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PII: S0890-6238(15)00012-X
DOI: http://dx.doi.org/doi:10.1016/j.reprotox.2015.01.011
Reference: RTX 7086

To appear in: *Reproductive Toxicology*

Received date: 11-8-2014
Revised date: 23-1-2015
Accepted date: 29-1-2015

Please cite this article as: Vigezzi L, Bosquiazzo VL, Kass L, Ramos JG, Muñoz-de-Toro M, Luque EH, Developmental exposure to bisphenol a alters the differentiation and functional response of the adult rat uterus to estrogen treatment, *Reproductive Toxicology* (2015), http://dx.doi.org/10.1016/j.reprotox.2015.01.011

Highlights

- Perinatal exposure to BPA increased the incidence of uterine abnormalities in rats
- The occurrence of glandular alterations[0] increased in BPA-exposed rats treated with E2
- ERα and PR subepithelial expression decreased in BPA-exposed rats treated with E2
- Perinatal exposure to BPA altered the response to E2 replacement therapy

Final Revised Version

DEVELOPMENTAL EXPOSURE TO BISPHENOL A ALTERS THE DIFFERENTIATION AND FUNCTIONAL RESPONSE OF THE ADULT RAT UTERUS TO ESTROGEN TREATMENT

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Grant support: This work was supported by Universidad Nacional del Litoral (Santa Fe, Argentina) (CAI+D program) and the Argentine Agency for the Promotion of Science and Technology. LV is a fellow and VB, LK, JGR and EHL are Career Investigators of the Argentine Council for Scientific and Technological Research (CONICET).

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ABSTRACT

We assessed the long-term effect of perinatal exposure to bisphenol A (BPA) on the rat uterus and the uterine response to estrogen (E2) replacement therapy. BPA (0.5 or 50 μg/kg/day) was administered in the drinking water from gestational day 9 until weaning. We studied the uterus of female offspring on postnatal day (PND) 90 and 360, and the uterine E2 response on PND460 (PND460-E2). On PND90, BPA-exposed rats showed altered glandular proliferation and α-actin expression. On PND360, BPA exposure increased the incidence of abnormalities in the luminal and glandular epithelium. On PND460-E2, the multiplicity of glands with squamous metaplasia increased in BPA50 while the incidence of glands with daughter glands increased in BPA0.5. The expression of steroid receptors, p63 and IGF-I was modified in BPA-exposed rats on PND460-E2. The long-lasting effects of perinatal exposure to BPA included induction of abnormalities in uterine tissue and altered response to E2 replacement therapy.

Key words: Bisphenol A, rat, uterus, uterine glands, steroid receptors, p63

Abbreviations

BPA: Bisphenol A; DES: diethylstilbestrol GD: gestational day; PND: postnatal day; E2: 17β-estradiol; BrdU: bromodeoxyuridine; α-SMA: smooth muscle α-actin; CK: cytokeratin; ERα: estrogen receptor alpha; ERβ: estrogen receptor beta; PR: progesterone receptor; IOD: integrated optical density; CT: cycle threshold; IGF-I: Insulin-like growth factor-I; IGF-IR: Insulin-like growth factor-I receptor

1. INTRODUCTION

Hormonal perturbation during fetal or neonatal development may predispose individuals to disease and/or dysfunction later in life [1-3]. Complete development of the rodent female reproductive tract occurs during the first two weeks of postnatal life [4, 5]. Along this period, the hormonal milieu is crucial for the correct organization and differentiation of the female reproductive tract, which occurs following a complex series of interactions between classical hormone receptors and signaling molecules that program target cells to respond appropriately to hormonal cues later in life [4, 5].

During the differentiation of the rodent female reproductive tract, the columnar (uterine) and squamous (cervicovaginal) epithelia express specific molecules such as p63 that are necessary to determine the type of epithelium [6]. TP63 is a p53-related gene that contains two alternative promoters, which give rise to transcripts that encode proteins with (TAp63) or without (ΔNp63) an amino-transactivating domain [7]. p63 is commonly expressed in cervical and vaginal cells but not in the uterine epithelium [5, 6]. However, it has been observed that developmental exposure to xenoestrogens can disturb the normal p63 expression pattern and proper uterine cytodifferentiation [8-10].

In the adult, the processes of uterine functional differentiation are also dependent on reciprocal stromal-epithelial interactions which are governed by sex steroids. For example, the mitogenic effects of E2 on the uterine epithelium are mediated indirectly through E2 binding to ERα in the stroma [11], which leads to epithelial proliferation through an unknown mechanism, probably involving paracrine effects of stromal growth factors. Since insulin-like growth factor-I (IGF-I) is produced predominantly in

the stroma and its receptor (IGF-IR) is mainly located in the uterine epithelium, it has been suggested that IGF-I could be a critical mediator of estrogen-induced stromal-epithelial interactions [12].

BPA is a prototypical endocrine disrupter, produced in large quantities for use in the manufacture of polycarbonate plastics and epoxy resins. The lowest observed adverse effects level (LOAEL) for BPA established by the United States Environmental Protection Agency (US EPA) is 50 mg/kg-d [13]. The established LOAEL was divided by an uncertainty factor of 1000 to provide a safety margin below the LOAEL for the permitted daily exposure limits. Therefore, the first safety standard set by the EPA and adapted by the FDA as a reference dose for BPA was calculated to be 50 μg/kg-d. This reference dose remains the current safety standard for BPA [14, 15]. Today, there are more than a hundred of studies showing sufficient evidence for low dose effects of BPA on estrogen sensitive organs[0] [1, 16-22].

Developmental exposure to xenoestrogen compounds, such as BPA, affects the uterine histology and uterine response to estradiol (E2) and progesterone in adulthood [23, 24]. The abnormal expression of steroid-sensitive genes would lead to a dysregulation of the hormonal signaling pathway.

Although there is abundant evidence about the long-term effects of BPA exposure in laboratory rodents, no studies have evaluated the additional effects of an estrogen treatment that mimics the replacement therapy used in menopausal women [25]. In the present work, we investigated the long-term effects of perinatal (gestation + lactation) BPA exposure on the uterus of cycling rats and ovariectomized (OVX) adult rats treated

with E2. BPA was administered by the oral route, the most relevant route of exposure to this chemical in the general population [26, 27].

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). Rats of a Wistar-derived strain bred at the Department of Human Physiology (School of Biochemistry and Biological Sciences, Universidad Nacional del Litoral) were kept in a controlled environment (22ºC ± 2ºC; 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 not evaluated; however, because food intake of control and BPA-treated rats was equivalent, we assumed that all animals were exposed to the same levels of phytoestrogens (see Kass et al. [28] for more information regarding food composition). 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.

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, pregnant rats were

weighed and randomly divided into three experimental groups: BPA-vehicle (0.001% ethanol), BPA0.5 (0.5 μg/kg-d, 99% purity, Sigma-Aldrich, Buenos Aires, Argentina) and BPA50 (50 μg/kg-d, Sigma-Aldrich), with 10-12 dams/group. BPA was administered in the drinking water from GD9 to weaning (Fig. 1A). BPA solution was prepared according to Kass et al. [28] and the dose was calculated on the basis of the average weight of dams 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 or two females from each litter were evaluated at each time point. 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 age-related effects of perinatal exposure to BPA on the rat uterus, a group of females (n=10-16 rats/group) were sacrificed in estrus on PND90 (young adults) and PND360 (adults) (Fig. 1A). In addition, to investigate whether perinatal exposure to BPA modified the response to a long-lasting treatment with E2, 12-month-old rats from the BPA-vehicle and BPA groups were ovariectomized (OVX) to avoid endogenous E2 variability, and then treated with an E2 replacement therapy (n=9-14 rats/group) for 90 days (Fig. 1A). 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. A group of OVX rats without any treatment (n=5) were implanted with sesame oil-filled capsules. The treatment with the E2 implants produces constant blood levels of E2 that are equivalent to E2 circulating levels during the rat estrous cycle [25]. This was verified by measuring E2 serum levels

(see section 2.10). This model is supposed to mimic the E2 replacement therapy via the use of patches in menopausal women [25]. To ensure exposure to constant E2 levels, the implants were changed every 30 days. After the treatment, the animals were sacrificed (PND460-E2 groups). Two hours before the autopsy, each rat was injected (ip) with the thymidine analog bromodeoxyuridine (BrdU; 60 mg/kg; Sigma-Aldrich).

2.3 Complementary experiments.

To evaluate whether the effects observed in the uterus of BPA-vehicle rats treated with E2 (PND460-E2 groups) were due to the E2 treatment, the age of animals, or a combination of both, we performed two complementary experiments (Fig. 1B): a) PND90 rats (n=8-11) were OVX and treated with E2 for 3 months (as described in section *2.2*) and sacrificed at the end of the treatment (PND190-E2); b) Cycling female rats (n=5) with no treatment were sacrificed on PND460.

2.4 Tissue sample collection.

The stage of the estrous cycle (proestrus, estrus, metestrus, or diestrus) of each 90- and 360-day-old rats was daily determined by vaginal smears [29] for at least 20 days prior to sample collection. The rats were autopsied in estrus (evaluated by vaginal smears, positive lordosis behavior and uterus histology [30]) and uterine tissue was collected and processed for different experimental purposes. For immunohistochemistry, one uterine horn of each rat was fixed in 10% buffered formalin for 24 h at room temperature and embedded in paraffin. For RNA extraction, the other uterine horn of each rat was immediately frozen in liquid nitrogen and stored at -80ºC.

2.5 Histology.

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). Uterine glands were classified using the criteria described by Gunin et al. [31] and McLachlan et al. [32]. To assess the incidence of epithelial or glandular abnormalities, the number of rats with at least one abnormality of the chosen type was divided by the total number of rats per group.

2.5.1 Morphometry.

The volume density of glands with squamous metaplasia was calculated by applying the formula given by Weibel [33]: Vv = Pi/P, where Vv is the estimated volume density of the object in study (glands with squamous metaplasia), 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 x 0.8 mm was inserted into a focusing eyepiece. The results were expressed as Vv x 1000.

2.6 Immunohistochemistry.

Uterine sections (5 μm in thickness) were deparaffinized and dehydrated in graded ethanol. BrdU incorporation to detect cells in the S phase of the cell cycle was evaluated as previously described [34]. Endogenous peroxidase activity and nonspecific binding sites were blocked. Primary antibodies against the proliferation marker p63, steroid receptors, basal and luminal cytokeratins (CK), vimentin and smooth muscle α-actin (α-SMA) were incubated overnight at 4ºC (Table 1). The reactions were developed using a streptavidin-biotin peroxidase method and diaminobenzidine (Sigma-Aldrich). 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). Negative controls for BrdU immunodetection were samples from rats that did not receive BrdU.

2.7 In situ detection of apoptosis.

To evaluate apoptosis, sections were analyzed for *in situ* detection of cells with DNA strand breaks using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) technique (ApopTag; Intergen Co., Purchase, NY, USA) as previously described [35].

2.8 Image analysis.

2.8.1 Cell proliferation and apoptosis.

Immunostained tissue sections were evaluated using an Olympus BH2 microscope (Olympus Optical Co. Ltd, Tokyo, Japan), with a Dplan 100X objective (numerical aperture = 1.25; Olympus). Incorporation of BrdU and apoptosis indices were quantitatively analyzed in all tissue compartments of the uterus [35].

2.8.2 Steroid receptors.

The images of tissue sections were captured with a Dplan 20X objective (numerical aperture, 0.65; Olympus). To measure the integrated optical density (IOD) of estrogen receptor alpha and beta (ERα and ERβ), and progesterone receptor (PR) immunostaining in the subepithelial stroma, images were analyzed using the Image Pro-Plus 4.1.0.1 system (Media Cybernetics, Silver Spring, MD, USA) [35]. At least 10 fields were recorded in each section, and three sections per rat were evaluated. The

238 subepithelial stromal compartment was delimited and the IODs were measured as
239 previously described [36]. Because the IOD is a dimensionless parameter, the results
240 were expressed as arbitrary units.
241
242 *2.8.3 Vimentin and α-SMA.*
243 The images were recorded as described above. The expression of vimentin was
244 evaluated in the periglandular stroma zone defined as the 10-μm-wide area around the
245 glands (from the basement membrane towards the outer layers), and the relative area
246 occupied by vimentin-positive cells was determined [37]. α-SMA expression was
247 measured as the proportion of the glandular perimeter occupied by cytoplasmic
248 projections of α-SMA-positive cells (linear density) [38].
249
250 *2.8.4 p63 and cytokeratins.*
251 The expression profiles of CK8 (luminal CK) and CK34bE12 (basal CK) were
252 qualitatively evaluated on three uterine sections. The expression of p63 was evaluated in
253 glands with squamous metaplasia and expressed as percentage of p63-positive glandular
254 cells.
255
256 *2.9 Reverse transcription and real-time quantitative PCR analysis.*
257 *2.9.1 RNA extraction and reverse transcription.*
258 Individual uterine horn samples were homogenized in TRIzol (Invitrogen, Carlsbad,
259 CA, USA), and RNA was prepared according to the manufacturer's protocol. The
260 concentration of total RNA was assessed by A260, and the samples were stored at -80ºC
261 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 [8].

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). The primer sequences used for amplification of TAp63, ΔNp63, IGF-I, IGF-IR and ribosomal subunit 18S (housekeeping gene) cDNAs are described in Table S1 (Supplementary data). For cDNA amplification, 5 µl of cDNA was combined with HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne; Biocientífica; Rosario, Argentina), and 10 pmol of each primer (Invitrogen) in a final volume of 20 µl. After initial denaturation at 95ºC for 15 min, the reaction mixture was subjected to successive cycles of denaturation at 95ºC for 15 sec, annealing at 54ºC (ΔNp63), 55ºC (TAp63 and IGF-I), 57ºC (IGF-IR) and 60ºC (18S) for 15 sec, extension at 72ºC for 15 sec and reading at 81ºC. 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 [39]. The CT for each sample was calculated using the Rotor-Gene Q – Pure Detection software (Version: 1.7, Quiagen; Tecnolab). Accordingly, fold expression over control values was calculated for each target by Relative Expression Software Tool V2.0.13 (REST; Quiagen; Tecnolab), which is specifically designed to analyze data from real-time PCR. REST calculates the relative expression ratio on the basis of the PCR efficiency and crossing point deviation (Δ CP) of the investigated transcripts and on a newly developed randomization test. This

test estimates how many times the investigated transcript increases or decreases relative to control, which is assigned a value of 1 [40]. No significant differences in CT values were observed for 18S between the different experimental groups.

2.10 Hormone assay

Serum levels of E2 were measured in blood samples of PND460-E2 rats by radioimmunoassay (RIA) after ethyl ether (Merck, Buenos Aires, Argentina) extraction [38]. The antibody was provided by G. D. Niswender, and the labeled hormone was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). Assay sensitivity was 4 pg/ml. Intra- e inter-assay coefficients of variation were 3.2% and 11% respectively.

2.11 Statistical analysis.

All data are expressed as the mean ± SEM. The incidence of uterine lesions was analyzed by the Fisher's exact test. For all the other variables, univariate Analysis of Covariance (ANCOVA) was applied to evaluate the sibling's contribution as a possible confounding factor in each time point evaluated (SPSS-PASW Statistics v. 18). In the variables studied, the covariate did not influence the responses to the treatment. Therefore, Kruskal-Wallis followed by Dunn's post-test was used to establish differences among experimental groups. $P \leq 0.05$ was accepted as significant.

3. RESULTS

3.1. BPA general effects (results summarized in Table S2)

As previously reported [28], exposure to BPA through drinking water did not produce signs of embryotoxicity (i.e., all pregnant dams successfully delivered their pups, and the number of live-born pups per litter was similar among groups, abnormal maternal or nursing behavior, or changes in the dam body weight gain or water consumption). The gestation length was unaltered since all dams delivered on GD23, and no gross malformations were observed in pups at delivery or weaning. The litter sex ratio showed no differences and was within the normal range (50% females and 50% males). Litter survival and weight gained during lactation was unaltered. The onset of puberty in female pups showed no significant differences between groups. All females on PND90 exhibited regular estrous cycles (5 days). On PND360 and PND460, intermittent extended periods of diestrus or extended periods of proestrus/estrus were occasionally observed. However, the patterns of estrous cycles did not differ between BPA-treated and BPA-vehicle group.

3.2. Uterine evaluation in rats perinatally exposed to BPA

3.2.1. Young adults (PND90)

Uterine histology showed characteristic signs of estrus stage, such as: luminal dilation and high percentage of apoptosis in the glands and luminal epithelium. No changes were observed in the two BPA-treated groups compared with the BPA-vehicle group. In the luminal epithelium, subepithelial stroma and muscular region, BrdU incorporation and apoptotic indexes showed no differences between groups. In the glandular epithelium, a significant decrease in proliferative activity was observed in animals exposed to BPA0.5 and BPA50 while apoptotic indexes were not modified (Fig. 2A-B). The vimentin and α-SMA protein expression in the stromal cells, surrounding the glandular epithelium was used to identify the fibroblastic and muscle immunophenotype of these cells. In the

BPA50-exposed group the percentage of glandular perimeter occupied by α-SMA-positive cells was significantly decreased (Fig. 2C). No differences were found in the areas occupied by vimentin-positive cells (Fig. 2D).

3.2.2. Adults (PND360)

Uterine histology showed characteristic signs of estrus stage. Morphological changes in the uterine luminal epithelium such as regions with cuboidal epithelium instead of tall columnar (Fig. 3B, double arrow) and abnormal cells (hypochromic nuclei and/or atypical arrangement, clear cytoplasm) (Fig. 3B-C, arrow) were observed. The incidence of these alterations was significantly higher in the BPA groups (BPA-vehicle 12.5% vs. BPA0.5 58.3% and BPA50 60%; Table 2).

In BPA-vehicle rats, different morphological types of uterine glands were observed: a) *normal glands* (round, oval or elongated shape with simple cuboidal epithelium) (Fig. 3D, arrowhead), b) *glands with squamous metaplasia* (two or three layers of cells, constituting a stratified epithelium) (Fig. 3D, arrow), c) *glands with cellular anomalies* (cylindrical epithelium, low nuclei/cytoplasm ratio, undefined cytoplasmic borders, or cells with dispersed chromatin and atypical arrangement) (Fig. 3E), and d) *cystic glands* (usually large size, enlarged lumen and flat epithelium) (Fig. 3F). The incidence of glands with cellular anomalies was higher in BPA50-treated rats than in BPA-vehicle ones (BPA-vehicle 31.3% vs. BPA50 90%; Table 2).

On PND360 the luminal and glandular epithelia were positive for ERα and CK8 (a marker of luminal cells), and negative for PR, basal CK (CK34βE12) and p63 (Fig. S1). Glands with squamous cell metaplasia were p63-positive and expressed basal and

luminal CKs. CK34βE12 was evident in basal cells, whereas CK8 was restricted to luminal cells. The expression of p63 was observed in more than one cellular layer of squamous metaplasia, mainly in basal cells, which expressed ERα. BPA exposure did not affect the expression pattern of any of the molecules evaluated.

3.3. Uterine response to estrogen in adult rats

3.3.1. Morphological features of uterine tissue

On PND460, the uterine tissue from OVX animals without any treatment exhibited signs of atrophy; the subepithelial stroma presented numerous cells with very narrow cytoplasm and picnotic nuclei. The uterine glands had a narrow lumen, and an oval or elongated shape with simple cuboidal epithelium, all features of atrophic glands. PND460-E2 rats without BPA exposure showed glands with squamous metaplasia, glands with cellular anomalies, cystic glands (abnormalities similar to those described in BPA-vehicle cycling rats on PND360) and with the presence of *glands with daughter glands*. These glands had various sizes and shapes –round, elongate, tortuous-, and formed daughter glands inside the epithelium or inside the mother gland lumen or on the outer surface of the mother gland, like budding glands (Fig.4). Interestingly, no daughter glands were observed on PND360.

To find out whether the glands with daughter glands observed in the uteri of PND460-E2 rats were due to the E2 treatment, the age of animals, or both, complementary experiments were performed. Uterine samples from PND190-E2 rats showed the characteristic signs of estrogenic stimulation but without glandular abnormalities. On the other hand, the uterine tissue from aged intact rats (PND460) showed glands with

385 daughter glands similar to those observed on PND460-E2 rats (PND460-E2 14.3%
386 *versus* PND460 25%).
387
388 *3.3.2. Glandular abnormalities in BPA-exposed rats*
389 In response to E2, the incidence of glandular abnormalities in the BPA-exposed groups
390 was higher. The incidence of cellular anomalies in glands was higher in the BPA50
391 group than in the BPA-vehicle group (BPA-vehicle 64.3% vs. BPA50 100%; Table 3).
392 The incidence of glands with daughter glands was higher in BPA0.5 rats than in BPA-
393 vehicle ones (BPA-vehicle 14.3% vs. BPA0.5 66.7%; Table 3). The percentage of rats
394 that exhibited uterine glands with squamous metaplasia increased in all experimental
395 groups of PND460-E2 (Table 3), without reaching statistical significance. However,
396 when we recorded the multiplicity (evaluated as volume density) of glands with
397 squamous metaplasia per rat, we found a significant increase in the BPA50 group
398 (Fig.5).
399
400 *3.3.3. Estradiol levels*
401 OVX rats without any treatment showed E2 levels below the detection limit of the
402 assay. No significant differences in E2 serum levels were observed in PND460-E2 rats
403 (BPA-vehicle 19.22 pg/ml ± 4.2; BPA0.5 19.3 pg/ml ± 9.8; BPA50 11.3 pg/ml ± 7.03).
404
405 *3.3.4. Steroid receptor expression and immunophenotype of uterine tissue*
406 As mentioned before, ovarian steroid hormones regulate proliferation and differentiation
407 of the uterine epithelium through binding to their stromal receptors. Therefore, ERα,
408 ERβ and PR expression was evaluated in the subepithelial stroma in all PND460-E2
409 groups. PR expression decreased in both BPA groups, while ERα expression was lower

in BPA50 rats (Fig. 6A and B). Regarding ERβ expression, no differences were observed (Fig. 6).

The immunophenotype of uterine glands in PND460-E2 rats presented a pattern similar to that found in PND360 rats (see Fig. S1). Moreover, BPA exposure did not affect the proliferative activity in PND460-E2 rats (Fig. S2).

3.3.5. Expression of p63 and IGF-1 in uterine tissue

RT-PCR results demonstrated that both ΔN- and TA-p63 isoforms were expressed in PND460-E2 rats (Fig. 7). TA-p63 was lower in BPA0.5 rats and higher in BPA50 ones (Fig. 7A). p63 immunostained cells/squamous metaplasia were significantly lower in BPA0.5 (50.9%) than in BPA50 rats (64.4%) (Fig. 7C).

To evaluate whether the IGF-I signaling pathway was implicated in the estrogen-induced effects on the glandular epithelium, RT-PCR assays were conducted in PND460-E2 rats. The expression of IGF-I and IGF-IR mRNA was lower in BPA0.5 rats (Fig. 8).

4. DISCUSSION

The endometrium is one of the main targets for estrogenic chemicals and there are many evidences about the adverse effect of xenoestrogens on the uterus [1, 8, 9, 41, 42]. In the present study, we demonstrated that perinatal exposure to low doses of BPA, such as the "safe dose" established by US EPA (equivalent to the high dose used here, BPA50), and a dose 100 times lower than the safe dose (BPA0.5), can reprogram the uterus

435 development and consequently alter its differentiation and its response to an estrogenic
436 therapy later in life. These effects were demonstrated in female offspring born from
437 dams exposed to BPA by using oral administration during pregnancy and lactation. The
438 oral route is the most relevant for BPA exposure in the general population [26, 27].
439
440 We showed that exposure to BPA during the perinatal period did not modify cell
441 apoptosis but reduced glandular cell proliferation on PND90 (young adults). In addition,
442 BPA50 exposure decreased α-SMA expression in stromal cells surrounding the uterine
443 glandular epithelium, without changes in vimentin expression. Thus, the decrease in
444 glandular cell proliferation could be due to the altered tissue organization in the
445 surrounding stroma affecting the epithelial–stromal communication. We have
446 previously shown that females exposed to diethylstilbestrol (DES) exhibit uterine
447 glandular alteration similar to that described here [8]. In the same sense, Ramos et al.
448 [37] have shown that, in BPA-treated rats, the phenotype of periductal stromal cells in
449 the prostate and the glandular cell function are affected. Similar results were found in
450 the peritubular cells surrounding the seminiferous tubules of *Caiman latirostris* exposed
451 to pesticides [43]. Altered organization of the periglandular myoid cells could modify
452 signals involved in the control of cell proliferation and survival as well as the
453 coordinated relationship between proliferative activity and cell death necessary to
454 maintain tissue histoarchitecture and functions [43].
455
456 On PND360 (adult rats), a few BPA-vehicle rats (12.5%) showed uterine tissue
457 abnormalities, including morphological changes in the uterine luminal epithelium such
458 as epithelial regions with abnormal cells and cuboidal instead of tall columnar
459 epithelium. In addition, BPA-vehicle rats showed different glandular lesions (cystic

460 glands, glands with cellular anomalies, and glands with squamous metaplasia). As
461 previously demonstrated, these uterine changes occur during the normal female aging
462 process [8]. The aging process is associated with a decrease in cell proliferation and in
463 the expression of steroid receptors within different endometrial cell types [44]. Khalyfa
464 et al. [45] demonstrated age-related changes in the expression of estrogen target genes
465 in the mouse uterus, which suggests that the modified expression of genes may play a
466 role in reproductive senescence and explain the decline in reproductive function in old
467 animals. Although these abnormalities were histologically similar to those seen in our
468 aged BPA-vehicle animals, the incidence of luminal epithelium alterations was higher in
469 BPA-treated rats. Other authors have shown similar uterine epithelial abnormalities in
470 rodents exposed to BPA [24, 46], suggesting that these changes may be specific BPA-
471 disruption patterns in the uterine epithelium. Moreover, BPA50-exposed rats showed a
472 higher incidence of glands with cellular anomalies. Our results are in agreement with
473 that reported by Newbold et al. [9, 47], who showed that neonatal treatment with BPA
474 induces pathologies in the adult rat uterus, including benign, premalignant, and
475 neoplastic changes.
476
477 Rats on PND460-E2 showed levels of E2 about 11-19 pg/ml, and no differences were
478 found between BPA-vehicle and BPA-exposed rats. These values are coincident with
479 serum physiological concentrations at the estrus stage in rats [48], showing that the E2
480 dose administered to the rats was in the range of low physiological values.
481
482 Female offspring perinatally exposed to BPA and treated with E2 in the adulthood
483 showed increased occurrence of glandular lesions. The BPA50 group showed higher
484 incidence of glands with cellular anomalies and multiplicity of glands with squamous

metaplasia, whereas the BPA0.5 group showed higher incidence of glands with daughter glands. We have previously demonstrated that rats perinatally exposed to DES show an altered response to E2 in the adulthood [8] similar to that of BPA-exposed rats. It is interesting to note that BPA-vehicle rats treated with E2 (PND460-E2), but not the PND190-E2 group, presented uterine glands with daughter glands and conglomerates of glands. Since aging intact rats (PND460) exhibit those alterations in uterine glands, these results would indicate that glands with daughter glands appear in aged animals in the presence of endogenous or exogenous estrogens. Gunin et al. [31] suggested that changes in the architecture of glands could be correlated with changes in mitosis orientation. Results presented here demonstrated that the presence of glands with daughter glands and conglomerates of glands are age-related. However, their higher incidence in animals exposed to BPA suggests that perinatal exposure to xenoestrogens 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 [31].

Stroma-epithelial interactions are critical for mediating the effects of hormones [11]. We demonstrated that PR expression decreased in response to E2 in uterine stromal cells in both BPA groups. Furthermore, we found decreased expression of ERα in BPA50 rats. 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 cystic endometrial hyperplasia [49].

509 The p63 transcription factor is transcribed from alternative splicing sites into two
510 isoforms either containing or lacking the N-terminal transactivation domain, *TA or ΔN*,
511 respectively. In adult tissues, p63 is expressed in stratified epithelia but not in single-
512 layered epithelia, suggesting a role for p63 in the development and maintenance of
513 stratified epithelia [50]. Little is known about specific functions of TAp63 and ΔNp63.
514 Koster et al. [50] demonstrated that TAp63 is the first p63 isoform expressed during
515 embryogenesis and that it is required for initiation of epithelial stratification. Our results
516 demonstrate that p63 expression was restricted to glands with squamous metaplasia.
517 Then, TAp63 was higher in the BPA50 group than in the BPA-vehicle one. This
518 increase in TAp63 would be mainly associated with the observed increase in the
519 multiplicity of glands with squamous metaplasia in BPA50 rats. Koster et al. [50]
520 observed that ectopic expression of TAp63 in simple lung epithelium *in vivo* promotes
521 the induction of squamous metaplasia. Based on these results, we suggest that the
522 increased p63 expression in BPA50 rats may be associated with increased multiplicity
523 of squamous metaplasia. Unlike the BPA50 group, rats exposed to BPA0.5 presented
524 lower expression of TAp63 mRNA associated with lower expression of p63 in glands
525 with squamous metaplasia.
526
527 In rodents, E2 induces epithelial cell proliferation by acting through the ERα located in
528 the stroma, which stimulates the secretion of growth factors such as IGF-I, which acts in
529 a paracrine fashion on uterine epithelial IGF-I receptor (IGF-IR) [12]. It has been shown
530 that mice perinatally exposed to estrogens develop endometrial hyperplasia associated
531 with alterations in signaling pathways involving IGF-I and IGF-IR [51]. In response to
532 E2, rats exposed to BPA0.5 showed a decrease in mRNA expression of IGF-1 and IGF-
533 IR. This suggests that the signaling pathway of IGF-I would not be associated with the

increased frequency of glands with daughter glands observed in BPA0.5 rats. More studies are needed to better characterize this phenomenon.

The present results provide further evidence that BPA ingestion by pregnant-lactating dams induces numerous abnormalities in the uterine tissues of offspring when the animals reach adulthood. Moreover, the adult uterine response to E2 was altered in the offspring perinatally exposed 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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690
691

Figure legends

Figure 1. Schematic representation of the experimental protocol used to study the effects of perinatal (gestation + lactation) exposure to bisphenol A (BPA) and E2 treatment on the uterus of young adult (PND90) and adult (PND360 or 460) females. A) Main experimental protocol. GD: gestational day, PND: postnatal day; E2: 17β-estradiol; OVX: ovariectomy. B) Complementary experiments, protocol used in non-exposed females to study uterine tissue in PND190 rats treated with E2 *versus* older adult rats (PND460) without E2 treatment.

Figure 2. Effects of perinatal exposure to BPA on uterine tissue of young adult rats (PND90). Proliferative (A) and apoptotic (B) rate quantified by immunohistochemistry and TUNEL respectively, in the uterine glandular epithelium. Quantification of αSMA (C) and vimentin (D) immunostaining in the uterine periglandular stroma. αSMA staining is expressed as linear density, which is the proportion of the glandular perimeter occupied by cytoplasmic projections of αSMA-positive cells. Vimentin is expressed as the integrated optical density (IOD), which is a linear combination between the average of immunostaining intensity and the relative area occupied by positive cells. Each column represents the mean ± SEM ($n>7$ per group). *, $P<0.05$ vs. the BPA-vehicle group.

Figure 3. Effects of perinatal exposure to BPA on the uterine luminal and glandular epithelium of adult rats (PND360). (A) Representative photomicrographs showing normal thickened uterine epithelium at estrus of BPA-vehicle rats (double arrow). In animals exposed to BPA (B-C) we observed regions with reduced thickness of the

epithelium (double arrow), hypochromic nuclei, clear cytoplasm and/or atypical cell arrangement (arrows). In adult rats, different types of uterine glands were observed: (D) glands with squamous metaplasia (arrow) and normal glands (arrowhead), (E) glands with cellular anomalies and (F) cystic glands. Scale bar: 50 μm.

Figure 4. Representative photomicrographs showing uterine gland abnormalities in BPA-vehicle rats treated with E2 (PND460-E2). The uterine glands show glands with daughter glands (A) and glands forming conglomerates (B). Scale bar: A 50 μm and B 75 μm.

Figure 5. Quantification of glands with squamous metaplasia in the uterus of PND460-E2 rats. Each column represents the mean ± SEM (n>8 per group) of the volume density (Vv) x1000. *, P<0.05 vs. the BPA-vehicle group.

Figure 6. Effects of perinatal BPA exposure on the immunohistochemical expression of steroid receptors in uterine tissue of PND460-E2 rats. (A) Photomicrographs showing detection of PR, ERα, and ERβ in the subepithelial stroma of uterine sections. These images were obtained from sections without hematoxylin counterstaining. Scale bar: 50 μm. (B) Quantification of steroid receptor immunostaining evaluated as integrated optical density (IOD). Results are expressed as relative units. Each column represents the mean ± SEM (n>8 per group). *, P<0.05 vs. the BPA-vehicle group.

Figure 7. Effect of perinatal exposure to BPA on p63 expression in the uterus of PND460-E2 rats. (A-B) Relative Tap63 and ΔNp63 mRNA levels measured by real-time RT-PCR. Samples were normalized to 18S expression and to control animals; a

value of 1 was assigned to the control group (REST 2009 software). (C) Immunohistochemistry expression of p63 protein in glands with squamous metaplasia of BPA-vehicle and BPA-exposed animals. *, P<0.05 vs. the BPA-vehicle group. Scale bar: 50 μm.

Figure 8. Effect of perinatal exposure to BPA on IGF-I and IGF-IR expression in the uterus of PND460-E2. Relative IGF-I and IGF-IR mRNA levels were measured by real-time RT-PCR. Samples were normalized to 18S expression and to control animals; a value of 1 was assigned to the control group (REST 2009 software). *, P<0.05.

Supplemental Figure S1. Photomicrographs showing immunohistochemical detection of ERα, PR, CK8, basal CK (CK34βE12) and p63 in the luminal epithelium and in the different types of glands in rats on PND360. Normal glands (black arrowhead), cystic glands (white arrowhead), glands with cellular anomalies (white arrow) and glands with squamous metaplasia (black arrow). Scale bar: 50 μm.

Supplemental Figure S2. Photomicrographs showing immunohistochemical detection of BrdU in uterine glands of PND460-E2 rats. BrdU incorporation of BPA-vehicle (A), BPA0.5 (B), and BPA50 (C) rats. Scale bar: 50 μm.

Table 1. Antibodies used for Immunohistochemistry

Primary Antibodies	Dilution	Supplier
Anti-PR (clone A0098)	1/500	Dako Corp. (Carpinteria, CA, USA)
Anti-ERα (clone 6F-11)	1/200	Novocastra, Newcastle upon Tyne, UK
Anti-p63 (clone 4A4)	1/100	Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA)
Anti-pan-CK basal (clone 34βE12)	1/100	Novocastra, Newcastle upon Tyne, UK
Anti-CK8	1/1600	The Binding Site Limited, (Birmingham, UK)
Anti-BrdU (clone 85-2C8)	1/100	Novocastra, Newcastle upon Tyne, UK
Anti-ERβ (clone EMRO2)	1/100	Novocastra, Newcastle upon Tyne, UK
Anti-Vimentin (clone V9)	1/50	Novocastra, Newcastle upon Tyne, UK
Anti-Smooth muscle α-actin (α-SMA clone αsm-1)	1/100	Novocastra, Newcastle upon Tyne, UK
Secondary Antibodies	**Dilution**	**Supplier**
Anti-mouse	1/80	Sigma (St. Louis, MO, USA)
Anti-goat	1/200	Sigma (St. Louis, MO, USA)
Anti-sheep	1/200	Sigma (St. Louis, MO, USA)

Table 2. Incidence of histologically diagnosed uterine abnormalities in adult rats (PND360) perinatally exposed to BPA

	Treatment		
	BPA-vehicle	**BPA0.5**	**BPA50**
luminal epithelium anomalies	2/16 (12.5%)	7/12 (58.3%)[a]	6/10 (60%)[a]
glands with squamous metaplasia	9/16 (56.3%)	10/12 (83.3%)	5/10 (50%)
glands with cellular anomalies	5/16 (31.3%)	7/12 (58.3%)	9/10 (90%)[a]
cystic glands	10/16 (62.5%)	8/12 (66.7%)	7/10 (70%)

Results are expressed as number of affected rats vs. total rats per group. [a] $p<0.05$ compared with the BPA-vehicle group.

Table 3. Incidence of histologically diagnosed uterine abnormalities in adult rats perinatally exposed to BPA and treated with E2 (PND460-E2 group)

	Treatment		
	BPA-vehicle	**BPA0.5**	**BPA50**
luminal epithelium anomalies	2/14 (14.3%)	0/9 (0%)	3/9 (33.3%)
glands with cellular anomalies	9/14 (64.3%)	8/9 (88.9%)	9/9 (100%)[a]
glands with daughter glands	2/14 (14.3%)	6/9 (66.7%)[a]	3/9 (33.3%)
glands with squamous metaplasia	10/14 (71.4%)	9/9 (100%)	8/9 (88.88%)
cystic glands	9/14 (64.3%)	9/9 (100%)	8/9 (88.9%)

Results are expressed as number of affected rats vs. total rats per group [a] $p<0.05$ compared with the BPA-vehicle group.

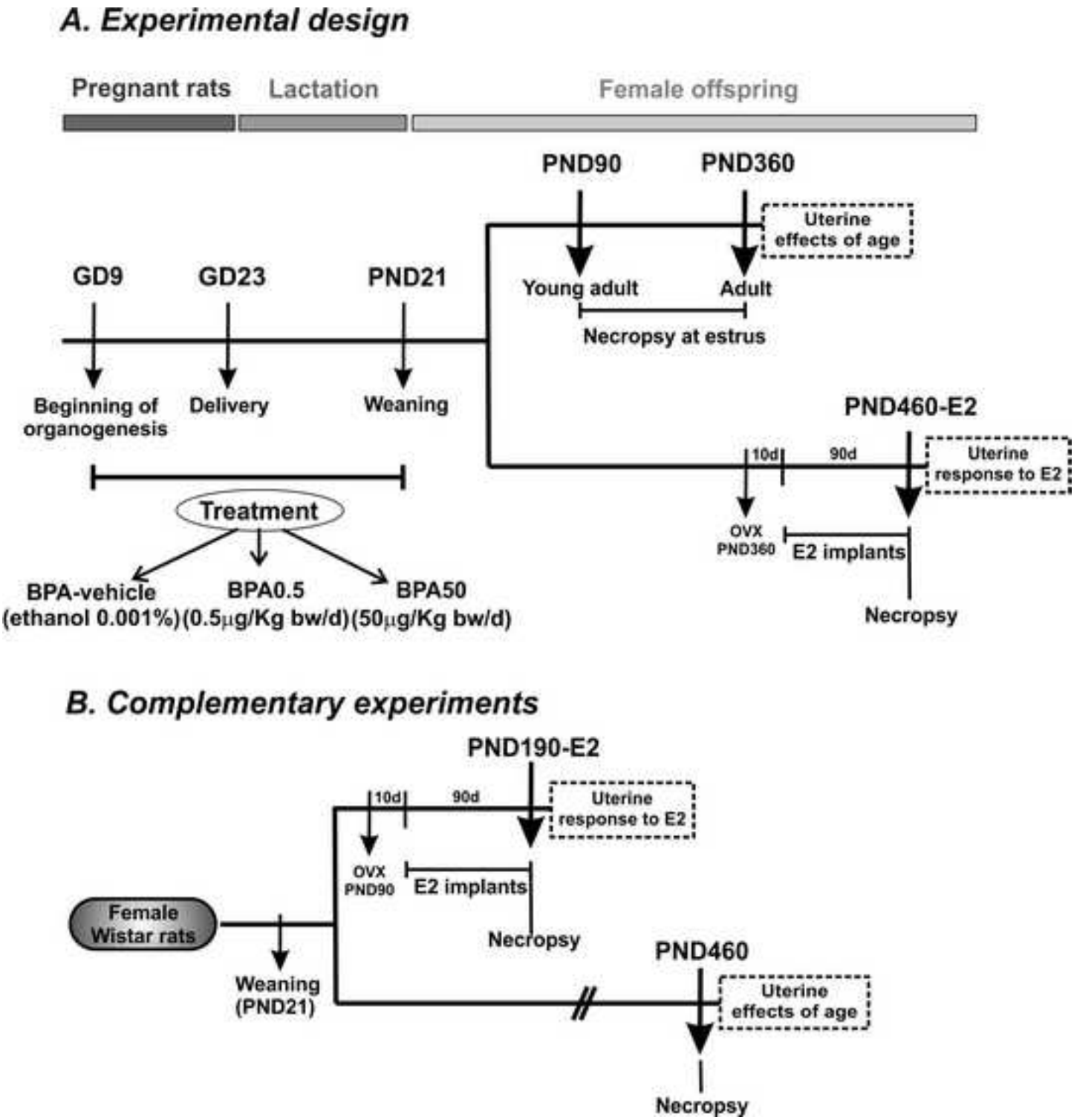


Figure 2

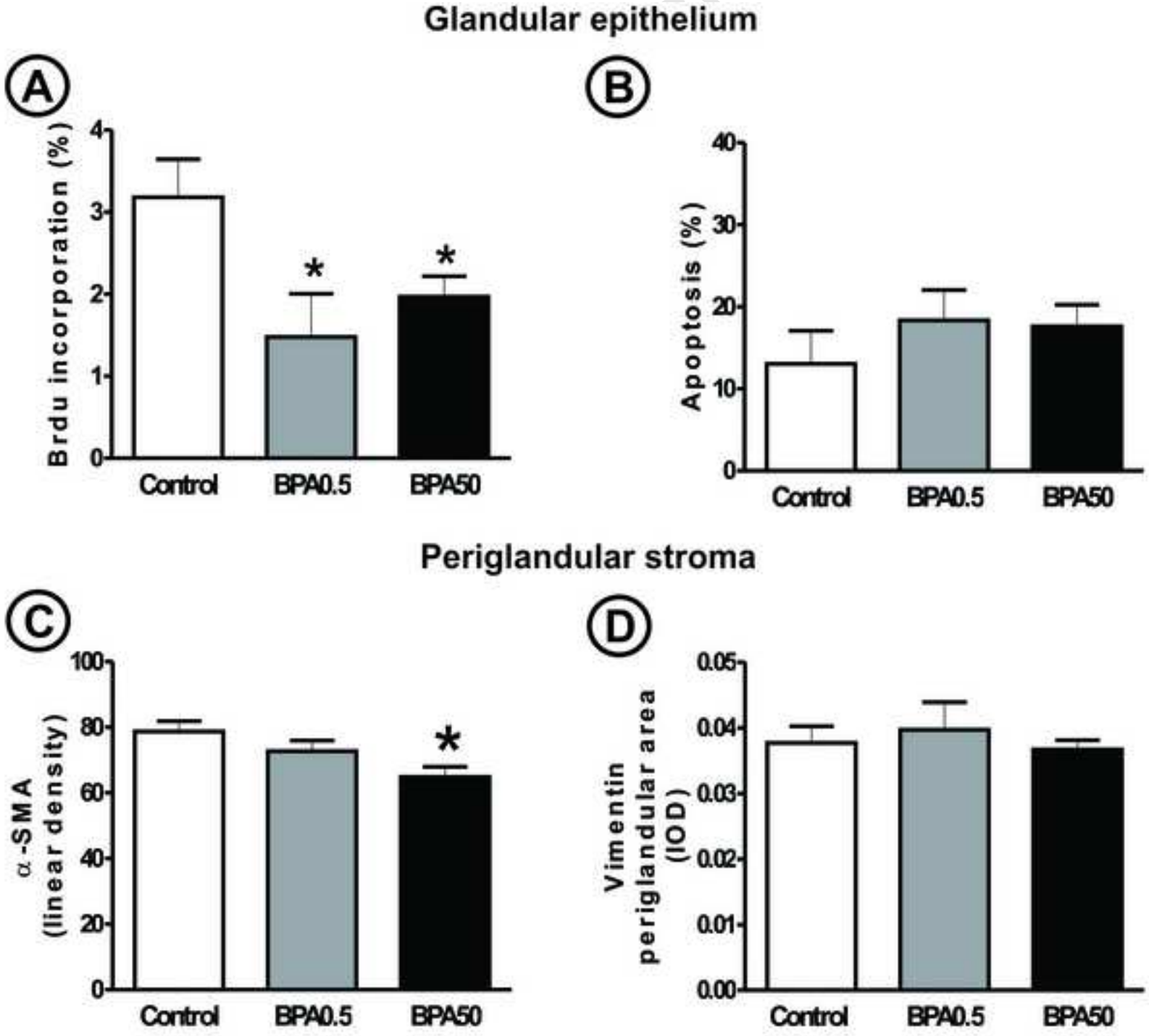

Glandular epithelium
A
Brdu incorporation (%)
0
1
2
3
4
*
*
Control
BPA0.5
BPA50
B
Apoptosis (%)
0
10
20
30
40
Control
BPA0.5
BPA50
Periglandular stroma
C
α-SMA (linear density)
0
20
40
60
80
100
*
Control
BPA0.5
BPA50
D
Vimentin periglandular area (IOD)
0.00
0.01
0.02
0.03
0.04
0.05
Control
BPA0.5
BPA50

Figure 3

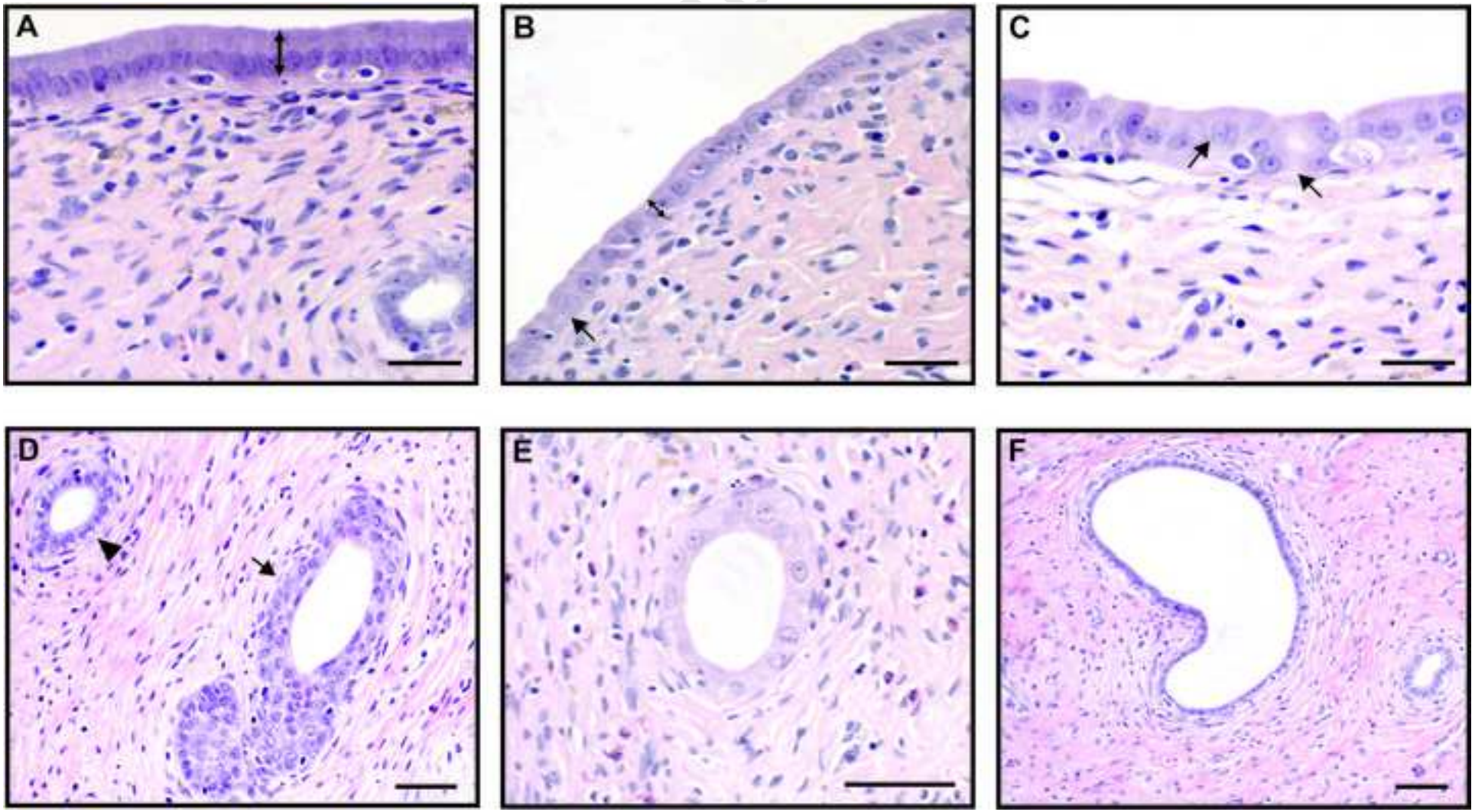


Figure 4

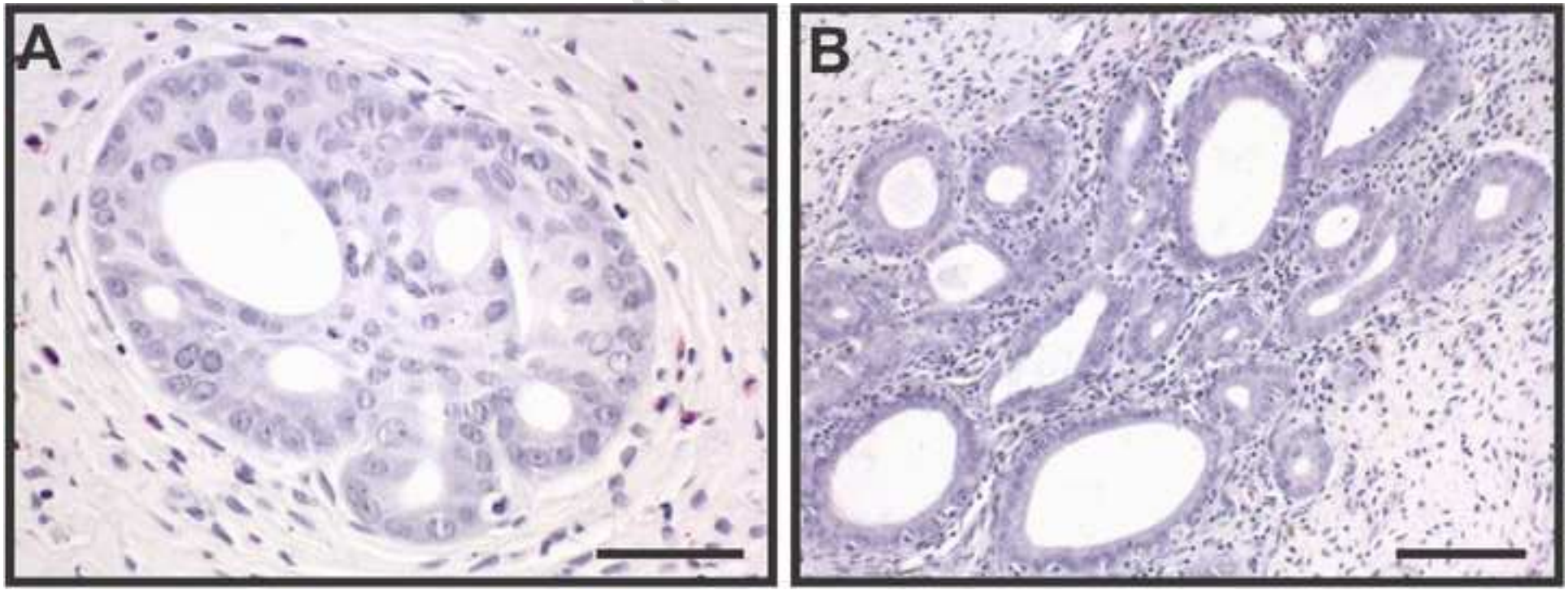


Figure 5

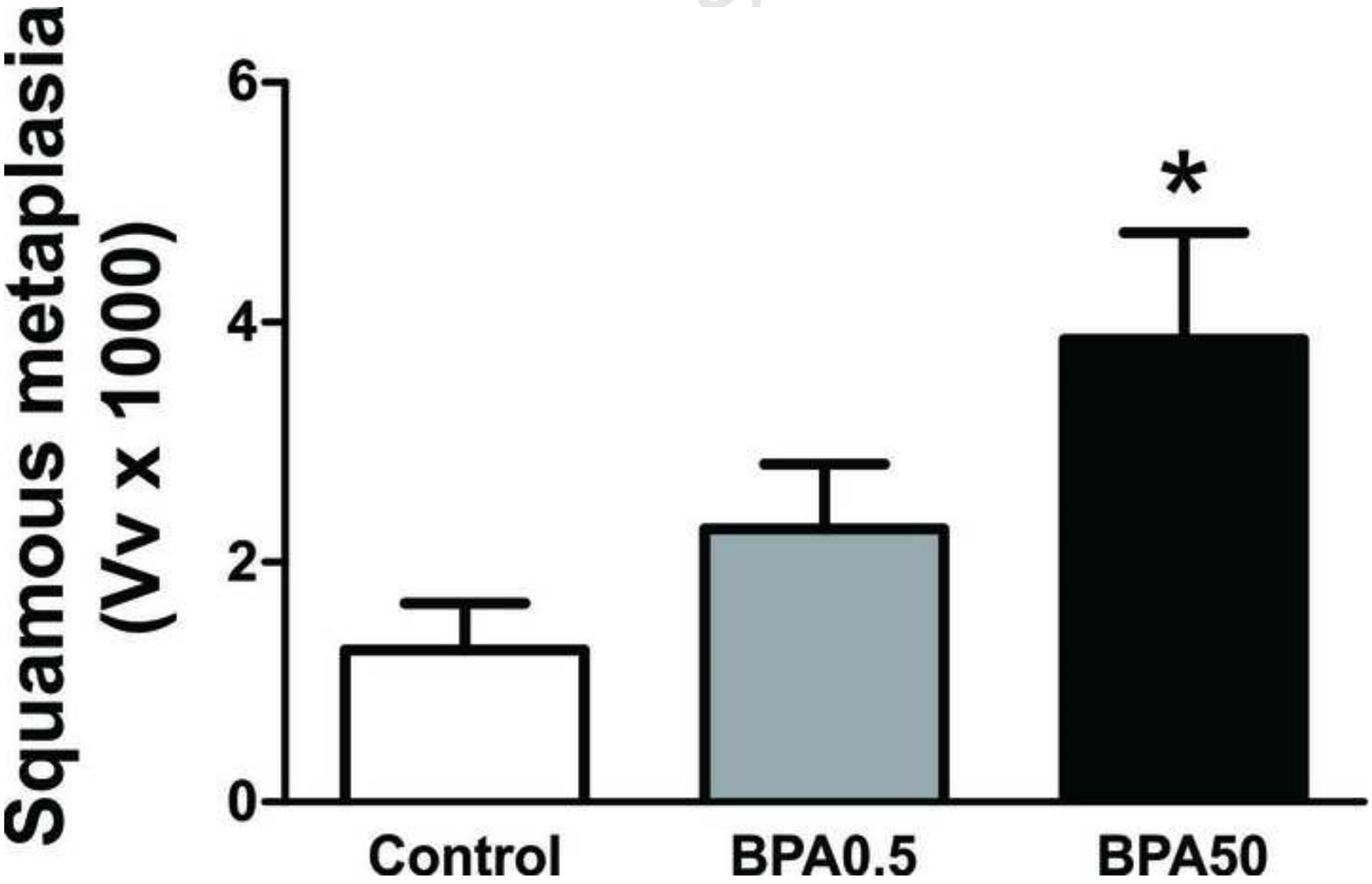


Figure 6

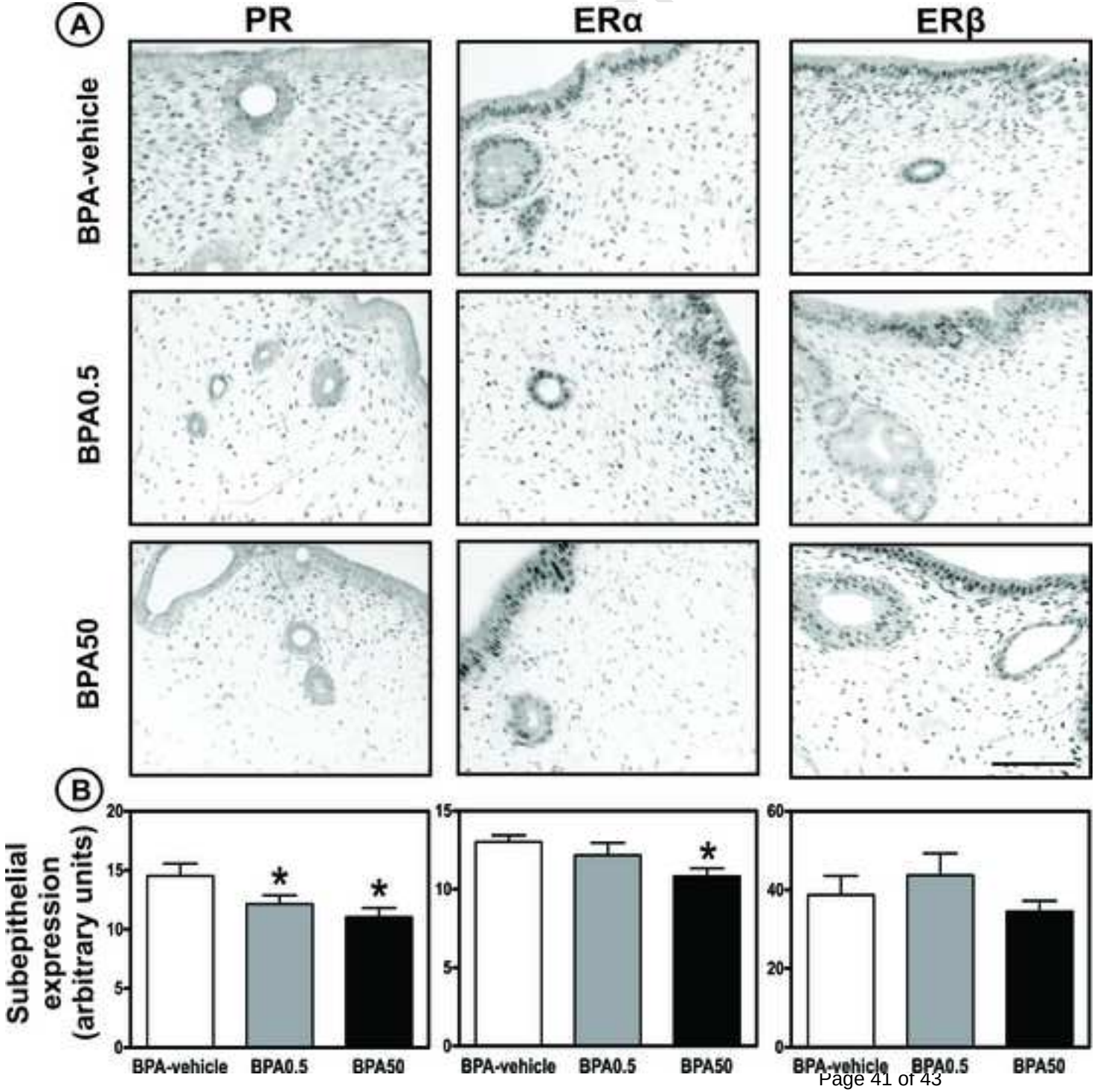

A
PR
ERα
ERβ
BPA-vehicle
BPA0.5
BPA50
B
Subepithelial expression (arbitrary units)
20
15
10
5
0
*
*
BPA-vehicle
BPA0.5
BPA50
15
10
5
0
*
BPA-vehicle
BPA0.5
BPA50
60
40
20
0
BPA-vehicle
BPA0.5
BPA50

Figure 7

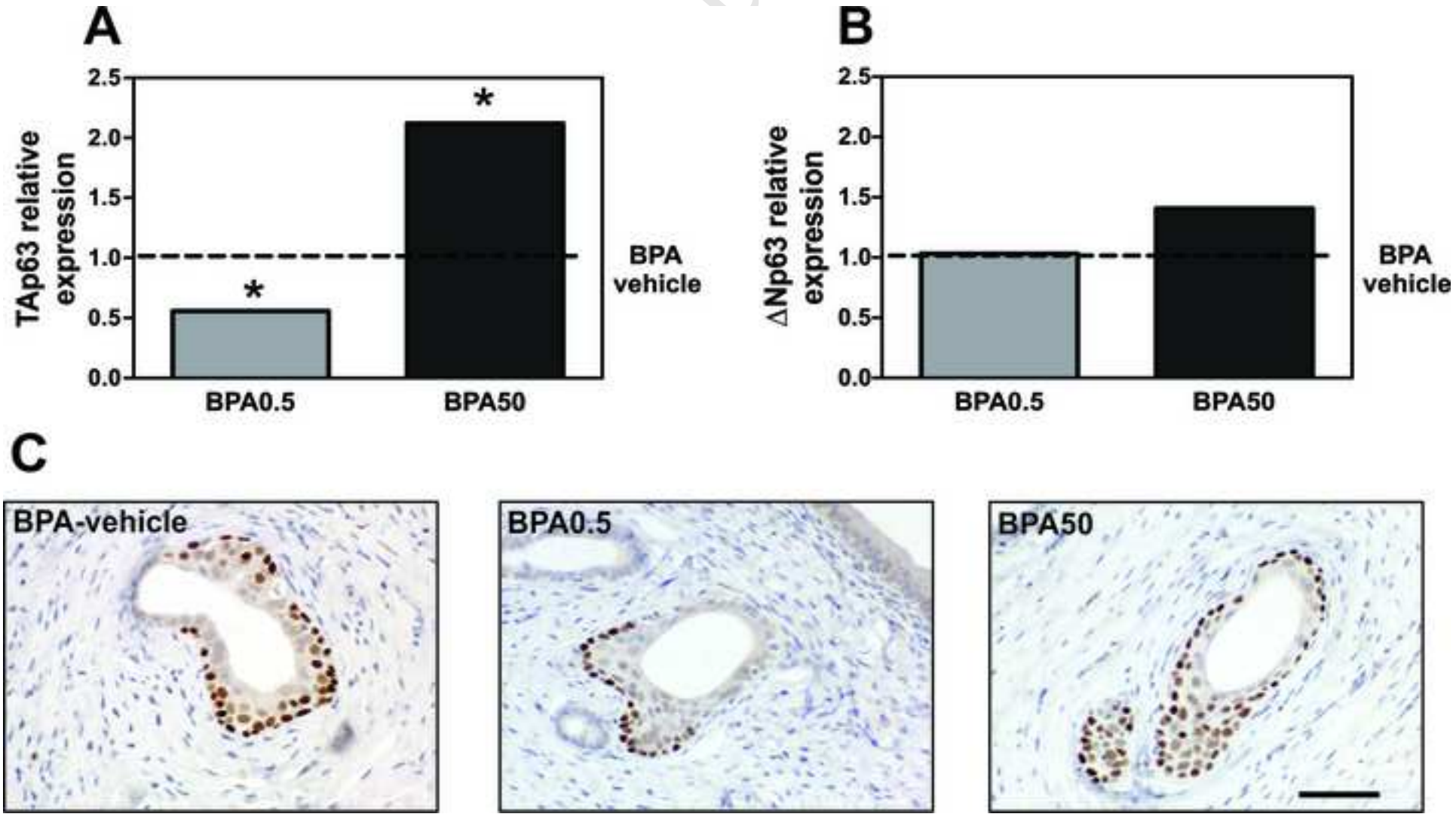
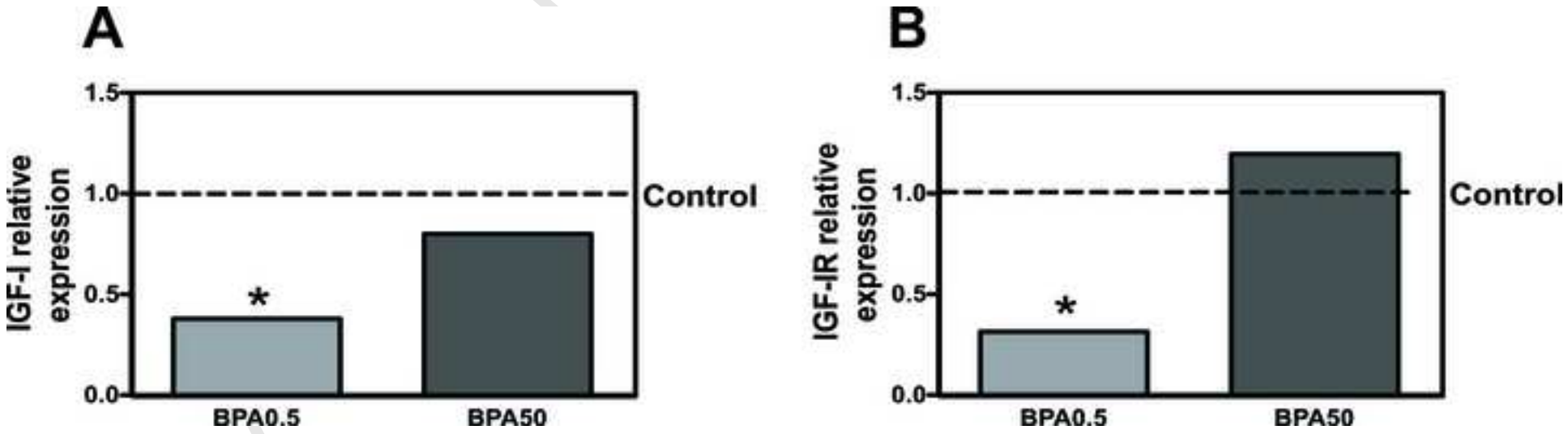

A
TAp63 relative expression
2.5
2.0
1.5
1.0
0.5
0.0
*
*
BPA0.5
BPA50
BPA vehicle
B
ΔNp63 relative expression
2.5
2.0
1.5
1.0
0.5
0.0
BPA0.5
BPA50
BPA vehicle
C
BPA-vehicle
BPA0.5
BPA50

Figure 8


A
IGF-I relative expression
1.5
1.0
0.5
0.0
Control
*
BPA0.5
BPA50
B
IGF-IR relative expression
1.5
1.0
0.5
0.0
Control
*
BPA0.5
BPA50