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Research Article

In Utero and Lactational Exposure to an Environmentally Relevant Mixture of Brominated Flame Retardants Induces a Premature Development of the Mammary Glands

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ABSTRACT

In utero and prepubertal development of the mammary glands occurs minimally in a hormone independent manner until puberty where maturation of the hypothalamic-pituitary-gonadal axis drives an extensive remodeling. Nevertheless, because the immature glands contain functional hormone receptors, they are especially vulnerable to the effects of endocrine disruptors, such as brominated flame retardants (BFRs). BFRs are widespread chemicals added to household objects to reduce their flammability, and to which humans are ubiquitously exposed. We previously reported that *in utero* and lactational exposure to BFRs resulted in an impaired mammary gland development in peripubertal animals. Here, we assessed whether BFR-induced disruption of mammary gland development could manifest earlier in life. Dams were exposed prior to mating until pups' weaning to a BFR mixture (0, 0.06, 20, or 60 mg/kg/day) formulated according to levels found in house dust. The mammary glands of female offspring were collected at weaning. Histo-morphological analyses showed that exposure to 0.06 mg/kg/day accelerates global epithelial development as demonstrated by a significant increase in total epithelial surface area, associated with a tendency to increase of the ductal area and thickness, and of lumen area. Significant increases of the Ki67 cell proliferation index and of the early apoptotic marker cleaved caspase-9 were also observed, as well as an upward trend in the number of thyroid hormone receptor α1 positive cells. These molecular, histologic, and morphometric changes are suggestive of accelerated pubertal development. Thus, our results suggest that exposure to an environmentally relevant mixture of BFRs induces precocious development of the mammary gland.

Key words: endocrine disruptors; brominated flame retardants; *in utero* and lactational exposure; mammary gland; precocious development; low-dose effects; proliferation; apoptosis; thyroid hormone receptor.

Brominated flame retardants (BFRs) are a family of chemicals broadly used in various consumer products such as plastics, electronics, textiles, and home furnishings to reduce their fire hazard (Alaee *et al.*, 2003). They are either bound to the matrix as reactive mixtures or dissolved in the polymer materials as additives (de Wit, 2002). Polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCDD) have been used extensively in North America (Allen *et al.*, 2008; Darnerud *et al.*, 2001; Stapleton *et al.*, 2008). As additive fire retardants, they easily migrate from products to the environment, resulting in ubiquitous contamination of homes and other indoor areas (Sjodin *et al.*, 2003). Due to their persistence, bioaccumulation and toxicity in animals and humans (Birnbaum and Staskal, 2004; Darnerud *et al.*, 2001), PBDE and HBCDD use, sale, offer for sale and import have been restricted in Europe and North America (Environment Canada, 2008; Environment and Climate Change Canada, 2011) and identified for global elimination by the Stockholm Convention. Despite the legislation, items containing these compounds are still present in homes and other indoor environments as well as in waste disposal, and thus remain sources of chronic environmental and human exposure (Abbasi *et al.*, 2015; Lucas *et al.*, 2018).

Exposure to PBDEs and HBCDD has been associated with a wide range of effects including endocrine disruption and reproductive/developmental alterations *in vitro* and *in vivo* (Ema *et al.*, 2008; Hallgren *et al.*, 2001; Lilienthal *et al.*, 2006; Szabo *et al.*, 2009; Talsness *et al.*, 2008; Tung *et al.*, 2016). In humans, non-occupationally related exposure occurs mostly through dust ingestion and consumption of contaminated food (Besis and Samara, 2012; Frederiksen *et al.*, 2009; Jones-Otazo *et al.*, 2005; Stapleton *et al.*, 2005; Wilford *et al.*, 2005). BFRs can be detected in serum, adipose tissue, placenta, umbilical cord, and breast milk (Chen *et al.*, 2018b; Gomara *et al.*, 2007; Mazdai *et al.*, 2003; Ryan and Rawn, 2014; Tang and Zhai, 2017; Toms *et al.*, 2009; Zhang *et al.*, 2017). Toddlers are the most exposed due to *in utero* and lactational exposure *via* their mother, in addition to their greater ingestion of dust given their closer proximity to ground and hand-to-mouth contacts (Ionas *et al.*, 2016; Linares *et al.*, 2015; Stapleton *et al.*, 2005; Zuurbier *et al.*, 2006).

The mammary gland develops through distinct stages regulated mainly by systemic hormones and local cues (Brisken and O'Malley, 2010; Macias and Hinck, 2012; Sternlicht *et al.*, 2006). *In utero* and early development is hormone independent (Brisken and O'Malley, 2010) and results in a gland composed of a rudimentary epithelial tree surrounded by a voluminous stroma in newborn rodents (Hovey *et al.*, 2002; Masso-Welch *et al.*, 2000). At the onset of puberty, systemic ovarian steroids induce exponential ductal growth and branching of the mammary glands as estrogen (ER) and progesterone receptors (PR) expression increases (Filgo *et al.*, 2016; Hovey *et al.*, 2002). This hormone stimulation promotes cell division and the formation of multilayered club-shaped terminal end buds (TEBs) at the end of the ductal trees (Paine and Lewis, 2017; Sternlicht, 2005). Thyroid hormone also contributes in regulating mammary growth (Neville *et al.*, 2002; Vonderhaar and Greco, 1979). TEBs are highly proliferative structures that drive epithelial elongation and ramification throughout the fat pad (Paine and Lewis, 2017; Sternlicht, 2006). As the ducts elongate and undergo side branching, apoptosis occurs in the stroma and in the center cells of the TEB to allow lumen formation (Humphreys *et al.*, 1996; Sreekumar *et al.*, 2017) and ductal invasion (Paine and Lewis, 2017). At adulthood, the ductal tree has reached the edge of the fat pad (Masso-Welch *et al.*, 2000). Milk-secreting structures named *alveoli* differentiate during pregnancy and lactation (Geddes, 2007; Neville *et al.*, 2002). At weaning, in the absence of suckling from the pups, the *alveoli* undergo apoptosis and the gland ultimately regresses to the pre-gestational stage (Hurley, 1989; Watson, 2006).

Given the major role of hormones in regulating mammary gland development, exposure to endocrine disruptors (EDs) can lead to mammary developmental abnormalities, altered lactation, and increased breast cancer risks (Fenton *et al.*, 2012; Macon and Fenton, 2013). The vulnerability of mammary gland development to the effects of EDs is higher during specific developmental stages (ie, embryonic life, puberty, and the pregnancy/lactation cycle) when the glands undergo extensive remodeling (Fenton, 2012; Macon and Fenton, 2013). Although the embryonic, neonatal, and prepubertal developmental stages occur independently of the regulation of systemic hormones, the early expression of functional receptors renders these immature glands also susceptible to EDs (Brown *et al.*, 1998; Brown and Lamartiniere, 1995; Fenton, 2006; Rudel *et al.*, 2011). Accordingly, in rodents, early exposure to EDs has been shown to impair timing of growth onset, TEB formation, *number* and persistence, and histological structure of the gland, in addition to increased susceptibility to carcinogens later in life (Macon and Fenton, 2013).

Previously we reported that gestational and lactational exposure to an environmentally relevant mixture of BFRs altered intercellular junctions and signaling in the lactating glands of dams (Dianati *et al.*, 2017). In addition, in the mammary glands of the offspring exposed *in utero* and through lactation to the same mixture, we observed a disruption of thyroid hormone receptor alpha1 (THRα1) homeostasis, of intercellular junctions and of the proliferation-apoptosis balance at PND 46 (Gouesse *et al.*, 2019). Hence, in the current study, we sought to assess whether this BFR-induced disruption of the mammary gland development was apparent earlier in life. Our findings highlight, for the first time that an *in utero* and lactational exposure to an environmentally relevant mixture of PBDEs and HBCDD results in a precocious mammary gland development in offspring before puberty (PND 21).

MATERIALS AND METHODS

Formulation of the BFR mixture. Formulation of the BFR mixture was described previously (Dianati *et al.*, 2017; Ernest *et al.*, 2012; Gouesse *et al.*, 2019). Briefly, three technical PBDE mixtures (DE-71, DE-79, and BDE 209) and one HBCDD mixture were combined to yield a ratio of PBDE congeners and HBCDD comparable to the median levels observed in Boston house dust (Allen *et al.*, 2008; Stapleton *et al.*, 2008). This BFR mixture was integrated into an isoflavone-free diet (Teklad Global 2019 diet; Harlan Laboratories, Madison, Wisconsin) with 4.3 g/kg corn oil. Diets were formulated to contain 0, 0.75, 250, or 750 mg of BFR mixture/kg and calculated to provide nominal doses of 0, 0.06, 20, and 60 mg/kg/day of body weight, respectively. The low dose was estimated to be a close approximation of maximum human exposure, based on a dust ingestion rate of 100 mg/day in children (16.5 kg body weight) and the scaling of dose from humans to rodents (1:6.9, human-to-rat body surface area ratio). Treatment with the BFR mixture did not alter dam food consumption or weight gain during the exposure period; analysis of the levels of PBDEs in various tissues revealed that BFR

ingestion achieved 74%–86% of the target doses for the three experimental conditions (Berger *et al.*, 2014; Ernest *et al.*, 2012; Poon *et al.*, 2014; Tung *et al.*, 2016).

Animals. All procedures and animal studies were done in accordance with the procedures and principles as provided by the Canadian Council on Animal Care and were reviewed and preapproved by the Health Canada Animal Care Committee (protocol no. 2012-015). Detailed treatment method and animal procedures were described previously (Tung *et al.*, 2016). In brief, virgin female Sprague Dawley rats were obtained from Charles River Laboratories (Charles River, St-Constant, QC, Canada). After one week of acclimatization to the control diet, female rats were randomly assigned to one of the four experimental groups and fed with a BFR supplemented diet for 2–4 weeks before mating. During this time, estrous cyclicity was evaluated by analyzing vaginal cytology. Females in proestrus were caged with proven breeder male Sprague Dawley rats (maintained on the control diet) overnight. After mating, females were returned to their assigned cages and provided with water and the appropriate dietary mixture *ad libitum*, during gestation and lactation. At postnatal day 4 (PND4; delivery = PND0, the 1st day pups were observed), litters were normalized to 8 pups (4 pups per sex if possible). Only 1 female pup from each litter was euthanized by exsanguination under isoflurane anesthesia at PND 21. The mammary glands were sampled and conserved separately depending on the downstream applications. Left inguinal mammary glands (pairs 4 and 5, respectively) were dissected, weighed, and immediately snap frozen before being stored at −80°C for subsequent Western blot analyses. Right inguinal mammary glands (pair 4) were immediately transferred onto slides, fixed in Carnoy's fixative (100% ethyl alcohol [EtOH], chloroform, glacial acetic acid; 6:3:1) and stained for whole-mount studies. Upper-thoracic and thoracic mammary glands (pairs 2 and 3, respectively) were excised, and either processed for paraffin embedding or embedded in Tissue-Tek O.C.T compound (VWR International, Ville Mont-Royal, QC, Canada) on dry ice and stored at −80°C, for subsequent histology and Masson's trichrome and immunofluorescence analyses.

Whole-mounts and carmine stain of mammary glands. Mammary glands were excised and processed for whole mount staining, as previously described (Plante *et al.*, 2011) with minor modifications. Briefly, the glands were first fixed for at least 2 days in Carnoy's solution (100% EtOH, chloroform, glacial acetic acid; 6:3:1) at room temperature, then washed in 70% EtOH for 1 h and rehydrated in water for 30 min. Then, the mammary glands were stained in carmine alum stain (2% carmine and 5% aluminum potassium sulfate in water) for a minimum of 2 days. The glands were progressively dehydrated through an EtOH series, cleared in xylene (minimum 2 days) and then mounted using Permount (Fisher Scientific, Burlington, Ontario, Canada). High-resolution images of mammary whole mounts were collected using a Zeiss SteREO Discovery.V20 microscope. Morphological features were quantified in images using ImageJ (ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, https://imagej.net/Downloads). Whole mounts from all animals ($n = 9$–14 per group) were analyzed.

Masson's trichrome stain. Mammary glands embedded in Tissue-Tek O.C.T compound (VWR) were cryosectioned (7 μm thickness) at −35°C and transferred onto slides. Then, cryosections were fixed in Bouin's solution overnight and rinsed with tap water for 10 min. The sections were successively incubated in Weigert's iron hematoxylin (10 min), in Biebrich scarlet-acid fuchsin (15 min), in phosphomolybdic-phosphotungstic acid (20 min), in aniline blue solution (5 min), and in 1% acetic acid (5 min). The slides were then dehydrated in 95% EtOH (5 min), absolute EtOH (5 min) and xylene (5 min), and mounted with Permount (Cedarlane, Burlington, Ontario, Canada). Cryosections from randomly picked animals ($n = 4$–6, depending on sections availability) were analyzed.

Western blots. Snap-frozen mammary glands were mechanically ground into a powder on dry ice. After weighing, powdered samples were homogenized in ice-cold triple detergent lysis buffer (pH 8) (Tris 50 mM, NaCl 150 mM, 0.02% sodium azide, 0.1% SDS, 1% Nonidet P40, 0.5% sodium deoxycholate) supplemented with 1.25 M NaF, 1 M $NaVO_3$, and Halt Protease and Phosphatase Cocktail Inhibitor (Fisher Scientific). The samples were sonicated and centrifuged at 13 000 rpm (10 min at 4°C) then the supernatants were aliquoted and stored at −80°C until further processing. All steps were performed on ice. Protein concentrations were determined using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, Illinois). For each sample, total protein was separated using SDS-PAGE gels (TGX Stain-Free FastCast Acrylamide kit, 7.5%, 10%, or 12%, BIO-RAD, Mississauga, Ontario, Canada) and transferred onto PVDF membranes using the Trans-Blot Turbo Transfer System (BIO-RAD). Following transfer, total lane proteins were imaged using the ChemiDoc MP imaging system (BIO-RAD). Membranes were blocked with TBS-Tween 0.1% supplemented with 3% bovine serum albumin (BSA) dissolved in TBS-Tween 0.1%. or 5% dry milk and incubated overnight at 4°C with the appropriate primary antibody (Supplementary Table 1) diluted in 5% dry milk or 3% BSA in TBS-Tween 0.1%, depending on manufacturers' recommendations. Next, blotted membranes were washed with TBS-Tween 0.1% 3 times for 5 min and probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (Supplementary Table 1). The chemiluminescent signals were revealed using Clarity Western ECL Blotting Substrate (BIO-RAD) and visualized using the ChemiDoc MP imaging system (BIO-RAD). Density of each protein band was quantified and normalized to the total proteins in the lane using ImageLab 6.0 software (BIO-RAD). Protein extractions were done and analyzed for all animals ($n = 9$–14 per group).

Immunofluorescence and image acquisition. Immunofluorescence staining and analyses were described previously (Dianati *et al.*, 2016, 2017; Gouesse *et al.*, 2019). Briefly, tissue cryosections (7 μm) were fixed in formaldehyde (4%) and blocked in 3% BSA dissolved in TBS-Tween 0.1%. Sections were incubated with the appropriate primary antibody (Supplementary Table 2) diluted in TBS-Tween 0.1% with 3% BSA for 60 min room temperature or overnight at 4°C, washed with TBS-Tween 0.1% and then incubated with the corresponding secondary antibody (Supplementary Table 2) for 60 min at room temperature. Tissue sections were then washed 3 times, for 5 min each, stained with 4′,6-diamidino-2-phenylindole (DAPI), and slides were mounted with Fluoromount-G (Cedarlane, Burlington, Ontario, Canada). Images were obtained with a Nikon A1R+ confocal microscope equipped with a laser spectral detector (Nikon Canada Inc., Mississauga, Ontario, Canada). All the analyses were done using NIS elements analysis software (version 4). For Ki67, ERα, PR, and THRα1, cell index or the percentage of positive cells was determined as the number of positive nuclei divided by the number of total nuclei (recognizable by DAPI staining) times 100 for each image. Cryosections from randomly picked animals ($n = 4$–6, depending on sections availability) were analyzed.

Statistical analyses. Only 1 PND21 female per litter was used for each experiment. All data were analyzed using GraphPad Prism, version 6.01. First, the normal distribution of each dataset and the presence of outliers were assessed with D'Agostino & Pearson omnibus normality test and ROUT test, respectively. Data that satisfied assumptions of normality and homoscedascity were analyzed using one-way ANOVA followed by Dunnett's multiple comparison tests. Otherwise, a Kruskal-Wallis test followed by a Dunn's multiple comparison test was utilized. Differences were considered as significant when p values were $\leq .05$.

RESULTS

In Utero *and Lactational Exposure to BFRs Induces Precocious Development of the Mammary Gland at Prepuberty*

We previously reported that *in utero* and lactational exposure to the low dose of the BFR mixture dysregulated mammary gland development in the offspring at PND 46 (Gouesse *et al.*, 2019). Hence, we wanted to assess whether BFR-induced disruption of mammary gland development was apparent earlier in life, in prepubertal animals at PND 21, at a stage when the gland grows isometrically with the body, independently of systemic hormones. The BFR treatments did not affect total body or mammary gland weights at PND 21 (Gouesse *et al.*, 2019). To gain further insight on the impact of this exposure on mammary gland development, whole mount and histological analyses were undertaken (Figure 1). We did not observe any significant effects of the treatments on epithelial elongation (Figure 1A) or on the average number of TEBs (Figure 1C). However, exposure to 0.06 mg/kg/day seems to accelerate global epithelial growth as demonstrated by a significant increase in total epithelial surface area (Figure 1B). Interestingly, BFR exposure did not affect the total number of ducts (Figure 2A) but it was associated with an increase in the average ductal area that approached statistical significance ($p = .08$) (Figure 2B), accompanied by a trend toward an increase in the average lumen area (Figure 2C) and the average ductal thickness (Figure 2D).

Low Dose Exposure to BFRs Hastens Ductal Formation and Maturation in Prepubertal Mammary Glands

Positive allometric development of the mammary gland is characterized by an important epithelial cell proliferation that leads to differentiation of TEBs and subsequently to newly formed ducts (Macias and Hinck, 2012; Paine and Lewis, 2017). As the cap and most of the body cells of the TEB proliferate to allow ductal elongation, the central body cell layer and stromal cells undergo apoptosis to ensure lumen formation and ductal invasion, respectively (Humphreys *et al.*, 1996; Paine and Lewis, 2017). Therefore, we evaluated whether the histomorphological changes observed in the mammary gland were accompanied by modulation of proliferation and apoptosis at the molecular level. Protein expression of proliferating cell nuclear antigen (PCNA) and the apoptotic marker Cleaved Caspase (CC)-9 was assessed. Levels of PCNA (Figure 3A) remained unaffected while exposure to the lowest dose of BFRs significantly increased CC-9 protein expression (Figure 3B).

At PND 21, the mammary gland is mainly composed of a small epithelial arborescence surrounded by a more voluminous stroma (Macias and Hinck, 2012). As total protein extracted from the whole mammary gland was assessed in our Western blot analyses, we hypothesized that protein quantification could be biased by the relative low amounts of epithelial proteins compared to stromal. Thus, we assessed proliferation at the histological level by analyzing Ki67 nuclear staining. Interestingly, Ki67 cell index was increased in the mammary glands of all treated pups; this increase was significant in the animals exposed to the 0.06 mg/kg/day dose (Figure 4). These changes in proliferation and apoptosis are consistent with the trends toward an increase the average lumen size (Figure 2C) and ductal thickness (ductal total area minus lumen area) (Figure 2D) we observed after low dose exposure to the BFR mixture.

BFR-Induced Precocious Development of the Mammary Gland Is Associated to an Upward Trend of Nuclear Thyroid Hormone Receptor α1

Extensive growth of the mammary gland is initiated at the onset of puberty, following maturation of the hypothalamic-pituitary-gonadal axis (Brisken and O'Malley, 2010). Increasing levels of ERs and progesterone induce ductal elongation and side branching through ERα and PR-B action, respectively (Brisken and Ataca, 2015; Brisken and O'Malley, 2010). Though not well characterized, thyroid hormones also regulate mammary gland outgrowth (Hovey *et al.*, 2002); disruption of the physiological balance of thyroid hormone has been associated with impaired mammary gland development (Vonderhaar and Greco, 1979). Thus, we assessed whether the BFR-induced premature development of the prepubertal gland was associated with dysregulation of signaling pathways of these hormones. As expected, PR protein levels were not detectable by Western blot (Kariagina *et al.*, 2007). THRα protein expression remained unchanged regardless of the treatment (Figure 5A); however, a significant decrease in ERα protein levels was observed in the pups exposed to the 20 mg/kg/day dose of BFRs (Figure 5B). As stated above, the prepubertal mammary gland is predominantly composed of stromal tissue that surrounds a less voluminous epithelium (Macias and Hinck, 2012). Hence, to further evaluate tissue levels and localization of these receptors, we undertook immunofluorescence analysis. ERα and PR nuclear staining were detected in a subset of epithelial cells and their expression patterns were not affected by BFR treatment (data not shown). THRα1 was present in a punctate pattern in the nucleus and cytoplasm of most epithelial cells and a few stromal cells (Figure 6). Quantification of positive nuclei demonstrated that the percentage of THRα1 positive cells tended to increase ($p = 0.1$) in the mammary glands of the pups exposed to the 0.06 mg/kg/day BFR dose (Figure 6).

DISCUSSION

Early Exposure to BFRs Induces Precocious Mammary Gland Development at PND 21

Previously, we demonstrated that *in utero* and lactational exposure to the lowest dose of an environmentally relevant mixture of BFRs dysregulated mammary gland development in the dams (Dianati *et al.*, 2017) and their offspring at PND 46, when stimulation by ovarian and other hormones drives morphological and functional changes in this organ (Gouesse *et al.*, 2019). Here, we examined whether the same exposure had an impact on the mammary gland prior to puberty. In contrast to the absence of any morphological modifications in PND 46 pups (Gouesse *et al.*, 2019), low dose exposure to BFRs induced a premature development of the overall mammary glands in PND 21 offspring, as demonstrated by a significant increase in epithelial surface area associated with a trend toward an increase in the average ductal area; these changes are characteristic of pubertal

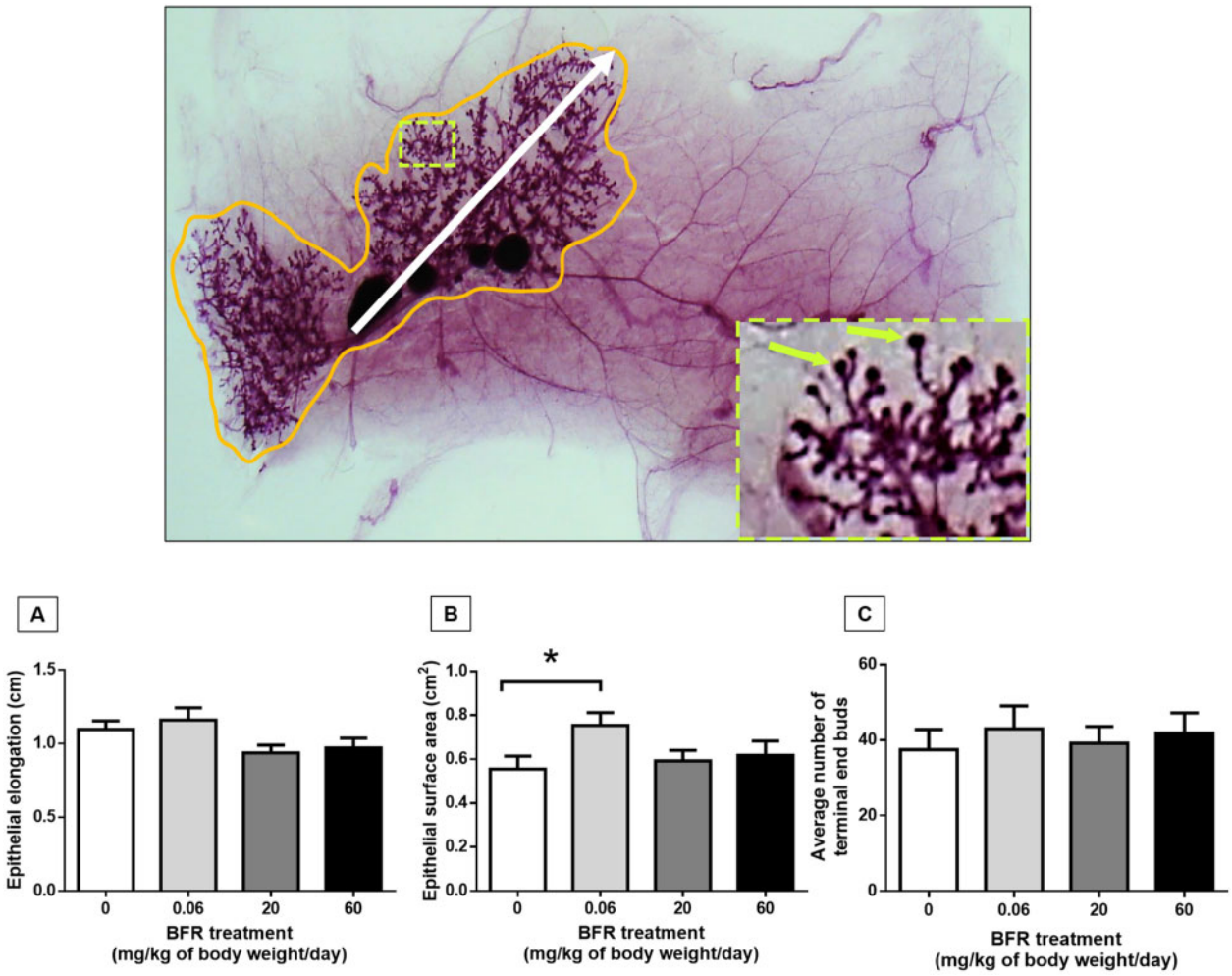

Figure 1. Low dose exposure to BFRs mixture increases the epithelial surface area of prepubertal mammary glands. Whole mounts of mammary glands of control pups or pups exposed *in utero* and through lactation to a BFRs mixture diet formulated to deliver a daily nominal BFR mixture dose of 0.06, 20, or 60 mg/kg of body weight/day were analyzed with ImageJ software. A, Epithelial elongation was measured from the lowest lymph node to the end of the longest branch (white arrow). B, Epithelial surface area was measured by tracing the best adjusted contour of the epithelium (orange line). C, The number of TEBs (peripheral bulb-like structures with area greater than 0.03 mm^2 [yellow arrow]) were counted by blinded volunteers for each animal. Histograms represent the mean of the groups ± SEM ($n = 9$–14). *p* values were calculated with a Kruskal-Wallis or an ANOVA statistical test. * indicates statistical significance, $p \leq .05$.

mammary development. During extensive pubertal development of the mammary glands, epithelial cells undergo important proliferation for TEB formation (Paine and Lewis, 2017). Major apoptosis occurs concurrently in the inner body cell layer of the TEBs and in the stroma, allowing for lumen formation and ductal invasion, respectively (Humphreys *et al.*, 1996; Paine and Lewis, 2017; Sreekumar *et al.*, 2017). Interestingly, we demonstrated that mammary glands from PND 21 pups exposed to the 0.06 mg/kg/BFR dose had significantly more epithelial cells expressing Ki67, a marker of cell proliferation, and higher levels of CC-9, an indicator of early apoptosis. This was associated to a trend toward increases in their average lumen size and ductal thickness. Such an increase in lumen size may result from death and regression of the central body cells of the TEB (Humphreys *et al.*, 1996). Other studies demonstrating that early exposure to EDs such as bisphenol A, bisphenol S, diethylstilbestrol, and nonylphenol can alter TEB formation and differentiation at puberty support our findings (Hovey *et al.*, 2005; Kolla *et al.*, 2018; Moon *et al.*, 2007; Munoz-de-Toro *et al.*, 2005). All together, these data indicate that low dose exposure to our BFR mixture induces precocious development of the prepubertal mammary gland at the morphologic and histologic level of organization, by modulating proliferation and apoptosis balance at the molecular level.

To our knowledge, only one previous *in vivo* study has reported the effects of a perinatal exposure to BFRs on mammary glands in prepubertal rodents. Unlike our results, this study showed that exposure to DE-71, a commercial mixture of PBDEs, delayed mammary outgrowth and branching at PND 21 (Kodavanti *et al.*, 2010). Besides the differences regarding the timing of exposure and the strains of the animals studied, the discrepancies observed between this study and our results could be due to the difference in the BFR mixture. DE-71 is a widespread pentabrominated mixture that has been shown to alter thyroid hormone homeostasis in rats (Ellis-Hutchings *et al.*, 2006; Szabo *et al.*, 2009; Zhou *et al.*, 2001, 2002) and elicit estrogenic activity *in vitro* (Meerts *et al.*, 2001; Mercado-Feliciano and Bigsby, 2008a,b) and *in vivo* (Mercado-Feliciano and Bigsby, 2008b). In contrast, our mixture is composed of 3 PBDEs (DE-71, DE-79, and BDE-209) and 1 HBCDD mixture (Allen *et al.*, 2008; Stapleton *et al.*, 2008) that can exhibit THR-, ER-, PR-, and AR-, agonistic, and anti-agonistic activities (Dang *et al.*, 2007; Hamers

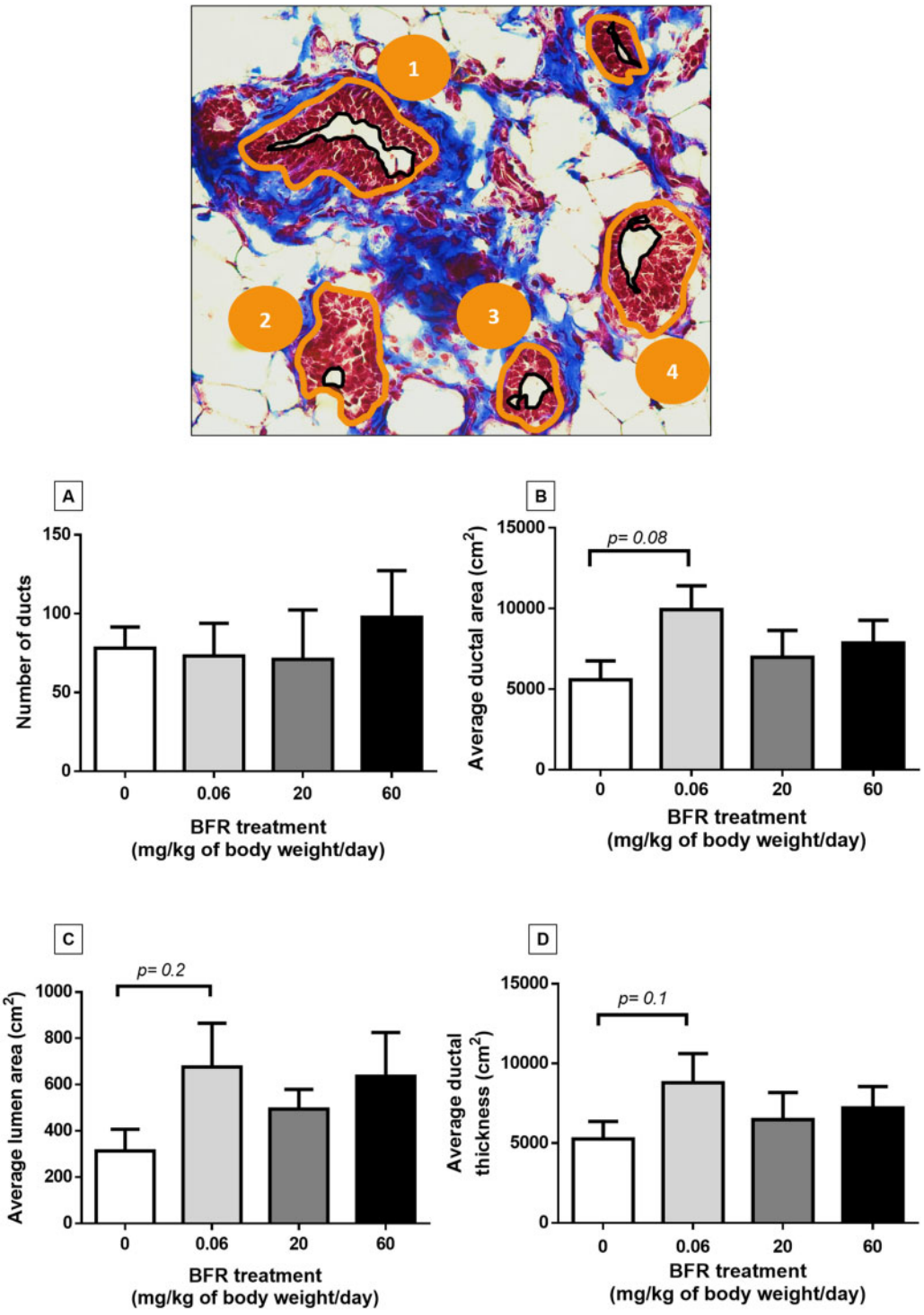

Figure 2. Low dose exposure to the BFR mixture tends to increase duct size. Mammary gland sections (7 μm) of control pups or pups exposed *in utero* and during lactation to a BFR mixture in diet formulated to deliver a daily nominal BFRs mixture dose of 0.06, 20, or 60 mg/kg of body weight/day, were fixed and stained with Masson's trichrome (luminal cells in red; extracellular matrix and fibroblast in blue; adipocytes remain uncolored [white]). The number of ducts in the entire gland cross section (A) and their respective average area (B) were determined by delineating the outline of the ducts (orange lines) using NIS-elements analysis software. The average lumen size (C) and ductal thickness (total ductal area [orange line] minus lumen area [black line]) (D) were determined using the same method. Histograms represent the mean of the group ± SEM ($n = 4$–6).

et al., 2006; Kojima *et al.*, 2009; Li *et al.*, 2010; Meerts *et al.*, 2001; Mercado-Feliciano and Bigsby, 2008a,b; Schriks *et al.*, 2006, 2007; Yamada-Okabe *et al.*, 2005). Thus, it is possible that these different mixtures of BFRs behave additively or even synergistically when used in combination (Diamanti-Kandarakis *et al.*, 2009). These diverse endocrine disruptive potencies of our BFRs mixture may explain the decrease of ERα protein levels observed in the 20 mg/kg/day treated pups. Other findings showing differential effects between exposure to a single ED versus a mixture of EDs support our results (Bunay *et al.*, 2018).

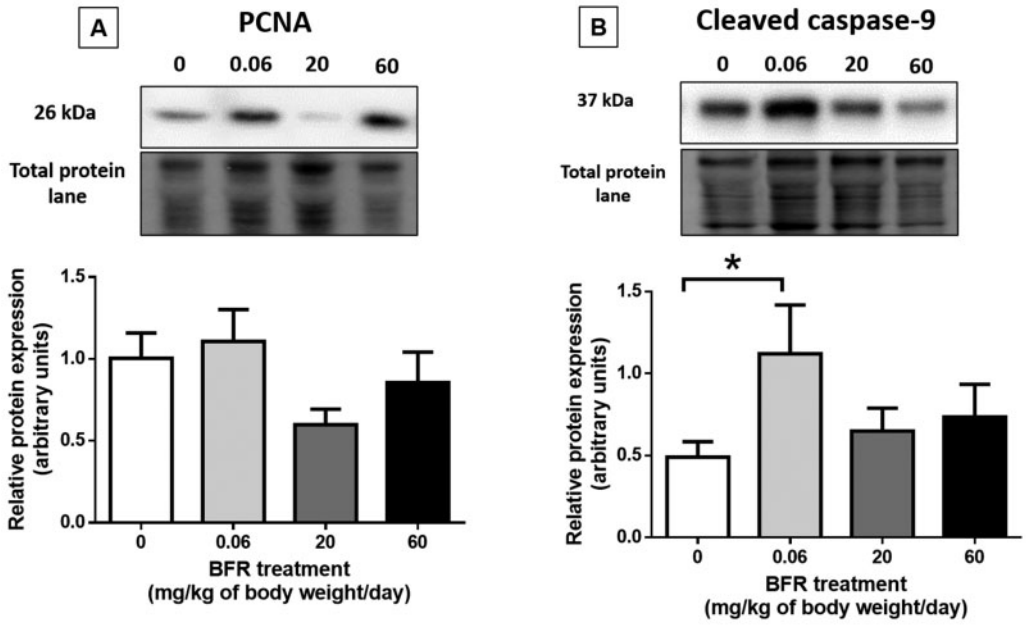

Figure 3. Exposure to the lowest dose of BFRs increases protein levels of the early apoptotic marker cleaved caspase-9. Semiquantitative Western blot of total proteins extracted from the mammary glands of control pups or pups exposed *in utero* and through lactation to a BFR dietary mixture formulated to deliver a daily nominal BFR mixture dose of 0.06, 20, or 60 mg/kg of body weight/day. Graphs show PCNA (A) and cleaved Caspase-9 (B) protein levels at PND 21. Histograms represent the means ± SEM ($n = 9$–14) for each band normalized to the total protein levels. p values were calculated with a Kruskal-Wallis statistical test or ANOVA. * indicates statistical significance, $p \leq .05$.

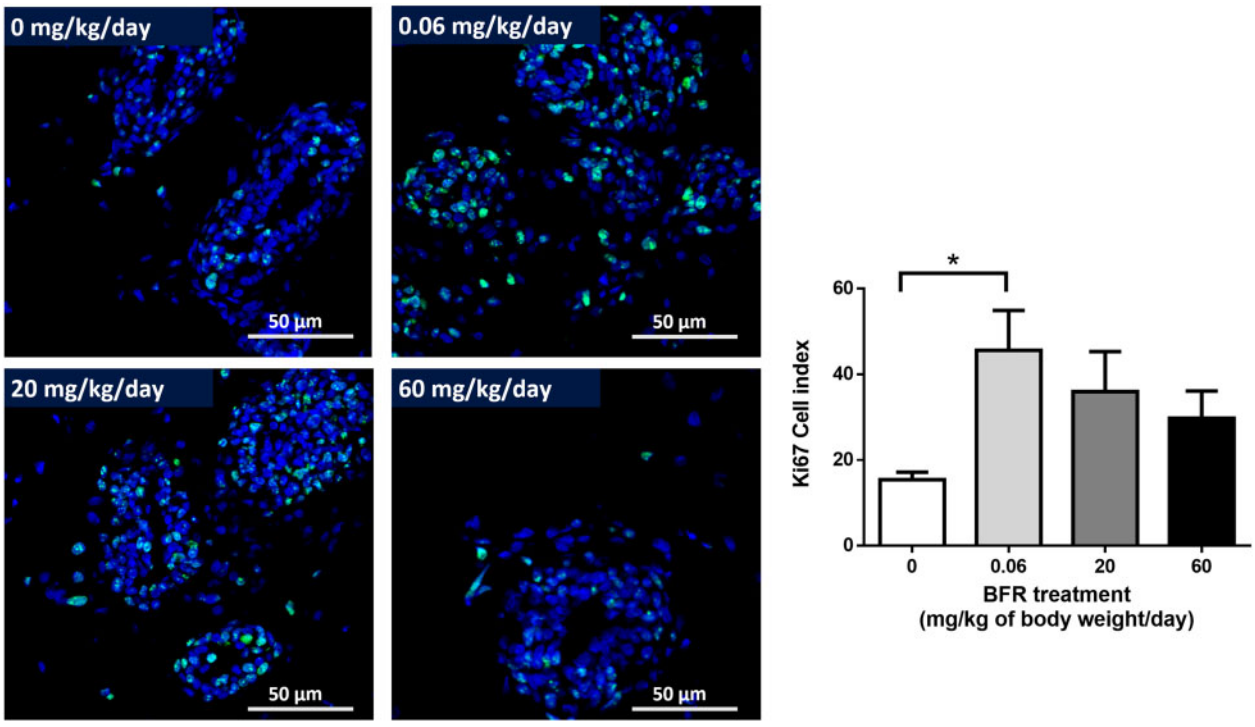

Figure 4. Low dose exposure to the BFR treatment enhances Ki67 cell index. Mammary gland cryosections (7 µm) from control or animal treated with 0.06, 20, or 60 mg/kg/day were processed for immunofluorescence staining. Ki67 (green) cell index was determined as a percentage of the number of positive nuclei against the number of total nuclei (DAPI) for each image. Images were obtained with a Nikon A1R+ equipped with a spectral detector and analyzed using NIS-elements analysis software. Histograms represent the mean of the group ± SEM ($n = 5$). * indicates statistical significance, $p \leq .05$.

Induction of Premature Development of the Mammary Gland Occurs Independently of Overall Maturation of the Reproductive System

Massive remodeling of the mammary glands occurs at the onset of sexual maturation or puberty (Howlin *et al.*, 2006). In humans, pubertal progression is assessed notably by the appearance of extensive mammary development (ie, thelarche), while in rodents it is conventionally defined by the occurrence of vaginal opening and estrus cyclicity (Vandenbergh, 1976). Histologic

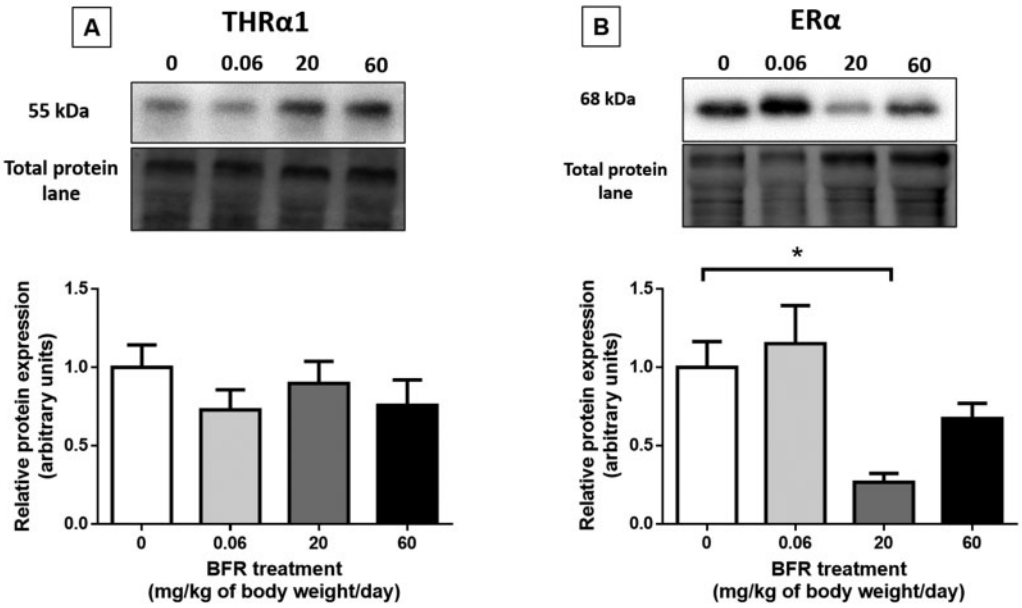

Figure 5. Exposure to BFRs mixtures does not affect thyroid hormone receptor alpha 1 but impairs estrogen receptor alpha protein levels. Semiquantitative Western blot of total proteins extracted from the mammary glands of control pups or pups exposed *in utero* and during lactation to a BFRs dietary mixture formulated to deliver a daily nominal BFRs mixture dose of 0.06, 20, or 60 mg/kg of body weight/day. Graphs show THRα1 (A) and ERα (B) protein levels at PND 21. Histograms represent the means ± SEM ($n = 9$–14) for each band normalized to the total protein level.

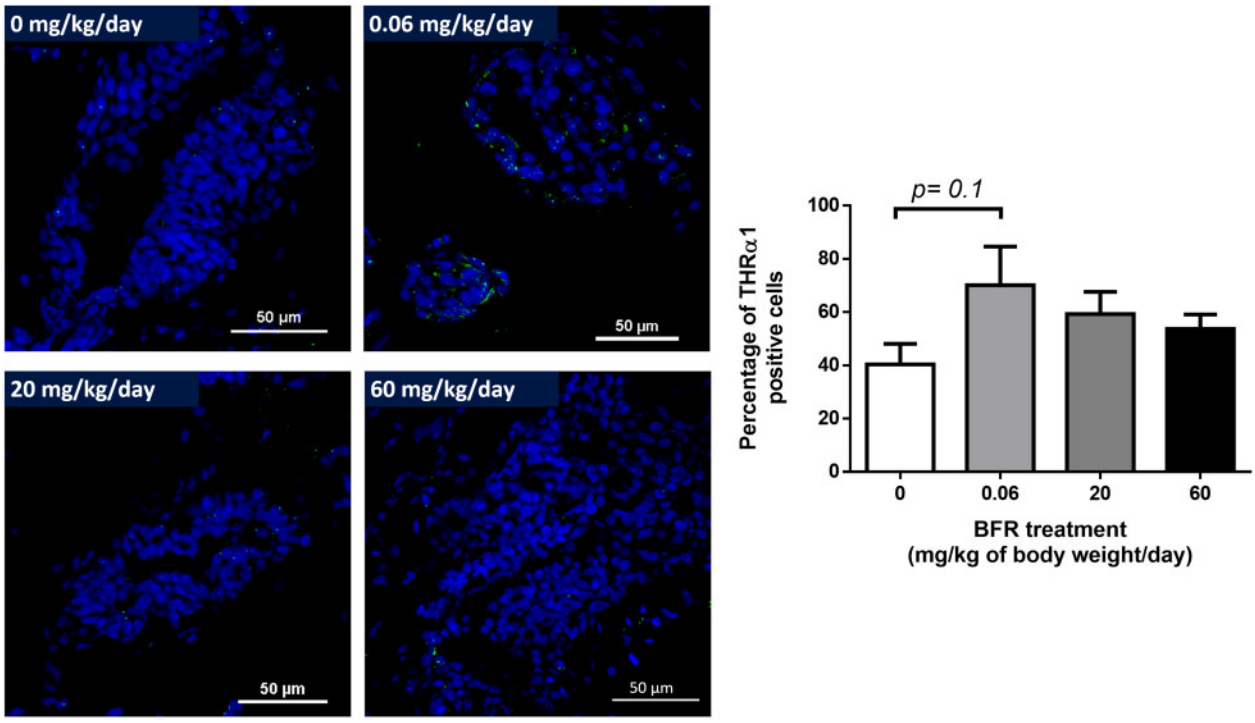

Figure 6. Exposure to a low dose BFR mixture tends to increase the number of cells positive for nuclear thyroid hormone receptor alpha 1. Mammary gland cryosections (7 µm) were processed for immunofluorescence staining from control 0 or animal treated with 0.06, 20, or 60 mg/kg/day. The number of cells positive for nuclear THRα1 (green) was determined as a percentage of the number of positive nuclei against the number of total nuclei (DAPI) for each image. Images were obtained with a Nikon A1R+ equipped with a spectral detector and analyzed using NIS-elements analysis software. Histograms represent the mean ± SEM ($n = 4$).

and morphologic changes in the ovaries and the uterus also serve as landmarks of sexual development in rodents (Picut *et al.*, 2014). Although we demonstrated that low dose exposure to BFRs led to premature mammary gland development at PND 21 in the prepubertal pups, changes in vaginal opening and estrus cyclicity were not observed in this group. However, pups exposed to 20 mg/kg/day had an advanced puberty, and disruption of ovarian follicular development were observed at the two highest doses (Allais *et al.*, 2020). The discrepancy between the low dose and non-monotonic effects of BFRs in the mammary

gland and the linear response observed in the ovaries could be due to variations in organization and in hormone responsiveness that are specific to these tissues (Vandenberg, 2014).

Similar to our findings, disruption of mammary gland development independently of any action on overall maturation of the reproductive system has previously been reported in rodents following exposure to EDs. Acute prenatal exposure to a low dose of an atrazine metabolite mixture resulted in a persistent delay in mammary gland development; this occurred without any effects on classical pubertal makers (ovary structure, vaginal opening, or estrus cyclicity) (Enoch *et al.*, 2007a). A delayed initiation of breast development was also observed in girls with higher prenatal and lactational exposure to dioxins, whereas the age at menarche was not affected (Leijs *et al.*, 2008). However, only a limited number of studies have assessed pubertal progression by analyzing mammary gland development and differentiation in rodents (Enoch *et al.*, 2007b; Fenton *et al.*, 2002, 2012; Markey *et al.*, 2001; Padilla-Banks *et al.*, 2006). Precocious and delayed puberty, as determined by the stage of breast development, has been associated with increased breast cancer risks and long-term health consequences (Fenton, 2006; Fenton *et al.*, 2012; Rudel *et al.*, 2011). Thus, our findings contribute to fill some of the gaps in the research that investigates the effects of exposure to EDs on mammary gland development and in the etiology of breast pathologies.

BFR-Induced Precocious Development of the Mammary Glands May Involve Interference With Thyroid Hormones Signaling

We showed that early exposure to the 0.06 mg/kg/day dose tended to increase the number of cells positive for nuclear THRα1 without affecting total protein levels from the whole mammary gland of PND 21 pups. Because it has been demonstrated that the expression of the various isoforms of ER and PR vary between tissue compartments and cell type in the mammary gland (Kariagina *et al.*, 2007; Saji *et al.*, 2000), we can speculate that similar differential tissue expression and cellular distribution of THRs could explain the divergences observed between total protein and nuclear localization quantification. Only a limited number of studies have investigated the expression and localization of THRs in the mammary gland of humans and animals. Cytoplasmic and nucleic staining of THRα1 was detected in mammary epithelium in human (Alyusuf *et al.*, 2014), and THRα1 and THRβ were present in the nuclei of both stromal and epithelial cells in cows (Capuco *et al.*, 2008). In Sprague Dawley rats, *THRα1* mRNA was expressed during puberty, pregnancy, and weaning, with the highest expression in the mammary glands of pubertal animals (Anguiano *et al.*, 2004). Although, to the best of our knowledge, no research has investigated THR tissue and subcellular expression and localization in the rat mammary gland across development, functional studies have demonstrated that thyroid hormones act as important regulators of mammary gland development and function (Neville *et al.*, 2002; Vonderhaar and Greco, 1979). Chemically induced hypothyroidism in mice, from weaning through adulthood, resulted in a delayed mammary gland development later at 3 months of age (Neville *et al.*, 2002; Vonderhaar and Greco, 1979). However, induction of hyperthyroidism caused an increased cell proliferation, extensive branching, and lobuloalveolar development in adult animals (Neville *et al.*, 2002; Vonderhaar and Greco, 1979). Moreover, thyroxine (T3) elicited a proliferative ER-like activity in several human breast cancer cell lines *in vitro* (Burke and Mcguire, 1978; de Launoit and Kiss, 1989; Dinda *et al.*, 2002; Mercado-Feliciano and Bigsby 2008a,b). Thus, the slight increase in the percentage of cells positive for THRα1 in the mammary gland of pups exposed to the 0.06 mg/kg/day dose is consistent with their greater number of proliferative cells and with the upregulation of morphometric endpoints related to ductal growth.

Downregulation of T4 plasma levels is one of the most described effects of BFR exposure, both in humans and experimental models (Ernest *et al.*, 2012; Hallgren *et al.*, 2001; Kim *et al.*, 2014; Kodavanti *et al.*, 2010; Kuriyama *et al.*, 2007; Tung *et al.*, 2016; van der Ven *et al.*, 2006; Zhou *et al.*, 2001, 2002). Such dysregulation may result from a direct effect of BFRs on thyroid hormone transport (Hamers *et al.*, 2006; Meerts *et al.*, 2001), metabolism (Szabo *et al.*, 2009; Tung *et al.*, 2016) and/or receptor binding (Kojima *et al.*, 2009; Qin *et al.*, 2019; Ren and Guo, 2013). In our study, PND 21 pups treated with 20 and 60, but not the 0.06, mg/kg/day doses of BFRs, had decreased serum T4 levels (Tung *et al.*, 2016). Similarly, pups exposed to the two highest, but not 0.06 mg/kg/day, exhibited an increased liver weight and higher metabolic activity (Tung *et al.*, 2016). Thus, changes observed in T4 levels at higher doses may result from liver cytotoxicity (Tung *et al.*, 2016). It has been proposed that EDs can behave like hormones that can be toxic at high doses but still influence biological endpoints at low, physiologically relevant doses (Vandenberg *et al.*, 2012; Welshons *et al.*, 2003). Thus, we speculate that though the low dose of our BFR treatment did not elicit quantifiable effects on T4 levels, it can still exert biological changes on thyroid signaling, as demonstrated by changes in THRα1-positive cell numbers. Such slight changes in thyroid signaling may result in an inappropriate hormone exposure and may contribute to the BFR-induced premature development observed at PND 21.

THRs bind to an element in the *Cx43* promoter in rat liver (Stock and Sies, 2000), stimulate *Cx43* gene expression in brook *trout* Sertoli cells (de Montgolfier *et al.*, 2011) and gap junctional intercommunication (GJIC) in rat liver cells (Stock *et al.*, 1998). Interestingly, we previously reported that *in utero* and lactational exposure to this BFR mixture resulted in increased protein levels of the highly phosphorylated (p) form of Cx43 in the mammary glands of PND 21 pups (Gouesse *et al.*, 2019). As Cx43 phosphorylation is usually associated with its localization at the cell membrane, and with effective GJIC (Lampe and Lau, 2004), this observation suggests that the BFR treatment increases cell-cell communication in the prepubertal pups. Collective migration of epithelial cells that retain their intercellular junctions contributes to the progression of TEBs into mammary stroma (Ewald *et al.*, 2008). Based on these observations, upregulated levels of p-Cx43 are coherent with the tendency to increase of the number of cells positive for THRα1; further, the implied increases of GJIC may contribute to ductal development driven by the TEBs at this stage.

However, we previously reported that the same treatment resulted in downregulated expression of p-Cx43 and this was associated with decreases in protein levels of THRα1 and of the apoptosis marker cleaved caspase-3 later at PND 46 (Gouesse *et al.*, 2019). These observations support the hypothesis that EDs can elicit different effects depending on the latency of exposure but also according to the developmental stage and hormone status of the individual (Diamanti-Kandarakis *et al.*, 2009). Similar "age-specific" effects have been observed in BPA-treated animals (Markey *et al.*, 2001). Together, these data suggest that thyroid hormone signaling plays an important role in regulating mammary gland development and appears as a prime target of BFRs. Nevertheless, further studies are needed to better define the specific mechanisms involved.

CONCLUSION

Overall, our findings suggest that *in utero* and lactational exposure to an environmentally relevant mixture of BFRs induces premature development of the mammary gland in prepubertal animals. Such precocious development has been associated with increased breast cancer risks later in life (Fenton, 2006; Group, 2004; Henderson and Feigelson, 2000). Disruption of thyroid hormone signaling, intercellular junction protein, and the proliferation-apoptosis balance appear as prime mechanisms for the effects of BFRs on the mammary gland in both PND 21 and PND 46 pups (Gouesse *et al.*, 2019). Effects of BFRs that are manifested at specific ages may be due to the substantial differences in architecture and in hormonal regulation between the "quiescent" prepubertal gland and the highly dynamic pubertal stage of mammary development (Brisken and O'Malley, 2010; Medina, 1996). Most of the mammary gland effects of BFRs resulted from low dose exposure, in prepubertal animals (current study), in older pups on PND 46 (Gouesse *et al.*, 2019), and in dams (Dianati *et al.*, 2017). Low-dose effects and non-monotonic response have been reported for BFR mixture (Berger *et al.*, 2014; Dianati *et al.*, 2017; Lefevre *et al.*, 2016; Tung *et al.*, 2017), and other technical PBDEs mixtures (Chen *et al.*, 2018a; Lilienthal *et al.*, 2006; Talsness *et al.*, 2008). These nonlinear response curves are common for hormones and EDs, and suggest that BFRs may induce opposite effects on independent processes that converge in a common quantifiable endpoint (Vandenberg, 2014; Vandenberg *et al.*, 2012). However, BFRs also triggers linear dose-response curves (Allais *et al.*, 2020; Berger *et al.*, 2014; Lefevre *et al.*, 2016; Tung *et al.*, 2016, 2017) that may result from tissue-specific hormone responsiveness (Vandenberg *et al.*, 2012). Taken together, these data suggest that our BFRs mixture may involve intricate mechanisms given their diverse potencies of EDs.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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