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Prenatal Bisphenol A exposure delays the development of the male rat mammary gland

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ABSTRACT

Our aims were to evaluate whether exposure to Bisphenol A (BPA) modifies the development of the male rat mammary gland (MG) and to evaluate whether this modification is gender specific. From gestational day 9, pregnant rats were exposed either subcutaneously to 0, 25 or 250 μg BPA/kgbw/day until parturition or orally to 0 and 64 μg BPA/kgbw/day until weaning. MG development was analyzed on postnatal days (PND) 5, 15 and 30. On PND30, steroid hormone receptor expression and mammary growth were also evaluated. On PND30, the exposure to 64BPA and 250BPA induced a delay in male MG development, evidenced by reduced ductal growth, decreased number of terminal structures and lower expression of androgen receptor (AR). In contrast, female mammary ductal growth was altered only by 250BPA. Regardless of the administration route and length of the exposure period, BPA induced a delay in MG development and modified AR expression in prepubertal male rats.

1. Introduction

Numerous synthetic compounds have the potential to alter developmental processes in animals and humans through their ability to affect the endocrine system. In rats, exposure to certain endocrine disrupting chemicals (EDCs) has been shown to alter the development and differentiation of the mammary gland [1–8]. These changes were seen when rats were prenatally, postnatally or perinatally (*i.e.* both pre- and postnatally) exposed, showing that the mammary glands are sensitive to EDCs during a wide time span of the development of the animal. Among EDCs, Bisphenol A (BPA), a monomer used in the manufacture of plastics, has received increased attention because of its widespread applications [9]. In addition, due to its environmental ubiquity, organisms may be exposed to BPA either chronically or during sensitive life stages. The estrogenic properties of BPA were first described in 1937 [10]. Since then, its hormonal activity has been confirmed and its adverse effects on hormone-dependent tissues demonstrated [9]. Moreover, several works have indicated that BPA may act as an androgen receptor antagonist [11,12] and that environmental chemicals are involved in the increasing occurrence of male reproductive dysfunction [13–15]. Strong evidence from animal studies supports the link between *in utero* exposure to EDCs and abnormal development of the male reproductive tract [16]. Recent studies have provided direct evidence that, despite differences in metabolism, the pharmacokinetics of BPA is extraordinarily similar in rodents, nonhuman primates and humans [17]. Given that human exposure to BPA is nearly continuous and likely occurs by both oral and non-oral routes, several routes of exposure should be used to analyze the effects of BPA on target organs [18].

Altered mammary gland maturation rates, delayed lumen formation, enhanced ductal growth and responsiveness to secondary estrogenic exposure, and increased susceptibility to carcinogenesis have been observed in BPA-treated female animals [19–22]. Although mammary epithelial growth also occurs in male rats and men [23], few laboratories have studied the effects of EDCs in male mammary glands [8,24–26]. Besides, a recent report has established that the rudimentary mammary gland of CD-1 male mice

Abbreviations: Alv, alveoli; AR, androgen receptor; BPA, Bisphenol A; BrdU, bromodeoxyuridine; DMSO, dimethylsulfoxide; EDCs, endocrine-disrupting chemicals; ER, estrogen receptor; F0, dams directly exposed to xenoestrogens; F1, F0 offspring; GD, gestational day; LN, lymph node; MG, mammary gland; N, nipple; OECD, Organization for Economic Cooperation and Development; PND, postnatal day; PR, progesterone receptor; sc, subcutaneously; TEBs, terminal end buds; TDs, terminal ducts; Vv, volume density.

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is sensitive to perinatal BPA exposure [27]. While some EDCs show earlier effects on male than in female mammary gland development [8], others lead to developmental changes only in male mammary glands [24]. Taking into consideration that the incidence of male breast cancer has increased over the last decades [28–30], changes in male mammary gland development as consequence of exposure to EDCs should be evaluated. Furthermore, effects of EDCs on male mammary gland development may serve as suitable markers of endocrine disruption. In this regard, evaluation of the male, but not the female, rat mammary gland is used by the Organization for Economic Cooperation and Development (OECD) as an endpoint in its guidelines for subchronic oral toxicity testing [23,31]. Moreover, the male rat mammary gland appears to be among the most sensitive endpoints evaluated when methoxychlor [32] or ethinylestradiol [33] are tested following the OECD guidelines. Therefore, the aim of this study was to evaluate the development of the male mammary gland in prepubertal offspring exposed to BPA prenatally (gestation) or perinatally (gestation and lactation) using the subcutaneous and the oral exposure route, respectively. In addition, to study possible gender specific differences in sensitivity to BPA, steroid hormone receptor expression, proliferative activity and mammary gland morphology were evaluated in samples from male and female offspring on postnatal day (PND) 30. This particular PND was chosen taking into consideration our previous data on the effects of prenatal BPA exposure on development and gene expression of the female rat mammary gland [5,20].

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. Animals were treated humanely and with regard for alleviation of suffering. Sexually mature female rats (90 days old) of a Wistar-derived strain bred at the Department of Human Physiology (School of Biochemistry and Biological Sciences, Universidad Nacional del Litoral, Santa Fe, Argentina) were used. Animals were maintained in a controlled environment ($22 \pm 2\,^{\circ}C$; 14 h of light from 06:00 h to 20:00 h) and had free access to pellet laboratory chow (16-014007 Rat-Mouse diet, Nutrición Animal, Santa Fe, Argentina). The concentration of phytoestrogens in the diet was not evaluated; however, because the food intake of control and xenoestrogen-treated rats was equivalent (data not shown), we assumed that animals of both groups were exposed to the same levels of phytoestrogens (see Kass et al. [7] for more information regarding food composition). To minimize exposure to other EDCs, rats were housed in stainless steel cages with sterile pine wood shavings as bedding and glass bottles with rubber stoppers to supply drinking water and oral treatments.

2.2. Experimental procedures

Females in proestrus were caged overnight with males of proven fertility. The day that sperm was found in the vagina was designated as gestational day 1 (GD1); the mating pair was separated and the pregnant female rats were housed individually in stainless steel cages. On GD9, corresponding to the beginning of organogenesis in the rat fetus, pregnant females (F0) were assigned to two different Experiments. In Experiment 1, BPA doses were chosen in a range that included the safe dose (50 μg/kg bw/day) established by the US Environmental Protection Agency [34]. For this experiment, the rats were exposed *in utero* (prenatal exposure) to 25 or 250 μg BPA/kg bw/day. In Experiment 2, a perinatal exposure to BPA (gestation plus lactation) was used. For this experiment, the animals were exposed only to the safe BPA dose (50 μg BPA/kg bw/day).

2.2.1. Experiment 1: prenatal exposure to BPA

On GD9, randomly chosen F0 rats were weighed and implanted subcutaneously (sc) with a miniature osmotic pump (model 1002; Alza Corp., Palo Alto, CA, USA); 10 dams were included in each group. Pumps were prepared to deliver either 50% dimethylsulfoxide (Control; DMSO, Sigma–Aldrich, Buenos Aires, Argentina), 25 μg BPA/kg bw/day (25BPA; 99% purity Sigma–Aldrich), or 250 μg BPA/kg bw/day (250BPA). BPA and DMSO were released continuously at a rate of 0.25 μl/h; from GD9 to parturition on GD23.

2.2.2. Experiment 2: perinatal exposure to BPA

On GD9, randomly chosen F0 animals were weighed and the oral treatment with BPA was started [7]. The BPA dose was calculated on the basis of the F0 dams' average body weight (250 g) and water consumption during pregnancy and lactation (50 ml). The stock solution of BPA (25 g/l, Sigma–Aldrich) was dissolved in ethanol (Merck Chemistry Argentina, Buenos Aires, Argentina). Immediately before administration, the BPA stock solution was diluted in distilled water (1:100) to a working solution of 0.25 g/l (2.5×10^5 μg/l) and then diluted again in BPA-free tap water (1:1000) to achieve the final concentration of 250 μg BPA/l corresponding to a dose of 50 μg BPA/kg bw/day. The BPA concentration in tap water samples was tested by gas chromatography–mass spectrometry using a very sensitive assay previously described [35]. All the tap water sample values were below the assay limit of detection (LOD = 38 ng/l).

The control group was exposed to a vehicle solution (0.001% of ethanol in water). Both treatments were administered until weaning on PND21. The glass bottles were rinsed and refilled twice a week with freshly prepared solutions in BPA-free tap water [35]. Ten dams were included in each group. To determine the actual dose administered the individual body weights and water consumption of F0 dams were recorded twice a week throughout treatment. After quantifying the daily water consumption and the body weight of the F0 dams throughout the experiment, the dose administered to the BPA group was, on average, 64 μg BPA/kg bw/day (64BPA, Supplementary data, Table S1).

A schematic representation of both Experiment 1 and Experiment 2 is shown in Fig. 1.

2.2.3. Time points analyzed

On PND1, F1 pups from Experiment 1 and Experiment 2 were weighed and sexed according to the anogenital distance. Litters of eight pups (preferably four males and four females) were left with F0 lactating mothers until weaning on PND21. The effects of BPA exposure were evaluated in male offspring on PND5, PND15 and PND30 and, in female offspring on PND30. One F1 pup per litter from each treatment group was assigned to each of the time points analyzed; the remaining male and female pups were assigned to other experiments. Samples from 10 F1 pups per group were analyzed in each time point. The onset of puberty was assessed by determining the day of vaginal opening in females and preputial separation in males.

Female rats from our colony reach puberty at around 39 days of age and prenatal exposure to 25BPA advances this event [20]. To avoid the influence of pubertal hormones in mammary gland development, prepubertal time points were chosen for this study.

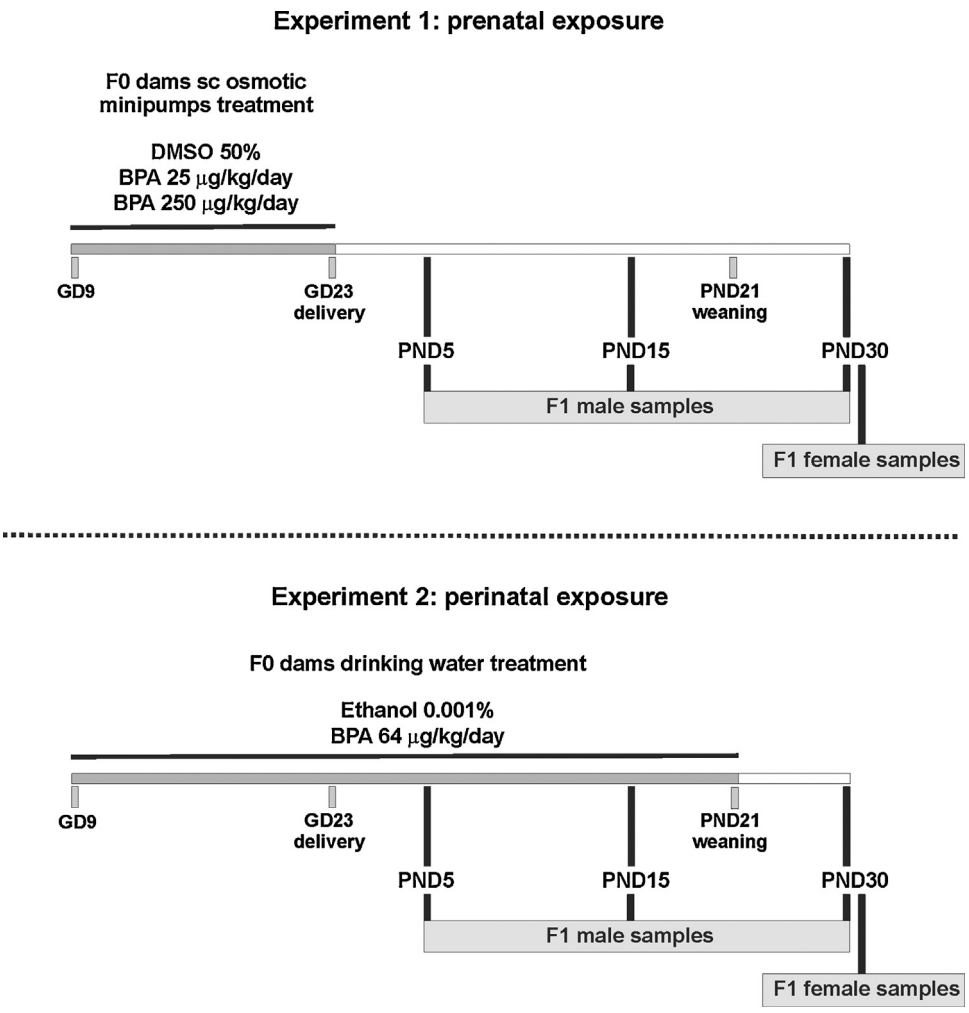

Fig. 1. Schematic representation of the experimental protocols used to study the effects of prenatal (upper panel) and perinatal (lower panel) BPA exposure on the mammary gland of male and female F1 offspring. In Experiment 1, BPA was delivered to F0 dams *via* subcutaneous (sc) osmotic minipumps whereas in Experiment 2, BPA was placed in the drinking water of the F0 dams. While male mammary gland samples were obtained on postnatal days (PND) 5, 15 and 30, female mammary gland samples were only obtained on PND30. GD: gestation day.

2.3. Tissue samples

On PND5 and PND15, both abdominal-inguinal male mammary gland chains were dissected out and processed for whole mounts [20]. On PND30, 2 h before necropsy, female and male rats were injected intraperitoneally (ip) with bromodeoxyuridine (BrdU; 6 mg/100 g bw; Sigma–Aldrich) to determine the proliferative index in the mammary gland. At necropsy, one abdominal-inguinal mammary gland chain was randomly chosen to be processed for whole mount, and the contralateral gland to be fixed in 10% (v/v) buffered formalin for 6 h at room temperature and embedded in paraffin [20].

2.4. Whole mount evaluation

Images of mammary gland whole mounts from PND5 were recorded using a Spot Insight V3.5 color video camera attached to an Olympus BH2 microscope (Olympus Optical Co. Ltd., Tokyo, Japan), whereas those from PND15 and PND30 were recorded using a Leica GZ6 stereomicroscope (Leica Inc., Buffalo, NY, USA). All images were analyzed with the Image Pro-Plus 4.1.0.1® system (Media Cybernetics, Silver Spring, MA, USA). The unequal illumination (shading correction) was corrected and the measurement system was calibrated with a reference slide. All evaluations were carried out blinded to treatment group on mammary gland number 4. The density of the mammary gland epithelium was measured by applying an orthogonal line grid mask on the whole mount image. On PND5 and PND15, the mammary gland density was measured in the whole gland (Fig. S1A, Supplementary data), whereas on PND30, the mammary gland density was measured in the area comprised by the outer 5 mm margin of the gland (Fig. S1B, Supplementary data) [36]. The ratio (Vv) between the intersections hitting the mammary parenchyma (Pp) and the total number of intersections occurring in the mammary gland (Tp, parenchyma and fat pad) was calculated as follows: $Vv \times 1000 = (Pp/Tp) \times 1000$. To assess the longitudinal growth of the mammary gland, two different measurements were recorded according with animal age. On PND5, the distance between the nipple and the mammary gland growing edge was measured (Fig. S1C), whereas on PND15 and PND30, the ductal extension was established by measuring the growth beyond the lymph node (LN). To this aim, we measured the distance between two parallel lines drawn on the mammary whole mount images, one tangential to the upper edge of the LN and the other at the end of the growing edge of the mammary gland (Fig. S1D). The number of terminal end buds (TEBs), which are teardrop-shaped ductal end structures that measure 100 μm or greater in diameter, were counted in all samples from PND15 and PND30 (Fig. S1E).

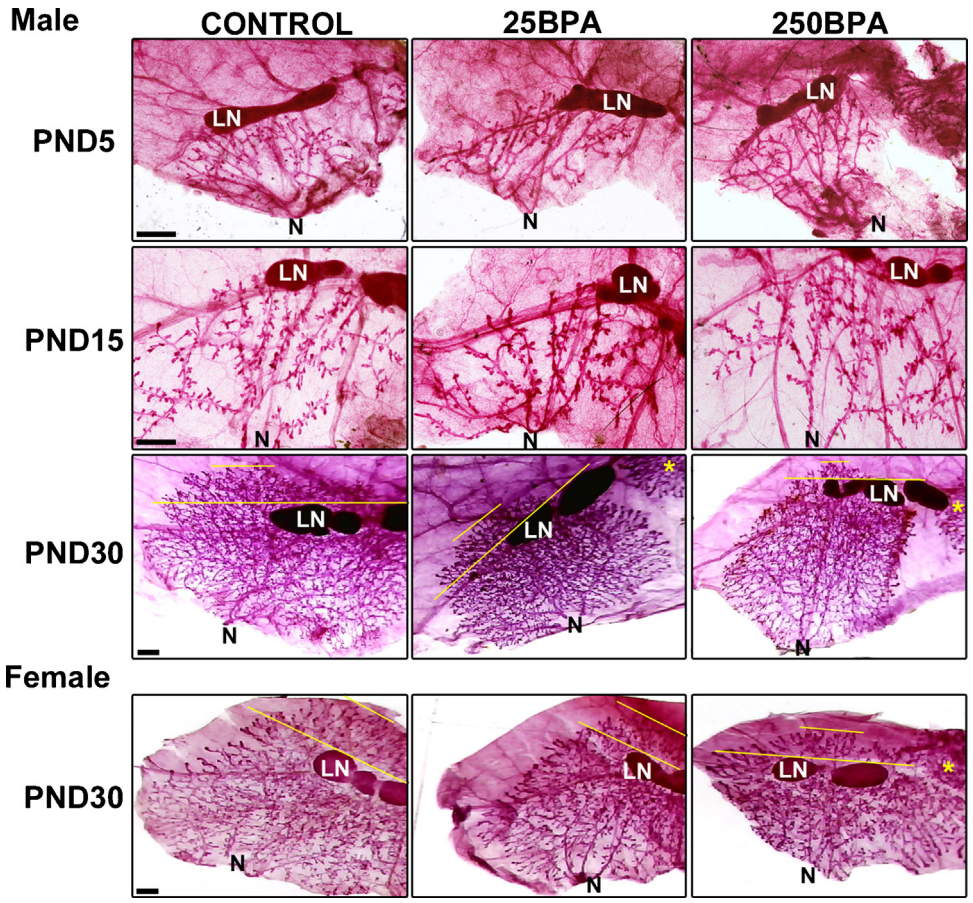

Fig. 2. Whole mounts images of male and female mammary glands of prenatally BPA-exposed offspring. The mammary whole mounts illustrate the mammary gland morphology on postnatal days (PND) 5, 15 and 30. The distance between the two parallel lines drawn on each PND30 image represents the longitudinal growth of the mammary gland on this day. N: nipple, LN: lymph node, asterisk (*): mammary gland number 5. Scale bar is 1000 μm in all images.

2.5. Immunohistochemistry

Consecutive 5 μm sections were immunostained to evaluate the proliferation index (BrdU-positive cells) and the expression of androgen receptor (AR), progesterone receptor (PR) and estrogen receptor alpha (ERα). Sections from two different depths were used to evaluate the expression of each protein. Immunoperoxidase staining was performed as previously described [20,37]. Sections were incubated overnight at 4 °C with primary antibodies against BrdU (clone 85-2C8, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK), AR (N-20, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), PR (PR A/B isoforms, Dako Corp., Carpinteria, CA, USA) and ERα (clone 6-F11, Novocastra Laboratories Ltd.). Anti-rabbit or anti-mouse (Laboratorio de Endocrinologia y Tumores Hormonodependientes, Santa Fe, Argentina) secondary antibodies (biotin-conjugated) were used [7], and reactions were developed using an avidin–biotin peroxidase method with diaminobenzidine (Sigma–Aldrich) as a chromogen substrate. Each run included negative controls in which the primary antibody was replaced with non-immune mouse or rabbit serum (Sigma–Aldrich). Two mammary tissue sections per animal were evaluated and at least 2000 cells per tissue section were analyzed. The percentage of BrdU, AR, PR and ERα expression in the epithelial cells of TEBs and terminal ducts (TDs) as well as ducts and alveoli (Alv) were quantified in each tissue section.

2.6. Statistical analysis

All data are expressed as the mean ± SEM. In Experiment 1 (prenatal exposure to BPA), Kruskal Wallis analysis was performed to obtain the overall significance, and Dunns *post hoc* test was used to compare each experimental group with the control group. In Experiment 2 (perinatal exposure to BPA), Mann–Whitney *U*-test was used to establish differences between the experimental and control groups. In both experiments, Wilcoxon matched-pairs signed rank test was used to analyze whether the mammary gland density was different between PND5 and PND30 in each group and, Mann–Whitney *U*-test was used to establish differences in AR expression between female and male animals in each group. In all cases, values with $p < 0.05$ were accepted as significant.

3. Results

Exposure of pregnant dams to BPA through the osmotic minipumps or in the drinking water neither affected gestation length nor produced signs of embryotoxicity (*i.e.*, the number of live-born pups per litter was similar among groups and no gross malformations were observed in F1 pups at delivery or weaning) (Supplementary data, Table S2). Besides, no abnormal maternal or nursing behaviors or changes in body weight gain in the mothers were observed (data not shown). In addition, neither male nor female pups reached puberty by PND30.

3.1. Experiment 1: prenatal exposure to BPA

3.1.1. Whole-mount analysis

The temporal pattern of mammary gland growth in the prepubertal control male is shown in the upper panel of Fig. 2. On PND30,

Table 1
Experiment 1 (prenatal exposure to BPA) whole mount analysis.

		Control	25BPA	250BPA
Male	**PND5**			
	MG density (Vv × 1000)	371.6 ± 4.7	370.8 ± 10.3	373.8 ± 18.3
	Distance between the Nipple and MG final edge (μm)	1764 ± 93	2140 ± 141	4022 ± 105*
	PND15			
	MG density (Vv × 1000)	342.3 ± 52.8	330.2 ± 11.4	300.0 ± 15.3
	Distance between the LN and MG final edge (μm)	13.8#	0	0
	TEBs (No.)	2.0 ± 0.6	2.2 ± 0.3	1.5 ± 0.9
	PND30			
	MG density (Vv × 1000)	541.7 ± 18.8	549.9 ± 31.6	465 ± 31.7
	Distance between the LN and MG final edge (μm)	1998 ± 357	1534 ± 240	629 ± 153*
	TEBs (No.)	31.2 ± 10.0	42.2 ± 8.3	20.38 ± 5.9
Female	**PND30**			
	MG density (Vv × 1000)	428.9 ± 175.1	540.1 ± 98.2	383.4 ± 95.6
	Distance between the LN and MG final edge (μm)	3642 ± 416	2240 ± 420	1400 ± 203*
	TEBs (No.)	63.7 ± 7.2	74.3 ± 8.6	49.2 ± 7.9

Values are expressed as mean ± SEM of 10 pups/group/day.
TEBs: terminal end buds.
* $p < 0.05$ between 250BPA and Control animals (Kruskal Wallis test).
# On PND 15, only in the control group, 1 out of 10 male pups showed a mammary gland (MG) that had grown beyond the lymph node (LN); in the other animals, the MG final edge reached the LN but did not extend beyond it.

a well-developed mammary gland tree is observed in both male and female rats.

Mammary gland whole mounts from offspring prenatally exposed to BPA were evaluated to assess ductal growth and gland density and differentiation (Table 1). On PND5, the ductal growth of the mammary gland tree was accelerated in 250BPA-exposed male rats compared with control animals as evidenced by a greater distance between the nipple and the mammary gland growing edge ($p < 0.05$). In contrast, on PND15, no differences between experimental groups were observed and ducts from all samples reached the LN, but did not extend beyond it except in 1 out of 10 control male animals. On PND30, the mammary gland from 250BPA-exposed males showed a significant delay in ductal growth, being the distance between the LN and the mammary gland edge at least three times shorter than in control males ($p < 0.05$). Although male mammary gland density was similar between groups of the same age, when temporal changes (from PND5 to PND30) in mammary gland density were compared, the significant increase ($p < 0.05$) exhibited in controls (541.7 ± 18.8 on PND30 *vs.* 371.6 ± 4.7 on PND5) and 25BPA-exposed male samples (549.9 ± 31.6 on PND30 *vs.* 370.8 ± 10.3 on PND5) was absent in samples from 250BPA-exposed males (465 ± 31.7 on PND30 *vs.* 373.8 ± 18.3 on PND5). In addition, on PND30, 250BPA-exposed male pups had fewer TEBs than controls, although no significant differences were found between them (Table 1 and Fig. 2).

The female mammary gland was only evaluated on PND 30 (Table 1 and Fig. 2). In the female pups, prenatal BPA exposure induced the same alterations in the mammary gland as in the male animals at the same age. The ductal growth of 250BPA-exposed female pups was delayed compared with control animals while the mammary gland density and the number of TEBs remained similar among groups. Although the mammary gland density was similar in male *vs.* female samples, the mammary gland ductal extension was greater in females than in males (Table 1, Fig. 2). In addition, by PND30, mammary glands number 4 and 5 grew together in female rats and not nearly to that extent in males (Fig. 2, the asterisks indicate mammary gland number 5).

3.1.2. Proliferation and steroid hormone receptor expression in the mammary gland

On PND30, samples from both male and female pups were analyzed to detect BPA-induced alterations in epithelial cell proliferation and/or expression of ERα, PR and AR. In samples from male offspring, the proliferation index (BrdU-positive cells) and ERα expression were similar between the different experimental groups (Table 2). The epithelial cells of the male mammary gland did not express PR on PND30 in any of the groups studied (Table 2). AR expression was found in ducts and terminal structures (Fig. 3). Although both doses of BPA had a lower total expression of AR than the control group, 250BPA-exposed males reached a significant decrease ($p < 0.05$). The lower levels of AR in 250BPA-exposed males were mainly due to a decreased ductal and alveolar epithelial cell expression (Fig. 3).

On PND30, proliferation indexes and the expressions of ERα, PR and AR in samples from female offspring were unaltered by BPA exposure (Table 2).

The proliferation index and ERα expression were similar between female and male animals regardless of the experimental group. In contrast, AR expression was higher in male rats than in female rats in the control (20.7 ± 4.0 *vs.* 10.3 ± 0.3, $p < 0.05$) and 25BPA groups (15.0 ± 1.6 *vs.* 7.8 ± 0.9, $p < 0.05$), whereas AR expression was similar between 250BPA-exposed males and females (12.2 ± 1.6 *vs.* 9.3 ± 0.5, $p > 0.05$).

3.2. Experiment 2: perinatal BPA exposure

3.2.1. Whole mount analysis

Representative images of mammary whole mounts of male and female pups perinatally exposed to BPA are shown in Fig. 4.

Mammary gland density in male pups was similar between 64BPA-exposed animals and controls when animals of the same age were compared (Table 3). While ductal growth of 64BPA-exposed males was similar to controls on PND5 and PND15, it was decreased on PND30 ($p < 0.05$; Table 3). The lower distance between the LN and the mammary gland edge shown by 64BPA-exposed males could be a consequence of the fewer TEBs shown on PND15 and PND30 compared to controls ($p < 0.05$, Table 3).

The whole mounts of female pups perinatally exposed to BPA were similar to those of the control group on PND30. Besides, regardless of the experimental groups, the overall development of the female mammary gland was greater than that of the male one (Table 3 and Fig. 4).

3.2.2. Proliferation and steroid hormone receptor expression in the mammary gland

On PND30, the male mammary gland showed no expression of PR. Besides, the perinatal exposure to BPA did not modify the proliferation index or ERα expression in the mammary

Table 2
Proliferation index and steroid receptor expression in male and female offspring prenatally exposed to BPA.

		Total			Ducts + Alv			TEBs + TDs		
	Biomarker[a]	Control	25BPA	250BPA	Control	25BPA	250BPA	Control	25BPA	250BPA
Male	BrdU	11.9 ± 1.5	10.3 ± 1.1	13.8 ± 1.1	5.7 ± 1.2	4.0 ± 0.4	6.4 ± 0.7	26.3 ± 2.0	24.6 ± 1.2	24.1 ± 2.0
	ERα	36.7 ± 0.7	35.0 ± 1.0	37.3 ± 0.5	37.1 ± 0.5	35.7 ± 1.3	37.4 ± 0.8	38.2 ± 3.3	32.4 ± 1.4	35.4 ± 1.2
	PR	ND	ND	ND	ND	ND	ND	ND	ND	ND
	AR	20.7 ± 4.0	15.0 ± 1.6	12.2 ± 1.6*	23.7 ± 4.6	16.0 ± 1.3	13.1 ± 1.8*	17.7 ± 4.2	20.3 ± 4.0	15.4 ± 1.5
Female	BrdU	13.4 ± 0.5	11.8 ± 1.3	11.5 ± 1.4	5.1 ± 0.2	4.2 ± 0.6	3.7 ± 0.5	25.6 ± 1.9	24.4 ± 1.7	23.9 ± 1.7
	ERα	35.8 ± 0.9	41.3 ± 5.7	39.5 ± 2.0	38.3 ± 1.6	40.4 ± 4.5	40.4 ± 1.7	30.9 ± 2.3	42.3 ± 7.2	38.6 ± 2.5
	PR	13.9 ± 3.1	9.7 ± 0.7	14.0 ± 2.6	13.8 ± 1.1	11.5 ± 1.2	14.9 ± 2.7	9.7 ± 0.7	8.9 ± 2.2	12.9 ± 2.8
	AR	10.3 ± 0.3	7.8 ± 0.9	9.3 ± 0.5	11.4 ± 0.4	10.2 ± 0.1	9.4 ± 0.9	8.8 ± 1.0	6.1 ± 1.5	8.3 ± 1.2

[a] All biomarkers were evaluated on post-natal day 30 (PND30). Results are expressed as percentage of positive epithelial cells.
* $p < 0.05$ between 250BPA-exposed animals and Control males (Kruskal Wallis test).PR expression was not detected (ND) in the male mammary gland on PND30.
Percentage values are expressed as mean ± SEM of 10 pups/group.
Alv: alveoli; TEBs: terminal end buds; TDs: terminal ducts.

epithelial cells (Table 4). As shown in Experiment 1, exposure to BPA significantly reduced AR expression in male samples. All the mammary gland structures had lower AR expression in 64BPA-exposed males ($p < 0.05$, Fig. 5). On the other hand, in the female offspring, the proliferation index and the expressions of ERα, PR and AR were similar between groups on PND30 (Table 4). There were no differences between males and females in proliferation and ERα expression; however, control males had higher AR expression than control females (20.5 ± 2.2 *vs.* 10.4 ± 0.8, $p < 0.05$). This difference between male and female AR expression was not observed in 64BPA-exposed animals (13.2 ± 1.6 *vs.* 9.8 ± 0.9, $p > 0.05$).

4. Discussion

Endocrine disruptors can have detrimental effects during specific stages of mammary gland development and no discernible effect during other life stages. Exposure during early development may have little observable effect until reproductive issues arise later in life, making it difficult to establish a cause–effect relationship between currently observed defects and previous exposures [12]. In both experiments conducted in the present work, significant differences in male mammary gland development were noticed on PND30, whereas only changes in ductal growth were evident on PND5 in the 250BPA group. Exposure to BPA induced a delayed mammary development on PND30 in male pups in both experiments, evidenced by reduced ductal growth and a decreased number of TEBs, together with decreased AR expression. A route dependency in the bioavailability and metabolism of BPA could be an important factor in determining its biologic activity. Pottenger et al. [38] have demonstrated that the relative bioavailability of BPA following oral administration is markedly lower than that following subcutaneous administration. In the male mammary gland, similar effects were observed on ductal growth and sex steroid receptor expression, regardless of the administration route and exposure period, implying that the parameters evaluated were not influenced by the differences in BPA bioavailability between experiments. In the female mammary gland, prenatal exposure to BPA (25BPA and 250BPA) induced morphological changes similar to those observed in males. On the other hand, perinatal exposure to 64BPA induced changes in the male mammary gland (*i.e.*: decreased ductal extension and fewer numbers of TEBs) that were not observed in the female mammary gland. In addition, in the female samples, the proliferation index and expression of the steroid hormone receptors were similar between groups. Since, neither the male nor the female pups reached puberty at PND30, it could be assumed that the morphological changes and/or protein expression observed in both experiments are not related to the hormonal changes associated with puberty.

Mammary epithelial growth occurs both in men and male rodents [23], and while male mice do not normally possess nipples, some mouse strains can have a rudimentary mammary gland tree [27]. The male rats of our Wistar-derived strain have a well-developed gland that resembles the female one. Although a sexual dimorphism in terms of mammary growth is insinuated on PND30,

Table 3
Experiment 2 (perinatal exposure to BPA) whole mount analysis.

		Control	64BPA
Male	**PND5**		
	MG density (Vv × 1000)	368.9 ± 11.6	379.4 ± 15.1
	Distance between the Nipple and MG final edge (μm)	1794 ± 167	1658 ± 192
	PND15		
	MG density (Vv × 1000)	401.2 ± 13.2	325.8 ± 9.1
	Distance between the LN and MG final edge (μm)#	0	0
	TEBs (No.)	2.6 ± 0.6	1.1 ± 0.2*
	PND30		
	MG density (Vv × 1000)	476.0 ± 12.0	530.2 ± 16.5
	Distance between the LN and MG final edge (μm)	1709 ± 180	1161 ± 175*
	TEBs (No.)	37.2 ± 3.1	18.6 ± 2.9*
Female	**PND30**		
	MG density (Vv × 1000)	522.1 ± 15.4	477 ± 18.7
	Distance between the LN and MG final edge (μm)	3570 ± 319.3	4207 ± 381
	TEBs (No.)	62.1 ± 3.7	65.1 ± 4.6

Values are expressed as mean ± SEM of 10 pups/group/day.
TEBs: terminal end buds.
* $p < 0.05$ between 64BPA and Control males (Mann–Whitney *U*-test).
# In all samples of PND15, the mammary gland (MG) final edge reached the lymph node (LN) but did not extend beyond it.

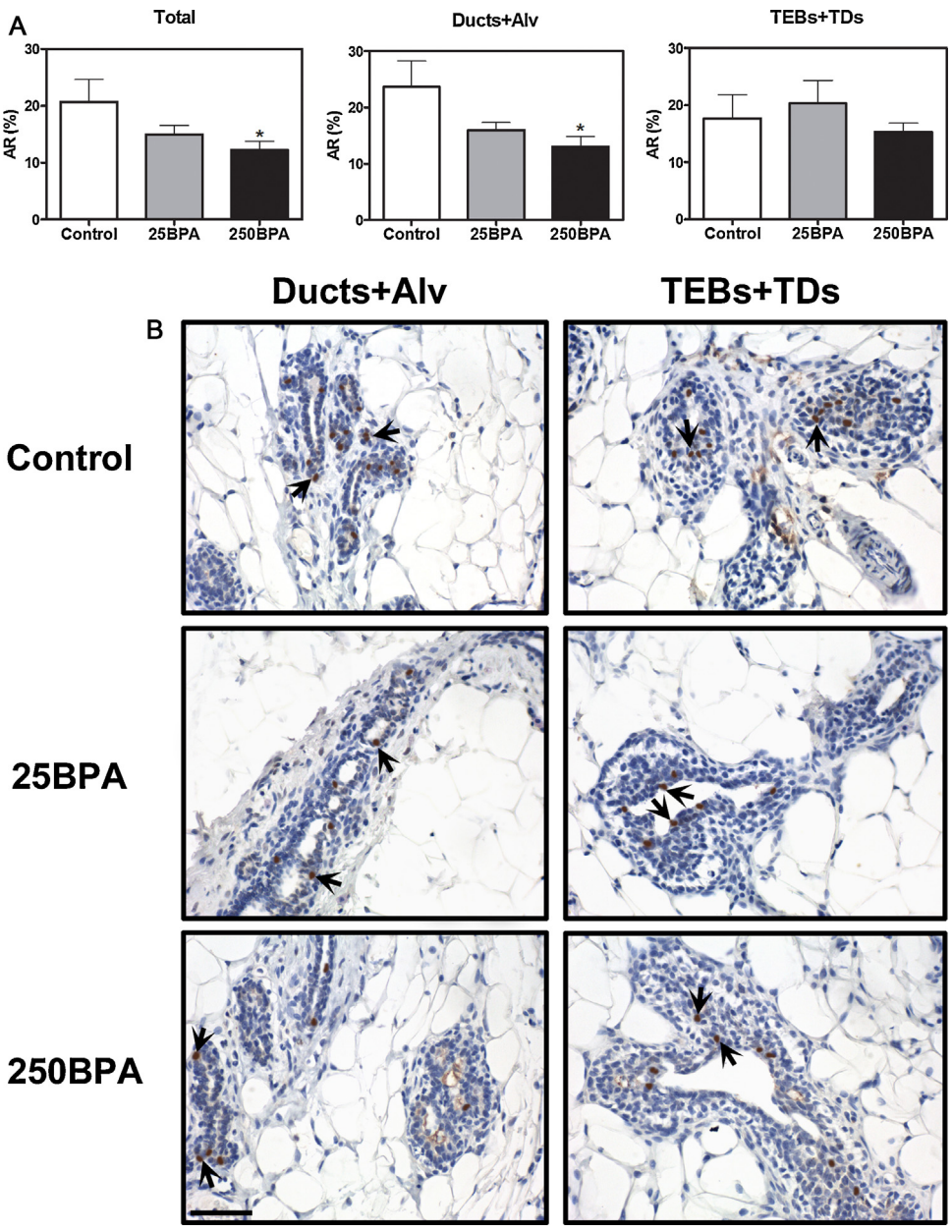

Fig. 3. AR expression in the mammary gland of prenatally BPA-exposed males on PND30. (A) Percentage of AR expression in the whole gland (total), ducts and alveoli (ducts + Alv) or in the terminal end buds and terminal ducts (TEBs + TDs). In 250BPA-exposed male offspring, the expression of AR in the mammary gland was decreased mainly in the epithelial cells of the ducts and Alv (*$p < 0.05$ Kruskal Wallis, followed by Dunns post-test). Bars represent the mean value ± SEM of 10 pups per experimental group. (B) Representative images of AR expression in the mammary gland of prenatally BPA-exposed male offspring on PND30. AR was expressed in the epithelial cells of all mammary structures, as shown by the arrows. Only the animals exposed to 250BPA expressed a decreased amount of AR significantly different from that of control animals. Scale bar = 50 μm.

the overall mammary gland tree of male and female prepubertal animals is similar. In some rat strains, it has been shown that the female and male mammary gland of adult rats is very different. The virgin female rat mammary gland is described as a tubuloalveolar structure, whereas the male gland is characterized by a predominance of lobulaveolar development [39,40]. In the current study, only the prepubertal period was analyzed and, accordingly to the age of the animal, the same epithelial structures (*i.e.*: ducts, Alv, TEBs, and TDs) are present in both female and male mammary glands of Wistar derived rats. However, a sexually dimorphic AR and PR expression pattern is observed in control animals. In fact, the male mammary gland shows higher AR expression than the female while PR was absent in male and present in female samples.

In the present work, different administration routes, doses and exposure periods were assessed to determine whether BPA modifies the development of the prepubertal male rat mammary gland. On PND30, male rats exposed prenatally to 250BPA and perinatally to 64BPA showed delayed mammary gland development, evidenced by reduced ductal growth and a decreased number of TEBs, whereas those exposed to 25BPA were indistinguishable from controls. Vandenberg et al. [27] showed that perinatal BPA exposure alters the male mouse mammary gland in adulthood and that the effects were age- and dose-specific. They found that the lowest doses were more effective in 3–4-month-old mice and that the highest doses were most effective in 12–15-month-old animals. The differences between our results and those obtained by Vanderberg et al. [27] could be attributed to the presence of a

Table 4
Proliferation index and steroid receptor expression in male and female offspring perinatally exposed to BPA.

		Total		Ducts + Alv		TEBs + TDs	
	Biomarker[a]	Control	64BPA	Control	64BPA	Control	64BPA
Male	BrdU	11.1 ± 1.6	11.5 ± 1.0	4.0 ± 0.7	5.8 ± 1.0	17.4 ± 2.5	18.1 ± 2.0
	ERα	34.5 ± 1.6	34.4 ± 1.4	37.1 ± 1.9	35.4 ± 1.4	31.0 ± 2.2	33.4 ± 1.8
	PR	ND	ND	ND	ND	ND	ND
	AR	20.5 ± 2.2	13.2 ± 1.6*	21.2 ± 2.6	15.0 ± 1.1*	19.3 ± 1.9	11.7 ± 1.9*
Female	BrdU	13.2 ± 0.5	12.9 ± 1.9	5.6 ± 0.6	4.5 ± 0.8	26.0 ± 2.1	22.7 ± 3.2
	ERα	41.4 ± 1.9	39.8 ± 2.8	42.4 ± 2.2	41.3 ± 4.1	39.9 ± 2.3	38.7 ± 2.3
	PR	18.6 ± 2.5	21.4 ± 1.0	19.0 ± 2.5	22.5 ± 0.8	18.1 ± 3.1	19.8 ± 1.3
	AR	10.4 ± 0.8	9.8 ± 0.9	11.0 ± 0.7	10.1 ± 1.0	9.6 ± 0.8	9.8 ± 1.0

[a] All biomarkers were evaluated on post-natal day 30 (PND30). Results are expressed as percentage of positive epithelial cells.
* $p < 0.05$ between 64BPA and Control males (Mann–Whitney *U*-test).
Percentage values are expressed as mean ± SEM of 10 pups/group.
PR expression was not detected (ND) in the male mammary gland on PND30.
Alv: alveoli; TEBs: terminal end buds; TDs: terminal ducts.

well-developed mammary tree in male rats compared to that in male mice, and/or to the different ages studied.

Estradiol, in combination with progesterone, is considered the main mitogen in the mammary gland during postnatal development [41]. However, the importance of the androgen signaling in the growth and development of the mammary gland is highlighted by the phenotype of prepubertal female AR-knockout mice, which exhibits a marked reduction in ductal extension, ductal branching, and epithelial cell proliferation [42]. In contrast, Peters et al. [43] observed an increase in cellular proliferation and branching in response to the AR inhibitor flutamide throughout the mammary gland of female postpubertal mice. In addition, the exposure to vinclozolin, a fungicide with antiandrogenic properties, has been shown to alter the peripubertal development of the female rat mammary gland [44,45]. These results suggest that endogenous androgen action in the breast is highly dependent on the stage of mammary gland growth [43] and the developmental period studied (pre- or postpuberty) [45]. The exposure to BPA during an early developmental stage of the mammary gland induced decreased AR expression in the parenchyma of male offspring on PND30. Besides, AR expression in male and female 250BPA- and 64BPA-exposed animals was similar. The decreased AR expression in males parallels a delayed mammary development, evidenced by reduced ductal growth and a decreased number of TEBs. These results are consistent with that reported by Yeh et al. [42] in female AR-knockout mice. It is interesting to point out that in male rats, BPA exposure not only changes AR levels in the mammary gland but also in other reproductive glands like the prostate [46,47]. We have previously demonstrated that prenatal exposure to BPA decreases AR expression in the prostate, while serum testosterone levels remain similar between control and BPA-exposed males and prostatic acid phosphatase expression decreases, suggesting alterations in prostatic functional activity [46,47]. Additional studies are needed to investigate whether BPA exposure turns on the latent ability of the well-developed male rat mammary gland to respond to a lactogenic stimulus that may produce milk [48] or predisposes the gland to the development of breast cancer. In this regard, it has been suggested that androgens can protect against the development of breast cancer through an inhibition of cell proliferation [49].

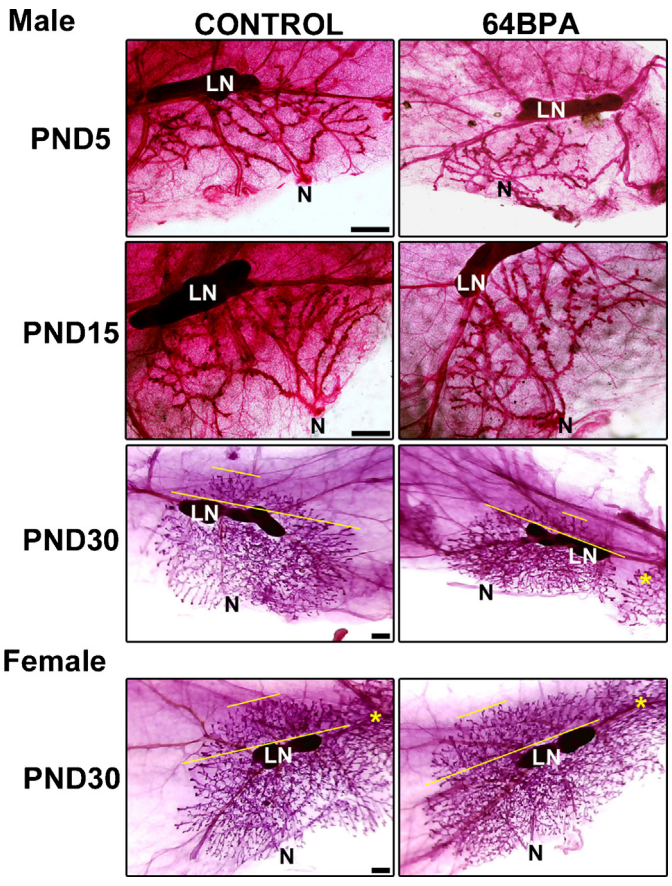

Fig. 4. Whole mount images of male and female mammary glands of perinatally BPA-exposed offspring. Representative images on postnatal days (PND) 5, 15 and 30 are shown. Mammary gland growth beyond the LN was quantified between the two parallel lines drawn on PND30 images. N: nipple, LN: lymph node, asterisk (*): mammary gland number 5. Scale bar is 1000 μm in all images.

Unlike the female mammary gland, on PND30, the male rat mammary gland did not express PR. BPA exposure neither induced PR expression in the male mammary gland nor modified AR expression in female samples. In contrast, both adult female [50] and prepubertal mice [19] exposed to BPA expressed higher levels of PR than control animals. It is noteworthy that the deregulation of PR expression in the mammary gland by BPA exposure appears to be species-specific as mice seem to be more sensitive than rats. In addition, BPA exposure did not affect proliferative activity in the epithelial compartment of either male or female rats on PND30. Moreover, ERα expression was similar between groups regardless of the gender of the animal. The results are consistent with that observed in BPA-exposed prepubertal female rodents on this particular postnatal day [19,20] and on 7–9 month-old male mice [27].

In conclusion, the male rat mammary gland is sensitive to endocrine disruptors, being the prepubertal stage a useful time point to test for potential endocrine disrupting effects of chemicals.

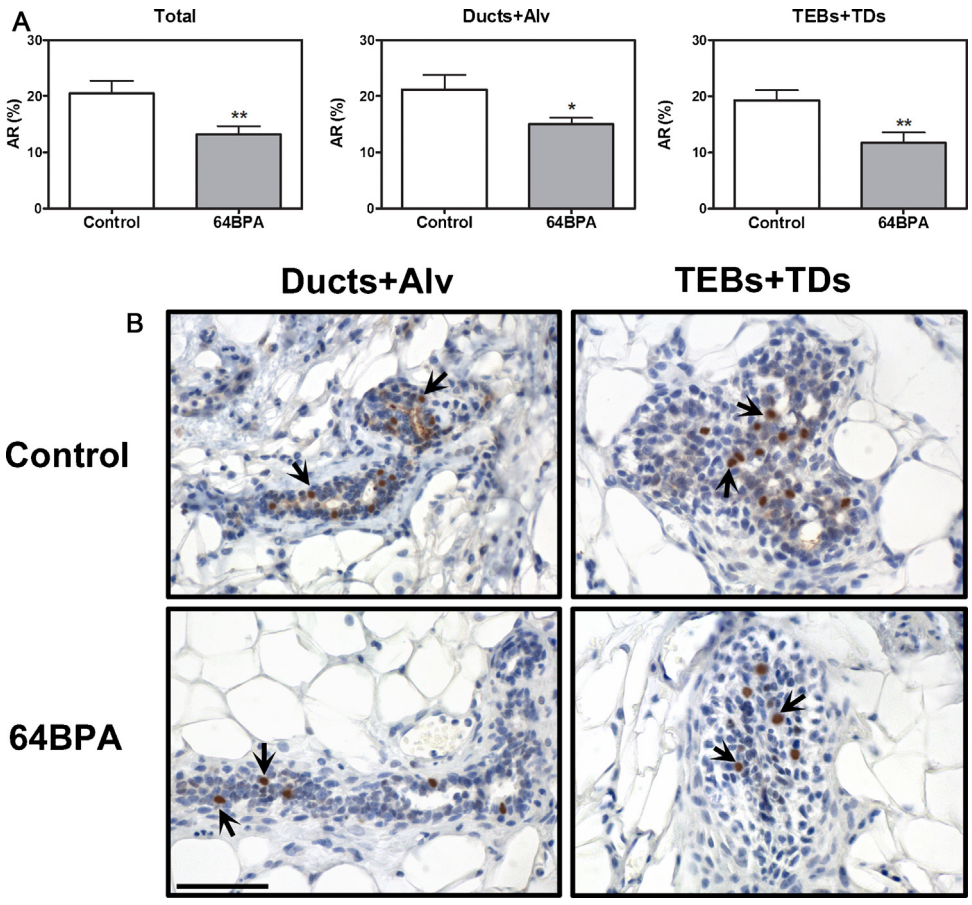

Fig. 5. AR expression in the mammary gland of perinatally BPA-exposed males on PND30. (A) Percentage of AR expression in the whole gland (total), ducts and alveoli (ducts + Alv) or in the terminal end buds and terminal ducts (TEBs + TDs). In every epithelial structure of the mammary gland, AR expression was consistently lower in BPA-exposed male pups than in control animals ($^{*}p < 0.05$ Mann–Whitney *U*-test). Bars represent the mean value ± SEM of 10 pups per experimental group. (B) Representative images of AR expression in the mammary glands of male pups on PND30 that were perinatally exposed to BPA. Epithelial cells of ducts, alveoli and terminal structures showed decreased AR expression after BPA exposure. The arrows indicate AR positive cells. Scale bar = 50 μm.

Our results show that, unlike that observed in females, BPA exposure not only induces a delay in male mammary gland development but also decreases AR expression. The results corroborate that the whole mount is an adequate tool to identify early effects on mammary gland development. Our findings highlight the importance of assessing different administration routes, doses and lengths of exposure to chemicals with endocrine activity since both the effects on the mammary gland and the moment at which this effect can be appreciable can be different.

Conflict of interest

The authors have nothing to disclose.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2014.02.001.

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