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Prenatal exposure to bisphenol A promotes angiogenesis and alters steroid-mediated responses in the mammary glands of cycling rats

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ABSTRACT

Prenatal exposure to BPA disturbs mammary gland histoarchitecture and increases the carcinogenic susceptibility to chemical challenges administered long after BPA exposure. Our aim was to assess the effect of prenatal BPA exposure on mammary gland angiogenesis and steroid hormone pathways in virgin cycling rats. Pregnant Wistar rats were exposed to either 25 or 250 μg/kg/day (25 and 250 BPA, respectively) or to vehicle. Female offspring were autopsied on postnatal day (PND) 50 or 110. Ovarian steroid serum levels, the expression of steroid receptors and their co-regulators SRC-3 and SMRT in the mammary gland, and angiogenesis were evaluated. At PND 50, all BPA-treated animals had lower serum levels of progesterone, while estradiol levels remained unchanged. The higher dose of BPA increased mammary ERα and decreased SRC-3 expression at PND 50 and PND 110. SMRT protein levels were similar among groups at PND 50, whereas at PND 110, animals exposed to 250 BPA showed a lower SMRT expression. Interestingly, in the control and 25 BPA groups, SMRT increased from PND 50 to PND 110. At PND 50, an increased vascular area associated with higher VEGF expression was observed in the 250 BPA-treated rats. At PND 110, the vascular area was still increased, but VEGF expression was similar to that of control rats. The present results demonstrate that prenatal exposure to BPA alters the endocrine environment of the mammary gland and its angiogenic process. Increased angiogenesis and altered steroid hormone signals could explain the higher frequency of pre-neoplastic lesions found later in life.

This article is part of a Special Issue entitled 'Endocrine disruptors'.

1. Introduction

Epidemiologic studies suggest that the intrauterine hormonal milieu may predispose an individual to carcinogenesis. An increased risk of breast cancer has been noted with twin dizygotic female birth, a marker of high estrogen exposure [1], while preeclampsia, a marker of low estrogen exposure, is associated with lowered risk [2]. Currently, concern about the effects of prenatal estrogen exposure has focused on exposure to environmental estrogens, which may affect mammary gland development and/or enhance the risk of breast cancer later in life [3].

Over the last 60–70 years, a plethora of synthetic hormonally active chemicals, named endocrine disruptors, has been released into the environment. Among them, bisphenol A (BPA) is receiving increased attention because is a chemical used widely in the production of several resins (e.g., epoxy, polyester, polysulfone, and polyacrylate), polycarbonate plastics (food and drinking package, baby formula bottles), flame retardants, and dental sealants [reviewed in 4]. Research has demonstrated that the mammary gland is affected by BPA treatment. In mice, BPA perinatal exposure altered mammary gland maturation rates, delayed lumen formation, enhanced ductal growth, promoted a pregnancy-like state, enhanced responsiveness to secondary estrogenic exposures [5]; while in BPA perinatal exposed rats, an increased susceptibility to carcinogenesis (via increased number of hyperplastic ducts) was observed [6]. These findings suggest that developmental exposure to BPA may lead to a predisposition to breast cancer later in life [6,7].

Abbreviations: BPA, bisphenol A; CV, coefficients of variation; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; E_2, 17β-estradiol; EPA, Environmental Protection Agency; ER, estrogen receptor; FSH, follicle stimulating hormone; GD, gestation day; H&E, hematoxylin and eosin; IOD, integrated optical density; LH, luteinizing hormone; LOAEL, lowest observed adverse effect level; P_4, progesterone; PND, postnatal day; PR, progesterone receptor; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; SRC-3, steroid receptor co-activator 3; sc, subcutaneous; T_A, total area; V_A, vascular area; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor.

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The increased incidence of hyperplastic ducts may be a consequence of misregulation in endocrine signaling pathways. Previously, we have demonstrated that prenatal exposure to BPA increases the sensitivity of the developing mammary gland to endogenous estrogen, thereby creating a permissive state that can lead to malignancy [5]. It is well established that steroid hormones can exert their effects by binding at specific receptors, and thereby modifying target gene expression. Steroid receptor-mediated gene regulation is complex, and depends on the recruitment of tissue-specific co-regulatory factors that differentially affect the interaction of receptors with their target genes. Progesterone receptor (PR) and estrogen receptor (ER)-regulated gene transcription are mediated through interactions with steroid receptor co-regulators, including the silencing mediators of retinoic acid and thyroid hormone receptor (SMRT) co-repressor, and proteins in the p160 steroid receptor co-activator (SRC) gene family [8]. The mammary gland, a target organ of ovarian steroids, expresses SRC-3 [9] and it was suggested that SRC-3 plays a role in PR- and/or ERα-mediated mammary gland development [10]. SMRT has been shown to play an active role in preventing tamoxifen from stimulating proliferation in breast cancer cells through repression of a subset of target genes involved in ERα function and cell proliferation [11]. Previous reports from our laboratory have demonstrated that postnatal BPA exposure affects the uterine responsiveness to steroid hormones in adulthood, possibly disrupting the transcription machinery's assembly of PR- and ER-dependent genes [12,13].

Moreover, inappropriate vessel growth underlies many pathological conditions, among them tumor growth and metastasis [14]. Numerous studies have shown that E_2 increases vascular endothelial growth factor (VEGF) expression, a key factor in blood vessels' growth [15,16]. VEGF partially regulates the angiogenic process, although angiogenesis is also affected by a diverse array of soluble mediators, matrix molecules, and accessory cells that function to orchestrate the growth, differentiation, and maturation of new capillaries [17]. Molecules that promote or inhibit angiogenesis can be produced by normal or tumor cells, can be mobilized from molecules in the extracellular matrix, or may be produced by cells recruited to the tumor side such as macrophages and mast cells [17]. Interestingly, BPA also increases VEGF expression in the female reproductive tract and the hypophysis [17,18].

We have previously demonstrated in rodent models [5,6] that increased susceptibility of the mammary gland to neoplastic transformation is anticipated by structural changes in the gland such as an increased proliferation/apoptosis ratio in both the epithelial and stromal compartments, a higher number of hyperplastic ducts and an increased number of mast cells surrounding the hyperplastic ducts. To better understand the processes involved in the aforementioned disruptions, in this study we assessed the effects of prenatal BPA exposure on mammary gland angiogenesis and steroid hormone pathways in virgin cycling rats.

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 ethical committee of the School of Biochemistry and Biological Sciences, Universidad Nacional del Litoral. Animals were treated humanely and with regard for alleviation of suffering. Sexually mature female rats (3 month-old) of a Wistar-derived strain bred at the Department of Human Physiology (School of Biochemistry and Biological Sciences, Santa Fe, Argentina) were used. Animals were maintained in a controlled environment ($22 \pm 2\,^{\circ}C$; 14 h of light from 0600 h to 2000 h) and had free access to pellet laboratory chow (Nutricion Animal, Rafaela, Argentina). The concentration of phytoestrogens in the diet was not evaluated; however, because food intake was equivalent for control and BPA-treated rats (unpublished data) we assumed that animals in the experimental and control groups were exposed to the same levels of phytoestrogens. To minimize other exposure to 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 procedures (Fig. 1)

Females in proestrous were caged overnight with males of proven fertility. The day that sperm was found in the vagina was designated day 1 of pregnancy [gestation day 1 (GD1)]. On GD8, corresponding to the beginning of organogenesis in the fetus, rats were weighed and implanted subcutaneously with a miniature osmotic pump (model 1002; Alza Corp., Palo Alto, CA, USA), which was prepared to deliver either 50% DMSO (vehicle-treated control; Sigma–Aldrich, Buenos Aires, Argentina), 25 μg BPA/kg/day (25 BPA; 99% purity Sigma–Aldrich), or 250 μg BPA/kg/day (250 BPA); 8–10 dams/group were included. BPA and DMSO were released continuously via the pump for 14 days (from GD8 to GD23) at a rate of 0.25 μl/h. After parturition (GD23), pups were weighed and sexed according to the anogenital distance and litters of eight pups (preferably four males and four females) were left with lactating mothers until weaning on postnatal day 21 (PND 21). The age at vaginal opening was not recorded in this experiment, but by PND 40 puberty onset had been attained by all female offspring. The effects of the treatment in female offspring at PND 50 and PND 110 were evaluated (one female per litter per time point in each group); the remaining females and males were assigned to other experiments. In spite of different administration routes, subcutaneous (sc) *versus* oral, we selected BPA doses taking as reference the lowest observed adverse effect level (LOAEL), and the safe dose established by the US Environmental Protection Agency (EPA). BPA doses used in this study, were lower than the LOAEL dose of 50 mg/kg/day; specifically, 25 BPA was equivalent to one-half of the safe dose established by the EPA (0.05 mg/kg/day) [19]. The other dose, however, was five-fold higher (250 BPA) than the safe dose.

2.3. Sample collection

To avoid estrous cycle related changes in the mammary growth and hormone serum levels, all samples were collected at diestrus I. Female rats from our colony exhibit a five-day estrous cycle, to determine the phases of each rat's estrous cycle, daily vaginal smears were evaluated for at least twelve days prior to sample collection [20]. Females were autopsied at the closest diestrus I to PND 50 (49–52) or PND 110 (110–114). At PND 50, we evaluated 9, 10 and 10 animals for DMSO, 25 BPA and 250 BPA, respectively. At PND 110, we evaluated 8, 9 and 10 animals for DMSO, 25 BPA and 250 BPA, respectively. Blood was collected and serum stored at $-80\,^{\circ}C$ until hormone assays were performed. One abdominal mammary gland from the 4th pair was obtained, fixed in 10% (v/v) buffered formalin, and embedded in paraffin and the contra lateral was kept for other studies.

2.4. Hormone assays

Serum levels of E_2 and progesterone (P_4) were determined by RIA using [2,4,6,7,16,17-^{3}H] E_2 and [1,2,6,7-^{3}H] P_4, respectively (Perkin–Elmer Life and Analytical Sciences Inc., Boston, MA, USA), as well as specific antibodies provided by Dr GD Niswender [21].

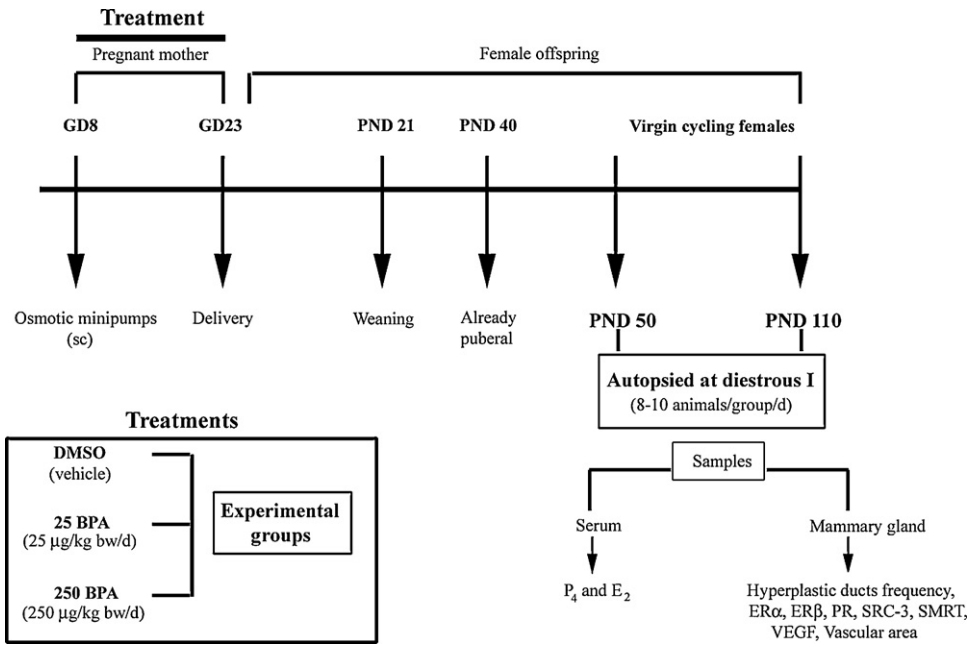

Fig. 1. Schematic representation of the experimental protocol used to study the effects of *in utero* exposure to BPA on the mammary gland of virgin cycling rats. GD: gestational day; PND: postnatal day; P_4: progesterone; E_2: estradiol.

Intra- and inter-assay coefficients of variation (CVs) for E_2 were 3.6% and 11% respectively; CVs for P_4 were 9.0% and 14.3%, respectively.

2.5. Immunohistochemistry

Consecutive 5-μm sections were immunostained to evaluate PR, ERα, ERβ, SMRT, SRC-3, VEGF and vWF. Sections from two different depths were used to evaluate the expression of each protein. Immunohistochemistry on mammary glands was performed as previously described [6]. Primary antibodies, including anti-PR (Dako Corp., Carpinteria, CA, USA), anti-ERα, anti-ERβ (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK), anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-von Willebrand factor (vWF; Dako, Glostrup, Denmark) were incubated overnight at 4 °C. To evaluate steroid receptor co-regulator expression, anti-SMRT and anti-SRC-3 affinity-purified rabbit polyclonal antibodies that had been generated and tested in our laboratory were used [13]. The avidin–biotin peroxidase method and chromogen diaminobenzidine (Sigma–Aldrich) were used (for more details see Supplemental Material).

2.6. Double immunofluorescence

Double labeling immunofluorescence assays were conducted using combinations of the antibodies listed above (ERα/SMRT, ERα/SRC-3). To minimize autofluorescence, sections were blocked with 10 mg/ml sodium borohydride (Sigma–Aldrich). This was followed by antigen retrieval using a microwave pre-treatment with sodium citrate buffer. The incubation with primary antibodies was performed overnight at 4 °C. The secondary antibodies (Invitrogen, Buenos Aires, Argentina), anti-rabbit Alexa Fluor 488 (green) and anti-mouse Alexa Fluor 546 (red), were incubated for 1 h and 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. All immunostained slides were investigated using an Olympus BX-51TRF microscope equipped for epifluorescence detection and with the appropriate filters (Olympus Optical Co., Ltd., Tokyo, Japan). Images were recorded using a high-resolution USB 2.0 Digital Color Camera (QImaging® Go-3, QImaging, Surrey, BC, Canada) [13].

2.7. Morphometry and quantification of protein expression

2.7.1. Proportion of hyperplastic ducts

The percentage of hyperplastic ducts (with four or more layers of epithelial cells) was quantified by examining H&E stained sections as has been previously described [6]. Each hyperplasia was classified as mild, moderate or florid (four, five or more than five layers of epithelial cells lining the ducts, respectively) [22]. To obtain the proportion of hyperplastic ducts, we evaluated four sections per mammary gland that were at least 30 μm apart from each other, and then we analyzed 50 ducts per section.

2.7.2. Relative vascular area

The area occupied by microvessels within the stroma surrounding mammary ducts ("normal" and hyperplastic) was determined. Images of sections immunostained with vWF were recorded and the total area (T_A) and the vessel area (V_A) were obtained. The relative vascular area was calculated as follows: $(V_A/T_A) \times 100$ (for more details see Supplemental Material).

2.7.3. Quantification of protein expression

Two mammary tissue sections per animal were evaluated and, a total 100 mm^2 ductal epithelial area was quantified per section. The ductal epithelial area analyzed per section was defined as the sampling area that captured most of the variations in the evaluated parameter within each group giving a result that was not affected by further sampling [23]. Moreover, as we were seeking for changes in mammary gland biomarker expression prior to the development of hyperplasia, we have quantified SMRT, SRC-3, ERα, PR and VEGF expression in "normal" ducts. Protein expression in ductal epithelial cells was measured as integrated OD (IOD) of immunostained slides. Image analysis was performed using the Image Pro-Plus 4.1.0.1® system (Media Cybernetics), as previously described [13] (for more details see Supplemental Material). The images of immunostained slides were converted to grey scale. The IOD was calculated as a linear combination of the average grey

intensity and the relative area occupied by positive cells. The results were expressed in arbitrary units. The percentages of ductal epithelial cells that expressed ERβ were quantified in two mammary tissue sections per animal and, at least 2000 cells were analyzed in each section.

2.8. Statistical analysis

All data were expressed as the mean ± S.E.M. Kruskal–Wallis analysis was performed to obtain the overall significance (testing the hypothesis that the response was not homogeneous across treatments), and the Dunn's posttest was used to compare each experimental group with the control group; values with $p < 0.05$ were accepted as significant.

3. Results

3.1. BPA exposure increased the percentage of hyperplastic ducts

At PND 50, the ductal epithelial layering of the mammary gland was unchanged due to treatment; however, at PND 110, animals exposed to 25 BPA exhibited not only an increased frequency of hyperplastic ducts ($p < 0.01$), but also a higher number of moderate and florid hyperplasias. Additionally, at PND 110, samples from animals exposed to 250 BPA showed a trend toward increased presence of hyperplastic ducts ($p = 0.06$) (Fig. 2).

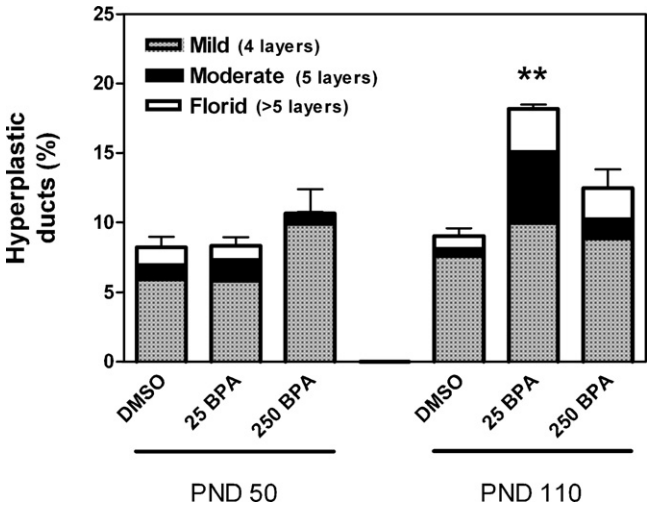

Fig. 2. Effects of prenatal exposure to BPA on ductal hyperplasia frequency and epithelial layering patterns of the mammary gland. Only ducts with 4 or more epithelial layers were considered hyperplastic. $^{**}p < 0.01$ *vs.* vehicle-treated control.

3.2. BPA exposure modified ovarian steroid pathways

During puberty, mammary gland ductal growth is closely regulated by E_2 and P_4, which act mostly via their nuclear receptors [24]. Therefore, in order to evaluate whether endocrine pathways were affected by prenatal BPA exposure, ovarian steroid levels and the expression of ERα, ERβ, PR, and co-regulator proteins were assessed.

At PND 50, *in utero* treatment with BPA resulted in significantly lower P_4 levels. However, at PND 110 there were no differences in P_4 levels among experimental groups. E_2 levels were not different between groups, regardless of the animal's age (Table 1). Regarding steroid receptors, exposure to the higher dose of BPA (250 BPA) increased the expression of ERα at PND 50 and PND 110 (Fig. 3A). Interestingly, PR and ERβ expressions were not altered after BPA exposure (Fig. 3B and Fig. 1 Supplemental Material, respectively).

To evaluate the effects of BPA treatment on the expression of steroid receptor co-regulators, we selected the co-activator SRC-3 and the co-repressor SMRT. SRC-3 was primarily found in the nuclei of epithelial cells in the alveolar buds and ducts of all evaluated samples, regardless of *in utero* treatment. When SRC-3 expression in the ducts was compared between the DMSO- and BPA-groups; we found that 250 BPA diminished SRC-3 expression at PND 50 and PND 110 (Fig. 3C). SMRT was also expressed in the epithelia of both ducts and alveolar buds, with a cytoplasmic staining pattern. Prenatal exposure to BPA did not affect SMRT ductal expression at PND 50, whereas at PND 110, decreased SMRT expression was observed in the mammary glands of 250 BPA exposed rats. On the

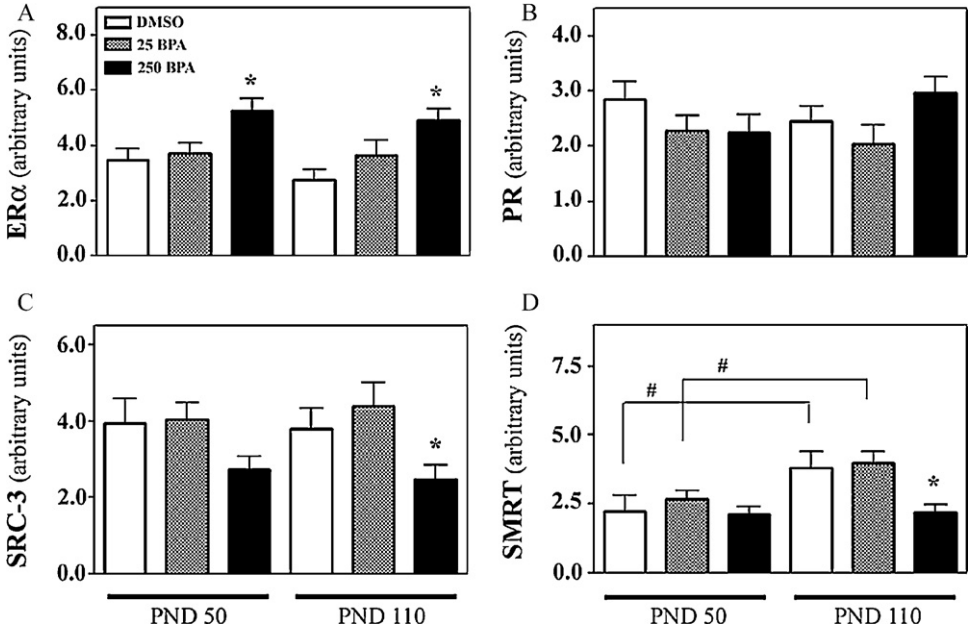

Fig. 3. Effect of BPA exposure on the expression of steroid receptors and co-regulators in the mammary gland. (A) ERα, (B) PR, (C) SRC-3, and (D) SMRT expression. Bars represent IOD mean values ± S.E.M. expressed as arbitrary units. $^{*}p < 0.05$ *vs.* control. In (D) the numeral sign (#) above the horizontal lines represents $p < 0.05$ between the designated groups.

Table 1
Serum concentrations of hormones.

Experimental groups	E_2 (pg/ml)		P_4 (ng/ml)	
	PND 50	PND 110	PND 50	PND 110
DMSO	21.59 ± 2.86	23.43 ± 1.79	25.04 ± 7.89	29.94 ± 8.60
25 BPA	24.41 ± 2.59	23.43 ± 0.99	12.40 ± 4.56*	28.90 ± 6.99
250 BPA	29.14 ± 2.28	26.71 ± 1.59	12.91 ± 4.52*	34.62 ± 6.97

* Statistically different relative to vehicle-treated control ($p < 0.05$).

other hand, when the temporal pattern of changes was observed, increased SMRT expression at PND 110 compared with PND 50 was found in the control (DMSO) and 25 BPA groups (Figs. 3D and 4).

3.3. Co-localization of ERα/SRC-3 and ERα/SMRT

Double-labeled immunofluorescence staining was used to detect the co-localization of ERα/SRC-3 and ERα/SMRT in the epithelial cells of the mammary gland; representative photomicrographs illustrating these results are shown in Fig. 5. The merged images were used to detect protein co-expression. Our dual immunofluorescence studies for ERα/SRC-3 showed that there was a nuclear co-localization of both proteins in the ductal epithelial cells of control females. SMRT was expressed in almost every epithelial cell, and several of these cells were also positive for ERα (Fig. 5).

3.4. BPA exposure increased mammary gland angiogenesis

To evaluate the influence of BPA treatment on the angiogenic process; we analyzed the relative vascular area in the stroma surrounding both, hyperplastic and non-hyperplastic ducts. At PND 50, the relative vascular area was higher in the 250 BPA group than in control rats, and at PND 110, animals exposed to either dose of BPA showed an increased relative vascular area (Fig. 6A). In order to establish the mechanisms responsible for the increased angiogenesis, two different pathways were explored. First, because higher cellularity in the hyperplastic ducts could generate a hypoxic environment that induces angiogenesis, the relative vascular area was quantified in the stroma surrounding "normal" and hyperplastic ducts from the mammary samples of BPA exposed animals. In contrast to our expectations, the relative vascular area in the stroma surrounding hyperplastic ducts was similar to that of the stroma of "normal" ducts in the same sample (data not shown). Second, the expression of VEGF by the epithelial cells was determined. There was a transient increase in VEGF expression at PND 50 in animals treated with 250 BPA (Fig. 6B).

4. Discussion

The present study examined the influence of prenatal BPA exposure on mammary gland steroid hormone pathways and two angiogenic-related parameters: vascular area and VEGF expression. The prenatal treatment with 250 BPA affected ERα, SRC-3, and SMRT expression as well as P_4 serum levels. Also, the vasculature of the mammary stroma was modified by BPA treatment. At PND 50, the vascular area was increased in 250 BPA-treated animals and this change was associated with a transient increase in VEGF expression by the epithelial cells. At PND 110, the vascular area was still increased relative to controls, but VEGF expression was similar to that of controls. Interestingly, the majority of the changes described in this paper occurred as a consequence of the exposure to the higher dose of BPA. Recently, using gavage feeding and the same doses as we did, it has been shown that the effects of prenatal exposure to BPA can be dose and time dependent based on up- or down-regulation of proliferation/differentiation-related genes, protein expression and morphological changes in the mammary gland [7,25–27]. BPA bioavailability using a sc route was higher than using oral administration [28]; however, it was suggested that in pregnant and neonatal rodents the administration route is irrelevant [29]. Therefore, animal studies that examine the effects of BPA low doses administered by non-oral routes during early development are as valid for assessing the potential impact of BPA on human and/or animal health as the studies involving oral administration [29].

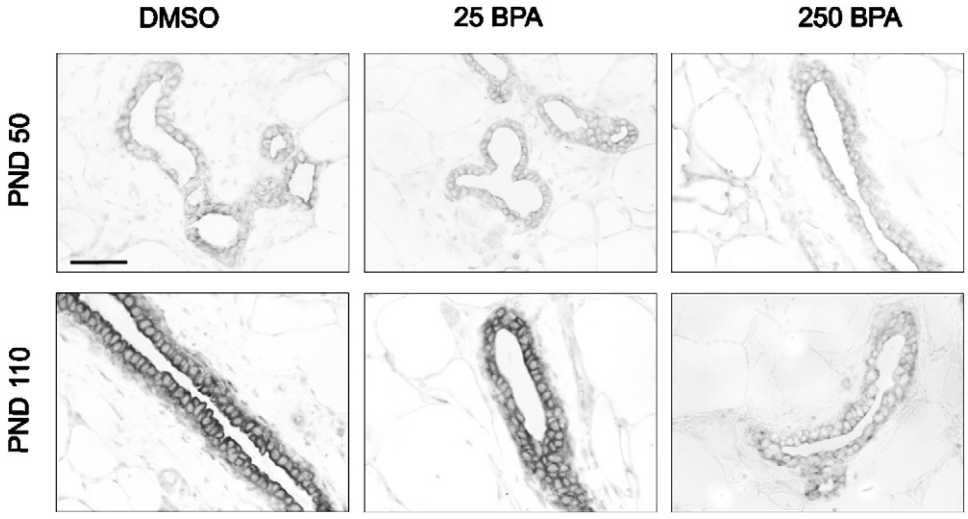

Fig. 4. Effects of BPA exposure on SMRT expression patterns. Representative photomicrographs depicting cytoplasmic SMRT staining in epithelial cells of ducts with normal appearance both in control (DMSO) and BPA groups. A significant rise in staining intensity from PND 50 to PND 110 was observed in DMSO and 25 BPA, whereas animals exposed to 250 BPA exhibited similar staining intensities between PND 50 and PND 110. Bar: 50 μm.

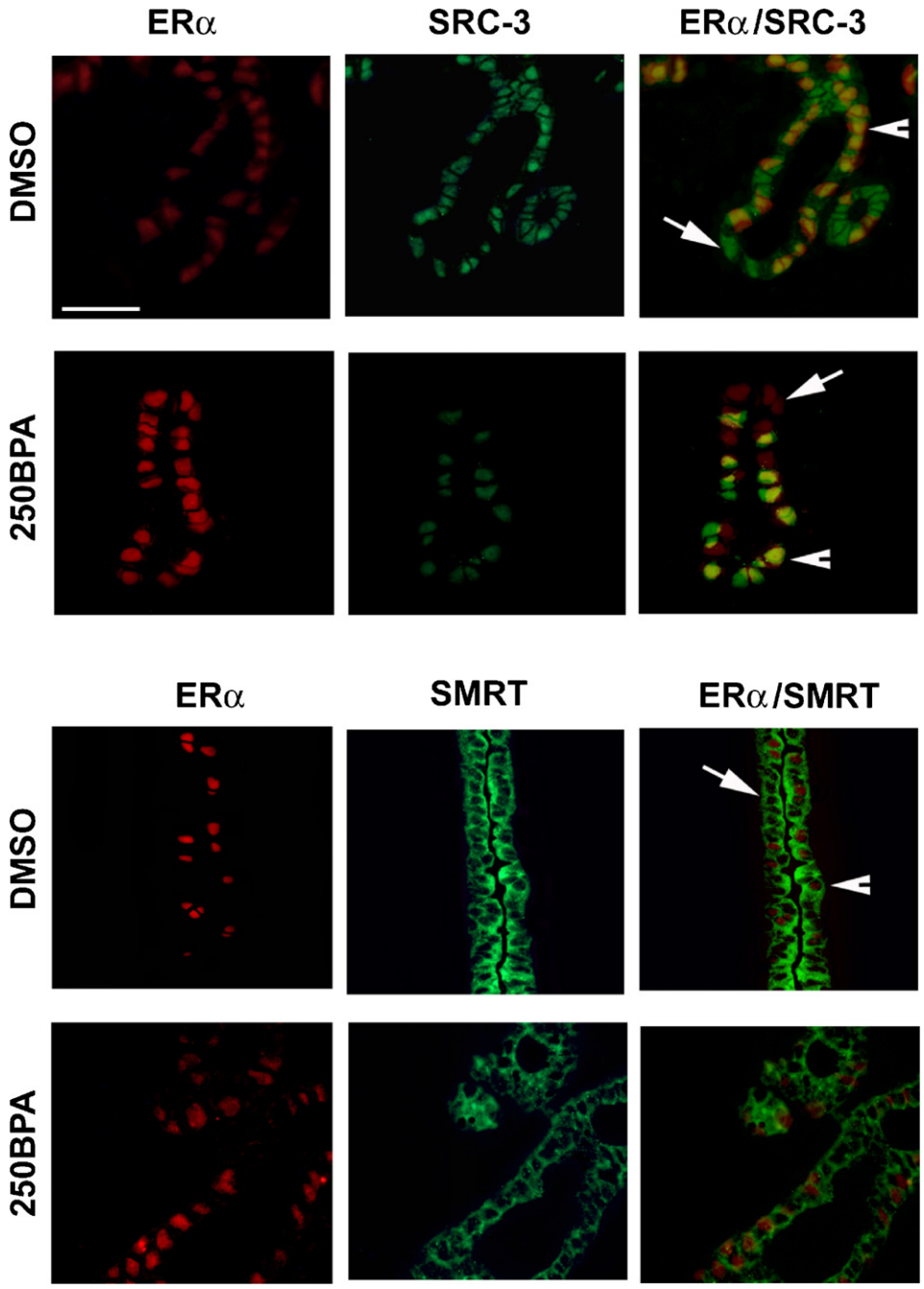

Fig. 5. Co-expression pattern of ERα and its co-regulators in the mammary gland. The selected photomicrographs show the range of variability in the expression of ERα and the co-regulators SCR-3 and SMRT between and within experimental groups. Upper panel: ERα/SRC-3 at PND 50 in control and 250 BPA groups. There was a nuclear co-localization of both proteins in ductal epithelial cells. Lower panel: ERα/SMRT at PND 110 in the control and 250 BPA groups. SMRT was expressed in the cytoplasm of epithelial cells. The arrows indicate single-labeled cells with either ERα, SRC-3, or SMRT and the arrowheads show the co-localization between the molecules. Bar: 50 μm.

The increased incidence of hyperplastic ducts does not seem to be a dose-dependent effect at PND 50 and 110. In fact, there are many published studies providing examples of BPA effects observed at lower doses instead of higher ones [12,26,30]. These types of dose–response relationships highlight the unreliability of assuming that the effect of exposure to low BPA doses can be extrapolated from the response to high doses. In a recent paper, Betancourt et al. [27] have demonstrated that prenatal exposure to 250 BPA shifts the window of susceptibility to a carcinogen in the rat mammary gland from PND 50 to PND 100. In addition, Markey et al. [31] have shown that while the exposure to 25 BPA produces a greater mammary ductal elongation, the treatment with 250 BPA has the opposite effect (delay of mammary gland growth) in 1 month-old CD-1 mice. However, when the animals were 6 month-old, both BPA groups had a larger development of the mammary tree than the control group. A temporal shift in mammary gland development induced by the exposure to BPA could explain our results. Since, a higher percentage of hyperplastics ducts in 250 BPA-treated animals were just observed on PND 180 as was shown by Durando [32], the modifications in ERα, SCR-3 and SMRT protein expressions seems to be previous events to the development of hyperplasia. Therefore, the incidence of hyperplastic ducts may be a consequence of misregulation in endocrine signaling pathways. As we previously reported, prenatal treatment with BPA modified mouse mammary gland response to E_2 [5] and, accelerated the onset of puberty by five days [6]. In our colony, female rats exhibit 5

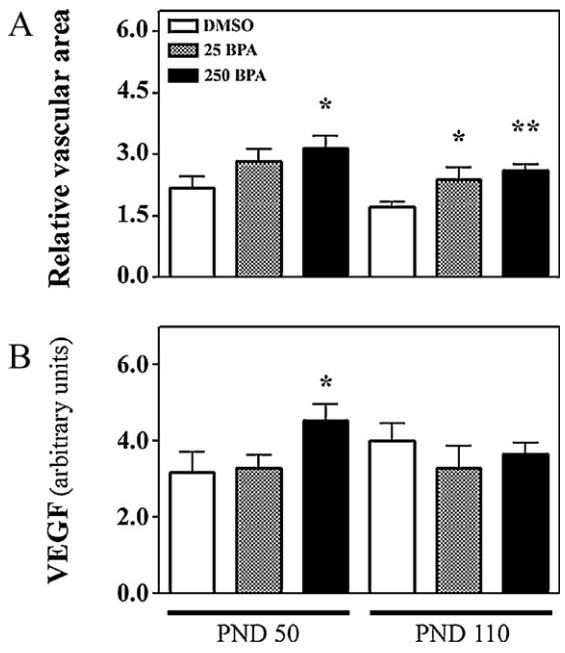

Fig. 6. Effects of prenatal exposure to BPA on mammary gland angiogenesis. (A) Vascular area, and (B) VEGF expression. Bars represent mean values ± S.E.M. $^{*}p < 0.05$; $^{**}p < 0.01$ *vs.* vehicle-treated control.

days estrous cycle, thus animals from the 25 BPA group advantaged control animals in one estrous cycle and could be exposed earlier to changes in their endogenous estrogens. Although our results do not show a direct relationship between the estrous cycle and the appearance of hyperplastic lesions in BPA animals, we cannot rule out that the mammary gland of those animals could be more sensitized to the development of premalignant lesions later in life. The longer the mammary gland is exposed to the proliferative action of E_2 the higher the chance of developing a malignant transformation of the cells [33].

BPA can act through different ERs in the target cells and it has been shown to have a higher affinity for ERβ than ERα [34,35]. BPA may also be working through one or more "non-classical" estrogen pathways, such as the membrane receptor GPR30 or other estrogen related receptors (ERRs) [36,37]. However, the specific receptors through which BPA acts in the whole animal have been reported to be different depending on the studied tissue, because the activity of BPA depends on the levels of expression of the different ER variants [35]. In the rodent mammary gland, ERα was expressed in both the epithelial and stromal compartments, ERβ expression was exhibited mainly by the epithelial cells, whereas PR was observed only in the epithelium [5,21,38]. The effects of prenatal BPA exposure on ERα and PR expression in the mouse mammary gland have been assessed [5,39]. No differences in the cellular distribution of ER protein or mRNA expression were found either in mouse female fetuses [39] or prepubertal females [5]; however, BPA induced the expression of PR in ductal cells clusters in prepubertal mice [5]. Although BPA has a higher affinity for ERβ than ERα, prenatal BPA exposure did not modify ERβ expression in our experimental model and its level of expression was similar to that reported by Saji et al. [38]. On the other hand, *in utero* BPA administration affected the pattern of expression of ERα and PR in the rat mammary gland differently than in mice. Here, an increased expression of ERα in 250 BPA exposed rats at both PND 50 and PND 110 was found while no differences in PR expression were observed. Recently, a differential response to P_4 and E_2 within the mammary gland epithelium of two different mouse strains it has been demonstrated [40]. Therefore, differences in PR and/or ERα expressions between mice [5,39] and rats (our present results) could be expected.

ER and PR actions are regulated by co-activators and co-repressors [8]. Recently, Jenkins et al. [7] have shown that BPA treatment administered orally during lactation induced a significant increase of SRC-3 and PR-A at PND 50. In spite of evaluating PR expression without assessing the individual contribution of each isoform, the absence of an increased expression of PR (PR-A plus PR-B) in the BPA-treated rats could be explained partially by the decreased expression of the co-activator SRC-3. The significant decrease in SRC-3 expression observed in the present study, but not in that of Jenkins et al. [7], may be a consequence of the difference in rat strains, BPA exposure schemes, and/or route of administration (i.e., postnatal *vs.* prenatal exposures, oral *vs.* sc administration), all of which could affect the metabolism and disposition of BPA. Given what we know about human, rat, and mouse mammary tissue developmental progression, the rat and mouse are adequate surrogates for human breast development. The use of different rodent species, strains, and inbred or outbred lines is useful to show different interactions between genetic and environment, helping to understand the variability in human responses to environmental exposure. Differences between strain susceptibility to BPA could also account for the observed disagreement between our results on hyperplastic duct incidence at PND 50 and that reported by Murray et al. [41] by using Wistar-Furth rats.

Deletion of SRC-3 in mice resulted in defects of mammary gland development, including retardation of ductal growth and penetration of ductal branches [9]. Giamas et al. [42] have shown that silencing of casein kinase δ isoform leads to reduced ERα transcriptional activity, despite increased ERα levels. Furthermore, SRC-3 protein levels were reduced by casein kinase δ silencing, in an E_2-dependent manner. In agreement, our results showed that SRC-3 protein levels were decreased in 250 BPA-exposed animals suggesting that the transcriptional activity of ERα in this group is reduced in spite of the higher expression of the protein found in these animals. SMRT, in conjunction with gene-specific and cell-dependent factors, is required for positively regulating agonist-dependent ERα transcriptional activity, and contributes to proliferation of ERα-positive breast cancer cells [43]. Thus the lower expression of SMRT found in the mammary gland of 250 BPA exposed rats at PND 110 could result in a decreased ERα signaling. However, whether the decreased SMRT transcriptional activity explains the lower percentage of hyperplastic ducts exhibited by 250 BPA animals at PND 110 compared with 25 BPA-treated rats, remains unknown.

The ovarian hormones play important roles in postnatal mammary gland morphogenesis. E_2 and P_4 levels reported here in DMSO control group were similar to that reported by Kaneko et al. [44] for diestrus rats. In agreement with previous results [5,7,26,27], E_2 serum levels did not differ among experimental groups. Although at PND 50, BPA-exposed animals exhibited lower P_4 levels than those who were unexposed; this change was not permanent, and at PND 110, all females had similar serum levels of P_4, showing that BPA can induce transitory effects on serum hormone levels [45]. This transient change could be the result of early organizational effects that are associated with the actions of exogenous or endogenous estrogens during organogenesis and development [46]. Recent experiments have shown that BPA disrupts granulose cell function *in vitro* lowering the P_4 output [47] and, inhibits follicle growth and decreases hormone production in adult ovarian antral follicles [48]. In addition, we have shown that neonatal exposure to BPA reduces the pool of primordial follicles in the rat ovary by stimulating the neonatal initial recruitment, associated with an increased proliferation rate likely mediated by an estrogenic pathway [49].

The formation of blood vessels is induced during mammary gland development and growth to support the metabolic needs

of the active tissue. Our present results showed an induced-BPA vascularization in virgin animals, an effect reported to be caused by estrogen [15,16]. Angiogenesis promoted by BPA exposure occurred regardless of the VEGF expression, confirming that this growth factor is not the only mediator of the angiogenic pathway in our model. VEGF expression has been induced in the reproductive tract by E_2 and BPA in prior studies [15,18,50]. Here we showed that, in BPA-treated animals, the increase in the vascular area was accompanied by a transient increase in VEGF expression. This result is in agreement with a previous report which suggested that, in the mammary gland, VEGF is partially responsible for angiogenesis [51]. On the other hand, it is known that mast cells are multifunctional effector cells that have been implicated in promoting angiogenesis in reproductive tissue [52] and within tumors [53]. Recently we described an increased number of mast cells in the stroma surrounding mammary gland hyperplastic ducts [6]. Surprisingly, in BPA-exposed animals, relative vascular area was as elevated in the stroma surrounding hyperplastic ducts as in the stroma of "apparently normal" ducts from the same sample, suggesting that the induction of angiogenesis could precede the increase in epithelial layers and might contribute to the proliferation/survival processes. The complexity of *in vivo* systems is reflected in sometimes unexpected results, and in some instances the mechanisms and interactions are even more intricate than originally thought.

In conclusion, our results show that BPA exposure during critical developmental periods induced modifications in the mammary gland endocrine environment and on angiogenesis that are dose- and time-specific.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2011.04.001.

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