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Hypothalamic-pituitary-ovarian axis perturbation in the basis of bisphenol A (BPA) reproductive toxicity in female zebrafish (*Danio rerio*)

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ABSTRACT

Thousands of safety-related studies have been published on bisphenol A (BPA), an ubiquitous environmental pollutant with estrogenic activity and many other potential biological effects. In recent years, BPA exposure has been shown to cause anovulation and infertility through irreversible alteration of the hypothalamic-pituitary-gonadal axis in several organisms, including fish and mammals. Recently, the European Chemical Agency classified BPA as a "substance of very high concern" because of its endocrine-disrupting properties, which have serious effects on human health. Given the risk of exposure to BPA as a pollutant in the environment, food, and drinking water, the objective of our study was to assess the effects of this compound on the adeno-hypophysis by means of a histopathological and morphometric study of the gonadotroph cells. In addition, using quantitative real-time PCR (qRT-PCR) assays, we analyzed the changes in the expression of Cyp19b (an aromatase gene). Zebrafish were randomly distributed into five groups: a control group and 4 treated groups which were exposed to different BPA concentrations (1, 10, 100 and 1000 μg/L). The effects of the different doses on Cyp19b mRNA molecules followed a non-monotonic curve, with the 1 and 1000 μg/L doses causing dramatic decreases in the number of *Cyp19b* transcripts while the doses of 10 and 100 μg/L caused important increases. The consequences might be deregulation of gonadotropic hormones causing degeneration of gonadotropic cells, as observed in BPA treated animals. This is the first study in which the gonadotroph cells have been evaluated using histomorphological endpoints after BPA exposure in zebrafish.

1. Introduction

There is a growing body of evidence that environmental exposure to endocrine-disrupting chemicals (EDCs) is ubiquitous and exerts potentially adverse health effects on humans (Hachfi et al., 2012). Consequently, there is an increasing need for consistent screening assays that specifically detect and characterize EDCs present in the environment (Sun et al., 2010; Flint et al., 2012). Bisphenol A (BPA), considered an EDC, is one of the most abundantly produced chemicals worldwide and an usual component of plastics and food containers. Over 3.5 million tons of BPA are produced each year, and more than 100 t are released into the atmosphere (Hoekstra and Simoneau, 2013; Fitzgerald et al., 2014). Changes in pH, mechanical abrasions, and heat accelerate hydrolysis of the ester bond linking BPA molecules in polycarbonate plastic, and repeated washing of polycarbonate products causes BPA to easily leach out and spread through the environment, even at moderate temperatures (Hoekstra and Simoneau, 2013; Huo et al., 2015; Inagaki et al., 2016). In the past few years, interest has increased because of its ubiquitous presence and thus the increasing exposure of humans and other organisms from the environment and food has become a public health problem (Shi et al., 2015); aquatic wildlife, in particular, appears to be at an increased risk, but humans and all animals may suffer adverse health effects from current BPA levels (Chapin et al., 2008; Sun et al., 2010; Rochester, 2013; Teeguarden and Hanson-Dury, 2013; Canesi and Fabbri, 2015). In June 2017, the European Chemical Agency (ECHA), supporting the decision made by France, classified BPA as a "substance of very high concern" because of its endocrine-disrupting properties, which have serious effects on human health. Because of its

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ability to mimic the body's own hormones and bind estrogen receptors, BPA interferes with the function of the endocrine system and alters overall female reproductive capacity by affecting the morphology and function of the oviduct, uterus, ovaries, and hypothalamus-pituitary-ovarian axis (HPO) (Ziv-Gal and Flaws, 2016). Normal folliculogenesis depends upon intra-ovarian androgens for the synthesis of estradiol, regulated by the neuroendocrine hypothalamic-pituitary-ovarian axis driven by the gonadotropin-releasing hormone (GnRH) neuronal network. The coordinated action of two gonadotropins, namely, luteinizing hormone (LH, GTHII in fish) and follicle-stimulating hormone (FSH, GTHI in fish), controls the levels of androgen and their conversion to estradiol. LH acts on the theca cells, inducing secretion of androstenedione. FSH acts on granulosa cells, promoting the conversion of androstenedione to estradiol by the action of aromatase. Estradiol levels must increase and predominate over androgen concentrations in order for follicles to develop (Ziv-Gal and Flaws, 2016). A fine balance exists, and excessive production of androgens as a consequence of disordered folliculogenesis results in poor follicle maturation and increased follicular atresia (reviewed in Baskind and Balen, 2016). The phenolic structure of BPA allows this compound to interact with estrogen receptors and estrogen signaling pathways, and it has been reported that BPA may be as effective as estradiol in triggering some receptor responses; it may also act as an androgen receptor antagonist (reviewed in Flint et al., 2012). Rodent and fish studies indicate that BPA exposure also affects FSH and LH levels, although the results are limited and inconclusive (Ziv-Gal and Flaws, 2016). The objective of the present study was to investigate the toxic effects of a wide range of BPA dosages, including a dose of 1 µg/L, at the pituitary level, which could render female *Danio rerio* unable to reach normal reproductive capacity. We assessed the histopathological consequences of BPA exposure for gonadotroph cells and analyzed the gene expression of brain aromatase (Cyp19b) at the transcript level, as this gene codes for the main aromatase in teleost fish brain (Diotel et al., 2010) and the primary enzyme required for estrogen production (Nakamura et al., 2003; Frisch, 2004). Both parameters are proposed as biomarkers of BPA exposure.

2. Material and methods

2.1. Fish exposure and sampling protocol

Sixteen-week-old female zebrafish *(Danio rerio)* (n = 105) were used. The experimental procedure was conducted at the Experimental Animal Service of the University of Córdoba, in concordance with the European Regulations for the Protection of Experimental Animals (Directive 2010/63/EU). The treated groups were exposed for 14 days (Organization for Economic Co-operation and Development -OECD-Guideline No. 204) to graded concentrations (1, 10, 100 and 1000 µg/L) of BPA (Sigma-Aldrich®). The concentrations were maintained constants by a continuous flow-through system using a pump which have guaranteed 10 water renewals/day. A control group (kept in unchlorinated tap water) completed the exposure design. After 2 weeks of exposure, the zebrafish were sacrificed by an overdose of an anesthetic solution of tricaine methanesulfonate (MS-222® 500 mg/L; Sigma-Aldrich) buffered with sodium bicarbonate (300 mg/L; Sigma-Aldrich). The heads of 30 animals (n = 6 per group) were dissected and fixed for histological analysis for the qualitative and quantitative evaluations. The heads of 45 additional zebrafish (n = 9 per group) were removed, immediately immersed in liquid nitrogen, and stored at −80 °C for qRT-PCR. The fish (n = 6 per group) used for the toxicological analysis (analytical BPA determinations) were dried with sterile gauze and then frozen and stored at −80 °C.

2.2. Analysis of BPA content

Whole-body homogenates were obtained for BPA quantification. They were homogenized with a ribolyser to 10.000 G in 1 ml tubes at a 1:2 ratio of tissue wet weight to buffer (50 mM Tris-HCl pH 7.4).

Samples were processed for extraction and purification and finally transferred into vials. A volume of 20 µl was injected into a LC-MS/MS system for BPA quantification using the technique employed by Molina et al. (2013).

2.3. Histological evaluation

2.3.1. Qualitative study

For the structural evaluation, samples were routinely processed for paraffin sections by fixation in 10% buffered-formalin solution, dehydration in a graded ethanol series, immersion in xylol and embedding in paraffin wax. Section (4 µm thick) of each block were stained with hematoxylin and eosin and used for the morphological evaluation.

For the ultrastructural study, small, randomly selected samples were first fixed in a 2% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.4, 4 °C overnight) and then refixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 30 min. After, samples were dehydrated in a graded ethanol series and embedded in Araldite. Semithin and ultrathin sections were cut on an LKB ultramicrotome at the Central Microscopy Research Facilities, University of Córdoba, Spain. Ultrathin sections were double stained with uranyl acetate and lead citrate. For the scanning electron microscope, the sample was critical point dried and then coated in colloidal gold. Ultrathin sections were viewed and photographed with a JEM 1400 transmission electron microscope.

2.3.2. Quantitative study

The morphometric study of the gonadotroph cells was conducted directly on images of the rostral pars distalis (RPD) of the adeno-hypophysis under the electron microscope, quantifying both the number and the size of the cells. Twenty small squares per animal were quantified for a total of 100 squares per group, each grid square being 100 µm per side, the total surface area analyzed per group being $100 \times 100 = 10{,}000\ \mu m^2$. We based our counts on cell identification by the pleomorphism of granules, measuring the nucleus surface so that we obtained the number of cells observed on that surface.

2.4. Isolation of RNA and synthesis of cDNA

Total RNA was isolated using Isol-RNA Lysis Reagent (5PRIME) and the Total RNA Cleanup/DNase Digestion kit to remove genomic DNA contamination; the success of the isolation and purification was verified by PCR amplification of the RNA samples without previous reverse transcription, using specific intraexonic primers designed for GAPDH (F: 5´-CCAGTACGACTCCACCCATGGAAA-3´; R: 5´-CGCTATAGACTG TGATTGCATGACCA-3´). PCR was performed using the following program: 95 °C for 2 min; 40 cycles of denaturing at 94 °C for 15 s, annealing at 65 °C for 30 s, and extension at 72 °C for 30 s; and a final extension step at 72 °C for 10 min.

Quantification of RNA was conducted by measuring optical density at 260 nm. An absorbance ratio of 2:1 at 260 nm and 280 nm was considered indicative of protein-free samples. The integrity of the RNA samples was determined with an Agilent 2100 Bioanalyzer (Agilent Technologies) and only samples with RIN values > 8.5 were used.

cDNA was generated from 1 µg total RNA per reaction with the QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer's protocol.

2.5. Primer design and amplification efficiency

Primers for the genes ActB, B2m, Hprt1, GusB, Nono and Rpl13 have been previously described (Molina et al., 2018). To design specific primers to quantify the mRNA transcripts of *Cyp19b*, we obtained the sequences from the GenBank database (http://www.ncbi.nlm.nih.gov/gene) and used the software Oligo 7 software (Molecular Biology Insights, Inc.). The *Cyp19b* primers (F: 5´-ACGTTACTGCTGCTG ACGGG