada) until satiety. Fish previously anesthetized in tricaine metasulfonate (MS-222, 100 mg/L) were weighed, placed in a flowing water board holder system and given an intraperitoneal injection using a precision animal health syringe 187 (SOCOREX, Swiss) and 2R2 hypodermic needles (Unimed, CH-12002 Lausanne, Switzerland). After injection, fish in the first experiment were tagged with color plastic tags (t-bar anchor tag) in front of the dorsal fin and moved into a flowing recovery tank system. They were then randomly placed in five 1000 L tanks (5 fish per treatment in each tank) under the same experimental conditions used during the acclimatization period. Fish from the second experiment were placed into duplicate 100 L glass tanks at a density of 6.8 kg/m^3, keeping the same experimental conditions as during the acclimatization period.

2.4. Fish sampling

Five fish were sacrificed before the experiment to evaluate their initial state of health and to get time zero measures (Time Zero, TZ control group). In both