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Neonatal exposure to bisphenol A reduces the pool of primordial follicles in the rat ovary

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ABSTRACT

We evaluated whether exposure to bisphenol A (BPA) disrupts neonatal follicle development in rats. From postnatal day 1 (PND1) to PND7, pups received corn oil (control), diethylstilbestrol (DES20: 20 μg/kg-d, DES0.2: 0.2 μg/kg-d), or BPA (BPA20: 20 mg/kg-d, BPA0.05: 0.05 mg/kg-d). We examined follicular dynamics, multioocyte follicles (MOFs) incidence, proliferation and apoptosis rates, expression of steroid receptors (ERα, ERβ, PR, AR) and cyclin-dependent kinase inhibitor 1B (p27) in PND8 ovaries. DES20, DES0.2 and BPA20-ovaries showed fewer primordial follicles and increased growing follicles. DES20-ovaries exhibited increased incidence of MOFs. Oocyte survival, AR, PR and apoptosis were not changed. Primordial and recruited follicles from BPA20-ovaries showed higher p27, whereas ERβ and proliferation were both increased in recruited follicles. ERα positive primary follicles increased in BPA 20-ovaries. Results show that BPA reduces the primordial follicle pool by stimulating the neonatal initial recruitment, associated with an increased proliferation rate likely mediated by an estrogenic pathway.

1. Introduction

The oocytes in an embryonic rodent ovary are arranged in large clusters called nests. After birth, selected oocytes in the nests undergo apoptosis, and the surviving isolated oocytes become surrounded by squamous pregranulosa cells and form the primordial follicle (follicular assembly). Primordial follicles remain arrested and static until they activate by undergoing the primordial to primary follicle transition [1]. The primordial to primary follicle transition is the first step by which the dormant primordial follicles are recruited into the growing follicle pool. This initial recruitment is a continuous and nonreversible process; a follicle continues to grow until its inevitable destruction by atresia or ovulation [2,3]. Therefore, the rate at which follicular assembly and initial recruitment occurs is of critical importance for the establishment and maintenance of the primordial follicle pool. This stockpile of primordial follicles represents the total population of germ cells available to mammalian females during their entire reproductive life [2,4].

The mechanisms that govern the onset of primordial follicle development have not been fully elucidated and present unique characteristics. Previous studies in rats have demonstrated that progesterone (P) inhibits primordial follicle assembly, whereas both estrogen (Eg) and P reduce the initial recruitment [3]. In previous studies using mice, P and Eg prevented primordial follicle assembly, but not subsequent development [5]. Recent observations have shown that normal folliculogenesis requires androgen receptor (AR)-mediated androgen action [6] and that cyclin-dependent kinase (Cdk) inhibitor 1B, commonly known as $p27^{kip1}$ or p27, suppresses primordial follicle endowment and activation and promotes follicle atresia [7].

Environmental awareness is rising about the consequences of exposure to endocrine-disrupting chemicals (EDCs) on reproduction of humans and wildlife [8,9]. Bisphenol A (BPA), a monomer used in the manufacture of plastics, has been demonstrated to act as an EDC with estrogen-like (xenoestrogen) actions in both "in vivo" and "in vitro" studies [9]. Today, BPA is one of the most highly produced chemicals worldwide, and it accounts for the majority of estrogenic activity that leaches from landfills into the surrounding ecosystem [10,11]. In the mammalian ovary, BPA has been shown to induce meiotic disturbances leading to meiotic aneuploidy [12,13], an increase in the percentage of ovarian tissue occupied by antral follicles, and a major incidence of ovarian hemorrhagic bursae [14]. In addition, postnatal exposure to high doses of BPA increased the appearance of multioocyte follicles (MOFs) [15]. Recently, in

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Caiman latirostris females prenatally exposed to a unique low dose of BPA (1.4 ppm), we demonstrated an increase in type III follicles and an augmented incidence of MOFs long after exposure [16]. The early stages of follicle development (follicle assembly and initial recruitment) have not been exhaustively explored as possible targets for the endocrine-disrupting activity of BPA. Therefore, the aim of this study was to investigate whether neonatal exposure to BPA is able to disrupt early follicle development in rats.

2. Materials and methods

2.1. Animals and treatments

Pups were obtained from timed-pregnant Wistar rats housed under a controlled environment (22 ± 2 °C; lights on from 06:00 to 20:00 h) with free access to pellet laboratory chow (Cooperación, Buenos Aires, Argentina) and tap water. DES and BPA were purchased from Sigma–Aldrich (Sigma–Aldrich S.A., Buenos Aires, Argentina). All rats were handled in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals issued by the US National Academy of Sciences, and all experiments were approved by the Institutional Animal Care Committee of the National University of Litoral (Santa Fe, Argentina). The concentration of phytoestrogens in the diet was not evaluated; however, because food intake was very similar in the control and experimental rats, we assumed that all animals were exposed to the same levels of food-borne phytoestrogens [17]. To minimize additional exposures to EDCs, rats were housed in stainless steel cages with wood bedding, and tap water was supplied in glass bottles with rubber stoppers surrounded by a steel ring.

Experimental design was carried out following a previously described protocol [18]. Ten timed-pregnant dams were used to collect offspring for each treatment. At delivery, pups were sexed according to anogenital distance and cross-fostered by distributing pups of each litter between different mothers. These actions allowed us to assign one animal per litter to each experimental group and to minimize the use of siblings, thereby avoiding potential litter effects. The cross-fostered litters were adjusted to ten pups (five females and five males whenever possible) and assigned to one out of five experimental groups: control (corn oil vehicle-treated pups), DES20 (female pups injected with DES 20 μg/kg), DES0.2 (DES 0.2 μg/kg), BPA20 (BPA 20 mg/kg) or BPA0.05 (BPA 0.05 mg/kg). All pups received sc injections every 48 h on PND1, 3, 5 and 7. The currently accepted lowest observed adverse effect level (LOAEL) for BPA in USA is 50 mg/kg-d, and that was used to calculate the current US Environmental Protection Agency (EPA) reference dose (FDA acceptable daily intake or ADI dose) of 50 μg/kg-d [19]. Experts from the peer review NTP-CERHR Bisphenol A Expert Panel concluded that 5 mg/kg-d is the cutoff dose for low-dose effects, regardless of the administration route, duration of exposure, or the age/life stage at which exposure occurred [20,21] http://cerhr.niehs.nih.gov/chemicals/bisphenol/bisphenol.pdf. Considering these guidelines, the dose of BPA20 used here is four times higher than the low-dose cutoff suggested by the US-NTP experts and 2.5-fold times lower than the LOAEL; meanwhile, the dose of BPA0.05 is 1000 times lower than the LOAEL, 100 times lower than the low-dose cutoff used by the US-NTP experts and similar to the reference dose or ADI level established by the US-EPA. Also, the route of administration, namely subcutaneous injections, was considered relevant for assessing the potential for developmental effects of BPA in humans because newborn mice do not demonstrate the rapid first pass metabolism of BPA that orally dosed adults do [21,22].

In the present study, DES was used as a positive control because it has been reported that after developmental exposure, an increase in the appearance of MOFs and the primordial to primary follicle transition occur [15,23,24,39]. The doses of DES used here (20 μg/kg or 0.2 μg/kg) were 5- or 500-fold lower than that given therapeutically to pregnant women [25]. No signs of acute or chronic toxicity were observed, and no significant differences in weight gain and anogenital distance between xenoestrogen-exposed and control pups were recorded. Alterations in maternal behavior were not detected between experimental groups. Pups were euthanized on PND8, ovaries were dissected, trunk blood was collected, and serum was stored at −20 °C for future studies. The number of animals per treatment group was at least 8.

2.2. Follicular dynamics

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 evaluated sections).

Regarding follicle population, after a pilot study we concluded that the percentages of follicles at each follicular stage obtained from the evaluation of two or three mid-diameter cross-sections were similar to the compiled values from all serial sections (see supplementary data). This conclusion agrees with the previously reported analyses [3,26]. Therefore, the percentage of follicles at each follicular stage was evaluated in at least three mid-diameter cross-sections. Only those 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, transitional, or preantral, following the morphological criteria previously described [3,27]. This classification is weighted toward earlier stages of follicular development. Primordial follicles consist of an oocyte partially or completely encapsulated by flattened squamous pregranulosa cells. Early primary follicles have initiated development and contain some cuboidal (enlarged) granulosa cells. Primary follicles are identified by a single layer of cuboidal granulosa cells around the oocyte. Transitional follicles have 1–2 layers and preantral follicles have more than 2 layers of cuboidal granulosa cells. In the present study, early primary follicles were categorized as part of the primary population because those follicles appear to grow continually in immature rats [28]. Consequently, early primary follicles were considered to be components of the growing or recruited follicle population (early primary, primary, transitional and preantral follicles).

Nest breakdown was evaluated in all stained sections from the whole ovary by counting follicles containing more than one oocyte enclosed within the granulosa cell layers (MOFs) [5,15,29]. Because MOFs were postulated to be oocyte clusters that did not separate and become enclosed individually in follicles [1], proportion of MOFs can be used as an index of the degree to which the process of primordial follicle assembly is compromised by inhibition of nest breakdown [29]. Nest breakdown was evaluated by two parameters [23]: (1) the percentage of rats with at least one MOF, where the presence of one MOF in a single ovarian section categorized each rat as positive for MOFs and (2) the MOF incidence, estimated as the percentage of MOFs/ovary.

Oocyte survival was evaluated as previously described [30] 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 ten to account for the proportion of the ovary not included in the sampling analysis [30].

2.3. Immunohistochemistry

Because DES was only used as a positive control for follicular dynamics and appearance of MOFs, ovaries from DES-exposed animals were not included in the immunohistochemistry studies. In control and BPA-treated groups, immunohistochemistry was performed to measure protein expression estrogen receptors alpha (ERα) and beta (ERβ), progesterone receptor (PR), AR, Ki67 and p27. At least two sections (5 μm thickness) at different depths from each ovary were immunostained as previously described [16]. After deparaffination, microwave pretreatment (antigen retrieval) was performed. Primary antibodies were incubated overnight at 4 °C at dilutions shown in Table 1. 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 employs diaminobenzidine (DAB) (Sigma) as a chromogen substrate. Except for determination of integrated optical density (IOD) of p27- and ERβ immunostained slides, samples were counterstained with Mayer hematoxylin (Biopur, Rosario, Argentina) and mounted with permanent mounting medium (PMyR, Buenos Aires, Argentina). Each immunohistochemical run included positive and negative controls. For negative controls, the primary antibody was replaced with non-immune mouse or rabbit serum (Sigma). For positive controls, ovaries from adult rats were processed as the neonatal specimens, immunostaining pattern reported by Drummond and colleagues were reproduced [31,32].

The proliferation rate of recruited follicles in the ovaries of control and BPA-treated animals was assessed as follows: (a) follicles with at least one Ki67-positive granulosa cell were defined as Ki67-positive and expressed as percentage of Ki67-positive follicles; (b) the percentage of proliferation (proportion of Ki67-positive granulosa cells with respect to total granulosa cells) was quantified for each follicle.

As expected, ERα protein was not expressed by granulosa cells; therefore, positive theca cells were considered to define ERα-positive follicles. For primary follicles, precursor theca cells were identified as the cells adjacent to the surface of the follicular basement membrane [33,34]. Follicles with at least one ERα-positive theca cell or ERβ-positive granulosa cell were defined as ERα- or ERβ-positive follicles, respectively. These results were expressed as percentage of ER-positive follicles for each follicle stage. In addition, ERβ and p27 expression levels were quantified measuring the IOD [35,36]. The images were recorded with a Spot Insight version 3.5 color video camera that was attached to an Olympus BH2 microscope, which used a Dplan 60× objective (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. Image analysis was performed using the Image Pro-Plus 4.1.0.1 system (Media Cybernetics, Silver Spring, MD). The images of 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. Judging from a careful

Table 1
Antibodies used for immunohistochemistry.

Target protein	Supplier	Antibody	Animal source	Concentration
ERα	Novocastra	ER-6F11	Mouse	1:60
ERβ	Zymed	ER-Z8P	Rabbit	1:200
PR	DAKO Corp	PR-A0098	Rabbit	1:500
AR	Santa Cruz Biotechnology	AR(N-20) sc-815	Rabbit	1:400
Ki67	DAKO Corp	Ki67-MIB-5	Mouse	1:10
p27	Santa Cruz Biotechnology	p27 (C-19) sc528	Rabbit	1:1200

examination of immunostained sections, AR and PR expression was unchanged between control and BPA-treated groups. Therefore, AR and PR were qualitatively evaluated.

2.4. Apoptosis

Follicular atresia was measured by evaluating either apoptotic granulosa cells or apoptotic oocytes by using the TUNEL technique (ApopTag® Plus Peroxidase In Situ Apoptosis Kit; Chemicon International Inc., Temecula, CA, USA) [35,37]. Briefly, after incubation with proteinase K (5.0 μg/mL) (Intergen Co., Purchase, NY, USA) for 10 min at 37 °C, sections were treated with hydrogen peroxide in phosphate-buffered saline for 10 min at room temperature to quench endogenous peroxidase activity. The sections were incubated with a mixture containing digoxigenin deoxynucleotide triphosphate, unlabeled deoxynucleotide triphosphate, and terminal transferase enzyme in a humidified chamber at 37 °C for 1 h. Subsequently, the reaction was visualized using anti-digoxigenin-peroxidase and DAB. Samples were counterstained with Mayer's hematoxylin, and then dehydrated and mounted with PMyR. The negative control slides were prepared similarly, except that distilled water was added instead of terminal transferase enzyme. For a positive control, we processed an involuted rat mammary gland collected 4 days after weaning.

2.5. Statistical analysis

Data were expressed as the mean and standard error of the mean (SEM). We performed a Kruskal–Wallis analysis to assess the overall significance (testing the hypothesis that the response was not homogeneous across treatments), and the Dunn post hoc test was used to compare each experimental group with the control group. $p < 0.05$ was accepted as significant. Proportions were analyzed with Fisher's exact probability test [38].

3. Results

3.1. Xenoestrogen exposure promoted the initial follicle recruitment and decreased the primordial follicle reserve

Fig. 1 shows the estimated percentages of primordial (Fig. 1A) and recruited follicles (Fig. 1B) in control and treated groups. Approximately 70% of the follicles in ovaries from control females were primordial follicles, whereas xenoestrogen-exposed females showed a significant decrease in percentage of primordial follicles (DES20: 56.9%, DES0.2: 51.4% and BPA20: 44.6%). This reduction in primordial follicles was associated with an increase in recruited follicles (Fig. 1B). The increase in the growing follicle population was at the expense of a significant rise in number of primary follicles (Table 2). Taken together, these results show that both doses of DES and BPA20 enhance the initial recruitment process by stimulating the transition of primordial to primary follicles.

Rats treated with DES20 exhibited an increased incidence of MOFs. This effect was found in both the percentage of animals exhibiting MOFs (Fig. 2A) and the percentage of MOFs in the total population of follicles (Fig. 2B). MOFs most often possessed 2–3 distinct oocytes in a single follicle that were consistently similar in size, and each appeared relatively healthy when examined with light microscopy. Most of the MOFs appeared similar to primor-

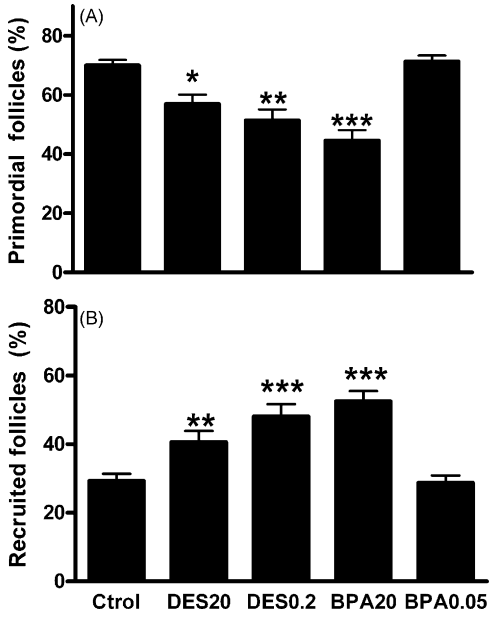

Fig. 1. Follicular recruitment in the ovaries of DES- or BPA-exposed neonatal rats. Ovaries from PND8 female rats were embedded in paraffin, and serial sections of 5 μm thickness were stained with picrosirius-hematoxylin. Percentages of primordial follicles (A) and recruited follicles (sum of early primary, primary, transitional and preantral follicles) (B) were estimated as described in M&M. Data were analyzed with Kruskal–Wallis followed by Dunn post hoc tests. Results were expressed as mean ± SEM. The number of animals evaluated per group was at least 8. $^{*}p < 0.05$ vs. control; $^{**}p < 0.01$ vs. control; $^{***}p < 0.001$ vs. control.

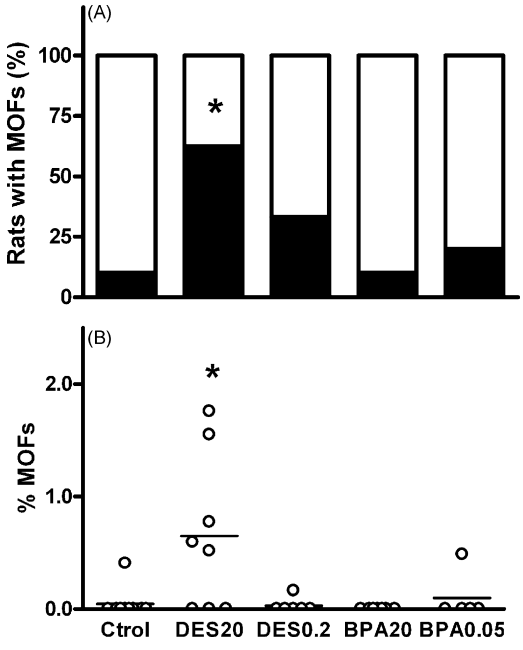

Fig. 2. Incidence of MOFs in ovaries of DES- or BPA-exposed neonatal rats. Ovaries from PND8 female rats were embedded in paraffin, and serial sections of 5 μm thickness were stained with picrosirius-hematoxylin. Percentage of rats positive for MOFs (A) and percentage of MOFs with respect to total number of follicles (B) were estimated as described in M&M. Proportions of rats positive for MOFs (A) were analyzed with Fisher's exact probability test and percentage of MOFs (B) with Kruskal–Wallis and Dunn post hoc tests. Data are expressed as mean ± SEM. The number of animals evaluated per group was at least 8. $^{*}p < 0.05$ vs. control.

Table 2
Number of oocytes and percentage of recruited follicles in ovaries from control, DES or BPA neonatal exposed rats.

Group	Oocytes/ovary (number)	Recruited follicles (%)	
		Primary	Transitional + preantral
Control	8213 ± 311.0	20.36 ± 1.89	8.99 ± 2.15
DES20	7503 ± 728.6	34.71 ± 3.39**	5.94 ± 1.71
DES0.2	7413 ± 2141	35.85 ± 4.76*	12.24 ± 2.03
BPA20	8713 ± 725.6	38.32 ± 2.26***	14.18 ± 1.41
BPA0.05	7066 ± 721.3	22.23 ± 0.82	6.49 ± 1.46

Ovaries from PND8 female rats were embedded in paraffin, serial sections of 5 μm thickness were stained with picrosirius-hematoxylin. Total number of oocytes/ovary and percentage of recruited follicles (the sum of primary, transitional and preantral follicles) were calculated as described in M&M. Data are expressed as mean ± SEM. The number of animals evaluated per group was at least 8.

* $p < 0.05$ vs. control.
** $p < 0.01$ vs. control.
*** $p < 0.001$ vs. control.

dial or primary-stage follicles, exhibiting a single layer of granulosa cells. DES0.2 or BPA treatments caused no significant increase of MOFs.

Oocyte survival was not modified following neonatal xenoestrogen exposure, as shown by the total number of oocytes/ovary (Table 2).

3.2. BPA exposure altered the expression of p27, ERα and ERβ

To gain insight into the pathway by which BPA exposure promotes initial follicle recruitment, we measured protein expression of p27, AR, PR, ERα and ERβ for each follicle stage in neonatal (PND8) ovaries.

The oocyte nuclei and granulosa cells of both primordial and recruited follicles at PND8 expressed p27 protein (Fig. 3A). p27 expression was increased in the granulosa cells of primordial, primary, transitional and preantral follicles from BPA20-treated females (Fig. 4A–D). Also, p27 protein levels increased in the oocytes of primordial and primary follicles from BPA20-treated animals (Fig. 4E–H). p27 expression was not altered in BPA0.05-treated rats.

AR was predominantly located in the granulosa cells of growing follicles, and AR expression increased gradually, reaching the highest levels in granulosa cells of preantral follicles. Theca and stroma cells also stained positive for AR (Fig. 3B). The AR expression was not modified by treatment with BPA (data not shown).

No expression of PR was observed either in interstitial or follicular ovarian cells from control and exposed rats. Therefore, neonatal xenoestrogen exposure was unable to induce early PR expression at PND8 (data not shown).

In controls, ERα was preferentially expressed in theca and interstitial cells, whereas ERβ was expressed in granulosa cells (Fig. 3C and D). In BPA20 animals, the percentage of primary follicles expressing ERα increased (Fig. 5A), whereas the percentage

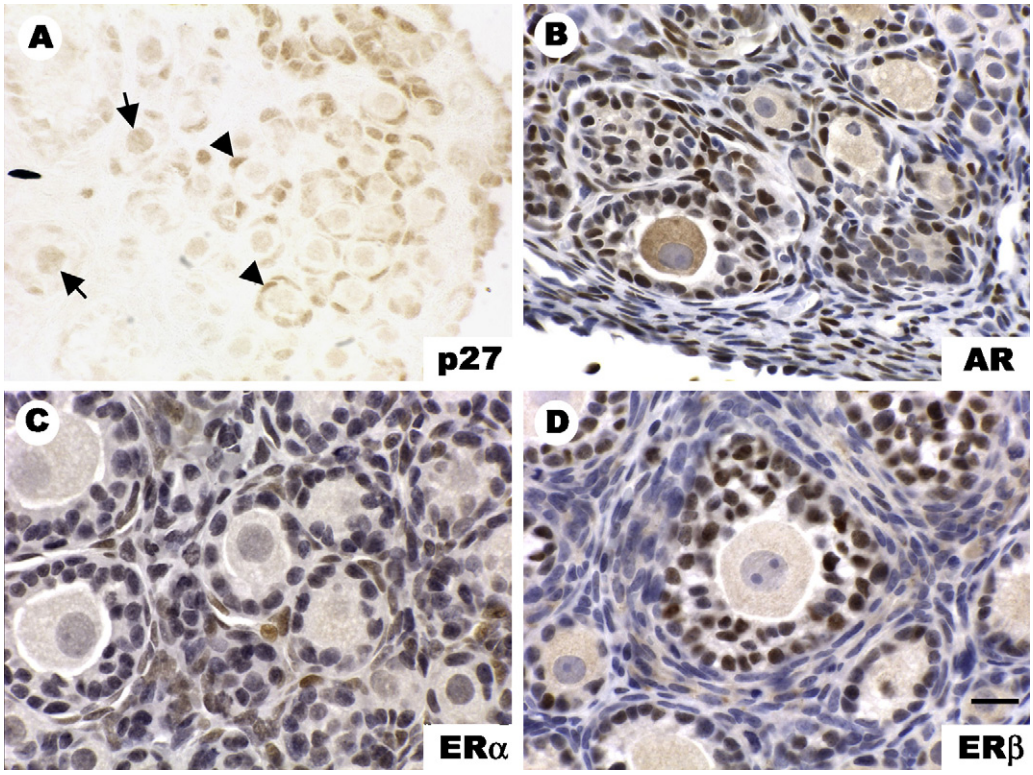

Fig. 3. Ovarian expression of cyclin-dependent kinase (Cdk) inhibitor 1B (p27), androgen receptor (AR), and estrogen receptors alpha (ERα) and beta (ERβ). Representative photomicrographs of ovarian sections immunostained with the specific antibodies detailed in Table 1. (A) p27-positive expression in oocyte nuclei (arrows) and granulosa cells (arrowheads) of primordial and recruited follicles. (B) AR-positive expression in granulosa cells of growing follicles and in theca and stroma cells. ERα (C) positive expression in thecal and interstitial cells and ERβ (D) positive expression in granulosa cells. Immunohistochemistry was developed by using DAB as chromogen substrate and counterstaining with Mayer hematoxylin. Scale bar = 25 μm.

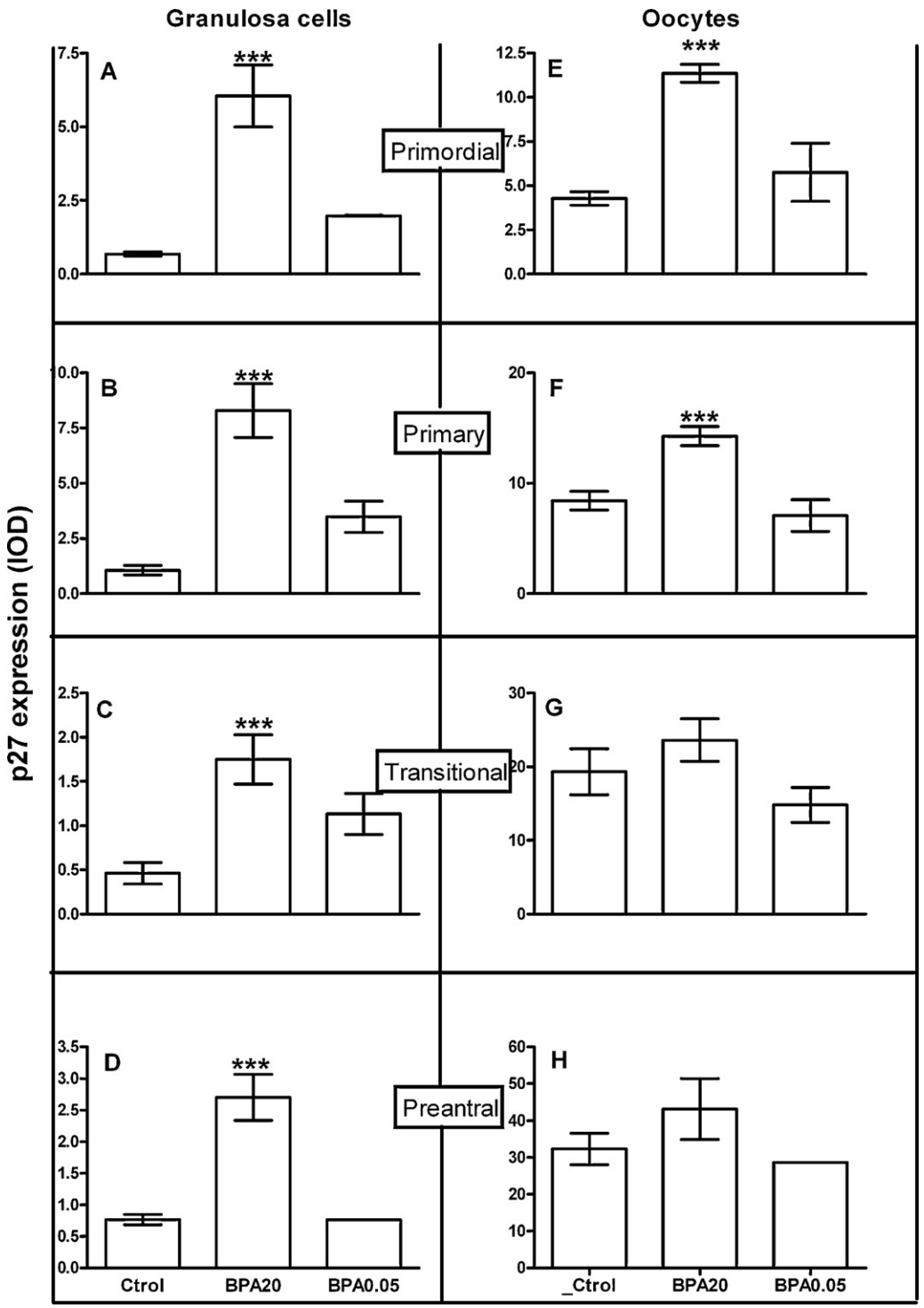

Fig. 4. Effect of neonatal BPA exposure on p27 expression in oocytes and granulosa cells of primordial and recruited follicles. Immunohistochemistry quantification was performed in digitized images of all fields for the entire tissue section. Images were converted to gray scale and IODs of the granulosa cells and oocytes of primordial (A, E), primary (B, F), transitional (C, G) and preantral (D, H) follicles were estimated and expressed as arbitrary units. Data were analyzed with Kruskal–Wallis followed by Dunn post hoc tests. Results were expressed as mean ± SEM. The number of animals evaluated per group was at least 8. $^{***}p < 0.001$ vs. control.

of primary ERβ-positive follicles was unchanged when compared to control (Fig. 5B). Remaining follicular populations showed no changes compared to control group (data not shown). Even though there was no change in the percentage of follicles expressing ERβ (ERβ-positive follicles) in the ovaries of BPA-treated rats, an increase in the amount of ERβ protein expressed by each follicle of ovaries from BPA20-treated rats was noted. This change in ERβ expression was not only observed in primary follicles but also in transitional and preantral follicles of BPA20-treated rats (Fig. 6A).

3.3. BPA exposure increased proliferation rate of recruited follicles

To establish whether the increase in p27 expression observed in BPA-exposed animals induced an increase in follicular atresia, the apoptotic index of the different follicle stages was determined. At

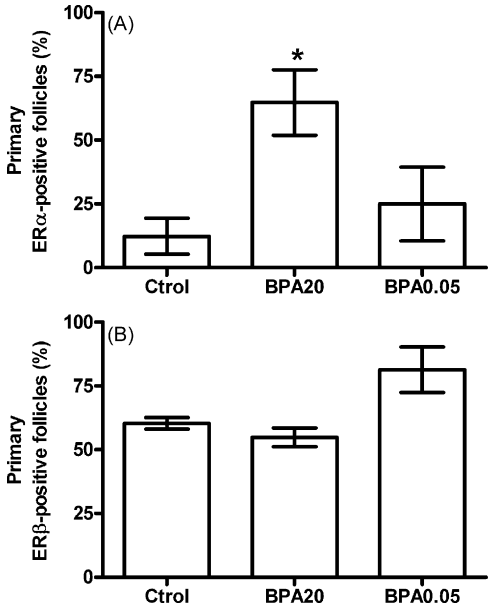

Fig. 5. Effect of neonatal BPA exposure on the percentages of primary follicles expressing ERα or ERβ. ERα- or ERβ-positive follicles were defined as follicles with at least one ERα-positive theca cell (A) or ERβ-positive granulosa cell (B), and expressed as percentages. Data were analyzed with Kruskal–Wallis followed by Dunn post hoc tests. Results were expressed as mean ± SEM. The number of animals evaluated per group was at least 8. $^{*}p < 0.05$ vs. control.

PND8, ovaries from controls showed few follicles containing apoptotic granulosa cells or apoptotic oocytes. BPA treatment did not modify the number of apoptotic cells (data not shown).

Furthermore, we examined the proliferation rate of each follicle type. Ki67-positive cells were detected in growing follicles at all stages. Both the flattened granulosa cells (present in early primary follicles) and the cuboidal granulosa cells (characteristic of all growing follicles) were able to express Ki67. The proportion of Ki67-positive follicles within each follicular stage in BPA-exposed females did not differ from controls. Conversely, the proliferation rate of granulosa cells (proportion of Ki67-positive granulosa cells/total of granulosa cells) was increased in the recruited follicles of ovaries from BPA20-treated animals (Fig. 6B).

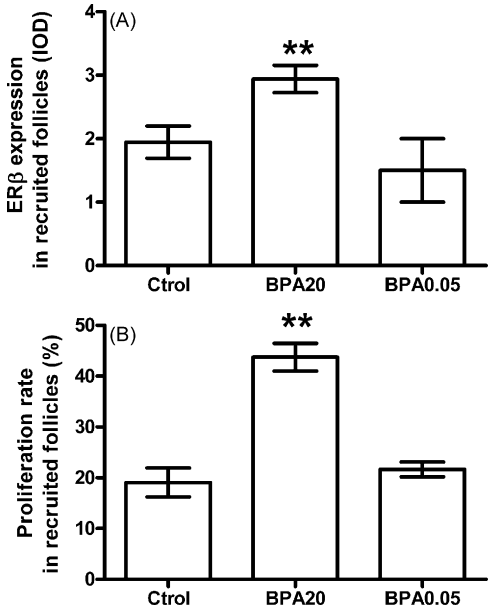

Fig. 6. ERβ expression and proliferation rate in recruited follicles in the ovaries from BPA-exposed neonatal rats. Digitized images of ERβ immunostained slides were converted to gray scale, the granulosa cell compartment was delimited, and the IOD was calculated (A). The proportion of Ki67-positive granulosa cells with respect to total granulosa cells was quantified for each follicle and expressed as proliferation rate (B). Data were analyzed with Kruskal–Wallis followed by Dunn post hoc tests. Results were expressed as mean ± SEM. The number of animals evaluated per group was at least 8. $^{**}p < 0.01$ vs. control.

4. Discussion

In the present study, we show that low doses of DES or BPA reduced the ovarian reserve of follicles. The reduction in the ovarian reserve of follicles induced by DES0.2 and BPA20 treatments was caused by the increased initial recruitment of primordial follicles, whereas the reduction in ovarian reserve induced by DES20 might be elicited by the contribution of both diminished follicular assembly and increased initial recruitment of primordial follicles. This stimulatory effect on the transition of primordial to primary follicles has been previously reported for DES [39]. BPA20-exposed animals exhibited a clear increase in both proliferation rate and ERβ expression in granulosa cells of primary, transitional and preantral follicles. An increase in ERα expression in terms of percentage of primary ERα-positive follicles was also observed. Interestingly, an isotype-selective ERβ agonist has been reported to cause a similar activation of early folliculogenesis, whereas an isotype-selective ERα agonist had no effect [40]. Taken together, these results suggest that follicle activation triggered by BPA exposure might be caused by an ERβ- and/or ERα-mediated stimulatory effect on early folliculogenesis. In addition, we found that BPA20 promotes a parallel increase in p27 expression that was not associated with follicular atresia.

In rodents, the pool of ovarian primordial follicles is established at birth [26,41,42]. Potential alterations that could result in an impoverished primordial follicle pool include an increase in apoptosis of the oocytes during nest breakdown, an increase in the number of oocytes trapped in MOFs and/or an increase in initial recruitment. Both doses of DES (DES20 and DES0.2) and the highest dose of BPA (BPA20) assayed here reduced the quantity of primordial follicles. This reduction was associated with an increase in the primordial to primary follicle transition. Because oocyte survival was not changed by any treatment and formation of MOFs was unchanged in DES0.2- or BPA20-treated rats, we may conclude that the reduction in the ovarian reserve of follicles induced by xenoestrogen exposure was caused by the increased initial recruitment of primordial follicles. This stimulatory effect on the transition of primordial to primary follicles has been previously reported for DES [39] but not for BPA. On the other hand, exposure to DES20 stimulated the primordial to primary follicle transition and also induced the appearance of MOFs. Therefore, the reduction in ovarian reserve induced by DES20 might be elicited by the contribution of both diminished follicular assembly and increased initial recruitment of primordial follicles.

It has been demonstrated that direct estrogenic effects on ovarian follicular development are mediated by ERs. Neonatal exposure to DES induces MOFs acting through ERβ [43] and/or ERα [44]. The use of E_2 or the selective ERβ agonist 8-vinylestra-1,3,5(10)-triene-3,17β-diol (8β-VE_2) in Gn-RH antagonist-treated juvenile mice or hypophysectomized juvenile Wistar rats caused an increase in the number of primary, preantral and antral follicles, together with an increase in proliferation and ERβ protein expression in granulosa cells of these growing follicles [40]. Concordant with these observations, our findings in BPA20-treated rats show an increase in recruited follicles that is associated with an increase in both proliferation rate and ERβ expression in the granulosa cells of primary, transitional and preantral follicles. In addition, an increase in the percentage of primary follicles expressing ERα was observed in

BPA20-treated rats, suggesting a role for the ERα-signaling pathway in the stimulatory effect of BPA on initial recruitment. Our results show a stimulatory effect on early follicular growth due to neonatal exposure to BPA, which suggests that BPA could stimulate proliferation of granulosa cells during neonatal development by acting through ERβ and/or ERα in the ovary. However, our results contrast with studies indicating that estrogenic compounds impede follicle assembly [3,5,41] and subsequent follicle development [3,45,46]. Moreover, BPA is considered a selective estrogen receptor modulator (SERM) with tissue- and species-specific effects [9,47]; acting in some cases as estrogen antagonist by interfering with the ERβ signaling pathway [13]. Although in present study BPA is causing an increase in the expression of ERβ, this does not necessarily mean it is acting as an agonist thereof. On the other hand, one potential marker for estrogen signaling, PR [48], was not changed after BPA or DES treatment.

In agreement with previous reports [15,23,39], here we found that DES inhibited primordial follicle assembly by induction of MOFs and stimulated subsequent follicle development. Previous studies on BPA have reported that MOFs were induced by postnatal injections of a large dose (100 mg/kg-d) but not a low dose (10 mg/kg-d) [15]. The doses of BPA assayed here were similar (BPA20) or lower (BPA0.05) to the lowest dose used by Suzuki and colleagues [15]. Accordingly, neither dose of BPA used here had a stimulatory effect on MOFs formation.

Recently, it has been demonstrated that p27 is a key molecule in the regulation of ovarian development in mice [7]. The immunohistochemical pattern of p27 expression described here was similar to the pattern described in the neonatal mouse ovaries. At PND8, p27-deficient ($p27^{-/-}$) mice showed a percentage of primordial follicles that was significantly lower (46%) than the percentage of primordial follicles in $p27^{+/+}$ ovaries (71%). Moreover, the percentage of activated follicles in PND8 $p27^{-/-}$ ovaries was higher (54%) than the percentage of activated follicles in PND8 $p27^{+/+}$ ovaries (29%). In addition, p27 deficiency in pregranulosa cells triggers pregranulosa cell proliferation and differentiation during follicle activation [7]. This report led us to expect that recruited follicles in BPA20-treated rats might have decreased levels of p27 protein. However, the majority of follicle stages showed an increase in p27 expression, revealing that the high initial recruitment of primordial follicles into the growing population caused by BPA would not be mediated by loss of p27. In addition, Rajareddy and colleagues [7] described p27 as an enhancer of follicle death from PND8 until sexual maturity through activation of the caspase-dependent apoptotic pathways. Accordingly, despite the p27 increase, we found that the apoptotic indexes of follicles from BPA-treated rats were not modified. Alternatively, increased p27 expression could lead to increased follicular atresia at later time points. An alternative explanation, based on the fact that p27 is one of several molecules that maintain the dormancy of primordial follicles [49], is that overexpressed p27 at PND8 might be part of a compensatory mechanism designed to control the follicular activation triggered by DES and BPA at a later stage.

There is scientific controversy over the definition of "low dose" studies regarding EDCs. A BPA expert panel considered studies that used doses that were less than LOAEL dose to be "low dose" studies [9,50]. Conversely, experts from the peer review NTP-CERHR Bisphenol A Expert Panel used 5 mg/kg-d as the cutoff dose for low-dose effects [20,21]. In the present study, effects associated with exposure to BPA were found in the ovaries of BPA20-treated rats but not BPA0.05-treated animals. As detailed in M&M, the dose of BPA20 is four times higher than the low-dose cutoff used by the peer review NTP-CERHR Bisphenol A Expert Panel and is 2.5-fold lower than the LOAEL. The reference dose or ADI level established by both the US-EPA and US-FDA is calculated based on the LOAEL. Regardless of the controversy about definition of low-dose BPA studies, we believe that studies showing effects of BPA at levels lower than the current LOAEL dose are highly relevant, as they may have an impact on the dose used to calculate the ADI dose.

There are multiple oocyte and granulosa cell-derived factors with which BPA might be interacting to alter neonatal follicular dynamics. Bone morphogenetic protein-15 (BMP-15) is an oocyte-derived factor, whereas kit ligand (KL) is a granulosa cell-derived factor; both of these factors have been shown to be crucial regulators of folliculogenesis [51,52]. Additionally, previous studies have shown that KL causes the phosphorylation and suppression of Foxo3a in oocytes, and loss of Foxo3a promotes primordial follicle activation [7,49]. On the other hand, emerging evidence highlights the importance of the activin/inhibin (Inh) system during early folliculogenesis. [53,54]. Moreover, neonatal DES or estradiol exposure decreased the number of small antral follicles, serum inhibin levels, activin β-subunit, and induced MOFs formation; demonstrating that activin subunits are targets of estrogenic action in the neonatal rodent ovary [55]. Further studies are necessary to fully elucidate how neonatal exposure to environmental estrogenic compounds disrupts the mechanisms behind neonatal initial recruitment of follicles.

In summary, our present findings demonstrate that BPA negatively impacts the pool of gametes in a neonatal rat ovary by acting as an activator of the initial recruitment of primordial follicles during the first week of postnatal life. BPA may mediate this activation of initial recruitment by increasing ERβ and/or ERα expression in granulosa cells of recruited follicles, which has been causally related to an increase in the exit of primordial follicles from a quiescent state [40]. Because all primordial follicles initiated to grow are programmed to undergo apoptosis unless rescued by FSH, menopause results from initial but not cyclic recruitment of follicles [2,56]. Therefore, the reduction in the primordial follicle stockpile reported in the present study allows us to hypothesize that BPA may lead to an early onset of menopause and consequently premature ovarian failure. Recently, it has been speculated that follicle activation during postnatal development is a potential target by which environmental EDCs alter follicle dynamics [57]. Our findings support this hypothesis and provide evidence that justifies the widespread concern about the potential role of EDCs in the increased worldwide incidence of reproductive abnormalities.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.reprotox.2010.07.008.

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