Toxicology and Applied Pharmacology 303 (2016) 101–109

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Global gene expression and morphological alterations in the mammary gland after gestational exposure to bisphenol A, genistein and indole-3-carbinol in female Sprague-Dawley offspring

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ARTICLE INFO

Article history:
Received 4 February 2016
Revised 6 May 2016
Accepted 7 May 2016
Available online 10 May 2016

Keywords:
Bisphenol A
Pregnancy
Mammary gland development and gene expression
Genistein and indole 3-carbinol

ABSTRACT

This study aimed to evaluate the modifying effects of dietary genistein (GEN) and indole-3-carbinol (I3C) on early mammary gland development in female Sprague-Dawley offspring born to mothers exposed to BPA during gestation. Pregnant rats were treated with BPA25 or 250 μg/kg bw/day from gestational days 10 to 21 with or without dietary intake of GEN (250 mg/kg chow) or I3C (2000 mg/kg chow). At post-natal day (PND) 21, female offspring from different litters were euthanized for mammary gland development and gene expression analyses. Our results indicated that prenatal exposure to BPA25 and 250 did not modify the ductal elongation of the mammary gland tree or the estrogen receptor alpha (ER-α) expression in terminal end buds (TEBs). However, BPA25-exposed offspring had a higher number of terminal structures (TEBs + TDs) and an increased mammary branching and cell proliferation index in TEBs. Besides that, BPA25 and 250 modulated the expression of several genes in the immature mammary gland that were not changed in a dose dependent manner and involved different clusters of up- and down-regulated genes. Furthermore, BPA25 and BPA250 + I3C-treated groups also had a higher number of enriched functional gene categories. In addition, maternal dietary GEN and I3C in association with BPA exposure produced specific gene expression alterations in the mammary gland and overcome the adverse effect of BPA25, decreasing the branching of the mammary gland. In conclusion, prenatal BPA exposure induced both morphological and gene expression modifications on the mammary gland that dietary intake of GEN and I3C reverted on BPA25-exposed animals.

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1. Introduction

Several chemicals found in our environment, food, and consumer products have been identified as potential endocrine disruptors, including natural and synthetic hormones, pesticides, plasticizers, and industrial byproducts which can interfere with hormone biosynthesis, metabolism, or result in a deviation from normal homeostatic control or reproductive status (Jenkins et al., 2012; Yoon et al., 2014).

Bisphenol A [BPA, 2,2,-bis(hydroxyphenyl)propane] is a synthetic monomer used in large quantities by industry for the manufacturing of polycarbonate plastic and epoxy resins, which are included in numerous household and consumer products (Rubin, 2011; Rochester, 2013). In addition, epoxy resins are frequently used as coating materials for food cans and metal lids, in dental sealants, and as finishing and coating materials for PVC pipes, whereas polycarbonate is used in the manufacture of household appliances, food packaging materials, plastic bottles, baby bottles, and many other products for human daily use (Rubin, 2011; Rochester, 2013). Because of their widespread industrial use, inadvertent presence of BPA has been detected in the environment leading to continuous exposure of the human population (Rubin, 2011; Rochester, 2013). Also, low doses of BPA have been frequently detected in the human serum, urine, breast milk and placental tissue (0.2 to 10 ng/ml; ~0.5–40 nM) (Ikezuki et al. 2002; Sun et al., 2004; Kuruto-Niwa et al., 2007; Vandenberg et al., 2010). In addition, several studies have reported the presence of BPA in the serum of pregnant women, umbilical cord blood, amniotic fluid, and fetal plasma, which

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http://dx.doi.org/10.1016/j.taap.2016.05.004
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suggests that embryos and fetuses are potentially exposed to BPA during pregnancy (Ikezuki et al., 2002; Engel et al., 2006; Edlow et al., 2012).

BPA has been categorized as an endocrine disruptor with weak estrogenic activity as demonstrated in different *in vitro* and *in vivo* studies (Rubin, 2011; Jenkins et al., 2012). Rodent studies have shown that BPA is easily transferred across the placenta and in breast milk, indicating a potential risk for fetuses and neonates (Takahashi and Oishi 2000; Zalko et al. 2003). The ability of early in life BPA exposure to influence mammary gland structure and/or carcinogenesis has been investigated in both female rats and mice (Muñoz-de-Toro et al. 2005; Murray et al., 2007; Durando et al., 2007; Moral et al., 2008; Jenkins et al., 2009; Betancourt et al., 2010; Acevedo et al., 2013).

US Environmental Protection Agency has established 50 μg/kg body weight/day of BPA as the "safe reference dose" but adverse effects to hormone-dependent organs have been described in different *in vivo* bioassay using BPA exposure at human relevant doses (reviewed by Teeguarden and Hanson-Drury, 2013). A wide range of epidemiological and experimental evidences point to the involvement of dietary compounds such as genistein (GEN) and indole-3-carbinol (I3C) in the prevention of many diseases, including breast cancer development (Higdon et al., 2007; Bradlow 2008; Taylor et al., 2009; Hilakivi-Clarke et al., 2010). GEN (4,5,7-trihydroxyisoflavone) is the main phytoestrogen found in soy, and human intake of this isoflavone in both conjugated (genistin) or unconjugated (aglycone) forms comes primarily from the soy-based infant formula and dietary supplements or soy foods such as tofu, soy milk, soy flour, textured soy protein, tempeh, and miso (Xiao, 2008; Taylor et al., 2009). GEN is often referred as a phytoestrogen because of its ability to bind to estrogen receptors (ERs) but displays weak estrogenic activity when compared to estradiol (Xiao, 2008; Taylor et al., 2009). GEN has showed beneficial effects against murine mammary carcinogenesis mainly when administered before or after carcinogen exposure (Bradlow 2008; Taylor et al., 2009; Hilakivi-Clarke et al., 2010).

Indole-3-carbinol (I3C) is a natural compound derived by hydrolysis from glucobrassicin found in high concentrations in vegetables of the Brassicaceae family, including broccoli, cauliflower, cabbage, and Brussels sprouts (Bradlow 2008, Weng et al., 2008). This natural compound has been investigated for its protective properties on the hormone-dependent tissue, including endometrium, prostate and breast (Bradlow 2008; Weng et al., 2008), probably by suppressing responsiveness to estrogen and decreasing the expression of ER alpha (ER-α) (Sundar et al., 2006; Wang et al., 2006). Also, I3C modifies the enzymatic metabolism of chemicals, resulting in antimutagenic/antigenotoxic and anticarcinogenic properties against various classes of chemical carcinogens (Bradlow 2008; Weng et al., 2008). In a clinical trial, daily intake of I3C at doses of 400 and 800 mg increased the levels of important metabolizing enzymes (glutathione-*S*-transferase and CYP1A2, and the 2-hydroxyestrone:16α-hydroxyestrone ratio) without causing any adverse effects (Reed, 2005).

To better understand the adverse effects of gestational BPA exposure on the early mammary gland development as well as the potential modifying effects of dietary GEN and I3C, the present study used morphological parameters of gland differentiation and global gene expression as biomarkers. Additionally, this study aimed at evaluating the modifying effects of the interaction between these compounds taking into consideration that they do not have the same properties on mammary gland development when given alone, in part because of their different affinities on ERs (Benninghoff and Williams, 2013;Yoon et al., 2014).

2. Materials and methods

2.1. Chemicals

BPA (CAS no 80–05-7, 99% purity), Dimethyl Sulfoxide (DMSO; CAS no 67–68-5, 99% purity) and I3C (CAS no 700-06-1, 99% purity) were purchased from the Sigma-Aldrich Co®, USA. GEN (99% purity) was kindly donated by DSM Nutritional Products Basel, Switzeland/DSM Nutritional Products Brazil Ltd.

2.2. Animals and experimental design

The animals were handled in accordance with Ethical Principles for Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Committee for Ethics in Animal Experimentation of the Faculty of Medicine, UNESP, Botucatu-SP, Brazil (Protocol n° 782).

Male and female Sprague-Dawely (SD) outbred rats were obtained from colonies under SPF-conditions from Multidisciplinary Center for Biological Investigation (CEMIB-UNICAMP, Campinas-SP, Brazil). The animals were housed in polypropylene cages with autoclaved white pine shavings and maintained in rooms under controlled environmental conditions (temperature 22 ± 2 °C, relative humidity 55 ± 20%, a 12/12 h light-dark cycle). All animals received phytoestrogen-free Nuvilab CR-1 commercial chow (Nuvital, PR, Brazil), named as basal diet, and filtered drinking water *ad libitum*, starting 2-wk. before the experiment beginning as an acclimation period.

After the acclimation period, 8-week–old female SD rats (n = 115) were mated to 12-week-old male SD rats (n = 55) by placing two females in a cage with one male (each female was designated for a different group). Daily vaginal smears were done and the day on which sperm was found in the vagina was designated gestational (GD) 0. Pregnant rats were randomly allocated to seven groups (n = 15/group) and from GD10 to GD21 they received daily 25 or 250 μg/kg body weight (bw) of BPA or vehicle (1% DMSO/canola oil) by gavage in a volume of 5.0 ml/kg (Brandt et al., 2014). During this period (GD0 to GD21), dams had *ad libitum* access to basal diet or diet containing GEN 250 mg/kg chow (Dave et al., 2005, Su et al., 2007) or I3C 2000 mg/kg chow (Yu et al., 2006; Brandt et al., 2014). From parturition to the end of the experiment, all animals continued on basal diet. The pregnant were daily weighted while lactating dams were weighed on alternating days; food and water consumption of dams were recorded during gestational period (GD0–GD21) and after parturition, the water and food consumption were recorded twice a week during the experimental period. Sexing of the offspring in each litter was performed on PND1. The litter size was reduced and standardized (8–10 pups per litter) for all groups (the gender ratio was kept as close to 1:1 as possible) and female offspring were weighted at PND 3, 10 and 21. At PND 21, 10 female offspring (F1)/group, (1 female/litter) were euthanized and abdominal mammary glands were removed and processed for whole-mounts, immunohistochemical analysis or gene expression. Morphological mammary gland analyses were performed without knowledge of treatment and independently evaluated by two different researchers.

2.3. Rationale for BPA, GEN and I3C doses

In this present study, BPA25 and 250 μg/kg bw/day doses were selected to represent one-half and four-fold higher than the daily tolerable dose of BPA (50 μg/kg bw/day) established by US EPA (reviewed by Teeguarden and Hanson-Drury, 2013).

GEN content in soy and soy foods ranges between 1.3 and 126 mg/kg (Mortensen et al., 2009). The estimated intake *per capita* of this isoflavone in the United States and United Kingdom is between 0.8 and 3.3 and 0.4–4.5 mg/day, respectively, whereas in Japan is approximately 8–50 mg/day (Mortensen et al., 2009). In addition, glucosinolate content in edible cruciferous vegetables ranges between 15 and 500 mg/kg of fresh weight (McNaughton and Marks, 2003). These vegetables contain glucobrassicin, an indolyl-methyl glucosinolate, which is a common intermediate form of I3C. The estimated intake *per capita* of glucobrassicin in the United States is 8.1 mg/day whereas in the United Kingdom is 19.4 mg/day and in some Asian countries is 45 mg/day (Hecht et al., 2004; Higdon et al., 2007). Taking into consideration

these values, we decided to supplement the chow with either GEN 250 mg/kg chow or I3C 2000 mg/kg chow to allow an estimated ingestion of GEN 5.5 mg/kg bw/day or I3C 45.0 mg/kg bw/day, respectively. These estimated doses are considered safe to developmental and reproductive studies in rats (Kang et al., 2002; Yu et al., 2006; Brandt et al., 2014).

2.4. Mammary gland whole mounts analysis

At necropsy, the right abdominal mammary glands were promptly dissected from the skin and spread onto a glass slide (10 samples per group using 1 female/litter). The mammary tissues were fixed in 10% phosphate buffered formalin for 24 h at room temperature and stained with Carmine Alum for two days at room temperature. Then, the mammary glands were rinsed in water and dehydrated in absolute alcohol, cleared in xylene and coverslipped with mounting media. Whole-mount images were captured and analyzed using a CellSens Standard Imaging System (Olympus, Japan). Analysis of mammary structures such as terminal end buds (TEBs) and terminal ducts (TDs) (Supplementary Fig. 1D) was done in the outer margin of mammary gland tree (Russo and Russo, 1996) by visual evaluation and computer-assisted image analysis (CellSens Standard program - Olympus Corporation – Japan). The mean number of TEBs + TDs by microscopic field was determined under a microscope Bx 53F (Olympus, Japan, 20× objective) (Supplementary Fig. 1D). Twenty microscopic fields per abdominal mammary gland, in 10 offspring/group (1 female/litter), were analyzed, resulting in 200 microscopic fields/group. The ductal extension of the mammary tree was also measured as the linear distance from the nipple to the furthest point of the ductal tree in 10 whole-mounts per group, using the Image J 1.46, range 8.044 pixels/mm program. In addition, the branching of the mammary gland was scored on a 1–3 subjective scale (1 = low branching and 3 = high branching, Supplementary Fig. 1A–C) using a DinoCapture 2.0 software (Dino-Lite Digital Microscope, *CA*, USA).

2.5. Immunohistochemical analysis

Left abdominal mammary glands were removed, fixed for 24 h in 10% phosphate buffered formalin and processed until paraffin embedded (10 samples per group using 1 female/litter). 5-μm-thick sections were either stained with hematoxylin–eosin (H&E) or immunostained to evaluate the expression of proliferating cell nuclear antigen (PCNA) and ER-α. Mammary gland sections on silane-coated slides were deparaffinized, and rehydrated with graded alcohol. Sections were subjected to antigen retrieval using Pascal pressure chamber with citrate acid buffer at pH 6.0 at 120 °C for 3 min. Endogenous peroxidase was blocked with 3% H_2O_2 in phosphate buffered saline (PBS) for 10 min in darkness. After washing with PBS, slides were incubated with nonfat milk in PBS for 60 min. Sections were then incubated with either mouse monoclonal anti-PCNA/PC10 (1:200 dilution) (DakoCytomation Denmark A/S, Glostrup, Denmark) or mouse monoclonal anti-ER-a/6F11 (1:50 dilution) (BioCare Medical, Concord, *CA*). Anti-mouse secondary antibodies with polymer/peroxidase (Max Polymer, Novolink TM, Novocastra TM, Leica Microsystems, Newcastle, UK) was used and reactions were developed with 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO). Harris's hematoxylin was used as counterstaining. PCNA and ER-α labeling indexes (LI%) in TEBs from abdominal mammary glands were calculated by the number of positively marked cells divided by the total number of cells scored (300 to 500 epithelial cells/animal).

2.6. RNA extraction

Total RNA was extracted from the abdominal left mammary gland (05 samples per group using 1 female/litter) using the RNeasy Lipid Tissue Mini Kit (QIAGEN Inc. USA Valencia, *CA* - USA) according to the manufacturer's instructions. The extracted RNA was stored at −80 °C. RNA was quantified in a spectrophotometer NanoVue (GE Healthcare UK Limited, UK) ($Abs_{260/280}$ value of 1.9 ± 0.07) and the integrity was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc. Life Sciences and Chemical Analysis Group, Santa Clara, *CA*, USA) (RIN value of 7.80 ± 0.45) under standard conditions.

2.7. Oligo microarrays

An aliquot containing 150 ng of total RNA was hybridized onto whole rat genome 4 × 44K oligo microarrays (G4131F, Agilent Technologies) using one color (Cy3) Low input Quick Amp labeling kit (Agilent Technologies, Inc. Life Sciences and Chemical Analysis Group, Santa Clara, *CA*, USA), according to manufacturer instructions. Briefly, total RNA plus spike-in controls were reverse transcribed into double stranded cDNA. The primers used for this reaction contained consecutive thymine bases attached to a T7 promoter that paired at the 5′ end of the first strand of cDNAs. Next, the T7 polymerase was added along with nucleotides labeled with fluorescent cyanine-3 (Cy3) dye, which amplified the anti-sense complementary RNAs (cRNA). Hybridizations were performed for 17 h at 65 °C using an automated system (SureHyb, GE Healthcare UK Limited, UK). Subsequently, the slides were washed with wash buffer solutions 1 and 2. The solutions Agilent's Stabilization and Drying also were used to protect cyanine probes against ozone degradation. The hybridization signals were captured using the GenePix 4000B scanner (Molecular Devices, LLC, Sunnyvale, *CA* USA).

2.8. Microarray data analysis

Data quantification and quality control were performed using Feature Extraction (FE) software version 15.5 (Agilent Technologies, Inc. Life Sciences and Chemical Analysis Group, Santa Clara, *CA*, USA). Expression data were loaded into *R*-environment (http://www.r-project.org) v.3.1.0 for the further steps. The background adjustment was performed by subtracting median background values from the median expression values. Data were log2-transformed and then normalized using quantile function from LIMMA (Linear Models for Microarray Analysis) package (Ritchie et al., 2015). Afterwards, spots flagged as positive or negative controls were excluded from the analysis, and the replicated probes were averaged.

Differentially expressed genes were identified by using the package multtest (Pollard et al. 2005). Genes were considered statistically significant with $p < 0.01$ and fold change >1.2 in each of the contrasts analyzed. In order to identify the effects of BPA *per se*, we compared BPA25 and BPA250 *vs.* Control group. Additionally, it was compared BPA25 + GEN *vs.* BPA25 and BPA250 + GEN *vs.* BPA25 to determine the effects of GEN in BPA-treated animals. Finally, it was compared BPA25 + I3C *vs.* BPA25 and BPA250 + I3C *vs.* BPA250 to determine the modifying effects of I3C in BPA-treated animals. The probes related to a valid Entrez Gene ID were selected.

Subsequently, the clusters of co-regulated genes were submitted to functional analysis using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) using the package RDAVIDWebService (Fresno and Fernández, 2013). Biological processes with non-adjusted $p < 0.05$ were considered statistically significant in each cluster. The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus through GEO Series accession number GSE68973 (Edgar et al., 2002) (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68973).

2.9. Cdkn1a gene expression by quantitative real- time PCR

Complementary DNA was synthesized using the High Capacity (Applied Biosystems, USA) kit as instructed by the manufacturer. Total RNA was reverse transcribed using 6 μL of random hexamer primer (10×), 6 μL of reaction buffer (10×), 2.5 μL of dNTPs (25×), and 3 μL

of Multiscribe enzyme (50 U/μL). This mixture was incubated at 25 °C for 10 min and then at 37 °C for 2 h, and it was kept at 4 °C. Subsequently, each cDNA was stored at −20 °C.

TaqMan/FAM-MGB probes and primers for *Cdkn1a* (*Rn00589996*_m1) were as used for amplification. Reactions were performed at 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. β-*actin* was used as an endogenous control. Taqman Universal PCR Master Mix was also obtained from Applied Biosystems, and quantitative real-time PCR was performed in duplicate in a 7500 FAST PCR system (Applied Biosystems, USA). Relative gene expression data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.10. Statistical analyses

Data of body weight and body-weight gain, food consumption (dams and female offspring), ductal length, number of mammary structures and PCNA and ER-α labeling indexes were analyzed by ANOVA and *post hoc* Tukey's test or Kruskal-Wallis test and *post hoc* Dunn's test. (1) For reproductive outcomes statistics were performed using the dam as the unit of measure; (2) For female offspring body weight and food consumption parameters, statistics were performed using the litter as the unit of measure; (3) For female offspring mammary parameters statistics were performed using 1 female/litter (selected randomly) as the unit of measure. Significant differences were assumed when $p < 0.05$. The statistical analyses were performed using Jandel Sigma Stat software for Windows Version 3.5, 2006 (Jandel Corporation, San Rafael, CA).

3. Results

3.1. Maternal and offspring performance

In the present study, no statistically significant changes in maternal body weight gain, food and water consumption and reproductive outcomes (*i.e.*, gestation length, litter size, and sex ratio) were observed during prenatal BPA exposure or when GEN or I3C was added to the diet (Table 1). In pregnant females receiving GEN or I3C, the estimated ingestion of these compounds during pregnancy (GD 12–20) were approximately 5.5 mg/kg/day and 45.0 mg/kg/day, respectively, and no alterations in offspring development were observed in these groups (Table 1). No statistical difference statistically was observed in maternal food and water consumption during lactation (data not shown).

3.2. Mammary gland whole mounts and immunohistochemistry analysis

To evaluate whether prenatal exposure to BPA and its association with dietary GEN and I3C could alter early mammary gland development, whole mount preparations were performed. No differences were found in the ductal length of the mammary gland tree among groups. However, the number of TEBs + TDs was significantly higher ($p < 0.05$) in BPA25 animals compared with control animals. Moreover, BPA25 animals had an increased mammary gland branching than control, BPA25 + GEN or BPA25 + I3C animals (Table 2).

Considering that cell proliferation and ER-α expression are indicators of mammary gland terminal differentiation (Russo and Russo, 1996), these biomarkers were quantified in TEBs from female offspring on PND 21. In fact, PCNA and ER-α immunostaining were clearly observed in the epithelial cells from abdominal mammary gland (Supplementary Fig. 2A and B). The mean values for PCNA and ER-α labeling indexes within TEBs in the various treatments groups are depicted in Table 2. ER-α labeling index was similar among different treatments whereas cell proliferation index tended to be higher ($p = 0.056$) in TEBs from BPA25 group when compared to the control group.

3.3. Gene expression profile

To elucidate whether prenatal exposure to BPA and its association with dietary GEN and I3C modify gene expression signature, global gene expression analysis was performed in the mammary gland at PND 21. After background correction and normalization, a nonspecific filter was applied to the data in order to filter out negative and positive control probes, and to average the replicate ones. The gene expression analysis in treatment groups showed a total of 41,012 probe-sets available for analysis after filtering. The group with the highest number of differentially expressed genes compared to its control was BPA250 + GEN with 386 genes, followed by BPA25 with 233, BPA250 with 191, BPA25 + GEN with 168, BPA250 + I3C with 135 and BPA25 + I3C with 118. BPA250 + GEN and BPA25 animals showed a higher number of up-regulated genes and down-regulated genes when compared with their respective controls (Table 3 and Supplementary data 1). The number of unique genes (non-shared genes among comparisons) modified in each treatment group was 220 and 178 (BPA25 *vs.* BPA250), 206, 147 and 94 (BPA25 *vs.* BPA25 + GEN *vs.* BPA25 + I3C) and 170, 361, 115 (BPA250 *vs.* BPA250 + GEN *vs.*

Table 1
Reproductive outcomes from dams and female pups in the different experimental groups[a].

Parameter	Group[b]						
	Control	BPA25	BPA250	BPA25 + GEN	BPA250 + GEN	BPA25 + I3C	BPA250 + I3C
Dams (Pregnancy)	15[d]	15	15	15	15	15	15
Gestation length (days)	22.08 ± 0.29	21.79 ± 0.43	21.92 ± 029	22.08 ± 0.29	21.83 ± 0.39	22.00 ± 0.00	21.79 ± 0.42
Litter size	10.00 ± 4.41	11.21 ± 2.83	11.25 ± 3.22	9.33 ± 2.96	10.42 ± 2.81	10.00 ± 3.78	10.00 ± 3.16
Sex ratio (F:M)	0.86 ± 0.41	1.11 ± 1.44	0.97 ± 0.58	1.92 ± 1.14	1.72 ± 1.83	1.00 ± 0.84	0.84 ± 0.70
Body weight (g) at GD20	376.40 ± 32.82	379.31 ± 19.89	382.10 ± 23.23	393.13 ± 31.20	382.55 ± 30.71	375.75 ± 22.30	395.38 ± 22.76
Body weight gain (g)[c]	127.70 ± 22.46	123.00 ± 19.32	124.40 ± 17.16	129.61 ± 21.01	125.60 ± 18.86	122.83 ± 11.11	123.19 ± 19.24
Food consumption (g/rat/day)[c]	65.94 ± 31.22	64.54 ± 25.63	64.08 ± 26.72	69.41 ± 34.91	62.45 ± 29.37	66.05 ± 21.55	69.08 ± 24.27
Water consumption (ml/rat/day)[c]	43.30 ± 3.55	42.32 ± 6.75	41.36 ± 5.41	45.95 ± 6.77	45.42 ± 3.67	48.38 ± 6.30	45.65 ± 7.04
GEN or I3C intake (mg/rat/day)[c]	–	–	–	5.78	5.16	44.03	46.05
Female pups (Lactation)	55[e]	68	62	65	65	58	69
Body weights (g)							
PND 03	8.02 ± 0.71	8.01 ± 0.61	8.26 ± 0.68	8.35 ± 1.01	8.27 ± 1.32	8.09 ± 0.72	7.93 ± 0.86
PND 10	19.52 ± 1.42	19.99 ± 1.12	21.06 ± 1.80	22.47 ± 4.11	21.65 ± 2.14	22.04 ± 1.00	20.47 ± 3.35
PND 21	48.93 ± 3.96	47.82 ± 4.23	48.06 ± 5.18	46.74 ± 5.02	46.76 ± 4.98	49.07 ± 5.34	43.79 ± 5.71

No statistical difference among groups.
[a] Values are mean + SD.
[b] BPA25 μg/kg bw/day; BPA250 μg/kg bw/day; BPA25 μg/kg bw/day *plus* GEN 250 mg/kg chow; BPA250 μg/kg bw/day *plus* GEN 250 mg/kg chow; BPA25 μg/kg bw/day *plus* I3C 2000 mg/kg chow and BPA250 μg/kg bw/day *plus* I3C 2000 mg/kg chow.
[c] From GD 0 to GD21.
[d] Number of pregnant dams.
[e] Number of female offspring.

Table 2
Ductal tree length, number of terminal ductal structures, mammary branching scores and ER-α and cell proliferation labeling indexes in TEB in the mammary gland in female offspring at the PND 21.

Group[a]	Number of animals	Morphological analysis					Labeling index (LI)	
		Ductal length (mm)	Number of TEBs	TDs	TEBs + TDs[b]	Branching score	ER-α (%)	PCNA (%)
Control	10	3.96 ± 1.09	2.59 ± 1.32 (20)[c]	1.87 ± 0.53 (18)	4.46 ± 1.38	1.70 ± 0.48	66.80 ± 4.98	71.11 ± 14.45
BPA25	10	4.93 ± 0.59	3.95 ± 0.91 (36)	3.38 ± 1.10[*] (32)	7.23 ± 1.16[*]	2.70 ± 0.48[**]	57.58 ± 9.36	86.14 ± 7.36[§]
BPA250	10	4.82 ± 0.66	3.81 ± 1.68 (33)	2.65 ± 0.81 (22)	6.56 ± 2.30	2.00 ± 0.67	63.40 ± 10.31	74.68 ± 17.33
BPA25 + GEN	10	3.53 ± 0.79	2.76 ± 1.01 (22)	2.76 ± 0.66 (26)	5.93 ± 1.36	1.80 ± 0.79	66.56 ± 7.81	68.21 ± 8.01
BPA250 + GEN	10	3.59 ± 0.85	3.12 ± 1.23 (24)	2.81 ± 0.91 (22)	5.93 ± 1.91	1.90 ± 0.74	75.58 ± 8.37	73.28 ± 10.07
BPA25 + I3C	10	3.82 ± 0.76	2.91 ± 1.42 (26)	2.32 ± 0.85 (21)	5.23 ± 2.03	1.80 ± 0.63	65.79 ± 4.63	73.34 ± 8.77
BPA250 + I3C	10	3.89 ± 0.52	2.55 ± 1.44 (23)	2.64 ± 0.72 (24)	5.19 ± 1.98	2.10 ± 0.74	73.16 ± 7.61	75.14 ± 5.14

Values are mean + SD.
[a] BPA25 μg/kg bw/day; BPA250 μg/kg bw/day; BPA25 μg/kg bw/day *plus* GEN 250 mg/kg chow; BPA250 μg/kg bw/day *plus* GEN 250 mg/kg chow; BPA25 μg/kg bw/day *plus* I3C 2000 mg/kg chow and BPA250 μg/kg bw/day *plus* I3C 2000 mg/kg chow.
[b] TEBs: terminal end buds; TDs: terminal ducts.
[c] Mean total number of terminal ductal structures/group.
[*] Different from control group, $p < 0.05$.
[**] Different from control, BPA25 + GEN and BPA25 = I3C.
[§] p = 0.056 from control.

BPA250 + I3C). In addition, BPA25 and 250 groups shared the highest number of common genes (Fig. 1).

DAVID functional analysis of the differentially expressed genes for treatments groups showing their respective clusters with enriched Gene Ontology (GO) terms were established (Supplementary data 2). A higher number of altered GOs were observed in BPA250 animals in relation to the BPA25 ones. In correlation to the mammary structure outcomes, BPA25 treatment leaded to the differential expression of 20 genes (cluster 04) that could be associated to mammary gland development, since they are related to branching morphogenesis of a tube, epithelium development, and epithelial cell differentiation, among others (Table 4). Among the altered gene, *Cdkn1a* was confirmed by qRT-PCR (Fig. 2). In BPA and I3C-treated groups a higher number of enriched cluster and functional gene categories in relation to the other groups were observed.

4. Discussion

Early in life exposures to endocrine-disrupting compounds (EDCs) may have deleterious long-term effects on the endocrine system and increase the risk for tumor development (Jenkins et al., 2012; Yoon et al., 2014). Among then, the *in utero* developmental window has been the subject of considerable studies because of its potential to modify subsequent lifetime risks for some chronic diseases (Jenkins et al., 2012; Osborne et al., 2014). The mammary glands arise during embryogenesis under both local and systemic influences; therefore, the exposure to xenoestrogen compounds such as BPA during this period may alter the epithelium/stroma signalization and increase the susceptibility of the epithelium to neoplastic transformation (Wadia et al., 2013; Soto et al., 2013). Thus, the present study investigated the modifying effects of prenatal BPA exposure and its association with maternal dietary GEN or I3C on mammary gland development and gene expression profile at weaning.

Table 3
Number of differentially expressed genes (DEGs) in mammary gland tissue from female offspring at PND 21.

Group[a]	DEGs	Genes[b]	
		Down-regulated	Up-regulated
BPA25 *vs.* control	233	105 (45%)	128 (55%)
BPA250 *vs.* control	191	117 (61%)	74 (39%)
BPA25 *vs.* BPA25 + GEN	168	99 (59%)	69(41%)
BPA250 *vs.* BPA250 + GEN	386	87 (22%)	199 (78%)
BPA25 *vs.* BPA25 + I3C	119	49 (41%)	70 (59%)
BPA250 *vs.* BPA250 + I3C	135	73 (54%)	62 (46%)

[a] BPA25 μg/kg bw/day; BPA250 μg/kg bw/day; BPA25 μg/kg bw/day *plus* GEN 250 mg/kg chow; BPA250 μg/kg bw/day *plus* GEN 250 mg/kg chow; BPA25 μg/kg bw/day *plus* I3C 2000 mg/kg chow and BPA250 μg/kg bw/day *plus* I3C 2000 mg/kg chow.
[b] Number (percentage) of down or up-regulated genes.

The oral treatment with BPA25 and 250 did not alter the body weight of dams and female offspring, reproductive performance or normal maternal or nursing behavior. Furthermore, the association of GEN or I3C with BPA did not modify these parameters. Similar findings on reproductive performance and neonatal outcomes have been recorded by others studies in which the same BPA doses were administered (Murray et al., 2007; Moral et al., 2008; Betancourt et al., 2010).

The differentiation status of the mammary gland is a critical factor for the risk of tumor development, and TEBs and TDs are considered prime targets for chemical carcinogens (Russo and Russo, 1996). Several endocrine disrupters and/or dietary occurring compounds can act as modulators on mammary gland development mainly on TEBs and ducts densities, resulting in lower or higher tumor susceptibility in adulthood (Russo and Russo, 1996; Hilakivi-Clarke, 2007). A suitable increase in TEB number or densities has been considered a major risk factor for increased susceptibility to chemically-induced mammary carcinogenesis in rodents (Russo and Russo, 1996). In our experiment, BPA25-exposed offspring had a higher number of TEBs + TDs, mammary gland branching and cell proliferation indexes in TEBs at PND 21 than control animals. These results are in agreement with other studies in which the adverse effects of BPA exposure on mammary gland maturation and development have been investigated in both female mice and rats (Muñoz-de-Toro et al. 2005; Murray et al., 2007; Durando et al., 2007; Moral et al., 2008; Jenkins et al., 2009; Betancourt et al., 2010; Ayyanan et al., 2011; Acevedo et al., 2013). In mice, BPA exposure increased the number of TEBs and the percentage of epithelial cells expressing the progesterone receptor on PND 30 (Muñoz-de-Toro, et al., 2005; Murray et al., 2007; Ayyanan et al., 2011). Furthermore, in SD rats Moral et al. (2008) also reported a significant increase in TDs and TEBs in BPA250-treated group when compared to control or control and BPA25 at PND 21 or PND 100, respectively. Depending on the experimental design, windows of exposure, dose range and rodent strain used BPA can induce either a dose-response or a U-response/non-monotonic dose response curve (Vandenberg, 2014; Camacho et al., 2015). In the present study, the intrauterine exposure to an environmentally low dose of BPA (25 μg/kg bw/day) rather than to a high dose (250 μg/kg bw/day) had an effective deleterious effect on the development of the mammary gland structures, cell proliferation in TEBs and global gene expression at PND 21.

Plasma concentrations of 50–800 ng/mL have been found for GEN in adults who consume modest amounts of soyfoods (Setchell and Cole,

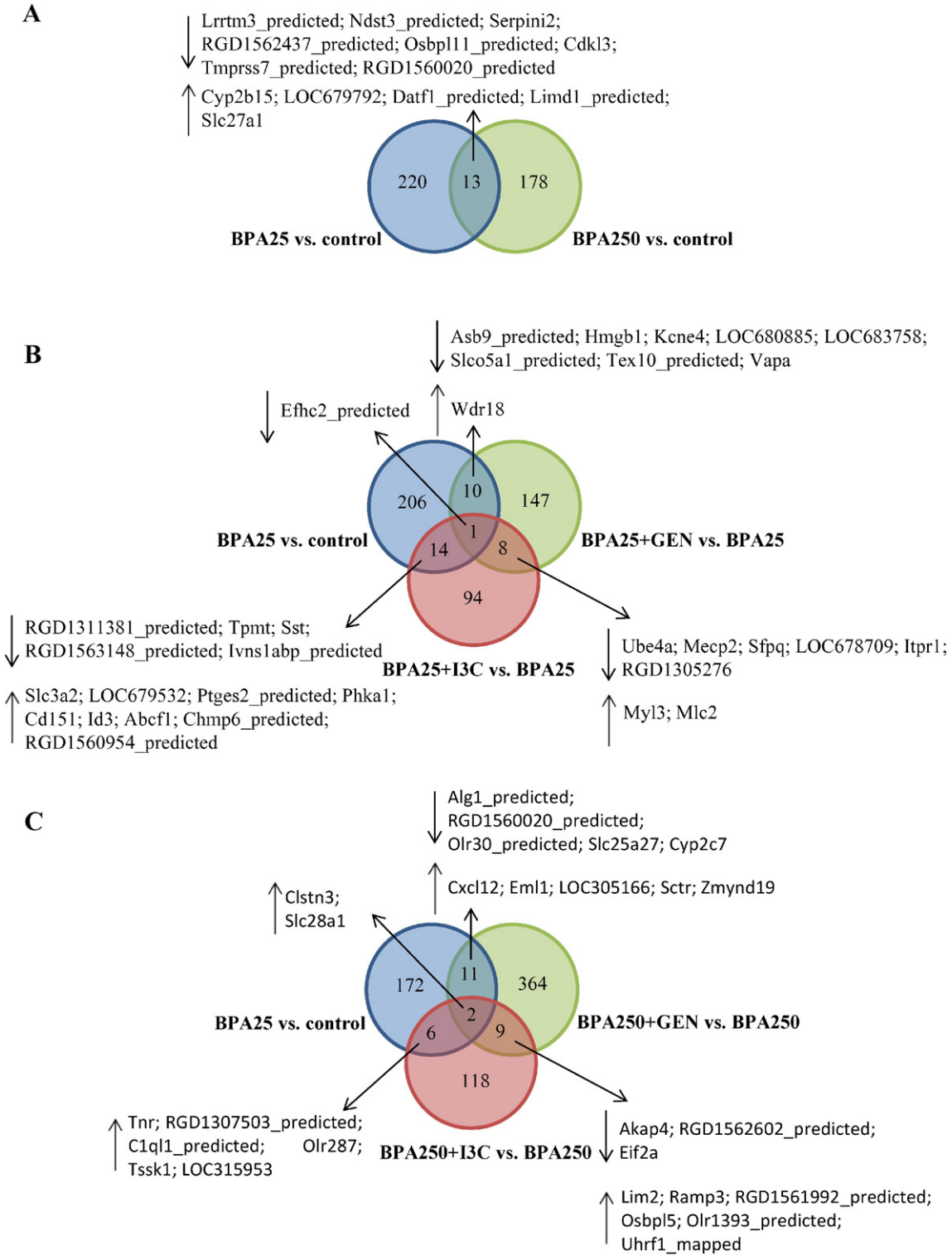

Fig. 1. Venn diagrams across different treatments. A) Control *vs.* BPA25 and BPA250; B) BPA25 *vs.* BPA25 + GEN and BPA25 + I3C, *Lrrtm3_predicted discordant between BPA25 and BPA25 + GEN; C) BPA250 *vs.* BP50 + GEN and BPA250 + I3C, *Pitx2 discordant between BPA250 and BPA250 + GEN; ↑ up-regulated genes; ↓ down-regulated genes.

2003). High intake of isoflavones increases 2-hydroxylation of estradiol, which results in estrogen derivatives that are mostly non- or weakly estrogenic (Xu et al., 2000) and lower risks for mammary cancer development due to epigenetic changes that result in alterations in the expression of genes that regulate mammary gland development (Hilakivi-Clarke et al., 2010). Fritz et al. (1998) showed that maternal dietary GEN (25 and 250 mg/kg chow) from conception until weaning significantly reduced the number of TEB and TD in the mammary epithelial tree in a dose response manner while diminished the susceptibility to mammary cancer induced by dimethylbenzo(a)anthracene (DMBA) administration in female SD rats at PND 50. In contrast, Su et al. (2007) did not observe a protective effect of maternal dietary GEN (250 mg/kg chow, GD4 until PND 0) against tumor development induced by *N*-methyl-*N*-nitrosourea (MNU) injection at PND 51. In addition, prepubertal combinational exposure through lactation (PND 2–20) to dietary GEN (250 mg/kg/chow) and BPA250 increased

Table 4
Genes and function present in the enriched Gene Ontology biological processes modulated in group BPA25-treated group in relation to control group.

Cluster 04	Function	Gene symbol	Gene name	Log ratio
GO:0048754	Branching morphogenesis of a tube	Gli3	GLI-Kruppel family member GLI3	−1.396849062
		Dag1	Dystroglycan1	1.267278348
		Cxcr4	Chemokine (C-X-C motif) receptor 4	1.873692277
		B4galt1_mappe d	UDP-Gal:betaglcnacbeta 1.4-galactosyltransferase, polypeptide 1 (mapped)	1.310962523
GO:0,060,429	Epithelium development	Gli3	GLI-Kruppel family member GLI3	−1.396849062
		Wdr77	WD repeat domain 77	1.544653162
		Dag1	Dystroglycan1	1.267278348
		Cxcr4	Chemokine (C-X-C motif) receptor 4	1.873692277
		B4galt1_mapped	UDP-Gal:betaglcnacbeta 1.4-galactosyltransferase, polypeptide 1 (mapped)	1.310962523
		Id3	Inhibitor of DNA binding 3	1.681040737
GO:0043068	Positive regulation of programmed cell death	Klk12	Glandular kallikrein 12, submandibular/renal	−1.428907864
		Zbtb16	Zinc finger and BTB domain containing 16	−1.814511484
		Cdkn1a	Cyclin-dependent kinase inhibitor 1A	−2.034175959
		Nupr1	Nuclear protein1	−1.705940956
		B4galt1_mapped	UDP-Gal:betaglcnac beta 1,4-galactosyltransferase, polypeptide 1 (mapped)	1.310962523
		Id3	Inhibitor of DNA binding3	1.681040737
		Dusp1	Dual specificity phosphatase1	1.539920298
GO:0010942	Positive regulation of cell death	Klk12	Glandular kallikrein 12, submandibular/renal	−1.428907864
		Zbtb16	Zinc finger and BTB domain containing 16	−1.814511484
		Cdkn1a	Cyclin-dependent kinase inhibitor 1A	−2.034175959
		Nupr1	Nuclear protein1	−1.705940956
		B4galt1_mapped	UDP-Gal:betaglcnac beta 1.4-galactosyltransferase, polypeptide 1 (mapped)	1.310962523
		Id3	Inhibitor of DNA binding 3	1.681040737
		Dusp1	Dual specificity phosphatase1	1.539920298
GO:0048610	Reproductive cellular process	Zbtb16	Zinc finger and BTB domain containing 16	−1.814511484
		Wdr77	WD repeat domain 77	1.544653162
		Cxcr4	Chemokine (C-X-C motif) receptor 4	1.873692277
		B4galt1_mapped	UDP-Gal:betaglcnac beta 1.4-galactosyltransferase, polypeptide 1 (mapped)	1.310962523
GO:0006950	Response to stress	Cdkn1a	Cyclin-dependent kinase inhibitor 1A	−2.034175959
		Tnfsf4	Tumor necrosis factor (ligand) superfamily, member 4	−1.644391065
		Gli3	GLI-Kruppel family member GLI3	−1.396849062
		Nupr1	Nuclear protein1	−1.705940956
		Hspa8	Heat shock protein 8	1.586261729
		B4galt1_mapped	UDP-Gal:betaglcnac beta 1.4-galactosyltransferase, polypeptide 1 (mapped)	1.310962523
		Kcnj8	Potassium inwardly-rectifying channel, subfamily J, member 8	1.272570797
		Slc27a1	Solute carrier family 27 (fatty acid transporter), member 1	1.6728672
		Id3	Inhibitor of DNA binding 3	1.681040737
		Ercc8_predicted	Excision repair cross-complementing rodent repair deficiency, complementation group 8 (predicted)	1.355009677
		Cxcr4	Chemokine (C-X-C motif) receptor 4	1.873692277
		Akr1b4	Aldo-keto reductase family 1, member B4 (aldose reductase)	1.390182225
		Ctsd	Cathepsin D	1.265916243
		Dna2l_predicted	DNA2 DNA replication helicase 2-like (yeast) (predicted)	1.289748637
		Dusp1	Dual specificity phosphatase1	1.539920298
		Loc681996	Similar to aha1, activator of heat shock 90kda protein atpase homolog 1	1.338701441
GO:0008285	Negative regulation of cell proliferation	Zbtb16	Zinc finger and btb domain containing 16	−1.814511484
		Cdkn1a	Cyclin-dependent kinase inhibitor 1A	−2.034175959
		Nupr1	Nuclear protein1	−1.705940956
		Gli3	GLI-Kruppel family member GLI3	−1.396849062
		Wdr77	WD repeat domain 77	1.544653162
		B4galt1_mapped	UDP-Gal:betaglcnac beta 1.4-galactosyltransferase, polypeptide 1 (mapped)	1.310962523
GO:0030855	Epithelial cell differentiation	Id3	Inhibitor of DNA binding 3	1.681040737
		Wdr77	Wd repeat domain 77	1.544653162
		Cxcr4,	Chemokine (C-X-C motif) receptor 4	1.873692277
		B4galt1_mapped	UDP-Gal:betaglcnac beta 1,4-galactosyltransferase, polypeptide 1 (mapped)	1.310962523
		Dag1	Dystroglycan1	1.267278348
		Cxcr4	Chemokine (C-X-C motif) receptor 4	1.873692277
		B4galt1_mapped	UDP-Gal:betaglcnac beta 1,4-galactosyltransferase, polypeptide 1 (mapped)	1.310962523

GO = Gene Ontology.

cell proliferation and reduced apoptosis in PND 21 rats, but reduced cell proliferation and increased apoptosis in PND50 rats (Wang et al., 2006). The results of the present study showed that gestational GEN associated with BPA treatment did not result in important changes in gene expression and mammary gland development in the female offspring at PND 21. However, mammary gland morphology and cell proliferation indexes in TEB in BPA25 + GEN group were more similar to the control group than BPA25 alone.

I3C has also showed protective effects against experimental mammary carcinogenesis when administered before or after carcinogen administration (Malejka-Giganti et al., 2007; Lubet et al., 2011). In hormone-dependent mammary cancer cell lines, I3C (10–250 μM) suppressed responsiveness to estrogen and decreased the expression of ER-α (Meng et al., 2000; Auborn et al., 2003; Wang et al., 2006; Marconett et al., 2012). In clinical trials, daily intake of I3C (300–800 mg) increased the levels of some important metabolizing enzymes (glutathione-*S*-transferase and CYP1A2, and the 2-hydroxyestrone:16-hydroxyestrone ratio) and estrogen metabolites without causing adverse effects (Michnovicz et al., 1997; Reed, 2005). In addition, some rodent studies showed that dietary I3C (2000 mg/kg), when administered during gestation or gestation/lactation periods, significantly reduced adverse effects of maternal BPA and dibenzo[*a*,*l*]pyrene exposures in prostate and lung at adulthood, respectively (Yu et al., 2006; Brandt et al., 2014). The results of the present study showed that gestational I3C

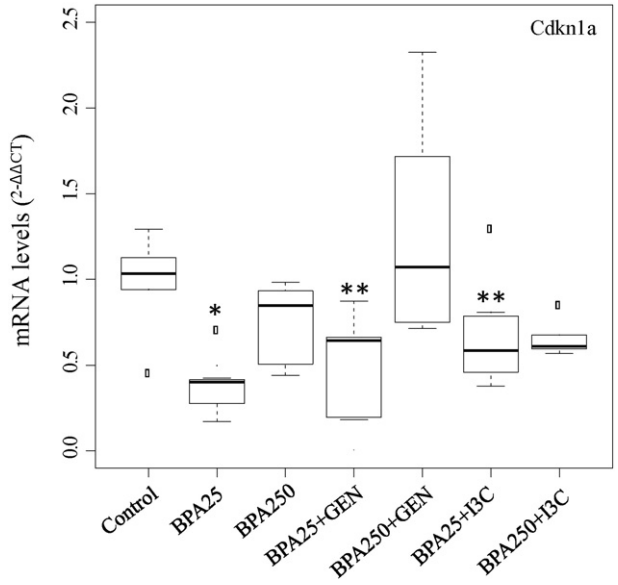

Fig. 2. Gene expression analysis by qRT-PCR for gene Cdkn1a. Values are Mean ± SD; * Significantly different from Control *vs.* BPA25 ($p < 0.02$); ** Significantly different from BPA25 *vs.* BPA25 + GEN and BPA25 *vs.* BPA25 + I3C($p < 0.02$).

associated with BPA treatments did not result in important changes in mammary gland development, but induced several important enriched gene functional categories at PND 21. In addition, mammary gland morphology and cell proliferation indexes in TEB in BPA25 + I3C group were more similar to the control group than BPA25 alone.

A few rodent studies have investigated the modulation of the gene expression signatures in the mammary gland from female offspring after maternal exposure to BPA, including the two doses and window of exposure evaluated in the present study (Moral et al., 2008; Wadia et al., 2013, Dhimolea et al., 2014; Camacho et al., 2015). Moral et al. (2008) reported that both doses of BPA (25 and 250 μg/kg bw/day) changed the gene expression signature in the mammary gland; BPA25 had the highest effect on the number of gene modulated at 50 days, whereas BPA250 had the highest influence on gene expression 100 days after maternal BPA administration. Dhimolea et al. (2014) investigated the gene expression profile and epigenetic alterations in the mammary gland from female Wistar-Furth rats exposed to BPA250 during GD 9 until PND1. The results of this study showed that fetal exposure to BPA induces large scale epigenetic changes in the rat mammary gland, with the majority of epigenetic changes occurring at PND21, while transcriptional analysis revealed that the majority of gene expression differences between BPA-treated and control animals were observed at PND50. Camacho et al. (2015) reported that several genes were found to be modulated in the mammary gland from female SD offspring at PND4 whose dams were exposed dose range (2.5–300,000 μg/kg bw/day) from GD6 until parturition. The findings of this study using microarray analysis in female mammary gland showed alterations in several genes in the "low BPA" dose, but the lack of a dose–response reduces the likelihood that these effects were causally linked to the treatment. Our results also indicated a lack of a dose-response considering the number of differentially expressed genes or cluster of genes altered, but a higher number of functional categories were observed in BPA250 in relation to the lower dose group (19 *vs.* 30, respectively). In BPA25 group, there is a cluster that present enriched GOs pointing to important gene categories associated to the mammary gland development (branching morphogenesis of the tube, epithelium development and epithelial cell differentiation and regulation of cell proliferation and death). In this functional category, the *Cdkn1a* (Cyclin-dependent kinase inhibitor 1A) was down-regulated while *Dag1* (Dystroglycan1) and *Cxcr4* (chemokine (C-X-C motif) receptor 4) were up-regulated. In special, the CDKN1A upregulation in BPA25 + GEN and BPA25 + I3C could be associated to protective effects of these natural compounds since that *Cdkn1a* gene is a tumor suppressor gene which contributes to mammary gland development and tumorigenesis (Cariou et al., 2000; Schaefer et al., 2010).

In conclusion, the findings of present study showed that prenatal BPA exposure BPA25, but not BPA250 induced morphological changes in the mammary gland at PND 21. These alterations were accompanied by modifications in gene expression in different functional categories associated with mammary gland development and branching. Dietary intake of GEN and I3C induced a different gene expression signature than BPA 25-exposed animals and countered its deleterious effect on the branching of the mammary gland on PND21. Considering that humans are exposed to thousands of chemicals through environment and diet, a critical analysis of modifying effects of complex chemical mixtures and their physiopathological effects in humans need to be undertaken to create a more reliable risk assessment of EDCs.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2016.05.004.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document related to this article can be found, in the online version.

Acknowledgements

Tony F. Grassi, Bruna F. Rossi, Marilia M. Quinalha and Lucas T. Bidinotto were recipients of fellowships from FAPESP (2009/17894-9; 2011/14655-3; 2011/06219-9; 2011/08523-7, respectively). Luis F. Barbisan was recipient of support research from FAPESP (2010/14110-4). We acknowledge Dra. Daisy Maria Fávero Salvadori (Botucatu Medicine School, UNESP) for useful microarray advice.

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