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Chronic effects of exposure to a pharmaceutical mixture and municipal wastewater in zebrafish

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ABSTRACT

Pharmaceuticals and personal care products (PPCPs) are discharged in municipal wastewater. Effects in aquatic organisms exposed to individual pharmaceuticals in the laboratory have raised concerns regarding the environmental impacts of PPCPs, yet environmental exposures are always to complex mixtures. In this study, adult zebrafish (*Danio rerio*) showed significantly decreased embryo production after a 6 week exposure to a pharmaceutical mixture (MIX; 0.5 and 10 $\mu g\,L^{-1}$) of acetaminophen, carbamazepine, gemfibrozil and venlafaxine and to diluted wastewater effluent (WWE; 5% and 25%). Atretic oocytes and altered ovarian histology were significantly increased in female zebrafish exposed to both concentrations of MIX or WWE, which indicates a direct effect on oocyte development that may account for reduced embryo production. Apoptosis within the thecal and granulosa cell layers was identified in female zebrafish with atresia. Exposures to MIX or WWE at both concentrations severely altered kidney proximal tubule morphology, but no histological impacts on other organs were observed. Exposure of embryos to MIX or WWE at the high concentration significantly increased the incidence of developmental abnormalities. Embryo mortality was elevated with exposure to the high concentration of MIX. These studies indicate that chronic exposure of fish to pharmaceutical mixtures and wastewater impacts reproduction and induces histopathological changes, similar to what we have previously seen with single compound exposures. These data suggest that fish populations exposed to pharmaceuticals discharged in wastewater are at risk of negative impacts to reproductive capacity and health.

1. Introduction

Many studies have documented low concentrations of pharmaceuticals and personal care products (PPCPs) in surface (Sacher et al., 1998), ground (Domagalski et al., 2007), and drinking (Benotti et al., 2008) water. The primary route of entry of PPCPs into the environment is via discharges of wastewater effluents (WWE) from municipal wastewater treatment plants (WWTPs). Pharmaceuticals are present in sewage as a result of the direct consumption and excretion of the parent compound or metabolites in human waste products (Halling-Sorensen et al., 1998), and to a lesser extent, by direct disposal of unused medications (Glassmeyer et al., 2009). Not all pharmaceutical compounds are removed effectively during wastewater treatment (Halling-Sorensen et al., 1998; Kolpin et al., 2002). Some products tend to persist while others are only marginally degraded in the WWTP, or by-pass treatment all together through sewage overflow (Stackelberg et al., 2004). The presence and the concentrations of pharmaceuticals in the aquatic environment are thus determined by prevalence of use, the rates of metabolism in humans, the effectiveness of wastewater treatment, and resistance to degradation in the aquatic environment. Nonetheless, there are strong similarities in North America and Europe in the types and concentrations of pharmaceuticals that have been detected in receiving waters. Concentrations typically are in the ng–$\mu g\,L^{-1}$ range (Benotti and Brownawell, 2007; Clara et al., 2005; Lau et al., 2002) and include analgesics, lipid regulators, antibiotics, beta blockers, anti-depressants and steroids (Clara et al., 2005; Kolpin et al., 2002; Lau et al., 2002).

Since pharmaceuticals are designed to induce specific biological responses in humans at low doses and vertebrate taxa share gene homology with high sequence similarity to humans (Gunnarsson et al., 2008; Huggett et al., 2005), laboratory and field based investigations with fish have focused on whether WWE and pharmaceuticals induce responses in teleosts that are analogous to the clinical effects of pharmaceuticals. Although the concentrations of

Abbreviations: WWE, wastewater effluent; MIX, pharmaceutical mixture containing acetaminophen carbamazepine gemfibrozil and venlafaxine; ACE, acetaminophen; CBZ, carbamazepine; GEM, gemfibrozil; VEN, venlafaxine.

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pharmaceuticals found in WWE and receiving waters are much lower than therapeutic doses, chronic exposures and the potential for some pharmaceuticals to bioaccumulate may lead to deleterious effects in aquatic organisms (Cleuvers, 2004). Studies with fish have correlated WWE exposure with elevated stress (Hignite and Azarnoff, 1977), variations in sex steroid levels in both juveniles and adults (Lishman et al., 2006), impaired gonadal development (Sedlak et al., 2000), gonadal intersex (Ings et al., 2011) and decreased reproduction (Lister et al., 2009; Schreck et al., 2001). Laboratory studies have shown that egg production is significantly reduced in sexually mature female zebrafish (*Danio rerio*) exposed to 50% treated wastewater for 7 days (Lister et al., 2009). Intersex and elevated vitellogenin have been documented in wild fish collected at sites downstream from WWE discharges (Brooks et al., 2006; Kavanagh et al., 2004; Vajda et al., 2008; Woodling et al., 2006).

Since natural (17β-estradiol) or synthetic (17α-ethynylestradiol) estrogens are present in WWE, steroids have been the presumed causative agent in many studies that have documented effects on fish reproduction and gonadal development (Tarrant et al., 2008; Vajda et al., 2008). Yet, these effects may not be a consequence of exposure to estrogens alone. Endocrine disrupting chemicals (EDCs) include compounds with multiple mechanisms of action that can impact a variety of physiological pathways. Several laboratory studies have examined the physiological consequences of exposure to non-estrogenic pharmaceuticals on aquatic vertebrates (Clara et al., 2004; David and Pancharatna, 2009; Mennigen et al., 2008; Mimeault et al., 2006). These studies indicate that multiple pharmaceuticals may have endocrine disrupting capacities that are not likely mediated by steroid receptors.

In our companion study, zebrafish were exposed for 6 weeks to 0.5 and 10 $\mu g\,L^{-1}$ acetaminophen (ACE), carbamazepine (CBZ), gemfibrozil (GEM), or venlafaxine (VEN) in single compound exposure experiments (Galus et al., in press). The concentrations were chosen based on reported levels in surface water (Andreozzi et al., 2003; Benotti and Brownawell, 2007; Kolpin et al., 2002; Metcalfe et al., 2003; Tixier et al., 2003). The high concentration was near the highest reported concentrations. Since complex PPCP mixtures are found in the environment, many pharmaceuticals are present with similar modes of action and the high concentration is similar to the concentration for all PPCPs within a single drug class. Thus, the concentrations were not meant to be an exact match to what is found at one wastewater treatment plant, or field site, but to represent concentrations that were reasonable and environmentally relevant. Chronic exposure of zebrafish to 10 $\mu g\,L^{-1}$ ACE or VEN, and 0.5 and 10 $\mu g\,L^{-1}$ CBZ or GEM significantly decreased fecundity (Galus et al., in press). Atretic oocytes and significantly altered ovarian histology were observed in female zebrafish exposed to CBZ and GEM at both concentrations, indicating a direct effect on oocyte development that may account for reduced reproduction (Galus et al., in press). Apoptosis within the theca and granulosa cells was identified in exposed female zebrafish with atretic oocytes by TUNEL positive staining. The incidence of follicular apoptosis was nearly 2-fold higher in exposed females than the controls. Pathological effects on kidney proximal tubules were observed histologically in both male and female zebrafish exposed to all pharmaceuticals at both concentrations. Liver histology was altered by ACE and GEM exposure. Parental exposure to pharmaceuticals did not increase developmental abnormalities, hatching success, or mortality in embryos. However, direct exposures of zebrafish embryos to all pharmaceutical compounds increased mortality and ACE exposure significantly increased developmental abnormalities (Galus et al., in press). Extrapolation of these results to the environment is difficult as pharmaceuticals are always present as complex mixtures in the environment and only a few studies have characterized the effects of pharmaceutical mixtures (Brian et al., 2006; Cleuvers, 2004; Thorpe et al., 2003). Since mixtures of PPCPs are found within WWE and different classes of pharmaceuticals have varying mechanisms of action (Cleuvers, 2004), it is possible that the effects of single pharmaceuticals cannot be replicated in a mixture of these compounds.

The present study was designed to investigate effects of chronic exposure to low concentrations of a mixture of pharmaceuticals (with different mechanisms of action) and diluted WWE on the reproduction, histopathology, and development of zebrafish. This study determines if a pharmaceutical mixture and a more complex effluent induce similar effects to those seen previously with single PPCP compounds. The compounds used in the pharmaceutical mixture were selected based on prior studies of single PPCP effects in zebrafish (Galus et al., in press) and were confirmed to be present in wastewater from the WWTP that provided the WWE for exposures. Effects found in single compound, mixture, and complex effluent exposures would be suitable endpoints for laboratory-based studies of mixture toxicity and drug–drug interactions and field studies downstream of WWE discharge.

2. Materials and methods

2.1. Wastewater

All samples of wastewater that were used for a preliminary survey of PPCPs in untreated and treated wastewater and for exposures of zebrafish to WWE were collected from a WWTP located in southern Ontario, Canada. The WWTP serves a population of approximately 480,000 through 2100 kilometers of sewers in a 40% combined and 60% separated sewer system. The WWTP uses secondary treatment with activated sludge. The average flow rate is 350 $ML\,day^{-1}$, with a hydraulic retention time of approximately 6.5 h.

2.2. Test chemicals

Pharmaceutical mixtures (MIX) were composed of equal concentrations of acetaminophen, carbamazepine, gemfibrozil, and venlafaxine. For stock solutions, pharmaceutical compounds (Sigma Aldrich, Toronto, ON, Canada) were dissolved in either reverse osmosis treated water (ACE and VEN) or in dimethyl sulfoxide (DMSO; GEM and CBZ) and diluted into 12 L (Experiment I) or 8 L (Experiment II) tanks with system water (distilled water with 12 $mg\,L^{-1}$ sodium bicarbonate and 60 $mg\,L^{-1}$ sea salts, Instant Ocean, Spectrum Brands, Madison, Wisconsin USA) for adult exposure experiments. PPCPs were diluted into 48 well plates with E3 media (5 $\mu g\,L^{-1}$ NaCl, 0.17 $\mu g\,L^{-1}$ KCl, 0.33 $\mu g\,L^{-1}$ $CaCl_2$ and 0.33 $\mu g\,L^{-1}$ $MgSO_4$) for embryo exposures. The nominal concentration of each pharmaceutical in the exposure tanks was 0 (control), 0.5 (low) or 10 (high) $\mu g\,L^{-1}$. The control and exposure tanks had a final concentration of carrier solvent (DMSO) of 0.004%. A water only control was not used and there were no differences in any measured endpoint (reproductive, developmental, histological, hormonal) across solvent and water controls during experimental exposures with ACE, CBZ, GEM or VEN alone (Galus et al., in press). The concentrations of PPCPs in the MIX were based on those used in single compound exposures in Galus et al. (in press).

For the treatments with wastewater effluent, final treated effluent was collected bi-weekly from the WWTP as a composite 24 h sample, transported to the lab and stored in the dark at 5 °C. WWE was diluted to 5% (low) or 25% (high) with system water or with E3 media for adult and embryo exposures, respectively. Control tanks and wells contained system water or E3 media alone.