Neonatal exposure to bisphenol A or diethylstilbestrol alters the ovarian follicular dynamics in the lamb☆

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ABSTRACT

We hypothesized that neonatal xenoestrogen exposure affects the ovarian follicular dynamics in lambs. Female lambs were exposed from postnatal day (PND) 1–14 to low doses of diethylstilbestrol (DES) or bisphenol A (BPA). At PND 30, the follicular dynamics and ovarian biomarkers (ERα, ERβ, AR, Ki67, p27) were evaluated. Lambs exposed to DES or BPA showed a decline in the stock of primordial follicles with stimulation of follicular development. BPA reduced ovarian weight and increased the number of multioocyte follicles. BPA promoted proliferation of granulosa/theca cells in antral follicles, and increased both the number of antral atretic follicles and p27 expression. Neonatal exposure to BPA or DES reduced the primordial follicle pool by stimulating their initial recruitment and subsequent follicle development until antral stage. In prepubertal lambs, the accelerated folliculogenesis resulted in increased incidence of atretic follicles. These alterations may affect the ovarian function in the adult.

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1. Introduction

Exposure to endocrine-disrupting compounds (EDCs) during critical periods of development can affect gonad formation and disrupt reproductive functions during adulthood [1]. Exposure of cattle and sheep to estrogenic forage, such as subterranean clover (*Trifolium subterraneum*), reduces conception rates, increases embryonic loss, and impairs ovarian function [2], leading to substantial reductions in livestock productivity [3,4].

Both diethylstilbestrol (DES) and bisphenol A (BPA) are considered EDCs and have been extensively studied using different animal models. DES is a synthetic estrogen with a stronger bioactivity than estradiol (E_2) [5]. In mice, postnatal DES exposure results in excessive multiocyte follicles (MOFs) and accelerated follicular development [6,7]. In the past, DES was widely used in human and veterinary medicine, and significant levels of DES may be present in the environment, mainly related to feed-lot areas [5].

BPA is used in numerous manufactured products. In 2004, the estimated BPA production in the United States was approximately 2.3 billion pounds, most of which was used in polycarbonate plastics and resins [8]. Because BPA has been shown to leach from containers into food and beverage products, and it is one of many contaminants in soil, surface water and sludge sewage. Therefore, BPA should be considered a potential health risk for animals and humans [9]. Many studies have clearly shown that BPA has estrogenic properties. In previous works, using rodent and reptile models, we have shown that early BPA exposure impairs the normal development of different endocrine-related tissues [10–20]. Additionally, *in utero* exposure to low doses of BPA disrupts early oogenesis in mice [21], and postnatal injections of BPA induce MOFs [22]. In contrast to laboratory animals and wildlife, the potential reproductive effects of EDC exposure in domestic animals have received little attention. In sheep, prenatal exposure to BPA reduces the birth weight of offspring, and the exposed-animals are hypergonadotropic and end their breeding season later during adulthood [23]. However, little is known regarding the effects of neonatal BPA or DES exposure on the developmental programming of the sheep ovary.

Because follicular differentiation gets completed in utero, all follicular classes (primordial to antral) are present in early postnatal lamb. Regardless of this early differentiation, EDC exposure during early postnatal life can have an impact on maintenance of ovarian reserve. In this study, we tested whether neonatal exposure to low doses of BPA or DES adversely affects the prepubertal lamb ovary. Specifically, the ovaries were studied on postnatal day 30 (PND 30) to assess whether exposure to xenoestrogens produces adverse effects on follicular development (follicle dynamics, total reserve of oocytes, induction of MOFs, and atretic follicles). In

addition, to investigate the potential signaling pathways underlying these effects we evaluated the expression of estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), androgen receptor (AR), Ki67 (a cellular proliferation marker), cyclin dependent kinase inhibitor 1B (commonly known as p27^{kip1} or p27), E_2 and testosterone (T) serum levels. Xenoestrogens can act through different steroid receptors in the target cells, thus the ontogeny of ovarian expression of ERα, ERβ and AR during the period of xenoestrogen exposure (PND1–14) was studied. Spatial and temporal patterns of expression of steroid receptors were described in neonatal ovaries of unexposed animals.

2. Materials and methods

2.1. Animals and experimental design

All procedures were revised and authorized by the Institutional Committee of Animal Use and Care of Universidad Nacional del Litoral. The experiments were conducted in an experimental farm belonging to the Universidad Nacional de Lomas de Zamora, Buenos Aires, Argentina. Corriedale ewes (2–4 years old) grazed pasture with a low rate of clover. During the breeding season, they were mated to Hampshire Down rams. No supplementary feeding was required during pregnancy and lactation. Female lambs selected for the experiments were born during August and September and from a single delivery (no twins were used). Phytoestrogen concentration in the pasture was not evaluated; however, because food intake was equivalent for control and treated animals, we assumed that all animals were exposed to the same levels of phytoestrogens. Mothers and offspring remained under natural conditions during the experiment. All experimental animals were weighed the day of birth and on PND 30.

After birth female lambs were randomly assigned to one of the following postnatal treatments: (1) controls given corn oil vehicle ($n = 10$ female lambs); (2) DES (Sigma, St. Louis, MO) at 5 μg/kg/day ($n = 6$); (3) BPA (99% purity, Aldrich, Milwaukee, WI) at 50 μg/kg/day ($n = 6$). The EPA-National Toxicology Program's Report of the Endocrine Disruptors-USA [24] has defined the lowest observed adverse effect level (LOAEL) dose for BPA as 50 mg/kg/day and the "safe dose" as 1000 times lower (50 μg/kg/day) [8,25]. Here we have used the safe dose of BPA. DES was used as positive control because developmental exposure to low doses of this compound induces MOFs and activates the primordial to primary follicle transition in mouse [6,7]. The dose of 5 μg/kg/day of DES used here was 20-fold lower than that given therapeutically to pregnant women, and according to previous reports, this is considered a low dose [26].

Treatments were given daily from PNDs 1 to 14 (day of birth: 1) by subcutaneous injections in the nape of the neck. Female lambs were weighed and ovaries were removed via a midline abdominal incision on PND 30 under ketamine (20 mg/kg, i.m.) and xylazine (0.1–0.2 mg/kg, i.m.) anesthesia. To evaluate the effects of neonatal exposure to DES or BPA on ovarian weight (g); the organ weight was adjusted for variations in lamb body weight (kg). No signs of acute or chronic toxicity were observed, and no significant differences in weight gain between xenoestrogen-exposed and control groups were recorded during the experiment (data not shown). In addition, to determine the ontogeny of the expression of steroid receptors during the early postnatal ovarian development in unexposed animals, we obtained neonatal ovaries at PNDs 1, 5, 10 and 30 ($n = 3$ lambs per time point) to describe the spatial and temporal pattern of expression of ERα, ERβ and AR. Peripheral blood serum samples were obtained for hormone assays. Ovaries were fixed in 10% buffered formalin for 6 h at room temperature and paraffin-embedded.

2.2. Morphometry

The whole ovary was serially sectioned (5 μm thick) and one slide out of every forty sections was stained with picrosirius–hematoxylin (200 μm interval between evaluated sections) and used for morphometry. The percentage of follicles at each stage was established under light microscope at 40× magnification. Follicles were classified following previously described morphological criteria [27]: primordial follicles, one layer of flattened granulosa cells; transitional follicles, one layer of a mixture of flattened and cuboidal granulosa cells; primary follicles, from one to less than two complete layers of cuboidal granulosa cells; small preantral follicles, from two to fewer than four complete layers of cuboidal granulosa cells; large preantral follicles, from four to fewer than six complete layers of cuboidal granulosa cells; and small antral follicles, more than five layers of cuboidal granulosa cells and a fully formed antrum, only small antral follicles showing an oocyte with the nucleus were scored to avoid counting twice the same follicle. The total number of follicles from each ovary was calculated as previously described [20,28]. Briefly, follicles were counted in every fortieth section (200 μm apart from each other) of the entire ovary and multiplied by a factor of forty to account for the proportion of the ovary not included in the sampling analysis. Finally, the percentage of follicles in different stages of each ovary was calculated relative to the total number of follicles.

In addition, nest breakdown was evaluated as previously described [22,29], counting follicles with more than one oocyte enclosed within the granulosa cell layers, which were defined as MOFs. MOF incidence was expressed relative to the total number of follicles.

2.3. Immunohistochemistry

Ovarian sections were used to evaluate protein expression of ERα, ERβ, AR, Ki67 and p27, following protocols published by our laboratory [30]. Immunostaining for steroid receptors was performed using a mouse anti-ERα (NCL-ER-LH2, clone CC4-5, 1:50 dilution, Novocastra Newcastle-upon-Tyne, UK), mouse anti-ERβ (NCL-ER-beta, clone EMR02, 1:25 dilution, Novocastra), and rabbit anti-AR (sc-816, 1:400 dilution, Santa Cruz Biotechnology Inc., CA) antibodies. To detect p27, a rabbit anti-p27 antibody (sc-528, 1:1200 dilution, Santa Cruz Biotechnology Inc.) was used. To evaluate cellular proliferation, we used an anti-Ki67 affinity-purified rabbit polyclonal antibody generated and tested in our laboratory. The protocol used to generate anti-Ki67 has been previously described [17]. Briefly, the Ki67 antigen was expressed in E. coli JM109 (Stratagene, GE Healthcare Bio-Sciences, Argentina SA, Buenos Aires, Argentina) as a glutathione-S-transferase fusion protein using a pGEX4T-3 vector (GE Healthcare Bio-Sciences). The Ki67 antigen included the region corresponding to aa 581–650 (accession number XP_215947). For immunohistochemical analysis, Ki67 antibodies were purified using antigen-linked affinity chromatography (Hi-Trap NHS activated HP column, GE Healthcare Bio-Sciences) and were used at a 1:250 dilution. The specificity of all antibodies was tested using Western blot analysis of protein extracts [31] obtained from intact uterine or gonad samples of ewes (data not shown).

Each immunohistochemical run included positive tissues and negative controls replacing the primary antibody with nonimmune serum (Sigma).

2.4. Evaluation of immunohistochemistry

Each immunohistochemical run included ovarian sections from different time points (PND 1, 5, 10, 30). For studying the expression of ERα, ERβ and AR and in order to assess staining pattern homogeneity we selected 3 sections, 200 μm apart from each other (ovaries from PND 1, 5 and 10) and 800 μm apart from each other at PND 30. No significant differences were found between sections of the same ovary. The intensity and distribution pattern of immunostained tissue and in each type of follicle was described. The steroid receptors were evaluated in the cortical and medullar regions. Cortical stroma was recognizable by the presence of densely packed stromal cells, the presence of primordial and early growing follicles and a low density of small blood vessels [32]. In the analysis of the cortical region, protein expression was assessed in the stroma and in follicular, theca cells, granulosa cells, and oocytes. Proliferative indexes for granulosa cells and theca cells were obtained considering the percentage of Ki67-positive cells relative to the total number of cells of each cellular type. All healthy follicles (without atretic signs) were evaluated; totaling at least 500 cells located in 5 different visual fields randomly chosen of each follicular stage (preantral and antral follicles). The field of view was positioned over the mural granulosa layer. 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 the rarity or absence of mitotic cells. In small antral atretic follicles, the remaining granulosa cell layer consisted almost entirely of pyknotic nuclei and apoptotic bodies. Even though these histomorphological features were found in most of the atretic follicles, in the present study, to avoid misclassification, atretic follicles were defined as those with ≤1% Ki67-positive granulosa cells [33]. The criteria established to evaluate proliferative indexes were followed to quantify p27 expression, as well.

2.5. Hormone assays

Serum samples were obtained and stored at −20 °C until use for hormone assays. Serum levels of E_2 and T were determined by RIA after ethyl ether extraction (Merck, Buenos Aires, Argentina) [34]. The antibodies were provided by Dr. G.D. Niswender, and labeled hormones were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). Assay sensitivities were 0.4 ng/ml and 1.6 pg/ml for T and E_2, respectively. Intra- and inter-assay coefficients of variation were, respectively, 6.96% and 10.44% for T and 3.6% and 11.0% for E_2.

2.6. Statistics

All data were calculated as 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. Analysis of ovarian weight was done comparing ovary (g)/lamb (kg) weight ratios. $P < 0.05$ was accepted as significant.

3. Results

3.1. Ovarian developmental expression of ERα, ERβ and AR

The ontogeny of ovarian ERα, ERβ and AR protein expression during the first month of lamb life was described in the cortical and medullar ovarian compartments. Differential patterns of expression of the steroid receptors were found throughout ovarian development (Fig. 1 and Table 1). Most of the ovarian medullar stroma cells showed positive expression of ERα at PNDs 1 and 5, while few positive cells were found in the cortical stroma at the

same time points. At PND 10, the ERα noticeably decreased and became almost negative in all cellular compartments by PND 30. No ERα protein was present in primordial follicles, and low expression in the granulosa cells of preantral follicles was found only at PND 1. The expression pattern exhibited by ERβ in medular and cortical stroma at PND 1 was similar to that described for ERα. From PND 5 to PND 10, ERβ expression increased, but it diminished at PND 30. Low expression of ERβ was detected on PND 1 in the granulosa cells of primordial follicles, and from PND 5 to PND 30, high expression of ERβ was found in the oocytes and in the granulosa cells of primordial and growing follicles. AR showed high expression in
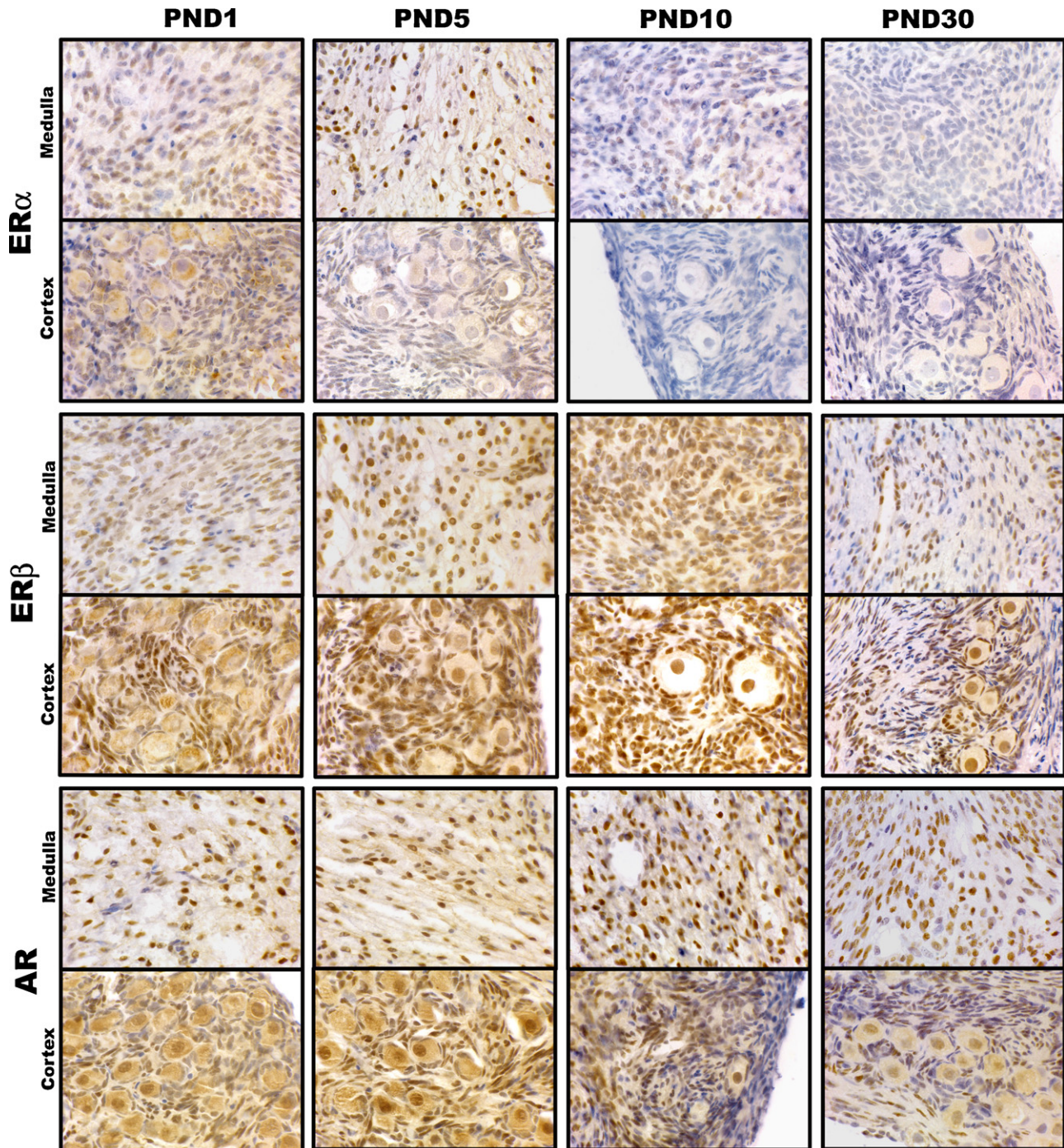

Fig. 1. Neonatal ontogeny of ovarian ERα, ERβ and AR protein expression from postnatal day 1 (PND 1) to PND 30. Representative photomicrographs showing the immunostaining pattern of the steroid receptors in the lamb ovary. Immunohistochemistry was developed using DAB and counterstaining with Mayer hematoxylin. Magnification: 600×.

Table 1
Neonatal expression of ERα, ERβ and AR in lamb ovary.

	PND1	PND5	PND10	PND30
ERα				
Medulla	++	+++	−/+	−
Cortical stroma	+	−/+	−	−
Primordial follicles	−	−	−	−
Growing follicles	+	−	−	−
Oocytes	−	−	−	−
ERβ				
Medulla	++	+++	+++	+
Cortical stroma	++	+++	+++	++
Primordial follicles	+	+++	+++	+++
Growing follicles	−	+++	+++	+++
Oocytes	−	+++	+++	+++
AR				
Medulla	+++	+++	+++	+++
Cortical stroma	++	+++	+++	++
Primordial follicles	++	++	+	−
Growing follicles	+++	+++	+++	+++
Oocytes	++	+++	++	+

Ovaries from untreated female lambs were obtained at postnatal day 1 (PND 1), PND 5, PND 10 and PND 30, then embedded in paraffin and immunostained with the specific primary antiserum. The evaluation of immunostaining was qualitatively performed in at least three sections/ovaries, as follows: negative (−), slightly positive (−/+), weakly positive (+), positive (++), and strongly positive (+++). The number of animals evaluated per time point was at least three.

the medullar stroma from PND 1 to PND 30, and cortical stroma also showed high AR expression but decreased slightly on PND 30. Granulosa cells of growing follicles showed high expression of AR at all studied time points. The highest expression of AR in the primordial follicles was observed on PNDs 1 and 5, decreasing gradually to PND 30.

3.2. Ovarian weight in xenoestrogen-exposed lambs

Neonatal exposure to BPA decreased the weight of the ovaries of lambs at PND 30 (Control: 0.036 ± 0.005 g/kg, BPA:

0.018 ± 0.019 g/kg, $p < 0.05$), whereas the ovarian weight was not affected in DES-exposed lambs (DES: 0.029 ± 0.006 g/kg).

3.3. DES and BPA increase the primordial-to-primary follicle transition and decrease the primordial follicle reserve

Ovaries from neonatally DES- or BPA-treated lambs showed altered follicular dynamics. The percentages of the different types of follicles in control and treated-lambs at PND 30 are shown in Fig. 2. Control ovaries had approximately 60% of primordial follicles, whereas ovaries from DES- or BPA-treated lambs had fewer primordial follicles (38% and 47%, respectively; $p < 0.05$). This reduction in the primordial follicle pool was associated with an increase in the two subsequent categories of growing follicles (transitional and primary). Therefore, DES and BPA enhanced the initial recruitment process by stimulating of the primordial-to-primary follicle transition (Fig. 2).

Even though in BPA-exposed lambs the ovarian weight decreased, no differences in the total number of follicles were recorded (Control: $28,930 \pm 3621$, DES: $24,333 \pm 2962$, BPA: $27,040 \pm 5630$). On the other hand, ovaries from lambs exposed to BPA exhibited an increased incidence of MOFs (Fig. 3A). MOFs often possessed two to three distinct oocytes in a single follicle, oocytes were consistently similar in size, and they looked healthy when examined under light microscopy. MOFs were mostly at the primordial stage with a single layer of granulosa cells (Fig. 3B) and were infrequently in other stages of growing follicles (Fig. 3C).

3.4. Effects of xenoestrogens on proliferation, p27 expression and atresia

Immunoreactivity for Ki67, a cell proliferation marker, was confined to cell nuclei (Fig. 4). Ki67 expression was observed in the granulosa and theca cells of preantral (small and large) and small antral follicles in all groups. Ovaries from BPA-exposed lambs showed a tendency to a higher proliferation rate in the granulosa cells of large preantral follicles. This trend became significant in the next stage of follicular development, showing an increase in
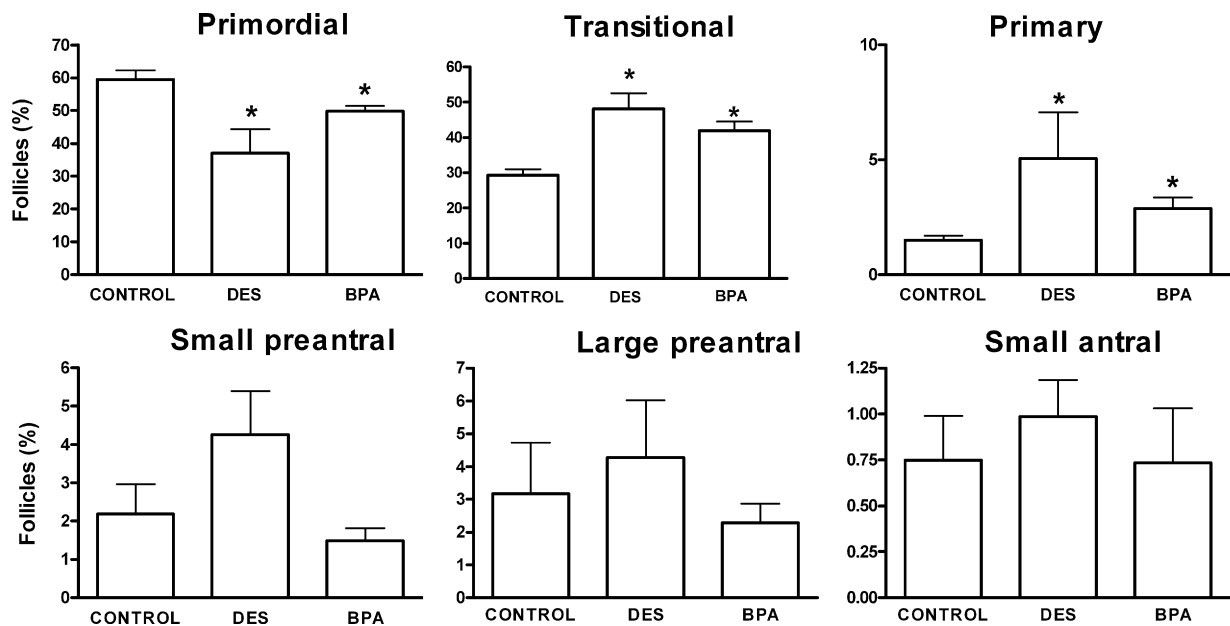

Fig. 2. Effect of neonatal exposure to DES or BPA on lamb ovarian follicular development. Serial sections of 5-μm thickness from paraffin-embedded PND 30 lamb ovaries were stained with picrosirius–hematoxylin to evaluate follicular dynamics as described in Section 2. Percentages of primordial, transitional, primary, small preantral, large preantral and small antral follicles were calculated. Following DES or BPA neonatal exposure, a significant decrease in primordial follicles together with an increase in recruited follicles was observed. Bars represent mean values ± SEM. Data were analyzed with Kruskal–Wallis followed by Dunn post hoc tests. Asterisks denote $p < 0.05$ vs. control.
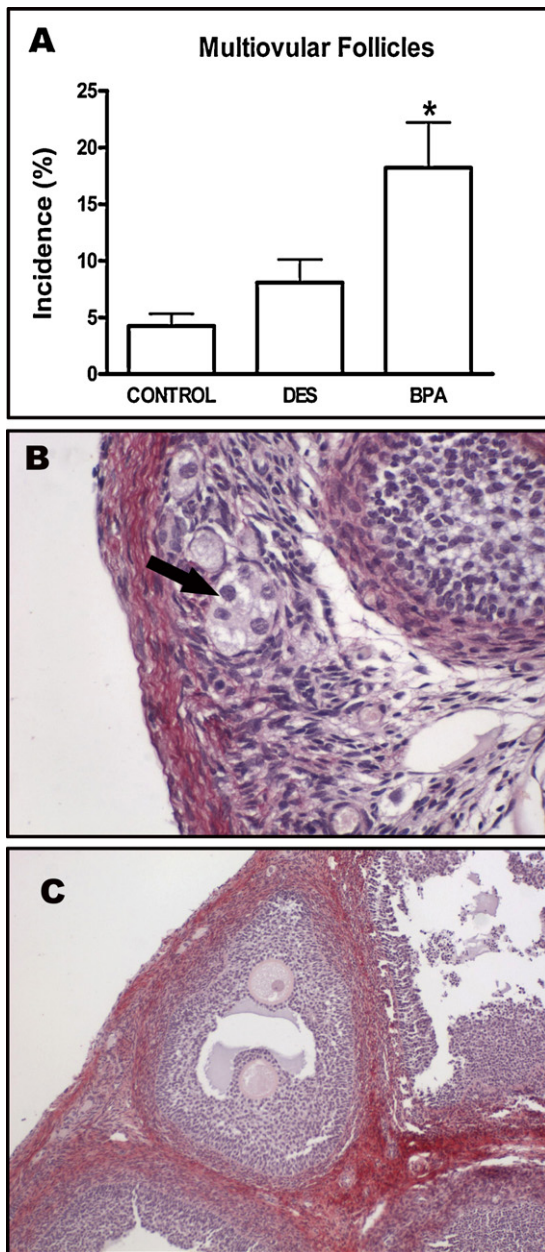
Fig. 3. Incidence of multivular follicles (MOFs) in the ovaries of neonatally DES- or BPA-exposed lambs. Ovaries from PND30 female lambs were serially sectioned and stained with picrosirius–hematoxylin. (A) Percentages of MOFs with respect to total number of follicles. Data are expressed as mean ± SEM. Data were analyzed with Kruskal–Wallis followed by Dunn post hoc tests. Asterisks denote $p < 0.05$ vs. control. Representative photomicrographs of a MOF at the primordial stage with three distinct oocytes in a single follicle (B) and a MOF in the small antral stage (C). Magnification: 400×.

the proliferation rate of both the granulosa and theca cells in small antral follicles (Figs. 4 and 5B). In DES-exposed animals, a significant increment in granulosa cell proliferation in both small (data not shown) and large preantral follicles was found (Fig. 5A), whereas in small antral follicles a high proliferation rate was observed, but this did not reach statistical significance (Figs. 4 and 5).

Immunostaining for p27 was confined to cell nuclei of granulosa cells and oocytes of all follicular stages (Fig. 4D–F). The staining of p27 was differentially localized in specific granulosa cell layers, showing a preferential expression on the basal part of the mural granulosa cells. Both DES- and BPA-exposed animals showed a higher expression of p27 in granulosa cells of small antral follicles (Figs. 4 and 6A).

BPA and DES promoted an increased number of small antral atretic follicles (Fig. 6B). It is important to highlight that this effect was associated with higher p27 expression; i.e., this protein was preferentially expressed in the population of atretic follicles.

3.5. Effects of xenoestrogen exposure on steroid receptor expression and ovarian steroidogenesis

At PND 30, the immunohistochemical expression pattern of ERα, ERβ and AR was not affected in neonatally BPA- or DES-exposed female lambs.

The effects of the neonatal xenoestrogen exposure on the steroidogenic activity of the ovary were studied by measuring the serum levels of E_2 and T in lambs at PND 30. Xenoestrogen exposure did not alter the serum levels of E_2 (Control: 15.24 ± 1.65 pg/ml, DES: 17.84 ± 1.88 pg/ml, BPA: 14.38 ± 1.44 pg/ml) or T (Control: 0.152 ± 0.011 ng/ml, DES: 0.137 ± 0.013 ng/ml, BPA: 0.125 ± 0.008 ng/ml).

4. Discussion

Most information regarding effects of environmental pollutants on ovarian development come from non-precocial species mainly rodent models. The aim of this study was to establish whether neonatal exposure to low doses of two widespread xenoestrogens, BPA and DES, can disrupt ovarian follicular dynamics in the lamb, a precocial species in which the follicular development trajectory is similar to humans [35,36]. Our results clearly show that subcutaneous BPA or DES injections from birth to PND 14 caused a decline in the stock of primordial follicles by stimulating follicular development and increasing follicular atresia at PND 30. In a prepubertal stage, an accelerated folliculogenesis must lead to increased follicular atresia. On the other hand, the exposure to BPA resulted in a lower ovarian weight and a higher incidence of MOFs. This increased incidence of MOFs may also have contributed to the decline in the pool of primordial follicles. Present study adds new data to the growing body of evidence on adverse effects following neonatal exposure to low doses of BPA or DES, and it is the first to demonstrate that xenoestrogen exposure promotes early follicular development not only in rodents, as we recently reported [20], but also in a species in which follicular assembly starts long before birth. Additionally, the present data show that the lamb ovary is sensitive to disruptions by EDC exposure during early postnatal life.

A previous work described the expression of steroid receptors in fetal, neonatal (4-week-old) and adult sheep ovary [35]. Since, in the present work, lambs were exposed to the EDC during the first two weeks of postnatal life, a description of ovarian steroid receptor protein expression during the early postnatal development (PND 1, 5, 10 and 30) was done. At PND30 (the only overlapping time-point), ours and Juengel et al. [35] results were almost identical with minor differences in ERα protein expression distribution. BPA and DES are both xenoestrogens that act mainly through estrogen receptors. BPA is commonly described as a "weak" estrogen that binds to the nuclear ERβ with approximately 7 times greater affinity than to ERα [37]. BPA also interacts with a variety of other cellular targets and acts as an androgen receptor antagonist [38]. On the other hand, DES is a potent synthetic estrogen that, in the past, was used to treat miscarriage and promote growth in cattle and sheep [5,26]. The early and high expression of ERβ throughout the first month of lamb life suggests that the ovarian alterations found in the DES and BPA groups may be mediated mainly through ERβ.

The BPA-exposed lambs showed decreased ovarian weight after normalization to body weights. This result is consistent with that
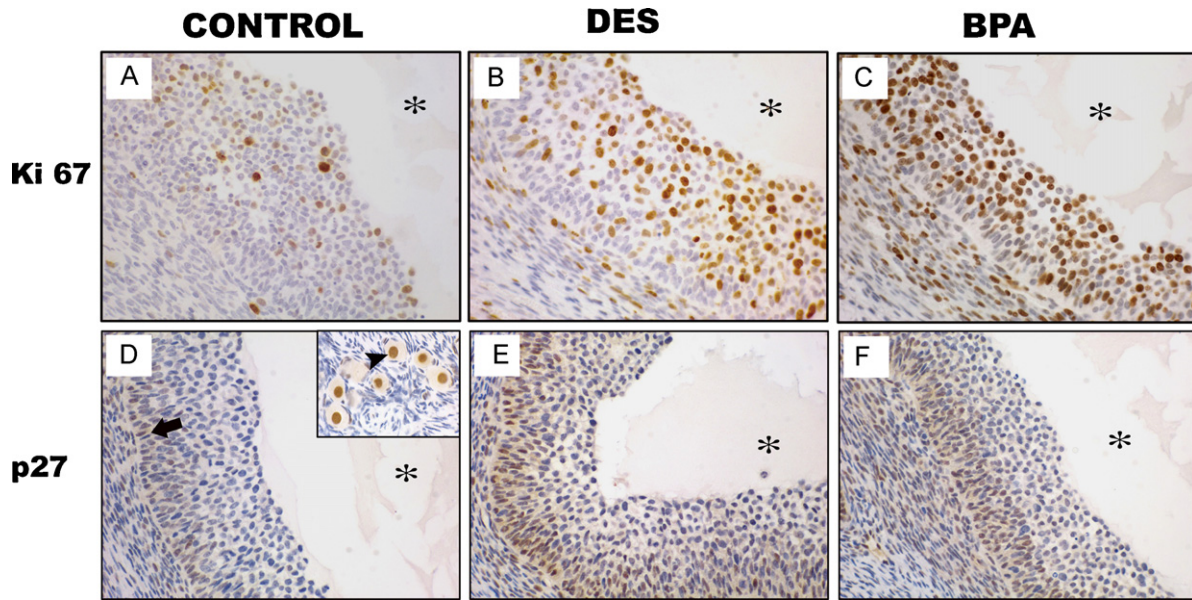
Fig. 4. Effect of neonatal exposure to DES or BPA on proliferation rate (Ki67 expression) and on the expression of cyclin-dependent kinase inhibitor 1B (p27) in ovarian follicles from PND 30 lambs. Representative photomicrographs of ovarian sections immunostained with Ki67- or p27-specific antibodies showing nuclear positivity in granulosa and/or theca cells of small antral follicles. (A) Ki67 expression was observed in the granulosa and theca cells of growing follicles. BPA-exposed lambs showed increased proliferation in both cellular compartments (C). (D) p27 expression was found in the oocyte nucleus at all follicle stages (inset, immunostained oocytes in primordial follicles) and in the granulosa cells of primordial and small antral follicles (arrow). DES- or BPA-exposed lambs showed and increase in p27 expression (E, F). Immunohistochemistry was developed by DAB and counterstaining with Mayer hematoxylin. *Fluid-filled cavity (*antrum*). Magnification: 600×.

found in neonatal rats treated with estradiol valerate, in which the ovarian size is smaller than in controls [39]. However, these findings contrast with those of Evans et al. [40], who reported that ovary weight is similar among female lambs treated i.m. (twice/week) with BPA, DES or octylphenol from PND 30 until PND 50. This discrepancy may be explained by considering the different administration routes, doses, frequencies, and, especially, the times of exposure.

Follicular development is under the coordinated regulation of factors from the oocyte and the somatic cells [41]. Follicular promotion or activation is an irreversible process by which primordial follicles initiate growth and follicular cells proliferate and change their morphology (primary follicle) [41,42]. A balance between inhibitory and stimulatory factors of systemic and/or local origin probably regulates this activation process in a gonadotropin-independent period [43]. We found a significant decrease in the reserve of primordial follicles associated with an increase in the transition of primordial follicles to primary in DES- or BPA-exposed lambs. This stimulus in the early stages of follicular growth also affected the more advanced stages of follicle development, as we found a higher rate of proliferation (measured by Ki67) of granulosa and/or theca cells in growing follicles of BPA- or DES-exposed animals (DES: large preantral follicles; BPA: small antral follicles). This altered pattern of follicular development, triggered by DES or BPA, suggests that the disruption of the normal "dialogue" between oocyte and granulosa cells could be the preferred mode of action when xenoestrogen exposure occurs in this early period of ovarian development. We have recently obtained similar results by exposing neonatal rats to DES or BPA [20], thus neonatal exposure to BPA or DES triggers early follicular development in both species.

The ovaries of female lambs exposed to DES or BPA also showed a higher percentage of antral atretic follicles. Thus, the population of antral follicles can be divided into two subpopulations: a subpopulation with a high proliferation rate and another with low proliferation rate or atretic follicles. Because the normal fate of growing follicles in prepubertal animals is atretic demise [44], our results suggest that the highest percentage of atretic follicles
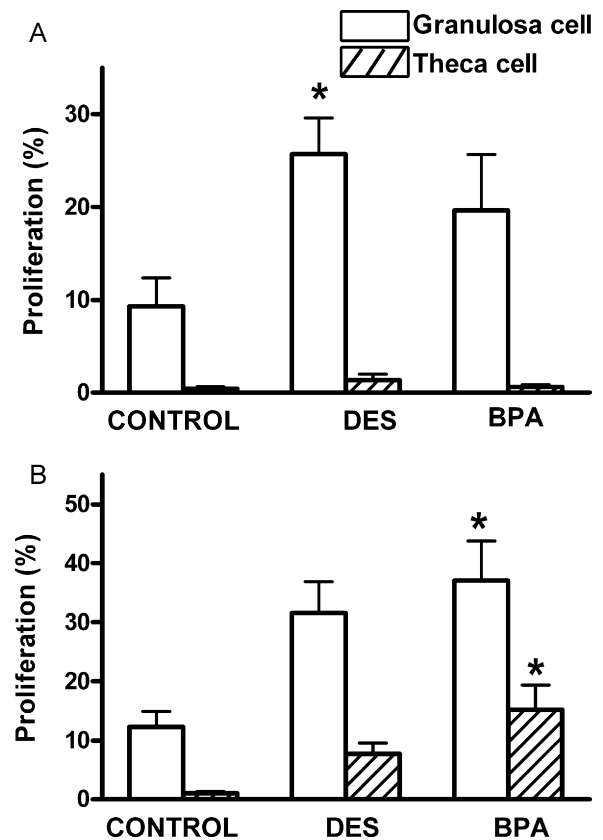

Fig. 5. Effect of neonatal exposure to DES or BPA on proliferation of granulosa and theca cells in large preantral follicles (A) and small antral follicles (B) from PND 30 lamb ovaries. Ki67 was used as proliferation marker. Results are expressed as the percentage of positive immunostained nuclei of each cellular subtype (atretic follicles were excluded in the Ki67 quantification). Bars represent mean values ± SEM. Data were analyzed with Kruskal–Wallis followed by Dunn post hoc tests. Asterisks denote $p < 0.05$ vs. control.
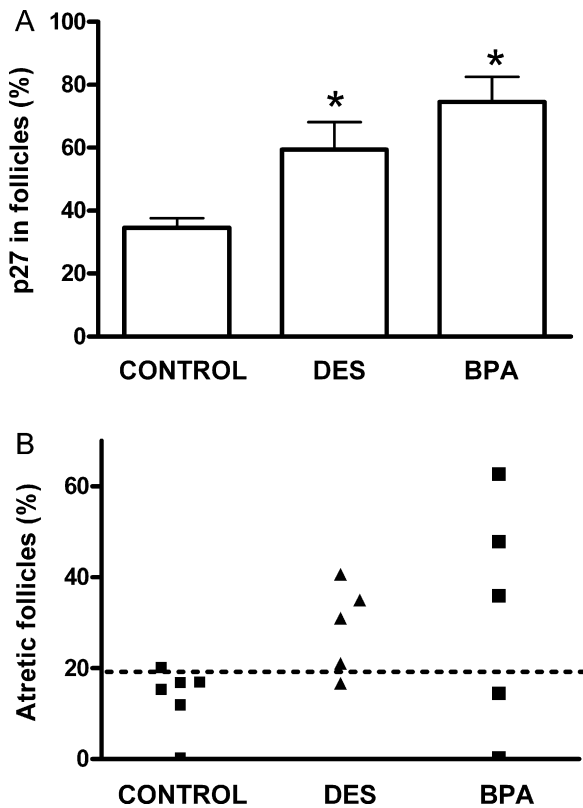
Fig. 6. Effect of neonatal exposure to DES or BPA on p27 expression and on atresia of small antral follicles from PND 30 lamb ovaries. (A) The quantification of p27 was expressed as the percentage of positive immunostained granulosa cells in small antral follicles. Bars represent mean values ± SEM. Data were analyzed with Kruskal–Wallis followed by Dunn post hoc tests. Asterisks denote $p < 0.05$ vs. control. (B) Individual values of the percentage of atretic small follicles in each female lamb were plotted in this graph. The dashed line represents the highest recorded proportion of atretic follicles in control lamb ovaries. Most of the DES- or BPA-exposed animals showed a percentage of atretic follicles higher than control females.

may be caused by the accelerated follicular development triggered by xenoestrogens. Interestingly, in BPA- or DES-exposed animals, we also found increased expression of p27 in oocytes and in the granulosa cells of small antral atretic follicles. Our results are consistent with those from Rajareddy et al. [45] who reported that, in mice, p27 expression in oocyte and pregranulosa/granulosa nuclei activates caspases that mediate follicle atresia. Taken together, all these data show that the exposure of neonatal lambs to BPA or DES alters ovarian dynamics by promoting follicular activation and increasing proliferation in preantral/antral follicles. In prepubertal animals, this wave of accelerated follicular development would cause an increased number of antral follicles that overexpress p27 and undergo follicular atresia.

The precise mechanisms that alter ovarian dynamics and their impact on adult ovarian function remain to be determined. Previously, it was suggested that the effects of a single dose of octylphenol may vary in relation to the stage of development at which sheep exposure occurs [46]. As xenoestrogens act via steroid receptors, they can have "organizational" or "activational" effects [47]. Organizational effects are those that occur during development and result in permanent changes in function, whereas activational effects are the temporary changes in the activity of a system that occur following exposure when the development of a system is complete. Further studies using the same xenoestrogen exposure schedule but evaluating ovarian alteration in adults will be necessary to define if the described effects are transient or permanent.

Another significant finding of the present study is the increased incidence of MOFs in the ovaries of lambs neonatally exposed to BPA. One of the major developmental events during ovarian folliculogenesis is the follicular assembly [44]. Because MOFs are oocyte clusters that did not separate and become enclosed individually in follicles [48], a 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 [28]. Because exposure to BPA stimulated the primordial to primary follicle transition and also promoted MOF occurrence, both events might be responsible for a reduction in ovarian follicular reserve. Increased incidence of MOFs has been observed in ovaries from mice prenatally or neonatally exposed to estrogenic compounds [22,49,50]. Studying the receptor type through estrogen exerts its influence over germ cell nest breakdown; Jefferson et al. [49] reported that ERβ-knockout mice exposed to estrogenic compounds do not form MOFs, whereas ERα-knockout or wild-type mice do. It is concluded that early folliculogenesis and the effects of estrogen on follicle development are mediated through ERβ [51]. Here, we found that primordial follicles did not express ERα, while the granulosa of preantral follicles expressed only low amounts during the BPA administration period. In contrast, follicles showed a high expression of ERβ from PND 5 onwards. Thus, neonatal exposure to BPA in lambs might extend the period of estrogenic stimulus acting through ERβ (likely in the granulosa cells), which could be responsible for the increased incidence in MOFs. This is surprising since it is considered that nest breakdown in sheep is completed before birth [36]. Our findings add new evidence to the hypothesis that continuous exposure to estrogen preserves germ cell nests probably via ERβ signaling, which leads to the assembly of pregranulosa cells around multiple oocytes. Previous BPA studies have reported that MOFs are induced by postnatal injections of a large dose (100 mg/kg/day) but not by a low dose (10 mg/kg/day) of BPA [22]. Here the induction of MOFs in lambs was found using an even lower dose of BPA (50 µg/kg/day, considered the "safe dose" according to EPA). The neonatal exposure of lambs to BPA altered oocyte nest breakdown, and this effect might influence female fertility because oocytes derived from MOFs have a reduced fertilization rate [52]. In contrast with the results obtained in mice and rats [6,20], neonatal exposure of lambs to DES did not induce the formation of MOFs. Even though, lambs neonatally exposed to DES showed altered follicular dynamics, it may be possible that MOF formation requires a different DES dose. Supporting this, we have found that neonatal exposure of rats to 20 µg/kg/day of DES induces MOFs but a 100-fold lower dose does not [20].

BPA exposure has been associated with recurrent miscarriage in women [53], and serum levels of BPA are greater in women with polycystic ovarian syndrome (PCOS) [54] compared to healthy controls. Our findings demonstrate that early postnatal exposure of sheep to BPA or DES decreased the proportion of primordial follicles, associated with a complementary increase of transitional and primary follicles; moreover, BPA increased the incidence of MOFs. These alterations suggest that postnatal exposure to BPA or DES accelerates follicular recruitment and reduces ovarian reserve. The reduced ovarian reserve may lead to an early cyclicity cessation and/or an increased incidence of premature ovarian failure (POF). Interestingly, this effect occurs in a species in which follicular assembly starts during embryonic development. Studies are being conducted to explore the effect of neonatal exposure to BPA or DES on sheep physiological events in which the ovary is involved, such as puberty, ovulatory and superovulatory response, luteal phase, and steroidogenic activity. Even though studying the adult reproductive performance of these exposed lambs will lead to clearer conclusions, present results suggest that early postnatal exposure to low doses of BPA or DES affects the developing lamb ovary in such a way as to reduce fertility of the adult sheep. Moreover,

these findings might provide valuable insight into the etiology of ovarian dysfunctions such as POF, PCOS, and altered cyclicity and fecundability.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

References

[1] Colborn T, vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 1993;101: 378–84.

[2] Adams NR. Detection of the effects of phytoestrogens on sheep and cattle. J Anim Sci 1995;73:1509–15.

[3] Adams NR, Sanders MR, Ritas AJ. Oestrogenic damage and reduced fertility in ewe flocks in South Western Australia. Aust J Agric Res 1988;39:71–7.

[4] Smith JF, Jagusch KT, Brunswick LF, Kelly RW. Coumestans in lucerne and ovulation in ewes. NZ J Agric Res 1979;22:411–6.

[5] McLachlan JA, Korach KS, Newbold RR, Degen GH. Diethylstilbestrol and other estrogens in the environment. Fundam Appl Toxicol 1984;4:686–91.

[6] Iguchi T, Takasugi N, Bern HA, Mills KT. Frequent occurrence of polyovular follicles in ovaries of mice exposed neonatally to diethylstilbestrol. Teratology 1986;34:29–35.

[7] Wordinger RJ, Derrenbacker J. In utero exposure of mice to diethylstilbestrol alters neonatal ovarian follicle growth and development. Acta Anat (Basel) 1989;134:312–8.

[8] NTP-CERHR. NTP-CERHR monograph on the potential human reproductive and developmental effects of bisphenol A. NTP CERHR MON, vol. 22. NTP-CERHR BPA monograph; 2008. p. i–III1.

[9] Welshons WV, Nagel SC, vom Saal F. Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. Endocrinology 2006;147:56–69.

[10] Muñoz-de-Toro M, Markey CM, Wadia PR, Luque EH, Rubin BS, Sonnenschein C, et al. Perinatal exposure to bisphenol A alters peripubertal mammary gland development in mice. Endocrinology 2005;146:4138–47.

[11] Ramos JG, Varayoud J, Sonnenschein C, Soto AM, Muñoz-de-Toro M, Luque EH. Prenatal exposure to low doses of bisphenol A alters the periductal stroma and glandular cell function in the rat ventral prostate. Biol Reprod 2001;65: 1271–7.

[12] Ramos JG, Varayoud J, Kass L, Rodríguez H, Costabel L, Muñoz-De-Toro M, et al. induces both transient and permanent histofunctional alterations of the hypothalamic–pituitary–gonadal axis in prenatally exposed male rats. Endocrinology 2003;144:3206–15.

[13] Durando M, Kass L, Piva J, Sonnenschein C, Soto AM, Luque EH, et al. Prenatal bisphenol A exposure induces preneoplastic lesions in the mammary gland in Wistar rats. Environ Health Perspect 2007;115:80–6.

[14] Monje L, Varayoud J, Luque EH, Ramos JG. Neonatal exposure to bisphenol A modifies the abundance of estrogen receptor alpha transcripts with alternative 5′-untranslated regions in the female rat preoptic area. J Endocrinol 2007;194:201–12.

[15] Stoker C, Rey F, Rodríguez H, Ramos JG, Sirosky P, Larriera A, et al. Sex reversal effects on Caiman latirostris exposed to environmentally relevant doses of the xenoestrogen bisphenol A. Gen Comp Endocrinol 2003;133:287–96.

[16] Stoker C, Beldoménico PM, Bosquiazzo VL, Zayas MA, Rey F, Rodríguez H, et al. Developmental exposure to endocrine disruptor chemicals alters follicular dynamics and steroid levels in Caiman latirostris. Gen Comp Endocrinol 2008;156:603–12.

[17] Varayoud J, Ramos JG, Bosquiazzo VL, Muñoz-de-Toro M, Luque EH. Developmental exposure to bisphenol A impairs the uterine response to ovarian steroids in the adult. Endocrinology 2008;149:5848–60.

[18] Rey F, Gonzalez M, Zayas MA, Stoker C, Durando M, Luque EH. Muñoz-de-Toro. Prenatal exposure to pesticides disrupts testicular histoarchitecture and alters testosterone levels in male Caiman latirostris. Gen Comp Endocrinol 2009;162:286–92.

[19] Bosquiazzo VL, Varayoud J, Muñoz-de-Toro M, Luque EH, Ramos JG. Effects of neonatal exposure to bisphenol A on steroid regulation of vascular endothelial growth factor expression and endothelial cell proliferation in the adult rat uterus. Biol Reprod 2010;82:86–95.

[20] Rodríguez HA, Santambrosio N, Santamaría CG, Muñoz-de-Toro M, Luque EH. Neonatal exposure to bisphenol A reduces the pool of primordial follicles in the rat ovary. Reprod Toxicol 2010;30:550–7.

[21] Susiarjo M, Hassold TJ, Freeman E, Hunt PA, Bisphenol A. exposure in utero disrupts early oogenesis in the mouse. PLoS Genet 2007;3:e5.

[22] Suzuki A, Sugihara A, Uchida K, Sato T, Ohta Y, Katsu Y, et al. Developmental effects of perinatal exposure to bisphenol-A and diethylstilbestrol on reproductive organs in female mice. Reprod Toxicol 2002;16:107–16.

[23] Savabieasfahani M, Kannan K, Astapova O, Evans NP, Padmanabhan V. Developmental programming: differential effects of prenatal exposure to bisphenol-A or methoxychlor on reproductive function. Endocrinology 2006;147: 5956–66.

[24] U.S. Environmental Protection Agency. Bisphenol A, CASRN-80-05-7, IRIS, Integrated Risk Information System; 1993. http://www.epa.gov/iris/subst/0356.htm.

[25] Melnick R, Lucier G, Wolfe M, Hall R, Stancel G, Prins G, et al. Summary of the National Toxicology Program's report of the endocrine disruptors low-dose peer review. Environ Health Perspect 2002;110:427–31.

[26] Newbold RR. Lessons learned from perinatal exposure to diethylstilbestrol. Toxicol Appl Pharmacol 2004;199:142–50.

[27] Lundy T, Smith P, OïConnell A, Hudson NL, McNatty KP. Populations of granulosa cells in small follicles of the sheep ovary. J Reprod Fertil 1999;115:251–62.

[28] Tilly JL. Ovarian follicle counts – not as simple as 1, 2, 3. Reprod Biol Endocrinol 2003;1:11.

[29] Britt KL, Saunders PK, McPherson SJ, Misso ML, Simpson ER, Findlay JK. Estrogen actions on follicle and early follicle development. Biol Reprod 2004;71:1712–23.

[30] Muñoz-de-Toro M, Maffini MV, Kass L, Luque EH. Proliferative activity and steroid hormone receptor status in male breast carcinoma. J Steroid Biochem Mol Biol 1998;67:333–9.

[31] Rodriguez HA, Kass L, Varayoud J, Ramos JG, Ortega HH, Durando M, et al. Collagen remodelling in the guinea-pig uterine cervix is associated with a decreased in progesterone receptor expression. Mol Hum Reprod 2003;9:807–13.

[32] Delgado-Rosas F, Gaytán M, Morales C, Gómez R, Gaytán F. Superficial ovarian cortex vascularization is inversely related to the follicle reserve in normal cycling ovaries and is increased in polycystic ovary syndrome. Hum Reprod 2009;24:1142–51.

[33] Jolly PD, Smith PR, Heath DA, Hudson NL, Lun S, Still LA, et al. Morphological evidence of apoptosis and the prevalence of apoptotic versus mitotic cells in the membrane granulosa of ovarian follicles during spontaneous and induced atresia in ewes. Biol Reprod 1997;56:837–46.

[34] Bosquiazzo VL, Ramos JG, Varayoud J, Muñoz-de-Toro M, Luque EH. Mast cell degranulation in rat uterine cervix during pregnancy correlates with expression of vascular endothelial growth factor mRNA and angiogenesis. Reproduction 2007;133:1045–55.

[35] Juengel JL, Heath DA, Quirke LD, McNatty KP. Oestrogen receptor α and β, androgen receptor and progesterone receptor mRNA and protein localisation within the developing ovary and in small growing follicles of sheep. Reproduction 2006;131:81–92.

[36] Juengel JL, Sawyer HR, Smith PR, Quirke LD, Heath DA, Lun S, et al. Origins of follicular cells and ontogeny of steroidogenesis in ovine fetal ovaries. Mol Cell Endocrinol 2002;191:1–10.

[37] Kuiper GG, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S, et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 1997;138:863–70.

[38] Wetherill YB, Akingbemi BT, Kanno J, McLachlan JA, Nadal A, Sonnenschein C, et al. In vitro molecular mechanisms of bisphenol A action. Reprod Toxicol 2007;24:178–98.

[39] Sotomayor-Zárate R, Dorfman M, Paredes A, Lara HE. Neonatal exposure to estradiol valerate programs ovarian sympathetic innervation and follicular development in the adult rat. Biol Reprod 2008;78:673–80.

[40] Evans NP, North T, Dye S, Sweeney T. Differential effects of the endocrine-disrupting compounds bisphenol-A and octylphenol on gonadotropin secretion, in prepubertal ewe lambs. Domest Anim Endocrinol 2004;26: 61–73.

[41] Binelli M, Murphy BD. Coordinated regulation of follicle development by germ and somatic cells. Reprod Fertil Dev 2010;22:1–12.

[42] Webb R, Campbell BK. Development of the dominant follicle: mechanisms of selection and maintenance of oocyte quality. Soc Reprod Fertil 2007;64:141–63.

[43] van den Hurk R, Zhao J. Formation of ovarian follicles and their growth, differentiation and maturation within ovarian follicles. Theriogenology 2005;63:1717–51.

[44] McGee EA, Hsueh AJW. Initial and cyclic recruitment of ovarian follicles. Endocr Rev 2000;21:200–14.

[45] Rajareddy S, Reddy P, Du C, Liu L, Jagarlamudi K, Tang W, et al. p27kip1 (cyclin-dependent kinase inhibitor 1B) controls ovarian development by suppressing follicle endowment and activation and promoting follicle atresia in mice. Mol Endocrinol 2007;21:2189–202.

[46] Sweeney T, Nicol L, Roche JF, Brooks AN. Maternal exposure to octylphenol suppresses ovine fetal follicle stimulating hormone secretion, testis size and sertoli cell number. Endocrinology 2000;141:2667–73.

[47] MacLusky NJ, Naftolin F. Sexual differentiation of the central nervous system. Science 1981;211:1294–303.

[48] Pepling ME. From primordial germ cell to primordial follicle: mammalian female germ cell development. Genesis 2006;44:622–32.

[49] Jefferson WN, Couse JF, Padilla-Banks E, Korach KS, Newbold RR. Neonatal exposure to genistein induces estrogen receptor (ER)alpha expression and multioocyte follicles in the maturing mouse ovary: evidence for ERbeta-mediated and nonestrogenic actions. Biol Reprod 2002;67:1285–96.

[50] Kipp JL, Kilen SM, Bristol-Gould S, Woodruff TK, Mayo KE. Neonatal exposure to estrogens suppresses activin expression and signalling in the mouse ovary. Endocrinology 2007;148:1968–76.

[51] Hegele-Hartung C, Siebel P, Peters O, Kosemund D, Müller G, Hillisch A, et al. Impact of isotype-selective estrogen receptor agonists on ovarian function. Proc Natl Acad Sci USA 2004;101:5129–34.

[52] Iguchi T, Fukazawa Y, Uesugi Y, Takasugi N. Polyovular follicles in mouse ovaries exposed neonatally to diethylstilbestrol in vivo and in vitro. Biol Reprod 1990;43:478–84.

[53] Sugiura-Ogasawara M, Ozaki Y, Sonta S, Makino T, Suzumori K. Exposure to bisphenol A is associated with recurrent miscarriage. Hum Reprod 2005;20:2325–9.

[54] Takeuchi T, Tsutsumi O, Ikezuki Y, Takai Y, Taketani Y. Positive relationship between androgen and the endocrine disruptor, bisphenol A, in normal women and women with ovarian dysfunction. Endocr J 2004;51:165–9.