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A deregulated expression of estrogen-target genes is associated with an altered response to estradiol in aged rats perinatally exposed to bisphenol A

Lucía Vigezzi [a, b], Jorge G. Ramos [a, c], Laura Kass [a, d], María V. Tschopp [a], Mónica Muñoz-de-Toro [a, d], Enrique H. Luque [a, b], Verónica L. Bosquiazzo [a, c, *]

[a] Instituto de Salud y Ambiente del Litoral (ISAL UNL-CONICET), Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina
[b] Cátedra de Fisiología Humana, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina
[c] Departamento de Bioquímica Clínica y Cuantitativa, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina
[d] Cátedra de Patología Humana, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina

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ABSTRACT

Here we assessed the effects of perinatal exposure to bisphenol A (BPA) on the uterine response to 17β-estradiol (E2) in aged rats. Pregnant rats were orally exposed to 0.5 or 50 μg BPA/kg/day from gestational day 9 until weaning. On postnatal day (PND) 360, the rats were ovariectomized and treated with E2 for three months. The uterine tissue of BPA50 and BPA0.5 rats showed increased density of glands with squamous metaplasia (GSM) and glands with daughter glands respectively. Wnt7a expression was lower in GSM of BPA50 rats than in controls. The expression of estrogen receptor 1 (ESR1) and its 5′- untranslated exons ESR1-O and ESR1-OT was lower in BPA50 rats. Both doses of BPA modified the expression of coactivator proteins and epigenetic regulatory enzymes. Thus, perinatal BPA-exposed rats showed different glandular abnormalities associated with deregulated expression of E2-target genes. Different mechanisms would be involved depending on the BPA dose administered.

1. Introduction

Bisphenol A (BPA) is one of the highest-volume chemicals produced worldwide. Current estimations indicate that more than eight billion pounds of BPA are produced annually and that approximately 100 tons may be released into the atmosphere each year (Rubin, 2011; Vandenberg et al., 2012). BPA is used in the manufacture of plastics and resins that are pervasive in our environment and in our daily lives (Rubin, 2011). A study carried out by the Center for Disease Control and Prevention in the United States detected BPA in the urine of >90% of the Americans sampled (Calafat et al., 2008). In addition, numerous studies have shown sufficient evidence for adverse effects of BPA at levels equal to or below the acceptable human intake dose (50 μg BPA/kg/day) established by the U.S. Environmental Protection Agency (EPA) (Fernandez et al., 2009; Peretz et al., 2014; Rochester, 2013; Tyl, 2014; Vandenberg et al., 2013; Varayoud et al., 2014; Vigezzi et al., 2015).

Exposure to BPA during uterine development is critical because it can affect the uterine histology and functional differentiation later in life (Bosquiazzo et al., 2010; Schonfelder et al., 2004; Suvorov and Waxman, 2015; Varayoud et al., 2008; Vigezzi et al., 2015). Sometimes, the effects of early exposure to BPA are not evidenced until adulthood, when hormone-dependent organs are exposed to particular endocrine situations such as pregnancy or exogenous hormonal treatment. Developmental exposures to BPA have been associated with altered hormonal signaling pathways

Abbreviations: BPA, bisphenol A; GD, gestational day; PND, postnatal day; E2, 17β-estradiol; NG, normal glands; GSM, glands with squamous metaplasia; GDG, glands with daughter glands; PR, progesterone receptor; ESR1, estrogen receptor 1; SRC, steroid receptor coactivator; Dnmt, DNA methyltransferase; EZH-2, Enhancer of zeste homolog 2; Hdac, Histone deacetylase; IOD, integrated optical density; CT, cycle threshold.

* Corresponding author. Instituto de Salud y Ambiente del Litoral (ISAL, CONICET-UNL), Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria, Paraje El Pozo, Casilla de Correo 242, 3000 Santa Fe, Argentina.
E-mail address: vlbosqui@fbcb.unl.edu.ar (V.L. Bosquiazzo).

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and increased susceptibility to dysfunction and/or disease in adult steroid-target organs including the mammary gland (Acevedo et al., 2013; Durando et al., 2007; Kass et al., 2012), ovary (Chao et al., 2012; Fernandez et al., 2010), uterus (Bosquiazzo et al., 2010; Newbold et al., 2007, 2009; Varayoud et al., 2014), prostate (Prins et al., 2008a; Ramos et al., 2001; Taylor et al., 2011) and liver (Weinhouse et al., 2014). In addition, it has been shown that perinatal (gestation + lactation) BPA exposure affects the long-term response to estrogen in target tissues such as the prostate (Prins et al., 2008b) and mammary gland (Wadia et al., 2007), increasing the susceptibility to carcinogenesis. Particularly in the uterus, we have previously demonstrated that rats perinatally exposed to BPA show an altered uterine response to 17β-estradiol (E2) in the adulthood (Vigezzi et al., 2015).

The development and differentiation of the uterine glands, or adenogenesis, is one of the main events that occur during the early postnatal period (Gray et al., 2001; Spencer et al., 2012). In rodents, uterine adenogenesis takes place from postnatal day (PND) 9 to PND15, and is characterized by epithelial invaginations or bud development followed by the formation of tubular glands lined by simple cuboidal epithelium (Cooke et al., 2013; Gray et al., 2001). *Wnt (wingless-type MMTV integration site family)* genes encode a large family of secreted growth factor proteins that play important roles in embryo development and adult tissue homeostasis (Ring et al., 2014). Specifically, Wnt7a and Wnt5a are involved in uterine adenogenesis (Cooke et al., 2013; Spencer et al., 2012). It has been shown that neonatal exposure to the endocrine disruptor diethylstilbestrol alters the expression of Wnt7a and Wnt5a and causes a range of uterine defects similar to those seen in knockout models (Dunlap et al., 2011; Hayashi et al., 2011; Mericskay et al., 2004; Miller and Sassoon, 1998). In addition, the absence of β-catenin, the main intracellular mediator of canonical Wnt signaling pathway, causes perturbation in the adenogenic process and produces squamous metaplasia in the luminal epithelium (Jeong et al., 2009).

In the adult, estrogens regulate numerous processes of uterine functional differentiation that depend on reciprocal stromal-epithelial interactions (Cunha et al., 2004), and one of their target genes is the progesterone receptor (PR) (Franco et al., 2012; Patel et al., 2015). It is known that uterine epithelial PR expression is regulated by stromal estrogen receptor 1 (ESR1) through a paracrine mechanism (Kurita et al., 2000). The Wnt family members are also specifically expressed in a hormone-dependent stromal-epithelial pattern (through stromal ESR1) in the adult uterus (Mericskay et al., 2004; Miller et al., 1998; Spencer et al., 2012). In addition, a dynamic Wnt expression pattern has been observed during the murine estrous cycle in response to changes in the circulating levels of steroid hormones (Mericskay et al., 2004).

The BPA-mediated abnormal expression of steroid-sensitive genes (such as PR and Wnt) would lead to a dysregulation of the hormonal signaling pathway with long-term negative consequences for adult uterine function and reproductive health (Suvorov and Waxman, 2015). Although the molecular pathways by which BPA developmentally reprograms gene expression are unknown, much evidence suggests that BPA could mediate its action through epigenetic mechanisms, including modification of DNA methylation profiles, changes in the expression or activity of DNA methyltransferases (Dnmts) and alteration of histone post-translational modifications (Bernal and Jirtle, 2010; Dhimolea et al., 2014; Kundakovic and Champagne, 2011; Xin et al., 2015). The patterns of methylation or histone modification necessary for normal tissue development are programmed during embryogenesis. Therefore, the developmental period is a critically sensitive window of vulnerability during which the epigenome is particularly vulnerable to hormonal dysregulation (Dolinoy et al., 2007; Thompson and Einstein, 2010; Vaiserman, 2014).

In the present work, we examined the expression of genes that could be implicated in the development of uterine gland abnormalities in response to a chronic E2 treatment, in aged rats perinatally exposed to BPA. Perinatal BPA exposure has been shown to dysregulate estrogen-target organs, including the uterus, but early-life priming for increased carcinogenic responses to estrogens later in life has only been shown in the prostate (Prins et al., 2008b) and breast (Wadia et al., 2007). Therefore, we asked whether a similar priming effect exists in the uterus.

2. Materials and methods

2.1. Animals

The experimental protocols were designed in accordance with the Guide for the Care and Use of Laboratory Animals issued by the U.S. National Academy of Sciences and approved by the ethics committee of the School of Biochemistry and Biological Sciences, Universidad Nacional del Litoral (Santa Fe, Argentina). We used an inbred Wistar-derived strain that was developed by mating a founding pair of Wistar rats and then sequential brother-sister mating for 20 generations (Departmento de Fisiología Humana, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral). Rats were kept in a controlled environment (22 °C ± 2 °C; 14 h of light from 0600 h to 2000 h) with free access to pellet laboratory chow (Nutrición Animal, Santa Fe, Argentina). The concentration of phytoestrogens in the diet was analyzed by HPLC at the Instituto Nacional de Tecnología Industrial (INTI, Buenos Aires, Argentina). The diet contained 400 mg/kg of phytoestrogens (210 mg/kg of daidzein and 190 mg/kg of genistein). These phytoestrogens came from the soy contained in the chow and are consistent with a soy protein content of approximately 23% in the diet. For more information regarding the food composition, see Altamirano et al. (2015); Andreoli et al. (2015); Kass et al. (2012). Even though the food intake of control and BPA-treated rats was equivalent and we assumed that all animals were exposed to the same levels of phytoestrogens, we cannot rule out that the effects observed in BPA-exposed rats were due to the combined exposure to BPA and chow phytoestrogens. To minimize the exposure to other endocrine-disrupting chemicals, rats were housed in stainless steel cages with sterile pine wood shavings as bedding. Tap water was supplied *ad libitum* in glass bottles with rubber stoppers.

2.2. Experimental design

We chose the perinatal exposure period based on strong literature about the greatest sensitivity of different steroid-target tissues during this exposure timing (Acevedo et al., 2013; Altamirano et al., 2015; Caserta et al., 2014; Kass et al., 2012; Maffini et al., 2006; Mendoza-Rodriguez et al., 2011; Rubin and Soto, 2009; Vandenberg et al., 2009; Vigezzi et al., 2015; Weinhouse et al., 2014). Females in proestrus were caged overnight with males of proven fertility. The day on which sperm was found in the vagina was designated day 1 of gestation (GD1). On GD9, which corresponds to the beginning of fetal organogenesis and does not interfere with the implantation process occurred on GD5 (Dey et al., 2004), pregnant rats were weighed and randomly divided into three experimental groups: Control (0.001% ethanol), BPA0.5 (0.5 μg/kg/day, 99% purity, Sigma−Aldrich, Buenos Aires, Argentina) and BPA50 (50 μg/kg/day, Sigma−Aldrich), with 10−14 dam s/group. BPA was administered in the drinking water from GD9 to weaning (Fig. 1). BPA solution was prepared according to Kass et al. (Kass et al., 2012). and the dose was calculated on the basis of the average weight of dams

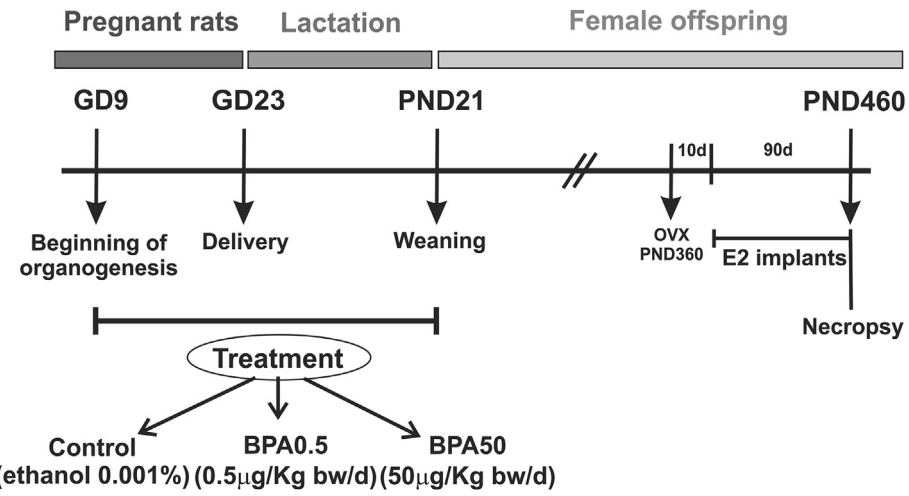

Fig. 1. Schematic representation of the experimental protocol used to study the effects of perinatal (gestation + lactation) oral exposure to bisphenol A (BPA) on the uterus of aged rats treated with E2. GD: gestational day, PND: postnatal day; E2: 17β-estradiol; OVX: ovariectomy.

and water consumption during pregnancy and lactation.

After parturition, pups were weighed and sexed according to the anogenital distance; litters of eight pups (preferably four males and four females) were left with lactating mothers until weaning on postnatal day (PND) 21. One female offspring per litter from each treatment group was assigned to this experiment. The remaining females and all males from each litter were assigned to other experiments. As an external index of female puberty onset, vaginal opening was monitored daily starting on PND30. To evaluate the effects of perinatal exposure to BPA on the uterine response to a chronic treatment with E2, on PND360, rats from the control and BPA-exposed groups were ovariectomized to avoid endogenous E2 variability, and then treated with an E2 replacement therapy for 90 days (Fig. 1). Rats were implanted (sc) with silastic capsules (outer diameter: 3.18 mm, inner diameter: 1.57 mm, 30 mm in length; Specialty Manufacturing, Midland, MI, USA) filled with 1 mg E2/ml dissolved in sesame oil. To ensure exposure to constant E2 levels, the implants were changed every 30 days. At the end of the treatment (PND460), the animals were sacrificed.

2.3. Sample collection

At autopsy on PND460, blood and uterine tissue were collected and processed for different experimental purposes. Serum samples were stored at −80 °C until hormone assays were performed. For immunohistochemistry (IHC), one uterine horn of each rat was fixed in 10% buffered formalin and embedded in paraffin. The other uterine horn of each rat was immediately frozen in liquid nitrogen and stored at −80 °C for protein and RNA extraction followed by Western blot and RT-PCR assays respectively.

2.4. Hormone assay

Serum levels of E2 were measured in blood samples by radioimmunoassay after extraction with ethyl ether (Merck, Buenos Aires, Argentina) (Kass et al., 2004). The antibody was provided by G. D. Niswender, and the labeled hormone was purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA, USA). Assay sensitivity was 4 pg/ml and intra- and inter-assay coefficients of variation were 3.2% and 11% respectively.

2.5. Histology and morphometry of glands

Uterine samples embedded in paraffin were cut into 5-μm sections, mounted on slides coated with 3-aminopropyl triethoxysilane (Sigma–Aldrich) and stained with hematoxylin and eosin for light microscopy (Olympus BH2, Tokyo, Japan). To estimate the extent of any changes in the uterus, glands were classified according to Gunin et al. (Gunin et al., 2001). and Vigezzi et al. (2015) as follows: (1) normal glands, (2) cystic glands, (3) glands with squamous metaplasia, (4) glands with daughter glands, (5) conglomerate of glands, (6) glands with cellular anomalies. The volume density of glands was calculated by applying the formula given by Weibel (Weibel, 1969): Vv = Pi/P, where Vv is the estimated volume density of the object in study (glands), Pi is the number of incident points over these glands, and P is the number of incident points over all cells in the studied population (stroma). To obtain the data for the point-counting procedure, a glass disk with a squared grid of 0.8 mm × 0.8 mm was inserted into a focusing eyepiece. The results were expressed as Vv x 1000.

2.6. Immunohistochemistry

Uterus sections (5 μm in thickness) were deparaffinized and dehydrated in graded ethanol. Endogenous peroxidase activity and nonspecific binding sites were blocked. Primary antibodies (Table S1, supplementary data) were incubated overnight at 4 °C. The reactions were developed using a streptavidin-biotin peroxidase method and DAB. Samples were mounted with permanent mounting medium (Eukitt, Sigma–Aldrich). Each immunohistochemical run included negative controls in which the primary antibody was replaced by non-immune goat serum (Sigma–Aldrich).

2.7. Generation of anti-Wnt7a and anti-Wnt5a and validation assays

For Wnt7a and Wnt5a, we generated affinity-purified rabbit polyclonal antibodies, following previously described protocols (Varayoud et al., 2008). The antigens were expressed in *Escherichia coli* JM109 (Stratagene Corp., La Jolla, CA, USA) as glutathione-S-transferase fusion proteins using a pGEX4T-3 vector (Stratagene Corp., La Jolla, CA). The Wnt7a antigen included the region corresponding to amino acids 194–283 of the rat sequence (accession

no. EDL91365.1), whereas the Wnt5a antigen included the region corresponding to amino acids 207-300 (accession no. AAV69750). Antibodies were purified using antigen-linked affinity chromatography (Hi-Trap NHS activated HP column; GE Healthcare, Buenos Aires, Argentina). For specificity validation tests, the antibodies against both Wnt proteins were preabsorbed by incubating 1 μg of antibody with 10–20 μg of the antigenic peptide for 24 h at 4 °C and both antibody–antigen complexes were assayed by Western blot and IHC in positive control tissues. In addition, the specificity of the anti-serum was tested by Western blot analysis of protein extracts from rat uterus and testis (Kirikoshi and Katoh, 2002) and by IHC of uterus and testis sections.

For Western blot, protein extractions were performed as previously described (Kass et al., 2012). Total protein concentration was determined using a colorimetric BCA Protein Assay Kit from Pierce Chemical Co. (Rockford, IL, USA). Equal amounts of protein (100 μg) from each sample were resolved by 15% SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad Argentina, Buenos Aires, Argentina) and reacted with anti-Wnt7a (dilution 1:200) and anti-Wnt5a (dilution 1:200). A peroxidase-conjugated anti-rabbit secondary antibody (Sigma–Aldrich) was used at a 1:400 dilution, and the reaction was visualized using diaminobenzidine (DAB, Sigma–Aldrich). Molecular weights were determined by comparison to molecular weight standards (Broad Range Protein Markers, Promega, Madison, WI, USA).

2.7.1. Characterization of anti-Wnt7a and anti-Wnt5a

The characterization of the antiserum obtained against Wnt7a and Wnt5a is shown in Fig. 2. A specific band of 39 kDa was detected with the anti-Wnt7a antibody in Western blot assays of both rat uterus and testis, whereas a specific band of 42 kDa was found using the anti-Wnt5a antibody only in the rat uterus (Fig. 2A). To evaluate the immunoreactivity in paraffin-fixed tissues, IHC assays on testis and uterus sections were performed (Fig. 2B and C). In control rats, Wnt7a was found in the nuclei and cytoplasm of glandular and luminal epithelium, whereas Wnt5a showed primarily cytoplasmic immunostaining with a weak nuclear staining in glandular and luminal cells. The stromal compartment showed negative immunostaining for both proteins (Fig. 2C). Specific staining was absent when the antibodies were preincubated with the corresponding peptide used as immunogen, indicating the specificity of the Western blot (Fig. 2A) and IHC staining (Fig. 2D).

2.8. Image analysis

Images from immunostained tissue sections were captured with a Dplan 20X objective (numerical aperture, 0.65; Olympus). Images were analyzed using the Image Pro-Plus 4.1.0.1 system (Media Cybernetics, Silver Spring, MD, USA) (Ramos et al., 2002). The expression of β-catenin, PR, Wnt7a and Wnt5a was evaluated by measuring the immunostaining integrated optical density (IOD) in all uterine glands present in each uterine section, and three sections per rat were evaluated. To measure the IOD of ESR1, SRC-1 and SRC-3 immunostaining in the uterine stroma, at least 10 fields were recorded in each section, and three sections per rat were evaluated. The IODs were measured as previously described (Varayoud et al., 2011). Since the IOD is a dimensionless parameter, the results were expressed as arbitrary units.

2.9. Reverse transcription and real-time quantitative PCR analysis

2.9.1. RNA extraction and reverse transcription

Individual uterine horn samples were homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA), and RNA was prepared according to the manufacturer's protocol. The concentration of total RNA was assessed by A260, and the samples were stored at −80 °C until needed. Equal quantities (1 μg) of total RNA were reverse-transcribed into cDNA with Moloney Murine Leukemia Virus reverse transcriptase (10 units; Promega, Madison, WI, USA) as previously described (Bosquiazzo et al., 2013).

2.9.2. Real-time quantitative PCR

Each reverse-transcribed product was diluted with ribonuclease-free water to a final volume of 60 μl and further amplified in triplicate using the Real-Time Rotor-Gene Q (Quiagen; Tecnolab; Buenos Aires, Argentina). An optimized real time-PCR protocol was used to analyze the mRNA expression levels of total ESR1; ESR1 transcripts containing the 5′ untranslated exons OS, ON, O, OT, and E1; DNA methyltransferase (Dnmt) 3a and Dnmt3b; *Enhancer of zeste homolog 2* (EZH-2); Histone deacetylase (Hdac) −1, -2 and −3 and L19 (housekeeping gene). The primer sequences designed for cDNA amplification are described in Table S2 (Supplementary data). For cDNA amplification, 5 μl of cDNA was combined with HOT FIREPol Eva Green qPCR Mix Plus (Solis BioDyne; Biocientífica; Rosario, Argentina), and 10 pmol of each primer (Invitrogen) in a final volume of 20 μl. Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. Controls containing no template DNA were included in all assays, yielding no consistent amplification. The relative expression levels of each target were calculated based on the cycle threshold (CT) method (Higuchi et al., 1993). The CT for each sample was calculated using the Rotor-Gene Q – Pure Detection software (Version: 1.7, Quiagen; Tecnolab) with an automatic fluorescence threshold (Rn) setting. No significant differences in CT values were observed for L19 between the different experimental groups. L19 was used to normalize the CT values of the genes evaluated. The relative expression levels of each target were calculated using the standard curve method (Cikos et al., 2007).

2.10. Statistical analysis

All data are expressed as the mean ± SEM. The expression of ESR1 5′ untranslated exons was analyzed using the *t* test. All the other variables studied were analyzed by Kruskal–Wallis test followed by Dunn's post-test to establish differences between experimental groups. $p < 0.05$ was accepted as significant.

3. Results

3.1. BPA general effects

As previously described (Altamirano et al., 2015; Kass et al., 2012; Vigezzi et al., 2015), exposure to BPA through the drinking water produced no signs of embryo toxicity, abnormal maternal or nursing behavior, or changes in dam body weight gain or water consumption. Furthermore, litter survival during lactation was unaltered and the onset of puberty in female offspring showed no significant differences between groups. All females perinatally exposed to BPA exhibited regular estrous cycles (5 days) when examined on PND90. These results are summarized in Table S3 (Supplementary data).

3.2. Estradiol levels

No significant differences in E2 serum levels were observed between experimental groups on PND460 (Control 19.22 ± 4.2 pg/ml; BPA0.5 19.3 ± 9.8 pg/ml; BPA50 11.3 ± 7.03 pg/ml).

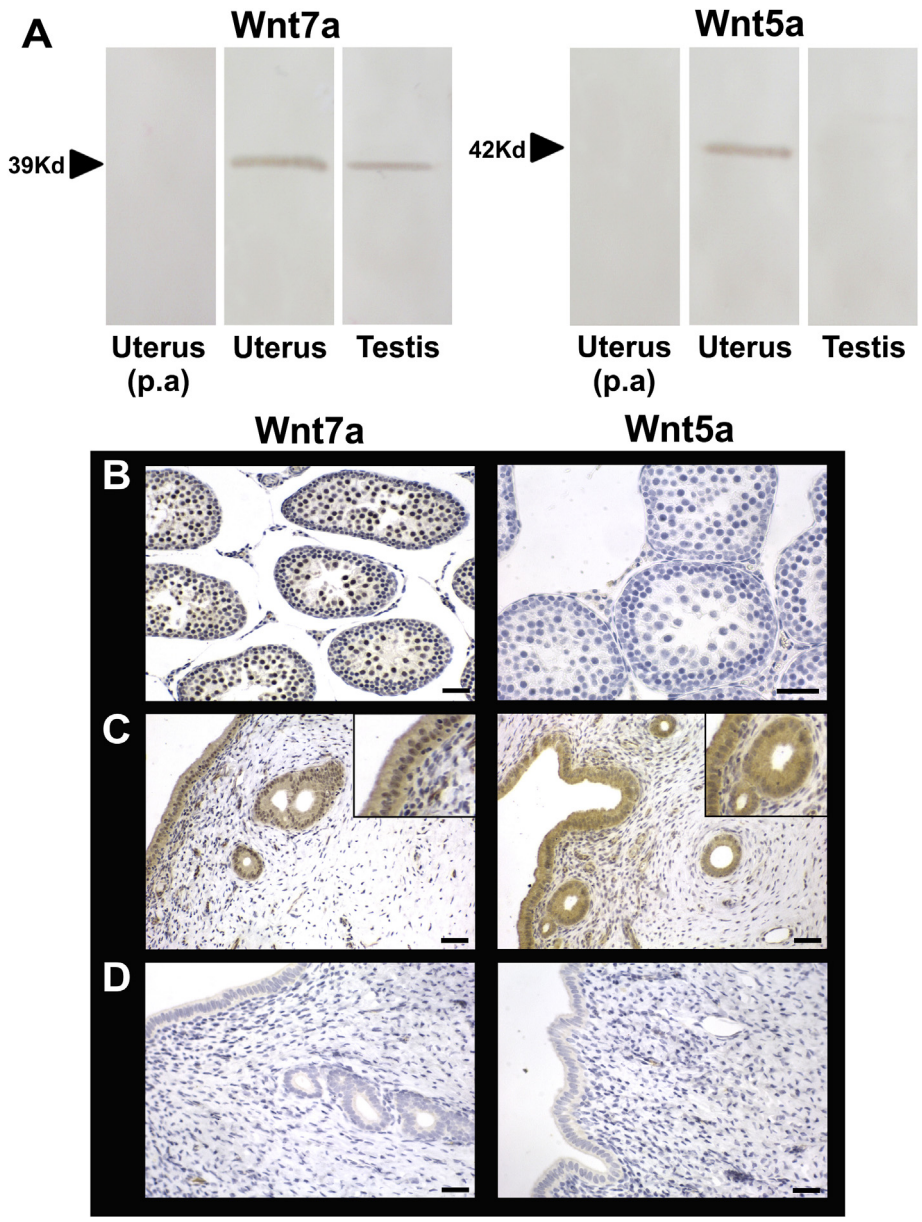

Fig. 2. Characterization of Wnt7a and Wnt5a antisera using rat uterus and testis. (A) In Western blot assays, Wnt7a native protein (39 kDa) was detected in the uterus and testis, and Wnt5a native protein (42 kDa) was detected in the uterus. Specific protein detection was absent in the uterus when the primary antibodies were preabsorbed (p.a) with the peptide used as immunogen. Immunoreactivity of the antibodies was evaluated by immunohistochemistry in tissue sections of testis (B) and uterus (C). (C) Positive staining for both Wnt7a and Wnt5a was found in glandular and luminal cells of uterine tissue; insets show that Wnt7a was expressed in the nuclei and cytoplasm, whereas Wnt5a expression was mainly cytoplasmic. (D) Specific staining was absent when the primary antibodies were preabsorbed with the peptide used as immunogen. Scale bars: 50 μm.

3.3. Morphological features of the uterine tissue

Although glandular abnormalities are frequently found in aged animals (Vigezzi et al., 2015), the volume density of glands with squamous metaplasia (GSM) was higher in BPA50 rats and the volume density of glands with daughter glands (GDG) was higher in BPA0.5 rats than in controls (Fig. 3).

3.4. Expression of molecules in uterine glands

3.4.1. Wnt7a and Wnt5a

To evaluate whether the increased density of glandular abnormalities in females perinatally exposed to BPA and then treated with E2 was associated with changes in glandular development-related genes, we measured the expression of Wnt7a and Wnt5a in normal glands (NG), GSM and GDG by IHC. Wnt7a expression was lower in GSM than in NG in all experimental groups (Fig. 4). In addition, Wnt7a expression in GSM of the BPA50 group was lower than that in GSM of the control group (Fig. 4). Wnt5a expression showed no differences between experimental groups (data not shown).

3.4.2. β-catenin

Then, protein expression of β-catenin, the main mediator of the Wnt signaling pathway, was analyzed. The IHC assay demonstrated that β-catenin was expressed in glandular and luminal cells and that it was localized mainly on the cellular membrane, with low cytoplasmic expression (data not shown). No differences between experimental groups were found in β-catenin expression in the

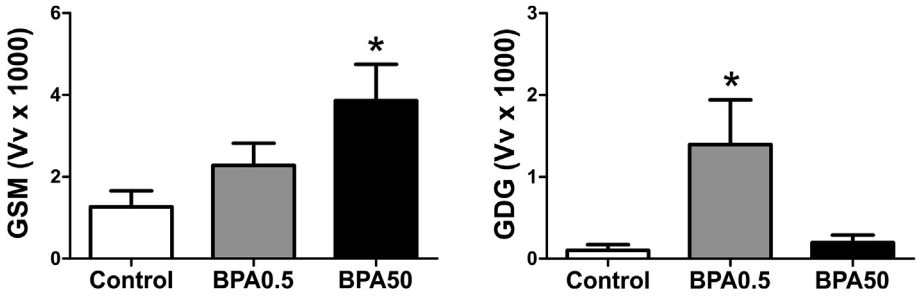

Fig. 3. Quantification of glands with squamous metaplasia (GSM) and glands with daughter glands (GDG) in uterine tissue of aged rats treated with E2. Each column represents the mean ± SEM of the volume density (Vv) x 1000 (n > 8 per group). *, p < 0.05 vs. the control group.

different glandular types analyzed (NG, GSM, or GDG, p > 0.05).

3.4.3. PR

As mentioned, PR plays critical roles in uterine function. Therefore, the expression of PR was evaluated in NG, GSM and GDG of all experimental groups. In control animals, PR protein expression was lower in GSM than in NG (NG: 196.1 ± 2.35; GSM: 159.3 ± 15.39, p < 0.05) without changes in GDG (187.0 ± 5.482). BPA did not modify the expression pattern of PR in the different types of glands (data not shown).

3.5. Expression of molecules in the uterine stroma

3.5.1. ESR1

Ovarian steroid hormones regulate functional uterine epithelial differentiation through binding to their stromal receptors. To assess whether alterations prompted by perinatal BPA exposure in response to E2 were associated with changes in ESR1 expression, we evaluated protein expression of ESR1 in the stroma of all groups. ESR1 expression was significantly reduced in BPA50 rats (Fig. 5A). Then, to know whether this decrease was due to changes at transcriptional level, we analyzed the relative ESR1 mRNA expression using a real-time PCR approach. BPA50 rats showed decreased ESR1 mRNA expression (Fig. 5B). Next, to determine whether the decrease in ESR1 mRNA was associated with changes in the transcriptional promoter usage, we studied the relative expression levels of all exons encoding the 5′ untranslated regions (UTR) of the rat gene. We determined that ESR1 gene transcription in the uterus was regulated by promoters OS, O, OT and E1, regardless of the treatment group. No expression of transcripts containing the ON exon was detected. Rats exposed to BPA50 presented lower ESR1-O and ESR1-OT transcription rates than controls (Fig. 5C).

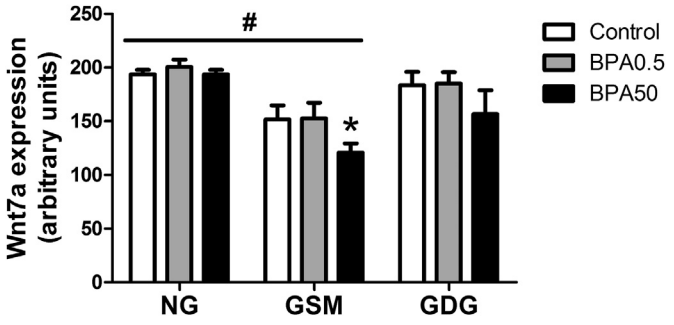

Fig. 4. Effect of perinatal BPA exposure on Wnt7a expression in uterine glands of aged rats treated with E2. Wnt7a immunostaining was evaluated as integrated optical density (IOD) in normal glands (NG), glands with squamous metaplasia (GSM) and glands with daughter glands (GDG). Each column represents the mean ± SEM (n > 8 per group). #, p < 0.05 vs. the NG.*, p < 0.05 vs. the control group.

3.5.2. Steroid receptor coactivators

To obtain more information regarding E2 pathways, the protein expression of two nuclear receptor coactivators, SRC-1 and SRC-3, was evaluated in the stromal compartment of the uterus. SRC-1 and SRC-3 expression was lower in both BPA groups (Fig. 6).

3.6. Dnmts, EZH-2 and Hdacs in uterine tissue

The disturbance in gene expression found in rats exposed to BPA could be influenced by alterations in epigenetic mechanisms. Here, the mRNA levels of Dnmt 3a and 3b, EZH-2, and Hdac-1,-2 and -3 were measured in the uterus of all groups. BPA50 increased the expression of Dnmt3a and Dnmt3b, whereas the expression of EZH-2 and Hdac-1 was higher in BPA0.5 rats (Fig. 7).

4. Discussion

The uterus is one of the main estrogen-sensitive organs, and much evidence has shown adverse effects of xenoestrogens on this tissue (Bosquiazzo et al., 2010, 2013; Newbold et al., 2009; Suvorov and Waxman, 2015; Varayoud et al., 2014; Vigezzi et al., 2015). This study provides new evidence about the ability of the environmental xenoestrogen BPA to long-term reprogram the uterine response to estrogens in aged rats, and supports evidence for possible mechanisms implicated in endocrine disruption by BPA. We demonstrated that perinatal exposure to BPA, at doses as low as the EPA “safe dose” (BPA50 group) and at a dose 100 times lower than that (BPA0.5 group), induced an altered uterine response to E2 in adult life. This was evidenced by increased density of glandular abnormalities associated with a deregulated expression of E2-target genes. In addition, altered epigenetic regulatory mechanisms could be implicated. These effects were observed in female offspring born to dams exposed to BPA by using oral administration during pregnancy and lactation. We used this route because it is the most relevant for BPA exposure in the general population (Christensen et al., 2012; Rudel et al., 2011).

Uterine glandular abnormalities, including GSM and GDG, were observed in aged control rats. In a previous work, we demonstrated that those types of glands appear in aged animals (Vigezzi et al., 2015). However, aged females perinatally exposed to BPA showed increased density of GSM and GDG, suggesting that BPA exposure may increase the susceptibility to develop this type of lesions in the adulthood. It is important to mention that these glandular lesions are considered preneoplastic alterations (Gunin et al., 2001; Nicolae et al., 2011). The mechanism by which squamous cell metaplasia develops in the endometrium is unknown; however, it has been associated with various conditions besides a senile endometrium, including chronic endometritis, endometrial hyperplasia, and endometrial carcinoma (Nicolae et al., 2011; Stefansson et al., 2006). Here, we demonstrated that GSM

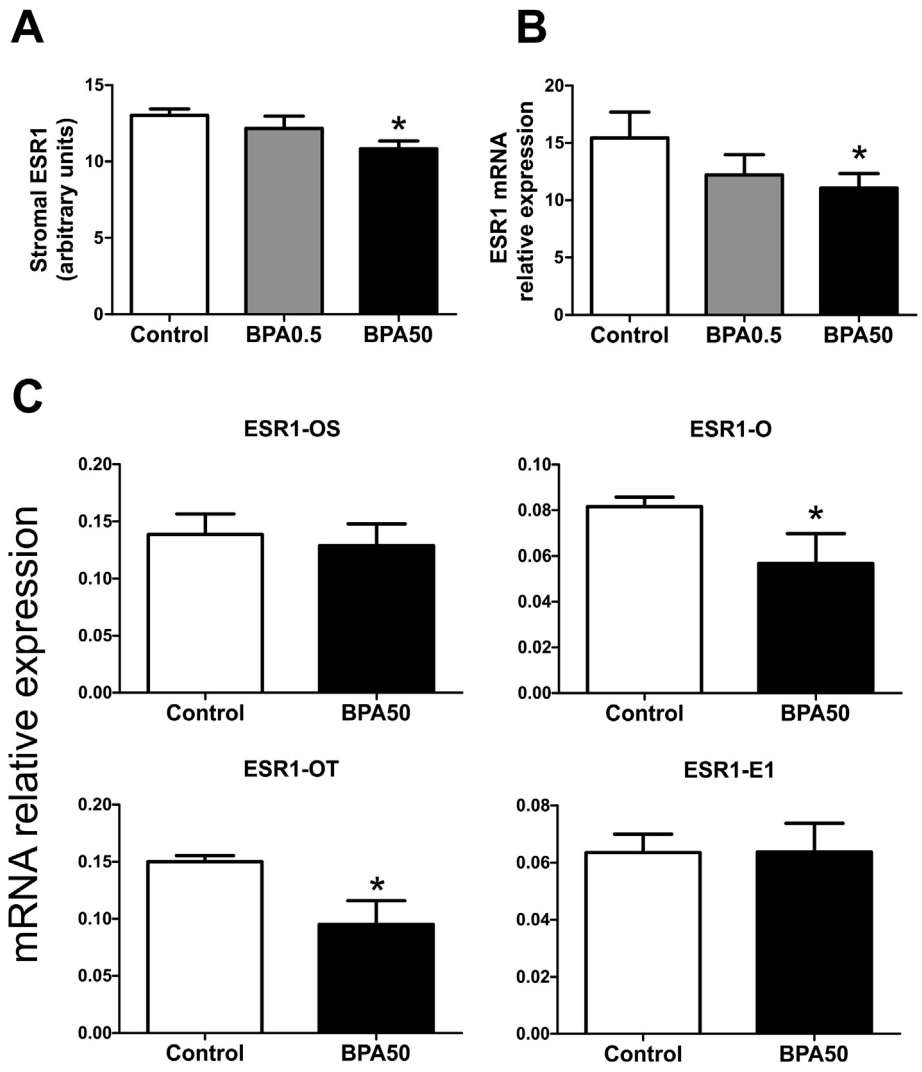

Fig. 5. Effect of perinatal BPA exposure on ESR1 expression in the uterus of aged rats treated with E2. (A) Immunohistochemical ESR1 expression was evaluated as integrated optical density (IOD) in the stromal compartment of the uterus. Each column represents the mean ± SEM (n > 8 per group). (B) The mRNA expression levels of total ESR1 and (C) specific 5′ UTR regions of the rat ESR1 gene were measured by real-time RT-PCR. Samples were normalized to L19 mRNA expression and the relative amounts of mRNA were calculated using the standard curve method. Each column represents the mean ± SEM (n > 8 per group).*, $p < 0.05$ vs. the control group.

presented a decreased expression of Wnt7a and PR compared with NG, suggesting that the down-regulation of these estrogen-target genes in the glandular epithelium could contribute to the development of GSM. We showed that BPA50 rats presented an increase in GSM density, and that these GSM exhibited even lower expression of Wnt7a than the GSM of the control group. It has been shown that the dysregulation of Wnt7a during the perinatal period leads to global disorganization of the uterine epithelium, which transforms into a squamous epithelium and induces a disruption of gland formation later in adult life (Kurita, 2011; Sassoon, 1999). In addition, Jeong et al. (2009) have shown that the conditional inactivation of β-catenin in the uterus of adult mice induces the development of a squamous epithelium, suggesting that the activation of the Wnt/β-catenin signaling pathway is necessary for the

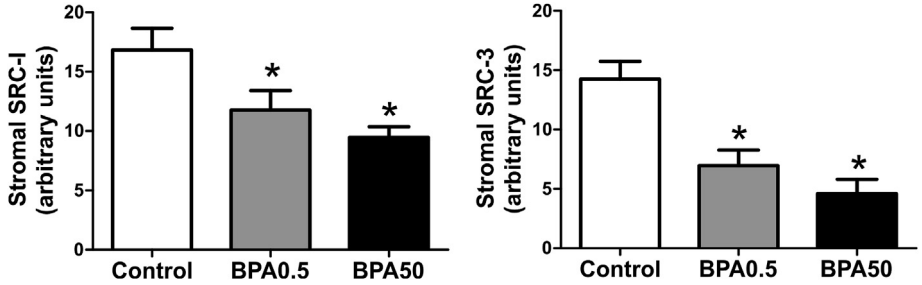

Fig. 6. Effect of perinatal exposure to BPA on the expression of SRC-1 and SRC-3 in uterine tissue of aged rats treated with E2. Immunohistochemical expression of SRC-1 and SRC-3 was evaluated as integrated optical density (IOD) in the stromal compartment of the uterus. Each column represents the mean ± SEM (n > 8 per group).*, $p < 0.05$ vs. the control group.

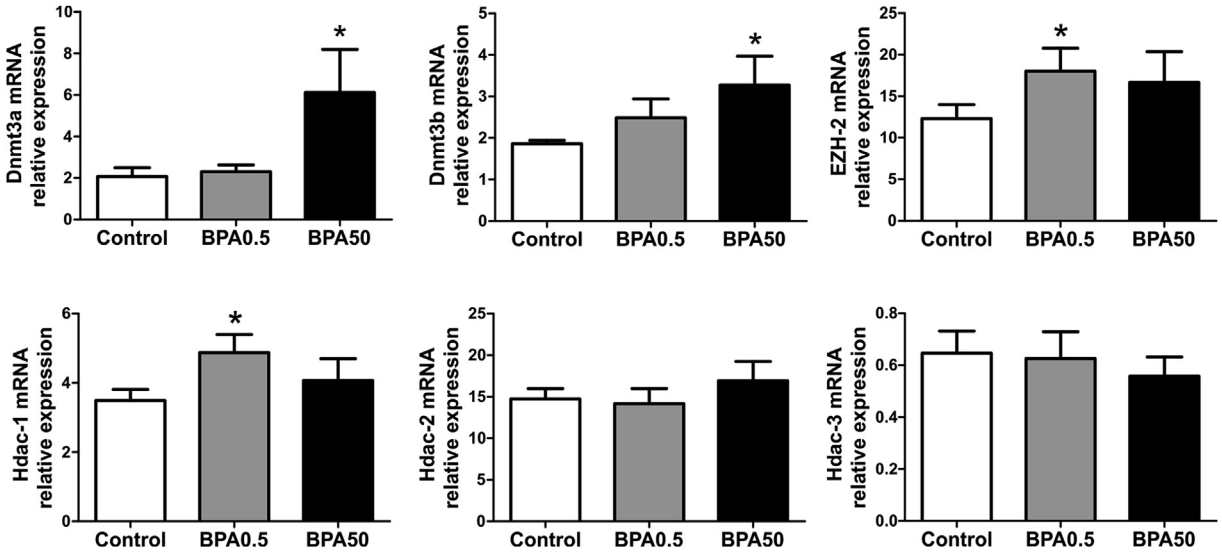

Fig. 7. Effect of perinatal exposure to BPA on the expression of Dnmts, EZH-2 and Hdacs in the uterus of aged rats treated with E2. The mRNA expression levels of Dnmt 3a and 3b, EZH-2, and Hdac-1, -2 and -3 were measured by real-time RT-PCR. Samples were normalized to L19 mRNA expression and the relative amounts of mRNA were calculated using the standard curve method. Each column represents the mean ± SEM (n > 8 per group).*, $p < 0.05$ vs. the control group.

maintenance of the normal epithelial structures of the uterus. Our results showed that perinatal exposure to BPA50 did not modify β-catenin expression. In addition, β-catenin was located in the membrane and cytoplasm of the glandular cells, suggesting that the Wnt/β-catenin pathway is inactive in those cells. On the other hand, Wnt signaling has been shown to be activated in the uterus at the estrous stage, and several Wnt members are differentially regulated along the rat estrous cycle (Miller et al., 1998; Yip et al., 2013). In addition, an altered expression of Wnt genes has been implicated in different pathological states in the reproductive system, including endometrial hyperplasia and cancer (Yip et al., 2013). Together, these results suggest that Wnt signaling could be differentially regulated in squamous metaplasia due to alterations in estrogen signaling mediated by BPA.

GDG, other age-related abnormalities, were found increased in BPA0.5 rats. Little is known about the mechanisms implicated in their formation (Vigezzi et al., 2015). Gunin et al. (2001) postulated that mitoses perpendicular to the basement membrane would probably form GDG. These authors also suggested that alterations in mitosis orientation are correlated with pre-cancerous changes induced by estrogen. However, the mechanism by which E2 or BPA could promote this morphological feature is still unknown. Further studies are needed to address this issue.

Numerous processes of uterine epithelial differentiation regulated by sex hormones are mediated through their receptors localized in the stroma (Cunha et al., 2004) (Varayoud et al., 2005). Altered patterns of expression of hormonal receptors have been suggested to play roles in the etiology of serious pathological alterations of the endometrium, concerning mainly the glands and the stroma, including endometrial hyperplasia and cancer (Hapangama et al., 2015; Jarzabek et al., 2013). Here, we demonstrated that ESR1 expression decreased in response to E2 in uterine stromal cells of the BPA50 group. Furthermore, the change observed in ESR1 protein was accompanied by a significant decrease in ESR1 mRNA. The mechanism associated with this change was the differential promoter usage, evidenced by a decrease in the relative abundance of the alternative spliced 5′UTR transcripts ESR1-O and ESR1-OT in BPA50 rats. Selective promoter usage in BPA-mediated ESR1 disruption has been previously demonstrated in the rat brain (Monje et al., 2007) and, differential promoter activity has been shown to be a key regulator of tissue-specific ESR1 expression (Donaghue et al., 1999; Hughes, 2006; Kato et al., 1998). In addition to the changes observed in the expression of ESR1, other key regulatory molecules could contribute to alterations in the expression of E2-target genes (PR and Wnt). Steroid receptor coregulators serve as partners for nuclear receptors, either enhancing or inhibiting hormone-dependent transcription (Hall and McDonnell, 2005). Here, decreased expression of the coactivator proteins SRC-1 and SRC-3 was found in the stroma of BPA-exposed rats. This would indicate a disruption in the transcriptional machinery of E2 target genes mediated by BPA. In the same sense, previous studies have shown that coregulator proteins are targets for the endocrine disruption by BPA exposure (Bosquiazzo et al., 2010; Salian-Mehta et al., 2014; Varayoud et al., 2008).

Epigenetic modifications such as DNA methylation and histone methylation/acetylation are carried out by Dnmts, histone-methylation enzymes and Hdacs respectively. During development, steroid hormones can induce permanent effects on gene activity and program target genes to respond to secondary hormonal cues later in life (Kundakovic and Champagne, 2011). This hormonal imprinting involves epigenetic regulation which can be passed from one cell generation to another and persist into adulthood (McLachlan, 2001). Therefore, epigenetic mechanisms represent one of the most plausible targets through which environmental estrogenic compounds such as BPA could exert their long-lasting effects (Cruz et al., 2014; Kundakovic and Champagne, 2011; Rezg et al., 2014). Developmental exposure to BPA has been previously associated with changes in the expression of Dnmts (Doshi et al., 2011; Kundakovic et al., 2013) and histone post-translational regulatory enzymes such as EZH-2 (Bhan et al., 2014; Doherty et al., 2010) in different sensitive tissues. However, there is very limited knowledge about the long-term epigenetic changes that are induced by BPA in the uterus (Suvorov and Waxman, 2015). In the present study, we showed changes in enzymes involved in the epigenetic regulatory machinery in BPA-exposed rats. Particularly, the expression of Dnmt3a and Dnmt3b was increased in BPA50 rats whereas the expression of Hdac-1 and the histone methylase EZH-2 was increased in BPA0.5 ones. We propose that BPA would act, through epigenetic pathways, as a predisposing factor which sensitizes the uterus to respond inappropriately to hormonal exposures (E2 in this study) later in life.

Further studies are needed to elucidate whether the alterations observed in the epigenetic regulatory enzymes in aged rats of the BPA + E2 groups are implicated in changes in specific estrogen-target genes such as ESR1, PR, coactivators or Wnt family members.

It is interesting to note that each of the two doses of BPA used in this work was associated with different alterations both in the histology of glands and at gene expression level. These results suggest that different mechanisms would be involved in the altered BPA-mediated response to E2 depending on the dose administered. This type of behavior has been previously reported for BPA (Monje et al., 2007; Vigezzi et al., 2015).

To our knowledge, this is the first study to demonstrate a statistically significant relationship between perinatal BPA exposure and increased glandular preneoplastic alterations associated with deregulated expression of E2-target genes, in the aged rat uterus following a chronic treatment with E2. Our results should serve to warn of a possible link between increasing age-related hormone-dependent pathologies in humans and altered uterine function due to perinatal exposure to BPA. A comprehensive analysis is needed to evaluate the potential hazards to humans and wildlife from exposure to BPA and other xenoestrogens at doses considered as "safe" or from the daily intake level established as acceptable by the U.S. EPA.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2016.02.010.

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