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Evaluation of toxicological endpoints in female zebrafish after bisphenol A exposure

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ABSTRACT

Given the importance of bisphenol A (BPA) as a xenoestrogen and its potential effects on human and animal health, we evaluated BPA exposure's short-term effects on follicular development, yolk protein vitellogenin (VTG) production and aromatase expression in female zebrafish. Histological modifications were observed along with increased presence of atretic follicles. Whole-body VTG concentration increased with the dose of BPA exposure. In contrast, expression of *Cyp19a* mRNA in the ovaries of BPA-exposed fish exhibited an apparent non-monotonic response curve, marked by downregulation at 1 μg/L BPA, upregulation at 10 μg/L BPA, and a return to downregulation at 100 μg/L BPA and higher doses. Ovaries only exhibited significant increases in follicular atresia and VTG concentration after exposure to 100 μg/L BPA and higher doses. Ovarian histopathology, aromatase *Cyp19a* transcript levels and whole-body VTG protein abundance may be good biomarkers for early detection of environmental BPA exposure.

1. Introduction

Endocrine-disrupting chemicals (EDCs) have attracted considerable worldwide scientific and public attention due to their potential adverse effects on exposed organisms. Xenoestrogenic endocrine-disrupting compounds are found in the environment as a result of industrial and manufacturing activities (Cheng et al., 2011). Among these compounds, bisphenol A (BPA) is one of the most highly produced chemicals worldwide. BPA is ubiquitously present as an environmental contaminant in rivers and drinking water, most likely due to the migration of plastic containers from industrial waste sites. In river water BPA levels were reported of 0.01–21 μg/L while higher concentrations were also detected near wastewater treatment plants or landfills (Crain et al., 2007; Kang et al., 2007; Naderi et al., 2014). BPA is widely used in the manufacturing of various polycarbonate plastics used in lacquer coatings of food cans and food and beverage containers. The widespread distribution and environmental persistence of xenoestrogen BPA indicates a strong potential for human and animal exposure. Because humans are exposed to this compound daily, the effects of BPA are relevant to public health (Graselli et al., 2010). This exposure primarily occurs via the hydrolysis of polycarbonate plastics and epoxy resins and results in low concentrations of free BPA in food and liquids. The presence of endocrine-disrupting chemicals in the environment reportedly disturbs the normal endogenous hormone pathway and interrupts reproductive development in wildlife species. These compounds can affect the reproductive regulation of the neuroendocrine system, often by mimicking or blocking endogenous hormones (Qin et al., 2013; Naderi et al., 2014).

BPA acts as an EDC by causing adverse biochemical and physiological changes that alter the histological structure of cells and modify the function of tissues and organs, interfering with reproductive efficiency. Within the reproductive system, ovarian granulosa cells have been documented as a target of BPA action (Graselli et al., 2010). It is well-known that granulosa cells play a crucial role in ovarian physiology through the production of estrogens, which depends on androgen production in theca cells in addition to other factors that interact with the oocyte during its development. Therefore, the disruption of their functional activities by BPA could have a significant impact on fertility (Graselli et al., 2010). Therefore, BPA's effects in ovaries could be evaluated by histology. However, molecular responses usually occur

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earlier than histological perturbations, which are considered to be higher-level biological responses. The yolk protein vitellogenin (VTG) has been widely used as an endpoint for many exposure studies on the estrogenic effect of BPA in fish (Holbech et al., 2006). Changes in VTG levels in females have been suggested as a complementary biomarker of potential reproductive disruption, as well as modifications in the sex hormone balance (Mandich et al., 2007).

Conversely, BPA, similar to some other EDCs, could also act as anti-androgens by binding to the androgen receptor and producing specific alterations in gene expression (López-Casas et al., 2012). The cytochrome P_{450} CYP19 (aromatase) is involved in the generation of estradiol from testosterone. In this way, CYP19 is considered a potential EDC target because the modulation of its expression and function can dramatically alter the rate of estrogen production (Cheshenko et al., 2008). Two different *Cyp19* genes, *Cyp19a* and *Cyp19b*, are expressed in many teleost fish, preferentially in the ovary and the brain, respectively. Recent *in vitro* studies showed that BPA downregulates *Cyp19a* mRNA and protein expression in rats (Lee et al., 2013), which is relevant to the evaluation of the expression of the aromatase genes in ovaries as a biomarker of BPA exposure.

The purpose of our study was to evaluate the estrogenic activity of BPA as a ubiquitous environmental contaminant through the assessment of different biomarkers in the zebrafish ovary. The toxicological endpoints were selected at different levels of biological organization and included histological modifications in ovaries affecting the number of atretic follicles, VTG measurements, and determinations of the aromatase *Cyp19a* mRNA levels in zebrafish exposed to BPA (1, 10, 100 and 1000 µg/L) for 14 days.

2. Materials and methods

2.1. Fish exposure and sampling protocol

Sixteen-week-old female zebrafish *(Danio rerio)* (n = 150; standard length: 4.17 ± 0.24 cm; 0.57 ± 0.14 g wet weight) were used. The treated groups were exposed (OCDE Guideline Nº 204, 1993) to graded concentrations (1, 10, 100 and 1000 µg/L) of BPA (Sigma-Aldrich®) for 14 days. A control group exposed to only unchlorinated tap water completed the exposure design. The research procedure was conducted in the Experimental Animal Service of the University of Córdoba (Spain) after approval by the animal care committee of the University of Córdoba (Spain) and in accordance with the European Regulations for the Protection of Experimental Animals (Directive 2010/63/EU).

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). Immediately afterward, the standard length (SL) and body weight (BW) of each animal were measured. The gonads from 30 animals (n = 6 per group) were dissected and fixed for histological analysis for qualitative and quantitative evaluations. To complete these experiments, each fish was necropsied by placing it in right lateral recumbency on the stage of a dissecting microscope. The ovaries of 45 additional zebrafish (n = 9 per group) were removed, immediately immersed in liquid nitrogen, and stored at −80 °C for qRT-PCR. For the vitellogenin analysis, another 45 fish were frozen and stored at −80 °C until further analysis. The final 30 fish (n = 6 per group) were dried with sterile gauze, frozen and stored at −80 °C until use in the toxicological analysis (analytical BPA determinations).

2.2. Light and electron microscopy

For light microscopy, the fixed ovaries of 6 animals from each experimental group were routinely processed for paraffin sectioning by fixation in 10% buffered formalin solution, dehydration in a graded series of ethanol, immersion in xylol and embedding in paraffin wax by routine techniques. Tissue sections of 4 µm were mounted. After deparaffinization, the sections were rehydrated, stained with hematoxylin and eosin, and mounted on microscope slides with Cristal/Mount (Paraplast, Oxford Labware, St. Louis, MO).

For electron microscopy, randomly selected gonad samples were first fixed in a 2% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.4, 4 °C, overnight) and later fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 30 min. After dehydration in a graded ethanol series and embedding in Araldite, semi-thin and ultra-thin sections were cut on an LKB ultramicrotome (LKB). The semi-thin sections were stained with toluidine blue, whereas the ultra-thin sections were double-stained with uranyl acetate and lead citrate. Tissue sections were examined in a JEM 1400 transmission electron microscope (TEM; JEOL, Ltd.).

2.3. Morphometric study

For the structural quantifications, the fixed gonads were cut into three sections. Each portion was later processed and embedded in paraffin, as for routine histology. The first section (4 µm thick) of each block was stained with hematoxylin and eosin and used for the stereological study.

The quantitative study was performed according to Molina et al. (2013). Each microscopic image was processed using the Visilog 5® software (Noesis). Quantification was performed in a blind manner by an observer experienced in the use of the analysis system (J.G.M.). The system was initially and regularly calibrated using a millimeter slide.

Atretic follicle identification was based on the determination of non-physiological alterations in four follicular components: oocyte, follicular cells, and pellucid zone. The numerical density (Q_A) of the atretic follicles in the plane was estimated using a test system consisting of sixteen rectangular counting frames superimposed onto each microscopic image. Thus, the number of profiles per area Q_A *(nucl/tis)* was estimated using the following equation:

$$\mathrm{est}Q_A(\mathrm{nucl/tis}) = \Sigma Q(\mathrm{nucl})/(\Sigma P(\mathrm{tis}) \cdot a/p),$$

where Q_A *(nucl/tis)* is the numerical density of follicular nuclei per ovary, *ΣQ(nucl)* is the total number of nuclear profiles counted within the counting frames of the area obtained from *ΣP(tis)* (i.e., the total number of points in the tissue), and *a/p* is the area associated with one point in the test system (in our study, $a/p = 125\ \mu m^2$).

2.4. Whole-body vitellogenin measurements

The VTG levels in whole-body homogenates (n = 9 per experimental group) were determined using a commercial enzyme-linked immunosorbent assay developed for *Danio rerio* (ELISA, Biosense Laboratories, Bergen, Norway) following the manufacturer's indications. A multiple range test was used to make multiple comparisons between groups.

2.5. Cyp19a transcript quantification by real-time qRT-PCR

2.5.1. RNA isolation

Nine zebrafish from each experimental condition were selected for this experiment. The total RNA from the ovaries of each animal was isolated using the Isol-RNA Lysis Reagent (5PRIME). Genomic DNA was removed by the Total RNA Cleanup with DNase Digestion kit using the Qiagen RNeasy Protocol (Qiagen). The absence of any remaining gDNA contamination was confirmed by PCR amplification of the RNA samples without previous retrotranscription. An Agilent 2100 Bioanalyzer (Agilent Technologies) was used to determine the RNA integrity number (RIN) (Schroeder et al., 2006). The RNA purity and concentrations were determined by spectrophotometry. Only high-quality RNAs with RIN values > 8.5 and A260:A280 ratios close to 2.0 were used for the subsequent experiments (Fleige and Pfaffl, 2006; Taylor