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Perinatal exposure to bisphenol A impacts in the mammary gland morphology of adult Mongolian gerbils

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Abstract

The endocrine disruptive effects caused by bisphenol A (BPA) are well known. Despite this, to date, evaluation of its long term effects is limited, meaning that there is still much to be unveiled in terms of alterations caused by perinatal exposure to BPA. Our aim was to determine if perinatal exposure to two different doses of BPA causes long term morphological and molecular alteration effects in the mammary gland (MG). We evaluated MG from Mongolian gerbil offspring exposed perinatally (during gestation and lactation) to 50 or 5000 µg/kg/day BPA. At 90 days of age the animals were subjected to a single dose of N-nitroso-N-methylurea in order to mimic a carcinogenic environment. At 6 months of age, animals in estrous were euthanized for morphological evaluation of the MGs. The MG architecture presented considerable changes in terms of detached epithelial cells, inflammation, glandular hyperplasia, and collagen fiber deposition. Furthermore, a higher index of epithelial cell proliferation was detected in comparison to the intact control group. In addition, we verified a higher molecular expression of EZH2 in the vehicle treated group, indicating that corn oil applied alone can alter the expression of this epigenetic biomarker. In conclusion, BPA perinatal exposure promotes significant changes in glandular cytoarchitecture and increases glandular epithelium proliferation rate, leading to the retention of stem-like properties. This event could compromise the fate and differentiation potential of mammary epithelium.

Keywords: BPA, estrogen, environment pollutant, morphologic alterations, phospho-histone-h3, EZH2

Introduction

Bisphenol A (BPA) is a compound used to manufacture plastics and resins. The main polycarbonates containing this compound are food and water storage containers. BPA residues may still be present in the epoxy resins used to make coatings inside food cans. The main concern is that BPA is released from its source to food and beverage stored in these containers (EFSA 2015). According to the European Food and Safety Authority (EFSA), the substance is known to induce morphological and proliferative changes in the mammary gland (MG). Consequences of BPA include estrogen-like effects that may induce a behavior similar to natural 17-β estradiol (Michałowicz, 2014); even more worrisome is the fact that BPA is able to bind to estrogen receptors, avoiding endogen estrogen access (Wetherill et al., 2007) and even causing distinct agonistic/antagonistic toxic effects (Zhuang et al., 2014). Some experiments have been developed in rodents and have revealed that, even at very low concentrations, BPA

may cause alterations related to hyperplasia in both male and female MGs (Mandrup et al., 2016).

During mammary organogenesis and development, estrogen signaling is critical; consequently, exposure to xenoestrogens during this period may cause lifelong negative impacts (Colborn et al., 1993). In rodents, the risk of MG tumor formation is considerably increased after in utero exposure to BPA (Durando et al., 2006). This mainly occurs because during this development period a dynamic environment takes place and the limits between epithelium and stroma with a basal membrane are ruptured (Cowin and Wysolmerski, 2010), facilitating free contact of precursor mammary epithelial cells with available BPA in the bloodstream.

In addition to nuclear receptor binding, the molecular mechanisms involved in BPA action in the reproductive tract include alterations in hormone metabolism, and genetic and epigenetic regulation (Acconcia et al., 2015). BPA is known to decrease expression of CYP11A1 and StAR, two rate-limiting factors for steroidogenesis, in rodent ovarian follicles and to increase the metabolism of natural hormones (Peretz and Flaws, 2013), these being important means by which this disruptor influences hormone-dependent signaling pathways.

Although altered morphological phenotypes are frequently seen in the microenvironment of exposed MGs (Hindman et al., 2017), other alterations may cause a more silent effect, consisting of epigenetic alterations that take longer to be evidenced at the morphological level but are persistent in changing gene expression (Doherty et al., 2010). These changes may modify chromatin structure and tumor suppressing regions can be methylated and lead to tumor formation (Jones and Baylin, 2002). Thus, environmental pollutants can trigger epigenetic changes in parents, that can be transferred to the offspring (Acconcia et al., 2015) and this risk has previously been linked to predisposition to diseases later in adulthood (Chiam et al., 2009).

Since mammary organogenesis requires these interactions between epithelium and stroma (Sakakura et al., 2013), the relationship between these compartments should also be explored and evaluated in terms of extracellular matrix deposition and alterations caused by BPA treatments. Many years ago the hypothesis was raised that mammary epithelial carcinogenesis arises from indirect effects of carcinogens on stroma (Steinmuller, 1971). Even in more recent years the possible roles of stroma signals have attracted interest in terms of other strands for cancer research and may consist of important targets for designing anti-cancer drugs (Sakakura et al., 2013).

The evaluation of different parameters in association is of extreme importance in terms of obtaining new knowledge regarding the pathological effects occasioned by carcinogenic compounds. Although many questions have been answered, there is still much to be explored regarding the long-term effects of BPA after in utero exposure. The use of an alternative experimental model, the Mongolian gerbil, has raised interest in the research field, suggesting an interesting species to assess the malignant effects of xenoestrogens in reproductive secreting organs such as the prostate (Facina et al., 2017; Gonçalves et al., 2017, 2013) and testes (Christante et al., 2018). Considering that endocrine disruptors have species-specific effects not yet widely explored in the MG, we decided to adopt this species to evaluate for the first time the consequences of BPA treatment. Thus, the aim of this experiment was to evaluate the long term deleterious effects caused by exposure to different doses of BPA during the early stages of development in the MG of Mongolian gerbils.

MATERIAL AND METHODS

Animals and ethics statement

A total of 20 pregnant Mongolian gerbils were used for this experiment. The animals were isolated in ventilated polysulfone cages and maintained in the Animal Breeding Center of the Institute of Biosciences, Humanities and Exact Sciences (IBILCE) of the São Paulo State University (UNESP). The temperature (24± 2ºC) and luminosity (12 h light/dark cycle) of the environment were controlled and all the equipment used was free from BPA, according to the manufacturer's specifications (Alesco, Monte Mor, Brazil). The animals received specific balanced feed (Presence, Paulínia, Brazil) and fresh filtered water ad libitum. The procedures were conducted according to the standards defined by the National Council of Animal Experiment Control (CONCEA) and were authorized by the Ethics Committee on the Use of Animals (CEUA) from IBILCE/UNESP (protocol number 113/2015).

Experimental design

The experimental design is illustrated in Figure 1. Briefly, the pregnant animals were divided into four groups according to the treatment received. The first group (IC) did not receive any treatment during the period; the second group (OC) was subjected to daily treatment with 0.1 ml of corn oil (Mazola, Mairinque, Brazil) by gavage; and the third (↓BPA, 50 µg/kg) and fourth (↑BPA, 5000 µg/kg) groups received daily doses of BPA also by gavage diluted in 0.1 ml of corn oil. The manipulations were performed from the 8th day of gestation until the last day of

lactation (total 46 days of treatment). The treatment was suspended only on the parturition day. On each day the gavage was performed during the morning.

After weaning, two females obtained from each offspring were used for future analysis; other individuals obtained in the same family were used in other experiments. The selected females were maintained under the previously described conditions until 90 days of age. At that point, half them received one single intraperitoneal application of N-nitroso-N-methylurea – NMU (1517, Sigma Aldrich St. Louis, USA); the other female from the same family was kept as a negative control for this treatment. After 90 further days, all animals were anesthetized with xylazine (3 mg/kg) and ketamine (10 mg/kg) and euthanized by decapitation. The procedures were performed during the morning with the animals always in the estrous phase of the cycle.

Histological processing and cytochemical staining

During the euthanasia procedures, the weight of the whole body, liver, and ovaries + uterus complex were evaluated. These organs were also evaluated for the possible presence of macroscopic lesions. The MGs were collected for analysis. The right abdominal gland was carefully removed and immediately fixed with paraformaldehyde 4% for 24 hours. Next, the samples were transferred to alcohol 70% and histologically processed under a semi enclosed system (TP1020, Leica Biosystems, Buffalo Grove, USA). The paraffin blocks were sliced (4 µm thick) and slides were mounted. Cytochemical staining was performed with hematoxylin and eosin (HE) as well as with picrosirius to evaluate the morphological alterations in tissues from different groups.

For the picrosirius technique, the slides were dehydrated and stained for 1h in Sirius Red solution (Direct Red 80 diluted in saturated picric acid solution). After washing, the slides were counterstained with hematoxylin for 10 minutes, dehydrated, and mounted.

Analysis and quantification of histopathological alterations

The incidence and multiplicity of lesions were evaluated in all animals. Three sections from different depths of each block were stained with HE and picrosirius (n=5) and digitized at 400x magnification using a BX61VS camera (Olympus Corporation, Tokyo, Japan) coupled to an Olympus VS120 Virtual Microscope Slide Scanning System (VS120-S5) from the same company.

Since neither malignant nor premalignant lesions have been previously described in the MG of Mongolian gerbils, the bases for our classification were publications describing these alterations in rats and mice (Medina, 2008; Rudmann et al., 2012; Russo et al., 2000; Singh et

al., 2000). Tissue sections were evaluated blindly according to the experimental group. The incidence of lumen with leukocytes or detached epithelial cells, as well as inflammation foci and hyperplasia were evaluated.

The picrosirius stained slides were analyzed in a light microscope coupled to a polarized light, which allowed identification of the birefringence of collagen fibers due to their different molecular composition. Thirty random microscopic fields per group were acquired and evaluated according to the birefringence spectrum. Stereological analysis was carried out to determine the frequency of collagen patterns according to the M130 multipoint test system (Weibel, 1963). Points coinciding with areas of yellow or red, epithelial cells or stroma were counted, for calculation of the frequency as a percentage over the total counted points. This analysis aimed to estimate the deposition of collagen fibers in the secreting structures of the MG.

Immunohistochemistry

The primary antibodies used were anti-α-actin (mouse monoclonal, 1A4, 1:100, sc-32251, Santa Cruz Biotechnology, Dallas, TX, USA); anti-phospho-histone H3 (P-H-H3, rabbit polyclonal, H3, 1:75,Ser10, 9701, Cell Signaling, Danvers, USA); and anti-enhancer of zeste homolog 2 (EZH2,rabbit monoclonal, 5246s, 1:100, Cell Signaling, Danvers, USA). Endogen peroxidases were blocked by immersing the slides in 3% H_2O_2 at room temperature for 30 minutes. Antigen retrieval was performed by immersing the tissue sections in 10mM citrate buffer pH 6.0; the temperature and time for retrieval were 96°C for 40 minutes for P-H-H3, 93°C for 20 minutes for α-actin, and 98°C for 75 minutes for EZH2. Next, nonspecific proteins were blocked using 10% normal goat serum + 1% bovine serum albumin (BSA) in TBS for EZH2, 5% BSA in TBS for P-H-H3, and 5% skimmed milk in PBS for α-actin. The sections were incubated overnight at 4°C (P-H-H3 and EZH2) or for 1h at room temperature (α-actin) before secondary antibodies (EZH2: Envision anti-rabbit, HRP, K4003, DAKO-Agilent, Santa Clara, USA; P-H-H3 and α-actin: Polymer kit Novocastra Novolink RE7230-CE, Leica Biosystems, Buffalo Grove, USA) were applied for 1h at room temperature. Subsequently, positive staining was detected using 3-30'-diaminobenzidine tetrahydrochloride (DAB) chromogen (NovolinkMaxDAB, RE7270-CE, Leica Biosystems, Buffalo Grove, USA) and counterstained with hematoxylin.

Determination of α-actin expression, cell proliferation index, and EZH2 positive cells

For α-actin, thirty random macroscopic fields were digitized at 400x magnification and stereological analysis was carried out to determine the frequency of myoepithelial cells according to the M130 multipoint test system.

Thirty microscopic fields per group (n=5) were analyzed to determine the relative frequency of P-H-H3 and EZH2 positive cells in MG tissue. The cell proliferation index and percentages of EZH2-positive cells were calculated by dividing the number of positive nuclei by the total number of nuclei counted. The results are expressed as percentages.

Protein expression quantification

The protein content of the left abdominal MG sample was extracted with a lysis buffer according to the protocol described by Li et al (2009); the protease activity was blocked by adding an inhibitor. Samples were smashed and left for extraction at 4ºC for 1h under agitation, before being centrifuged for 20 minutes at 14000 rpm at 4ºC. The extracts were stored at -80°C until analysis. For quantification, a BCA Protein Assay Kit (23227 Thermo Fischer, Rockford, IL, USA) was used and the absorbance was read in an absorbance microplate reader (SPECTROstar Omega, BMG Labtech, Ortenberg, Germany).

EZH2 was quantified in MG samples by Western blotting. This marker was selected because of its association to aggressive breast cancer and regulation of metastasis and survival of cancer cells (Chien et al., 2018). A total protein amount of 15 µg was placed in each well in the SDS page gel and subjected to electrophoresis (120 V for 10 minutes then 170 for 30 minutes). The bands were transferred to a PVDF membrane (100 V for 1 hour). Blots were blocked using BSA 5% in TBS + 0.1% Tween (TBSt) for 1 hour at room temperature. Overnight incubation with anti-EZH2 (rabbit monoclonal 5246s Cell Signaling, Danvers, MA, USA, 1:1000) and GAPDH (mouse monoclonal MB374 Millipore, Burlington, MA, USA, 1:10000) diluted in BSA 1% in TBSt was performed. Then the membranes were washed in TBSt and incubated at room temperature for 1 hour under agitation with horseradish peroxidase conjugated secondary antibodies (goat anti-mouse 115-035-062 or goat anti-rabbit 115-035-144, Jackson Immunoresearch, West Grove, PA, USA, 1:10000). After washing, the membrane antibody detection was revealed with ECL Substrate Pierce (32109 ThermoFischer, Rockford, IL, USA) and Super Signal (34094 ThermoFischer, Rockford, IL, USA) reagents. The membranes were revealed in a Fusion machine (Fusion Solo VilberLourmat, Marne-la-Vallée, France) and the band densities were analyzed in a densitometry program – Image J (version 1.52a, Wayne Rasband, NIH, USA).

Statistical analysis

The statistical analysis was performed using the GraphPad Prism 5.0 software (GraphPad 5.0, Inc, CA, USA). The quantitative analysis was based on parametric (ANOVA) or non-parametric (Kruskal-Wallis) analysis followed by Tukey's or Dunn's multiple comparison tests, respectively. Results were considered significant at the $p<0.05$ level.

RESULTS

Histopathological alterations

Evaluation of the animals during the maintenance period did not reveal the presence of any macroscopic lesions in the regions of the MGs when palpation was performed. During euthanasia procedures, there were no differences between the groups regarding the weights obtained; the presence of a swollen uterus and some cysts in the ovaries being the only noteworthy alteration observed in some cases.

The incidence of morphological alterations showed some significant differences between the evaluated groups (Figure 2). The values are shown in Table 1. In summary, the highest values were found in the ↓ BPA group, where a higher incidence of leukocytes was present in comparison to the OC and ↑ BPA groups (Figure 2A,B). The incidence of detached epithelial cells was also higher in the ↓ BPA group without NMU in comparison to all other groups non-treated with NMU (Figure 2C-E)

Signs of inflammation were characterized by the presence of inflammatory cells among the conjunctive tissue or through the epithelial structures (Figure 2G-H). This was more frequent in the ↓ BPA group non-treated with NMU in comparison to both the IC groups.

Hyperplasia was characterized by the presence of two or more layers of epithelial cells in the acini or ducts, sometimes covering the totality of the structure (Figure 2J-K). Its incidence differed between the ↑ BPA and IC groups and was also higher in the ↑ BPA and ↓ BPA groups in comparison to the OC (Figure 2I).

The results showed that, with the exception of hyperplasia in the OC group and inflammation in the ↓ BPA group, no differences were found between animals treated or not with NMU regarding morphological alterations. This led us to perform the remaining evaluations only in animals from the control subgroup (not treated with this compound) to evaluate the effects caused by each treatment.

The deposition of collagen in the stroma and among the adipose tissue was quantified and is shown in Figure 3. A higher incidence of collagen was found in the IC (21.5±2.8) in comparison with the OC (12.5±0.7) and ↑ BPA (13.4±0.5) groups. The ↓ BPA group (18.7±1.9) did not differ from any other group.

Expression of α-actin

The maintenance of physiological morphology in the secretory units was complemented with α-actin identification by means of immunohistochemistry. As expected, the myoepithelial cells were present in the compartment surrounding the basal lamina; no staining was found in the other compartments. The analysis showed that, in general, the structures were well preserved and with the myoepithelial layer maintained, therefore there were no differences between the groups regarding the presence of this protein in the tissue evaluated. Illustrative pictures and the quantification of α-actin are shown in Figure 4.

Cell proliferation index

The quantification of positive cells for P-H-H3 revealed that all groups treated with BPA demonstrated increased proliferation indices in comparison with the intact control group. The means ± standard error as well as illustrative pictures are shown in Figure 5.

Expression of EZH2: immunohistochemistry and Western Blotting

The quantifications and illustrative photomicrographs of EZH2 are shown in Figure 6. Although a slight increase in positive cells was observed in the OC group, the statistical analysis did not show any differences between the treatments in terms of positive cells for EZH2 (Figure 6A). This led us to go further through the analysis and evaluate the total amount of this protein in the extracts obtained from the samples through western blotting. The results demonstrated that the amount of protein expressed in the MGs from the group treated with oil was significantly higher than the intact control group (Figure 6C).

DISCUSSION

The results described in the present study showed that perinatal exposure to 50 or 5000 µg/kg of BPA induced an increase in the incidence of morphological alterations in the MGs after 6 months of age: detached epithelial cells and leukocytes in lumen, inflammation, collagen deposition, and hyperplasia modifications were described. Despite that, the gland architecture as observed through myoepithelial cell presence and organization was not altered in any of the groups. A prominent difference, however, was present in the proliferation index of both BPA

treated groups in comparison to the intact control group. Another interesting finding was the increase in EZH2 expression in the OC group, suggesting that treatment with the vehicle usually adopted for dilution and administration of lipophilic compounds increases the risk of breast cell malignancy and cancer development (Doherty et al., 2010).

The presence of detached cells was high in the ↓ BPA and low in the OC group (both without NMU). Although the presence of detached cells indicates that apoptosis is taking place in the gland – according to the morphology – the accumulation in lumen may indicate deregulations related to malignant progression (Green and Streuli, 2004). The main mechanism for their removal is phagocytosis by macrophage or neighbor cells (Gardai et al., 2006; Monks et al., 2008), which is in accordance with the increased presence of leukocytes in the MG lumen of the ↓ BPA group.

An important rise in the incidence of leukocytes in lumen was present in the ↓ BPA group, with or without NMU. Immune system cells are known to protect tissue against pre-malignant alterations, destroying cells and suppressing tumor growth (Hanahan and Coussens, 2012). The higher incidence of lumen with leukocytes could indicate that cells from the immune system may have been recruited, aiming to control a possible tumorigenic environment, controlling the presence of luminal apoptotic epithelial cells, for example. Similarly, inflammation was more frequently present in the ↓ BPA group in comparison to the IC. This corroborates the results of Fischer et al (2016) who stated that mice perinatally treated with this compound demonstrated deregulation of inflammatory cytokines that may lead to disordered cell growth (Fischer et al., 2016). The authors suggest that this may occur due to changes in the immune response targeting cancer cells.

As shown in Figure 2, hyperplasia was the most frequent alteration found. This lesion is considered a sentinel sign of malignancy, since the proliferation of epithelial cells, sometimes filling the lumen, is the earliest sign of a ductal or lobular carcinoma in situ (Keely, 2011; Singh et al., 2000). This alteration occurs due to a reduction in the apoptotic levels of ductal cells that may be related to reduced pro-apoptotic factors (Mailleux et al., 2007). This corroborates the previously proposed hypothesis of an apoptotic/phagocytic unbalance, possibly leading to malignant progression.

The ability of perinatally administered BPA to cause hyperplasia leading to ductal carcinoma has already been seen in Wistar rats (Gomez et al., 2017). In that experiment the authors described the presence of a typical lobular hyperplasia in a higher incidence after treatment with lower concentrations (0.5 µg/kg/day) versus high concentrations (50µg/kg/day) of BPA.

Similarly to rats (Soto et al., 2008) other species, such as mice (Vandenberg et al., 2008) and rhesus monkeys (Tharp et al., 2012), showed proliferative disturbances in the adult MG after BPA perinatal exposure. In the present study, the main differences were present between the↑ BPA with NMU and control groups without NMU (IC and OC), suggesting that although the ↓ BPA group showed higher impacts regarding other morphological alterations, a larger hyperplastic response was given by the 5000 µg/day exposure to BPA.

Although the majority of experimental groups demonstrated higher means of hyperplasia incidence after treatment with NMU, only the OC group presented a significant difference. In the same way, the incidence of inflammation was higher only in the ↓ BPA group without NMU in comparison to the non-NMU-treated group. Since previously published experiments described the presence of malignancy and tumors in Mongolian gerbils after NMU intraperitoneal administration (Gonçalves et al., 2013, 2010, Quintar et al., 2017), we expected the presence of neoplasia in this experiment. However, as previously described experiments did not test the use of NMU in female gerbils we hypothesize that in this case the time necessary to develop mammary neoplasia is longer, female rats (Cha et al., 1994) usually show macroscopic lesions after 90 days of NMU administration. Nevertheless, it is known that susceptibility to NMU carcinogenesis may vary according to species (Medina, 2007) and that mice do not develop mammary tumors unless hormonally stimulated; therefore, the hormonal milieu at the time of NMU treatment influences most the incidence and type of tumor (Guzman et al., 1992). Another hypothesis is that perinatal exposure to BPA induces a marked action, in terms of morphological alteration, during specific hormonal stages such as gestation and lactation. These findings suggest that, in Mongolian gerbils, the development of female mammary tumors requires more time to occur or a higher dose of NMU.

The absence of substantial differences after NMU treatment and higher lesion incidences in the BPA treated groups led us to focus attention on the effects caused only by this hormonal disruptor. Other morphological and molecular markers were then evaluated exclusively in the animals not exposed to NMU.

The deposition of collagen fibers in the stroma indicates that the state of the architecture and function of the gland and extracellular matrix (ECM) remodeling is directly related to the proliferative and invasive state of tumors (Keely, 2011). This is the reason why so much importance is given to periodic mammographic examinations in adult women (Huo et al., 2015). We chose picrosirius staining, which gives red/yellow birefringence according to the crosslinking stage of the collagen fibers. Our results showed higher percentages of collagen

deposition in the ECM of the IC group, probably meaning that the capacity of ECM to maintain its function of tumor suppression was decreased in the OC and ↑ BPA groups. On the other hand, the gland architecture in terms of the basal membrane was maintained, as shown by α-actin marking and no differences on its expression index were found between the groups. According to Thompson et al. (2017), there is a higher expression of α-actin in the MG of caveolin-1 knockout mice, a protein that has been linked to tumor aggressiveness; in addition the expression of collagens was upregulated. The authors, though, justify this finding by the presence of additional ducts in the knockout model and not an increase in the thickness of the myoepithelial layer. For us the absence of an aberrant ECM may be a factor that contributed to avoid tumor progression since an adequate environment for tumor initiation was not present.

BPA is known for its developmental disturbance and the majority of the alterations are occasioned by its estrogen-agonist activities. It is probable that this is the reason why the significant result obtained referred to the higher incidence of proliferative cells in the treated groups, as shown by the P-H-H3 quantification in the epithelial mammary structures. The phosphorylation of histone H3 is directly linked to cell division (Hans and Dimitrov, 2001). Histones are fundamental compounds that contribute to build nucleosomes (Arents et al., 1991) and they phosphorylate both during transcriptional activation, when chromatin is uncondensed (Nowak and Corces, 2000), and during cell division with a compacted chromatin (de la Barre et al., 2000). Thus, P-H-H3 becomes a valuable tool to quantify cell proliferation since phosphorylation is detected in the heterochromatin at the late G2 phase and spreads through all chromosomes as mitoses develops (Hans and Dimitrov, 2001). This has been successfully adopted as a proliferation marker (Chow et al., 2017; Elmaci et al., 2017). It has previously been stated that exposure to xenoestrogens during fetal development induces alterations that change control of cell proliferation (Durando et al., 2006); thus, our results corroborate the hypothesis that perinatal exposure to BPA increases the sensitivity of the MG to endogenous estradiol, creating a permissive situation that facilitates malignancy (Muñoz-de-Toro et al., 2005).

Finally a last factor that raised interest in terms of MG alteration was the expression of EZH2 in the parenchyma. Epigenetic regulation produces heritable changes, as well in gene expression. EZH2 is the catalytic subunit of the polycomb repressive complex 2 (PRC2), a histone methyltransferase involved in tumorigenesis (Doherty et al., 2010). In this way, EZH2 is able to provide the methyltransferase activity resulting in the transcriptional silencing of target genes (Gulati et al., 2018). Mice with overexpression of EZH2 developed a disorganized ductal epithelium (Li et al., 2009). EZH2 knockout mice present a delay in ductal elongation during

puberty and in lobuloalveolar expansion in pregnancy (Michalak et al., 2013). No differences were found between the groups in the immunohistochemistry analysis; however, we verified that gestational and lactational exposure to corn oil caused a significant increase in the expression of EZH2, as evidenced by western blotting. This oil has been widely applied as a vehicle for administration of lipophilic compounds. Previously described experiments showed that gavage administration of corn oil produced elevated corticosterone levels in comparison to methylcellulose, Tween, or water used as vehicles (Brown et al., 2000). The authors add that administration with lipid vehicles induces activation of a stress response. We believe that there may be an interaction between BPA and the vehicle used, sometimes causing an alteration in the usual effects. A recent publication has described the protective effect in the liver when sesame oil is used for BPA administration (Elhamalawy et al., 2018). Corn oil is known to cause alterations in gene expression profiles in other organs, while no histopathological and cell counting alterations are seen (Geng et al., 2012). In this way, there is a possibility that the corn oil alone caused some alterations in EZH2 gene expression that did not reflect in other parameters evaluated.

The importance of evaluating the effects of BPA in experimental and clinical assays consists of raising attention turned towards this compound, aiming to reduce more and more the permitted limits of use in industries. Even though nowadays very low concentrations are present in plastic compounds, there are still high plasma and serum levels in the human population all over the world (Thoene et al., 2018). The United States Environmental Protection Agency (EPA) stated that BPA has the third priority place in terms of toxicity (Reif et al., 2010). Thus the more information available regarding the long term effects of this compound in the body the more attention will be given to this subject.

Our findings demonstrated that Mongolian gerbils showed considerable morphological alterations in MGs after perinatal exposure to BPA; furthermore, an increase was observed in the proliferation index, which could lead to the retention of stem-like properties. This event could compromise the fate and differentiation potential of mammary epithelium. In addition, we verified a higher molecular expression of EZH2 in the vehicle treated group, indicating that corn oil applied alone can also alter the expression of this epigenetic biomarker. Thus, the presence of BPA in the microenvironment of perinatal development negatively affects the architecture of MG and corn oil alone is a bigger threat in terms of epigenetic alterations in mammary tissue.

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Conflict of interest

The authors declare that there are no conflicts of interest.

REFERENCES

Acconcia, F., Pallottini, V., Marino, M., 2015. Molecular Mechanisms of Action of BPA. Dose-Response 13, 1559325815610582. https://doi.org/10.1177/1559325815610582.

Arents, G., Burlingame, R.W., Wang, B.C., Love, W.E., Moudrianakis, E.N., 1991. The nucleosomal core histone octamer at 3.1 A resolution: a tripartite protein assembly and a left-handed superhelix. Proc. Natl. Acad. Sci. U. S. A. 88, 10148–10152.

Brown, A.P., Dinger, N., Levine, B.S., 2000. Stress produced by gavage administration in the rat. J. Am. Assoc. Lab. Anim. Sci. 39, 17–21.

Cha, R.S., Thilly, W.G., Zarbl, H., 1994. N-nitroso-N-methylurea-induced rat mammary tumors arise from cells with preexisting oncogenic Hras1 gene mutations. Proc. Natl. Acad. Sci. U. S. A. 91, 3749–3753. https://doi.org/10.1073/pnas.91.9.3749.

Chiam, K., Tilley, W.D., Butler, L.M., Bianco-Miotto, T., 2009. The dynamic and static modification of the epigenome by hormones: A role in the developmental origin of hormone related cancers. Biochim. Biophys. Acta - Rev. Cancer 1795, 104–109. https://doi.org/https://doi.org/10.1016/j.bbcan.2008.12.001.

Chien, Y.C., Liu, L.C., Ye, H.Y., Wu, J.Y., Yu, Y.L., 2018. EZH2 promotes migration and invasion of triple-negative breast cancer cells via regulating TIMP2-MMP-2/-9 pathway. Am. J. Cancer Res. 8 (3), 422–434.

Chow, K., Tse, K., Cheung, C., Wong, K., Cheung, A.N.Y., Wong, R.W.C., Chan, A.N.H., Yuen, N.W.F., Ngan, H.Y.S., Ip, P.P.C., 2017. The mitosis-specific marker phosphohistone-H3 (PHH 3) is an independent prognosticator in uterine smooth muscle tumours: an outcome-based study. Histopathology 70, 746–755. https://doi.org/10.1111/his.13124.

Christante, C.M., Pinto-Fochi, M.E., Negrin, A.C., Taboga, S.R., Góes, R.M., 2018. Effects of gestational exposure to di-n-butyl phthalate and mineral oil on testis development of the Mongolian gerbil. Reprod. Fertil. Dev. https://doi.org/10.1071/RD17482.

Colborn, T., vom Saal, F.S., Soto, A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ. Health Perspect. 101, 378–384. https://doi.org/10.1289/ehp.93101378.

Cowin, P., Wysolmerski, J., 2010. Molecular Mechanisms Guiding Embryonic Mammary Gland Development. Cold Spring Harb. Perspect. Biol. 2(C), a003251. https://doi.org/10.1101/cshperspect.a003251.

de la Barre, A.-E., Gerson, V., Gout, S., Creaven, M., Allis, Cd., Dimitrov, S., 2000. Core histone N-termini play an essential role in mitotic chromosome condensation. EMBO J. 19, 379–391. https://doi.org/10.1093/emboj/19.3.379.

Doherty, L.F., Bromer, J.G., Zhou, Y., Aldad, T.S., Taylor, H.S., 2010. In utero exposure to diethylstilbestrol (DES) or bisphenol-A (BPA) increases EZH2 expression in the mammary gland: an epigenetic mechanism linking endocrine disruptors to breast cancer. Horm. Cancer 1, 146–155. https://doi.org/10.1007/s12672-010-0015-9.

Durando, M., Kass, L., Piva, J., Sonnenschein, C., Soto, A.M., Luque, E.H., Muñoz-De-Toro, M., 2006. Prenatal bisphenol a exposure induces preneoplastic lesions in the mammary gland in wistar rats. Environ. Health Perspect. 115, 80–86. https://doi.org/10.1289/ehp.9282.

European Food and Safety Authority (EFSA), 2015. Scientific Opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs. EFSA J. 13, 3978. https://doi.org/10.2903/j.efsa.2015.3978.

Elhamalawy, O.H., Eissa, F.I., El Makawy, A.I., EL-Bamby, M.M., 2018. Bisphenol-A Hepatotoxicity and the Protective Role of Sesame Oil in Male Mice. Jordan J. Biol. Sci. 11 (4), 461-467.

Elmaci, I., Altinoz, M.A., Sari, R., Bolukbasi, F.H., 2017. Phosphorylated Histone H3 (PHH3) as a

Novel Cell Proliferation Marker and Prognosticator for Meningeal Tumors: A Short Review. Appl. Immunohistochem. Mol. Morphol. 26 (9), 627-631. https://doi.org/10.1097/PAI.0000000000000499.

Facina, C.H., Campos, S.G.P., Gonçalves, B.F., Góes, R.M., Vilamaior, P.S.L., Taboga, S.R., 2017. Long-term oral exposure to safe dose of bisphenol A in association with high-fat diet stimulate the prostatic lesions in a rodent model for prostate cancer. Prostate 78, 152–163. https://doi.org/10.1002/pros.23458

Fischer, C., Mamillapalli, R., Goetz, L.G., Jorgenson, E., Ilagan, Y., Taylor, H.S., 2016. Bisphenol A (BPA) Exposure In Utero Leads to Immunoregulatory Cytokine Dysregulation in the Mouse Mammary Gland: A Potential Mechanism Programming Breast Cancer Risk. Horm. Cancer 7, 241–251. https://doi.org/10.1007/s12672-016-0254-5

Gardai, S.J., Bratton, D.L., Ogden, C.A., Henson, P.M., 2006. Recognition ligands on apoptotic cells: a perspective. J. Leukoc. Biol. 79, 896–903. https://doi.org/10.1189/jlb.1005550.

Geng, X., Li, B., Zhang, L., Song, Y., Lin, Z., Zhang, Y., Wang, J., 2012. Corn oil as a vehicle in drug development exerts a dose-dependent effect on gene expression profiles in rat thymus. J. Appl. Toxicol. 32, 850–857. https://doi.org/10.1002/jat.2773.

Gomez, A.L., Delconte, M.B., Altamirano, G.A., Vigezzi, L., Bosquiazzo, V.L., Barbisan, L.F., Ramos, J.G., Luque, E.H., Muñoz-de-Toro, M., Kass, L., 2017. Perinatal Exposure to Bisphenol A or Diethylstilbestrol Increases the Susceptibility to Develop Mammary Gland Lesions After Estrogen Replacement Therapy in Middle-Aged Rats. Horm. Cancer 8, 78–89. https://doi.org/10.1007/s12672-016-0282-1.

Gonçalves, B.F., Campos, S.G.P. De, Zanetoni, C., Scarano, W.R., Jr, L.R.F., Amorim, R.L., Góes, R.M., Taboga, S.R., 2013. ANew Proposed Rodent Model of Chemically Induced Prostate Carcinogenesis : Distinct Time-Course Prostate Cancer Progression in the Dorsolateral and Ventral Lobes 1213, 1202–1213. https://doi.org/10.1002/pros.22669.

Gonçalves, B.F., de Campos, S.G.P., Góes, R.M., Scarano, W.R., Taboga, S.R., Vilamaior, P.S.L., 2017. Dual action of high estradiol doses on MNU-induced prostate neoplasms in a rodent model with high serum testosterone: Protective effect and emergence of unstable epithelial microenvironment. Prostate 77, 970–983. https://doi.org/10.1002/pros.23353.

Gonçalves, B.F., Zanetoni, C., Scarano, W.R., Góes, R.M., Vilamaior, P.S.L., Taboga, S.R., Campos, S.G.P., 2010. Prostate carcinogenesis induced by N-methyl-N-nitrosourea (mnu)

in gerbils: Histopathological diagnosis and potential invasiveness mediated by extracellular matrix components. Exp. Mol. Pathol. 88, 96–106. https://doi.org/https://doi.org/10.1016/j.yexmp.2009.09.017.

Green, K.A., Streuli, C.H., 2004. Apoptosis regulation in the mammary gland. Cell. Mol. Life Sci. 61, 1867–1883. https://doi.org/10.1007/s00018-004-3366-y.

Gulati, N., Béguelin, W., Giulino-Roth, L., 2018. Enhancer of zeste homolog 2 (EZH2) inhibitors. Leuk. Lymphoma 59, 1574–1585. https://doi.org/10.1080/10428194.2018.1430795.

Guzman, R.C., Osborn, R.C., Swanson, S.M., Sakthivel, R., Hwang, S.-I., Miyamoto, S., Nandi, S., 1992. Incidence of c-Ki-ras activation in N-methyl-N-nitrosourea-induced mammary carcinomas in pituitary-isografted mice. Cancer Res. 52, 5732–5737.

Hanahan, D., Coussens, L.M., 2012. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 21, 309–322. https://doi.org/10.1016/j.ccr.2012.02.022.

Hans, F., Dimitrov, S., 2001. Histone H3 phosphorylation and cell division. Oncogene 3, 3021–3027. https://doi.org/10.1038/sj.onc.1204326.

Hindman, A.R., Mo, X.M., Helber, H.L., Kovalchin, C.E., Ravichandran, N., Murphy, A.R., Fagan, A.M., St. John, P.M., Burd, C.J., 2017. Varying Susceptibility of the Female Mammary Gland to In Utero Windows of BPA Exposure. Endocrinology 158, 3435–3447. https://doi.org/10.1210/en.2017-00116.

Huo, C.W., Chew, G., Hill, P., Huang, D., Ingman, W., Hodson, L., Brown, K.A., Magenau, A., Allam, A.H., McGhee, E., Timpson, P., Henderson, M.A., Thompson, E.W., Britt, K., 2015. High mammographic density is associated with an increase in stromal collagen and immune cells within the mammary epithelium. Breast Cancer Res. 17, 79. https://doi.org/10.1186/s13058-015-0592-1.

Jones, P.A., Baylin, S.B., 2002. The fundamental role of epigenetic events in cancer. Nat. Rev. Genet. 3, 415-428. https://doi.org/10.1038/nrg816.

Keely, P.J., 2011. Mechanisms by which the extracellular matrix and integrin signaling act to regulate the switch between tumor suppression and tumor promotion. J. Mammary Gland Biol. Neoplasia 16, 205-219. https://doi.org/10.1007/s10911-011-9226-0.

Li, X., Gonzalez, M.E., Toy, K., Filzen, T., Merajver, S.D., Kleer, C.G., 2009. Targeted

Overexpression of EZH2 in the Mammary Gland Disrupts Ductal Morphogenesis and Causes Epithelial Hyperplasia. Am. J. Pathol. 175, 1246–1254. https://doi.org/10.2353/ajpath.2009.090042.

Mailleux, A.A., Overholtzer, M., Schmelzle, T., Bouillet, P., Strasser, A., Brugge, J.S., 2007. BIM regulates apoptosis during mammary ductal morphogenesis, and its absence reveals alternative cell death mechanisms. Dev. Cell 12, 221–234. https://doi.org/10.1016/j.devcel.2006.12.003.

Mandrup, K., Boberg, J., Isling, L.K., Christiansen, S., Hass, U., 2016. Low-dose effects of bisphenol A on mammary gland development in rats. Andrology 4, 673–683. https://doi.org/10.1111/andr.12193.

Medina, D., 2008. Premalignant and malignant mammary lesions induced by MMTV and chemical carcinogens. J. Mammary Gland Biol. Neoplasia 13, 271–277. https://doi.org/10.1007/s10911-008-9086-4.

Medina, D., 2007. Chemical carcinogenesis of rat and mouse mammary glands. Breast Dis. 28, 63–68. https://doi.org/10.3233/BD-2007-28107.

Michalak, E.M., Nacerddine, K., Pietersen, A., Beuger, V., Pawlitzky, I., Cornelissen-Steijger, P., Wientjens, E., Tanger, E., Seibler, J., van Lohuizen, M., Jonkers, J., 2013. Polycomb group gene Ezh2 regulates mammary gland morphogenesis and maintains the luminal progenitor pool. Stem Cells 31, 1910–1920. https://doi.org/10.1002/stem.1437.

Michałowicz, J., 2014. Bisphenol A – Sources, toxicity and biotransformation. Environ. Toxicol. Pharmacol. 37, 738–753. https://doi.org/https://doi.org/10.1016/j.etap.2014.02.003.

Monks, J., Smith-Steinhart, C., Kruk, E.R., Fadok, V.A., Henson, P.M., 2008. Epithelial cells remove apoptotic epithelial cells during post-lactation involution of the mouse mammary gland. Biol. Reprod. 78, 586–594. https://doi.org/10.1095/biolreprod.107.065045.

Muñoz-de-Toro, M., Markey, C.M., Wadia, P.R., Luque, E.H., Rubin, B.S., Sonnenschein, C., Soto, A.M., 2005. Perinatal Exposure to Bisphenol-A Alters Peripubertal Mammary Gland Development in Mice. Endocrinology 146, 4138–4147. https://doi.org/10.1210/en.2005-0340.

Nowak, S.J., Corces, V.G., 2000. Phosphorylation of histone H3 correlates with transcriptionally active loci. Genes Dev. 14, 3003–3013. https://doi.org/10.1101/gad.848800.

Peretz, J., Flaws, J.A., 2013. Bisphenol A down-regulates rate-limiting Cyp11a1 to acutely inhibit steroidogenesis in cultured mouse antral follicles. Toxicol. Appl. Pharmacol. 271, 249–256. https://doi.org/https://doi.org/10.1016/j.taap.2013.04.028.

Quintar, A.A., Gonçalves, B.F., Taboga, S.R., Maldonado, C.A., 2017. The mongolian gerbil (*Meriones unguiculatus*) as a model for inflammation-promoted prostate carcinogenesis. Cell Biol. Int. 41, 1234–1238. https://doi.org/10.1002/cbin.10789.

Reif, D.M., Martin, M.T., Tan, S.W., Houck, K.A., Judson, R.S., Richard A.M., Knudsen, T.B., Dix, D.J., Kavlok, R.J., 2010. Endocrine Profiling and Prioritization of Environmental Chemicals Using ToxCast Data. Environ. Health Perspect. 118, 1714–1720. https://doi.org/10.1289/ehp.1002180

Rudmann, D., Cardiff, R., Chouinard, L., Goodman, D., Küttler, K., Marxfeld, H., Molinolo, A., Treumann, S., Yoshizawa, K., 2012. Proliferative and Nonproliferative Lesions of the Rat and Mouse Mammary, Zymbal's, Preputial, and Clitoral Glands. Toxicol. Pathol. 40, 7S–39S. https://doi.org/10.1177/0192623312454242.

Russo, J., Russo, Russo, I.H., 2000. Atlas and Histologic Classification of Tumors of the Rat Mammary Gland. J. Mammary Gland Biol. Neoplasia 5, 187-200. https://doi.org/10.1023/A:1026442305758.

Sakakura, T., Suzuki, Y., Shiurba, R., 2013. Mammary stroma in development and carcinogenesis. J. Mammary Gland Biol. Neoplasia 18, 189–197. https://doi.org/10.1007/s10911-013-9281-9.

Singh, M., McGinley, J.N., Thompson, H.J., 2000. A comparison of the histopathology of premalignant and malignant mammary gland lesions induced in sexually immature rats with those occurring in the human. Lab. Investig. 80, 221-231. https://doi.org/10.1038/labinvest.3780025.

Soto, A.M., Vandenberg, L.N., Maffini, M. V, Sonnenschein, C., 2008. Does breast cancer start in the womb? Basic Clin. Pharmacol. Toxicol. 102, 125–133. https://doi.org/10.1111/j.1742-7843.2007.00165.x.

Steinmuller, D., 1971. A Reinvestigation of Epidermal Transplantation during Chemical Carcinogenesis. Cancer Res. 31, 2080-2084.

Tharp, A.P., Maf, M. V, Hunt, P.A., Vandevoort, C.A., Sonnenschein, C., Soto, A.M., 2012.

Bisphenol A alters the development of the rhesus monkey mammary gland 109, 8190–8195. https://doi.org/10.1073/pnas.1120488109.

Thoene, M., Rytel, L., Nowicka, N., Wojtkiewicz, J., 2018. The state of bisphenol research in the lesser developed countries of the EU: a mini-review. Toxicol. Res. (Camb). 7, 371–380. https://doi.org/10.1039/C8TX00064F.

Thompson, C., Rahim, S., Arnold, J., Hielscher, A., 2017. Loss of caveolin-1 alters extracellular matrix protein expression and ductal architecture in murine mammary glands. PLoS One 12, e0172067. https://doi.org/10.1371/journal.pone.0172067.

Vandenberg, L.N., Maffini, M. V, Schaeberle, C.M., Ucci, A.A., Sonnenschein, C., Rubin, B.S., Soto, A.M., 2008. Perinatal exposure to the xenoestrogen bisphenol-A induces mammary intraductal hyperplasias in adult CD-1 mice. Reprod. Toxicol. 26, 210–219. https://doi.org/10.1016/j.reprotox.2008.09.015.

Weibel, E.R., 1963. Principles and methods for the morphometric study of the lung and other organs. Lab Invest 12, 131–155.

Wetherill, Y.B., Akingbemi, B.T., Kanno, J., McLachlan, J.A., Nadal, A., Sonnenschein, C., Watson, C.S., Zoeller, R.T., Belcher, S.M., 2007. In vitro molecular mechanisms of bisphenol A action. Reprod. Toxicol. 24, 178–198. https://doi.org/10.1016/j.reprotox.2007.05.010.

Zhuang, S., Zhang, C., Liu, W., 2014. Atomic Insights into Distinct Hormonal Activities of Bisphenol A Analogues toward PPARγ and ERα Receptors. Chem. Res. Toxicol. 27, 1769–1779. https://doi.org/10.1021/tx500232b.

Figure 1. Experimental design. Pregnant females (n=5) were left as intact control (IC), or subjected to corn oil (OC), 50 µg/kg BPA (↓BPA), or 5000 µg/kg BPA (↑BPA) daily treatments from the 8th day of gestation until the end of lactation. At 90 days of age the females from offspring obtained were divided into two groups (n=5) and half them were treated with a single dose of NMU. The animals were euthanized at 180 days of age. BPA: bisphenol A; NMU: N-nitroso-N-methylurea.

Figure 2. Incidence of morphological alterations (mean±SEM) observed in the mammary glands (n=5). Different letters in A, C, F, and I indicate different results. Parametric (A, D, F: ANOVA followed by Tukey's test) and non-parametric (I: Kruskal-Wallis followed by Dunn's multiple comparison test) values were considered significant when *p<0.05*. Leukocytes (B, arrow) and epithelial cells (D-E) released in the lumen, presence of inflammatory cells among the stroma (G-H, arrows) and presence of two or more layers of epithelial cells in acini and ducts (J-K) were quantified among the secretory structures of the mammary gland.

Figure 3. Percentages (mean±SEM) of collagen deposition incidence in the mammary tissue of animals evaluated in different groups (n=5). Pictures from groups OC (B-C) and IC (D-E). ANOVA followed by Tukey's Multiple Comparison test, *p<0.05*. Bars: 20 µm in A, 40 µm in D.

Figure 4. Percentages (mean±SEM) of α-actin expression incidence in the epithelium of mammary tissue of animals evaluated in different groups (n=5). Pictures from groups ↓ BPA (B), ↑ BPA (D), and IC (C). ANOVA followed by Tukey's Multiple Comparison test, *p<0.05*. Bars: 50 µm.

Figure 5. Percentages of positive cells for phospho histone-H3 (P-H-H3) in the different groups (A). There was a higher incidence of positive cells in the↓ BPA group (B) followed by the ↑ BPA (C) and intact control (D) groups. ANOVA followed by Tukey's Multiple Comparison test, *p<0.05*, n=5. Bars: 50 µm in B-C and 20 µm in D.

Figure 6. Percentages of positive cells (A, n=5), illustrative pictures (B,D), and relative density (C, n=4) of EZH2 in the mammary gland from animals from different treatments. Different letters represent statistically different results. ANOVA followed by Tukey's Multiple Comparison test (*p<0.*05). Bars: 50 µm.

Table 1. Incidences (Mean±SEM) of histopathological alterations found in the evaluated mammary glands.

Group	Leukocytes in the acini	Detached epithelial cells	Inflammation	Hyperplasia
IC without NMU	1,4±0,5^{a,b}	4,5±2,0^{a,b}	2,3±0,7^{a}	9,3±0,6^{a,b}
IC with NMU	1,4±0,5^{a,b}	8,5±1,0^{a,c}	4,5±0,4^{a,b}	15,3±0,7^{a,b,c}
OC without NMU	0,4±0,2^{a}	1,8±0,61^{b}	7,5±1,2^{b,c}	4,8±1,2^{a}
OC with NMU	0,0±0,0^{a}	6,5±0,43^{a,b,c}	5,9±0,54^{b}	17,8±1,9^{b,c}
↓ BPA without NMU	5,3±1,0^{b}	11,8±2,0^{c}	9,2±0,6^{c}	18,2±2,2^{b,c}
↓ BPA with NMU	5,2±2,0^{b}	6,1±0,8^{a,b,c}	5,7±0,7^{b}	12,9±1,0^{a,b,c}
↑ BPA without NMU	0,3±0,2^{a,b}	4,6±1,1^{a,b}	6,[illegible]±0,3^{b,c}	14,8±2,1^{a,b,c}
↑ BPA with NMU	2,5±0,8^{a,b}	4,0±1,0^{a,b}	7,7±[illegible],4^{b,c}	19,4±0,8^{c}

^{a,b,c}Similar letters in the same column indicate statistically similar results.