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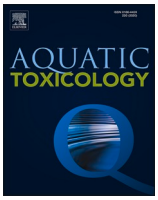

Immunohistochemical expression of aromatase cyp19a1a and cyp19a1b in the ovary and brain of zebrafish (*Danio rerio*) exposed to different concentrations of bisphenol A

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ABSTRACT

Bisphenol A (BPA) is used to produce plastic and plastic derived products in multitude of daily utensils, being one of the industrial compounds most widely used. This endocrine disrupting chemical (EDCs) is a well-known environmental pollutant released into the aquatic environment from industrial wastewater, sewage sludge or landfill leachate. Aromatases are considered potential targets of EDCs with characteristics that make them suitable biomarkers of exposure to their effects. The main objective of our study was to evaluate the expression of cyp19a aromatase as a toxicological endpoint after BPA exposure through the identification and assessment of alterations of the main cells responsible for cyp19a1a and cyp19a1b expression in the zebrafish ovary and brain using different concentrations of BPA in water. Immunohistochemistry was used to analyze the expression of these enzymes in female zebrafish exposed and not exposed to different concentrations of BPA (1, 10, 100 and 1000 μg / L) in water (n = 6/group) for 14 days. The results obtained in this study showed that the cyp19a aromatase system, involved in the synthesis of steroid compounds, is specially located in distinct oocyte stages in the ovary (cyp19a1a) and in radial glial cells of the brain (cyp19a1b). An overexpression of these aromatases was observed after BPA exposure in zebrafish, peaking from a concentration of 10 μg/L and showing to be good biomarkers of exposure to identify the early effects of low BPA concentrations. To our knowledge, this study is the first to localize and quantify the expression of cyp19a1a and cyp19a1b in the cells of brain and ovary after fish exposure to different BPA concentrations in water.

1. Introduction

Endocrine disrupting chemicals (EDCs) are being detected in almost all aquatic sources tested due to their continuous production as well as their environmental persistence (Hoffmann and Kloas, 2012). EDCs act on hormonal functions, disrupting the reproductive, nervous and immune systems, as well as driving cells to cancer transformation (Kolatorova et al., 2017).

Bisphenol A (BPA) is one of the most important EDCs nowadays, because it is one of the most widely used industrial compounds (Environment Canada, 2008; Galloway et al., 2010), with annual global production of over 3.5 million tons, and more than 100 tons released into the atmosphere, which means that BPA is currently widespread (Flint et al., 2012; Hoekstra and Simoneau, 2013; Corrales et al., 2015; Shi et al., 2019). Around 90% of the total manufactured BPA is used to produce plastic and plastic derived products in multitude of daily utensils (Eladak et al., 2015). Although the diet is the main source of BPA exposure in mammals, this EDCs is as well a ubiquitous pollutant in the environment and, as for many other chemicals, the rivers, lakes and ground waters are the major sinks (Bhandari et al., 2015; Canesi and

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Fabbri, 2015), producing waterborne BPA the most relevant estrogenic effects on fish (Kang et al., 2007). Therefore, many aquatic species, such as fish, amphibians, aquatic reptiles and mammals, are exposed daily to high concentrations of BPA (Bandhari et al., 2015; Canesi and Fabbri, 2015). Nowadays, exposure to BPA is a growing concern because of its effects on public and animal health (Kim et al., 2017; Delclos et al., 2018), especially at the reproductive level. Thus, the European Agency for Chemical Substances and Mixtures (ECHA) has added BPA to its list of chemicals of great concern because of its endocrine disrupting properties that can cause serious effects to human health and has announced future restrictions on the use of this substance (ECHA, 2017) European Agency for Chemical Substances and Mixtures (ECHA) 2021.

Negative effects of BPA on female reproductive system have been documented by several authors. Santangeli et al. (2016) and Molina et al. (2018a) reported that BPA was able to alter the reproductive system of females zebrafish (*Danio rerio*). In fathead minnows (*Pimephales promelas*), an ovarian impairment and ovulation reduction was also reported (Sohoni et al., 2001). Similarly, BPA decreased 17β-estradiol (E2) level in medaka (*Oryzias latipes*) (Huang et al., 2018) and reduced ovarian maturation in goldfish (*Carassius auratus*) (Wang et al., 2019). In this sense, BPA has demonstrated to bind estrogen receptors given its affinity for the nuclear estrogen receptors (α and ß), the orphan nuclear estrogen receptor (ERR) (Bulayeva and Watson, 2004; Matsushima et al., 2007), the transmembrane G protein-coupled estrogen receptor (tGPR30) (Eckstrum et al., 2016) or the aromatases themselves (Molina et al., 2018a; Faheem and Bhandari, 2021). Thus, there are various biomarkers to assess the effects of BPA exposure in females, such as follicular atresia, vitellogenin synthesis and determination of aromatase expression, among others (Villeneuve et al., 2012; Canesi and Fabbri, 2015; Molina et al., 2018a).

Aromatase is an enzyme complex found in all vertebrates and plays a particularly important role in the reproductive activity of animals. Most teleosts such as zebrafish have two structurally distinct cyp19 genes: cyp19a1a, which is predominantly expressed in the gonads, and cyp19a1b, which is mainly found in neuronal tissues (Sawyer et al., 2006; Cheshenko et al., 2007). Expression of cyp19 aromatase is necessary for normal sexual behavior, playing an important role in gonadal maturation and catalyzing the conversion of androgen to estrogen (Conley and Hinshelwood, 2001; Kallivretaki et al., 2007; Alharthy et al., 2017). These enzymes are considered potential targets of EDCs such as BPA, with characteristics that make them suitable biomarkers of activating or inhibitory effects (Cheshenko et al., 2008; Kallivretaki et al., 2007; Lee et al., 2013). There are various indications about EDC interference with the aromatase cyp19 system in fish that could lead to reproductive system problems (Fenske and Segner, 2004). Firstly, BPA has affinity for both cyp19a1a and cyp19a1b genes (encoding ovary and brain aromatase respectively) (Faheem and Bhandari, 2021). Moreover, it has been reported that ovarian aromatase downregulation produced by low doses of BPA causes decrease of 17-β estradiol serum concentration (Lee et al., 2013), which could produce a disruption in estrogen-related biological processes (Faheem and Bhandari, 2021). The exact mechanism by which BPA acts on cyp19a is not entirely clear. Faheem and Bhandari (2021) suggest that BPA disrupts neuroendocrine regulation of reproduction by acting on hypothalamic gonadotropin, kisspeptins and aromatases mRNA; while Molina et al. (2018a) reported that low doses of BPA could have a direct negative action on the transcriptional regulation of cyp19a expression in granulosa cells.

To the authors' knowledge, the evaluation of expression of cyp19a1a and cyp19a1b in brain and ovary cells of fish after exposure to different BPA concentrations has yet to be performed. Moreover, the exposure to BPA in water rather than by transcutaneous route is a technical advantage, since BPA is frequently detected as an environmental pollutant in rivers and seas (Juan-García et al., 2015; Kim et al., 2017) and could be an important indicator to evaluate aquatic ecosystems health (Bhandari et al., 2015).

Therefore, the hypothesis of this study was that exposure to BPA, a common aquatic pollutant, could interfere with the aromatase cyp19 system and give rise to unsuitable functioning of the reproductive system in fish. For this, we identified and evaluated the expression of cyp19a1a and cyp19a1b in the main target cells in the zebrafish ovary and brain as a toxicological endpoint after exposure to different concentrations of BPA in water.

2. Materials and methods

2.1. Animals

Sixteen-week-old female zebrafish (*Danio rerio*) sexually mature ($n = 30$) with a standard length of 4.17±0.24 cm and wet weight of 0.57±0.14 g, from the Experimental Animal Service of the University of Córdoba, were used in this study. This species of fish was added by Directive 2010/63/EU to the list of animals that can be used in experimental procedures (Annex I) and is increasingly utilized in animal experimentation as an alternative to mammals.

Many studies have used the fish biomodels to understand the causes of endocrine disruption, in which aquatic animals are often exposed to multiple sources of EDCs coming from domestic and industrial effluents, so that diverse cases of abnormal development possibly due to EDC exposure have been reported (Eggen et al., 2003; Segner et al., 2003; Goksoyr, 2006). The zebrafish is a common biomodel in animal experimentation, which has become an increasingly popular system, particularly in ecotoxicological and reproduction studies, because of its suitability for the study of water pollution (Nüsslein-Volhard and Dahm, 2002). Moreover, it confers many advantages, such as its small size, rapid development, short ovarian cycles, and has been widely used in recent years to study the effects of EDCs, particularly BPA (Le Fol et al., 2017; Molina et al., 2018a,b; Pinto et al., 2019; Sun et al., 2020).

2.2. Experimental design

Zebrafish obtained from the same aquarium were randomly allocated into five experimental groups ($n = 6$ animals/group): a control group (kept in non-chlorinated tap water) and four treated groups, each one exposed to graded concentrations (1, 10, 100 and 1000 μg/L) of BPA (Sigma-Aldrich®, Steinheim, Germany). The procedure was carried out using glass tanks; each aquarium had a capacity of 30 L, and contained 20 L of water. The concentrations were maintained constants by a continuous flow-through system using a pump which have guaranteed 10 water renewals/day. Photoperiod of 14 light hours and 10 dark hours was used; mean temperature of the water and the test room were maintained respectively at 26.4 ± 1.1 °C and 25.4 ± 1.1 °C, dissolved oxygen concentration was above 80% of the air saturation value by throughout the test. In addition, all the water quality parameters were controlled during the testing period. The mean pH value was 7.47 ± 0.1, the conductivity was maintained at 32.5μS / cm, the measured water hardness ranged between 110.3 ppm and 111.1 ppm and the alkalinity recorded was 80.1 ppm. Ammonium and nitrites showed levels close to zero. After 2 weeks of exposure, all the zebrafish were euthanized using an anesthetic overdose of tricaine methanesulfonate (MS-222® 500 mg/L; Sigma-Aldrich®, Steinheim, Germany) buffered with sodium bicarbonate (300 mg/L; Sigma-Aldrich®, Steinheim, Germany), according to the recommendations of FELASA (European Federation for Laboratory Animal Science Associations, using an euthanasic method recommended by current legislation (Directive 2010/63/EU).

The gonads and heads were immediately dissected and fixed in 10% buffered formalin (pH=7.2) for 24 h and Bouin's solution for 8 h, then routinely processed and embedded in paraffin wax for immunohistochemical study.

The BPA concentrations used for exposure (0, 1, 10, 100 and 1000 μg/L) were selected in accordance with previous studies performed with different fish species (Mandich et al., 2007; Villeneuve et al., 2012), as

well as those conducted with zebrafish (Molina et al., 2018a,b). BPA concentrations lower than 200 μg/L are considered low for aquatic organisms, given that it is a commonly found environmental pollutant in wastewater discharges (vom Saal et al., 2006). Nevertheless, these concentrations are enough to induce significant adverse effects in the behavior and locomotor movements of teleost fish (Inagaki et al., 2016). For this reason, our study considered a wide variety of BPA doses, ranging from the lowest concentration (1 μg/L), frequently detected as an environmental pollutant, to the highest (1000 μg/L), mainly detected in industrial areas where this chemical is produced (Azevedo et al., 2001; Kang et al., 2007; Huang et al., 2012).

2.3. Ethics statement

The experimental procedure was approved by the animal care committee of the University of Cordoba (Spain) and conducted by the Experimental Animal Service, in accordance with European Regulations for the Protection of Experimental Animals (Directive 2010/63/EU).

2.4. Histopathological study

Formalin-fixed samples were processed routinely, dehydrated in a graded series of ethanol, immersed in xylol and embedded in paraffin wax, using an automatic processor. Sections were cut at 3 μm and stained with hematoxylin-eosin for standard procedures.

2.5. Immunohistochemical analyses

Paraffin-embedded sections (3 μm) of ovary and brain tissue samples were routinely processed for immunohistochemistry (IHC), using a modified version of the avidin-biotin-peroxidase complex (ABC) method described by Risalde et al. (2015). Briefly, the tissue sections were dewaxed and rehydrated, and endogenous peroxidase activity was exhausted by incubation with 0.3% hydrogen peroxide in methanol for 30 min at room temperature (RT). For antigen retrieval, the samples were incubated in 0.1 M tri-sodium citrate dihydrate (Merck®, Darmstadt, Germany) (pH 6) in an oven at sub-boiling temperatures for 15 min. Sections were rinsed three times in PBS (pH 7.2) for 10 min and covered with 20% normal goat serum (Vector Laboratories, Burlingame, CA) in 0.01 M phosphate buffered saline (PBS) for 30 min at RT. The sections were then incubated with polyclonal primary homologous antibody, cyp19a1a (Anaspec, CA, USA) or cyp19a1b (Pellegrini et al., 2007) diluted 1:100 in 10% normal goat serum overnight at 4 °C. After incubation, slides were washed in PBS (three times x 5 min each), then incubated with the biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories, CA, USA) diluted at 1:200 in PBS containing 10% normal goat serum for 30 min at RT. After three further 5 min washes in PBS, samples were incubated with the ABC complex (Vectastain® ABC Elite Kit, Vector Laboratories, CA, USA) for 1 h at RT. All tissue sections were rinsed in 0.05 M Tris-buffered saline (TBS) and incubated with the chromogen solution (NovaRED® Substrate Kit, Vector Laboratories, CA, USA). Finally, slides were counterstained with Harris' hematoxylin.

For immunohistochemical detection of aromatase, positive control tissues were obtained from specific pathogen-free zebrafish not exposed to BPA. For negative controls, specific primary antibodies were replaced by non-immune rabbit sera (DakoCytomation, Glostrup, Denmark).

2.6. Cell counting

Identification of cells immunostained with the different primary antibodies was based on their localization, morphological characteristics and cell size. To estimate the number of immunolabeled cells, these were independently examined by two blinded and experienced observers, a veterinary pathologist and an investigator (M.A.R. and R.M.). For it, cell counts were performed in 20 × 0.2 mm^2 fields chosen by randomly drawing diagonal lines in the organ, in one slide for each animal. The final results were the mean values obtained from both observers, which were expressed as the number of positive cells per 0.2 mm^2.

2.7. Statistical analysis

Immunolabeled cells against the two primary antibodies in the ovary and brain were expressed as mean ± standard error (SE) of the number of positive cells per 0.2 mm^2. A Shapiro-Wilk test was first performed to assess the normality of data. As data were not normally distributed, a non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test were used to compare the results of zebrafish exposed and not exposed to different BPA concentrations ($n_{total} = 30/n_{group} = 6$). Statistical analyses were performed with GraphPad Prism V.6 software (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

None of the zebrafish died or presented lesions during the study. There were no significant differences in body weight or length between the control group and those exposed to BPA.

3.1. Histologic evaluations of effects of BPA in ovary and brain of zebrafish

There were no remarkable gross or histopathologic lesions in the ovaries of the control group. However, although no macroscopic lesions were detected in the ovaries of BPA exposed group, several microscopic alterations were observed. Thus, while the ovaries of fish exposed to low BPA concentrations (1 and 10 μg/L) maintained their structure and the type of lining cells, the ovaries of fish exposed to high BPA concentrations (100 and 1000 μg/L) showed an increased oocyte apoptosis and atretic ovarian follicles, mainly at the highest BPA concentration (Fig. 1). These findings were observed in the pre-vitellogenic oocytes when the ooplasm size was drastically reduced and appeared as a dark stained mass (Fig. 1). The main characteristics of the atresia of cortical alveoli, early vitellogenic and vitellogenic oocytes were the formation of peculiar marks in the cytoplasm and the irregular appearance of the zona radiate (Fig. 1). On the other hand, histopathological evaluation interestingly evidenced a high number of primary oocytes in zebrafish exposed to the highest concentration of BPA (1000 μg/L) (Fig. 1).

The brain of control and BPA exposed groups does not present remarkable gross or histopathologic lesions (data not shown).

3.2. Effects of BPA in the expression of cyp19a1a and cyp19a1b in ovary

In the mature ovary of adult zebrafish, cyp19a1a is mainly immunolocalized in the ooplasm of primary oocyte, cortical alveolus oocyte and lightly in vitellogenic oocyte, as well as peri-follicular cells (granulosa and thecal cells) of pre-vitellogenic and vitellogenic oocytes, and occasionally in interstitial cells (Fig. 2). These immunoreactive cells showed remarkable changes in cyp19a1a expression when the zebrafish were exposed to different BPA concentrations in water. The quantification of *ooplasm* immunoreactive to cyp19a1a in the ovary showed that BPA induced pre-vitellogenic oocytes-specific overexpression of aromatase, which was statistically significant at a BPA exposure range of 10 to 1000 μg/L with respect to control and 1 μg/L BPA exposure groups (Fig. 3). In the *granulosa cells*, cyp19a1a expression was minimal in the control group and 1 μg/L BPA exposure group, while groups exposed to BPA concentrations from 10 to 1000 μg/L showed significant dose-dependent increased expression of this aromatase (Fig. 3). With respect to the *theca cells*, there was a significant non-linear increase in cyp19a1a expression after exposure to low BPA doses, but decreasing significantly at the highest BPA concentration used (1000 μg/L) (Fig. 3). The ovarian *interstitial cells* followed a similar trend to the oocytes, showing a significant increase of cyp19a1a-immunopositive cells in the

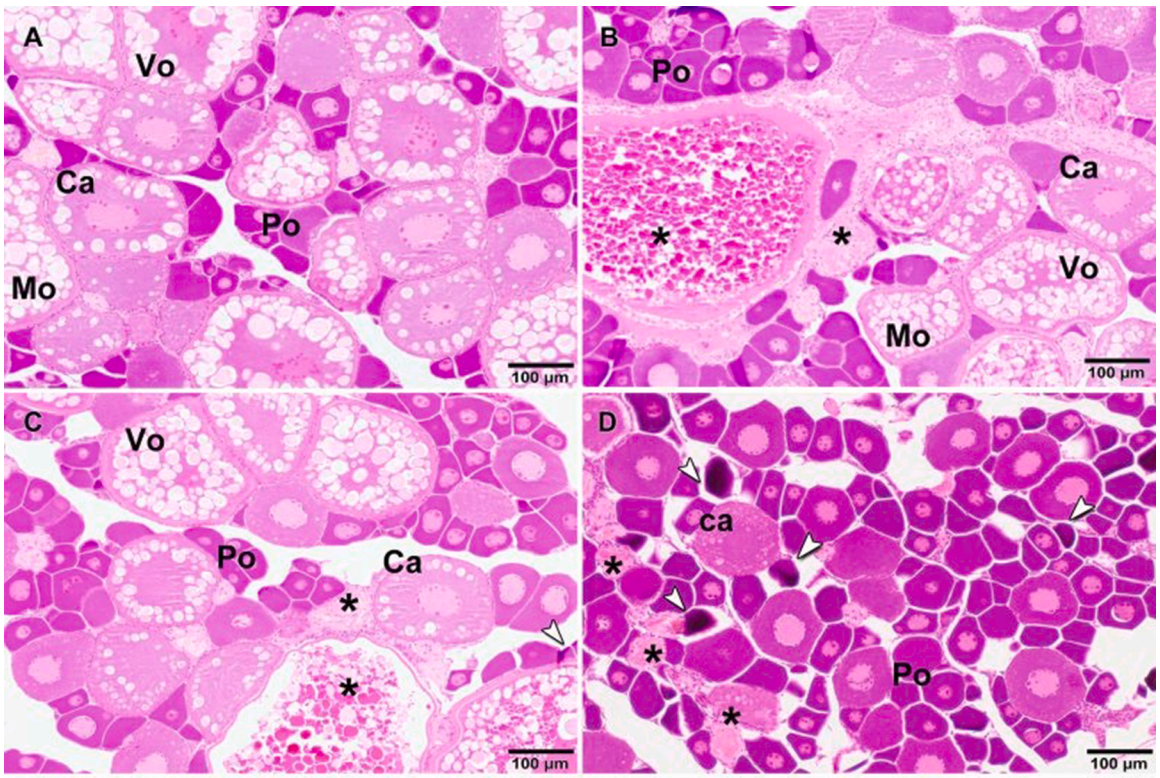

Fig. 1. Histopathological changes in the zebrafish ovary exposed to different concentrations of BPA in water. A) Ovarian parenchyma of control zebrafish not exposed to BPA with numerous follicles at different stages of maturation, without histopathological lesions. B) Occasional presence of atretic follicles (asterisk) in ovary from zebrafish exposed to 10 µg/L of BPA. C) Presence of some atretic follicles (asterisk) and occasional presence of primordial follicles apoptosis (arrow) in ovary from zebrafish exposed to 100 µg/L of BPA. D) Multiple primary oocytes, primordial follicles apoptosis (arrows) and atretic follicles (asterisk) in ovary from zebrafish exposed to 1000 µg/L of BPA. Po-Primary oocyte; Ca-oocyte in cortical alveolus stage; Vo-vitellogenic oocyte; Mo-mature oocyte. Hematoxilin-eosine stain.

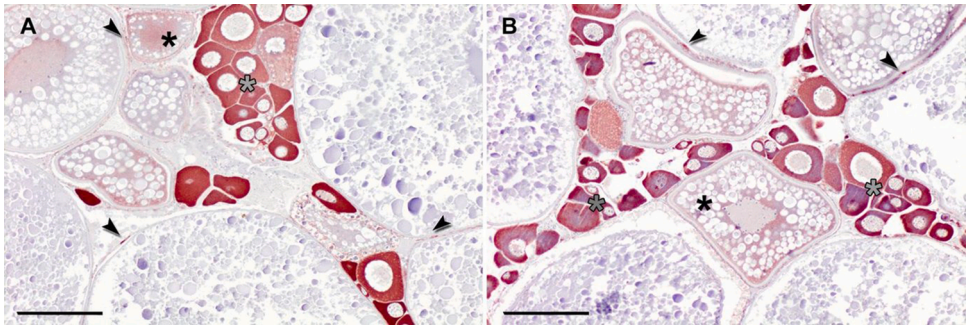

Fig. 2. Cyp19a1a expression in the zebrafish ovary. Ooplasm of primary oocytes (gray asterisks) and peri-follicular cells of pre-vitellogenic and vitellogenic oocytes (arrows) were strongly stained against anti-cyp19a1a antibody, as well as lightly in ooplasm of vitellogenic oocyte of the ovary from zebrafish control (A) and exposed to 10 µg/L of BPA (B). Immunohistochemistry (ABC method). Bars: 100 µm.

groups of exposure to high BPA concentrations (from 10 to 1000 µg/L) compared with the control and 1 µg/L BPA exposure groups (Fig. 3).

Aromatase cyp19a1b-reactive cells were detected only in the ooplasm of pre-vitellogenic oocytes in the ovary of the control zebrafish group or groups exposed to different BPA concentrations. Cyp19a1b expression in the ovary was scant and no significant changes in its expression were observed after the fish were exposed to different concentrations of BPA ($p = 0.33$) (Fig. 4).

3.3. *Effects of BPA in the expression of cyp19a1a and cyp19a1b in brain*

The radial glial cells were identified as the main cells responsible for cyp19a1b expression in the telencephalon, preoptic area and hypothalamus of zebrafish. These cells immunolabeled for the anti-cyp19a1b antibody showed a granular intracytoplasmic immunostaining (Fig. 5). The *radial glial cells* exposed to BPA showed a significant non-linear increase in cyp19a1b expression, with levels peaking at 10 µg/L of BPA, decreasing after significantly at the highest BPA concentration used (1000 µg/L) (Fig. 5).

The control group and those exposed to different BPA concentrations showed an absence of cyp19a1a expression in the brain by immunohistochemistry (data not shown).

4. Discussion

The most widely accepted hypothesis is that EDCs such as BPA can interfere with the action of steroid hormones on the hypothalamus-pituitary-gonadal axis, impairing the biosynthesis of sex steroids in the

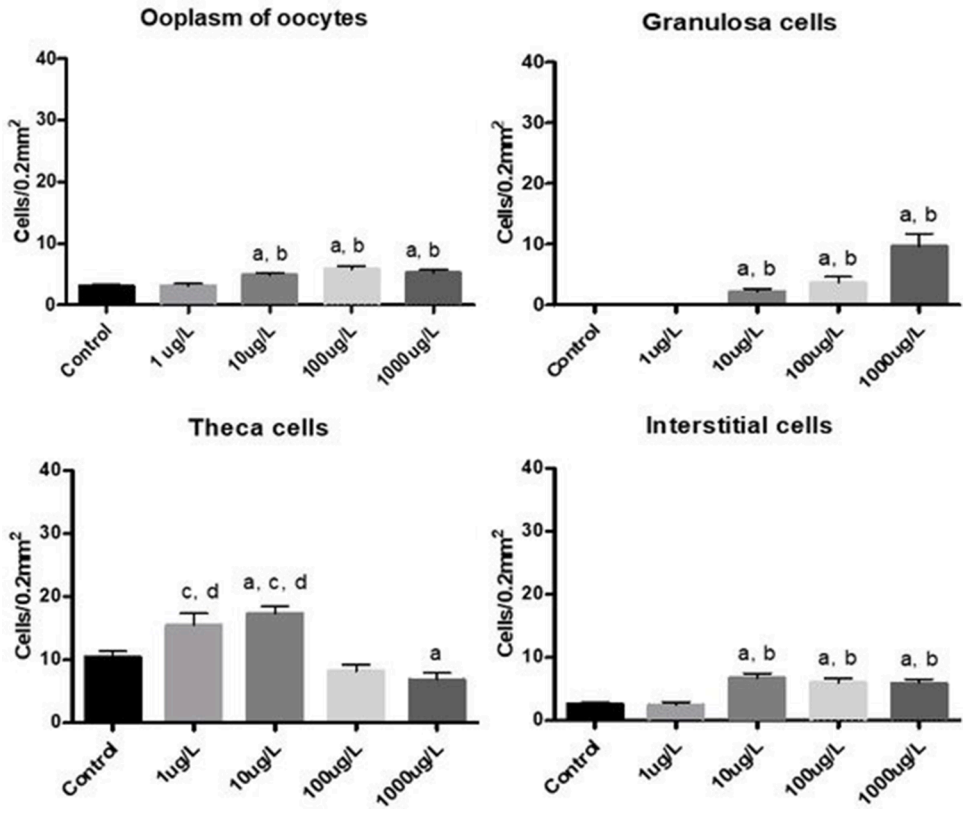

Fig. 3. Response of cyp19a1a expression in the zebrafish ovary after exposure to different BPA concentrations. Means ± standard errors of the ooplasm of oocytes, as well as granulosa, theca and interstitial cells immunopositive for cyp19a1a in the ovary from control zebrafish and groups exposed to 1, 10, 100 and 1000 μg/L of BPA ($n = 6$ per group). [a]significant differences vs control ($p<0.05$); [b]significant differences vs 1 μg/L ($p<0.05$); [c]significant differences vs 100 μg/L ($p<0.05$); [d]significant differences vs 1000 μg/L ($p<0.05$) (Kruskal-Wallis test followed by Dunn's multiple comparisons test for non-parametric distributions).

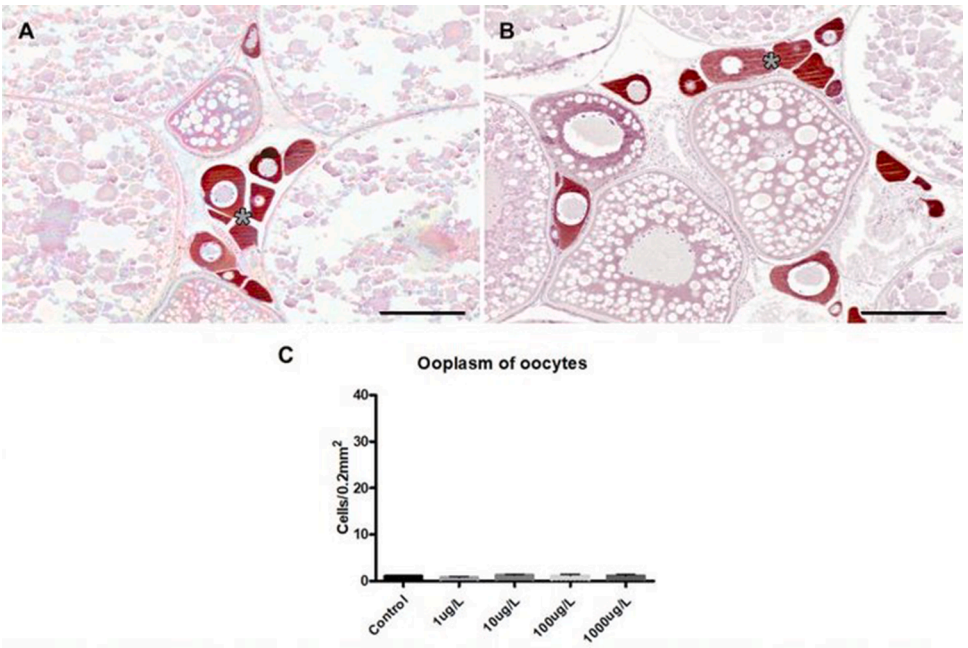

Fig. 4. Cyp19a1b expression in the zebrafish ovary. Ooplasm of numerous primary oocytes was the main immunoreactive localization against anti-cyp19a1b antibody (asterisks) in the ovary of zebrafish control (A) and exposed to 10 μg/L of BPA (B). Immunohistochemistry (ABC method). Bars: 100 μm. C) Means ± standard errors of the ooplasm of oocytes immunopositive for cyp19a1b in the ovary from control zebrafish and groups exposed to 1, 10, 100 and 1000 μg/L of BPA ($n = 6$ per group).

gonads, which could have consequences at the reproductive level (Muriach, 2012). This study evaluated the effects of BPA on aromatases cyp19a1a and cyp19a1b expression in different ovary and brain cell populations from zebrafish exposed to different concentrations of BPA in water.

In general, our study corroborates the well-known behavior of BPA with respect to its action on the biosynthesis of steroid hormones, showing a non-linear response in which low doses lead to remarkable aromatase expression, but whose action decreases or is maintained at higher concentrations (Myers et al., 2009a). This kind of response in some cell types appears to follow the characteristic U-shaped curve of EDC behavior on hormone systems. In order to prove this phenomenon, it would be necessary to extend our study using higher doses than the maximum established, waiting for another increase in enzymatic expression.

Histopathological findings detected in zebrafish after BPA exposure

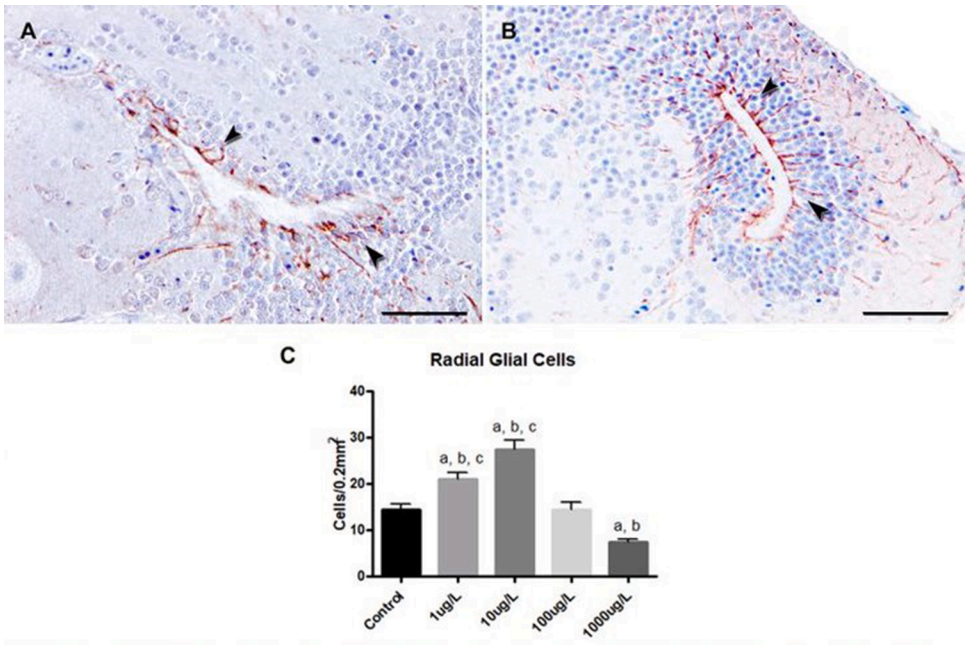

Fig. 5. Response of cyp19a1b expression in the zebrafish brain after exposure to different BPA concentrations. The radial glial cells were the main immunoreactive cells against the anti-cyp19a1b antibody (arrows) in the brain from zebrafish control (A) and exposed to 10 μg/L of BPA (B). Immunohistochemistry (ABC method). Bars: 50 μm. C) Means ± standard errors of radial glial cells immunopositive for cyp19a1b in the brain from control zebrafish and groups exposed to 1, 10, 100 and 1000 μg/L of BPA (n = 6 per group). [a]significant differences vs control ($p<0.05$); [b]significant differences vs 100 μg/L ($p<0.05$); [c]significant differences vs 1000 μg/L ($p<0.05$) (Kruskal-Wallis test followed by Dunn's multiple comparisons test for non-parametric distributions).

were localized in the ovary. BPA induced a dose-dependent increase in primordial follicular atresia, coinciding with that observed in zebrafish, common carp (*Cyprinus carpio*) and *Catla catla* (Mandich et al., 2007; Faheem et al., 2017; Migliaccio et al., 2018; Molina et al., 2018a). This histopathological finding, together with an increased number of primary oocytes observed in zebrafish exposed to the highest concentration of BPA (1000 μg/L), are indicative of reduced maturation of oocytes and the alteration of normal oogenesis (Qin et al., 2013; Bhandari et al., 2015). It suggests that BPA affects reproductive functions at high concentrations, thus interfering in the fertility of females (Mandich et al., 2007; Molina et al., 2018a). In this context, cyp19a1a has a main role in the synthesis of estrogens and consequently in the endocrine regulation of oocyte's growth during vitellogenesis, being necessary for female sexual development (Cheshenko et al., 2008; Dranow et al., 2016). Moreover, inadvertent exposure to environmental pollutants, and specifically to EDCs, may be one of the factors that would interfere with the reproductive success changing population dynamics of wildlife species through disruption of their reproductive abilities (Flint et al., 2012; Faheem and Bhandari, 2021). Therefore, monitoring cyp19a1a expression in BPA-exposed fish will help determine the sensitivity to this EDC and elucidate the mechanisms responsible for the effects of this disruptor.

The identification of aromatases in main ovary cells was evidenced since cyp19a1a was detected in the cytoplasm of interstitial cells and follicular layer cells (theca and/or granulosa cells), as well as in the ooplasm of pre-vitellogenic oocytes of zebrafish (Rodriguez-Mari et al., 2005; Zapater et al., 2012; Caulier et al., 2015; Hinfray et al., 2018). These results are consistent with several studies that also reported the presence of cyp19a1a in the germinal compartment of different fish species (Park et al., 2008; Gohin et al., 2011; Raghuveer et al., 2011; Zapater et al., 2012; Caulier et al., 2015; Hinfray et al., 2018). The presence of aromatase in interstitial cells, which are required for the synthesis of estrogens and progestogens, has also been reported, but their role in the cyp19a1a expression is not elucidated (Sunobe et al., 2005; Wang et al., 2007; Ruksana et al., 2010; Caulier et al., 2015). On the other hand, interestingly, cyp19a1b immunolabeling was found in zebrafish ovaries but only in the ooplasm of pre-vitellogenic oocytes. This cyp19a1b expression was previously described in studies on the ovary of different fish species (Kobayashi et al., 2004; von Schalburg et al., 2013; Caulier et al., 2015). The role of this molecule in ovarian germ cells is not yet known, but cyp19a1b has been previously detected in unfertilized eggs from different fish, including zebrafish (Callard et al., 2001; Sawyer et al., 2006; Shanthanagouda et al., 2014).

Cyp19a1a expression in ovary showed a non-linear response to BPA exposure dose in zebrafish, which could be due to receptor saturation by high concentrations of estrogenic compounds with affinity for their binding sites. These results are consistent with other studies performed on adult female rats (Lee et al., 2013) and rare minnows *Gobiocypris rarus* (Liu et al., 2012). Likewise, in a previous study with the same biomodel, we used qRT-PCR, a more sensitive technique to evaluate the expression of cyp19a mRNA in the ovaries exposed to BPA concentrations of 1, 10, 100 and 1000 μg/L (Molina et al., 2018a). This study showed an apparent non-monotonic response curve marked by down-regulation at the lowest BPA concentration, upregulation at 10 μg/L and downregulation at the higher doses, being these results in agreement with those reported in the theca cells studied in this work. The granulosa cells however showed a dose-dependent increase in cyp19a1a expression following exposure to BPA, which could disturb the ovarian differentiation and affect the ovary maintenance (Wang and Orban, 2007; Dranow et al., 2016), since these cells have a decisive role in the synthesis of estrogens, and BPA would act mimicking the role of these compounds (Cheshenko et al., 2008; Muriach et al., 2012). This BPA effect on cyp19a1a expression, contrary to what occurs with cyp19a1b in brain, does not seem to be estrogen response elements (ERE)-dependent (Callard et al., 2001). Indeed, potential binding of BPA to other transmembrane G protein-coupled and orphan receptors could activate a non-genomic cAMP-responsive element binding protein (CREB)-mediated mechanism (Quesada et al., 2002) through cAMP/PKA signaling that, in turn, was responsible for this increase of cyp19a1a expression in the ovary (Cheshenko et al., 2008; Faheem and Bhandari, 2021). By contrast, cyp19a1b overexpression was not observed in the ovary in this study after BPA exposure, unlike that previously described, which could be due to sensitivity of the technique used or to the differences in the base level of circulating coregulators other than estrogens (Shanthanagouda et al., 2014).

Cyp19a1b gene is most strongly expressed either in the developing and adult brain of both sexes, contains ERE in its sequence and is estrogen-sensitive (Cheshenko et al., 2008). In our study, a clear non-linear trend was observed in the increase of cyp19a1b expression in fish brain following exposure to different BPA concentrations. Our

results coincided with non-monotonic dose–response curves reported for adverse effects with a number of EDCs (Welshons et al., 2003; Myers and Hessler 2007), including BPA (Wetherill et al., 2007; Molina et al., 2018b). It also diverged with other previous works showing that exposure to BPA can induce significant dose-dependent overexpression of cyp19a1b in brain (Chung et al., 2011; Shanthanagouda et al., 2014), where only a temporal interplay of negative and positive feedback on cyp19a1b was observed.

Immunohistochemical studies revealed strong cyp19a1b expression in the radial glial cells of telencephalon, preoptic area and hypothalamus. This elevated expression of cyp19a1b in radial glial cells suggests the existence of a high local production of estradiol under certain physiological conditions (Menuet et al., 2005; Mouriec et al., 2009). Radial glial cells persist in the brain of adult fish, in contrast to mammals where disappear following embryonic neurogenesis, a characteristic that could be related to their capacity as progenitor cells that initiate a cell differentiation program (mostly into neurons) in the adult fish brain (Ekstrom et al., 2001; Grandel et al., 2006; Le Page et al., 2006; Pellegrini et al., 2007). On the other hand, radial glial cells are also the target cells of a long list of EDCs with estrogenic activity (Pellegrini et al., 2007; Chung et al., 2011; Brion et al., 2012; Cano-Nicolau et al., 2016; Hinfray et al., 2018), so that fish brain remains potentially as highly sensitive to sex hormones and xenobiotics during the fish's entire life. Thus, this study shows that BPA induces an overexpression of cyp19a1b in radial glial cells, which subsequently could affect to the production of estrogens and to natural repair mechanisms in the brain.

With respect to concentrations used, BPA concentrations lower than 200 μg/L are considered low for aquatic organisms, given that it is a commonly found environmental pollutant in wastewater discharges (vom Saal et al., 2006). Nevertheless, these concentrations are enough to induce significant adverse effects in the behavior and locomotor movements of teleost fish (Inagaki et al., 2016), some authors defined about 12 μg/L or lower as the environmentally relevant concentration in surface waters (Flint et al., 2012). Several studies indicate that BPA was detected in surface water samples ranging from 0.07 to 4 μg/L (Azevedo et al., 2001) and in surface seawater, where ranged from 0.19 μg/L up to 370 μg/L in industrial effluents (Fukazawa et al., 2001; Heemken et al., 2001; Huang et al., 2012), being the highest BPA levels reported in landfill leachate sites at concentrations up to 17 mg/L (Flint et al., 2012). For this reason, our study considered a wide variety of BPA doses, ranging from the lowest concentration (1 μg/L), frequently detected as an environmental pollutant, to the highest (1000 μg/L), mainly detected in industrial areas where this chemical is produced (Azevedo et al., 2001; Kang et al., 2007; Huang et al., 2012). Our results evidenced that the effect of exposure to BPA on the aromatase expression in ovary and brain of zebrafish peaked from a concentration of 10 μg/L, using immunohistochemical tools. However, modifications in the aromatase expression at 1 μg/L have been observed with this experimental model using RT-PCR, a more sensitive technique (Molina et al., 2018a). Aquatic organisms exposed to lower BPA concentrations may be affected by this compound (Chen et al., 2015), so it could be interesting to investigate BPA effects at concentrations below 1 μg/L to identify earlier biomarkers of exposure.

This study has some limitations for the interpretation of results. The toxicity of BPA as well as its adverse effects on the hormonal systems of living organisms have been proven. However, we did not determine hormone levels and variations in them after exposure to different BPA concentrations so that our findings could be compared with the earlier studies to improve understanding of the effects of BPA on animals and humans. It would also be necessary to perform the study over a longer period of time in order to assess the long-term effects of BPA on cyp19a expression in zebrafish.

5. Conclusions

The results obtained in this study showed that the cyp19a aromatase system, involved in the synthesis of steroid compounds, is specially located in distinct oocyte stages in the ovary (cyp19a1a) and in radial glial cells of the brain (cyp19a1b). Cyp19a expression seems to be most affected by BPA in the ovary than in brain, which means that its effect could lead to the development of reproductive dysfunctions. The effect of exposure to BPA on the expression of aromatases peaked from a concentration of 10 μg/L, being cyp19a1a and cyp19a1b good biomarkers of exposure to identify the early effects of low BPA concentrations in ovary and brain, respectively. Future studies to evaluate the BPA effects at lower concentrations are required to establish possible preventive ecotoxicology measures.

Author contributions

María A. Risalde: conceptualization, data curation, formal análisis, investigation, methodology, validation, writing original draft ± review & editing; ***Ana Mª Molina:*** conceptualization, data curation, formal análisis, investigation, methodology, validation, visualization; writing original draft ± review & editing; ***Antonio J. Lora:*** investigation, methodology, writing ± review & editing; ***Nahum Ayala:*** investigation, methodology, writing ± review & editing; ***Jose C. Gómez-Villamandos:*** conceptualization, investigation, resources, supervisión, writing ± review & editing; ***Mª Rosario Moyano:*** conceptualization, investigation, funding acquisition, project administration, resources, supervision, visualization, writing ± review & editing.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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