Endocrine-Active Compound Evaluation: Qualitative and Quantitative Histomorphological Assessment of Zebrafish gonads after Bisphenol-A exposure

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Abstract

There is great social concern about the risk involved from exposure to BPA as an endocrine disrupter in humans, as well as the possible repercussion of this chemical on the environment. In this study, the short-term effects of BPA at a gonadal level were assessed by means of different biomarkers in a model animal organism in vogue, the zebrafish (*Danio rerio*). For this purpose, 60 female zebrafish aged 16 weeks were used. These were exposed for 14 days in aquariums (following OECD Directive no.24) to BPA concentrations of 1, 10, 100 and 1000 µg/l, in addition to a control batch. After the exposure period, the zebrafish were sacrificed and samples taken for a histopathological study by light and electron microscopy and morphometric analysis. During the 14 days of exposure, water samples were taken from the aquariums to analyze the BPA levels. The BPA concentration in the fish and the water was determined by LC-MS/MS.

The gonads of the zebrafish exposed to the BPA had a normal external appearance and there were no variations in their size or body weight. An accumulation of BPA was produced in the zebrafish tissues, and this increased as the BPA concentration to which the fish were exposed did. In the histopathological and morphometric studies, multiple alterations were observed in the zebrafish ovaries, particularly highlighting the vacuolization of the follicular cytoplasm, a great degeneration of all the cell components, and an important increase in the percentage of atretic follicles as from concentrations of 100 and 1000 µg/l of BPA, verified by morphometry.

These data indicate that morphological endpoints are sufficiently sensitive to individuate precocious effects of environmental concentration of BPA on gonads after two weeks of exposure.

Keywords: Bisphenol-A; Endocrine-Disruption; Zebrafish; Atretic Follicles; Histomorphology

1. Introduction

Bisphenol A (2,2-bis (4-hydroxyphenyl) propane) (BPA), is one of the chemicals most produced in the whole world, with an annual production of over 2 million tonnes (Lang et al., 2008). It is extensively employed in a great variety of consumer products, a large number of which enter into contact with food (Wen-Tien, 2006). It is also present as an environmental contaminant in rivers and drinking water, probably due to the migration of plastic containers from industrial rubbish heaps (Kolpin et al., 2002; Coors et al., 2003). BPA is an endocrine disrupter, which can mimic the body's own hormones and may lead to negative health effects. Its action of mechanism is based on its binding to estrogenic receptors, presenting an estrogenic activity at the same time as it induces dysfunctions in reproduction and development at a neurological level and in the immunological system. Also, recently, this compound has been related to cardiovascular disorders, diabetes and obesity in humans.

The European Union has agreed to ban the presence of BPA in plastic feeding bottles for its possible harmful effects on children's health (E.U.Directive 2011/8). However, for the moment, the European Commission has no plans to further restrict the use of BPA and has agreed to maintain the Admissible Daily Intake (ADI) of BPA for humans at 0.05 mg/kg/day. However, the considerable amount of research on the action of BPA on health reflects certain doubts (EFSA, 2008).

The importance of BPA as an environmental contaminant and the risk it entails due to its low biodegradability rate and its bio-accumulation in the trophic chain have caused

the European Parliament to recently include it as a substance whose toxicity should be evaluated. The European Commission will have until 2013 to decide whether 13 new substances should be added to the inventory, among which BPA is included. Hence the importance of an assessment of the danger of this chemical as a contaminant.

Zebrafish are suitable for assessing toxic effects of chemicals on development and reproduction, because test protocols have already been established, including OECD guidelines (OECD 204, 210, 212), 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.

Histopathological examinations may provide an insight into the nature of reproductive impairments (Miles-Richardson et al., 1999; Metcalfe et al., 2000). At the fourth meeting of the OECD Task Force on Endocrine Disrupter Testing and Assessment (EDTA) in Paris on May 12, 2001, it was generally agreed that histopathology should be adopted as a core endpoint in the assessment of estrogen-active compounds (Segner et al., 2003).

The histopathological assessment of fish reproductive organs can be divided into 2 separate components: the evaluation of gonads for abnormal findings and gonad staging. Gonad staging involves the assessment of germinal cell type proportions in order to identify the potential effects of exogenous or endogenous chemicals on gametogenesis. Cell type proportions can be obtained by morphometry of zebrafish ovaries (Wolf et al., 2004).

2. Material and methods

2.1. Fish exposure and sampling protocol

16 week-old female zebrafish *(Danio rerio)* (n=60; standard length; 4,173±0,239 cm; 0,568±0,139 g wet weight) were used. Treated groups were exposed for 14 days (OECD Guideline No. 204) to graded concentrations (1, 10, 100 and 1000 µg/L) of BPA (Sigma Aldrich®, St. Luis, EE.UU.) under flow through conditions (10 water renewals/day) and photoperiod of 16 light hours: 8 dark hours. Water temperature was 26±1º C and dissolved oxygen was maintained above 60% of saturation level by continuously aerating the test solution. Zebrafish were fed twice a day with a non estrogenic granulated diet (Supervit® minigranulated, Tropical, Chorzow, Poland). A control group (unchlorinated tap water) completed the exposure design.

After 2 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), next and immediately, standard length (SL) and body weight (BW) were measured.

Gonads from 50% of animals from each group were dissected and fixed for histological analysis by qualitative and quantitative evaluations. 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 dissecting in a caudal to cranial direction, while applying very gentle traction to the oviducts (Wolf et al., 2004). The rest of the fish from each group were frozen and stored at -80º C for analytical BPA determinations.

2.2. Analysis of BPA content

2.2.1. Water:

Three times a week water from each tank was sampled, keeping it frozen until its analytical verification of BPA exposure concentrations. Prior to the analysis, the samples were thawed and subsequently processed for their determination, injecting 20 µl sample in the LC–MS/MS system

2.2.2. Fishes:

Whole body homogenates were obtained for BPA quantification. Their maceration was carried out with a homogenizer (Ribolyser®) to 10.000 G in tubes of 1 ml, in a 1:2 wet weight/ buffer volume (50 mM Tris-HCl ph 7,4).

Samples were processed for their extraction and purification, and finally transferred into vials. 20 µl was injected in the LC-MS/MS system for the BPA determination. The HPLC system consisted of two Varian 210 series pumps, a Varian 410 series autosampler, a Metahcem Tecn degasificator with four channels in line. A Synergi Hydro RP 80 Å, 150 x 2 mm 4 µ Phenomenex column was used for the chromatographic separation with a AQ C18 4 x 2 mm Phenomenex precolumn. For the HPLC–MS/MS experiments, a 1200 L Varian triple quadruple mass spectrometer was used. The IS voltage was 1400 V, temperature drying gas 350º, pressure drying gas 30 psi. Mass spectrometry analyses were performed in the MRM mode (Multiple Reaction Monitoring). For the MRM experiments, the collision gas was argon. The mobile phases for negative ionisation were 0.05% TEA (triethylamine) in Milli Q water (A) and acetonitrile (B) with a gradient from 100% A to 100% B in 21 min at 200 µl/min.

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152 2.3. Qualitative Histomorphological Assessment: Light and electron microscopy

153 For the structural evaluation, the fixed ovaries were routinely processed for paraffin
154 sections by fixing in 10% formaldehyde, dehydrating in graded series of ethanol,
155 immersing in xylol and embedding in paraffin wax. Every tenth section (4 µm thick) of
156 each block was stained with haematoxylin and eosin and used for the morphological
157 study.

158 For the ultrastructural study, small randomly selected samples of gonads were primarily
159 fixed in a 2% glutaldehyde solution in 0.1M phosphate buffer (pH 7.4) overnight at 4º C
160 and then refixed in 1% osmium tetroxide in 0.1M phosphate buffer (pH 7.4) for 30 min.
161 After dehydration in graded ethanol series and embedding in Araldite, semithin and
162 ultra-thin sections were cut on an LKB ultramicrotome. Semithin sections were stained
163 with toluidine blue, whereas ultra-thin sections were double-stained with uranyl acetate
164 and lead citrate. Ultra-thin sections were viewed and photographed in a Philips CM10
165 transmission electron microscope.

166

167 2.4. Quantitative Histomorphological Assessment: Morphometric study

168 For the morphological study, the fixed gonads were cut into three sections. Each portion
169 was then processed and embedded in paraffin as for routine histology. The first section
170 (4µm thick) of each block was stained with haematoxylin and eosin and used for the
171 stereological study.

172 The quantitative study was performed using an image analysis system consisting of a
173 Leitz Ortholux triocular microscope connected by means of a SONY SSC-C370P® colour
174 video camera to an IBM-compatible personal computer equipped with a frame
175 grabber board. Each specimen was sampled in a systematic manner for the selection of

176 microscopic images that were then digitized; a 100x lens (N.A. 1.25) was used for this
177 procedure. An average of 50 microscopic fields per slab was chosen in each specimen.
178 Each microscopic image was processed using Visilog 5® software. Quantification was
179 performed by an observer experienced in the use of the analysis system (J.G-M) but
180 with no previous knowledge of which group was being analysed. The system was
181 initially, and regularly, calibrated using a millimetre slide.
182 The numerical density (Q_A) of each type of follicle in the plane was estimated using a
183 test system consisting of sixteen rectangular counting frames superimposed onto each
184 microscopic image. Thus, the number of profiles per area Q_A *(nucl/tis)* were estimated
185 according to:
186 $est\ Q_A(nucl/tis) = \Sigma Q(nucl) / (\Sigma P(tis) \bullet a/p)$

187 where Q_A *(nucl/tis)* is the numerical density of follicular nuclei per ovary tissue,
188 $\Sigma Q(nucl)$ is the total number of nuclear profiles counted within the counting frames
189 of the area obtained from $\Sigma P(tis)$ as the total number of points which hit the tissue,
190 multiplied by a/p as the area associated with one point on the test system (in our
191 study, $a/p = 125$ μm^2).
192
193 2.5. Statistical analysis
194 Data were analysed using the statistical programme Statgraphic (Centurion XVI®) to
195 determine BPA effects on every exposed group. ANOVA (test- F) was used to
196 demonstrate if significant differences between the averages existed. The Fisher LSD
197 post hoc test was used to perform multiple comparisons between groups. Results are
198 expressed as mean values ± standard deviation (SD) and $P<0.05$ was considered to be
199 significant.
200

3. Results

3.1 Macroscopic findings

There was no fish mortality during the 14-day study period. No macroscopic findings were found, and no significant differences in mean BW and SL were detected between the control and the BPA-exposed animals.

3.2 BPA levels

The data of the BPA concentrations in the water coincided with the nominal ones and no significant results were obtained in this respect. This agreed with prior studies, in which no degradation of BPA in water was observed (Dorn et al., 1987).

Table 1 shows the levels of BPA concentration in the zebrafish at day 14 after 1, 10, 100 and 1000 µg/L of BPA exposure. Whereas non-detected BPA levels were found in the control group, an increase in the BPA concentration was observed in the zebrafish exposed to graded concentrations of BPA. In the treated groups, data reported showed significant differences from the control group. While none were detected between treated groups at 1, 10 and 100 µg/L, there were significant differences between these groups and the highest BPA concentration (1000 µg/L) exposure group.

3.3 Qualitative Histomorphological Assessment: Light and electron miscroscopy

The parenchyma of the ovary corresponding to the **control** group showed images of an extensive development of its follicles, displaying a correct distribution of all its elements. In this group, the interstitium gave the typical images of this tissue component

in the species being studied, with the presence of abundant connective cells and blood vessels (Fig. 1).

The **primordial follicle**, the first phase of the follicular evolution of the oocyte, was integrated by the oocyte covered by a single layer of flat follicular cells, which established a simple flat epithelium surrounded by a sharp basement membrane. The oocyte integrating the primordial follicle had a spherical, vesicular and large nucleus, in an eccentric situation with respect to the cytoplasm, and a highly developed nucleolus. The cytoplasm of the oocytes, which had a very intense basophilia under the electron microscope, displayed huge amounts of ribosomes.

The transition of a primordial follicle to a **cortical alveolar follicle** involved a series of changes in the oocyte, in the follicular epithelium, and in the connective tissue surrounding it. The beginning of the transition was marked by a notable increase in the size of the oocyte in relation to the primordial follicles. This is because it was starting a period of growth which implicated the nucleus, the nucleolus and the cytoplasm, accompanied by the transformation of the follicular epithelial cells from their flattened shape into cubic cells. In the peripheric areas of the oocyte's cytoplasm, vitelline vesicles appeared, which made the nucleus become compressed in the central area. The size of the nucleus was even more apparent than in the primordial follicles. It had undulating borders and possessed a homogeneous chromatin and with a lower basophilia than the cytoplasm.The number and size of the nucleoli were increased, incrementing their basophilia and becoming situated in the periphery of the nucleus (perinucleolar state).

The **Vitellogenic follicles** were formed by the oocyte surrounded by a thick pellucid membrane, with the follicular cells constituting a cylindrical epithelium. They were

249 located inside the ovarian parenchyma, close to the primordial follicles, and were an
250 intermediate step between the cortical alveolar follicles and the mature ones.
251 The nuclei were maintained in the centre of the oocyte, with undulated borders, their
252 chromatin being homogeneous and with a scant basophilia. In their centre, numerous
253 nucleoli were seen, which were mainly arranged in the periphery of the nucleus,
254 although they were also found in the rest of it. Morphologically, they were spherical,
255 very basophilic and had a hollow in the centre.
256 The pellucid membrane entirely surrounded the oocyte. It was very thick and
257 homogeneous with a manifest acidophilia. The enveloping epithelium was formed by
258 scant layers but stood out for being constituted by an epithelium of cubic cells.
259 The **Mature follicles** were comprised of oocytes highly transformed in relation to the
260 primordial follicle. These follicles possessed a thin envelope of flat cells. They were
261 distributed throughout the ovary, occupying most of it, and represented the oocytes
262 which, in the final phase, were released outside. In them, mainly, the cytoplasm stood
263 out since in these follicles a masking of the nucleus took place.
264 The cytoplasm was very well developed, of a large size and was occupied practically in
265 its totality by large vitelline granules and by lipidic vacuoles. The pellucid layer was
266 maintained, although its thickness had notably diminished, and although it kept its
267 acidophilia, its border was uneven. The layer of follicular cells was thinner due to a
268 decline in the number and thickness of its cells.
269 In the **Atretic follicles** we, can mainly highlight phagocytosis and invasion from the
270 follicular cells of the cytoplasmic rests of the oocyte. This triggered the atresia and re-
271 absorption of the ovarian follicles with the destruction of the nucleus, the folding and
272 dissolution of the pellucid zone, the disorganization and liquefaction of the vitelline
273 vesicles, and the destruction of the lipidic granules.

The fish treated with **1** and **10 µg/L of bisphenol-A** presented all the types of follicles described in the control group (primordial, cortical alveolar, vitellogenic, mature and atretic). All these follicles maintained the composition and the type of lining cell (Fig.2).

The primordial follicles showed an apparently normal morphology with a strong basophilia in their oocytes, although, unlike the control group, they presented a certain vacuolization in the peripheric zone of the oocyte.

Neither did the cortical alveolar follicles exhibit any alterations worth mentioning when comparing them to the control group, only certain vacuolizations, together with the initiation of the formation of the pellucid zone in the fish treated with 10 µg/L of bisphenol-A.The nuclei of the oocyte had numerous nucleoli, a basophilic cytoplasm, and the presence of vitelline vacuoles beginning their formation was noted.

The vitellogenic follicles also showed an apparently normal morphology, with a highly developed, homogeneous and acidophilic pellucid membrane, with abundant vitelline vesicles, and, above all, lipidic drops. In fish of these batches, there were few atretic follicles.

In the zebrafish treated with **100 µg/L of bisphenol-A** the different ovarian follicles were also observed (Fig.3).

There were few primordial follicles although they kept their structure. They were hypertrophic and displayed a marked vacuolized cytoplasm, although they maintained their intense basophilia.

Although the cortical alveolar follicles maintained their components the same as the previous ones, they had become larger, and abundant vacuolizations were observed.

There were very many vacuolizations also in the pellucid zone of the mature follicles with wide spaces between the follicular cells of the oocytes, and disorganizations.

The gonads of these fish treated with this dose of bisphenol-A (100 µg/L) displayed abundant atretic follicles, with all their components disorganized and degenerated.

The study of the zebrafish group treated with **1000 µg/L of bisphenol-A**, the same as in the previous study, showed the different types of follicles in their gonads although more marked alterations were observed (Fig.4).

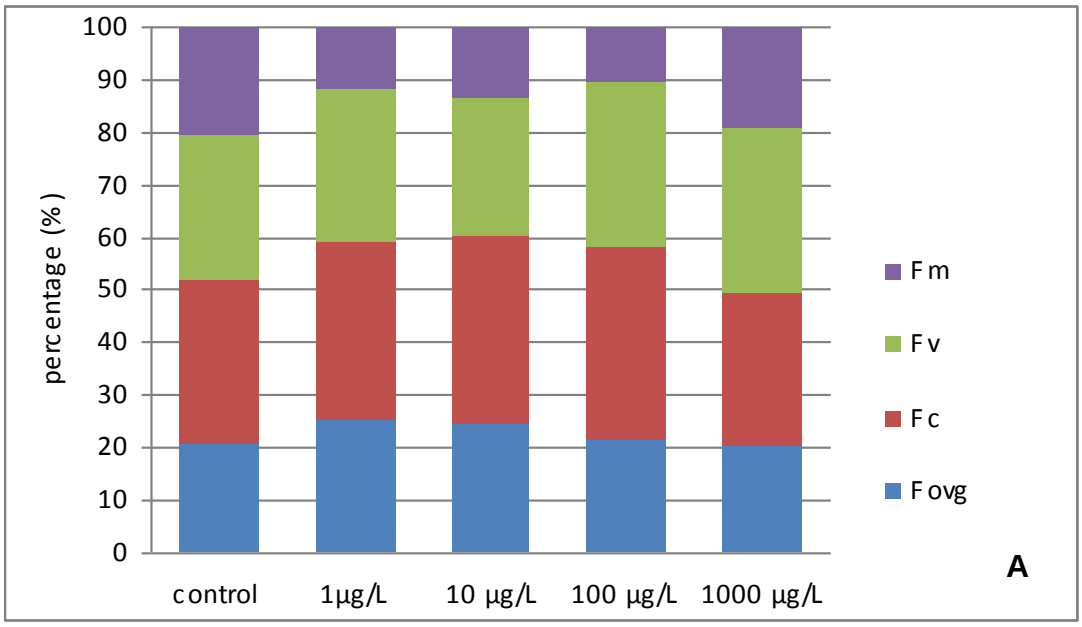
Fewer primordial follicles were noted than in the gonads of fish from the previous batches, although they were larger in size. What stood out was the great increase in vacuolizations in the cytoplasm, highly destructured nuclei, and extensive degenerative processes.

The cytoplasm of the oocytes in the vitellogenic follicle was seen to be also highly vacuolised, with a degeneration and vacuolization of the pellucid zone. In this batch, abundant atretic follicles were observed with a degradation of all their components.

3.4 Quantitative Histomorphological Assessment: Morphometric study

Data related to gametogenic cells are represented in Figure 5A, where different percentages of each type of follicle in each study group are reported. Significant differences have been observed between the different study groups with respect to each type of follicle counted.

Regarding the results obtained in the morphometric study, the percentages of atretic follicles were clearly affected by BPA exposure (Figure 5B). A significant increase in the density of the atretic follicles was observed in fish exposed to graded concentrations of BPA, reporting significant differences from the control group, and between the dosed groups.



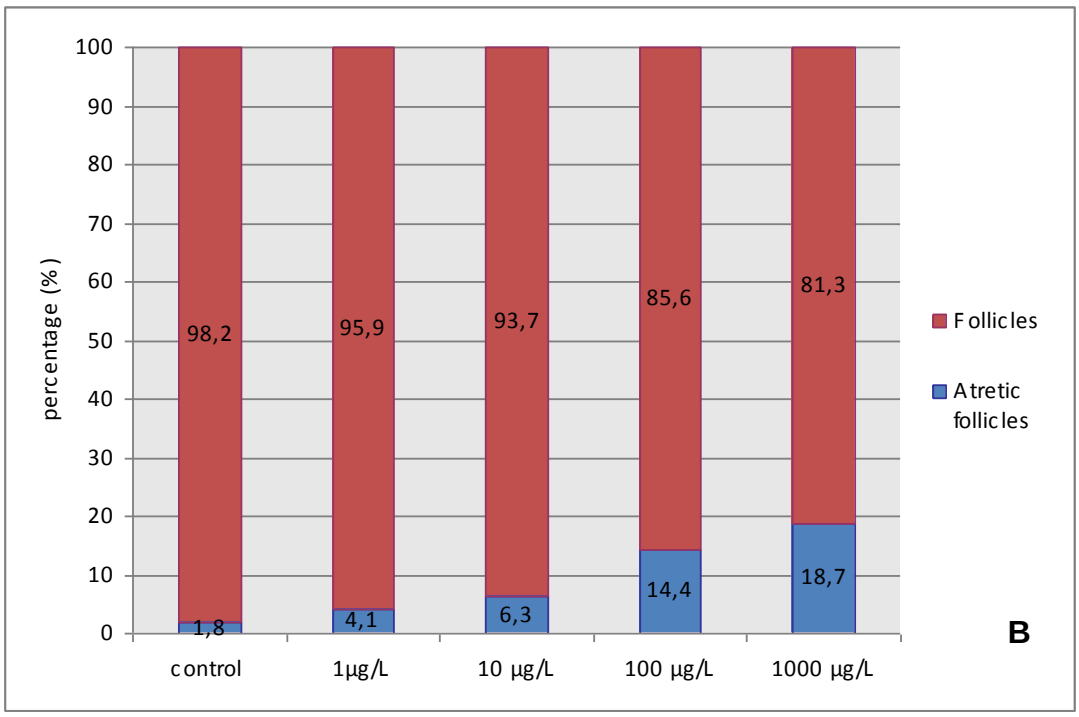


Fig. 5. A: Different type follicle distribution in each group. Primordial (Fovg), cortical alveolar (Fc), vitellogenic (Fv), mature (Fm); B: Atretic follicle percentage in the different groups of the study.

4. Discussion

The equilibrium between the estrogens and the androgens is decisive for the complete development of the gonads so that their evaluation as a biomarker of gonadal development and possible alterations demonstrating the estrogenic action of BPA is of extraordinary interest.

There are very many studies on rodents as a model for the evaluation of the disrupting action of BPA (Rodriguez et al., 2010; Karavan et al., 2012), although they are not so frequent in work using fish, and, more specifically, in zebrafish, as an experimental model to evaluate the estrogenic action of BPA. Zebrafish are one of a group of small fish species that can be kept in the laboratory, are easily exposed to endocrine-disrupting chemicals in tank water at different stages in their life cycle, and exhibit measurable sensitivity to endocrine-disrupting chemicals, including sexual dimorphism (Van den Belt et al., 2001; Orn et al,. 2003; McGonnell et al., 2006).

This study has focused on the action of BPA on zebrafish ovaries by observing the alterations in the structure of the germinal cells and follicular atresia, as well as the modifications in the relative proportion of germinal cells.

The adverse effects produced by BPA on gonadal development in fish have been described by other authors in carp in highly polluted rivers (Patino et al., 2003; Mandich et al., 2007) finding serious alterations in their gonadal morphology starting from environmental concentrations (1µg of BPA/L).

Concentrations of BPA have been used by means of a continuous flow system in the water of aquariums, i.e. 1, 10, 100 and 1000 µg/L of BPA, very similar doses to those employed by different authors in diverse fish species (Lindholst et al., 2000; Ishibashi et al., 2005; Mandich et al., 2007; Villaveuve et al., 2012). Coinciding with previously reported data, no mortalities occurred during the BPA exposure period (Villaneuve et

al., 2012). Nor were any differences obtained with regard to the weight and length of the animals after the study period, the same as was found by Mandich et al. (2007). But, however, the macroscopic aspect of the gonads remained apparently normal in our study, whereas the other authors, after the period of exposure to 17β-estradiol, noted a gelatinous appearance in the ovaries (Wolf et al., 2004).

In our study, a bio-accumulation of BPA was produced in the fish's tissues, which increased at the same time as the BPA concentration to which they were exposed did.

With regard to the follicular growth in the ovaries, in the first two batches (1 and 10 µg/L of BPA) we found the same types of follicles as those observed in the control group, all of them clearly seen, and they maintained their structure and the type of lining cells, coinciding with what was observed by other authors (Mandich et al., 2007). In the zebrafish exposed to 100 µg/L of BPA we found some abnormality in their ovaries, such as the vacuolization of the cytoplasm of the primordial follicles, and the irregular borders of the nucleus of the vitellogenic follicles. At doses of 1000 µg/L of BPA, in all the types of ovarian follicles, we observed a degradation of their cell components, where an interstitial fibrosis characterized by the presence of connective fibrous tissue inside the ovarian stroma, as well as granulomatous infiltrations, stood out. These alterations were similar to those observed by Mandich et al. (2007) in carp exposed to the same concentrations,which developed alterations in their connective stroma, and an increase in eosinophilic granulocytes. Similar lesions have been described in ovaries produced by estrogen-active compounds like 17β-estradiol (Wolf et al. 2004). This similarity between the histological modifications induced by both compounds leads us to believe in an estrogenic action of BPA at the ovarian level.

As for the quantification of the follicles, we observed that as the concentration exposure increased, there was a decline in the percentage of primordial follicles, the same as was

observed by Rodríguez et al. (2010) in a study made in rats, whereas the percentage of recruited follicles (sum of cortical alveolar, vitellogenic and mature follicles) increased. These results indicate that the reduction in the ovarian reserve of follicles induced by xenoestrogen exposure was caused by the increased initial recruitment of primordial follicles, demonstrating that BPA negatively impacts by acting as an activator of the initial recruitment of primordial follicles (Rodriguez et al., 2010). Similarly, this stimulatory effect on the transition of primordial to cortical alveolar follicles has been previously reported for other estrogen-active compounds like diethylstilbestrol (Wordinger et al., 1989).

In the OECD Directive of 2009, we find a series of diagnosis criteria in the gonadal histopathology of females in relation to the analysis of the action of potentially estrogenic compounds. Among the primary diagnosis objectives, an increase in follicular atresia was found to be a marker of the gonadal histopatholgical harm from the action of these compounds (OECD, 2009). Follicular atresia, whether caused by physiological stimuli or environmental stress, entails the degeneration of follicles at any stage of follicular development, although atretic follicles are often most noticeable in the later development stages (Wolf et al., 2004).

In our study, we quantified the percentage of atretic follicles in the ovary of the females at different exposure concentrations of BPA and we verified that in the control there was a small percentage of atretic follicles, 1.8%, a justifiable datum since follicular atresia is a physiological mechanism, which appears in the ovary of vertebrate animals. But as the exposure concentrations increased (100 and 1000 μg/L of BPA), an increment was produced in the percentage of atretic follicles with respect to the control, reaching values of above 10% of the total of ovarian follicles (14.4 and 18.7%, respectively). In this sense, we coincide with other authors who, similarly, observe this

dose-dependent increment in atretic follicles, and even a more marked one, reaching up to 57.1% of follicular atresia in carp at the highest BPA exposure dose (Mandich et al., 2007).

When exposing fathead minnows to the estrogenic action of 17β-estradiol (2.780 ng/L), 18% of atretic follicles was obtained (Wolf et al. 2004) compared to their physiological percentage, which ranged from 1.6% to 5% (Mc Cormick et al., 1989; Miles-Richardson et al., 1999). The percentage of atretic follicles obtained after exposure to estradiol was similar to that observed in our study when exposing the zebrafish to the highest BPA concentration (100 µg/L).

Also, Weber et al. (2003) exposed zebrafish to a concentration of 100µg/L of 4-nonylphenol, observing an increase in the percentage of atretic follicles with respect to the control group. Also, Spano et al. (2004), after exposing adult goldfish to 100 and 1000 µg/L of atrazine for 3 weeks, noted a higher proportion of atretic follicles in these exposed batches than in the control (20 and 25% of the follicles, respectively), and this revealed that exposure to other estrogen-active compounds induces an increase in the percentage of follicular atresia. This was the same as occurred in our experiment after exposure to BPA, indicating that its estrogenic action is not as intense as that of other estrogen-active compounds previously reported.

These data indicate that morphological endpoints are sufficiently sensitive to individuate precocious effects of environmental concentration of BPA on gonads after two weeks of exposure. BPA triggers alterations in follicular development, causing an increase in the percentage of atretic follicles in zebrafish ovaries, which intensifies as the BPA concentration to which the fish are exposed rises. Our results demonstrate that histopathology and gonadal morphometry are sensitive markers for the analysis of BPA

in zebrafish, as well as the latter's adaptation as an experimental model organism in the assessment of the effects of BPA and other chemical compounds as an endocrine disrupter.

6. Acknowledgments

The authors wish to thank the Spanish Junta de Andalucía (P09-AGR-514) for their financial support for this study.

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Tables

BPA concentration (µg/L)					
	Control	1	10	100	1000
BPA concentration (µg/g)	nd	0.046±0.005*	0.071±0.015*	0.570±0.034*	29.562±4.076*

Table 1. Zebrafish-BPA levels (µg/g) expressed by mean±SD
Nd: Non detected
*Significantly different from the control with $p<0.05$

Figure1

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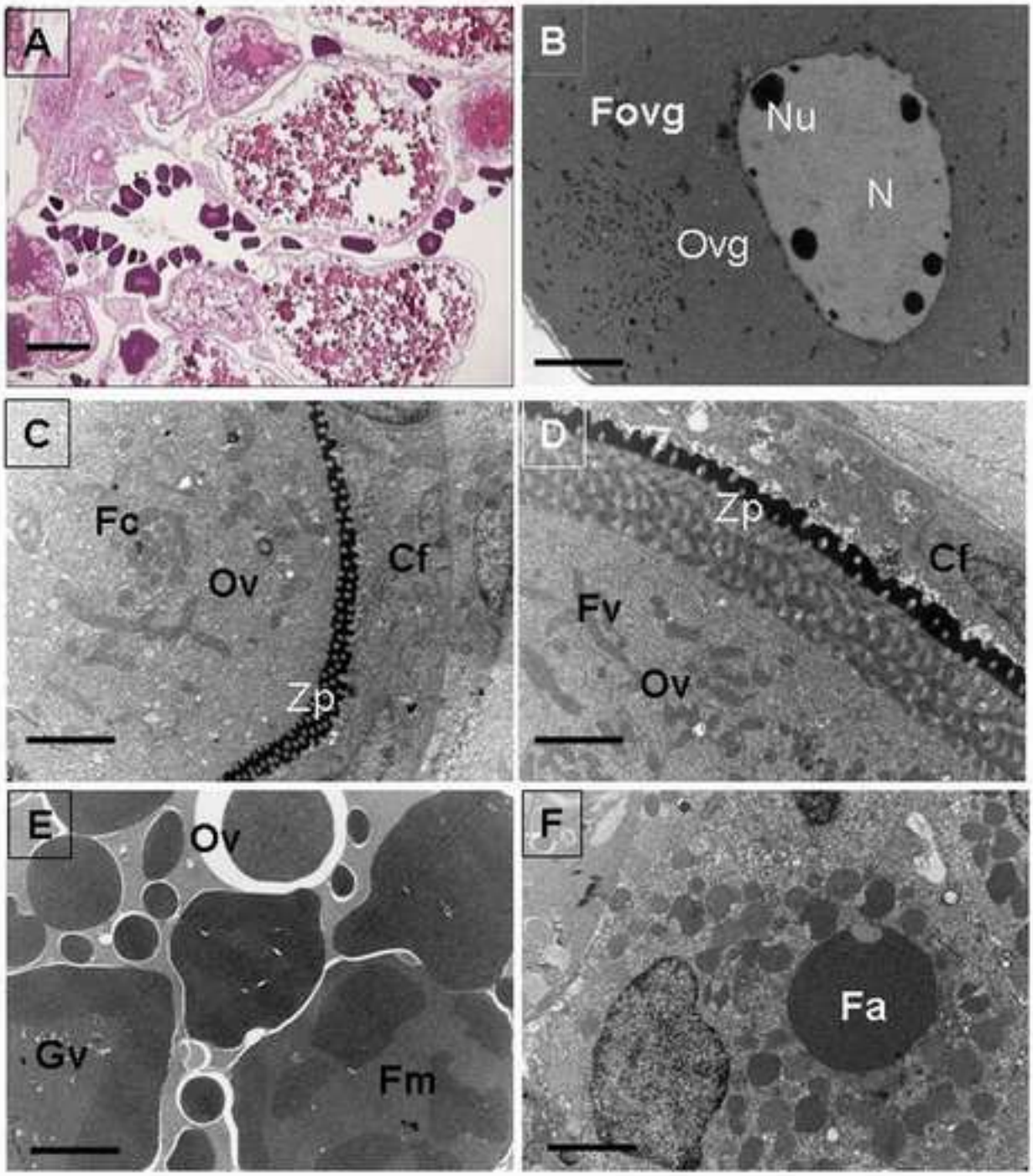

Fig. 1. Ovary of zebrafish control

A: Light microscope. Bars, 100 µm. **B, C, D, E, F**: Ultrastructural observations. Bars, 10 µm.

A. View of ovary in which the different apparently normal follicles can be seen.

B. View of primordial follicle with oogonia (Fovg), with the ovule showing (Ovg) a homogeneous nucleus (N) and abundant nucleoli (Nu), and the cytoplasm homogeneous and dense at the electrons.

C. View of cortical alveolar follicle (Fc) with ovule (Ov), pellucid zone (Zp) and apparently normal follicular cells (Cf).

D. View of vitellogenic follicle (Fv) with ovule (Ov), pellucid zone (Zp) and apparently normal follicular cells (Cf).

E. View of mature follicle (Fm). The ovule (Ov) and apparently normal vitelline granules.

F. View of apparently normal atretic follicle (Fa).

Figure2

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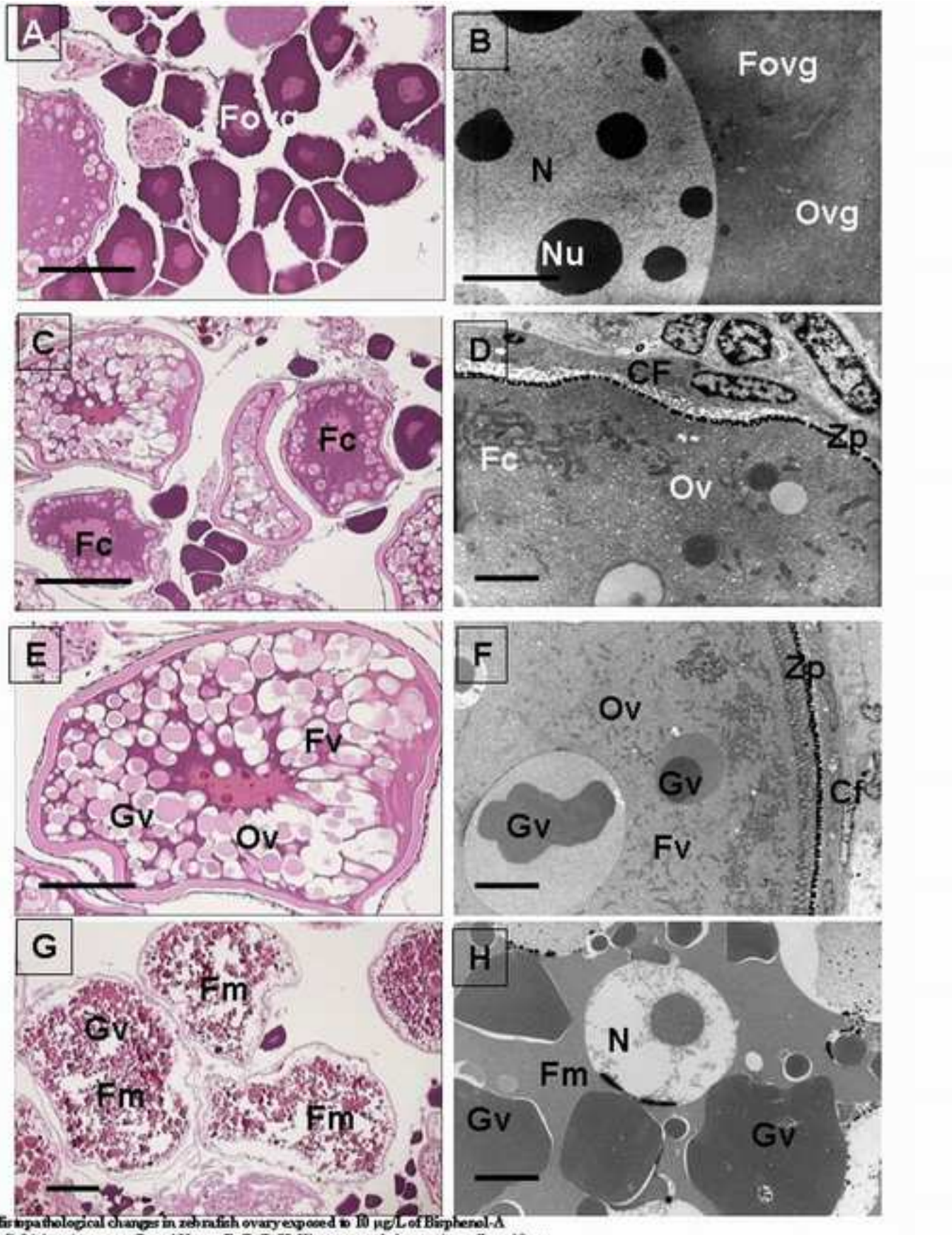

Fig. 2. Histopathological changes in zebrafish ovary exposed to 10 μg/L of Bisphenol-A

A, C, E, G: Light microscope. Bars, 100 μm. **B, D, F, H**: Ultrastructural observations. Bars, 10 μm.

A. Ovarian parenchyma with numerous primordial follicles with apparently normal oogonia with a marked basophilia of the oogonia (Fovg). **B**. Primordial follicles with oogonia (Fovg). Nucleus (N) with abundant nucleoli (Nu) and a homogeneous and dense cytoplasm (Ovg). **C**. Apparently normal cortical alveolar follicles (Fc). **D**. View of cortical alveolar follicles (Fc) in which apparently normal ovules (Ov), follicular cells (Cf) and the pellucid zone (Zp) are seen. **E**. View of vitellogenic follicle (Fv). The ovule (Ov) shows numerous vitelline granules (Gv). **F**. View of vitellogenic follicle (Fv). A normal oocyte (Ov) with abundant vitelline granules, and a normal pellucid zone (Gv) and follicular cells (Cf). **G**. View of mature follicles (Fm), with abundant vitelline granules (Gv). **H**. View of mature follicle (Fm), showing the nucleus (N), and apparently normal vitelline granules (Gv).

Figure3

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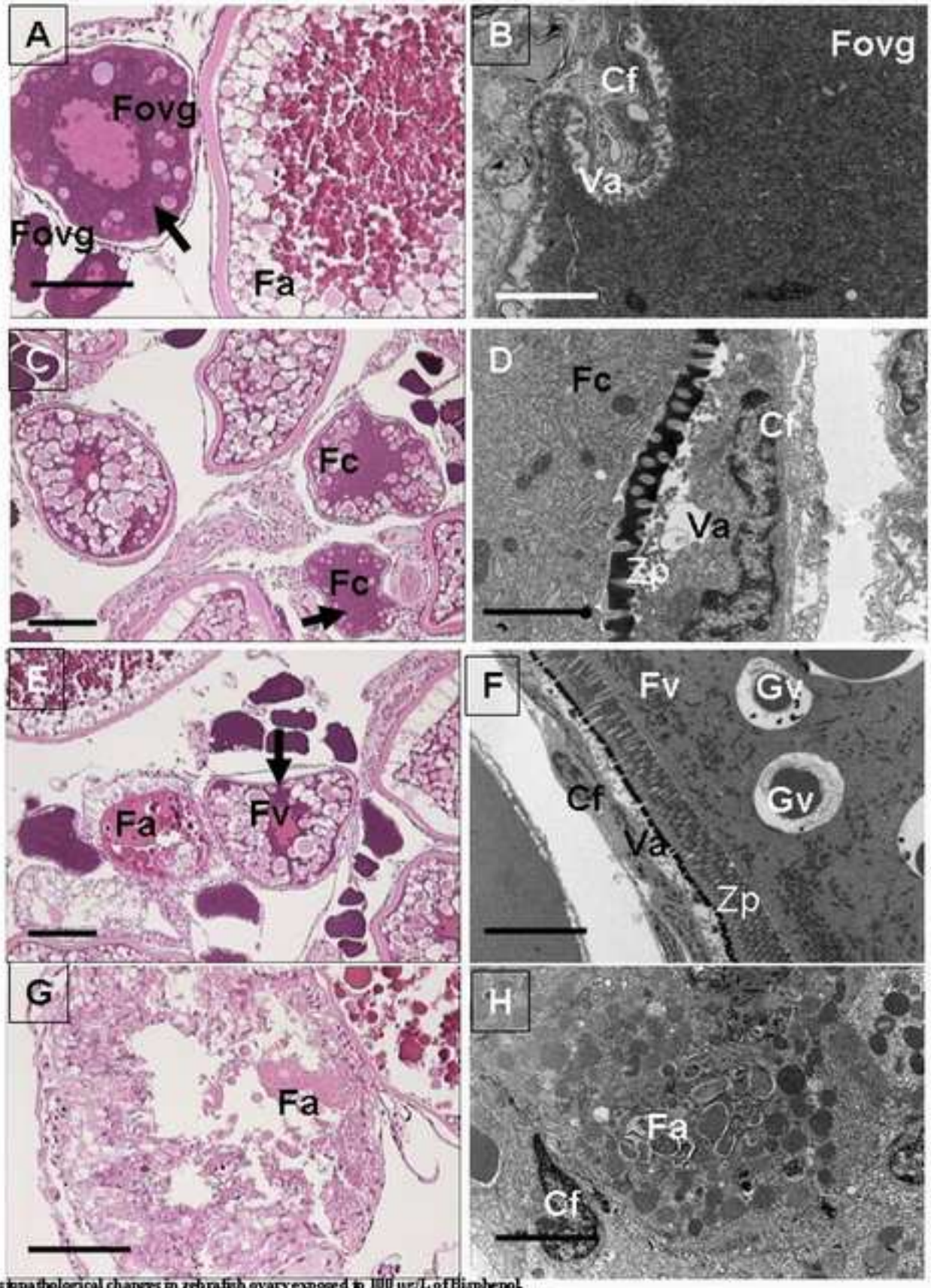

Fig. 3. Histopathological changes in zebrafish ovary exposed to 100 µg/L of Bisphenol.
A, C, E, G: Light microscope. Bars, 100 µm. **B, D, F, H**: Ultrastructural observations. Bars, 10 µm.
A. View of primordial follicles with oogonia (Fovg), hypertrophic and vacuolized oocytes are seen (arrow), and an atretic follicle (Fa). **B**. View of Primordial follicles with oogonia (Fovg), detachment of the follicular cells (Cf) of the oocyte, with vacuolization zones (Va). **C**. View of cortical alveolar follicles (Fc), in some of which vacuolizations are highlighted (arrow). **D**. View of cortical alveolar follicles (Fc) with separations of the follicular cells (Cf), of the oocytes and vacuolizations (Va) in the pellucid zone (Zp). **E**. View of vitellogenic follicle (Fv) with detachments of the follicular cells appearing (arrow) next to an atretic follicle (Fa). **F**. View of vitellogenic follicle (Fv) with disorganizations of the pellucid zone (Zp), vacuolizations (Va), vitelline granules (Gv) and detachment of the follicular cells (Cf). **G**. View of atretic follicle (Fa) with all its components disorganized and degraded. **H**. View of atretic follicle (Fa) with alterations in the follicular cells (Cf) and all their components.

Figure4

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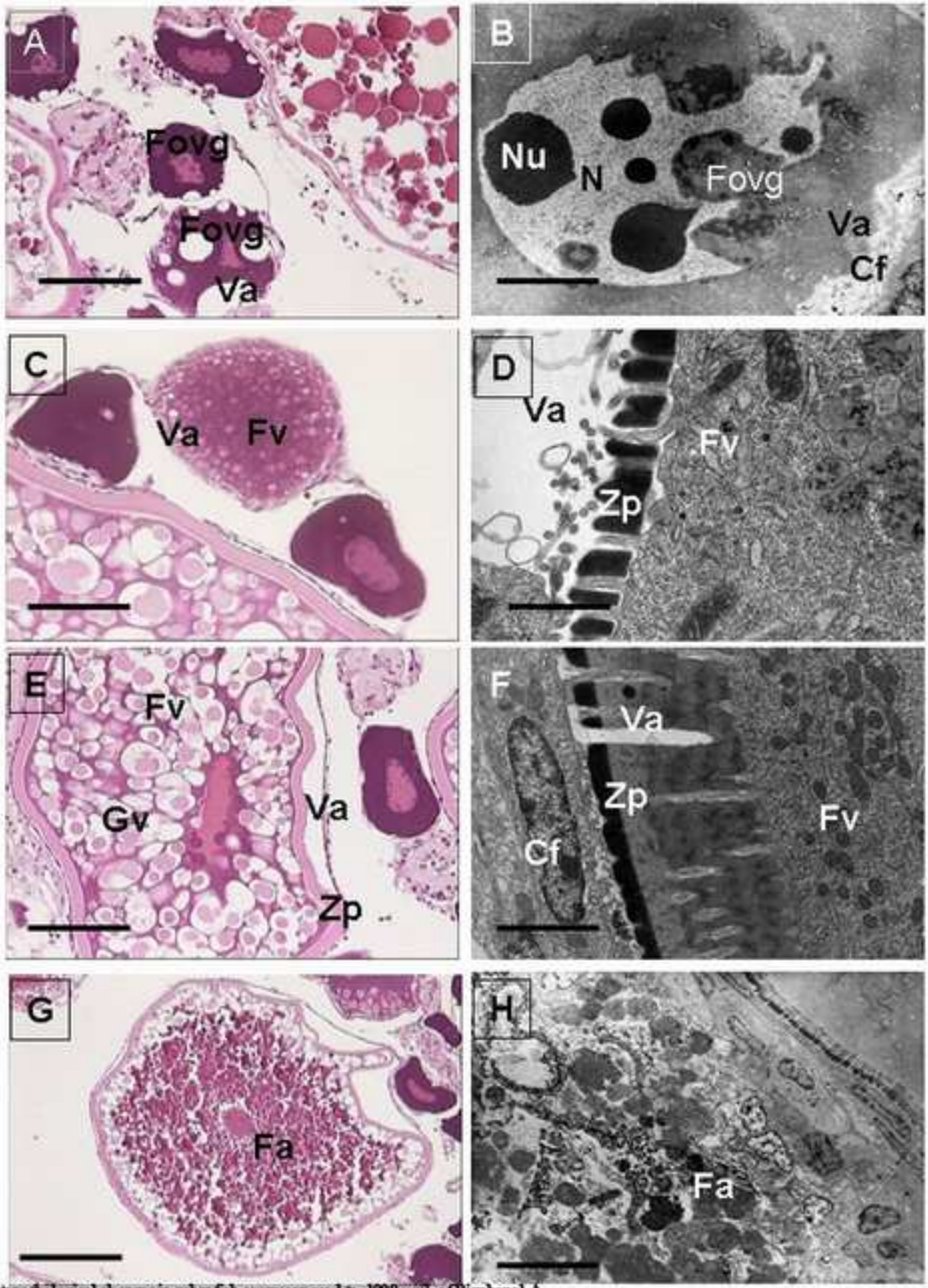

Fig. 4. Histopathological changes in zebrafish ovary exposed to 1000 µg/L of Bisphenol-A

A, C, E, G: Light microscope. Bars, 100 µm. **B, D, F, H**: ultrastructural observations. Bars, 10 µm.

A. View of Primordial follicles with oogonia (Fovg), with numerous vacuolizations (Va). **B**. View of Primordial follicles with oogonia (Fovg), the nucleus (N) highly disorganized with presence of nucleole (Nu), vacuolizations (Va) and follicular cells (Cf) in degenerative processes. **C**. View of vitellogenic follicles (Fv) with a highly vacuolized cytoplasm of the oocyte (Va). **D**. View of vitellogenic follicles (Fv), with vacuolizations (Va), and degradation in the pellucid zone (Zp). **E**. View of the vitellogenic follicle (Fv), with a vacuolized (Va) pellucid zone (Zp) and numerous vitelline granules (Gv). **F**. View of vitellogenic follicle (Fv) with degradation and vacuolization (Va) of the pellucid zone (Zp), and detachment of the follicular cells (Cf). **G- H**. View of atretic follicles(Fa), with all their components degraded.

graphic (Figure5)
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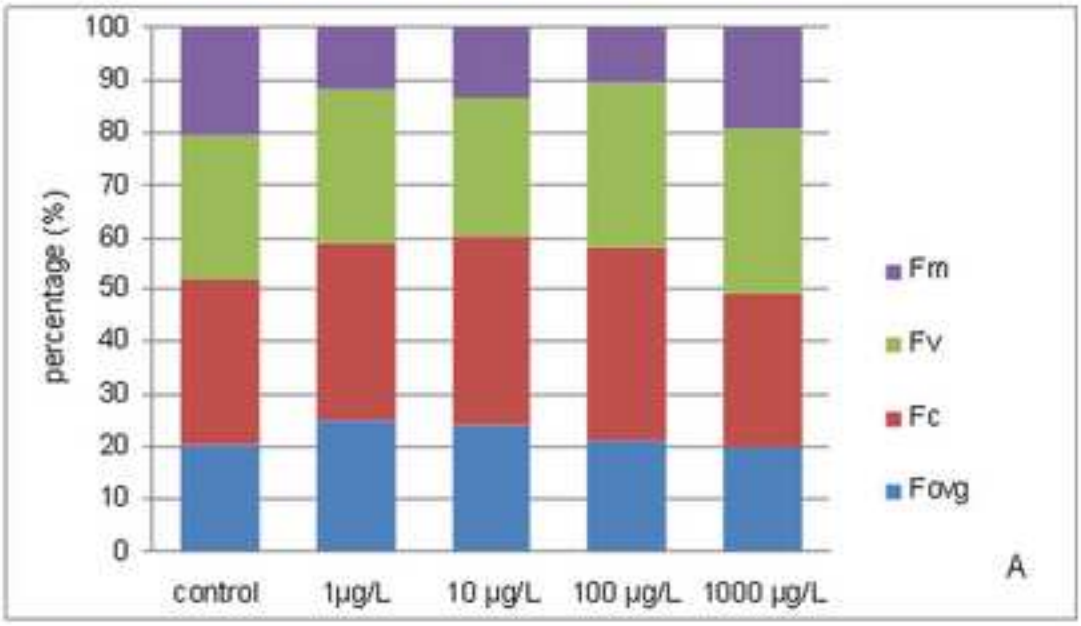

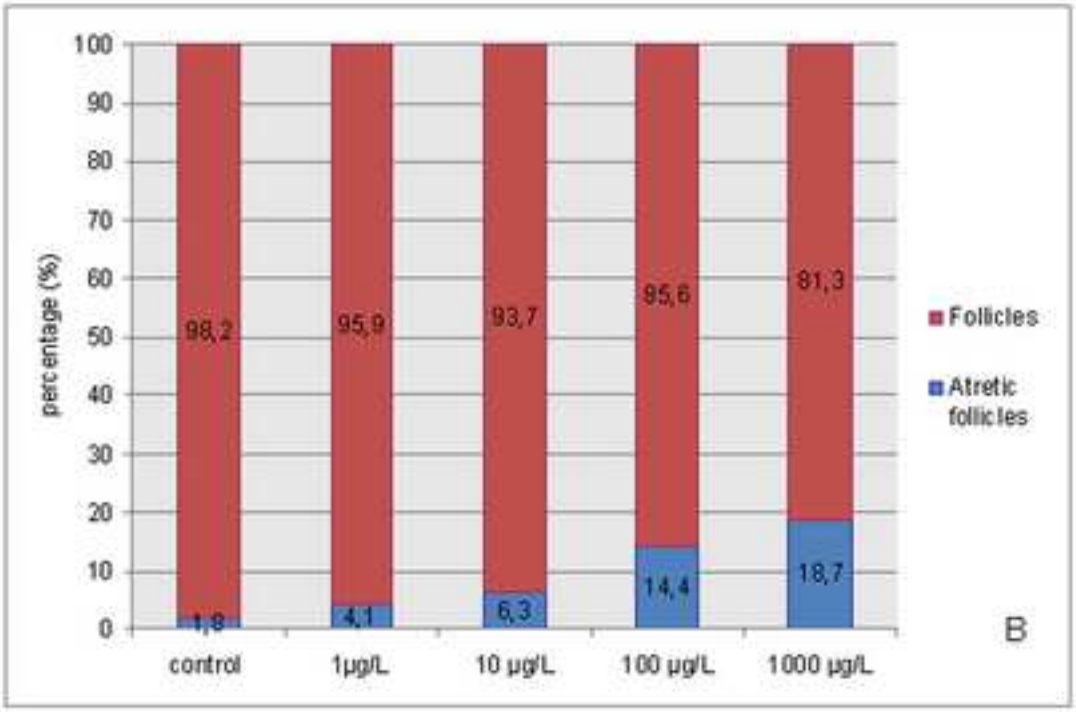

Fig. 5. A: Different type follicle distribution in each group. Primordial (Fovg), cortical alveolar (Fc), vitellogenic (Fv), mature (Fm); B: Atretic follicle percentage in the different groups of the study