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# Ovarian dysfunctions in adult female rat offspring born to mothers perinatally exposed to low doses of bisphenol A

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ABSTRACT

The study of oral exposure to the environmental estrogen bisphenol A (BPA) during the perinatal period and its effects on ovarian functionality in adulthood has generated special interest. Thus, our objective was to investigate ovarian folliculogenesis and steroidogenesis in adult female rat offspring born to mothers exposed to low doses of BPA (BPA50: 50 μg/kg day; BPA0.5: 0.5 μg/kg day) by the oral route during gestation and breastfeeding. Ovaries from both BPA-treated groups showed reduced primordial follicle recruitment and a greater number of corpora lutea, indicating an increased number of ovulated oocytes, coupled with higher levels of mRNA expression of 3β-hydroxysteroid dehydrogenase and serum progesterone. BPA50-treated animals had lower expression of androgen receptor (AR) at different stages of the growing follicle population. BPA0.5-treated rats evidenced an imbalance of AR expression between primordial/primary follicles, with higher mRNA-follicle-stimulating hormone receptor expression. These results add to the growing evidence that folliculogenesis and steroidogenesis are targets of BPA within the ovary.



## 1. Introduction

A link between ovarian dysfunctions and altered fertility has been clearly associated with an abnormal follicle development, the requirement of maintenance of a constant stream of growing follicles and/or impaired steroidogenesis [1,2]. Ovarian dysfunctions associated with altered fertility have also been linked with alterations in the expression of steroid receptors and the cyclin-dependent kinase inhibitor 1B or p27 [3–11]. All types of estrogen receptor beta (ERβ) knockout mice (BERKO) [3], estrogen receptor alpha (ERα) knockout mice (ERKO) [4,5], most androgen receptor (AR) knockout (ARKO) females [6–9], PR-null mice [10], and p27-deficient mouse ovaries [11] have altered folliculogenesis associated to defective fertility.

Bisphenol A (BPA) is recognized as one of several endocrine disrupting chemicals (EDCs) with estrogenic activity and thus cataloged as an environmental estrogen. EDCs represent a challenge, as their effects depend on both the level and timing of exposure, being especially critical when exposure occurs during development [12]. BPA is used in a variety of common consumer products, such as baby bottles, lunch boxes, toys, water pipes, and other materials [13,14]. Although many studies have reported effects of BPA on early stages of ovary development, the consequences of these effects on ovarian functionality in adulthood are still unclear [15]. In addition, greater attention has been paid to exposures during the perinatal period (i.e., the period comprised of gestation and early postnatal period) because altered organizational programming can confer increased susceptibility for diseases later in life [16].

Previously, we and others have demonstrated that BPA administered via the sc route modifies neonatal folliculogenesis, decreases reproductive capacity and increases the rate of abortions [17–20]. However, because food and beverages account for the majority of daily human exposure, the primary source of exposure to BPA for most people is through the oral route [21,22]. Some studies have shown abnormalities associated with the oral administration of BPA [23,24], including impaired fertility [25,26]. However, the ovarian dysfunctions related to this route of administration in pregnant females during the perinatal period and the consequences in the pups when they are adults have not been exhaustively addressed. Therefore, the objective of this study was to investigate the effects on ovarian function in adult female offspring born to mothers that were exposed to low doses of BPA by the oral route during gestation and breastfeeding. To explore

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potential mechanisms, we investigated different signaling pathways related to folliculogenesis and steroidogenesis (expression of ERα, ERβ, AR, p27, steroidogenic enzymes and gonadotropin receptors).

## 2. Materials and methods

### 2.1. Animals

The experimental protocols were designed in accordance with the Guide for the Care and Use of Laboratory Animals issued by the U.S. National Academy of Sciences and approved by the ethical committee of the School of Biochemistry and Biological Sciences, Universidad Nacional del Litoral, Santa Fe, Argentina. Animals were treated humanely and with regard for alleviation of suffering. Sexually mature female rats (90 days old) of a Wistar-derived strain bred at the Department of Human Physiology (School of Biochemistry and Biological Sciences, Universidad Nacional del Litoral, Santa Fe, Argentina) were used. Animals were maintained in a controlled environment (22 ± 2 °C; 14 h of light from 0600 h to 2000 h) and had free access to pellet laboratory chow (16-014007 Rat-Mouse diet, Nutrición Animal, Santa Fe, Argentina). The concentration of phytoestrogens in the diet was not evaluated; however, because the food intake for control and xenoestrogen-treated rats was equivalent (data not shown), we assumed that the animals in the experimental and control groups were exposed to the same levels of phytoestrogens. To minimize other exposures to EDCs, rats were housed in stainless steel cages with sterile pine wood shavings as bedding, and glass bottles with rubber stoppers were used to supply drinking water and oral treatments.

### 2.2. Experimental procedures

Females in proestrus were caged overnight with males of proven fertility. The day that sperm was found in the vagina was designated day 1 of gestation (GD1). On GD9, corresponding to the beginning of organogenesis in the fetus, pregnant rats (F0) were weighed and oral treatment with BPA or vehicle was begun.

#### 2.2.1. Dosing solution

Doses were calculated based on the dams' average body weight and water consumption during pregnancy and lactation. Stock solutions of BPA (25 mg/ml, 99% purity, Sigma–Aldrich, Buenos Aires, Argentina) were dissolved in ethanol (Merck Chemistry Argentina, Buenos Aires, Argentina). Immediately before administration, serial dilutions in water were performed to a final concentration of 2.5 μg BPA/l (BPA0.5) and 250 μg BPA/l (BPA50) corresponding to the theoretical doses of (a) 0.5 μg BPA/kg bw/day (BPA0.5) and (b) 50 μg BPA/kg bw/day (BPA50), respectively. The control group was exposed to a vehicle solution (0.001% ethanol in water). Cage bottles were rinsed and refilled twice a week with freshly prepared solutions [25].

#### 2.2.2. Experimental design

On GD9, the F0 dams were randomly assigned to the following experimental groups: BPA0.5, BPA50 and control (10–12 dams were included in each group). All treatments were administered in the drinking water of F0 dams from GD9 to weaning on postnatal day 21 (PND21). To determine the actual dose administered the individual body weights and water consumption of F0 dams were recorded twice a week throughout the treatment (See Table 1 in Supplementary data). The higher BPA dose used in this experiment (BPA50) was equivalent to the safe dose established by the US Environmental Protection Agency (50 μg/kg bw/day) [27], and the other dose was a hundred-fold lower (BPA0.5). The latest dose was chosen in accordance with the estimated daily intakes of BPA for human population published in NTP-CERHR Monograph on BPA. Thus, BPA0.5 is a representative dose of the estimated daily intake of BPA in infant breast-fed population [22].

After parturition, F1 pups were weighed and sexed according to the anogenital distance, and litters of eight pups (preferably four males and four females) were left with F0 lactating mothers until weaning on PND21; the remaining females and males were assigned to other experiments. At weaning, one F1 female per litter from each treatment group (exposed to the xenoestrogen transplacentally and through the milk) were transferred to a BPA-free environment until the end of the experiment; the remaining females and all males were assigned to other experiments.

**Table 1**
Estrous cycle.

| | Control | BPA0.5 | BPA50 |
|---|---|---|---|
| Estrous percentage | 21.01 ± 1.362 | 18.54 ± 2.362 | 18.91 ± 1.166 |
| Estrous cycle length (days) | 5.208 ± 0.150 | 5.963 ± 0.593 | 6.339 ± 0.521 |

Data were expressed as mean ± SEM. The number of animals evaluated per group was at least 10. Statistical significance: *: $p < 0.05$ vs. control.

### 2.3. Estrous cycles, tissue and blood sample collection

To determine whether the exposure to BPA involved abnormalities in estrous cycles, vaginal smears were taken daily from vehicle- and BPA-treated females from PND45 to PND90 [28]. Data were analyzed and expressed as estrous cycle duration (days) and estrous percentage (% of time in estrus relative to total duration of the estrous cycle).

The animals were autopsied on PND90 ($n$ = 10–12 dams/group), in the morning of estrus (evaluated by vaginal smears and positive lordosis behavior). Blood and ovaries from F1 dams were obtained. Blood serum samples were stored at −80 °C until hormone assays were performed, and one ovary was fixed in 10% (v/v) buffered formalin and embedded in paraffin. The contralateral ovary was snap-frozen in liquid nitrogen and kept at −80 °C for RT-PCR analysis.

### 2.4. Follicular dynamics

Follicular dynamics was evaluated following protocols previously described [17,20]. Briefly, ovaries were fixed in 10% buffered formalin for 6 h at room temperature and embedded in paraffin. The whole ovary was serially sectioned (5 μm thick) and one slide out of every ten sections was stained with picrosirius-hematoxylin for morphological observation (50-μm interval between the evaluated sections). The numbers of corpora lutea (CL) and follicles at each developmental follicular stage were determined. Only follicles where the nucleus could be seen were counted to prevent counting the same follicle multiple times. Follicles were classified as primordial, early primary, primary, preantral or antral, following the morphological criteria previously described [17,20]. Early primary, primary, preantral and antral follicles were considered to be components of the growing or recruited follicle population. The number of CL has been used as indicative of [29] and in coincidence with natural or superstimulated ovulation rate [7–9]. To avoid the repeated counting of CL that could overlap, we measured the diameter of CL in all sections and then we calculated its mean diameter. We found that CL in average had 1017.2 μm. Based on this, the number of CL per ovary was quantified in three sections separated 1000 um from each other. All healthy follicles (without atretic signs) were evaluated. Follicles classified as healthy showed a granulosa cell layer that appeared compact and well organized, with closely apposed cells, numerous mitotic

figures, and only occasional or rare pyknotic cells. In contrast, follicular atresia was characterized by widespread disintegration of the granulosa cell layer and rare or absent mitotic cells. In small antral and antral atretic follicles, the remaining granulosa cell layer consisted almost entirely of pyknotic nuclei and apoptotic bodies. Multioocyte follicles were also evaluated by counting follicles containing more than one oocyte enclosed within the granulosa cell layers. Both the number of follicles for each follicular stage and the number of multioocyte follicles were counted in all stained sections obtained from the whole ovary and normalized by the number of sections counted [30,14].

Oocyte survival was evaluated as previously described [31,17] by determining the total number of oocytes per ovary. Oocytes were counted in every tenth section of the entire ovary and multiplied by a factor of 10 to account for the fact that nine-fifths of the ovary was not analyzed. In addition, the size of the preantral and antral follicles was measured by determining the maximum diameter of these follicles in each ovary. The analysis was performed on digitized images recorded with a Spot Insight version 3.5 color video camera attached to an Olympus BH2 microscope. The diameter was measured using the basic tools on Image Pro-Plus 4.1.0.1 system (Media Cybernetics, Silver Spring, MD, USA) in 300 preantral and 100 antral follicles, in each ovary, averaged per rat, and then averaged for each treatment group .The results are expressed in μm.

### 2.5. Immunoperoxidase stains

In ovaries from the control and BPA-treated groups, immunohistochemistry was performed to evaluate protein expression of ERα, ERβ, AR, and p27 as we previously described [17]. At least three sections (5 μm thick) at different depths from each ovary were immunostained as previously described [32,17]. After deparaffinization, microwave pretreatment (antigen retrieval) was performed. Primary antibodies were incubated overnight at 4 °C at the dilutions shown in Table 2 (see Supplementary data). Biotinylated anti-mouse IgG (Sigma–Aldrich Argentina S.A., Buenos Aires, Argentina) or anti-rabbit IgG (Sigma) were used as secondary antibodies. Reactions were developed by the streptavidin-biotin peroxidase labeling method, which uses diaminobenzidine (Sigma) as a chromogen substrate. Except for the determination of the integrated optical density (IOD) of ERβ- and AR-immunostained slides, samples were counterstained with Mayer hematoxylin (Biopur, Rosario, Argentina) and mounted with permanent mounting medium. Each immunohistochemical run included positive tissues and negative controls replacing the primary antibody with nonimmune serum (Sigma). The primary antibodies used are the same that we reported and validated in previous publications, reproducing the immunostaining pattern reported by us and Drummond [17,58,59]. Briefly, we observed p27-positive expression in oocyte nuclei and granulosa cells of primordial and recruited follicles; AR-positive expression in granulosa cells of growing follicles, being highest in small preantral and early antral follicles; ERα positive expression mainly in the cal and interstitial cells and ERβ positive expression in granulosa cells.

**Table 2**
Average size of preantral and antral follicles.

| | Control (μm) | BPA 0.5 (μm) | BPA 50 (μm) |
|---|---|---|---|
| Preantral | 3282 ± 85.11 | 4598 ± 1054 | 3655 ± 407.0 |
| Small antral | 5533 ± 308.3 | 5453 ± 180.6 | 5914 ± 367.2 |
| Big antral | 18981 ± 1492 | 22531 ± 2678 | 20394 ± 1544 |

Data were expressed as mean ± SEM. The number of animals evaluated per group was at least 10. Statistical significance: *: $p < 0.05$ vs. control.

As previously described, all immunostained sections were exhaustively examined at low magnification, and proteins showing no changes between control and BPA-treated groups were qualitatively expressed [17]. Careful examination of the immunostained sections showed that ERα and p27 expressions were unchanged between control and BPA-treated groups. Therefore, ERα and p27 were qualitatively evaluated [17]. ERβ and AR expression levels were quantified measuring the IOD [33,17]. The images were recorded with a Spot Insight version 3.5 color video camera attached to an Olympus BH2 microscope (all fields for the entire section were recorded and four sections per animal were evaluated). The microscope was set up properly for Koehler illumination. Correction of unequal illumination (shading correction) and calibration of the measurement system were done with a reference slide. Images were analyzed using the Image Pro-Plus 4.1.0.1 system (Media Cybernetics). The images of the immunostained slides were converted to gray scale, and the different cell types were delimited (oocyte and granulosa cells). IOD was calculated as a linear combination between the average gray intensity and the relative area occupied by positive cells. Because IOD is a dimensionless parameter, the results were expressed as arbitrary units. Parameters were determined, averaged per rat, and then averaged for each treatment group.

### 2.6. Real-time PCR

An optimized reverse transcription-qRT-PCR protocol was used to analyze the relative expression levels of Cyp11, Cyp17, Cyp19, 3β-hydroxysteroid dehydrogenase (Hsd3b), follicle-stimulating hormone receptor (Fshr) and luteinizing hormone receptor (Lhcgr) mRNA in the ovaries obtained. Ovaries from each experimental group (control, BPA0.5 and BPA50) were individually homogenized in TRIzol (Life Technologies, NY, USA), and RNA was prepared according to the manufacturer's protocol. The concentration of total RNA was assessed by A260, and RNA was stored at –80 °C until needed. Equal quantities (1 μg) of total RNA were reverse-transcribed into cDNA according to Ramos et al. [34]. Primer pairs used to amplify Cyp11, Cyp17, Cyp19, Fshr, Lhcgr and L19 (housekeeping gene) cDNAs are shown in Table 3 (see Supplementary data). cDNA levels were detected using qRT-PCR with a Rotor-Gene Q cycler (Qiagen Instruments AG, Hombrechtikon, Switzerland) and HOT FIRE Pol EvaGreen Qpcr Mix PlusS (Solis BioDyne; Biocientífica, Rosario, Argentina). After initial denaturation at 95 °C for 15 min, the reaction mixture was subjected to successive cycles of denaturation at 95 °C for 15 s, annealing at 54 °C (for Cyp11), 56 °C (for Hsd3b), 59 °C (for Cyp17), 54 °C (for Cyp19), 52 °C (for Fshr), 53 °C (for Lhcgr) or 60 °C (for L19) for 15 s, and extension at 72 °C for 15 s. The product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. All PCR products were cloned using a TA cloning kit (Invitrogen) and specificity was confirmed by DNA sequencing (data not shown). Controls containing no template DNA were included in all assays, yielding no consistent amplification. A sample without reverse transcriptase was included to detect contamination by genomic DNA. For each analysis, a standard curve was prepared from eight serial dilutions of a standard sample containing equal amounts of cDNA from the different experimental groups, as previously reported [35]. All standards and samples of each independent experiment were assayed in triplicate. The efficiency of the PCR reactions was assessed for each target by amplification of serial dilutions (over five orders of magnitude) of cDNA fragments of the transcripts under analysis. Accordingly, fold expression over control values was calculated for each target by the relative standard curve methods, following our previously described protocols [36,37].

### 2.7. Hormone assays

Serum levels of estradiol (E2) and progesterone (P4) were measured in blood samples by radioimmunoassay (RIA) according to the manufacturer's instructions (DSL-4800, Beckman Coulter Ultra-Sensitive Estradiol RIA, IMMUNOTECH; IM1188, Beckman Coulter RIA Progesterone, IMMUNOTECH). All assays were performed in triplicate.

The E2 kit used rabbit anti-E2 serum and iodinated E2. The primary antiserum cross-reacts 2.4% with estrone, 0.64% with estriol, 0.21% with 17β-estradiol, 2.56% with 17β-estradiol-3-glucoronide, 0.17% with estradiol-3-sulfate, and 3.4% with D-equilenin. Goat anti-rabbit gamma globulin serum and polyethylene glycol were used as the precipitating second antibody reagent. The sensitivity of the assay was 2.2 pg/ml. The intra- and inter-assay coefficients of variation were 8.9% and 12.2%, respectively.

The P4 assay uses tubes coated with anti-P4 antibodies and iodinated P4. The antibodies cross-react 10% with 5α-dihydrotestosterone, 5% with 19-nortestosterone, 2% with 11β-hydroxytestosterone and 2% with methyltestosterone. The intra- and inter-assay coefficients of variation for P4 were 8.15% and 8.66%, respectively. The assay sensitivity was 0.03 ng/ml.

### 2.8. Statistics

Data are expressed as the mean and standard error of the mean (SEM). Values from hormone assays were not normally distributed and were log-transformed prior to statistical analysis [38,39]. All data are expressed as the means ± SEM. ANOVA was performed to obtain the overall significance, and Dunnett's post-hoc test was used to compare each experimental group with the control group. In all cases, values with $p < 0.05$ were accepted as significant.

## 3. Results

Direct exposure to BPA or vehicle in the drinking water produced no signs of embryotoxicity (i.e., all pregnant dams successfully delivered their pups, and the number of live-born pups per litter was similar in the three groups), abnormal maternal or nursing behavior, or changes in body weight gain in the F0 dams (data not shown). The length of gestation was unaltered, and no gross malformations were observed in F1 pups at delivery or weaning. In addition, the analysis of the estrous cycle of F1 females showed no changes between animals from control and BPA-treated groups, as evidenced by the length of the estrous cycle or the percentage of time that animals spent in estrus (Table 1).

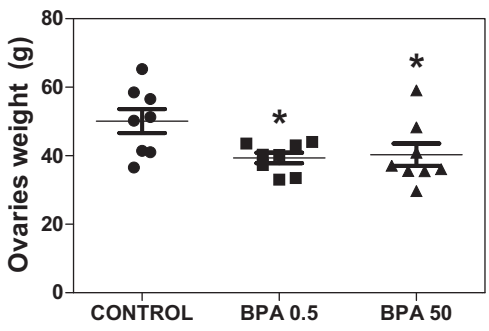


**Fig. 1.** The wet weight of ovaries in PND90 of females born to mothers that were perinatally exposed to vehicle (Control), safe dose (BPA 50) or 100-fold lower dose of BPA (BPA 0.5). Data were expressed as mean ± SEM. The number of animals evaluated per group was 8. Statistical significance: *: $p < 0.05$ vs. control.

### 3.1. Ovaries from animals exposed to BPA had lower weights and showed a decrease in initial follicle recruitment

The body weights of animals were similar between groups. However, the wet weight of ovaries was much smaller in animals exposed to BPA than in those exposed to vehicle (Fig. 1).

Fig. 2 shows the numbers of primordial and recruited follicles (primary, preantral and antral) in the control and treated groups. As expected, most of the follicles in the ovaries from control females were primordial follicles, and BPA-exposed females showed similar values. A significant decrease in the number of growing follicles was observed in both BPA-treated groups (Control: 7.634 ± 0.983; BPA0.5: 5.013 ± 0.550*; BPA50: 5.101 ± 0.485*). This decrease in the number of growing follicles was at the expense of a significant decrease in the number of primary follicles. Taken together, these results show that both doses of BPA inhibit the initial recruitment process by reducing the transition of primordial to primary follicles.

As observed in Fig. 2, the decrease in the transition of primordial to primary follicle had no effects on ulterior folliculogenesis at preantral and antral stages or incidence of atretic follicles. To investigate whether a possible alteration could occur in the size of individual follicles rather than in the amount of follicles comprising the population, then we measured the size of preantral and antral follicles. Table 2 summarizes these results, showing that preantral and antral follicles of females exposed to BPA reached sizes similar to those reached by controls.

We also evaluated oocyte survival by measuring the total number of oocytes in the whole ovary and observed no changes in the total population of oocytes between control and BPA-treated groups (Fig. 3).

### 3.2. Animals exposed to BPA showed both increased number of CL and P4 levels

We determined the number of CL in ovaries from control and BPA-exposed animals. Exposure to BPA elicited an increase in the number of ovulated oocytes, as evidenced by the higher number of CL in the BPA-treated groups when compared to the control group (Fig. 3).

To establish whether the higher number of CL in treated animals was correlated with abnormal levels of P4, we measured the serum concentration of this hormone. Animals treated with either dose of BPA had higher circulating levels of P4 than untreated animals (Fig. 3). In contrast, E2 levels showed no changes between vehicle and BPA-treated animals (data not shown).

### 3.3. Steroidogenic enzyme Hsd3b in ovaries of PND90 females was increased by perinatal exposure to BPA

Using quantitative real-time PCR, we analyzed the expression levels of ovarian steroidogenic enzymes in response to perinatal treatment with BPA. Exposure to BPA caused no alterations in the mRNA expression levels of Cyp11, Cyp17 or Cyp19. Instead, we observed increased levels of mRNA expression of Hsd3b with both doses of exposure to BPA (Fig. 4).

### 3.4. Recruited follicles showed low levels of AR without changes in ERα, ERβ or p27 protein expression

To gain insight into the pathway by which BPA exposure might alter follicular development and ovulation, we evaluated protein expression of p27, AR, ERα and ERβ for each follicle stage in adult (PND90) ovaries.

p27 was expressed in the oocyte nuclei and granulosa cells of both primordial and recruited follicles on PND90 (Fig. 5A). ERα was

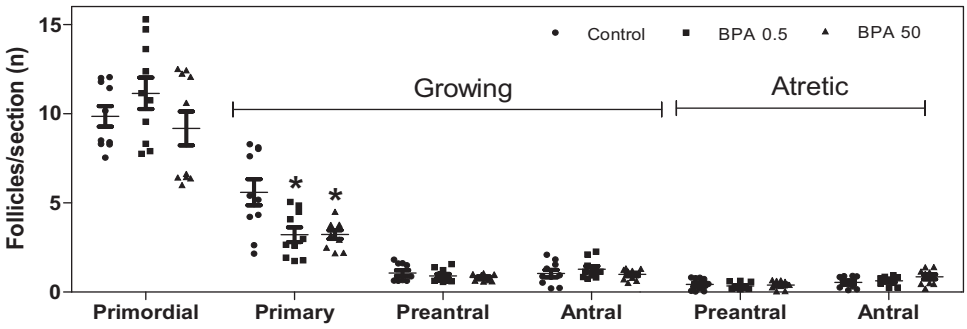


**Fig. 2.** Follicular recruitment in the ovaries of females born to mothers that were perinatally exposed to Control or BPA. Ovaries from PND90 female rats were embedded in paraffin. The whole ovary was serially sectioned (5 μm thick) and one slide out of every ten sections was stained with picrosirius-hematoxylin for morphological observation (50 μm interval between sections). Numbers of primordial follicles, primary, preantral and antral follicles were calculated as described in M&M. Data were expressed as mean ± SEM. The number of animals evaluated per group was at least 10. Statistical significance: *: $p < 0.05$ vs. control.

expressed in granulosa and theca cells of growing follicles, as well as in stromal/interstitial cells (Fig. 5C). Neither p27 nor ERα expression was altered in the follicles of BPA-treated rats (data not shown).

Both ERβ and AR were predominantly located in the granulosa cells of growing follicles and their expression increased gradually, reaching the highest levels in granulosa cells of antral follicles (Fig. 5B and D). Theca and stroma cells also stained positive for AR. Expression of ERβ in follicle populations of BPA-treated animals was unchanged when compared with ovaries from vehicle-treated rats (Fig. 6). In contrast, AR expression was significantly reduced in primary, preantral and antral follicles of ovaries from BPA50-treated rats and in primary follicles from BPA0.05-treated rats. In contrast, in primordial follicles of ovaries from BPA0.05-treated rats, AR expression was increased (Figs. 7 and 8).

### *3.5. mRNA expressions of both gonadotropin receptors Fshr and Lhcgr were not altered by exposure to BPA*

To investigate whether BPA could alter follicle sensitivity to gonadotropin, we measured the mRNA expression of Lhcgr and Fshr. Fig. 9 displays the ovarian expression of Lhcgr and Fshr mRNA determined by quantitative RT-PCR. Fshr showed an increase in the ovaries from BPA0.5-treated rats whereas Lhcgr mRNA expression showed no changes in response to perinatal exposure to BPA (Fig. 9).

## 4. Discussion

The present results showed ovarian dysfunctions in adult female offspring born to mothers exposed to low doses of BPA during the perinatal period (i.e., from day 9 of gestation until the end of lactation). These dysfunctions comprised a greater number of CL, indicating an increased number of ovulated oocytes, coupled with higher levels of serum P4. In addition, our results suggest that the two doses of BPA tested might affect ovulation through a different mechanism: a lower expression of AR in ovaries of animals exposed to BPA50 and increased sensitivity to FSH in animals exposed to BPA0.5.

Several effects of BPA on ovarian development have already been reported. These effects comprise induction of polyovular follicles [40,20,32], cysts [41], aneuploidy [42], persistent estrus, increased incidence of blood-filled ovarian bursae and tissue occupied by antral follicles [43], alteration of the steroidogenic capacity [15], in the hypothalamic–pituitary–gonadal axis [44], and alteration of folliculogenesis [17,14]. Different expert panels have highlighted the need to conduct studies designed to evaluate the use of oral and dietary exposure routes. In this sense, Wang et al. [26] observed that in utero low-dose BPA oral exposure disrupts germ cell nest breakdown and reduces the size of the primordial follicle pool in neonates (PND4). However, researchers have also emphasized the need of using doses of BPA similar to human exposure levels and establish effects of early exposure in adulthood, mainly based on the fact that humans can be

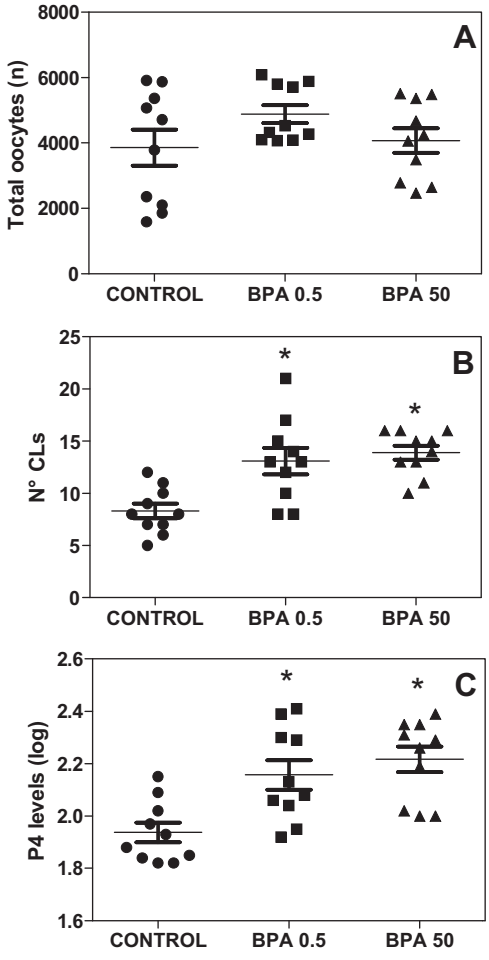


**Fig. 3.** Ovarian function and BPA treatments, (A) total number of oocytes in the ovaries of control and BPA-exposed rats. Total oocytes were calculated as described in M&M. (B) Total number of CLs in the ovaries of control and BPA-exposed rats. The number of CL per ovary was quantified in three sections separated 1000 μm from each other. (C) Levels of serum progesterone of control and BPA-exposed rats. Progesterone levels were calculated as described in M&M. Data was expressed as mean ± SEM. The number of animals evaluated per group was 10. *: $p < 0.05$ vs. control.

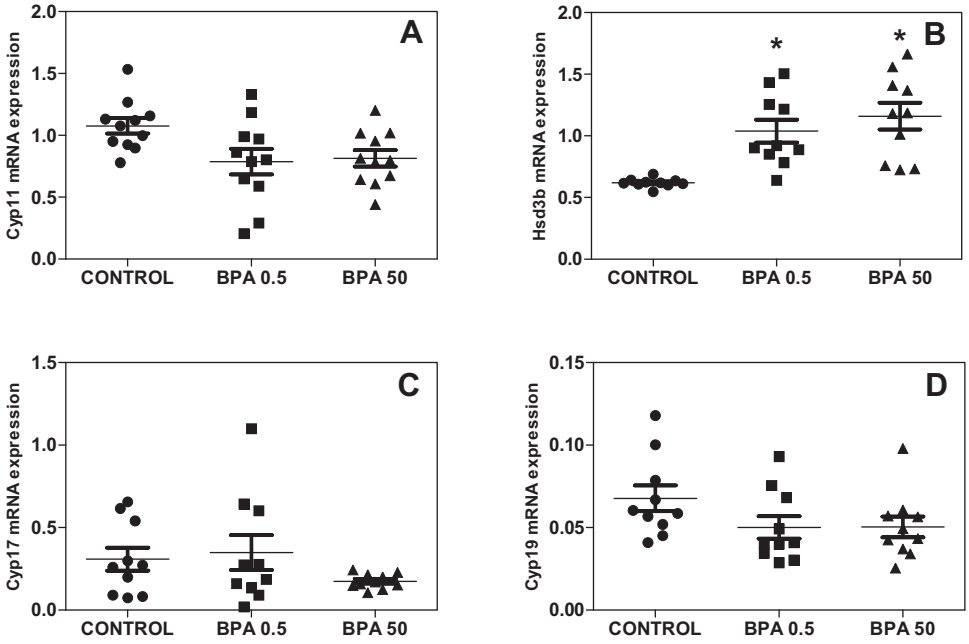


**Fig. 4.** Quantitative real-time PCR analysis of the mRNA levels of steroidogenic enzymes of ovaries from rats perinatally exposed to BPA. Relative mRNA levels of Cyp11 (A), Hsd3b (B), Cyp17 (C) and Cyp19 (D) were measured by quantitative real time PCR and normalized to L19. Data are expressed as mean ± SEM. *: $p < 0.05$ vs. control.

considered the primary targets of BPA exposure by contaminated foods and drinks, and dental materials [15]. Herein, three-month-old female rat offspring born to mothers that were orally exposed to 50 and 0.5 μg/kg BPA during the perinatal period showed an increase in the number of CL. Our data are not in agreement with results recently published, reporting that adverse effects induced by oral administration of BPA are observed only at the two highest doses (100,000 and 300,000 μg/kg bw/day) of BPA [45]. However, several differences in the administration scheme of BPA (mothers administered from gestation day 6 until the start of labor, and then

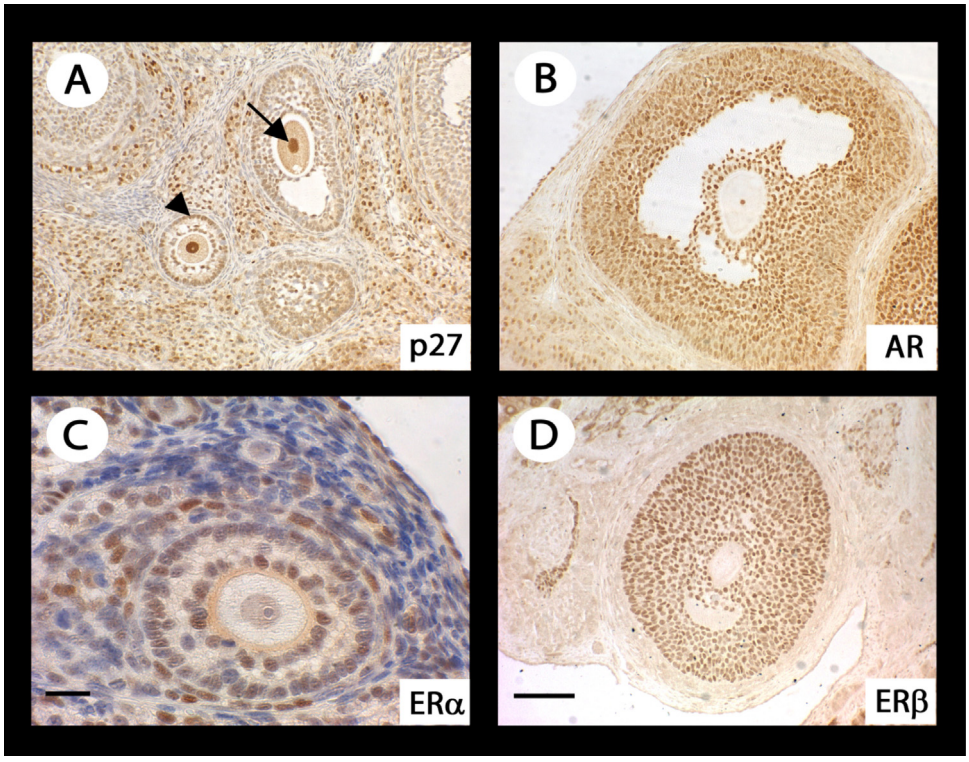


**Fig. 5.** Ovarian expression of cyclin-dependent kinase (Cdk) inhibitor 1B (p27), androgen receptor (AR), and estrogen receptors alpha (ERα) and beta (ERβ) in control rats. Representative photomicrographs of ovarian sections immunostained with the specific antibodies detailed in Table 1: (A) p27-positive expression in oocyte nuclei (arrows) and granulosa cells (arrowheads) of recruited follicles; (B) AR-positive expression in granulosa and theca cells of growing follicles and stromal cells; (C) ERα positive expression in theca, granulosa and interstitial cells; (D) ERβ positive expression in granulosa cells. Immunohistochemistry was developed by using DAB as chromogen substrate and counterstaining with Mayer hematoxylin. Scale bar = 25 μm (a, b y d); 10 μm (c).

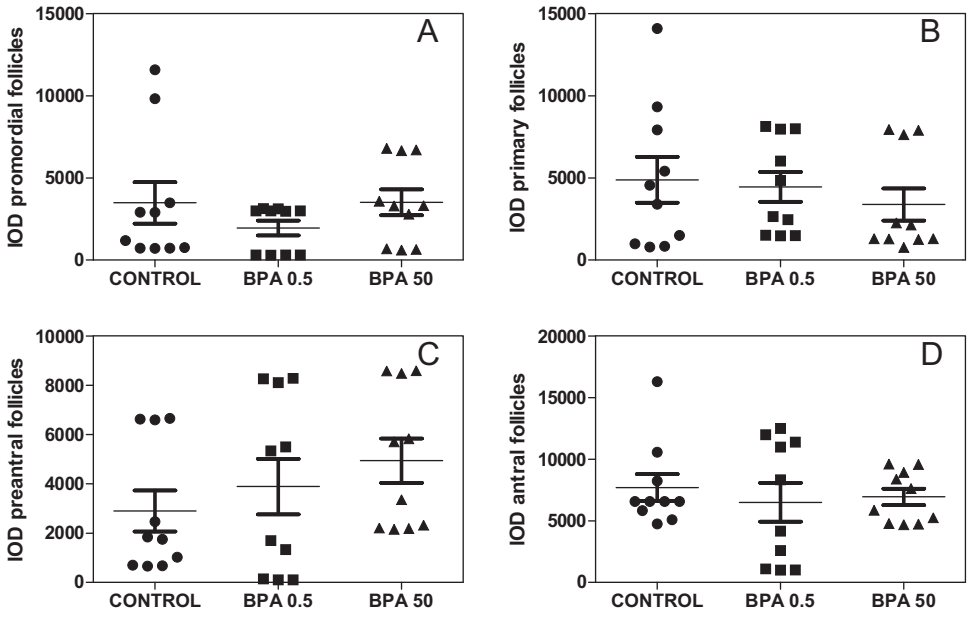


**Fig. 6.** Effect of BPA exposure on ERβ expression in granulosa cells of primordial and recruited follicles. Immunohistochemistry quantification was performed in digitized images of all fields for the entire tissue section. Images were converted to gray scale and IODs of the granulosa cells of primordial, primary, preantral and antral follicles were calculated and expressed as arbitrary units as described in M&M. Data are expressed as mean ± SEM. The number of animals evaluated per group was 10. $p < 0.05$ vs. control.

their pups directly dosed from day 1 after birth to termination on PND90) can account for the differences observed. Taken together, our data add strong evidence that BPA acts on early stages of ovarian development with functional consequences in adulthood.

We also found increased P4 levels in three-month-old female offspring born to mothers that were perinatally exposed to both doses of BPA. However, the BPA effects on gonadal steroidogenesis may largely depend on the exposure time and doses tested [46]. Several studies using in utero exposed rats [47], neonatally exposed rats [19] or lambs [20] did not prove BPA effects on steroidogenesis. In contrast, other studies have found that BPA decreases P4 levels in adult mice during early pregnancy [48] or inhibits the expected decrease in P4 levels on gestational day 21 compared with gestational day 18 [25]. In an in vitro study with porcine granulosa cells, depending on the dose given, BPA caused both an increase and a decrease in basal and FSH-induced P4 production [49]. On the other hand, it has been reported that exposure to BPA results in greater aromatase expression level and synthesis of estrogen in female pups [23]. In our present study, the changes in P4 serum levels were accompanied by changes in mRNA levels of steroidogenic enzyme Hsd3b. This result, together with the high number of CL that we simultaneously observed in ovaries of PND90 ovaries, could explain the increased P4 serum levels observed in BPA-treated rats.

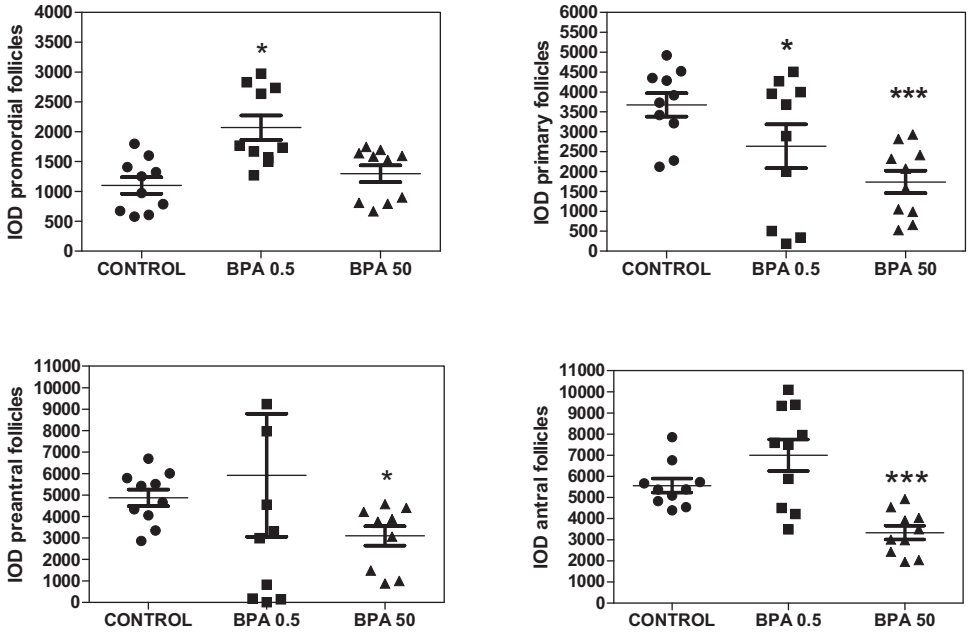


**Fig. 7.** Effect of BPA exposure on AR expression in granulosa cells of primordial and recruited follicles. Immunohistochemistry quantification was performed in digitized images of all fields for the entire tissue section. IODs were calculated and expressed as arbitrary units as described in M&M. Data are expressed as mean ± SEM. The number of animals evaluated per group was 10. *$p < 0.05$ vs. control; ***: $p < 0.001$ vs. control.

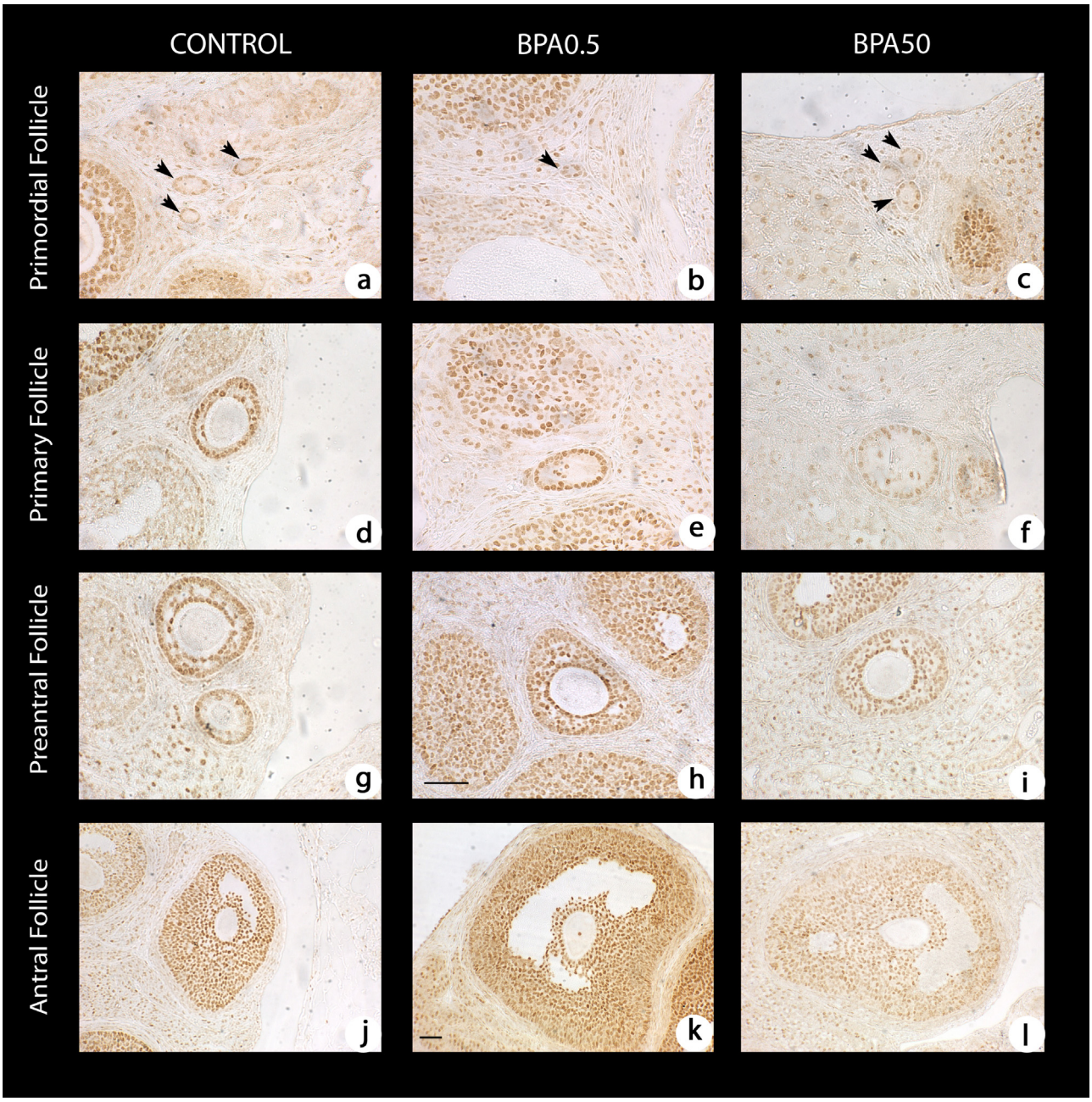


**Fig. 8.** AR expression at each follicle stage in ovaries from vehicle and BPA-exposed animals. Representative photomicrographs of AR expression of primordial (a–c), primary (d–f), preantral (g–i) and antral (j–l) follicles in control, BPA 0.5 and BPA 50 groups. Scale bar = A–I: 40×, J–L: 20×.

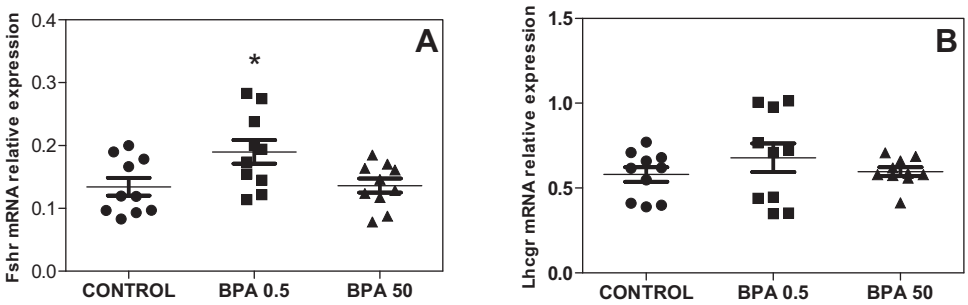


**Fig. 9.** mRNA expression of Fshr and Lhcgr of ovaries from rats perinatally exposed to BPA. Relative mRNA levels of Fshr (A) and Lhcgr (B) were measured by quantitative real time PCR and normalized to L19 expression. Data are expressed as mean ± SEM. *: $p < 0.05$ vs. control.

In ovaries from perinatally BPA50-treated female rats, we observed an increase in the total number of CL, indicating an increased number of ovulated oocytes. To identify potential underlying mechanisms, we investigated the expression of sex steroid receptors and p27. The ovarian expression of ERα, ERβ, and p27 was unchanged. However, AR expression was significantly reduced in preantral and antral follicles of ovaries from BPA50-treated rats. It is now increasingly realized that a critical balance in androgen actions through AR is necessary for the maintenance of the ovulation rate in physiological levels [50,51]. Ware showed that large dosages of antiandrogens decrease the ovulation number while low dosages significantly enhance the ovulatory response [52]. Given our results, the decrease in AR mainly in preantral and antral follicles might result in a slight reduction in androgen action, with a consequent increase in the ovulation rate of BPA50-treated females. We also found an increase in the number of CL of BPA0.5-treated females but no changes in the expression of AR in these animals. However, we found increased mRNA-FSHR expression in ovaries from BPA0.5-treated females. This high mRNA-Fshr expression might result in follicles exhibiting a higher FSH sensitivity, leading to an advance in follicular maturation and polyovulation [53]. Similarly, at the hypothalamic–pituitary level, BPA exposure results in the up-regulation of the mRNA expression levels of FSH in both male and female pups [23]. Taken together, our results suggest that BPA could affect ovulation through different mechanisms according to the dose assayed.

We have previously found that BPA, administered via sc in the postnatal period, acts as an enhancer of folliculogenesis, increasing the percentage of growing follicles in rats and lambs [17,20]. In contrast, BPA intraperitoneally injected for one week in the prepubertal period decreases the numbers of all follicle types and CL, and the number of atretic follicles significantly increases on PND35 [14]. Herein, we found a reduced number of growing follicles when BPA was administered during the perinatal period by the oral route. This differential action of BPA adds to the evidence that EDCs action differs according to the organism studied, route of administration, dosage, and timing of development in which it is studied [12]. On the other hand, an apparent discrepancy emerges, because we found increased number of CL together with a reduced number of growing follicles. However, it has been pointed out that the number of ovulations does not depend on the size of dynamic reserve of small antral follicles but on the mechanisms of follicular selection [54]. Our results support this concept since the decrease in the number of growing follicles we found in this experiment is caused by a decrease in the transition from primordial to primary follicles, which is not transferred to the subsequent stages of follicular development. Moreover, given the stimulatory role of androgen through AR in primordial follicle recruitment [51], the reduction of primordial to primary follicle transition associated with AR reduction in primary follicles of BPA50-treated females could be a brake applied to the departure of primordial follicles. This is in agreement with the quorum sensing model, which postulates the occurrence of compensatory mechanisms leading to the preservation of a healthy range of follicle number in the primordial reserve [55]. In contrast, in the BPA0.5-treated rats, an increase in AR in primordial follicles and a decrease in primary follicles were simultaneously observed. In this case, an imbalance in AR expression between primordial/primary stages could alter this transition. Taking into account the concepts discussed above, the AR reduction in antral follicles could enhance the number of ovulated oocytes, whereas an unbalanced AR expression between primordial/primary stages could be an attempt to slow down initial recruitment from primordial follicles. In concordance, there is increasing emphasis that androgens might differentially regulate each stage of follicular development [51].

In accordance with Nah et al., we found a decrease in the weight of ovaries in BPA-treated animals. However, unlike these authors, we observed no changes in the estrous cyclicity. This discrepancy may be due to several differences in the experimental design [56]. As it has been previously emphasized, ovarian cyclicity and fertility are not invariably compromised by steroid disruptive actions, pointing to an impressive resiliency of the reproductive axis to insult by exogenous estrogenic compounds [57]. In summary, female rats born to mothers perinatally treated with BPA by the oral route exhibited an increased number of CL with defective folliculogenesis, increased P4 levels and altered expression of AR and Fshr. These results add to the growing body of evidence that folliculogenesis and steroidogenesis are targets of EDCs within the ovary. They also highlight the importance of studying EDCs because our society continues to release large amounts of industrial chemicals into our environment. In addition, studying how EDCs affect the ovary may influence the political and regulatory handling of EDCs.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jsbmb.2015.11.016.

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