Ecotoxicology and Environmental Safety 101 (2014) 168–176

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Dehydroabietic acid (DHAA) alters metabolic enzyme activity and the effects of 17β-estradiol in rainbow trout (*Oncorhynchus mykiss*)

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ARTICLE INFO

Article history:
Received 19 June 2013
Received in revised form
12 November 2013
Accepted 27 November 2013
Available online 15 January 2014

Keywords:
Dehydroabietic acid
Estradiol
Chronic exposure
Metabolic enzyme
Sorbitol dehydrogenase
Vitellogenin

ABSTRACT

Recent studies have shown that dehydroabietic acid (DHAA), a resin acid present in pulp and paper mill effluent, affects liver energy metabolism and may have anti-estrogenic effects in fish. A chronic-exposure toxicity experiment using immature rainbow trout (*Oncorhynchus mykiss*) was conducted in order to assess the endocrine disrupting and liver metabolic effects of the model estrogen 17β-estradiol (E2) and the wood extractives DHAA and β-sitosterol (BS), regularly present in pulp and paper mill effluents. Exposure to 5 ppm of E2 significantly increased hepatosomatic index (HSI), vitellogenin (VTG) and plasma sorbitol dehydrogenase (SDH). This latter effect was reduced by mixing E2 with DHAA, indicating that DHAA does not cause its endocrine disrupting effects indirectly due to liver damage. Exposure to 0.5 ppm of DHAA as well as all the DHAA mixed treatments caused significant increases in liver citrate synthase (CS), activity after 7 days, however, the fish returned to control values by 28 days. Results indicate that DHAA may alter metabolic enzyme activity as well as alter the effects of E2 in juvenile rainbow trout.

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1. Introduction

Many studies have demonstrated that pulp and paper mill effluent can affect fish, more specifically at the reproductive level (Hewitt et al., 2008). An increase in ethoxyresorufin-O-deethylase (EROD) activity has been regularly observed in fish exposed to pulp and paper mill effluents (Wartman et al., 2009; Orrego et al., 2009). Generally EROD is used as a biomarker for exposure to polycyclic aromatic hydrocarbons (PAHs) and organochlorines (Whyte et al., 2000). A reduction in gonad size has also been seen across many species of fish exposed to pulp and paper mill effluent (Lowel et al., 2005). A study by Orrego et al. (2011b) demonstrated that many types of pulp and paper mill effluent (treated and untreated) have the potential to cause embryo-toxicity (mainly delaying hatching time, and decreased hatchability) in the embryos of three different species (rainbow trout (*Oncorhynchus mykiss*), American flagfish (*Jordanella floridae*) and Japanese medaka (*Oryzias latipes*)). Another effect attributed to pulp and paper mill effluents is a change in the production of the egg yolk precursor protein vitellogenin (VTG) (Kovacs et al., 2005; Orrego et al., 2006), with induction of VTG in males and immature females indicative of endocrine disruption (Jensen and Ankley, 2006). The effects of pulp mill effluent on VTG in fish are quite variable; some studies in Canada and Chile have shown that exposure to pulp mill effluent increased plasma VTG in male fathead minnows (*Pimephales promelas*) and immature female rainbow trout (Kovacs et al., 2005; Orrego et al., 2006), while others in the USA have shown a reduction in plasma VTG in female largemouth bass (*Micropterus salmoides*) and no effect in males (Sepulveda et al., 2003). Either way, pulp and paper mill effluents have the potential to cause significant endocrine disruption.

It is proposed that most pulp and paper mill effluent effects arise from three main types of compounds: resin acids (e.g. abietic acid), isoflavonoids (e.g. genistein) and phytosterols (e.g. sitosterol and stigmastanol) (Hewitt et al., 2008). A detailed review of individual effluent compounds and mill operating conditions was published by Hewitt et al. (2008). However, effluent quality can vary substantially between mills using different manufacturing processes, mill furnish, operating conditions and effluent treatment, which contributes to the wide variety of observed effects between mills (Orrego et al., 2011a).

Resin acids are a class of wood extractives, naturally present in wood and found in higher levels in conifers (Hernandez et al., 2008). Resin acid effluent concentrations vary greatly between mills and represent some of the most acutely toxic chemicals in pulp mill effluents (Oikari et al., 1982). Biological treatment of resin acids during secondary treatment of pulp mill effluent is inconsistent (Wang et al., 1995). While the modernization of mills has significantly reduced the amount of resin acids emitted, they are still found in measurable levels in the receiving water and sediment downstream of mills (Orrego et al., 2009).

Dehydroabeitic acid (DHAA) is the most abundant resin acid and is acutely toxic to fish (Oikari et al., 1983). DHAA is a common pollutant of softwood pulp and paper mill effluent, and ~90

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0147-6513/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ecoenv.2013.11.027

percent of it is generally removed through secondary treatment (Hewitt et al., 2006). Because of this, many researchers deem it unlikely to cause effects. However, DHAA can still be measured in fish downstream of pulp and paper mills due to the constant loading of resin acids like DHAA into the environment even at low levels (Leppanen et al., 1998). The highest concentrations are accumulated in the bile, blood and liver of trout as DHAA ultimately accumulates in the bile to be eliminated (Oikari et al., 1982). In fact, the presence of resin acids in the bile of fish has been shown to be a good indicator (biomarker) of exposure to pulp and paper mill effluent (Lindesjoo et al., 2002). Recent research by Oikari et al. (2010) has been focused on showing the ability of DHAA to accumulate in sediment and to impact the environment.

Although not a strong endocrine disruptor, recent studies have suggested that DHAA may act as an anti-estrogen, such as reducing the production of vitellogenin in fish exposed to estradiol (Orrego et al. 2011b). However, the ability of DHAA to alter reproductive function in fish varies as a study by Chrisanson-Heiska et al. (2008a) saw a decrease in VTG levels in male zebrafish exposed to 50 μg/L but no change in female fish. As a result, resin acids such as DHAA may be a factor in the variance of observed endocrine disrupting effects due to pulp mill effluent exposure. However, the exact mechanism by which DHAA affects the endocrine system is unknown, and should be investigated. The anti-estrogenic activity of DHAA may not be mediated by the intracellular estrogen receptor (Teresaki et al., 2009). Since DHAA is known to be hepatotoxic, it may cause an indirect reproductive disruption as a result of liver damage (Orrego et al., 2010). Liver damage may cause suppression of the reproductive system if the body consider the fish unfit for such activities. As VTG is specifically produced in the liver, liver damage may impair the livers function to produce VTG.

Sorbitol dehydrogenase (SDH) activity in the plasma or serum of fish is directly linked to liver cell damage in fish, and is a useful biomarker of liver damage (Dixon et al., 1987). Serum/plasma SDH is frequently used to indicate mammalian liver damage (Raghavendra and Rao, 2000); it has also been commonly used in fish toxicity studies (Dixon et al., 1987; Holdway et al., 1994; Mukherjee and Jana, 2007). SDH analysis is preferred to utilizing other liver enzymes such as LDH, glutamate oxaloacetate transaminase or glutamate pyruvate transaminase, since SDH is not elevated by other organ diseases but rather is specific to liver damage (Dixon et al., 1987). SDH was more responsive at lower levels of toxicant exposure than other common measures of liver health such as hepatosomatic index (HSI) or histopathology (Dixon et al., 1987). Using SDH and VTG as biomarkers would allow the investigation of whether DHAA's anti-estrogenic effect is indirect due to liver cell damage.

DHAA has also been implicated in metabolic energy disruption in trout hepatocytes (Rissanen and Krumschnabel, 2003). Disrupted liver energy metabolism via altered LDH and CS enzyme activities was observed following a single intra-peritoneal (IP) injection of rainbow trout with Chilean pulp mill effluent (Orrego et al., 2011a). Studies have shown that changes in respiratory enzyme activities may be used as biomarkers of xenobiotic exposure in aquatic organisms (Gagnon and Holdway, 1998; Ozmen et al., 2008). As DHAA may affect energy metabolism it may play a role in the observed disruption of liver metabolic enzymes following pulp and paper mill effluent exposure.

This study seeks to determine the effect of DHAA on energy metabolism using metabolic biomarkers and to determine if DHAA acts as an anti-estrogen by causing liver damage. It is hypothesized that the phytosterol β-sitosterol (BS), and 17β-estradiol (E2) will show an induction of vitellogenin (VTG) levels and an increase in hepatosomatic index (HSI) after exposure. Furthermore, it is expected that simultaneous injection of DHAA+E2 and DHAA+BS will reduce the effects found in fish injected with E2 or BS alone.

2. Methods

2.1. Chemicals

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich or Fisher Scientific.

2.2. Experimental animals and conditions

A total of 184 female juvenile rainbow trout *Oncorhynchus mykiss* (92.4 ± 18.8 g) were obtained from the local hatchery and transported to the fish lab at UOIT. They were acclimated for two weeks in 12.1 ± 1 °C flow through, dechlorinated water in 1500 L tanks and held at a density of 7.5 kg/m^3 and at a flow rate of 3 L/min. Fish were approximately 1 year old at the start of the experiment. Photoperiod was a constant 16 h light, 8 h dark with 1/2 h of simulated dawn and dusk included in the light phase. Fish were fed once a day with 5.0 mm trout pellets (5PT, Martin Mills) until satiated.

2.3. Test chemical preparation

Test chemicals were prepared prior to injection. The DHAA and β-sitosterol used in this experiment, had been previously extracted from pulp mill effluent through solid phase extraction and estimated to be >95 percent pure (Orrego et al., 2009, 2010). Test compounds were first dissolved in acetone (including the control), allowing the compounds to be mixed with corn oil for injection. The acetone was allowed to evaporate off in a fume hood and afterward the bottles were capped and sealed. The test compounds included the steroid standard 17β-estradiol at 5 mg/kg wet weight (E2), one pulp mill phytosterol standard β-sitosterol at 5 mg/kg wet weight (BS), 3 different DHAA concentrations at 5.0, 0.5 and 0.05 mg/kg wet weight, 3 mixed DHAA and E2 concentrations at 5 mg/kg E2+5.0, 0.5 and 0.05 mg/kg wet weight DHAA respectively, a DHAA+BS mixed at 5 mg/kg BS+5 mg/kg DHAA wet weight, and a carrier control of intra-peritoneal injected corn oil, CO. All prepared injection solutions were stored at −20 °C and thawed and vortexed prior to injection.

2.4. Dose calculation and intra-peritoneal injection

The concentrations chosen were based on those used in previous studies (Orrego et al., 2009, 2010). A dilution of DHAA was also included ranging from 5 mg/kg DHAA to 0.05 mg/kg in order to determine if the effect observed in previous studies was dose dependent. The doses were determined immediately prior to injection based on individual fish weight (100 μl/100 g of body weight, Rottmann et al., 1991).

A total of 184 juvenile rainbow trout *Oncorhynchus mykiss*, were used in this 28 day pulse-dose toxicity experiment. Fish were anesthetized in tricaine methanesulfonate (MS-222, 100 mg/L buffered 1:2 with sodium bicarbonate), weighed, placed in a tray with flow through water and given an intra-peritoneal injection using a precision syringe (#187, SOCOREX) fitted with 22 G single use sterile needle (BD). After injection, fish were tagged with sequentially numbered and color coded Floy™ T-Bar anchor tags. Fish were then separated into replicate groups and placed in two 1500 L tanks divided in the following manner; 3 sampling periods, 2 replicates per treatment, 18 fish per treatment, 90 fish per tank, and 7.5 kg/m^3 density under the same experimental conditions used during the acclimatization period. The only differences from the acclimation period were that water temperature was 13.0 ± 1.06 °C and fish were fed twice per day 5.0 mm trout pellets until satiated.

2.5. Fish sampling/re-injection

Four fish were sacrificed prior to starting the experiment to evaluate their initial state of health and to get time zero measurements of enzyme activity, condition factor (K), hepatosomatic index (HSI) and gonadosomatic index (GSI). Groups of six fish per treatment were sampled after 7, 14, and 28 days after injection. Fish were anaesthetized in buffered tricaine then placed ventral side up on a dissection tray and euthanized by exsanguination in accordance with CCAC Guidelines. Blood was collected from the caudal vein using a 22G1 disposable needle and a 4 mL heparinized vaccutainer (BD). The liver and gonads were then extracted, weighed, placed in 2 mL cryotubes, flash frozen in liquid nitrogen and stored at −80 °C. The collected blood vaccutainers were spun at 3000 rpm at 4 °C for 10 min, the supernatant removed and placed in 1.5 mL micro-centrifuge tubes and these tubes were stored at −80 °C. The remaining trout in each tank not sampled were re-injected with their respective doses weekly, on days 7 (120 fish remaining), 14 (60 fish remaining) and 21 (60 fish remaining).

It should be noted that there were 3 mortalities during the 28 day exposure. All occurred on or shortly after the second injection, two fish from 5 ppm DHAA/BS (one from replicate a, one from replicate b) and one fish from the 5 ppm DHAA/E2 treatment. Mortality occurred after receiving their second injection. As a result of

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