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# Impaired ovarian response to exogenous gonadotropins in female rat offspring born to mothers perinatally exposed to Bisphenol A

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ABSTRACT

The ovary is sensitive to disruption by the environmental estrogen Bisphenol A (BPA). Our aim was to investigate whether perinatal exposure to BPA (50 μg/kg day), orally administered, affects ovarian response to exogenous gonadotrophins (PMSG or PMSG + hCG) in prepubertal female offspring. An altered response to gonadotrophins was observed in BPA-exposed rats. Increased proportion of antral follicles, altered levels of ovarian steroidogenic enzymes, gonadotropin receptors, AR and ERβ were observed in PMSG group. Besides that, in response to PMSG + hCG, a persistent high Fshr mRNA expression and a decreased number of follicles with high expression of PR before ovulation were observed. After ovulation, there was an increase in antral atretic follicles, reduced Lhcgr mRNA expression and high serum levels of E2. Therefore, an early exposure to a low dose of BPA during perinatal period induces ovarian changes leading to an altered response to exogenous gonadotropin treatment later in life.



## 1. Introduction

Numerous chemicals in the environment possess estrogenic activity and are classified as endocrine-disrupting compounds (EDCs) [1]. Some of these chemicals may alter gonadal morphogenesis and functional differentiation, affecting reproduction if exposure occurs during critical periods of development [2]. One of these chemicals is Bisphenol A (BPA), an EDC with estrogenic and antiandrogenic activity [3], and one of the highest-volume chemicals produced worldwide, since it is used in polycarbonate plastics, resins, papers, implanted medical devices and other medical equipment [4]. BPA has also been detected in a variety of environmental samples, including water, sewage leach, indoor and outdoor air samples, and dust [5]. Since BPA has been shown to leach from containers into food and beverage products and proved to be one of the multiple contaminants included in the soil, this compound should be considered a potential health risk for animals and humans [4,6].

It has been described that women with high concentrations of BPA in serum and urine show a poor ovarian response to in vitro fertilization treatment, probably by an impairment on ovarian function [7–10]. The ovary is one of the reproductive organs sensitive to disruption by BPA. As with many other EDCs, BPA effects on the ovary depend on both the dose and timing of exposure, route of entry to the body and species assessed [11–18]. Particularly, the study of oral exposure to BPA during the perinatal period (i.e. the period comprised between gestation and the early postnatal period) has generated special interest, because altered organizational programming can result in increased susceptibility for diseases later in life [19,20]. In this sense, we have recently shown that 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) show ovarian dysfunctions, including a greater number of corpora lutea (CL), coupled with higher levels of serum progesterone (P4) and concomitant altered expression of androgen receptor (AR) and follicle-stimulating hormone receptor (Fshr) [21]. The effects of oral perinatal exposure to BPA on ovarian response to exogenous gonadotropins have not been exhaustively addressed. Based on these reasons, we selected the ovarian response to an exogenous gonadotropin treatment as a tool to further study ovarian functionality in immature rats born to mothers perinatally exposed to BPA. The aim of the present study was to investigate whether the perinatal exposure to BPA affects

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folliculogenesis and steroidogenesis in response to an exogenous treatment of gonadotropins in prepubertal rats.

## 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 ($22 \pm 2\,^{\circ}C$; 14 h of light from 06:00 to 20:00 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, we assumed that all the animals were exposed to the same levels of phytoestrogens [see Kass et al. [22] for more information regarding food composition]. 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 [23], 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 250 μg BPA/l corresponding to the theoretical doses of 50 μg BPA/kg bw/day (BPA50). 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.

#### 2.2.2. Experimental design

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 body weights and water consumption of F0 dams were recorded twice a week throughout the treatment (see Table 1 in supplementary data). The 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). This "safe" dose of BPA was obtained by dividing the lowest observed adverse effect level (LOAEL) dose of 50 mg/(kg day) by a safety factor of 1000 [3] and was the only dose used for present study.

On GD9, the F0 dams were randomly assigned to the experimental groups: BPA50 or control (5–6 dams per group). To ensure random distribution to allocate animals in the treatment groups, we proceeded as follows: pregnant rats (F0) were treated with either vehicle (N = 6) or BPA (N = 5) in the drinking water from gestational day 9 (GD9) to weaning on postnatal day 21 (PND21). These pregnant rats were born from different mothers. After parturition, F1 pups were weighed and sexed according to the anogenital distance, and litters of eight pups (preferably four females and four males) were left with F0 lactating mothers until weaning on PND21; the remaining females and males were assigned to other experiments. At weaning, the female F1 offspring (exposed to the BPA transplacentally and through the milk) were transferred to a BPA-free environment.

On PND30, F1 females were subjected to the treatment with exogenous gonadotropins. Each one of the F1 females from each F0 mother was assigned to study a different time of the gonadotrophins treatment, according to the description in Fig. 1.

The researchers were blind to the treatments. One of the researchers carried out the distribution of the animals in order to avoid the inclusion of sisters in the same group. This researcher didn't take part in the measurements of the variables of the study. Confounding variables were taken in account. Baseline comparisons were done to confirm that animals were not different among groups, regarding age, body mass weight, etc.

#### 2.2.3. Tissue and blood sample collection

On PND30, blood was obtained from F1 females to determine the basal FSH and LH levels prior to treatment with exogenous gonadotropins. Then, F1 females from each F0 dam of both experimental groups (control n = 6; BPA50 n = 5)were treated with Pregnant Mare Serum Gonadotropin (PMSG. Ecegon® Biogénesis Bagó S.A. Buenos Aires, Argentina). Beginning on PND30 (in our colony, PND45 is the mean of beginning of puberty), PMSG was administered sc every 24 h for 3 consecutive days (PND30, 31 and 32). Each rat received a total dose of 10 UI/ml of PMSG. To evaluate the ovarian status after PMSG treatment, one of the F1 females from each F0 dam (control n = 6; BPA50 n = 5) were euthanized on PND33, 24 h after the last PMSG injection. Ovaries were dissected and one was used for histology and the other stored at $-80\,^{\circ}C$ for RNA extraction. Trunk blood was collected and serum was stored at $-20\,^{\circ}C$. The remaining three F1 females, previously treated with PMSG, received an ip injection of 25 UI/ml of hCG (Ovusyn, Syntex, Buenos Aires, Argentina), 24 h after the last PMSG injection. Then, to evaluate the ovarian response to hCG, ovaries were collected at 7, 14 and 22 h post-injection of hCG and processed for different experimental purposes (see below). One ovary was fixed for histology and the other stored at $-80\,^{\circ}C$ for RNA extraction. Serum was obtained at 22 h post-hCG injection, separated and stored at $-20\,^{\circ}C$ until hormone assay (Fig. 1).

The vehicle or BPA treatments, exogenous gonadotropins treatments and surgeries were performed by some of the authors, who randomly assigned a number to each sample. Thus, the others authors who analyzed the samples don't knew which sample was about.

### 2.3. Superovulation and oocyte count

The oocytes released in response to the superstimulatory protocol were counted in the ovaries obtained at 14 and 22 h post-hCG injection. The oocyte cumulus masses were surgically harvested from the oviducts and placed in Dulbecco's modified Eagle's medium: F12 (Gibco®, Life Technologies, Invitrogen). After treating with 0.3% hyaluronidase (Sigma) for 30 min at $37\,^{\circ}C$, the naked oocytes were categorized and counted.

### 2.4. Follicular dynamics

Follicular dynamics was evaluated following protocols previously described [11,21]. Briefly, ovaries were fixed in 10% buffered formalin and embedded in paraffin. The whole ovary was seri-

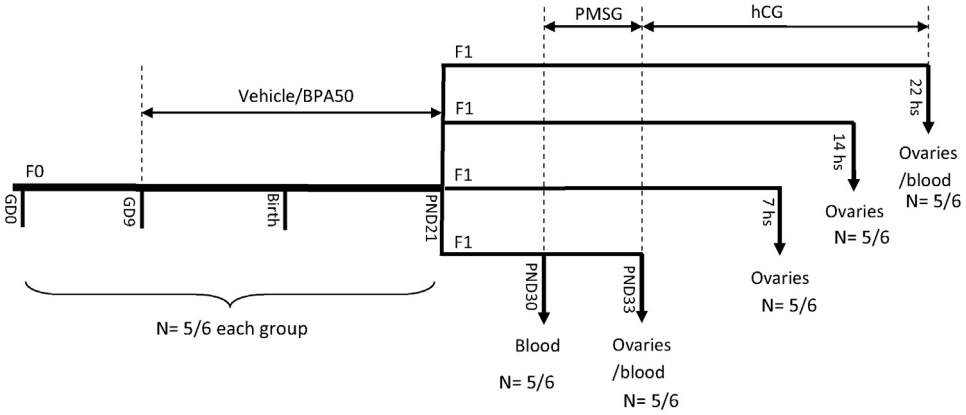


**Fig. 1.** Experimental design.
F0 dams were treated with either vehicle (N = 6) or the "safe dose" of bisphenol A (BPA): 50 μg/kg bw/day (BPA50) (N = 5) in the drinking water from gestational day 9 (GD9) to weaning on postnatal day 21 (PND21). After birth, F1 pups were weighed and sexed according to the anogenital distance, and litters of eight pups (preferably four females and four males) were left with F0 lactating mothers until weaning on PND21. At weaning, the female F1 offspring were transferred to a BPA-free environment. On PND30, F1 females from each F0 dam (N = 5/6) were subjected to the treatment with exogenous gonadotropins, and samples were obtained at different times (perpendicular arrows indicate both the tissue and the time of collection).

ally sectioned (5 μm thick) and one out of every ten sections was stained with picrosirius-hematoxylin for morphological observation (50-μm interval between the evaluated sections). The numbers of CL and follicles at each developmental follicular stage were determined. Only follicles where the nucleus of the oocyte was seen were counted to prevent counting the same follicle multiple times. Follicles were classified as primordial, primary, preantral or antral, following the morphological criteria previously described [11,12,21]. Primary, preantral and antral follicles were considered to be components of the growing or recruited follicle population. The follicles for each follicular stage were categorized as healthy or atretic, as previously published [14,21]. In brief, follicles classified as healthy showed a granulosa cell layer that appeared compact and well organized, with closely apposed cells, several 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 and the remaining granulosa cell layer consisted almost entirely of pyknotic nuclei and apoptotic bodies. All the follicles were counted in the largest three stained sections obtained from the whole ovary and normalized by the number of sections counted [21]. As previously published [21], 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 984.6 um. Based on this, the number of CL per ovary was quantified in three sections separated 1000 um from each other. The results are expressed as number of CL/section.

### 2.5. Immunoperoxidase stains

Immunohistochemistry was performed in ovaries from Control and BPA50 to evaluate protein expression of estrogen receptor beta (ERβ), AR and progesterone receptor (PR). At least three sections (5 μm thick) at different depths from each ovary were immunostained as previously described [11,21]. 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-rabbit IgG (Sigma) was used as secondary antibody. 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's hematoxylin (Biopur, Rosario, Argentina). Each immunohistochemical run included positive tissues and negative controls replacing the primary antibody with nonimmune serum (Sigma). The primary antibodies used are the same as those reported and validated in previous publications [11,21,24,25].

ERβ and AR expression levels were quantified by measuring the IOD [11,21,26]. 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 two 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, Silver Spring, MD, USA). The images of immunostained slides were converted to gray scale, and the different cell types were delimited (oocyte and granulosa cells). The IOD was calculated as a linear combination between the average gray intensity and the relative area occupied by positive cells. Because the 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.

PR expression was evaluated only in the ovaries obtained 7 h after the hCG injection since it is the moment when PR expression peaks [27–29]. Thus, three types of follicles were identified: follicles with low, medium or high PR expression. The number of each follicular type was counted in at least three sections (5 μm thick) at different depths from each ovary and normalized by the number of sections counted.

### 2.6. Real-time PCR

An optimized reverse-transcription qRT-PCR protocol [21] was used to analyze the relative expression levels of cytochrome P450scc (Cyp11), 17a-hydroxylase (Cyp17), P450aromatase (Cyp19), Fshr and luteinizing hormone receptor (Lhcgr) mRNA in the ovaries of the experimental groups. The 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

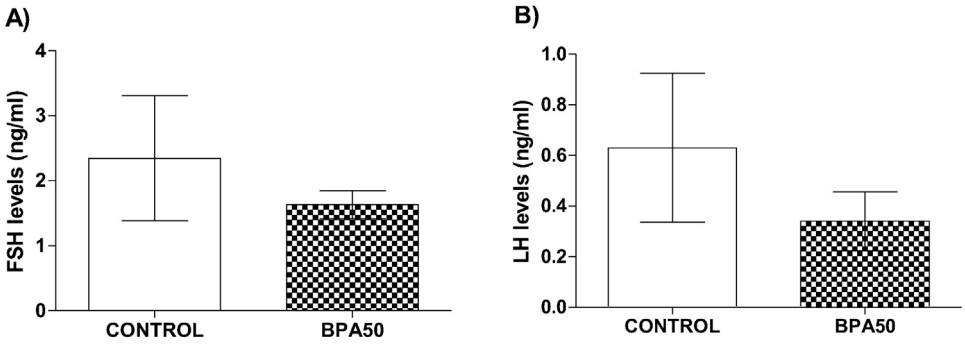


**Fig. 2.** Levels of serum gonadotropins in control and BPA-exposed rats.
Serum LH and FSH levels in females born to mothers perinatally exposed to vehicle (Control) or BPA (BPA50). Trunk blood was collected on PND30, before the first dose of PMSG. Serum levels of FSH (A) and LH (B) were calculated as described in M&M. The number of animals evaluated was 6 (Control) and 5 (BPA50). Data are expressed as mean ± SEM.

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), 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. 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 [21,30]. 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, as previously described [21,30].

### 2.7. Hormone assays

Serum levels of estradiol (E2), P4 and testosterone (T) 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; IM119, Beckman Coulter RIA Testosterone direct, IMMUNOTECH). All assays were performed in triplicate. The sensitivity of the E2 assay was 2.2 pg/ml, and the intra- and inter-assay coefficients of variation were 8.9% and 12.2%, respectively. The sensitivity of the P4 assay was 0.03 ng/ml, and the intra- and inter-assay coefficients of variation were 8.15% and 8.66%, respectively. The sensitivity of the T assay was 0.02 ng/ml, and the intra- and inter-assay coefficients of variation were 5.6% and 15.9% respectively. LH (3.5%) and FSH (7.2%) levels in blood samples were measured by RIA at the Instituto de Biología y Medicina Experimental, IBYME-CONICET, Buenos Aires. Argentina. The outcome assessors that performed the RIA assays were blind to the treatment groups. The samples they received were identified with a number, without any reference to the animal or treatment group.

### 2.8. Statistics

Data are expressed as the mean and standard error of the mean (SEM). Studentís unpaired two-tailed *t*-test was performed to compare experimental groups. The outliers were analyzed with Graph Pad software (http://www.graphpad.cpm/quickcalcs/Grubbs1.cfm); this program uses Grubbs' test. In all cases, values with $p < 0.05$ were accepted as significant. Assumptions of normality and heterogeneity of variance were met. T-test was made with GraphPad Prism software. This software tests automatically normality and variance. No animals were lost during the experiment.

**Table 1**
Serum levels of the sex steroids estradiol (E2), progesterone (P4) and testosterone (T), in control and BPA-exposed rats after PMSG treatment.

| Sex steroids | Control | BPA50 |
|---|---|---|
| E2 (pg/ml) | 642.80 ± 129 | 665.90 ± 109 |
| P (ng/ml) | 58.60 ± 0.71 | 60.32 ± 1.86 |
| T (ng/ml) | 0.86 ± 0.15 | 1.08 ± 0.07 |

Values are expressed as mean ± SE. The number of animals evaluated was 6 (Control) and 5 (BPA50).

## 3. Results

To know the hormonal status prior to the treatment with exogenous gonadotropins, LH and FSH levels were measured on PND30. No differences were observed in LH and FSH levels between control and treated animals (Fig. 2).

### 3.1. Ovarian status after exogenous PMSG treatment in BPA-treated rats

#### 3.1.1. Follicular dynamics

Fig. 3 shows the numbers of primordial and growing follicles (primary, preantral and antral) in the control and BPA-treated groups. A significant increase in the number of growing follicles was observed in the BPA-treated group (control: 7.91 ± 1.12, BPA50: 11.33 ± 1.31), specifically at antral stage (Fig. 3A). This increase coexisted with no changes in the number of atretic follicles and with a trend to a reduction in the size of the primordial follicles reserve. The increase in the number of antral follicles was at the expense of a significant increase in the subpopulation of small antral follicles with no changes in the number of large antral follicles (Fig. 3B). These results show that the ovaries of females born to mothers perinatally exposed to BPA have increased folliculogenesis at the small antral stage after PMSG treatment.

#### 3.1.2. Gene expression

The expression levels of ovarian steroidogenic enzymes and gonadotropin receptors were evaluated using quantitative real-time PCR. The levels of Cyp17, Cyp19, Fshr and Lhcgr in ovaries from animals born to mothers perinatally exposed to BPA were markedly increased after PMSG treatment (Fig. 4).

#### 3.1.3. Serum levels of sex steroids

To establish whether the changes found in folliculogenesis and mRNA expression of steroidogenic enzymes were correlated with abnormal levels of steroids, we measured the serum concentration of sex steroids. E2, P4 and T levels showed no differences between vehicle- and BPA-treated animals after PMSG treatment (Table 1).

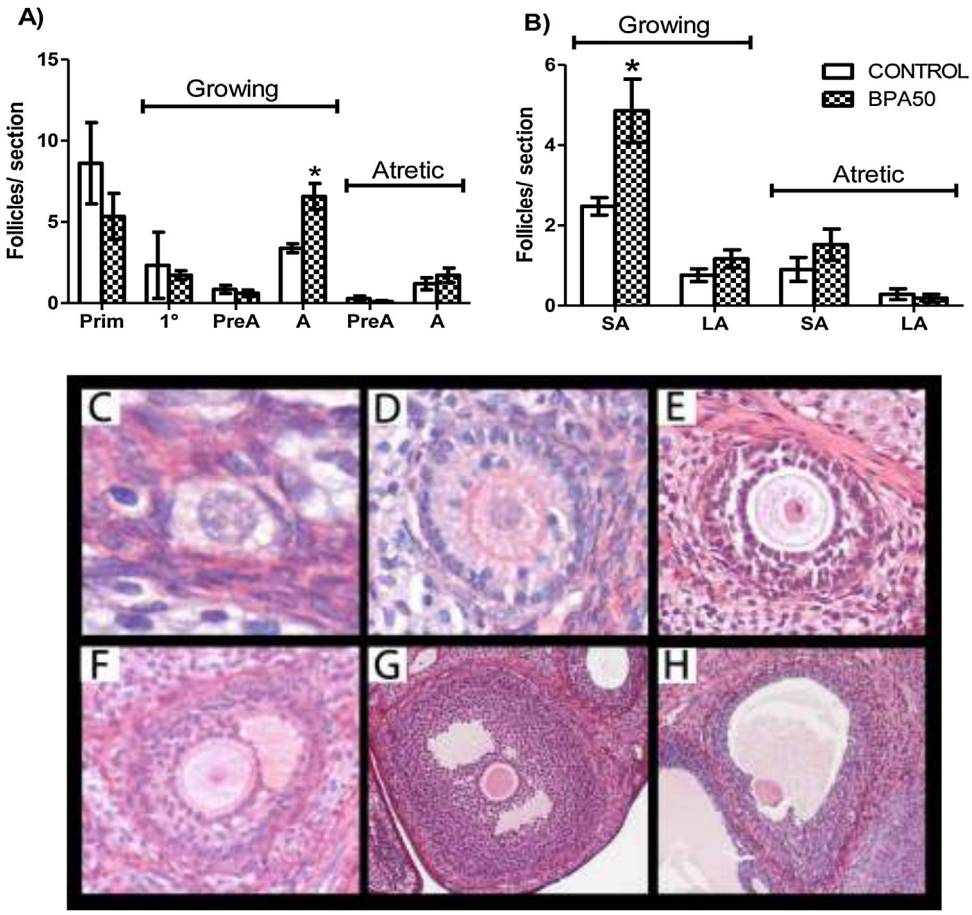


**Fig. 3.** Follicular development after PMSG treatment.
Ovaries of females born to mothers perinatally exposed to vehicle (Control) or BPA (BPA50) were evaluated on PND33, 24 h after the last injection of PMSG. The results of folliculogenesis were expressed based on all follicle stages (A) or only antral stages (B), distinguishing healthy (F;G) and atretic follicles (H) in both cases. The whole paraffin-embedded ovary was serially sectioned (5 μm thick) and one out of every ten sections was stained with picrosirius-hematoxylin for morphological observation (50 μm interval between sections). Numbers of primordial (Prim, C), primary (1°, D), preantral (PreA, E), antral (A), small antral (SA, F) and large antral (LA, G) follicles were counted and expressed as described in M&M. The number of animals evaluated was 6 (Control) and 5 (BPA50). Data are expressed as mean ± SEM. *: $p < 0.05$ vs. control. Magnification: C: 60×; D, E y F: 40×; G y H: 20×.

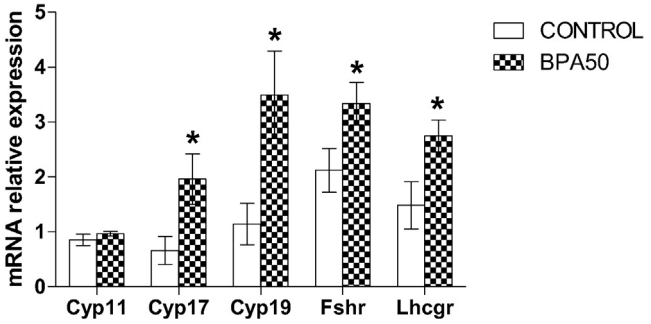


**Fig. 4.** mRNA levels of steroidogenic enzymes and gonadotropin receptors in response to PMSG treatment.
Ovaries of females born to mothers perinatally exposed to vehicle (Control) or BPA (BPA50) were evaluated on PND33, 24 h after the last injection of PMSG. The mRNA expression of cytochrome P450scc (Cyp11), 17a-hydroxylase (Cyp17), P450aromatase (Cyp19), follicle-stimulating hormone receptor (Fshr) and luteinizing hormone receptor (Lhcgr) were quantified by real-time RT-PCR, as described in M&M. The samples were normalized to L19 expression and to the control animals. The expression over control values was calculated for each target by relative standard curve methods. The number of animals evaluated was 6 (Control) and 5 (BPA50). Data are expressed as mean ± SEM. *: $p < 0.05$ vs. control.

### 3.1.4. Sex steroid receptors

To gain insights into the pathway by which BPA exposure might alter follicular development, we evaluated protein expression of AR and ERβ in the population of antral follicles. Briefly, we observed AR-positive expression in granulosa cells of growing follicles, being highest in small preantral and early antral follicles; PR- and ERβ-positive expression was mainly localized in granulosa cells. In both the vehicle- and BPA-treated groups, 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. AR and ERβ expression in granulosa cells of antral follicles from ovaries from females born to mothers perinatally exposed to BPA was increased after PMSG treatment (Fig. 5).

## 3.2. Effects of BPA on the ovarian response to exogenous PMSG + hCG treatment

### 3.2.1. Ovulation, luteinization and follicle growth

To further assess the consequences on ovarian function in females born to mothers perinatally exposed to BPA, we next determined the oocyte production per female after a superovulatory protocol with exogenous gonadotropins. The number of ovulated oocytes and CL showed no changes at 22 h after the hCG injection between BPA- and vehicle-exposed animals (Fig. 6A and B, respectively). However, the CL of the ovaries of the BPA-treated group were smaller than those in the control group (Fig. 6C).

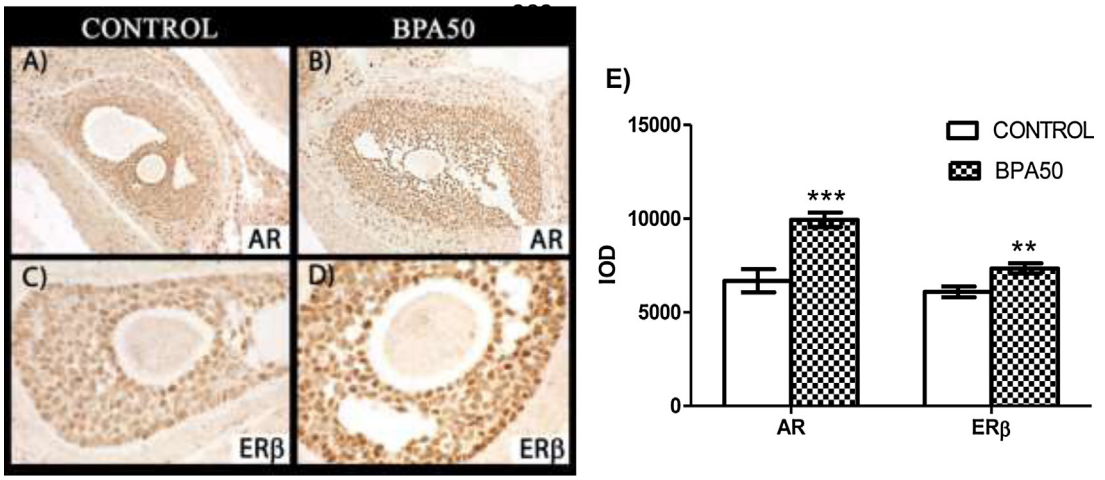


**Fig. 5.** ERβ and AR protein expression in antral follicles after PMSG treatment.
Ovaries of females born to mothers perinatally exposed to vehicle (Control: A, C) or BPA (BPA50: B, D) were collected on PND33, 24 h after the last injection of PMSG. ERβ (C, D) and AR (A, B) protein expression was quantified measuring the integrated optical density (IOD) of granulosa cells of antral follicles. The number of animals evaluated was 6 (Control) and 5 (BPA50). Data are expressed as mean ± SEM. **: $p < 0.01$ vs control; ***: $p < 0.001$ vs. control. Magnification: A and B: 20×; C and D: 40×.

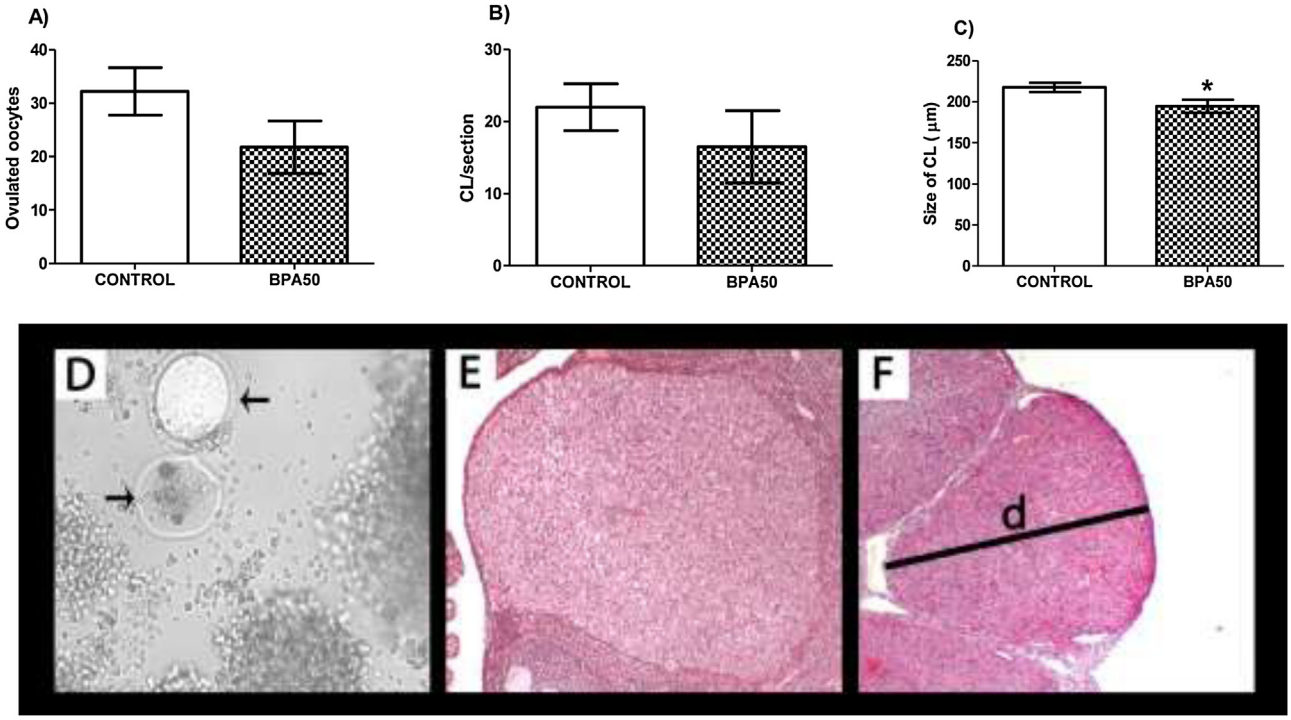


**Fig. 6.** Effects of perinatal exposure to BPA on ovulated oocytes and corpora lutea (CL) at 22 h after hCG injection.
To study the ovarian response to gonadotropins, females born to mothers perinatally exposed to vehicle (Control) or BPA (BPA50) were injected with PMSG and hCG. Ovaries were collected on PND34, 22 h after the last injection of hCG. The number of ovulated oocytes (A, D), number of CL (B, E) and size of CL (C, F) were evaluated 22 h after hCG injection, as described in M&M. The number of animals evaluated was 6 (Control) and 5 (BPA50). Data are expressed as mean ± SEM. *: $p < 0.05$ vs. control. Arrow: oocyte. d: diameter. Magnification: D: E y F: 10×.

Fig. 7 shows the numbers of preantral and antral follicles in the control and BPA-treated groups. In females born to mothers exposed to BPA, hCG injection elicited an increase in the number of small antral atretic follicles but no differences in other follicular types (Fig. 8).

### 3.2.2. Gene expression

The superovulatory treatment induced a reduction in Lhcgr mRNA expression in ovaries of females born to mothers exposed to BPA. There was no difference in mRNA levels of Cyp11, Cyp17, Cyp19 or Fshr (Fig. 8).

### 3.2.3. Serum levels of sex steroids

Serum levels of E2, P4 and T were determined by RIA. Serum levels of E2 in females born to mothers perinatally exposed to BPA were increased at 22 h after hCG injection, whereas those of P4 and T showed no changes (Table 2).

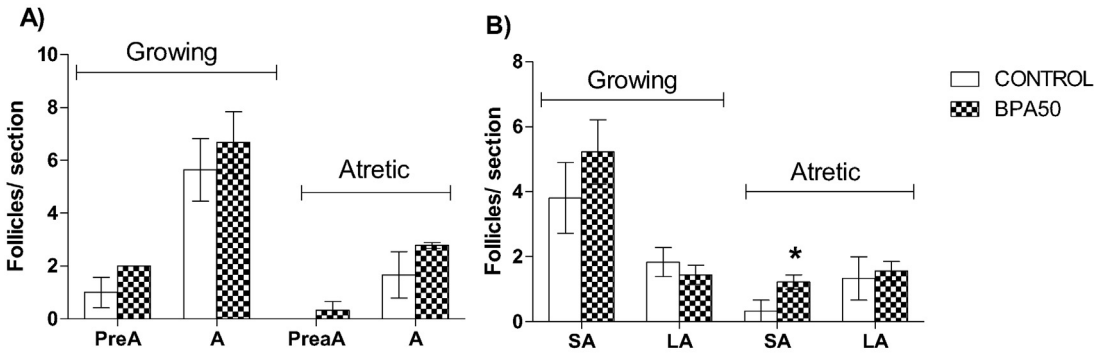


**Fig. 7.** Effects of perinatal exposure to BPA on preantral and antral follicles at 22 h after hCG injection.
To study the ovarian response to gonadotropins, female rats exposed to BPA or vehicle were injected with PMSG and then with hCG. Ovaries were collected on PND34, 22 h after the last injection of hCG. Folliculogenesis was evaluated based on preantral and antral follicle stages (A) or only antral stages (B), distinguishing healthy and atretic follicles in both cases. Ovaries were embedded in paraffin. The whole ovary was serially sectioned (5 μm thick) and one out of every ten sections was stained with picrosirius-hematoxylin for morphological observation (50 μm interval between sections). The numbers of preantral (PreA), antral (A), small antral (SA) and large antral (LA) follicles were counted and expressed as described in M&M. The number of animals evaluated was 6 (Control) and 5 (BPA50). Data are expressed as mean ± SEM. *: $p < 0.05$ vs. control.

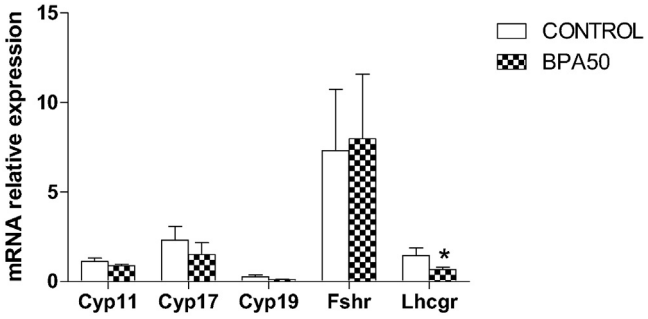


**Fig. 8.** mRNA levels of steroidogenic enzymes and gonadotropin receptors at 22 h after hCG injection.
Females born to mothers perinatally exposed to vehicle (Control) or BPA (BPA50) were injected with PMSG and then with hCG. Ovaries were collected on PND34, 22 h after the last injection of hCG. Then, RNA expression of cytochrome P450scc (Cyp11), 17a-hydroxylase (Cyp17), P450 aromatase (Cyp19), follicle-stimulating hormone receptor (Fshr) and luteinizing hormone receptor (Lhcgr) were quantified by using real-time RT-PCR, as described in M&M. The samples were normalized to L19 expression and to the control animals. The expression over control values was calculated for each target by relative standard curve methods. The number of animals evaluated was 6 (Control) and 5 (BPA50).Data are expressed as mean ± SEM. *: $p < 0.05$ vs. control.

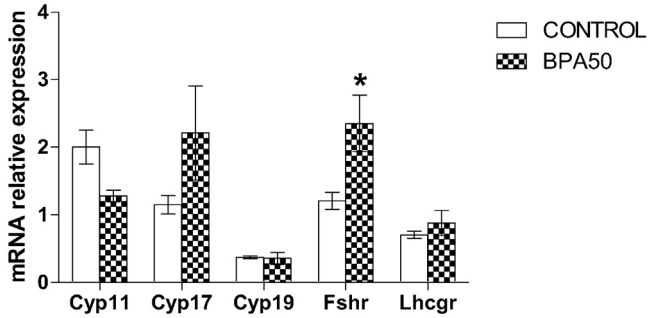


**Fig. 9.** mRNA levels of steroidogenic enzymes and gonadotropin receptors at 7 h after hCG injection.
Females born to mothers perinatally exposed to vehicle (Control) or BPA (BPA50) were injected with PMSG and then with hCG. Ovaries were collected 7 h after the last injection of hCG. Then, mRNA expression of cytochrome P450scc (Cyp11), 17a-hydroxylase (Cyp17), P450 aromatase (Cyp19), follicle-stimulating hormone receptor (Fshr) and luteinizing hormone receptor (Lhcgr) were quantified by using real-time RT-PCR, as described in M&M. The samples were normalized to L19 expression and to the control animals. The expression over control values was calculated for each target by relative standard curve methods. The number of animals evaluated was 6 (Control) and 5 (BPA50). Data are expressed as mean ± SEM. *: $p < 0.05$ vs. control.

**Table 2**
Serum levels of E2, P4 and T of control and BPA-exposed rats after PMSG + hCG treatment.

| Sex steroids | Control | BPA50 |
|---|---|---|
| E2 (pg/ml) | 422.40 ± 228.10 | 599.80 ± 8.24* |
| P (ng/ml) | 62.50 ± 2.14 | 62.21 ± 1.46 |
| T (ng/ml) | 0.59 ± 0.09 | 0.67 ± 0.05 |

Values are expressed as mean ± SE. The number of animals evaluated was 6 (Control) and 5 (BPA50).
* $p < 0.05$ vs. control.

### 3.2.4. Sex steroid receptors

AR and ERβ protein expression in ovaries of females born to mothers perinatally exposed to BPA showed no differences after hCG treatment (data not shown).

### 3.2.5. Ovarian parameters at intermediate stages (7/14 h) after gonadotropin treatment

Based on the results of follicular dynamics after PMSG, i.e., increased population of antral follicles with higher levels of expression of gonadotropin receptors, we also expected a higher rate of ovulated oocytes in response to hCG treatment. Conversely, the number of ovulated oocytes in response to gonadotropin treatment was similar between control and BPA-treated animals (see Figs. 3, 4 and 6). Thus, we explored possible changes that could be present at intermediate stages to clarify this apparent controversy.

To establish if ovulation could take place earlier than 22 h after the hCG injection in BPA–treated animals, we measured the number of ovulated oocytes at 14 h after hCG administration. We found no differences in the number of ovulated oocytes between control and BPA-treated animals at this time (Control: 17.80 ± 9.07; BPA50: 14.50 ± 4.57).

Comparison of the results of gene expression observed after treatment with PMSG (Fig. 4) with those found after PMSG + hCG administration (Fig. 8) led us to wonder how long the high expression levels persist. Thus, we quantified gene expression at 7 h after hCG administration and the results showed that all genes normalized their expression at this moment except for Fshr, which persisted increased in BPA-treated animals (Fig. 9).

Finally, we quantified the expression of PR protein at 7 h after hCG injection. It has been clearly established that PR is necessary for normal ovulation and that there is a peak expression of PR at 7 h after hCG injection [27]. Interestingly, a reduced number of follicles

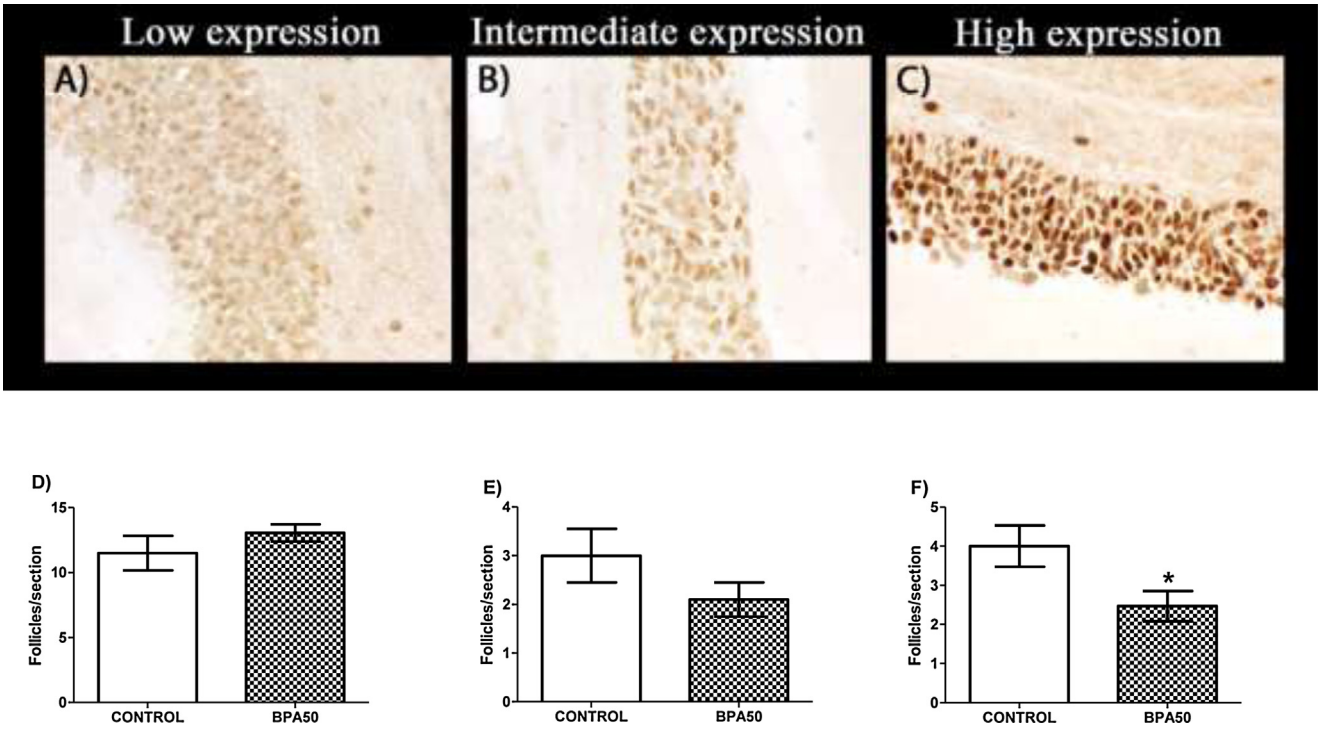


**Fig. 10.** Number of follicles with low (A, D), intermediate (B, E) and high (C, F) expression of PR at 7 h after hCG injection.
Protein expression of PR was immunohistochemically assessed in granulosa cells of antral follicles at 7 h after hCG administration. Immunostaining was evaluated in at least three sections/ovary and follicles were assigned to one of the following categories: follicles with low (A, D), intermediate (B, E) or high (C, F) expression of PR, as described in M&M. The number of animals evaluated was 6 (Control) and 5 (BPA50). Data are expressed as mean ± SEM. *: $p < 0.05$ vs. control. Magnification: A, B and C 40×.

with high expression of PR was observed in ovaries from females born to mothers perinatally exposed to BPA (Fig. 10).

## 4. Discussion

The study of oral exposure to the environmental estrogen BPA during the perinatal period (the period comprised of gestation and the early postnatal period) has generated special interest, because altered organizational programming can lead to increased susceptibility for diseases later in life [19,20]. In this study, we investigated whether the perinatal exposure to BPA affects folliculogenesis and steroidogenesis in response to an exogenous treatment of gonadotropins in prepubertal rats. Our results showed an altered ovarian response after exogenous PMSG in immature female rat offspring born to mothers perinatally exposed to BPA50. This altered response included an increase in the population of antral follicles, altered mRNA levels of steroidogenic enzymes and gonadotropin receptors, and altered protein expression of AR and ERβ. On the other hand, after PMSG + hCG treatment, we found a persistent high expression of Fshr mRNA together with a decreased number of follicles that have a high expression of PR before ovulation. After ovulation, an increase in the number of small antral atretic follicles and smaller CL, a reduced expression of Lhcgr mRNA, and high serum levels of E2 were observed.

In rats, we have previously demonstrated that low doses of subcutaneous BPA injections from birth to PND8 cause a reduction of the primordial follicle pool by stimulating the neonatal initial recruitment associated with an increased proliferation rate, an effect that is likely mediated by an estrogenic pathway [11]. In sheep, we have shown that neonatal exposure to BPA50, by the subcutaneous route, reduces the primordial follicle pool by stimulating their initial recruitment and subsequent follicle development until antral stage, resulting in an increased number of atretic follicles [12]. We have also found that, in response to oFSH treatment, lambs neonatally exposed to low doses of BPA (50 or 0.5 mg/kg/day) by the subcutaneous route show an impaired ovarian response to gonadotropin treatment, with a lower number of follicles ≥2 mm in diameter, together with a lower number of atretic follicles and no increase in E2 serum levels [30]. Recently, in three-month old female rat offspring born to mothers orally exposed to BPA, we demonstrated an inhibition of the transition of primordial to primary follicles and a greater number of CL coupled with higher levels of P4 [21]. In the present study, the extent of follicular development after exogenous PMSG treatment was enhanced by BPA, a fact evidenced by the higher population of antral follicles in ovaries of females born to mothers perinatally exposed to BPA. These results, as well as several findings from other authors [13–16,18,20,31,32], highlight that follicular development is a sensitive target of BPA disruption, since these given the effects are found with different routes of administration, different doses and time of exposure, and even in different species.

The promotion of follicular development found in the present study could be explained by an increased susceptibility of follicles to FSH, due to the previous exposure to BPA. In this regard, the higher level of Fshr mRNA in the ovaries of offspring treated with BPA support the idea that exposure to BPA enhances follicular susceptibility to FSH, because Fshr expression has been identified as an indicator of increased susceptibility to FSH [33]. It has also been established that one of the actions of FSH mediated through its receptor is to stimulate the expression of steroidogenesis enzymes to increase E2 synthesis and to induce Lhcgr expression in granulosa cells of antral follicles [34]. Thus, steroidogenic enzymes and Lhcgr expression are considered FSH-dependent differentiation markers [33]. In the present study, Cyp17, Cyp19 and Lhcgr mRNA levels were increased in the ovaries of females exposed to BPA. Moreover, another indication that exposure to BPA enhances follicular susceptibility to FSH comes from analyzing the expression of AR and ERβ, both of which showed increased expression after treatment with

PMSG in the ovaries of females perinatally exposed to BPA. AR and ERβ modulate the differentiation response of the follicles to FSH. Regarding AR, different authors have demonstrated that androgens, acting through their receptors, increase follicular sensitivity to FSH by increasing Fshr levels, and also contribute to the physiological actions of FSH during early follicular development by amplification of the intracellular cAMP [35,36]. As regards ERβ, in mice null for ERβ, it has been established that one role of ERβ is to amplify and modulate the response of granulosa cells to FSH [34], 2005. Taken together, all these results support the idea that follicles of ovaries of BPA-exposed animals are more susceptible to FSH action. It should be noted that, although the expressions of Cyp17 and Cyp19 were increased after treatment with PMSG, serum levels of E2, P4 and T were unchanged at this time. However, we observed higher levels of E2 later, in response to hCG.

As already described, the ovary of females exposed to BPA in the perinatal period exhibited a greater number of antral follicles and increased expression of Lhcgr after treatment with PMSG. Based on this background, we expected to find a greater number of ovulated oocytes in response to hCG. However, no changes were observed in the ovulation rate or in the number of CL in the BPA-treated group versus the control group. A possible explanation for this apparent controversy is a premature ovulation. However, as established by quantifying the ovulation rate at 14 h after hCG injection, ovulation did not occur prematurely. Instead, we found an increase in follicular atresia elicited by the hCG injection. Interestingly, this increased atresia was evident specifically in the population of small antral follicles, the same population that was increased at the time of the hCG injection. Another issue to be taken into account is the smaller size of CL at 22 h after the hCG injection. Taken together, these results suggest that a fraction of the population of small antral follicles was able to ovulate, whereas the remaining fraction was destined to atresia.

The present results suggest that different mechanisms might be leading the follicles to atresia. Loss of AR in the later stages of follicular development might be part of a paracrine mechanism that affords protection against premature luteinization and atresia [35–37]. On the other hand, one of the mechanisms by which follicles enter atresia is high intracellular levels of cAMP [35,38], which is the mediator of Fshr and Lhcgr actions [39]. Therefore, we can suggest that the increased expression of AR, Fshr and Lhcgr at the time of hCG injection and the persistence of high levels of Fshr, even at 7 h post-hCG observed in BPA-treated animals, might have an impact on the entry of follicles to atresia. On the other hand, ovaries of animals treated with BPA showed fewer antral follicles with high expression of PR at 7 h after hCG injection. In the rat ovary, mRNA PR is induced quickly and briefly by the LH peak. This induction occurs selectively in granulosa cells of pre-ovulatory follicles destined to ovulate [27–29]. Several studies have shown that a successful ovulation depends on this PR signaling induced by LH. For instance, it has been demonstrated that a PR antagonist (RU486) blocks ovulation in rats [40] and mice [41]. Consequently, the loss of follicles expressing high levels of PR found in BPA-treated animals in response to hCG might result in a lower number of follicles able to ovulate, leading to atresia.

In summary, the present results show that perinatal exposure to a low dose of BPA conditions the ovarian responsiveness to treatment with exogenous gonadotropins in prepubertal rats. Studies are under way to investigate if offspring exposed to BPA in the same way as in the present experiment have an impaired ovarian response associated with endogenous gonadotropins. It has been described that women with high concentrations of BPA show a high risk of infertility and poor ovarian response to in vitro fertilization treatment, probably by an impairment on the quality of oocytes and ovarian function [7–10]. Accordingly, results of the present study suggest that an early exposure to BPA during the prenatal and postnatal stages of life might induce changes in ovarian follicles that alter their response to gonadotropins later in life.

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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.reprotox.2017.06.050**.

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