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Exposure to a Brazilian pulp mill effluent impacts the testis and liver in the zebrafish

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ABSTRACT

While many studies have shown that pulp mill effluents can affect ovarian physiology in fish, far fewer studies have considered the effects in males. We conducted a lab study to examine the effects of effluent from a Brazilian pulp and paper mill on hepatic and testicular morphology and various aspects of testicular physiology in the zebrafish *Danio rerio*. Males were exposed to lab water (control) or 4% effluent for 14 days. Effluent exposure did not affect testis size as measured by the gonadosomatic index, but contributed to morphological changes in the seminiferous tubules. The number of cysts with histopathological changes was elevated in effluent-exposed fish and the number of cysts containing spermatids was significantly reduced. The testis of effluent exposed fish had reduced levels of lactate, elevated lactate dehydrogenase activity, increased levels of reactive oxygen species and reduced levels of phosphorylated P38 mitogen-activated protein kinase (pP38 MAPK). Separate studies showed that the addition of lactate to testicular tissue incubated *in vitro* increased the activation of P38 MAPK. Effluent exposure also increased vacuolization, necrosis, apoptosis, hyperemia, and fat infiltration of the hepatocytes. Collectively, we provide evidence of short term effects of pulp mill effluent on testicular and hepatic physiology and biochemistry in the zebrafish.

1. Introduction

For > 30 years, there have been reports that effluents from some pulp and paper mills have the potential to negatively affect fish populations. Many of these studies have shown effects of pulp mill effluents on reproduction (*e.g.* Munkittrick et al., 1998; Hewitt et al., 2008; van den Heuvel, 2010). Studies involving wild fish, *in situ* experiments, and laboratory *in vivo* tests conducted on a world-wide basis have documented reductions in sex steroid hormone levels, gonad size and fecundity, alterations in secondary sex characteristics, and delayed sexual maturity associated with exposure to pulp mill effluents or its constituents (*e.g.* Tana and Nikunen, 1986; Van Der Kraak et al., 1992; Kovacs et al., 2013). Other studies have shown that the liver is also affected by exposure to pulp mill effluents as evidenced by changes in gene expression, metabolism and morphology (Khan, 2010; Orrego et al., 2011; Costigan et al., 2012).

The purpose of this study was to evaluate the potential of treated effluent from the Klabin pulp and paper mill in Santa Catarina State in Brazil to affect liver and testicular morphology and the biochemistry of the testis in the zebrafish (*Danio rerio*). This was interest because few studies have tested effluents from South American pulp mills, and Brazilian mills in particular, for effects on fish. As the specific chemicals responsible for the biological effects of pulp mill effluents are not known, it was of interest to determine if Brazilian tree species or the mill process used would contribute to the toxicity of this effluent. As well, relative to studies conducted with female fish, few studies examine the responses of male fish to pulp mill effluents. In this study, adult zebrafish were exposed *in vivo* to effluent for 14 days and effects on testicular and hepatic structure were determined by histological evaluation. As well, a suite of biochemical responses within the testis were determined including measurement of lactate content, lactate dehydrogenase activity, total reactive species, and the amounts of P38 mitogen-activated protein kinase (P38 MAPK) and phosphorylated P38 mitogen-activated protein kinase (pP38 MAPK).

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2. Material and methods

2.1. Chemicals

Histological resin was obtained from Leica Biosystems (Nussloch, Germany). The indicator of reactive oxygen in cells 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Acrylamide and bis-acrylamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-P38 (sc-728) and anti-pP38 (sc-17852-R) were obtained from Santa Cruz Biotechnology (Dallas TX, USA). Peroxidase conjugated anti-rabbit IgG and the Immobilion™ Western Chemoluminescent Horseradish Peroxidase (HRP) substrate were obtained from Millipore (St. Charles, MO; Temecula, CA, USA). The test kits for Lactate (Ref: 138) and LDH (Ref: 086) were purchased from Labtest (Lagoa Santa, Minas Gerais, Brazil). All other chemicals were of analytical grade.

2.2. Pulp and paper mill effluent

Effluent was collected from the Klabin pulp and paper mill located in Correia Pinto in Santa Catarina State, Brazil. Pinus is used as the feedstock for the mill which uses a conventional Kraft bleaching process with the following sequence - O, C1, E1, D1, E2, where O represents the extended delignification, C1 the molecular chlorine treatment, E1 the first alkaline extraction, D1 the first extraction using chlorine dioxide and E2 second alkaline extraction. The effluent was treated in an aerated lagoon system with oxygen application and a 7 day retention time. The lagoon is exclusively for the treatment of the effluents generated by the pulp mill. After treatment, the effluent was released in the Canoas river. Effluent was collected after the last step of treatment and immediately before release to the river.

2.3. Animal care, maintenance and exposure

Male zebrafish weighing between 200 and 300 mg were used in this study. They were obtained from a commercial producer (Botiquarium Enterprise, Florianópolis, SC, Brazil) and housed in aquaria supplied with dechlorinated water pH 7.4 at 28 °C under a 12L:12D photoperiod. The fish were fed once a day.

All experiments were conducted using protocols approved by the Brazilian College of Animal Experimentation (Protocol CEUA/PP00968). For the *in vivo* exposures, male zebrafish (6 animals per group) were exposed semi-statically to lab water or 4% effluent in a 15L-aquarium with aeration. The experiments were 14 days in duration and the water was changed every two days. Testing of the effluent at a 4% dilution was based on previous studies of the toxicity of effluent from this mill on tilapia (*Oreochromis niloticus*) and reflected local riverine (fluvial) concentrations of the effluent (Zunino and Soares, 2007).

2.4. Sample collection

Fish were immobilized in ice cold water, euthanized by spinal transection and weighed. The testes were removed, weighed and separated for histology studies or homogenized for biochemical or Western blot analysis. The gonadosomatic index (GSI) was calculated as the testis weight/whole fish body weight × 100.

2.5. Histological analysis

For histological analysis, the testes and livers from six zebrafish from both control and effluent-exposed treatments were fixed in Bouin's solution for 24 h at room temperature. The samples were then dehydrated through a graded series of ethanol solutions and embedded in historesin. Serial sections (4 μm thickness) were cut and 12 to 20 slides with eight sections per slide were prepared for each specimen. The slides were stained with Hematoxylin–Eosin–Phloxyne and examined using a light microscope. The histological slides were analyzed qualitatively and quantitatively. For the testes, the numbers of cysts with histopathological changes and the number of cysts containing spermatids were counted on four different randomly chosen areas for each specimen. Each analyzed area was 0.0943 mm^2. As well, the number of spermatozoa was counted in four sections on two slides per testis (0.000885 mm^2).

2.6. Measurement of total lactate content and lactate dehydrogenase (LDH) activity

Testes collected after the 14 day exposure period were homogenized in cold Cortland's Buffer (124 mM NaCl, 5 mM KCl, 1.7 mM $CaCl_2$·2H2O, 3.4 mM NaH_2PO_4, 2.1 mM $MgCl_2$, 1.91 mM $MgSO_4$, 11.9 mM $NaHCO_3$, pH 7.6), centrifuged (2 min at 2000 × *g*) and the supernatant was collected. The samples were then analyzed for total lactate content and lactate dehydrogenase (LDH) activity by spectrophotometry based on the methods of Bergmeyer, 1983 and Burtis et al., 2007, respectively.

2.7. P38 and pP38 MAPK measurement

The amounts of P38 and pP38 MAPK were determined by polyacrylamide gel electrophoresis and immunoblotting. Testes were obtained from animals exposed *in vivo* for 14 days to effluent. In other tests, testes were obtained from untreated zebrafish and incubated *in vitro* for 30 min in Cortland's buffer supplemented with 10 mM sodium lactate to analyze the direct effect of lactate on P38 activation. The testes were homogenized in a lysis solution containing 2 mM EDTA, 50 mM Tris–HCl pH 6.8, 1 mM Na_3VO_4, 0.2% triton X-100, 0.5 mM dithiothreitol, 1 mM benzamidine. The total protein concentration was determined based on Lowry et al., 1951. For the electrophoresis analysis, samples were boiled for 3 min in 25% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris–HCl, pH 6.8. Equal protein concentrations (50 μg/μl) were loaded onto 12% polyacrylamide gels and separated by discontinuous SDS-PAGE. Separated proteins were transferred to nitrocellulose membranes for 1 h at 100 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol and 0.25% SDS). The nitrocellulose membranes were incubated for 2 h in blocking solution (TBS: 0.5 M NaCl, 20 mM Trizma, plus 5% bovine serum albumin) and then incubated overnight at 4 °C with anti-P38 (1:100) or anti-pP38 (1:200). Membranes were incubated for 2 h with anti-rabbit IgG (1:1000) and immunoreactive bands were visualized using the Immobilon™ Western Chemiluminescence HRP substrate kit (Zanatta et al., 2011). The images of the band were captured using the ChemiDoc MP system and the optical densities determined with Image Lab Software (Bio-Rad).

2.8. Determination of testicular ROS

The total ROS generation in testes was detected using the fluorogenic probe H_2DCFDA. Testes were collected from fish that had been exposed to control water or 4% effluent for 14 days. The testes were homogenized in sodium phosphate buffer (20 mM NaH_2PO_4 and 140 mM KCl; pH 7.4), centrifuged (960 × *g*, 10 min, 4 °C) and the supernatant incubated in the presence of 1 mM H_2DCFDA for 30 min at 37 °C. H_2DCFDA oxidation was measured by spectrophotometry (excitation in 485 and emission in 520 nm) using the methods described by Halliwell and Whiteman (2004). The results were expressed as fluorescence units (UF)/μg of protein.

2.9. Effluent chemistry

Total phenols were determined using the colorimetric method based on the phenol and 4-aminoantipyrine (4-AAP) reaction. This approach uses 0.21 M sodium persulfate as a catalyst at pH 10 in ammonium