

# Neonatal exposure to xenoestrogens impairs the ovarian response to gonadotropin treatment in lambs

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## Abstract

Bisphenol A (BPA) and diethylstilbestrol (DES) are xenoestrogens, which have been associated with altered effects on reproduction. We hypothesized that neonatal xenoestrogen exposure affects the ovarian functionality in lambs. Thus, we evaluated the ovarian response to exogenous ovine FSH (oFSH) administered from postnatal day 30 (PND30) to PND32 in female lambs previously exposed to low doses of DES or BPA (BPA50: 50 µg/kg per day, BPA0.5: 0.5 µg/kg per day) from PND1 to PND14. We determined: i) follicular growth, ii) circulating levels of 17β-estradiol (E<sub>2</sub>), iii) steroid receptors (estrogen receptor alpha, estrogen receptor beta, and androgen receptor (AR)) and atresia, and iv) mRNA expression levels of the ovarian bone morphogenetic protein (BMPs) system (*BMP6*, *BMP15*, *BMPR1B*, and *GDF9*) and FSH receptor (*FSHR*). Lambs neonatally exposed to DES or BPA showed an impaired ovarian response to oFSH with a lower number of follicles ≥ 2 mm in diameter together with a lower number of atretic follicles and no increase in E<sub>2</sub> serum levels in response to oFSH treatment. In addition, AR induction by oFSH was disrupted in granulosa and theca cells of lambs exposed to DES or BPA. An increase in *GDF9* mRNA expression levels was observed in oFSH-primed lambs previously treated with DES or BPA50. In contrast, a decrease in *BMPR1B* was observed in BPA0.5-postnatally exposed lambs. The modifications in AR, *GDF9*, and *BMPR1B* may be associated with the altered ovarian function due to neonatal xenoestrogen exposure in response to an exogenous gonadotropin stimulus. These alterations may be the pathophysiological basis of subfertility syndrome in adulthood.

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## Introduction

Numerous chemicals in the environment possess estrogenic activity and are classified as endocrine-disrupting compounds (EDCs; McLachlan *et al.* 1984). Some of these chemicals may alter gonadal morphogenesis and functional differentiation, affecting reproduction if exposure occurs during critical periods of development (Colborn *et al.* 1993).

Both diethylstilbestrol (DES) and bisphenol A (BPA) are EDCs that have been extensively studied using different animal models. DES is a synthetic estrogen with a stronger bioactivity than 17β-estradiol (E<sub>2</sub>) (McLachlan *et al.* 1984). In the past, DES was widely used in human and veterinary medicine, and significant levels were reported in the environment, mainly related to feedlot areas (McLachlan *et al.* 1984). On the other hand, BPA is one of the highest volume chemicals produced worldwide, as it is used in polycarbonate plastics, resins, papers, implanted medical devices, and other medical

equipment (Welshons *et al.* 2006, Shelby 2008). BPA has also been detected in a variety of environmental samples, including water, sewage leach, indoor and outdoor air samples, and dust (Vandenberg *et al.* 2007). As 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 (Welshons *et al.* 2006).

The lamb ovary is sensitive to disruption by EDC exposure during intrauterine life (Adams *et al.* 1988, Adams 1995, Savabieasfahani *et al.* 2006, Fowler *et al.* 2008) or during early postnatal life (Rivera *et al.* 2011). In sheep, a precocial species, we have previously demonstrated that low doses of s.c. BPA or DES injections from birth to postnatal day 14 (PND14) cause a decline in the stock of primordial follicles by stimulating follicular development and increasing follicular atresia (Rivera *et al.* 2011). We also found that exposure to BPA results in a lower weight of the lamb ovaries and a higher incidence

of multiovular follicles (MOFs) on PND30 (Rivera *et al.* 2011). These adverse effects may be mediated through abnormal early protein levels of ovarian estrogen receptors (ERs) and could alter ovarian function and female fertility (Rivera *et al.* 2011). Nagel & Bromfield (2013) suggested that BPA can directly bind to both ERs and increase endogenous estrogen levels via upregulation of aromatase enzyme, increasing the overall estrogenic effects during development.

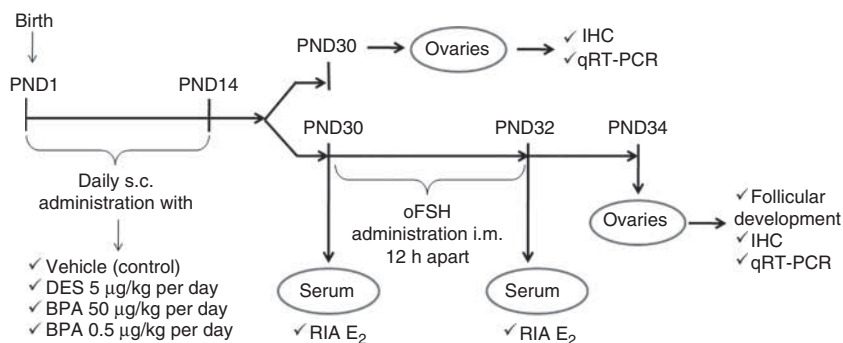
Various models have been used to test endocrine disruption of ovarian function in rodents, primates, and other species. One of the most widely used ovarian endocrine-disruption models is the immature animal primed with exogenous hormones (Petroff *et al.* 2000, Sekiguchi *et al.* 2003). This animal model allows detecting dysfunctions in the development of growing follicles that will reach the pre-ovulatory stage, the number of corpora lutea and ova shed, and the levels of ovarian hormones. In addition, the use of this procedure to investigate female reproductive toxicity certainly simplifies and reduces the time-consuming properties of routine experiments (such as evaluation of the estrous cycle, spontaneously ovulated ova, etc.) and allows the development of toxicological procedures to elucidate the mechanisms of toxicants, which impair the female reproductive system (Sekiguchi *et al.* 2003). Based on these reasons, we selected the ovarian response to an exogenous gonadotropin treatment as a tool to study ovarian functionality in immature lambs neonatally exposed to xenoestrogens. In this study, we investigated whether the neonatal exposure to low doses of BPA or DES adversely affects the ovarian response to an exogenous treatment of ovine follicle-stimulating hormone (oFSH) in prepubertal lambs and examined its possible association with abnormalities in steroid receptor pathways. Moreover, as one of the potential mechanisms underlying the ovarian response to oFSH treatment may reside in the bone morphogenetic protein (BMP) system that controls follicular dynamics and ovulation rate (Fabre *et al.* 2006), the mRNA expression of *BMP6* and *BMP15*, growth and differentiation factor 9 (*GDF9*), and BMP receptor 1B (*BMPR1B*) was also evaluated.

## Material and methods

### Animals and experimental design

All the procedures were revised and authorized by the Institutional Committee of Animal Use and Care of Universidad Nacional del Litoral (Santa Fe, Argentina). 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 with Hampshire Down rams. No supplementary feeding was required along pregnancy and lactation. Female lambs selected for the experiments were born during August and September from a single delivery (no twins were used). The phytoestrogen concentration in the pasture was not evaluated; however, because food intake in control and treated animals was equivalent, we assumed that all animals were exposed to the same levels of phytoestrogens. Mothers and offspring remained under natural conditions during the experiment.

After birth, female lambs were randomly assigned to one of the following postnatal daily treatments (Fig. 1), from PND1 (this being the day of birth) to PND14, by s.c. injections in the nape of the neck: i) corn oil vehicle (controls;  $n=18$ ), ii) DES (Sigma–Aldrich) at 5  $\mu\text{g}/\text{kg}$  per day ( $n=13$ ), iii) BPA50 (99% purity, Sigma–Aldrich) at 50  $\mu\text{g}/\text{kg}$  per day ( $n=16$ ), and iv) BPA0.5 at 0.5  $\mu\text{g}/\text{kg}$  per day ( $n=9$ ). Although the s.c. route of administration for EDC is not the natural mode of exposure, we selected this method to be certain of the dose administered to the animals. The postnatal model of exposure to xenoestrogens has been extensively used in our laboratory in both rodents (Monje *et al.* 2007, 2009, 2010, Ramos *et al.* 2007, Varayoud *et al.* 2008, Bosquiazzo *et al.* 2010, Rodríguez *et al.* 2010) and lambs (Rivera *et al.* 2011) and has been demonstrated as a persuasive paradigm to study short- and long-term consequences of neonatal exposure to hormonally active substances. On the other hand, the route of administration is an important issue to determine BPA health risks in animal models. In fetuses and neonates, Taylor *et al.* (2008) observed low levels of the enzyme that conjugate BPA (uridine diphosphate-glucuronosyl-transferase), implying that both oral and non-oral administration of BPA during neonatal life provide the same internal active dose.



**Figure 1** Schematic representation of the experimental protocol used to study the effects of early postnatal diethylstilbestrol (DES) or bisphenol A (BPA) exposure on the ovary of lambs. Daily administration was carried out by s.c. injections. PND, postnatal day; IHC, immunohistochemistry; qRT-PCR: quantitative real-time PCR.

The EPA-National Toxicology Program's Report of the Endocrine Disruptors-USA (U.S.EPA 1993) has defined the LOAEL dose for BPA as 50 mg/kg per day and the 'safe dose' as 1000 times lower (50 µg/kg per day) (Melnick *et al.* 2002, Shelby 2008). In this work, we used the safe dose of BPA and a dose 100 times lower. DES was used as a positive control because it has been reported that developmental exposure to low doses of this compound induces MOFs and activates the primordial-to-primary follicle transition in mice (Iguchi *et al.* 1986, Wordinger & Derrenbacker 1989), rats (Rodríguez *et al.* 2010), and lambs (Rivera *et al.* 2011). The dose of 5 µg/kg per day of DES used in this study is considered a low dose (Newbold 2004), being 20-fold lower than that given therapeutically to pregnant women.

On PND30, lamb ovaries from the experimental groups (control  $n=6$ ; DES  $n=4$ ; BPA50  $n=5$ ) were removed via a midline abdominal incision under ketamine (20 mg/kg, i.m.) and xylazine (0.1–0.2 mg/kg, i.m.) anesthesia. The remaining lambs from each experimental group (control  $n=12$ ; DES  $n=9$ ; BPA50  $n=11$ ; BPA0.5  $n=9$ ) were treated with multiple doses of oFSH (Ovagen, ICPbio Ltd, Auckland, New Zealand) starting on PND30. Each lamb received a total dose of 8.8 mg of oFSH. oFSH was administered every 12 h by i.m. injection for 3 consecutive days (at 0800 and 2000 h, PND30, PND31, and PND32). This treatment protocol was adapted, with minor modifications, from that described previously by Kelly *et al.* (2005). Forty hours after the last oFSH injection (PND34), ovaries were exhibited by medial laparotomy under general anesthesia (ketamine + xylazine). Follicles  $\geq 2$  mm in diameter on the ovarian surface were counted under a stereomicroscope (Olympus) to establish the oFSH response. Then, ovaries were collected, cut into halves, and processed for different experimental purposes. For immunohistochemistry, ovarian halves were fixed in 10% buffered formalin for 6 h at room temperature and paraffin embedded. For RNA extraction, the other ovarian halves were immediately frozen in liquid nitrogen and stored at  $-80$  °C. Peripheral blood was collected by jugular venipuncture before the first oFSH administration (PND30 at 0800 h) and before the last one (PND32 at 2000 h). Serum was separated and stored at  $-20$  °C until hormone assay.

### Hormone assays

Blood samples were allowed to clot for 1 h at room temperature. Serum was then collected and stored at  $-20$  °C for hormone analysis. Serum  $E_2$  levels were determined by a double-antibody RIA procedure (DSL-4800; Beckman Coulter Ultra-Sensitive Estradiol RIA, Inc., Webster, TX, USA) (Taylor *et al.* 2000, Carpenter *et al.* 2003), validated for use with ovine samples. The RIA used rabbit anti- $E_2$  (polyclonal) serum and iodinated estradiol. The primary antiserum cross-reacts 2.4% with estrone, 0.64% with estriol, 0.21% with  $17\alpha$ -estradiol, 2.56% with  $17\beta$ -estradiol-3-glucuronide, 0.17% with estradiol-3-sulfate, and 3.4% with  $D$ -equilenin. Goat anti-rabbit gamma globulin serum and polyethylene glycol were used as the precipitating second antibody reagent. The sensitivity of the assay was 2.2 pg/ml. The intra- and interassay coefficient of variation values were 8.9 and 12.2% respectively.

### Immunohistochemistry and TUNEL assay

Ovarian sections (5 µm thick) on PND30 and PND34 (40 h after the last oFSH administration) were used to evaluate protein levels of estrogen receptor alpha (ESR1), estrogen receptor beta (ESR2), androgen receptor (AR), and Ki67, following protocols published by our laboratory (Rivera *et al.* 2011). To evaluate follicular atresia, we used two different approaches: i) the determination of granulosa cell proliferation by Ki67 immunodetection and ii) the evaluation of granulosa cell apoptosis by TUNEL assay.

Steroid receptors were immunostained using anti-ESR1 (NCL-ER-LH2, clone CC4-5, 1:50 dilution, Novocastra, Newcastle-upon-Tyne, UK), anti-ESR2 (NCL-ER-beta, clone EMR02, 1:25 dilution, Novocastra), and anti-AR (sc-816, 1:400 dilution, Santa Cruz Biotechnology, Inc.) antibodies. For granulosa cell proliferation, we used an anti-Ki67 affinity-purified rabbit monoclonal antibody generated and tested in our laboratory (Varayoud *et al.* 2008, Rivera *et al.* 2011). The specificity of each antibody was tested using western blot analysis of protein extracts (Rodríguez *et al.* 2003) 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 non-immune serum (Sigma–Aldrich).

Apoptotic cells in follicular sections were evaluated by TUNEL assay using the In Situ Cell Death Detection Kit, POD (Roche), following the manufacturer's instructions. To minimize autofluorescence, tissue sections were blocked with 10 mg/ml sodium borohydride (Sigma–Aldrich) and then pretreated with microwave at 350 W (Citrate 0.01 M pH 6). Thereafter, sections were rinsed in PBS, immersed in a buffer containing 3% BSA (Sigma–Aldrich), and 20% normal horse serum for 20 min to block non-specific binding sites. Then, samples were incubated with TUNEL reaction mixture: terminal deoxynucleotidyl transferase (TdT) and fluorescein (FITC)-labeled nucleotide mixture (fluorescein-dUTP) for 60 min at 37 °C in a humidified chamber in the dark. After rinsing with PBS, sections were mounted in Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA) with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Fluka, Sigma–Aldrich) and stored in the dark at 4 °C. The detection of DNA fragmentation was conducted using an Olympus BX-51 microscope equipped for epifluorescence with the appropriate filters (Olympus). Cells containing fragmented nuclear chromatin exhibited green nuclear staining. Images were recorded using a High-resolution USB 2.0 Digital Color Camera (QImaging Go-3, QImaging, Surrey, BC, Canada). As a negative control, sections were processed without TdT. For positive control, the involuting rat prostate after the second day of castration was processed in the same way as the experimental samples (Ramos *et al.* 2002).

### Evaluation of immunohistochemistry

To study the protein levels of ESR1, ESR2, and AR, we selected three sections, 800 µm apart from each other (Rivera *et al.* 2011). No significant differences regarding the immunohistochemistry pattern were found between sections of the same

ovary. 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 (Delgado-Rosas *et al.* 2009). In the analysis of the cortical region, protein levels were assessed in the stroma and in different cellular compartments of the follicles (theca cells, granulosa cells, and oocytes). Immunostaining was evaluated using the following score: negative (–), slightly positive (–/+), weakly positive (+), positive (++), and strongly positive (+++).

### Evaluation of atretic follicles

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. Although follicular atresia could be characterized by histomorphological features, atretic follicles were defined as those with  $\leq 2\%$  Ki67-positive granulosa cells in this study (Jolly *et al.* 1997, Rivera *et al.* 2011). To confirm the percentage of atretic follicles, the granulosa apoptotic cells detected by TUNEL were counted on the whole area of each ovarian section. Follicles were considered atretic if they contained more than 2% of TUNEL/positive granulosa cells (Jolly *et al.* 1997).

### Quantitative real-time PCR

An optimized RT-qRT-PCR protocol was used to analyze the relative expression levels of *BMP6*, *BMP15*, *BMPR1B*, *GDF9*, and FSH receptor (*FSHR*) mRNA in ovaries obtained on PND30 or after stimulation with oFSH on PND34. Ovaries from each experimental group (control, BPA0.5, BPA50, and DES) were individually homogenized in TRIzol (Life Technologies), and RNA was prepared according to the manufacturer's protocol. The concentration of total RNA was assessed by A260, and RNA was stored at  $-80^{\circ}\text{C}$  until use. Equal quantities (4  $\mu\text{g}$ ) of total RNA were reverse transcribed into cDNA according to Ramos *et al.* (2007). Primer pairs used to amplify *BMP6*, *BMP15*, *BMPR1B*, *GDF9*, *FSHR* and the ribosomal protein 18S (housekeeping gene) cDNAs are shown in Table 1. cDNA levels were detected by qRT-PCR using a Rotor-Gene Q cyler (Qiagen Instruments AG) and HOT FIRE Pol EvaGreen Qpcr Mix PlusS (Solis BioDyne; Biocientifica, Rosario, Argentina).

**Table 1** Primers and PCR products for real-time quantitative PCR.

Genes	Primer sequence (5'–3')	Product size (pb)	GenBank accession number
<i>BMP6</i>	Forward: CTCTACGTGAGCTTCCAGGACCT Reverse: TCTCCGTCACAGTAGTTGGCAGC	83	DQ192014.1
<i>BMP15</i>	Forward: ATGGTCCTCCTGAGCATCCTTAG Reverse: CTGCCCTACCTGTGTCAATTGG	87	NM_001114767
<i>BMPR1B</i>	Forward: TCTACACTTTGGTTATCAGC Reverse: TTTGTATCCTCTCTTGTTCAT	95	NM_001009431
<i>GDF9</i>	Forward: TAGAGGTTCTGTATGATGGG Reverse: ATGCCTTATAGAGCCTCTTC	90	NM_001142888
<i>FSHR</i>	Forward: CCAACAACCTGCTATACATC Reverse: GTGCCTAATACCTGTGTTGG	103	NM_001009289

*BMP6*, bone morphogenetic protein 6; *BMP15*, bone morphogenetic protein 15; *BMPR1B*, BMP receptor 1B; *GDF9*, growth and differentiation factor 9; *FSHR*, follicle-stimulating hormone receptor.

After initial denaturation at  $95^{\circ}\text{C}$  for 15 min, the reaction mixture was subjected to successive cycles of denaturation at  $95^{\circ}\text{C}$  for 15 s, annealing at  $59^{\circ}\text{C}$  (for *BMP6*),  $54^{\circ}\text{C}$  (for *BMP15*),  $52^{\circ}\text{C}$  (for *BMPR1B*),  $53^{\circ}\text{C}$  (for *FSHR* and *GDF9*), or  $55^{\circ}\text{C}$  (for r18S) for 15 s, and extension at  $72^{\circ}\text{C}$  for 15 s. The product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. All PCR products were cloned using a TA cloning kit (Invitrogen) and specificity was confirmed by DNA sequencing (data not shown). Controls containing no template DNA were included in all assays, yielding no consistent amplification. A sample without reverse transcriptase was included to detect contamination by genomic DNA. For each analysis, a standard curve was prepared from eight serial dilutions of a standard sample containing equal amounts of cDNA from the different experimental groups as reported previously (Varayoud *et al.* 2008). All standards and samples of each independent experiment were assayed in triplicate.

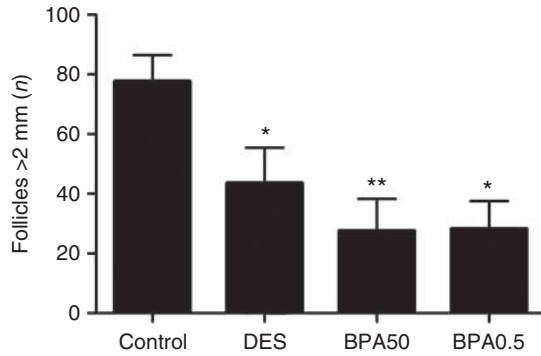
### Statistical analysis

All data are expressed as mean  $\pm$  s.e.m. We performed a one-way ANOVA to assess the overall significance, and differences between the treatments and the control group were determined using Dunnett's post test. For hormone measurement, and as data were not normally distributed, we use the Kruskal–Wallis followed by Dunn's *post-hoc* test.  $P < 0.05$  was considered statistically significant.

## Results

### Ovarian response to exogenous oFSH treatment

Control prepubertal lambs responded to oFSH treatment on PND34, showing a mean of 78 follicles  $\geq 2$  mm (Fig. 2). Lambs exposed to both doses of BPA or DES and treated with oFSH showed a significantly lower number of follicles  $\geq 2$  mm on the ovarian surface ( $C = 77.6 \pm 8.8$  vs BPA50 =  $27.5 \pm 10.7$  vs BPA0.5 =  $28.2 \pm 9.3$  vs DES =  $43.5 \pm 11.9$ ) (Fig. 2). The percentage of antral atretic follicles in all xenoestrogen-exposed lambs was lower than that in controls (Fig. 3). Figure 4 illustrates the ovarian surface of representative samples in the different experimental groups. The ovaries from prepubertal



**Figure 2** Effect of exogenous treatment (oFSH) on ovarian follicular response on PND34 in neonatally xenoestrogen-exposed lambs. Follicles  $\geq 2$  mm were recorded on the ovarian surface. Bars represent mean values  $\pm$  S.E.M. One-way ANOVA followed by Dunnett's post-test. \* $P < 0.05$  or \*\* $P < 0.01$  vs control.

lambs on PND30, without oFSH treatment, showed the expected atrophic small size (Fig. 4A). As expected, the ovaries from lambs treated with oFSH showed larger and highly hemorrhagic follicles (Fig. 4B). The ovaries from lambs treated neonatally with DES or BPA were unable to respond to stimulation with exogenous oFSH, as evidenced by a lower number of large follicles in the ovary (Fig. 4C and D).

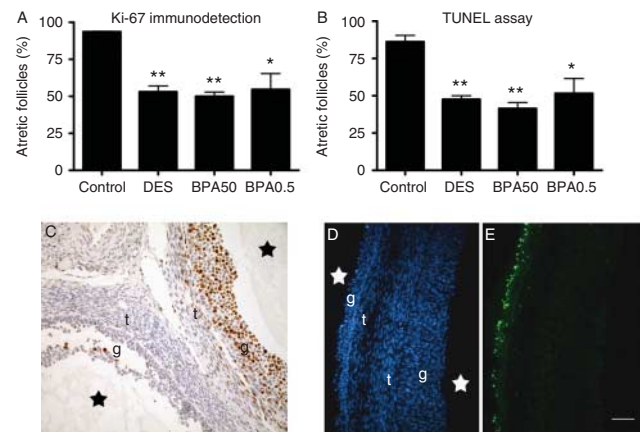
We have also investigated the ovarian steroidogenic response to oFSH treatment by measuring the serum  $E_2$  levels. In control lambs, not exposed to xenoestrogens,  $E_2$  levels increased significantly in response to oFSH (PND30  $2.5 \pm 0.7$  pg/ml vs PND32  $44.9 \pm 18.8$ ) (Fig. 5). Basal  $E_2$  levels on PND30 were not affected by the xenoestrogen treatment (PND30; C =  $2.5 \pm 0.7$  pg/ml vs DES =  $5.3 \pm 0.6$  vs BPA50 =  $5.3 \pm 1.9$  vs BPA0.5 =  $3.8 \pm 1.1$ ;  $P > 0.05$ ), although the response to oFSH stimulation was impaired. In accordance with the alteration in the follicular development in xenoestrogen-exposed lambs described earlier in this study, characterized by a lower number of large follicles, no increase was found in the serum levels of  $E_2$  following oFSH treatment (Fig. 5).

### Potential mechanisms underlying impaired ovarian response to oFSH treatment

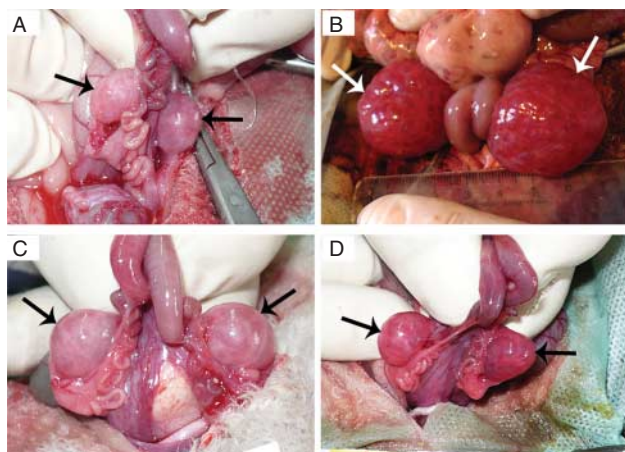
To gain insight into the mechanisms that impaired the follicular response to oFSH treatment in lambs exposed to xenoestrogens, protein levels of sexual steroid receptors were compared between ovaries obtained on PND30 and PND34 by immunohistochemistry. In PND30 ovaries, ESR1 was not detected, whereas ESR2 and AR were highly expressed in granulosa and theca cells of antral follicles. DES or BPA exposure did not change the protein level pattern of steroid receptors observed in controls (Table 2), thus demonstrating that DES or BPA themselves are unable to disrupt the protein level of these receptors in PND30 ovaries. In PND34 ovaries, we found no detectable level of ESR1 protein in

response to exogenous oFSH, whereas ESR2 was highly expressed in both granulosa and theca cells of antral follicles (Table 2); however, no differences were found in the protein level pattern observed following stimulation with oFSH (Fig. 6). In contrast, oFSH treatment increased AR protein level in small antral follicles in PND34 control lambs (Fig. 6 and Table 2). However, oFSH induction of AR was impaired in ovaries from lambs previously exposed to xenoestrogens (Table 2). Representative immunohistochemical photomicrographs of AR in ovaries from DES- or BPA-exposed lambs showed disruptive protein level of this steroid receptor in both theca and granulosa cells (Fig. 6).

Another potential mechanism underlying impaired ovarian response to oFSH treatment in xenoestrogen-exposed lambs may reside in the BMP system. Therefore, we assessed the response of the BMP system and *FSHR* after stimulation with oFSH. We observed that previous exposure to DES or BPA did not change the mRNA levels of *BMP6*, *BMP15*, or *FSHR* after stimulation with oFSH. Instead, we found a significantly high level of *GDF9* mRNA in ovaries from oFSH-stimulated lambs previously treated with DES or BPA50 (Fig. 7A). Then, to investigate whether the expression levels of *GDF9* were abnormally high before oFSH stimulus, we measured mRNA levels of *GDF9* in ovaries of 30-day-old lambs previously treated with DES or BPA50. We observed that *GDF9* mRNA levels were already abnormally high on



**Figure 3** Effect of exogenous treatment (oFSH) on the number of antral atretic follicles on PND34 in neonatally xenoestrogen-exposed lambs. Percentage of antral atretic follicles detected by Ki67 proliferation marker in granulosa cells (A) or granulosa cell apoptosis by TUNEL assay (B). In (C), an antral atretic follicle and a healthy antral follicle detected by Ki67 proliferation marker are shown; compare the high proliferation index in the granulosa of the healthy follicle (right) with the low percentage in the granulosa of the antral atretic follicle (left). Photomicrographs showing *in situ* TUNEL assay with DAPI nuclear blue fluorescence (D) and apoptotic cells displaying green fluorescence (E). TUNEL was positive predominantly within the granulosa of atretic follicles (left) (E) and negative within the granulosa of healthy follicles (right). Bars represent mean values  $\pm$  S.E.M. One-way ANOVA followed by Dunnett's post-test. \* $P < 0.05$  or \*\* $P < 0.01$  vs control. Scale bars, 50  $\mu$ m for all panels. g, granulosa; t, theca; star, antrum.



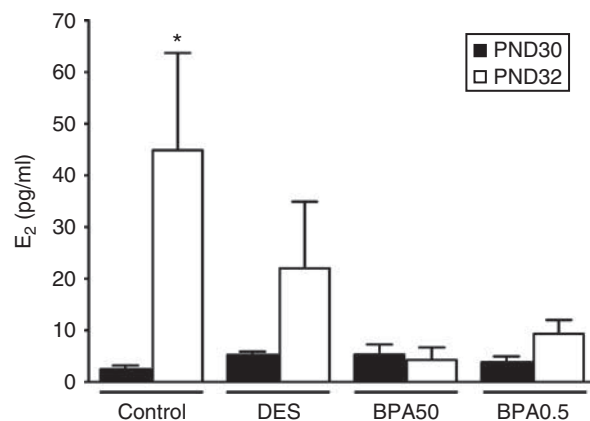
**Figure 4** Representative photographs of ovaries from prepubertal lambs. Ovaries from control lambs without any treatment on PND30 (A) and following oFSH administration on PND34 (B). Lambs neonatally exposed to DES (C) or BPA50 (D) and treated with oFSH at PND34. Arrows indicate the ovaries.

PND30 in BPA50-treated lambs (Fig. 7B) and that, following stimulation with oFSH, the significant differences prevailed (Fig. 7A). In DES-treated lambs, no difference with control in *GDF9* was observed on PND30. In addition, we found, on PND34, a decreased expression of *BMPR1B* in ovaries from oFSH-stimulated lambs previously treated with the lowest dose of BPA tested (BPA0.5, Fig. 7A).

## Discussion

Most studies on the effects of environmental pollutants on ovarian development and function have relied on *in vitro* systems or rodent models (Rodríguez *et al.* 2010, Peretz *et al.* 2011) and thus need to be validated in other animal models (Veiga-Lopez *et al.* 2014). To conduct this experiment, we used sheep, a precocial species in which the reproductive developmental trajectory follows a timeline similar to that of humans (Padmanabhan *et al.* 2007, Padmanabhan & Veiga-Lopez 2013). In sheep and humans, full follicular differentiation occurs before birth, unlike in rodents, where it occurs postnatally (Padmanabhan *et al.* 2007, 2010, Padmanabhan & Veiga-Lopez 2013). In this study, we demonstrated that early postnatal exposure of lambs to BPA or DES decreased the ovarian response to exogenous oFSH in the prepubertal age, demonstrating decreased follicular development and decreased estradiol production. Moreover, present results allow us to postulate a link between these ovarian disorders and abnormalities of the BMP system and a deficient FSH-induced AR increase in the population of small antral follicles. These results indicate that lamb ovaries are sensitive to disruptions by EDC exposure in early postnatal life, and that these effects may be responsible for fertility problems, including a failure in the superstimulation response.

Previously, we showed a lower ovarian weight and altered follicular development in lambs postnatally exposed to BPA or DES from PND1 to PND14, and that both BPA and DES are able to reduce the primordial follicle pool by stimulating their initial recruitment and subsequent development until antral stage (Rivera *et al.* 2011). We reported similar results in a rodent model (Rodríguez *et al.* 2010). Herein, we found that follicles of lambs neonatally exposed to BPA or DES are unable to respond to the stimulatory effect of oFSH. Following oFSH stimulation, the ovaries from non-exposed lambs responded with a significant increase in follicular development, evidenced by the high number of follicles > 2 mm in diameter. However, when lambs have been previously exposed to BPA, the follicular development was drastically reduced. Previously, we have also demonstrated that the same BPA or DES postnatal treatment induces a high incidence of MOFs, suggesting that follicular assembly may be active during early postnatal life in lambs. This was surprising as most studies have suggested that a defined and finite pool of primordial follicles exists at birth in lambs (Juengel *et al.* 2002, Padmanabhan *et al.* 2007). However, based on a recent report that has proposed a new mechanism for generation of MOFs in the postnatal rat ovary, we cannot rule out the possibility that MOFs in xenoestrogen-treated lambs are generated by fusion of adjacent growing follicles (Gaytán *et al.* 2014). It is interesting to note that a similar increase in the incidence of MOFs has been demonstrated in caimans (Stoker *et al.* 2008) and rats (Rodríguez *et al.* 2010) exposed to BPA, the rat being a species in which follicular assembly continues after birth. Both abnormal preantral folliculogenesis and high incidence of MOFs are potentially related to the appearance of fertility syndromes in human adulthood (Franks *et al.* 2008, Asimakopoulos *et al.* 2013).



**Figure 5** Serum  $E_2$  levels in lambs neonatally exposed to xenoestrogens and treated with exogenous oFSH. Serum levels in samples on PND30 before the first dose of oFSH and before the last injection on PND32. All experimental groups were treated with six doses of oFSH. Kruskal–Wallis followed by Dunn's *post-hoc* test, comparisons were made between PND30 vs PND32, \* $P < 0.05$ .

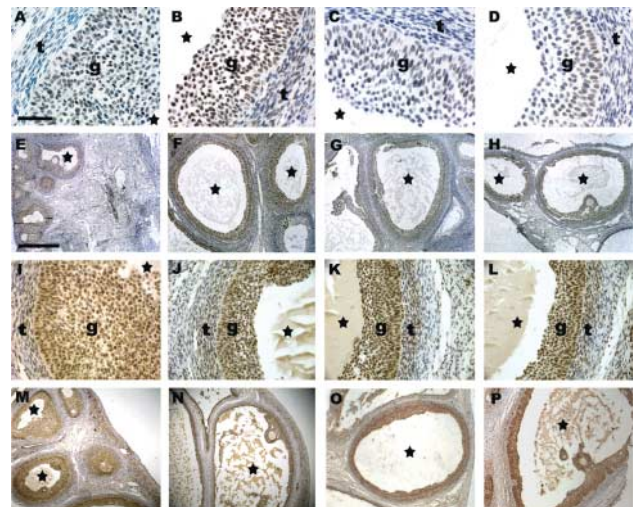
**Table 2** Expression of ESR1, ESR2, and AR in antral follicles in ovaries from lambs neonatally exposed to xenoestrogens (PND30) and following treatment with oFSH (PND34).

	PND30			PND34			
	C	DES	BPA50	C+oFSH	DES+oFSH	BPA50+oFSH	BPA0.5+oFSH
ESR1							
Granulosa	–	–	–	–	–	–	–
Theca	–	–	–	–	–	–	–
ESR2							
Granulosa	+++	+++	+++	+++	+++	+++	+++
Theca	++	++	++	++	++	++	++
AR							
Granulosa	++	++	++	++++	++	++	++
Theca	+	+	+	++	+	+	+

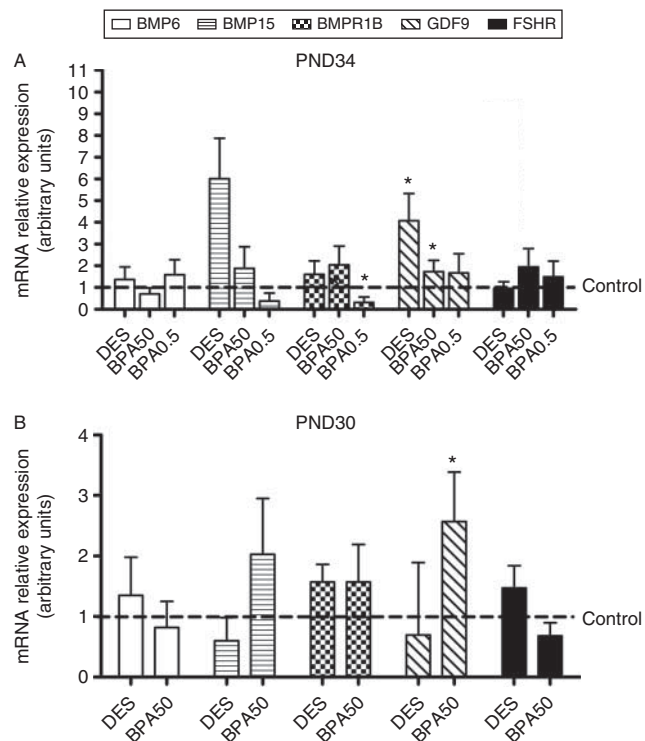
From birth to postnatal day 14 (PND14), lambs were exposed to DES (5 µg/kg per day), BPA (50 µg/kg per day or 0.5 µg/kg per day), or vehicle (C). Another group of exposed lambs were stimulated with oFSH (described in M&M). On PND30 and PND34, steroid receptors were immunohistochemically analyzed. Immunostaining was qualitatively evaluated in at least three sections/ovary, as follows: negative (–), slightly positive (–/+), weakly positive (+), positive (++), and strongly positive (++++). At least three lambs were evaluated at each time point.

Superovulation is a reproductive practice applied to many mammalian species whereby exogenous gonadotropins are used to increase follicular development or the ovulation rate with the expectation to generate greater numbers of embryos. This technique is used both in adults (multiple ovulation and embryo transfer (MOET)) (Wray & Goddard 1994) and in prepubertal females (juvenile *in vitro* embryo transfer (JIVET)) (Kelly *et al.* 2005). It has long been known that follicles of 4- to 8-week-old lambs are particularly sensitive to gonadotropin administration using protocols developed for adult animals (Worthington & Kennedy 1979, Armstrong *et al.* 1994, Ptak *et al.* 1999). On the other hand, ovarian stimulation has proved to be a simple and useful tool to detect alterations in rodent reproductive organs and to study likely changes in the mechanisms of hormonal action induced by certain substances (Sekiguchi *et al.* 2003). In this study, we applied a protocol of oFSH-ovarian stimulation in prepubertal lambs and demonstrated that early postnatal exposure of BPA or DES impaired the ovarian functional response to oFSH treatment. Moreover, lambs exposed to xenoestrogens showed an increased follicular atresia rate (measured by histomorphology, Ki67 proliferation, and TUNEL *in situ* apoptosis assay) after oFSH treatment. The failure of the lamb ovarian response to treatment was found using a ‘safe dose’ of BPA and a 100-fold lower dose at early postnatal exposure. Several factors, such as healthy, nutritional, and reproductive status, genetic factor, age, stress, hormone used, and dose, may affect the success of superovulatory treatment in females (Mapletoft *et al.* 2002). According to the present results, an additional factor such as the xenoestrogen exposure during a critical developmental period may affect the ovarian response to exogenous hormonal treatment. Despite improvements in superovulatory treatments, ovarian responsiveness remains highly variable between individuals and difficult to predict (Rico *et al.* 2009). This variability in the superovulatory response may be explained by different individual levels of exposure to xenoestrogens.

As ovarian dysfunctions associated with altered fertility have also been linked with alterations in the protein levels of sex steroid receptors (Britt & Findlay 2002, Drummond 2006, Prizant *et al.* 2014), we measured these molecules in the ovaries from the lambs exposed to DES or BPA. We found no changes in ovarian ESR1 or ESR2 protein levels in both unstimulated (PND30) and oFSH-stimulated (PND34)



**Figure 6** Representative photomicrographs of ovarian sections showing AR (A, B, C, D, E, F, G, and H) and ERB (I, J, K, L, M, N, O, and P) immunostaining in neonatally xenoestrogen-exposed lambs treated with exogenous oFSH. (A and E) Control lambs on PND30 (non-treated with oFSH) show AR-positive nuclear immunostaining in granulosa and/or theca cells of antral follicles. (B and F) Control lambs following oFSH administration on PND34 show increased AR protein levels. DES- (C and G) or BPA50- (D and H) neonatally exposed lambs treated with oFSH on PND34 did not show any increase in AR protein level in either cellular compartment of antral follicles. ERB protein was highly expressed in both granulosa and theca cells of antral follicles in controls not treated with oFSH (I and M); the protein level showed no differences in controls treated with oFSH (J and N) and in BPA (K and O)- or DES (L and P)-exposed lambs. Scale bars: 60 µm for A, B, C, and D and I, J, K, and L, and 300 µm for E, F, G, and H and M, N, O, and P. g, granulosa; t, theca; star, antrum.



**Figure 7** Quantitative real-time PCR analysis of the levels of *BMP6*, *BMP15*, *BMPR1B*, *GDF9*, and *FSHR* mRNA expression of ovaries from lambs neonatally exposed to xenoestrogens. (A) Ovaries from PND34 lamb after oFSH treatment. (B) PND30 lambs without oFSH treatment. The amounts of mRNA in each experimental group are indicated as values relative to those of control lambs (dashed line). The columns and error bars represent the means  $\pm$  S.E.M. One-way ANOVA followed by Dunnett's post-test. \* $P < 0.05$  vs controls.

ovaries of vehicle-treated lambs vs xenoestrogen-treated lambs. On the other hand, AR protein level in antral follicles of lamb exposed to xenoestrogens showed a significant change. The induction of AR protein level in response to oFSH was lower when the lambs were previously exposed to DES or BPA. It is known that androgens have a stimulatory effect on follicular development in rodents and large farm animals, including ewes (Smith *et al.* 2009, Prizant *et al.* 2014). In fact, in the absence of functional ARs in granulosa cells, follicle progression from preantral to antral stage is inhibited and preantral follicles become atretic (Sen & Hammes 2010, Prizant *et al.* 2014). Therefore, the decreased AR induction in response to oFSH specifically observed in antral follicles of DES- and BPA-exposed lambs could explain the lower follicular development found in these animals. Unexpectedly, we simultaneously found a low percentage of atretic antral follicles. Two different AR-mediated pathways regulating follicular atresia and follicular development have been recently described in granulosa cells (Sen *et al.* 2014). On the other hand, given the disruption in AR induction in response to oFSH found in xenoestrogen-treated

lambs, it is probable that any stimulatory effect on follicle growth acting through the AR pathway is at least attenuated. Some factors belonging to the BMP system are associated with follicular development and ovarian steroidogenesis (Fabre *et al.* 2006) and act through the AR pathway. In this sense, we found that both DES and BPA50 disrupt *GDF9* mRNA expression, with higher expression from PND30 onwards. It has been reported that *GDF9* controls ovarian follicular development from the preantral stage to the early antral stage by upregulating follicular androgen biosynthesis and that the specific AR antagonist flutamide suppresses *GDF9*-induced preantral follicle growth (Orisaka *et al.* 2009). Therefore, we can hypothesize that the increased levels of *GDF9* found in the ovaries from xenoestrogen-treated lambs (assuming that protein and mRNA have the same pattern of expression) affected the expected stimulatory effect of oFSH on follicular development due to the low AR protein levels in antral follicles. Taken together, the present results suggest that the low protein levels of AR induced by BPA or DES exposure could adversely affect AR-mediated stimulatory effects on follicular development, without affecting follicular atresia.

Although it is known that mouse follicles exposed *in vitro* to BPA show altered ovarian steroidogenesis due to decreased levels of key enzymes that regulate estradiol biosynthesis pathway (Peretz *et al.* 2011), the complex mechanism causing these effects remains yet to be determined. Recently, a study conducted on sheep has demonstrated that prenatal BPA exposure alters fetal ovarian steroidogenic gene and microRNA expression in an age-dependent manner (Veiga-Lopez *et al.* 2013). In our experiment, although the basal levels of  $E_2$  were not affected in lambs neonatally exposed to BPA or DES, the stimulatory response to oFSH was impaired. Differences in experimental design may explain differences in the results. Interestingly, Vitt *et al.* (2000) demonstrated that *GDF9* suppresses both FSH-induced progesterone and estradiol production in rat follicles. The high values of *GDF9* expression detected in the present work in xenoestrogen-treated lamb ovaries could explain, at least in part, the failure of antral follicles to respond to oFSH. In addition, the low number of antral follicles could also explain the diminished capability of ovaries from lambs previously exposed to DES or BPA to synthesize estradiol. Moreover, ovaries from lambs exposed to the lowest dose of BPA and treated with oFSH showed a decreased expression of *BMPR1B*. *BMPR1B* is expressed by granulosa cells and oocytes from the primary to the late antral follicle stages and acts as a receptor for various BMP factors (ten Dijke *et al.* 2003, Fabre *et al.* 2006). It has been described that a single mutation in the coding sequence of *BMPR1B* is responsible for the hyperproliferic phenotype of Booroola ewes (McNatty *et al.* 2001, Mulsant *et al.* 2001, Souza *et al.* 2001). Then, Campbell *et al.* (2003) reported that ovaries of  $Fec^{B/-}$  (Booroola) ewes contain mainly small



follicles with a low number of medium-size follicles and no large follicles after 3 days of FSH infusion. Interestingly, mice deficient in BMPR1B are infertile and show impaired estradiol synthesis (Souza *et al.* 2002) and decreased Cyp19 expression (Yi *et al.* 2001). Our results indicate that the alterations in estradiol levels and folliculogenesis observed in response to oFSH in lambs previously exposed to the lowest dose of BPA could be explained by an attenuated action of some BMP factors due to the decreased expression of ovarian BMPR1B.

Although some researchers have shown that xenoestrogen exposure affects hypothalamic–hypophyseal function (Monje *et al.* 2010), it appears that xenoestrogen treatment may also affect the response to oFSH stimulus acting directly on the ovary. Xenoestrogen can interfere with endogenous estrogens by either mimicking or blocking their responses via non-genomic and/or genomic signaling mechanisms (Viña *et al.* 2012). As mentioned earlier in this study, the protein levels of ESR1 and ESR2 in ovaries of PND30 and PND34 did not differ between the experimental groups even following oFSH treatment. However, we cannot exclude that xenoestrogens are causing changes in the functionality of the receptors belonging to the genomic pathway or are acting on the non-genomic pathway (Viña *et al.* 2012). ESR1 was not detected in lamb ovaries at this age and our results are slightly different from those of other authors (Juengel *et al.* 2006) that show immunostaining in surface epithelium and granulosa cells of preantral and antral follicles. Differences may be due to the primary antiserum used. Disruption of estrogens' actions through the non-genomic pathway can alter functional end points such as cell proliferation, peptide hormone release, catecholamine transport, and apoptosis, among others. BPA has been found to be a 'weak' inducer of estrogenic activity via the genomic pathway; however, BPA is equipotent with E<sub>2</sub> in its ability to initiate rapid non-genomic responses from membrane receptors (Wozniak *et al.* 2005). However, more studies are needed to know the mechanistic effects of xenoestrogens altering the ovarian function. The adverse effects of the neonatal exposure to xenoestrogens are usually observed later in the female life, impairing different reproductive events such as puberty onset, cyclicity, or implantation (Durando *et al.* 2007, Monje *et al.* 2010, Varayoud *et al.* 2014). If the changes observed in this study (failure in response to oFSH in ovarian follicular development, increased follicular atresia, and failure in steroidogenesis response) due to the disrupting effect of xenoestrogens were organizational (permanent), they could negatively affect the adult sheep reproductive function and the ovarian response to a superovulatory treatment in animal practice.

Organogenesis is a highly regulated process, including precise exposure to steroid hormones at specific times during development. The present results describing

altered ovarian functions in response to an exogenous gonadotropin stimulus add to a growing body of evidence reporting xenoestrogen-induced abnormalities in sheep (Veiga-Lopez *et al.* 2013, 2014). In addition, recent studies have demonstrated that tall women treated with estrogens in adolescence are at increased risk of infertility in later life and their fecundity is reduced (Hendriks *et al.* 2012). Our results indicated signs of primary ovarian insufficiency with concomitant early follicle pool depletion. Taking into account that, in our model, xenoestrogen-exposed lambs showed similar results to that reported in women, we may suggest that the decreased fertility in domestic animals naturally exposed to xenoestrogens is due to an impaired ovarian response.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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