Aquatic Toxicology 160 (2015) 76–86

Contents lists available at ScienceDirect

Aquatic Toxicology

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

Chronic effects of clofibric acid in zebrafish (*Danio rerio*): A multigenerational study

Ana M. Coimbra[c,d,1], Maria João Peixoto[a,d,1], Inês Coelho[a], Ricardo Lacerda[a], António Paulo Carvalho[a,b], Manuel Gesto[a,e], Angeliki Lyssimachou[a], Daniela Lima[a], Joana Soares[a], Ana André[a], Ana Capitão[a], Luís Filipe C. Castro[a,b], Miguel M. Santos[a,b,*]

[a] *CIMAR/CIIMAR, Interdisciplinary Centre for Marine and Environmental Research, University of Porto, Rua dos Bragas 177, 4050-123 Porto, Portugal*
[b] *FCUP, Faculty of Sciences University of Porto, Department of Biology, Rua do Campo Alegre, 4169-007 Porto, Portugal*
[c] *Centre for The Research and Technology of Agro-Environmental and Biological Sciences (CITAB), University of Trás-os-Montes e Alto Douro (UTAD), Quinta de Prados, 5000-801 Vila Real, Portugal*
[d] *Department of Biology and Environment, Life Sciences and Environment School (ECVA), University of Trás-os-Montes e Alto Douro (UTAD), Quinta de Prados, 5000-801 Vila Real, Portugal*
[e] *Department of Functional Biology and Health Sciences, Faculty of Biology, University of Vigo, As Lagoas-Marcosende s/n, 36310, Vigo, Spain*

ARTICLE INFO

Article history:
Received 12 September 2014
Received in revised form 8 January 2015
Accepted 15 January 2015
Available online 16 January 2015

Keywords:
Lipid metabolism
Endocrine disruption
Multigenerational
Reproduction
Emerging pollutant
Epigenetic

ABSTRACT

Clofibric acid (CA) is an active metabolite of the blood lipid lowering agent clofibrate, a pharmaceutical designed to work as agonist of peroxisome proliferator-activated receptor alpha (PPARa). It is the most commonly reported fibrate in aquatic environments with low degradation rate and potential environmental persistence. Previous fish exposures showed that CA may impact spermatogenesis, growth and the expression of fat binding protein genes. However, there are limited data on the effects of chronic multigenerational CA exposures.

Here, we assessed chronic multigenerational effects of CA exposure using zebrafish (*Danio rerio*) as a teleost model. Zebrafish were exposed through the diet to CA (1 and 10 mg/g) during their whole lifetime. Growth, reproduction-related parameters and embryonic development were assessed in the exposed fish (F1 generation) and their offspring (F2 generation), together with muscle triglyceride content and gonad histology. In order to study the potential underlying mechanisms, the transcription levels of genes coding for enzymes involved in lipid metabolism pathways were determined.

The results show that chronic life-cycle exposure to CA induced a significant reduction in growth of F1 generation and lowered triglyceride muscle content (10 mg/g group). Also, an impact in male gonad development was observed together with a decrease in the fecundity (10 mg/g group) and higher frequency of embryo abnormalities in the offspring of fish exposed to the lowest CA dose.

The profile of the target genes was sex- and tissue-dependent. In F1 an up-regulation of male hepatic *pparaa*, *pparb* and *acox* transcript levels was observed, suggesting an activation of the fatty acid metabolism (provided that transcript level change indicates also a protein level change). Interestingly, the F2 generation, raised with control diet, displayed a response pattern different from that observed in F1, showing an increase in weight in the descendants of CA exposed fish, in comparison with control animals, which points to a multigenerational effect.

1. Introduction

The development of modern medicine and the continuous fabrication of new drugs have increased human life expectancy across the globe but have also resulted in the release of significant amounts of pharmaceuticals into the environment. Consequently, the presence of these compounds in aquatic ecosystem is an ongoing concern (Daughton and Ternes, 1999; Fent et al., 2006; Heberer, 2002; Corcoran et al., 2010; Rodrigues et al., 2006; Santos et al., 2010; Sárria et al., 2011). Pharmaceuticals are

* Corresponding author at: CIMAR/CIIMAR, Interdisciplinary Centre for Marine and Environmental Research University of Porto, Rua dos Bragas 177, 4050-123 Porto, Portugal. Tel.: +351 223401812; fax: +351 223390608.
E-mail addresses: acoimbra@utad.pt (A.M. Coimbra), santos@ciimar.up.pt (M.M. Santos).
URL: http://www.utad.pt (A.M. Coimbra).
[1] Equal contribution.

http://dx.doi.org/10.1016/j.aquatox.2015.01.013
0166-445X/

designed to be biologically active, to have metabolic stability and to pass through biological membranes. When these properties are combined, their removal during wastewater treatment is limited (Fent et al., 2006), allowing these compounds to enter the aquatic environment and potentially resulting in chronic exposure of aquatic non-target organisms. Among aquatic animals, fish are a prime potential target, susceptible to both bioconcentration and biomagnification of water contaminants, since they can be exposed to municipal effluents throughout their lifetime.

Clofibric acid (CA), an active metabolite of the blood lipid lowering agent clofibrate, (Daughton and Ternes, 1999; Fent et al., 2006), was one of the first pharmaceuticals to be reported in water samples (Hignite and Azarnoff, 1977). Over the years, its presence has been detected in different countries' wastewaters, surface waters, groundwater, tap water and seawater (Buser et al., 1998; Heberer et al., 2002; Heberer and Stan, 1997; Hignite and Azarnoff, 1977; Stumpf et al., 1999; Ternes, 1998). Environmental concentrations of this compound range from 1–2 ng/L in the North Sea (Buser et al., 1998), to 270 ng/L in Berlin drinking water, and up to 4 μg/L in groundwater (Heberer and Stan, 1997). Furthermore, CA has a low degradation rate which might potentiate environmental persistence (Wu et al., 2012). Clofibrate belongs to the class of fibrates along with bezafibrate, fenofibrate, ciprofibrate and gemfibrozil (Staels et al., 1998). Fibrates are used to decrease plasma triglyceride and cholesterol levels and their mode of action involves several mechanisms, namely: induction of lipoprotein lipolysis; induction of hepatic fatty acid uptake and reduction of hepatic triglyceride production; increased removal of low-density lipoprotein (LDL) particles; reduction in neutral lipid (cholesteryl ester and triglyceride) exchange between very-low-density lipoproteins (VLDL) and high-density lipoproteins (HDL); increase in HDL production and stimulation of reverse cholesterol transport (Staels et al., 1998). Fibrates, including CA, also regulate the expression of proteins (including enzymes) involved in lipid transport and metabolism by acting as transcription activators of the nuclear receptor heterodimer peroxisome proliferator-activated receptors (PPARs)-retinoid X receptors (RXRs) (Schoonjans et al., 1996), being designed to act as agonists of PPARa (Ibabe et al., 2005b).

Considering their effects in mammalian lipid metabolism, these pharmaceuticals might also interfere with lipid homeostasis and growth of non-target exposed animals, like fish as recently shown by the regulation of the genes coding for fatty acid-binding proteins (Venkatachalam et al., 2012; Venkatachalam et al., 2013) and the enzyme fatty acyl-coenzyme-A oxidase (FAO) involved in fatty acid oxidation (Weston et al., 2009). Previous studies on the effects of CA on the growth of fish were inconclusive as both reduced growth (Owen et al., 2010; Raldua et al., 2008) or no effects at all have been observed following exposure (Owen et al., 2010). However, CA appears to have an effect on cholesterol levels, the precursor of all sex steroid hormones. In fact, exposure of the grass carp (*Ctenopharyngodon idella*), fathead minnow (*Pimephales promelas*) and zebrafish to fibrates reduced plasma cholesterol levels and consequently induced hypocholesterolemic effects (Du et al., 2008; Owen et al., 2010; Velasco-Santamaria et al., 2011). Furthermore, waterborne exposures to gemfibrozil (goldfish, *Carassius auratus*) and to CA (fathead minnow) induced a decline in testosterone concentrations (Mimeault et al., 2005; Runnalls et al., 2007). Likewise, the exposure of zebrafish to bezafibrate through the diet suppressed the levels of 11-ketotestosterone (11KT) (Velasco-Santamaria et al., 2011). Additionally, studies in fathead minnow showed that exposure to fibrates reduced both sperm count and motility, suggesting a potential impact in males (Runnalls et al., 2007) and studies of Weston et al. (2009) show that CA exposure for 21 days at a concentration of 108.91 mg/L impacts egg production. Fish can be continually exposed to these chemicals in their natural environment, through diffuse sources such as STWs, and therefore may suffer from gradual, prolonged exposure effects that may be difficult to detect. In fact, the constant, multi-generational exposure of aquatic life has unknown consequences at the population level (Sumpter, 2005).

Here we assessed chronic multigenerational effects of CA exposure in fish. Although CA is the most commonly reported and persistent fibrate in aquatic ecosystems, the risk toward teleost fish is still poorly understood and contradictory findings have previously been reported. Hence, this study aimed at (a) assessing the multigenerational impact of CA exposure in fish; (b) comparing the findings with the literature on CA effects in mammals, and (c) relating the adverse effect endpoints with the underlying mechanisms of action.

2. Materials and methods

2.1. Fish maintenance and reproduction

Adult wild-type zebrafish (*Danio rerio*), obtained from local suppliers in Singapore, were used as breeding stocks. Zebrafish were maintained under controlled temperature (28 ± 1 °C) and photoperiod 14:10 h (light:dark) in dechlorinated and aerated water in a recirculation system with both mechanical and biological filters. Water parameters (i.e., dissolved oxygen, nitrates and ammonium) were monitored twice a week, whereas temperature was monitored daily. The fish were fed *ad libitum* twice a day with a fish based diet, prepared according to Carvalho et al. (2006), and every other day with a *Artemia* supplement.

In order to obtain the fish for the exposure assay (F1 generation) and for the multigenerational assays (F2 and F3 generations), in the afternoon before breeding, groups of males and females were independently housed in cages with a net bottom covered with glass marbles (Soares et al., 2009). In the following day, 1.5 h after the beginning of the light period, breeding fish were removed and the eggs were collected and cleaned. Fertilized eggs were randomly allocated (F1 generation) or allocated according to their parenthood (F2) generation. F3 generation was maintained only up to 6 days post fertilization (dpf) to evaluate embryonic development according to Soares et al. (2009).

2.2. Experimental design

In order to study the effects of clofibric acid (CA) on teleost fish, a multigenerational life-cycle study was performed with zebrafish (Fig. 1).

In F1 generation, 5 dpf eleuthero-embryos ($n = 330$) were randomly allocated to 5 L aquaria kept in a water-bath inside 30 L aquaria to keep temperature constant. At 20 dpf, the number of fish was adjusted to 100 and the fish were allocated to the corresponding 30 L aquaria. This number was further reduced to 30 fish per aquarium at 60 dpf. For the exposure experiments, seven flow-through systems were used (Soares et al., 2009) with an approximate flow rate of 40 L/day: three replicates for control fish and two replicates for each CA concentration. CA was dosed through the diet, at nominal contents of 1 and 10 mg/g of food and the exposure period was from 5 to 140 dpf. These contents were selected based on human therapeutic doses and according to previous studies that used a similar range of bezofibrate and clofibrate contents incorporated in the diet in zebrafish (Velasco-Santamaria et al., 2011; Venkatachalam et al., 2012; Venkatachalam et al., 2013). CA was incorporated in the diet using acetone as vehicle. Diet from control treatments received acetone only (Santos et al., 2006). Zebrafish were fed twice a day with the previously described diet, and the amount of food was adjusted