ISSN: 0001-5113 AADRAY	ACTA ADRIAT., 55(1): 57 - 64, 2014	ORIGINAL RESEARCH PAPER

Structural and ultrastructural evaluations of zebrafish ovaries after exposure to 2, 3, 7, 8 - Tetrachlorodibenzo-*p*-dioxin (TCDD)

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The morphological consequences of long-term exposure to low doses of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin in the ovaries were investigated in 50 adult female zebrafish at structural and ultrastructural levels. Animals were exposed to graded concentrations of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (10, 40, 100 and 270 ppb) for 21 days, then zebrafish were sacrificed by an overdose of anaesthetic solution tricaine methanesulfonate, and immediately samples were taken for morphological evaluation. At lower concentrations of exposure there was no evidence of morphological modifications, while at higher concentrations (100 and 270 ppb) we frequently observed degeneration and inflammation. Significant increases in follicular atresia were observed among all groups ($p<0.05$). These results indicate that long-term exposure to low doses of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin are able to induce morphological damage on the ovaries, which could then produce adverse effects on fish reproductive health.

Key words: TCDD, zebrafish, ovaries, histopathology, follicular atresia

INTRODUCTION

Halogenated aromatic hydrocarbons (HAHs) constitute a class of global environmental contaminants. 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD) is the most toxic member of the class of polyhalogenated, diaromatic hydrocarbons. It is a lipophilic compound persistent in the environment and known to bioaccumulate and biomagnify in the food chain. TCDD is a by-product of the manufacture of herbicides, insecticides and disinfectants and human exposure comes in the form of waste (KING-HEIDEN *et al.,* 2006; ZODROW *et al.,* 2004). The environmental toxicant TCDD is a potent endocrine disruptor with the ability to affect several biologic processes, including reproduction, by acting directly on the ovary like a potent ovarian toxicant

(BHATTACHARYA, P. & A.F. KEATING, 2012; KARMAN *et al.,* 2012; KING-HEIDEN *et al.,* 2006; ZODROW *et al.,* 2004).

The main benefits of using zebrafish as a toxicological model regard their small size, husbandry, and early morphology and welfare. The zebrafish model organism is increasingly used to assess drug toxicity and safety, and numerous studies confirm that mammalian and zebrafish toxicity profiles are strikingly similar, indicating that zebrafish can be used to predict toxic responses in other species (KING-HEIDEN *et al.,* 2005; MCGRAATH, P. & C.Q. LI, 2008; SEOK *et al.,* 2008).

Fish are among the vertebrates most sensitive to the toxicity of TCDD. Previously, the toxicity of TCDD to fish has been investigated in short-term studies at exposure concentrations greater than those found in the environment.

Zebrafish are suitable for assessing toxic effects of chemicals on development and reproduction, because test protocols have already been established, including OECD guidelines (OECD 210, 212, 230) (OECD, 2009, 1998, 1992) that recommend zebrafish for chemical toxicity assessments (as well as in Annex 1 of Directive 2010/63/EU, relative to the protection of animals used for scientific purposes).

In the OECD Directive of 2009, we find a series of diagnosis criteria in the gonadal histopathology of females relative to the analysis of the actions of potentially estrogenic compounds. Among the primary diagnosis objectives, an increase in follicular atresia was found to be a marker of gonadal histopathology from the action of these compounds (OECD, 2009).

The goal of this study was to determine the histopathologic effects of TCDD by light and electron microscopy, on zebrafish ovaries after 21 days of exposure.

MATERIAL AND METHODS

Sixteen-week-old female zebrafish *(Danio rerio)* (n=50), were randomly distributed into 5 groups (n=10/group). Animals were maintained at a photoperiod of 16 light hours: 8 dark hours. Water temperature was 26±1ºC and dissolved oxygen was maintained above 60% of saturation level. Treated groups were exposed for 21 days (OECD 230) to graded concentrations (10, 40, 100 or 270 ppb) of TCDD (Sigma Aldrich®) contained in food. A control group was fed twice a day with a non-estrogenic granulated diet (Supervit® minigranulated, Tropical, Chorzow, Poland) to complete the exposure design.

The research procedure was carried out after approval by the animal care committee of the University of Córdoba (Spain) and in concordance with the European Regulations for the Protection of Experimental Animals (Directive 2010/63/EU).

After 3 weeks of exposure, zebrafish were sacrificed by an overdose of anaesthetic solution tricaine methanesulfonate (MS-222® 500 mg/L; Sigma Aldrich®, St. Luis, EE.UU) buffered with sodium bicarbonate (300 mg/L; Sigma-Aldrich®, St. Luis, EE.UU). Ovaries were dissected and fixed for histological analysis. Each fish was necropsied by placing it in right lateral recumbency on the stage of a dissecting microscope. The left body wall was removed to excise the gonads, which were then dissected in a caudal to cranial direction, while applying very gentle traction to the oviducts (WOLF *et al.,* 2004).

Light and electron microscopy

For the light microscopic evaluation, the fixed ovaries were routinely processed for paraffin sections by fixing in 10% formaldehyde, dehydrated in graded series of ethanol, immersed in xylol and embedded in paraffin wax. Every tenth section (4 µm thick) of each block was stained with haematoxylin and eosin and used for the morphological study.

For the ultrastructural study, small randomly selected samples of gonads were fixed in a 2% glutaldehyde solution in 0.1M phosphate buffer (pH 7.4) overnight at 4ºC and then refixed in 1% osmium tetroxide in 0.1M phosphate buffer (pH 7.4) for 30 min. After dehydration in a graded ethanol series and embedding in Araldite, semithin and ultra-thin sections were cut on an LKB ultramicrotome (Central Microscopy Research Facilities, University of Córdoba, Spain). Semi-

thin sections were stained with toluidine blue, whereas ultra-thin sections were double-stained with uranyl acetate and lead citrate. Ultra-thin sections were viewed and photographed in a Philips CM10 transmission electron microscope (Philips Export B.V., Eindhoven, The Netherlands).

Morphometric study

The quantitative study was performed using an image analysis system consisting of a Leitz Ortholux triocular microscope connected by means of a SONY SSC-C370P® color video camera attached to an IBM-compatible personal computer equipped with a frame grabber board (Pinnacle System. California, USA). Each specimen was sampled in a systematic manner for the selection of microscopic images that were then digitized; a 40x lens (N.A. 1.25) was used for this procedure. An average of 50 microscopic fields per slab was chosen in each specimen.

Each microscopic image was processed using Visilog 5® software (Noesis. Saint Aubin, France). Quantification was performed by an observer experienced in the use of the analysis system (J.G-M) but with no previous knowledge of which group was being analyzed. The system was initially, and regularly, calibrated using a millimetre slide.

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 according to:

$$est\ Q_A(nucl/tis) = \Sigma Q(nucl) / (\Sigma P(tis) \bullet a/p),$$

where Q_A *(nucl/tis)* is the numerical density of follicular nuclei per ovary tissue, $\Sigma Q(nucl)$ is the total number of nuclear profiles counted within the counting frames of the area obtained from $\Sigma P(tis)$ as the total number of points which hit the tissue, multiplied by *a/p* as the area associated with one point on the test system (in our study, $a/p = 125\ \mu m^2$).

Statistical analysis

Data were analysed using the statistical program Statgraphic (Centurion XVI®, Warrenton, VA.) to determine TCDD effects for every exposed group. ANOVA (F-test) was used to demonstrate whether significant differences existed among the averages. The Tukey's test was used to perform multiple comparisons among groups and $P<0.05$ was considered to be significant.

RESULTS

The parenchyma of the ovary corresponding to the control group showed images with an ample development of its follicles, exhibiting a normal distribution of all its elements. In this control group, the interstitium displayed images characteristic of the species, with the presence of abundant active connective cells and blood vessels (Fig. 1).

In fish treated with 10 and 40 ppb of TCDD, all the follicles (primordial, cortical alveolar, vitellogenic, mature and atretic) maintained their composition and type of coating cells, similar to the control group.

In the groups of zebrafish treated with 100 and 270 ppb of TCDD, the different types of follicles are shown in the gonads although alterations were observed, these being most notable in fish treated with 270 ppb (Fig. 2).

Primordial follicles were observed less frequently than in the gonads of the fish from previous groups, although they were larger. A large increase in vacuolations was observed in the cytoplasm. There were highly disrupted nuclei with considerable degenerative processes.

In the cortical alveolar follicles, the same modifications were observed as in the previous follicles, and although the two components of the pellucida area were pronounced; it was disorganized and even disintegrated, showing follicular cells falling off.

The vitellogenic follicles showed an even greater degree of degeneration, with a loss of their structure and, above all, disintegrations in the pellucida area.

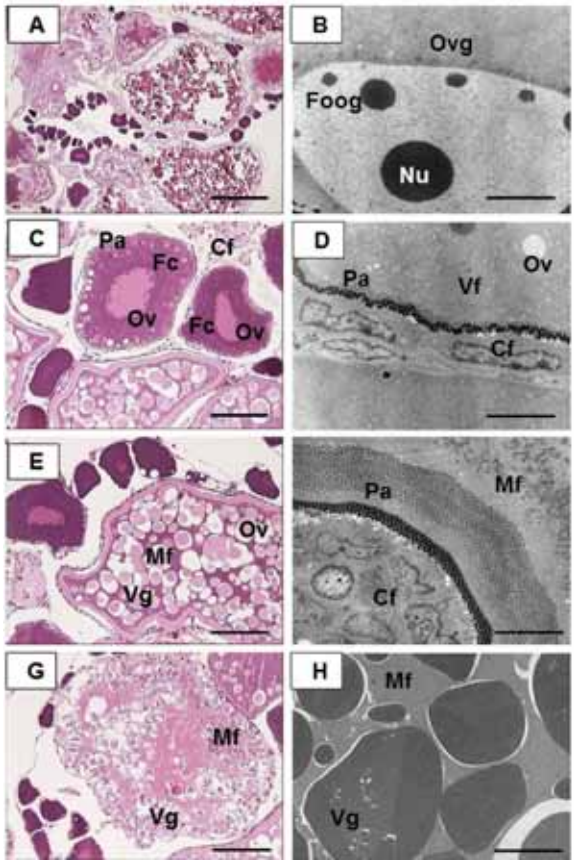

Fig. 1. Control zebrafish ovary

A, C, E, G: Light microscopy (H&E stain). Bars, 100 µm. B, D, F, H: Ultrastructural observations. Bars, 10 µm.

A. Ovary in which apparently normal follicles are seen

B. Primordial follicle with oogonia (Foog), with the oocyte standing out (Ovg) with a homogeneous nucleus and abundant nucleoli (Nu), and the homogeneous and dense cytoplasm

C. Cortical alveolar follicle (Fc) with oocyte (Ov), zona pellucida area (Pa) and apparently normal follicular cells (Cf).

D. Vitellogenic follicle (Vf) with oocyte (Ov), pellucida area (Pa) and apparently normal follicular cells (Cf)

E. Mature follicle (Mf). The ovule (Ov) and apparently normal vitellogenic granules (Vg) are prominent

F. Mature follicle (Mf), with follicular cells (Cf) and a normal pellucida area (Pa)

G. Mature follicles (Mf), with abundant vitellogenic granules (Vg)

H. Mature follicle (Mf), with apparently normal vitellogenic granules (Vg) observed

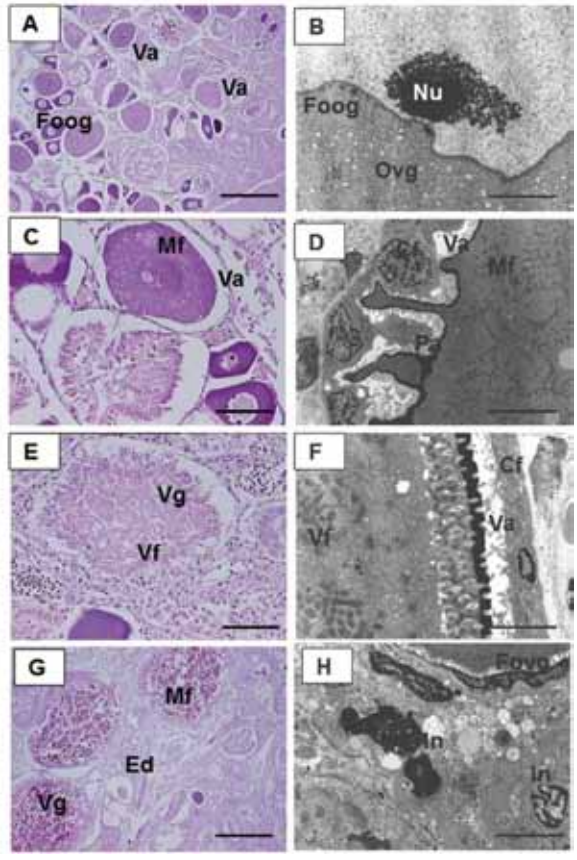

Fig. 2. Zebrafish ovary exposed to 270 ppb of TCDD

A, C, E, G: Light microscopy (H&E stain). Bars, 100 µm. B, D, F, H: Ultrastructural observation. Bars, 10 µm.

A. Primordial follicles with oogonia of different sizes and that are highly vacuolated (Va)

B. Primordial follicles with oogonia (Foog), nucleus containing a highly disorganized nucleolus (Nu) with degenerative processesC Mature follicles (Mf) with the cytoplasm of the vacuolated/ ooocyte (Va) and destruction of all its components

D. Mature follicles (Mf), with vacuolations (Va) between the oocytes and the follicular cells (Cf), and a degradation of the zona pellucida (Pa)

E. Vitellogenic follicle (Vf), in which numerous vitellogenic granules (Vg), are disintegrating

F. Detail of vitellogenic follicles (Vf), with vacuolations (Va) and separations between the oocytes and the follicular cells (Cf)

G. Mature follicle (Mf) with abundant vitelllogenic granules (Vg), edema in the interstitium (Ed) and numerous atretic follicles

H. Ovarian interstitium (Fovg), with abundant inflammatory cells (In)

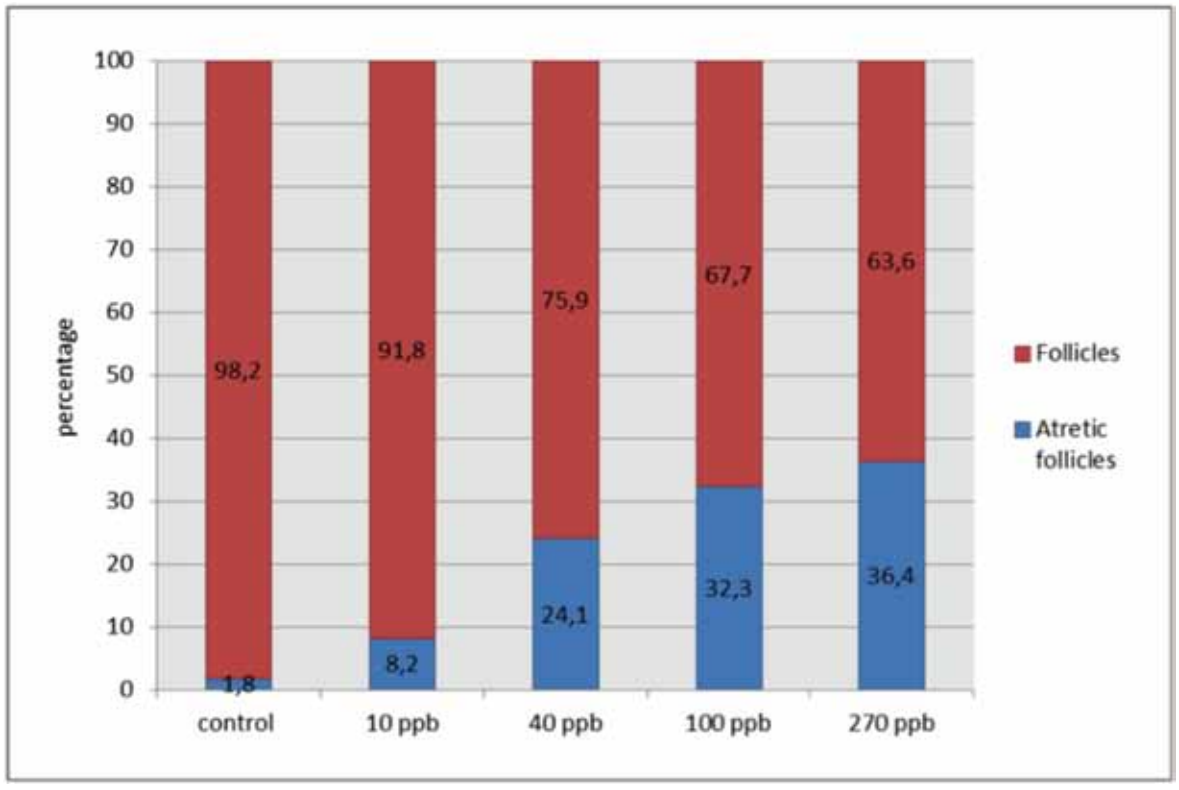

Figure 3. Atretic follicles percentage in the different groups of the study

DISCUSSION

The gonads of these fish treated with 100 and 270 ppb TCDD exhibited abundant atretic follicles, with all their components were disorganized and degenerated, with the presence of inflammatory cell infiltrates. Thus, most of the mature follicles either distintegrated or they were prematurely atretic.

A significant increase in the density of the atretic follicles was observed in fish exposed to graded concentrations of TCDD. The control group showed nearly a 2% of atretic follicles, the lowest dose group showed an increase of follicular atresia reaching above 8%. From the 40 ppb concentration, a higher increase of the follicular atresia was found reaching above 36% in the highest concentration group. The percentages of atretic follicles were clearly increased (Fig. 3), and there were significant differences between the dosed and control groups.

Polychlorinated dibenzodioxins, environmental contaminants known to adversely affect reproduction, are persistent in the environment and known to bioaccumulate and biomagnify in the food chain. These chemicals have been proven to exert an effect on non-target species. TCDD is the by-product of industrial processes and pyrolytic reactions. Numerous studies show that TCDD is an endocrine disruptor and potent ovarian toxicant (BHATTACHARYA, P. & A.F. KEATING, 2012; KARMAN *et al.,* 2012).

In this study, we have exposed female zebrafish to TCDD during 21 days as OECD guidelines (OECD 230) have previously established, in order to evaluate the histopathologic effects on their ovaries by light microscopic and ultrastructural analyses.

In our study we have observed how, at lower doses, (10 and 40 ppb), no modifications in the ovarian structure were observed, with the follicular composition kept normal as in the control

group. This differed from what was observed by other authors, who, after 20 days of exposure to 40 ppb, observed ovarian necrosis (KING-HEIDEN *et al.,* 2006).

Follicular development in zebrafish was attenuated by TCDD exposure, as has been described in mammals where follicular maturation was mitigated, and the number of antral and preantral follicles was reduced (BHATTACHARYA, P. & A.F. KEATING, 2012; PETROFF, *et al.,* 2001). There were frequent examples of degeneration and disintegration in the cortical alveolar and vitellogenic follicles, starting with exposure at 100 ppb, congruent with what was observed by other authors (KING-HEIDEN *et al.,* 2006, 2005; SAKAMOTO *et al.,* 2003). These lesions intensified with the exposure concentration unlike what was observed by other authors who did not associate the pathological modifications observed with any dose level (WALTER *et al.,* 2000).

However, the inflammation that we observed at higher exposure concentrations coincided with that observed by other authors, who frequently reported inflammation and associated it with degeneration (WALTER *et al.,* 2000).

Several studies suggest that a sublethal exposure to TCDD perturbs reproduction (KING-HEIDEN *et al.,* 2012, 2006, 2005; PETROFF *et al.,* 2001). In the ovaries of the exposed animals we noted an increase in the proportion of atretic follicles, although some atresia is physiologically normal in control animals. This was also the case in the group of animals exposed to the lower concentration (10 ppb); and it increased concomitantly with augmented exposure concentrations, in agreement with what was observed by other authors (KING-HEIDEN *et al.,* 2006). Our data reported significant differences ($p<0.05$) between all exposed groups and the control group, and among all the groups exposed to TCDD, showing a significant dose-dependent increase. This differed from other studies in which follicular atresia was not described (SAKAMOTO *et al.,* 2003).

CONCLUSIONS

Our results suggest that TCDD induces degeneration of follicles, causing a disruption in follicular development and an induction of follicular atresia, indicating that long-term exposure to low concentrations of TCDD produces adverse effects on reproductive health. These results show us that it is necessary to continue such long-term, low-dose investigations further and at a gonadal level, so as to elucidate their possible repercussions on fish fertility due to exposure to this endocrine disruptor.

ACKNOWLEDGMENTS

The authors wish to thank the Institut für Pathologie Stiftung Tierärztliche Hochschule Hannover (Germany), especially Dr Wolfgang BAUMGÄRTNER and the electron microscopy technician, for their assistance and encouragement during the ultrastructural analyses.

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Received: 03 September 2013
Accepted: 11 March 2014

Procjene strukture i ultrastrukture ovarija vrste *Danio rerio* nakon izlaganja 2, 3, 7, 8 – tetraklorodibenzo-p-dioksinu (TCDD)

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SAŽETAK

Morfološke konsekvence ovarija po dugotrajnijem izlaganju niskim dozama 2, 3, 7, 8-tetraklorodibenzo-p-dioksina su utvrđene na 50 odraslih jedinki vrste *Danio rerio* i to na strukturnoj i ultrastrukturnoj razini. Jedinke su bile izložene stupnjevanim koncentracijama 2, 3, 7, 8-tetraklorodibenzo-p-dioksina (10, 40, 100 i 270 ppb) tijekom 21 dana, i tada su jedinke bile žrtvovane uporabom letalne doze anestetske solucije tricain metansulfonata, i odmah su jedinke uzete za morfološku procjenu. Utvrđeno je da pri nižim koncentracijama izloženosti nema morfoloških modifikacija, dok su pri višim koncentracijama (100 i 270 ppb) utvrđene učestale degeneracije i upale. Značajan porast folikularne atrezije je utvrđen unutar svih grupa ($p<0.05$). Ovi rezultati ukazuju na to se da po dugom izlaganju ovarija niskim dozama 2, 3, 7, 8-tetraklorodibenzo-p-dioksina mogu inducirati morfološke promjene ovarija, koje tada mogu imati negativni utjecaj na normalnu reprodukciju vrste.

Ključne riječi: TCDD, *Danio rerio*, ovariji, histopatologija, folikularna atrezija