Revised Version

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

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Short title: Xenoestrogens impair ovarian response 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 Follicle Stimulating Hormone (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.day, BPA0.5: 0.5 μg/kg.day) from PND1 to PND14. We determined: a) follicular growth, b) circulating levels of E_2, c) steroid receptors (ERA, ERB, AR) and atresia, d) mRNA expression levels of the ovarian bone morphogenetic protein (BMPs) system (*BMP6*, *BMP15*, *BMP receptor type 1B*, *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 together with a lower number of atretic follicles and no increase in E_2 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.

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 17B-estradiol (E_2) (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, since it is used in polycarbonate plastics, resins, papers, implanted medical devices and other medical equipment (Welshons *et al.* 2006, NTP-CERHR 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). Since BPA has been shown to leach from containers into food and beverage products and proved to be one of the multiple contaminants included in the soil, this compound should be considered a potential health risk for animals and humans (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 subcutaneous 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 PND 30 (Rivera *et al.* 2011). These adverse effects may be mediated through abnormal early protein levels of ovarian estrogen receptors and could alter ovarian function and female fertility (Rivera *et al.* 2011). Nagel & Bromfield (2013) suggested that BPA can directly bind to both estrogen receptors (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 model 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. Besides, 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. Here, we investigated whether the neonatal

exposure to low doses of BPA or DES adversely affects the ovarian response to an exogenous treatment of ovine FSH (oFSH) on prepubertal lambs and its possible association with abnormalities in steroid receptor pathways. Moreover, since 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 bone morphogenetic protein-6 and -15 (*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 to 4 years old) grazed pasture with a low rate of clover. During the breading 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.

126 After birth, female lambs were randomly assigned to one of the following postnatal daily
127 treatments (Fig. 1), from PND1 (this being the day of birth) to PND14, by subcutaneous
128 injections in the nape of the neck: 1) corn oil vehicle (controls; n= 18), 2) DES (Sigma-
129 Aldrich, St. Louis, MO, USA) at 5 μg/kg/day (n= 13), 3) BPA50 (99% purity, Sigma-
130 Aldrich, Milwaukee, WI, USA) at 50 μg/kg.day (n= 16), and 4) BPA0.5 at 0.5 μg/kg.day
131 (n= 9). Although the sc route of administration for EDC is not the natural via of exposure,
132 we selected this via because we were certain of the dose that goes into the animals. The
133 postnatal model of exposure to xenoestrogens has been extensively used in our laboratory
134 in both rodents (Monje *et al*. 2007, 2009, 2010; Ramos *et al*. 2007; Varayoud *et al*. 2008;
135 Bosquiazzo *et al*. 2010; Rodriguez *et al*. 2010) and lambs (Rivera *et al*. 2011) and has been
136 demonstrated as a persuasive paradigm to study short- and long-term consequences of
137 neonatal exposure to hormonally active substances. On the other hand, the route of
138 administration is an important issue to determine BPA health risks in animal models. In
139 fetuses and neonates, Taylor *et al*. (2008) observed that the low levels of the enzyme that
140 conjugates BPA (uridine diphosphate-glucuronosyltransferase) implies that both oral and
141 non-oral administration of BPA during neonatal life give the same internal active dose.
142
143 The EPA-National Toxicology Program's Report of the Endocrine Disruptors-USA
144 (U.S.EPA 1993) has defined the LOAEL dose for BPA as 50 mg/kg/day and the "safe
145 dose" as 1000 times lower (50 μg/kg/day) (Melnick *et al.* 2002, NTP-CERHR 2008). In
146 the present work, we used the safe dose of BPA and a dose 100 times lower. DES was used
147 as positive control because it has been reported that developmental exposure to low doses
148 of this compound induces MOFs and activates the primordial to primary follicle transition
149 in mice (Iguchi *et al.* 1986, Wordinger & Derrenbacker 1989), rats (Rodriguez *et al.* 2010)
150 and lambs (Rivera *et al.* 2011). The dose of 5 μg/kg/day of DES used here 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, im) and xylazine (0.1–0.2 mg/kg, im) 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 hours by im injection for 3 consecutive days (at 0800 and 2000 h, PND30, 31 and 32). This treatment protocol was adapted, with minor modifications, from that previously described 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 ≥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 -80ºC. 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 -20ºC until hormone assay.

Hormone assays

Blood samples were allowed to clot for 1 h at room temperature. Serum was then collected and stored at -20ºC for hormone analysis. Serum E_2 levels were determined by a double-

antibody radioimmunoassay 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 17α-estradiol, 2.56% with 17β-estradiol-3-glucoronide, 0.17% with estradiol-3-sulfate, and 3.4% with D-equilenin. Goat anti-rabbit gamma globulin serum and polyethylene glycol were used as the precipitating second antibody reagent. The sensitivity of the assay was 2.2 pg/ml. The intra- and interassay coefficients of variation were 8.9% and 12.2%, respectively.

Immunohistochemistry and TUNEL assay

Ovarian sections (5 μm thick) on PND30 and 34 (40 h after the last oFSH administration) were used to evaluate protein levels of estrogen receptor alpha (ERA), estrogen receptor beta (ERB), androgen receptor (AR) and Ki67, following protocols published by our laboratory (Rivera *et al.* 2011). To evaluate follicular atresia, we used two different approaches: a) the determination of granulosa cell proliferation by Ki67 immunodetection and b) the evaluation of granulosa cell apoptosis by TUNEL assay.

Steroid receptors were immunostained using anti-ERA (NCL-ER-LH2, clone CC4-5, 1:50 dilution, Novocastra Newcastle-upon-Tyne, UK), anti-ERB (NCL-ER-beta, clone EMR02, 1:25 dilution, Novocastra), and anti-AR (sc-816,1:400 dilution, Santa Cruz Biotechnology Inc., CA, USA) antibodies. For granulosa cell proliferation, we used an anti-Ki67affinity-purified rabbit polyclonal 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 (Rodriguez *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 nonimmune serum (Sigma-Aldrich).

Apoptotic cells in follicular sections were evaluated by TUNEL assay using the In Situ Cell Death Detection Kit, POD (Roche, Mannheim, Germany), 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% bovine serum albumin (BSA, Sigma–Aldrich) and 20% normal horse serum for 20 min to block non-specific binding sites. Next, samples were incubated with TUNEL reaction mixture: terminal deoxynucleotidyl transferase (TdT) and fluorescein (FITC)-labeled nucleotide mixture (fluorescein-dUTP) for 60 min at 37 ºC in a humidified chamber in the dark. After rinsing with PBS, sections were mounted in Vectashield (Vector Laboratories, Inc., Burlingame, CA) with 4´,6-diamidino-2-phenylindole dihydrochloride (DAPI; Fluka, Sigma-Aldrich) and stored in the dark at 4ºC. 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 to the same way as the experimental samples (Ramos *et al.* 2002).

Evaluation of immunohistochemistry

To study the protein levels of ERA, ERB 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, here atretic follicles were defined as those with ≤2% Ki67-positive granulosa cells (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. Atretic follicles contained more than 2% of TUNEL-positive granulosa cells (Jolly *et al.* 1997).

Quantitative real-time polymerase chain reaction (qRT-PCR)

An optimized reverse transcription-qRT-PCR protocol was used to analyze the relative expression levels of *BMP6*, *BMP15*, *BMPR1B*, *GDF9* and follicle stimulating hormone 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, NY, USA), and RNA was prepared according to the manufacturer's protocol. The concentration of total RNA was assessed by A260, and RNA was stored at –80°C until needed. Equal quantities (4 μg) 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 using qRT-PCR with a Rotor-Gene Q cycler (Qiagen Instruments AG, Hombrechtikon, Switzerland) and HOT FIRE Pol EvaGreen Qpcr Mix PlusS (Solis BioDyne; Biocientifica, Rosario, Argentina). After initial denaturation at 95°C for 15 min, the reaction mixture was subjected to successive cycles of denaturation at 95°C for 15 s, annealing at 59°C (for *BMP6*), 54°C (for *BMP15*), 52°C (for *BMPR1B*), 53°C (for *FSHR* and *GDF9*) or 55°C (for r18S) for 15 s, and extension at 72°C for 15 s. The product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. All PCR products were cloned using a TA cloning kit (Invitrogen) and specificity was confirmed by DNA sequencing (data not shown). Controls containing no template DNA were included in all assays, yielding no consistent amplification. A sample without reverse transcriptase was included to detect contamination by genomic DNA. For each analysis, a standard curve was prepared from eight serial dilutions of a standard sample containing equal amounts of cDNA from the different experimental groups as previously reported (Varayoud *et al*. 2008). All standards and samples of each independent experiment were assayed in triplicate.

Statistics

All data were calculated as the mean±SEM. We performed a one-way- ANOVA to assess the overall significance and differences between the treatments with the control group were determined using the Dunnett's post test. For hormone measurement, and since data were not normally distributed, we use Kruskal-Wallis followed by Dunn's post-hoc test. $P < 0.05$ was accepted as significant.

RESULTS

Ovarian response to exogenous oFSH treatment

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

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±0.7 pg/ml vs. PND32 44.9±18.8) (Fig. 5). Basal E_2 levels on PND30 were not affected by the xenoestrogen treatment (PND30; C= 2.5±0.7 pg/ml vs. DES= 5.3±0.6 vs. BPA50= 5.3±1.9 vs. BPA0.5= 3.8±1.1; $p>0.05$), although the response to oFSH stimulation was impaired. In accordance with the alteration of the follicular development in xenoestrogen-exposed lambs described above, 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, ERA was not detected, whereas ERB 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 showing 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 ERA protein in response to exogenous oFSH, whereas ERB 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, 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 find out 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 PND30 in BPA50-treated lambs (Fig. 7B) and that, following stimulation with oFSH, the significant differences remained (Fig. 7A). In DES-treated lambs, no difference with control in *GDF9* was observed on PND30. In addition, we found a decreased expression of *BMPR1B* in ovaries from oFSH-stimulated lambs on PND34 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 (Rodriguez *et al.* 2010, Peretz *et al.* 2011) and thus need to be validated in other animal models (Veiga-Lopez *et al.* 2014).

To perform 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). Here, we demonstrated that early postnatal exposure of lambs to BPA or DES decreased the ovarian response to exogenous oFSH in prepubertal age, showing 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 demonstrate 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 (Rodriguez *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 greater than 2 mm in diameter. However, when lambs were previously exposed to BPA, the follicular development was drastically reduced. Previously, we also demonstrated that the same BPA or DES postnatal treatment induces a high incidence of MOFs, suggesting

374 that follicular assembly may be active during early postnatal life in lambs. This was
375 surprising since most studies have suggested that a defined and finite pool of primordial
376 follicles exist at lamb birth (Juengle *et al.* 2002, Padmanabhan *et al.* 2007). However,
377 based on a recent report that proposes a new mechanism for generation of MOFs in the
378 postnatal rat ovary, we cannot rule out the possibility that MOFs in xenoestrogen-treated
379 lambs are generated by fusion of adjacent growing follicles (Gaytán *et al*. 2014). It is
380 interesting to note that a similar increase in the incidence of MOFs has been demonstrated
381 in caimans (Stoker *et al.* 2008) and rats (Rodriguez *et al.* 2010) exposed to BPA, being the
382 rat a species in which follicular assembly continues after birth. Both abnormal preantral
383 folliculogenesis and high incidence of MOFs are potentially related to the appearance of
384 fertility syndromes in human adulthood (Franks *et al.* 2008, Asimakopoulos *et al.* 2013).
385
386 Superovulation is a reproductive practice applied to many mammalian species whereby
387 exogenous gonadotropins are used to increase follicular development or the ovulation rate
388 with the expectation to generate greater numbers of embryos. This technique is used both
389 in adults (Multiple Ovulation and Embryo Transfer-MOET) (Wray & Goddard 1994) and
390 in prepubertal females (Juvenile In Vitro Embryo Transfer - JIVET) (Kelly *et al.* 2005). It
391 has long been known that follicles of 4- to 8-week-old lambs are particularly sensitive to
392 gonadotropin administration using protocols developed for adult animals (Worthington CA
393 & Kennedy JP 1979, Armstrong *et al.* 1994, Ptak *et al.* 1999). On the other hand, ovarian
394 stimulation has proved to be a simple and useful tool to detect alterations in rodent
395 reproductive organs and to study likely changes in the mechanisms of hormonal action
396 induced by certain substances (Sekiguchi *et al.* 2003). Here, we applied a protocol of
397 oFSH-ovarian stimulation in prepubertal lambs and showed that early postnatal exposure
398 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 with 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.

Since 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 ERα or ERβ protein levels in both unstimulated (PND30) and oFSH-stimulated (PND34) ovaries of vehicle- 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 up-regulating 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 to be

449 determined. Recently, a study conducted on sheep demonstrated that prenatal BPA
450 exposure alters fetal ovarian steroidogenic gene and microRNA expression in an age-
451 dependent way (Veiga-Lopez *et al.* 2013). In our experiment, although the basal levels of
452 E_2 were not affected in lambs neonatally exposed to BPA or DES, the stimulatory response
453 to oFSH was impaired. Differences in experimental design may explain differences in the
454 results. Interestingly, Vitt *et al.* (2000) demonstrated that GDF9 suppresses both FSH-
455 induced progesterone and estradiol production in rat follicles. The high values of *GDF9*
456 expression detected in the present work in xenoestrogen-treated lamb ovaries could
457 explain, at least in part, the failure of antral follicles to respond to oFSH. In addition, the
458 low number of antral follicles could also explain the diminished capability of ovaries from
459 lambs previously exposed to DES or BPA to synthesize estradiol. Moreover, ovaries from
460 lambs exposed to the lowest dose of BPA and treated with oFSH showed a decreased
461 expression of *BMPR1B*. BMPR1B is expressed by granulosa cells and oocytes from the
462 primary to the late antral follicle stages and acts as a receptor for various BMP factors
463 (Dijke *et al.* 2003, Fabre *et al.* 2006). It has been described that a single mutation in the
464 coding sequence of the *BMPR1B* is responsible for the hyperprolific phenotype of
465 Booroola ewes (Mulsant *et al.* 2001, Souza *et al.* 2001, McNatty *et al.* 2001). Then,
466 Campbell *et al.* (2003) reported that ovaries of $Fec^{B/-}$ (Booroola) ewes contain mainly
467 small follicles with a low number of medium size follicles and no large follicles after 3
468 days of FSH infusion. Interestingly, mice deficient in BMPR1B are infertile and show
469 impaired estradiol synthesis (Souza *et al.* 2002) and decreased Cyp19 expression (Yi *et al.*
470 2001). Our results suggest that the alterations in estradiol levels and folliculogenesis
471 observed in response to oFSH in lambs previously exposed to the lowest dose of BPA
472 could be explained by an attenuated action of some BMP factors due to the decreased
473 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). Here, as mentioned before, the protein levels of ERA and ERB 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). ERA 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 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_2 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 like puberty onset, cyclicity or implantation (Durando *et al.* 2007, Monje *et al.* 2010, Varayoud *et al.* 2014). If the changes observed in the present 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 shown 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 showed 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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758

FIGURE LEGENDS

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 done by sc injections. PND: postnatal day; IHC: immunohistochemistry; qRT-PCR: quantitative real-time polymerase chain reaction.

FIGURE 2. Effect of exogenous treatment (oFSH) on ovarian follicular response on PND34 in neonatally xenoestrogen-exposed lambs. Follicles ≥ 2 mm were recorded on the ovarian surface. Bars represent mean values ± SEM. One-way- ANOVA ~~Kruskal-Wallis~~ followed by Dunnett's post-test. Asterisks denote $p<0.05$ * or $p<0.01$** vs. control.

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) *versus* 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 ± SEM. One-way- ANOVA ~~Kruskal-Wallis and~~ followed by Dunnett's post-test. Asterisks denote

p<0.05 * or p<0.01** vs. control. Scale bars, 50μm 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.

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 6 doses of oFSH. Kruskal-Wallis followed by Dunn's post-hoc test, comparisons were made between PND30 vs. PND32, * p<0.05.

FIGURE 6. Representative photomicrographs of ovarian sections showing AR **(A - H)** and ERB **(I - 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 ~~expression~~. DES- **(C and G)** or BPA50- **(D and H)** neonatally exposed lambs treated with oFSH on PND34 did not show increased in AR protein level ~~expression~~ in either cellular compartment of antral follicles. ERB protein was highly expressed in both

granulosa and theca cells of antral follicles in controls non-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: 60μm for A - D and I - L, and 300 μm for E – H and M - P. g, granulosa; t, theca; star, antrum.

FIGURE 7. Quantitative real-time PCR analysis of the mRNA 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 ± SEM. One-way- ANOVA followed by Dunnett's post-test. *$p < 0.05$ vs. controls.

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

Gene	Primer sequence (5'-3')	Product size (pb)	GenBank Access number
BMP6	Forward: CTCTACGTGAGCTTCCAGGACCT	83	DQ192014.1
	Reverse: TCTCCGTCACAGTAGTTGGCAGC		
BMP15	Forward: ATGGTCCTCCTGAGCATCCTTAG	87	NM_001114767
	Reverse: CTGCCCTACCTGTGTCATTTGG		
BMPR1B	Forward: TCTACACTTTGGTTATCAGC	95	NM_001009431
	Reverse: TTTGTATCCTCTCTTGTCAT		
GDF9	Forward: TAGAGGTTCTGTATGATGGG	90	NM_001142888
	Reverse: ATGCCTTATAGAGCCTCTTC		
FSHR	Forward: CCAACAACCTGCTATACATC	103	NM_001009289
	Reverse: GTGCTTAATACCTGTGTTGG		

Abbreviations: *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.

Table 2. Expression of ERA, ERB 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**
ERA							
Granulosa	-	-	-	-	-	-	-
Theca	-	-	-	-	-	-	-
ERB							
Granulosa	+++	+++	+++	+++	+++	+++	+++
Theca	++	++	++	++	++	++	++
AR							
Granulosa	++	++	++	++++	++	++	++
Theca	+	+	+	++	+	+	+

From birth to postnatal day 14 (PND14), lambs were exposed to DES (5 μg/kg/day), BPA (50 μg/kg/day or 0.5 μg/kg/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.

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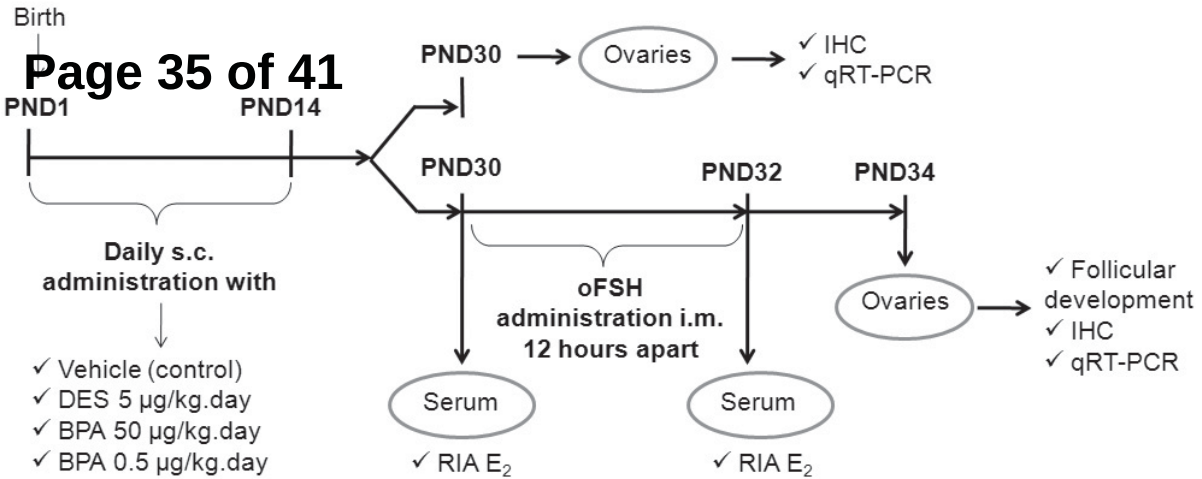
Birth
PND1
PND14
Daily s.c. administration with
✓ Vehicle (control)
✓ DES 5 µg/kg.day
✓ BPA 50 µg/kg.day
✓ BPA 0.5 µg/kg.day
PND30
Ovaries
✓ IHC
✓ qRT-PCR
PND30
PND32
PND34
oFSH administration i.m. 12 hours apart
Serum
✓ RIA E_2
Serum
✓ RIA E_2
Ovaries
✓ Follicular development
✓ IHC
✓ qRT-PCR

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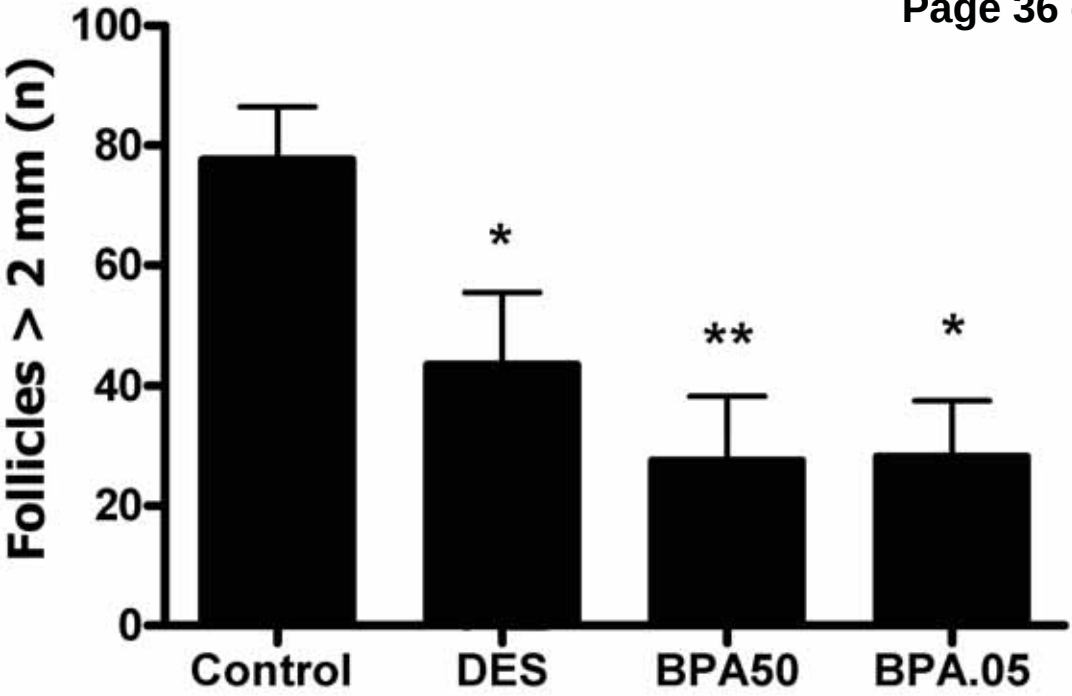
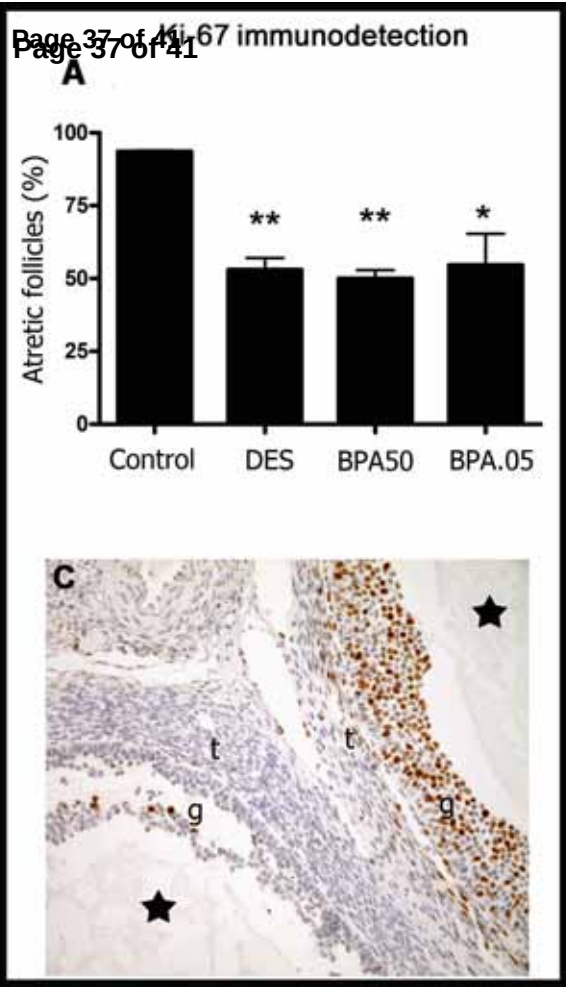
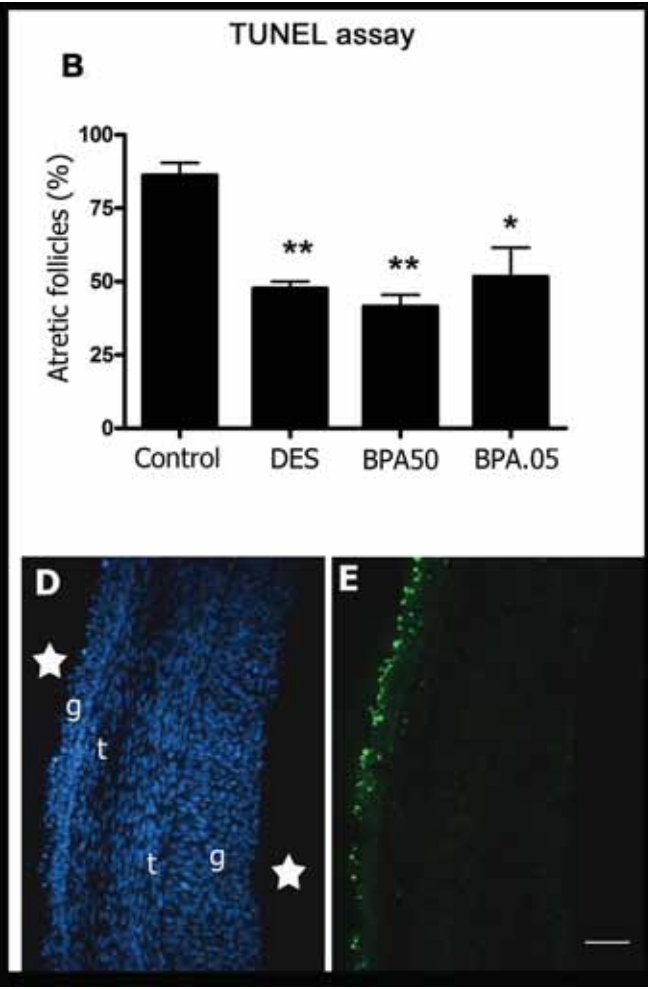



Ki-67 immunodetection
A
Atretic follicles (%)
100
75
50
25
0
**
**
*
Control
DES
BPA50
BPA.05
C
t
t
g
g


TUNEL assay
B
Atretic follicles (%)
100
75
50
25
0
**
**
*
Control
DES
BPA50
BPA.05
D
g
t
t
g
E

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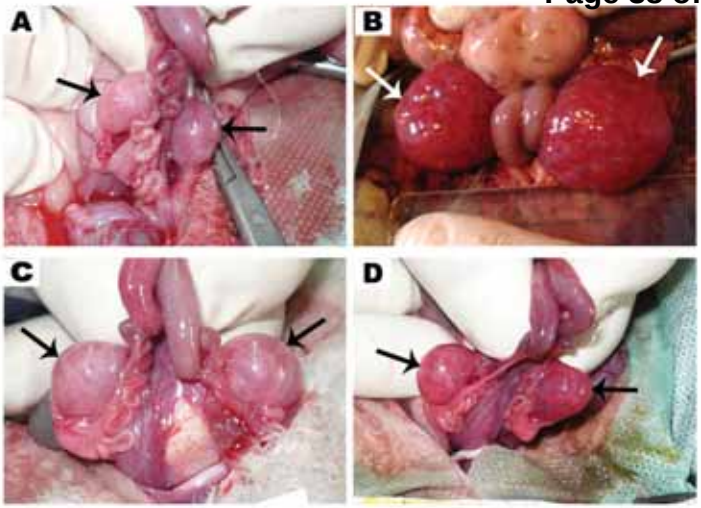

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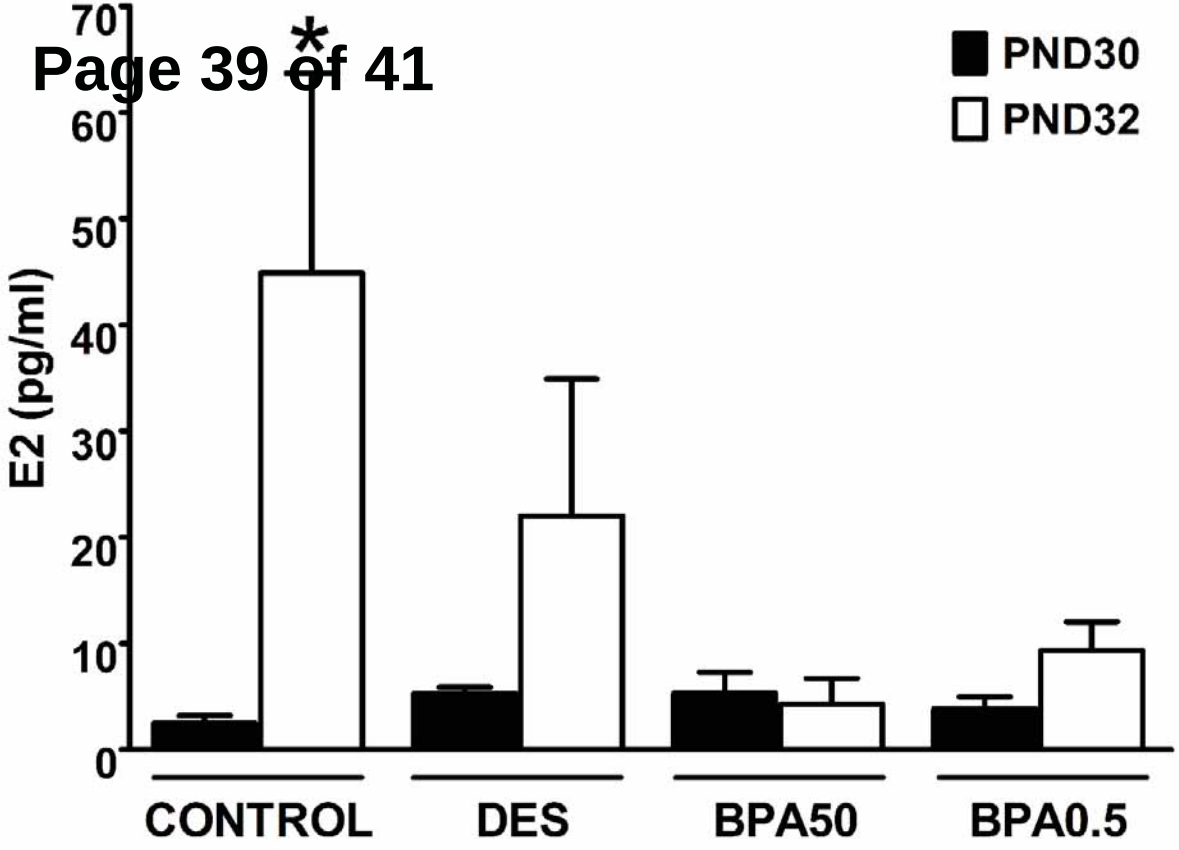
70
60
50
40
30
20
10
0
E2 (pg/ml)
*
PND30
PND32
CONTROL
DES
BPA50
BPA0.5

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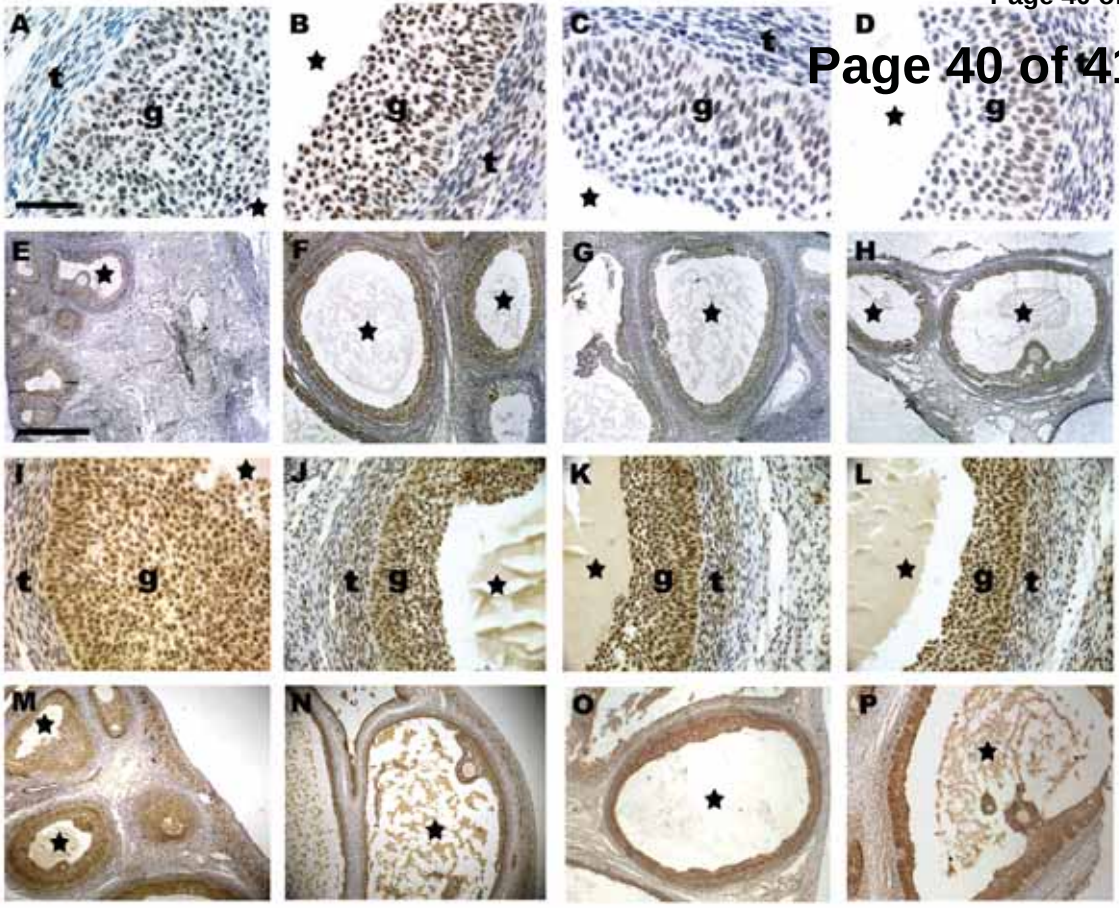

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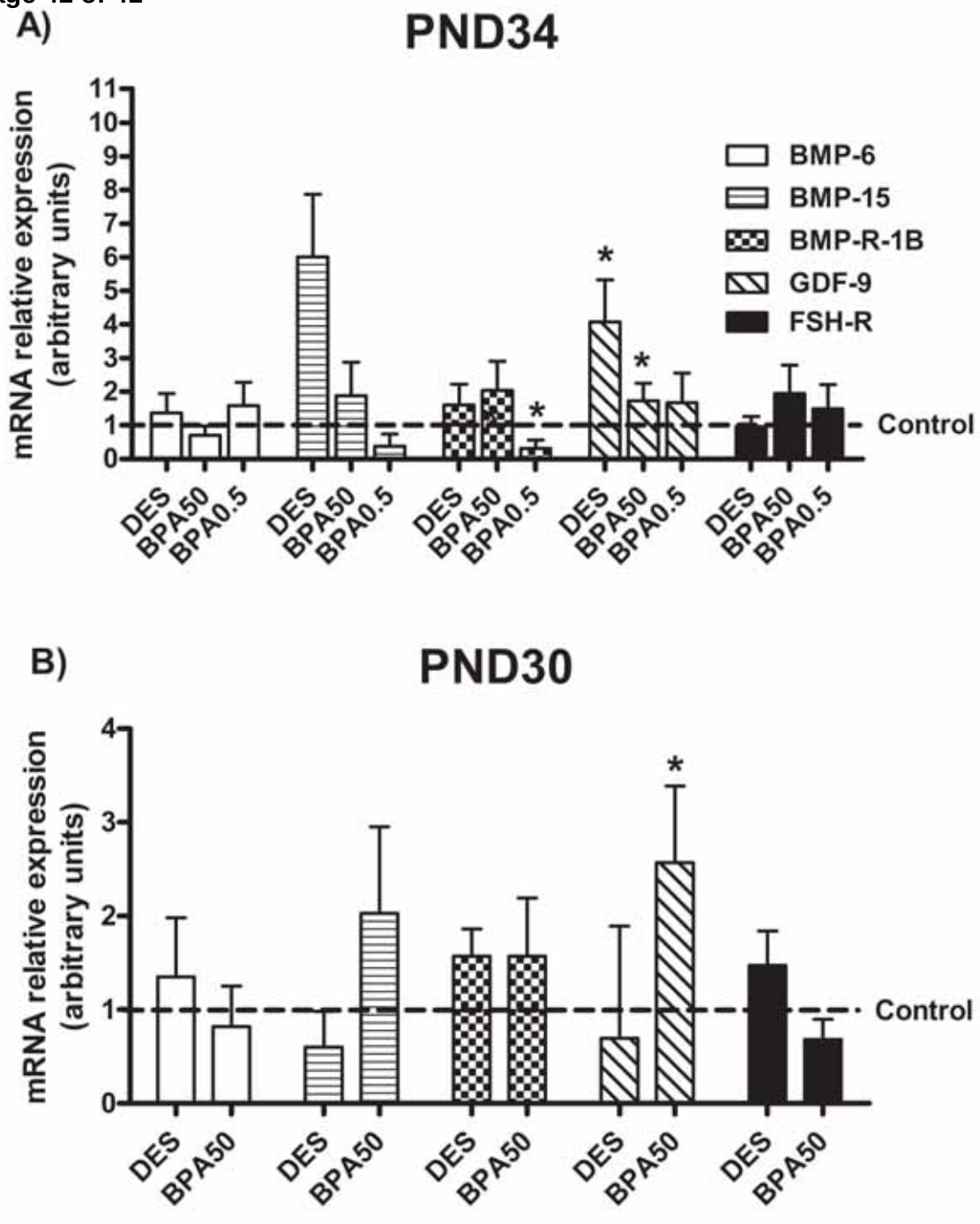
A)
PND34
mRNA relative expression (arbitrary units)
11
10
9
8
7
6
5
4
3
2
1
0
BMP-6
BMP-15
BMP-R-1B
GDF-9
FSH-R
Control
*
*
*
DES
BPA50
BPA0.5
B)
PND30
mRNA relative expression (arbitrary units)
4
3
2
1
0
Control
*
DES
BPA50