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Hormone receptor expression in aging mammary tissue and carcinoma from a rodent model after xenoestrogen disruption

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ABSTRACT

Aims: Hormone receptors are the main markers applied for prognosis of breast cancer subtypes. Among modulators, exogenous chemical agents known as endocrine disruptors interact with certain receptors, triggering molecular pathways or increasing their expression. Bisphenol A (BPA), a xenoestrogen, interacts with several hormone receptors. Thus, our aim was to characterize the hormone receptor status in the mammary gland (MG) of aged female Mongolian gerbils exposed to BPA in pregnancy and lactation.
Methods: We evaluated the expression of receptors for estrogens (ERα and ERβ), progesterone (PR), prolactin (PRL-R), HER2/ErbB2, and androgen (AR) in normal and hyperplastic mammary tissue and in carcinomas developed after BPA exposure.
Key findings: BPA-exposed MG presented increased ERα, whereas ERβ, PR, and PRL-R showed lower expression. AR and HER2/ErbB2 showed similar expression in normal and hyperplastic tissue from control, vehicle, and BPA groups. Both receptors were found in cytoplasm and nucleus in BPA-induced carcinoma. We demonstrate the presence of EZH2 expression, an epigenetic and epithelial-mesenchymal transition (EMT) marker, with a high H-score in BPA-exposed MG, which was associated with poor prognosis of cancer. Co-localization of ERα and EZH2 was present in normal and carcinoma features, corroborating the installation of ERα-positive mammary cancer associated with the EMT process. Enhanced EZH2 in BPA-exposed mammary tissue could decrease ERβ expression and promote tumorigenesis progress through HER2/ErbB2.
Significance: The present study proposes the Mongolian gerbil as an experimental model for mammary carcinogenesis studies, based on BPA disruption that triggers a phenotype of increased ERα/HER2 positivity and depletion of ERβ/PR expression.

1. Introduction

The receptor-hormone binding signal is the base of hormone-responsive organs, such as the prostate and breast [1–3]. This signal alters molecular pathways that could accelerate pathological processes of cancer installation [4]. The mammary gland is one of the most susceptible organs to hormonal regulation [5–7]. Diagnosis of neoplastic lesions in this tissue is commonly based on the expression of key markers, such as estrogen receptors (ERα and ERβ), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2/ErbB2) [1,8,9]. Variations in the expression of these receptors in mammary gland neoplastic lesions are related to impacts in tissue homeostasis through proliferative activity [10] and changes in signaling for establishment of a tumor microenvironment t [4]. Specifically, a

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decline or absence of these three receptors characterizes a triple negative (ER-/PR-/HER2-) tumor, which is one of the most aggressive types of breast cancers [11]. A new tumor subtype (quadruple negative) has been considered based on the absence of androgen receptor (AR) expression [12,13], which has a relevant role in healthy tissue [14] and breast cancer [15]. Thus, the decreased expression of hormone receptors in neoplastic mammary tissue represents a poor prognosis for breast cancer, indicating low responsiveness to hormonal therapies [16–18].

Recently, efforts have been made to describe an effective relationship between compounds known as endocrine disruptors (ED) and breast cancer [19–21]. EDs act by pathways that mimic or modulate the action of hormones and factors [22,23] to develop carcinogenesis. Among them, xenoestrogens have great relevance because they act mainly through ER binding [24]. However, bisphenol A (BPA), a ubiquitous xenoestrogen, acts by interacting with several other hormone receptors for disruption [25]. Furthermore, studies have suggested that the receptor-related action of BPA goes beyond direct binding interaction: it further promotes expression or silencing of genes related to these receptors [26], and consequently impacts the expression of these proteins [27].

Indeed, epigenetic changes have been linked to BPA, as strong evidence of the endocrine disruption of this compound that leads to tumorigenesis [28]. Among these epigenetic alteration pathways [29], methylation is one of the main mechanisms of BPA disruption [30,31]. Enhancer of zeste homolog 2 (EZH2) is a methyltransferase of histone H3 (in lysine 27) from the polycomb protein group [32]. It largely modulates the expression of genes that encode relevant proteins for tumor progression [33,34] including some linked to estrogen-like compounds [27,35]. This modulation is particularly important when exposure to BPA occurs during the perinatal development susceptibility window [28,36].

For better understanding of these proliferative lesion mechanisms the establishment of new rodent species as experimental models is relevant. The hypothesis that the Mongolian gerbil (*Meriones unguiculatus*) is an adequate model for these experiments is based on previous data and consistent evidence from our research group, with a focus on prostatic and mammary tissues [36–38]. Thus, we present analysis of the hormone receptor expression patterns in normal and hyperplastic mammary gland and in carcinoma induced by xenoestrogen BPA, allied with evaluation of the epigenetic marker EZH2. For this, the object of study was the late repercussions of BPA in females exposed during phases of major mammary morphological changes: pregnancy and lactation.

2. Materials and methods

2.1. Experiments and ethics

The present study was conducted at the Animal Breeding Center of the Institute of Biosciences, Humanities and Exact Sciences (IBILCE, UNESP, São Paulo). Twenty female Mongolian gerbils, 3 months of age, were bred with fertile males. After birth the first litter was discarded in order to induce a second pregnancy in females. Eight days later, the females were divided into 4 experimental groups: control (gavage, water), vehicle (gavage, corn oil vehicle), ↓BPA (gavage, 50 μg/kg of BPA), and ↑BPA (gavage, 5000 μg/kg of BPA). The BPA dosage in the ↓BPA group reflects the daily safe exposure as considered by the US EPA [39] and EFSA [40], while the ↑BPA group depicts overexposure to the compound. The pregnant females were subjected to gavage daily, from the 8th day of gestation until the end of lactation, comprising 39 days of exposure. After weaning the mothers were kept in polysulfone isolators with water and balanced food *ad libitum* until 18 months of age, characterizing an aging period. Euthanasia was performed in animals with the exclusive presence of cornified cells in the vaginal smear, performed to standardize the hormonal status among animals of the experiment, since this impacts on the mammary tissue morphology. The abdominal mammary glands were removed and fixed whole in 4% paraformaldehyde for 24 h, and then embedded in paraffin in a Leica Semi Enclosed System (TP1020, Leica Biosystems).

The experiment followed the standards and protocols for animal experimentation of the National Council of Control and Animal Experimentation (CONCEA, Brazil) and was approved by the local Ethics Committee on the Use of Animals (CEUA, IBILCE, UNESP), number 217/2019.

2.2. Immunohistochemistry procedures and analysis

The fixed samples were sectioned at 4 μm thick and placed on silanized slides. They were then assigned to routine immunohistochemistry (IHC). Slides were deparaffinized and hydrated and before being subjected to antigen retrieval in 10 mM citrate buffer (97 °C) for 40 min. Subsequently, sections were destined to peroxidase blockage in H_2O_2 5% diluted in methanol for 20 min. Blocking of non-specific proteins was performed in 10% bovine serum albumin for 30 min. Antibody incubation was performed overnight with the following primary antibodies: estrogen receptors – anti-ERα (mouse monoclonal, sc-8005, 1:50, Santa Cruz Biotechnology) and anti-ERβ (rabbit polyclonal, PA1-310B, 1:50, Invitrogen, ThermoFisher), progesterone receptor – anti-PR (mouse monoclonal, 1:50, GTX22765, GeneTex), anti-HER2/ErbB2 (mouse monoclonal, 3B5, ab16901, 1:75, Abcam), prolactin receptor – anti-PRL -R (rabbit monoclonal, D4A9, 1:100, Cell Signaling Technology), androgen receptor – anti-AR (mouse monoclonal, sc-7305, 1:75, Santa Cruz Biotechnology), and enhancer of zeste homolog 2 – anti-EZH2 (rabbit monoclonal, D2C9, 1:75, Cell Signaling Technology). The slides were then incubated with post-primary antibody and polymer (Novolink™ polymer detection system 1, Leica Biosystems Newcastle 118 Ltd., Newcastle, United Kingdom). Steps were interspersed with PBS or TBS wash buffers. Positive staining was detected by DAB chromogen (3-30′-diaminobenzidine tetrahydrochloride, Novolink™ DAB, RE7270-CE, Leica Biosystems, Buffalo 121 Grove, USA) and counterstaining was performed with hematoxylin.

For analyses of immunohistochemical expression of ERα, ERβ, PR, and AR in the mammary gland, 15 microscopy fields were randomly selected for each animal ($n = 5$ per group). Incidence of the following structures with different morphological patterns was quantified: normal alveoli (1 or 2 layers of epithelial cells), hyperplastic foci (alveoli with more than 3 layers of epithelial cells), and carcinomas/neoplasia (regions with no lumen, showing non-polarized cells and with no basal delineation). Cells showing positive staining for each marker were counted, and the percentage of positive epithelial cells in relation to the total number of epithelial cells in the microscopic field was provided. These automated analyses were performed in QuPath software (Version 0.1.2, an open-source pathology software platform). In addition, the incidence of EZH2 expression was evaluated in entire tissue sections (cells/mm^2) and the H-score of EZH2 immunostaining was taken into account for quantifying the intensity of labeling in cells throughout the section, as described by Vougiouklakis et al. (2020). Both analyses were performed and standardized by QuPath software.

2.3. Immunofluorescence assay

Immunofluorescence was performed to detect localization of HER2/ErbB2 and AR, and co-localization of ERα and EZH2. Antigen retrieval and nonspecific protein blockage were performed as stated above for IHC. Primary antibody incubation was performed overnight (4 °C). Incubation was then carried out with the following specific fluorochrome conjugated secondary antibodies: anti-mouse FITC (sc-2010, 1:100, Santa Cruz Biotechnology, USA) and anti-rabbit Texas Red (sc-2780, 1:100, Santa Cruz Biotechnology, USA), for 1 h at room temperature. The slides were stained and mounted with DAPI (Fluoroshield™ with DAPI, F6057, histology mounting medium, Merck, Darmstadt, Germany) for nuclear fluorescence. The sections were analyzed with a Zeiss

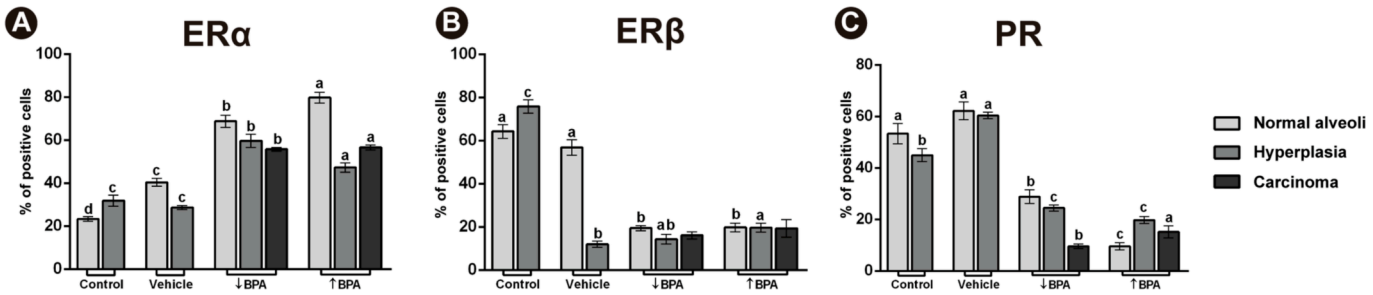

Fig. 1. Incidence of ERα, ERβ, and PR expression (percentages) in normal and hyperplastic tissues and carcinoma regions of mammary gland exposed to BPA. (A) ERα; (B) ERβ; (C) PR. Carcinomas were absent in mammary glands from control and vehicle groups. Graphical values are expressed as mean ± SEM and different letters (a, b and c) indicate significant differences among treatment groups in each morphological pattern (normal, hyperplasic or carcinoma) (A, C: ANOVA test followed by Tukey's test; B: Kruskal-Wallis test followed by Dunn's test).

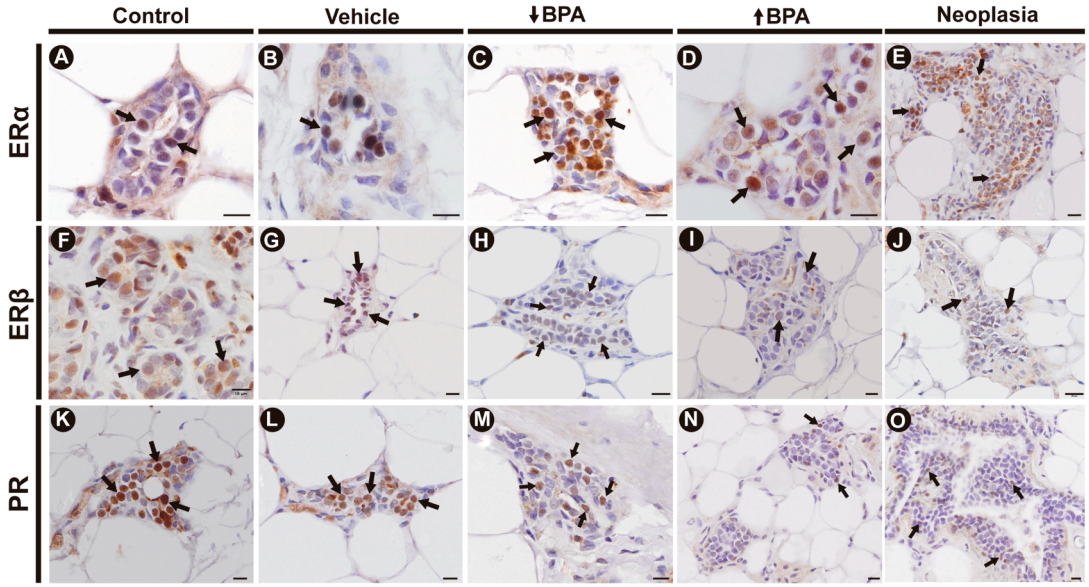

Fig. 2. ERα, ERβ, and PR expression in mammary gland from different groups. (A-E) ERα. Normal epithelium in control and vehicle (A, B) presented low expression of ERα (arrows) in comparison to both BPA groups (C, D); this marker was also highly expressed in neoplastic structures disrupted by BPA (E). (F-J) ERβ. Nuclear expression (arrows) of ERβ was enhanced in normal alveoli of control and vehicle groups (F, G). However, its expression in ↓BPA (H) and ↑BPA (I) groups was rare. Also, neoplasia of BPA groups showed diminished ERβ expression (J). (K-O) PR. Expression of PR (arrows) was enhanced in control and vehicle (K, L), decreased in ↓BPA (M), and drastically reduced in ↑BPA (N) groups. Note sparse and reduced expression in neoplasia of BPA-exposed mammary gland. Scale Bars: (A, B, D, F) 10 μm; (C, G-O) 20 μm; (E) 30 μm;

AX10 Fluorescence Microscope (Zeiss, Oberkochen, Germany) coupled to AxioVision (Zeiss) software.

2.4. Statistical analysis

The analysis of data normality was performed by the Kolmogorov-Smirnov test. Data of ERα, PR, and EZH2 (parametric data) were analyzed by one-way ANOVA followed by Tukey's test. For ERβ and AR (non-parametric data), the Kruskal-Wallis followed by Dunn's tests were applied. $P < 0.05$ value was considered for statistically significant differences between groups. The statistical analyses were performed in GraphPad Prism 5.00 software for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

3. Results

3.1. ERα, ERβ and PR expression

The expression of ERα, ERβ, and PR was quantified in epithelial cells of normal and hyperplastic tissue, and in multifocal carcinoma features in mammary gland. The percentages of cells expressing these receptors over the total number of epithelial cells are shown in Fig. 1(A) for ERα, Fig. 1(B) for ERβ, and Fig. 1(C) for PR, where different letters (a, b and c) indicate significant differences among treatment groups in normal, hyperplasic or carcinoma patterns. In addition, Fig. 2 presents the nuclear expression of ERα in Fig. 2(A-E), ERβ in Fig. 2(F-J), and PR in Fig. 2 (K-O), in normal, hyperplastic, and carcinoma structures. Control and vehicle groups presented the lowest values of ERα-positive cells (Fig. 2 (A-B)) in both normal and hyperplastic tissues; the ↓BPA group presented the highest percentage in hyperplasia (59.67 ± 3.11) and ↑BPA showed the highest rates in normal alveoli (79.81 ± 2.52) (Fig. 2(C-D), respectively). In BPA-induced carcinoma >50% of cells were ERα-positive in both groups (Fig. 2(E)).

ERβ and PR presented opposite patterns of expression in BPA groups in comparison to ERα. The percentages of ERβ and PR positive cells are shown in Fig. 1(B-C). ERβ was highly expressed in normal alveoli (Fig. 2 (F)) and hyperplastic tissues from the control group (64.27 ± 3.22 and 75.87 ± 3.05, respectively), while its expression drastically decreased in tissues from BPA groups (Fig. 2(H-J)). Percentage of PR-positive cells also decreased in mammary gland of BPA groups (Fig. 1(C)). Normal and hyperplasic structures in control and vehicle groups presented around 50% of epithelial cells expressing PR (Fig. 2(K-L)). However, in BPA

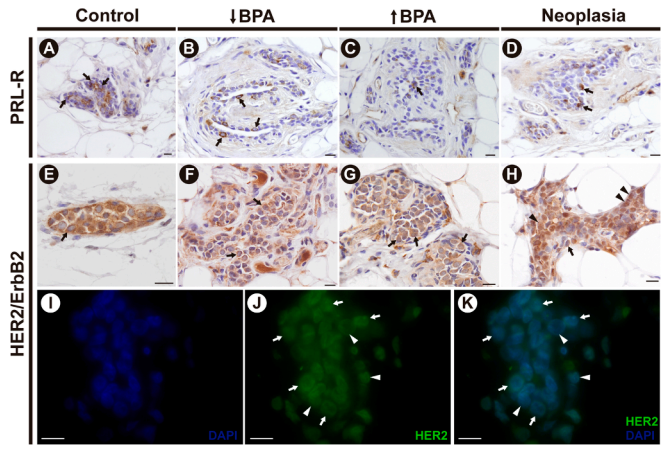

Fig. 3. PRL-R and HER2/ErbB2 expression in BPA-disrupted mammary gland. (A-D) PRL-R. Note cytoplasmic expression (arrows) in control group in A, compared to scarce expression in normal alveoli of ↓BPA (B) and in hyperplasia of ↑BPA (C) groups, as in neoplastic regions (D). (E-K) HER2/ErbB2. Control group presented HER2/ErbB2 positivity in cytoplasm (arrows) of all epithelial compartment (A), as well as in ↑BPA and ↓BPA groups (F, G). However, in neoplastic structures of BPA group, nuclear (arrowheads) staining for HER2/ErbB2 was observed (H). Immunofluorescence assay enabled observation of the nuclear (arrowheads) and cytoplasmic (arrows) localization of HER2/ErbB2 that occurred in normal alveoli of BPA disrupted groups (I-K). Scale Bars: 20 μm.

groups (Fig. 2(M-N)), there was a lower rate of normal epithelium in ↑BPA compared to ↓BPA. Carcinomas from the ↓BPA group presented the lowest percentages of PR positive cells (Fig. 2(O)). These three receptors did not show significant expression in stromal cells.

3.2. PRL-R, HER2/ErbB2 and AR expression

The expression of PRL-R and HER2/ErbB2 showed a visible decrease in mammary tissue of BPA groups (Fig. 3). A diffuse cytoplasmic pattern of staining required the use of a particular qualitative method to quantify the expression of these receptors in the mammary tissue. Normal epithelium in the control group showed cytoplasmic localization of PRL-R (Fig. 3(A)). However, in normal (Fig. 3(B)) and hyperplasic (Fig. 3(C)) alveoli from BPA groups, the PRL-R positive staining was scarce. A similar pattern was observed in neoplastic cells (Fig. 3(D)). HER2/ErbB2 expression was similar in normal and hyperplasic structures from all groups (Fig. 3(E-G)). However, in multifocal neoplasia from BPA groups, cytoplasmic and nuclear expression of HER2/ErbB2 were observed (Fig. 3(H)), which was an uncommon feature in normal epithelium from the control group (Fig. 3(E)), but often observed in disrupted and normal alveoli from BPA groups (Fig. 3(F-G)). These aspects were confirmed by immunofluorescence (Fig. 3(I-K)).

The percentages of AR-positive cells among groups quantified in epithelial compartments from normal and hyperplastic glands and carcinoma are shown in Fig. 4 (A, where a, b and c indicate significant differences among treatment groups in normal, hyperplasic or carcinoma patterns). Control and vehicle groups presented a lower incidence of nuclear staining for AR (Fig. 4(B)). Hyperplastic structures presented more than 50% of AR-positivity staining in the ↓BPA group (Fig. 4(C)).

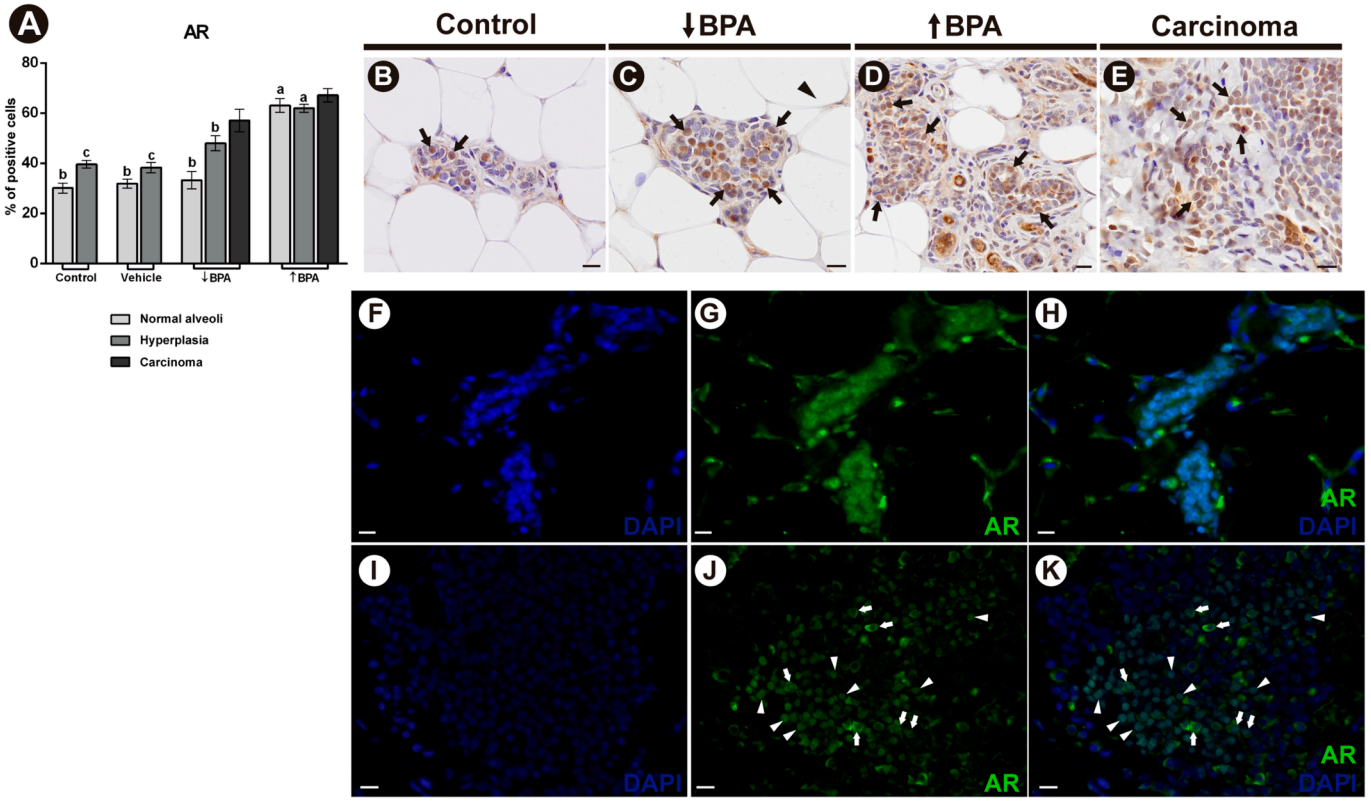

Fig. 4. AR expression and localization in mammary gland. (A) Incidence (percentage) of AR positive cells. Graphical values are expressed as mean ± SEM and different letters (a, b and c) indicate significant differences among treatment groups in each morphological pattern (normal, hyperplasic or carcinoma) (ANOVA test followed by Tukey's test). (B-E) AR expression (arrows) in mammary gland tissue: lower in normal alveoli of control group (B), enhanced in hyperplasia of ↓BPA (C) and in normal tissue and hyperplasia of ↑BPA (D). Neoplasia/carcinoma regions presented high expression of AR (arrows). (F-K) Localization of AR in BPA groups. Normal alveoli of ↑BPA group presented high expression in nucleus (arrowheads) and cytoplasm (arrows) of epithelial cells (F-H), as well as in carcinoma of ↓BPA group (I-K). Scale Bars: 20 μm.

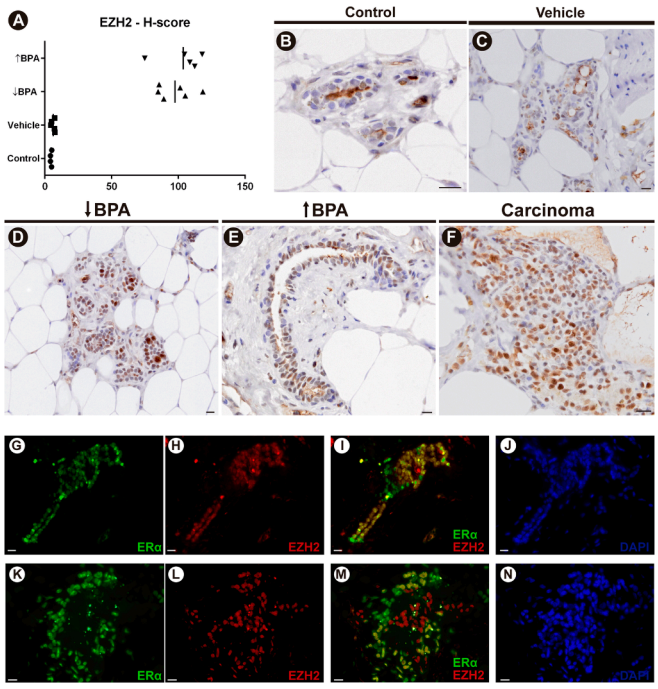

Fig. 5. EZH2 expression. (A) H-score of EZH2. Both BPA groups presented high indices of H-score. (B-F) IHC for EZH2. (B) Control and (C) vehicle groups presented less EZH2 staining in mammary gland tissue. (D-E) Normal and hyperplasic alveoli presented heterogenous stain intensity for EZH2, reflecting in the H-score of these groups. (F) Carcinoma/neoplasia showed EZH2 positivity in almost every cell in different intensities. (G-N) Immunofluorescence assay for co-localization (yellow) of ERα (green) and EZH2 (red). (J and N) Shows the total nuclei in the same section with DAPI. Scale Bars: 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The highest rates were observed in the ↑BPA group in both normal and hyperplasic epithelium (Fig. 4(D)), with statistically different percentages from other groups. Carcinomas presented the highest percentages of AR-positive cells in each BPA exposed group (Fig. 4(E)). AR expression was observed in stromal cells among normal and hyperplastic alveoli in BPA groups (Fig. 4(D)). Furthermore, in the immunofluorescence assay it was possible to observe the cytoplasmic and nuclear localization of AR in normal tissue (Fig. 4(F-H)) and carcinoma regions (Fig. 4(I-K)) from BPA groups.

3.3. EZH2 expression

The EZH2 H-score expression is shown in Fig. 5(A). In control and vehicle groups (Fig. 5(B-C)), mammary tissue presented a low incidence of EZH2 positive cells (1.56 ± 0.18 and 1.80 ± 0.23 cells/mm^2, respectively). Normal mammary tissue and carcinoma from BPA groups presented enhanced positivity for EZH2 (↓BPA: 41.12 ± 3.29 and ↑BPA: 43.62 ± 4.17 cells/mm^2) (Fig. 5(D-F)). Immunoreactive intensity (Fig. 5 (D, F)) was stronger in BPA groups, which presented elevated H-scores (↓BPA: 97.44 ± 5.42 and ↑BPA: 103.7 ± 7.51) in comparison with control and vehicle groups (control: 4.68 ± 0.26 and vehicle: 6.41 ± 0.62). In BPA groups, nuclear co-localization of ERα and EZH2 were observed in normal alveoli (Fig. 5(G-J)) and in carcinoma regions (Fig. 5 (K-N)).

4. Discussion

The present study describes the hormone receptor standards in the tumorigenic process induced by BPA in the mammary gland of aged female gerbils. ERα expression was more frequent than PR and ERβ in carcinomas and in normal and hyperplastic structures from BPA exposed individuals. This demonstrates that the multifocal carcinoma developed in response to this xenoestrogen tends to be ERα-positive, similarly to what is most commonly described in breast cancer [42]. We also emphasize the positivity of BPA-induced neoplastic regions for HER2/ErbB2, as these receptors are applied in the clinical diagnosis, for understanding the malignant and metastatic status that may occur [43,44].

According to previous descriptions, BPA presents molecular interaction with other hormone receptors, such as PR and AR [45,46]. Furthermore, an epigenetic action is assigned to BPA-disruptive processes that lead to tumor installation [28,47,48]. In this aspect, BPA interacts with ERα-promoting regions through DNA methylation, increasing its expression in cells [49] and making it persistent [50]. It also increases ERα translocation to the nucleus [51]. This epigenetic imprinting [51] was observed by the marking and incidence of EZH2, an epigenetic marker [52] in the mammary tissue. Furthermore, we suggest that this mechanism may have been a major candidate for the installation of ERα + cancer in mothers exposed to both BPA dosages, shown in Fig. 6-1. Clinically, EZH2 overexpression is related to the increased incidence of hyperplasia and alveoli disarray [34] in addition to chemotherapy resistance [53]. H-score values were also increased in the BPA exposed animals, defining a pathological status of neoplasia [41]. The action of EZH2 impacts the protein machinery of normal cells during BPA endocrine disruption due to its methyltransferase activity, causing hypomethylation of histone H3 (Fig. 6-1) [28,30]. This EZH2 mechanism acts on cell signaling pathways linked to the estrogen receptor and its coregulatory elements [27,35], being an attenuating factor for the progression of ERα positive carcinomas, as observed in the present study. This crosstalk between ERα and EZH2 may be related to the EMT phenotype observed in neoplastic cells disrupted by BPA (Fig. 6-2), since EZH2 is an inducer of this process [54–56].

BPA action is based on the disruption of epigenetic relationships in mammary gland [57], leading to the hypothesis that these changes could be the reason for ER positivity in mammary carcinoma. BPA disruption is not only related to the onset, but also to the progression of mammary cancer, since this ED alters more than 170 breast cancer-related genes [51], some of them linked to HER2/ErbB2 signaling [51], and the main pathways are triggered by ERα [58] (Fig. 6-2). In general, the alterations caused by BPA are selective to pathways related to genes under ERα transcriptional regulation [58], associated with apoptosis inhibition [59] and EMT process induction [60,61], as observed in the present study (Fig. 6-3).

Endocrine disruption by BPA led to the development of mammary carcinoma in aged female gerbils with PR, ERβ, and PRL-R loss of expression. This suggests that BPA modulates the response to certain hormones through depletion or exacerbation of hormonal-specific response, such as proliferation and expression of matrix remodeling proteins [58]. Under normal conditions, PR expression is mediated by ERα through the induction of estrogens [62], which was not observed in the present study. This demonstrates a pathway selectivity associated with BPA disruption in the expression of genes modulated by ERα [63], as found in ER+ breast cancer stem-like cells [64]. Changes in PR expression modulate molecular pathways preferentially activated by the progesterone-PR binding signal [17,65] (Fig. 6-1). This promotes a molecular crosstalk with ERα, decreasing estrogenic responses in neoplastic cells [17,66]. Among these signs, the main progesterone pathway in mammary gland is the establishment of a distinct alveolar structure and differentiation of the terminal end buds during estrous/menstrual cycles [67,68]. The absence of PR in breast cancer is associated with the progression of an irreversible state determined by the EMT process (Fig. 6-4), associated with an invasive phenotype [69] and poor prognosis [70]. Furthermore, in the present study the loss of PR enabled the classification of the developing cancer as luminal B [71], which is positive for ER and HER2/ErbB2.

Regarding ERs, the present study demonstrated that even in regions with normal and hyperplastic alveoli, expressive depletion in ERβ in

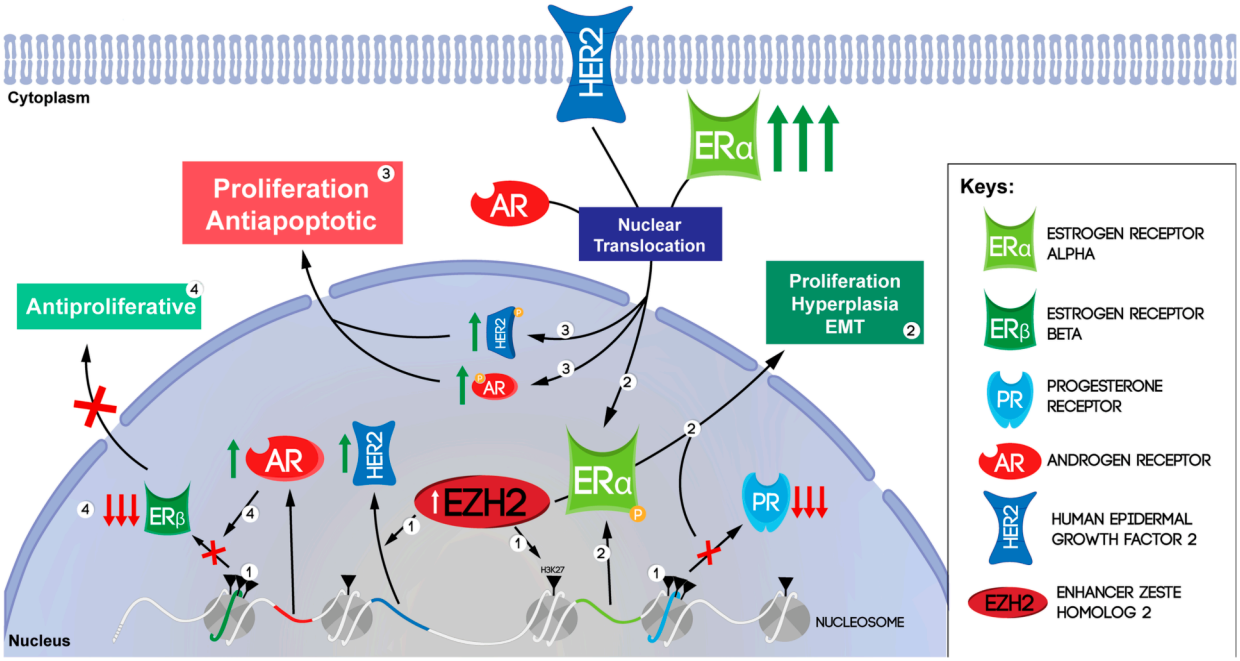

Fig. 6. Role of hormonal receptor expression in aged gerbil mammary gland under BPA disruption. (1) EZH2 mediated BPA disruption: EZH2 overexpression indicates the enhanced methylation of histone H3 in (H3K27). This contributes to increasing (green arrows) or decreasing (red arrows) expression of hormonal receptors – modifying HER2/ErbB2 expression. (2) Hyperplasia/EMT process: in the BPA exposed mammary gland, EZH2 expression promotes increased ERα expression and its nuclear translocation. ERα overexpression increases molecular pathways of proliferation causing hyperplasia and the EMT process in disrupted cells. Also, low expression of PR contributes to proliferative disorders in mammary tissue. (3) Proliferative/antiapoptotic action: BPA increases the translocation of AR and HER2/ErbB2 to the nucleus, which activates/inhibits cell proliferative/antiapoptotic pathways. (4) Deregulation in proliferative pathways: BPA reduces ERβ positive cells, impacting in the AR expression and, consequently, blocking the antiproliferative control for tissue homeostasis. Antiproliferative action: BPA promotes decreased expression and increases AR expression which contributes to the decrease in ERβ, and consequently the antiproliferative process. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

comparison to ERα was observed. As previously mentioned, BPA acts selectively through induction pathways linked to ERα, which are maintained through endocrine disruption. Differently, ERβ is associated with antiproliferative pathways [72]. We observed a higher incidence of ERβ expression in the control group, probably due to perimenopause regression and aging [73], against a drastic decrease in the BPA groups. ERβ presents a protective function in normal hormone-responsive organs [74] and is less expressed in female mammary gland after perinatal exposure to BPA [36,75]. Loss of ERβ leads to diminished regulatory control of cell maintenance processes in normal breast epithelium and promotes premalignant growth [76] linked to invasiveness in breast cancer [77]. Furthermore, ERβ is highly expressed under homeostatic conditions in mammary gland [72] while ERβ (*ESR2*) gene silencing occurs by hypermethylation events [78] (Fig. 6-4). The therapeutic implications of ERβ expression in breast cancer treatment demonstrate resistance to endocrine therapy [18,79].

ARs are relevant for the normal development of hormone-responsive organs [80,81] and/or neoplastic lesions [82]. Although there is little investigation on breast cancer development, efforts in the last decade defined relevant and conflicting parameters for the prognosis of breast cancer related to this receptor [83,84]. As in the present study, more than 90% of mammary tumors present AR expression, this being considered an interesting therapeutic target [85]. In addition, according to Farmer and collaborators [86], AR and ER positivity define the development of luminal breast cancer, corroborating the characterization made from the expression of PR.

As suggested for prostate cancer cells [87], the increase in mammary gland androgen receptors induced by BPA dosages may present an interaction through molecular pathways that alter ERβ expression (Fig. 6-2). Furthermore, AR has the ability to antagonize ERα processes by crosstalk with PR [83,88]; however, these pathways may be hampered, since PR showed low expression in BPA induced carcinomas. Oncogene activation mechanisms and cofactors for AR in prostate cancer are similar to those of ERα in the breast, and when expressed in the same context they can compete and increase the mitogenic activation capacity of cells [89]. Thus, we suggest that proliferation in BPA-induced carcinoma may be triggered through AR and ERα proliferative pathways, both highly expressed in the present study.

Additionally, the expression and nuclear detection of AR and HER2/ErbB2 in carcinoma regions suggest a co-regulation [90], with the ability to indirectly modulate cell proliferation [91] (Fig. 6-3). Both receptors are commonly found in the cytoplasm in their non-phosphorylated/non-ligand-bound form [88,92]. When both events occur, translocation to the nucleus promotes effects in cells [93,94], such as proliferation and invasiveness, in addition to risk of cancer recurrence [89]. This increased nuclear AR is associated with estrogenic effects [88]. BPA could also present pathways for tumor progression by overexpression of HER2/ErbB2 through epigenetic reprogramming, since EZH2 participates in the tumorigenesis process mediated by this receptor [95]. Indeed, some anticancer therapies aim to decrease nuclear translocation of AR and HER2/ErbB2, aiming to decrease the progression of effects through oncogenes [12,96]. Thus, pathways activated by these receptors and mechanisms associated with tumor growth are affected by age, tumor size, and sentinel lymph node characteristics [97]. This correlates with the present study, since aging is the main factor for the development of neoplasia in the gerbil model [98].

Several works identify features of mammary cancer progression in non-genetically modified experimental models from exposure to BPA [36,75,99–101]. In most cases, there is evidence of neoplastic processes after induction with carcinogenic compounds, such as MNU [26,102]. Among these models, the most widely used in studies of BPA-induced neoplasia/preneoplasia is the Sprague-Dawley rat [103,104]. Recently, our research group has focused efforts on experiments related to mammary gland of the Mongolian gerbil and observed the effects BPA

endocrine disruption in female offspring [36,105,106]. This species is used as a model for prostate [38,107] and gastric carcinogenesis [108], as females have developed prostate glands [109]. Furthermore, aging is a phase that greatly increases the incidence of neoplasia in hormone-responsive glands, such as the prostate [37,110].

The female gerbil is prone to the development of neoplasia in old age [37,111], such as adrenal dysregulation and ovarian tumors [112]. In addition, due to hormone-responsive variations in several susceptibility windows [113], the development of hormone-sensitive cancers in aging can be a useful application in this experimental model. Interestingly, in previous studies, the non-monotonicity of BPA effects at different concentrations found in Sprague-Dawley rats [114,115], occurred in gerbil mammary gland [36,105], and similar effects are observed in both dosages for tumor development and progression.

Malignant pathological processes in gerbils are attributed to events related to hormone receptors in organs of the reproductive system [116]. Carcinogenic development should reach spontaneous relevance [117] for observation of the progression and tumor establishment induced by hormonal pathways [116,118] or hormone-like pathways, such as by endocrine disruptors [119]. Thus, the effects observed in this study present the gerbil as an interesting model for the study of carcinogenesis in mammary tissue.

In summary, BPA-induced carcinoma with increased ERα positivity, loss of ERβ, PR, and PRL-R, and increasing nuclear incidence of HER2/ErbB2 and AR in mammary gland of aged female gerbils, defining the mechanisms of this ED in carcinogenesis (Fig. 6). Both BPA dosages triggered similar effects related to modulation of hormonal receptors, important to the progression of mammary cancer. In addition, the Mongolian gerbil is proposed as an experimental model for carcinogenesis induced by BPA, and useful for the study of endocrine disruption mechanisms and effective hormonal therapies for mammary neoplasia.

CRediT authorship contribution statement

Thalles Fernando Rocha Ruiz: Conceptualization, Investigation, Methodology, Writing manuscript.

Simone Jacovaci Colleta: Conceptualization, Investigation, Methodology.

Débora Aparecida Pires de Campos Zuccari: Provision of study materials, reagents, materials.

Patrícia Simone Leite Vilamaior: Resources and Critical review, commentary or revision – including pre-or postpublication stages.

Ellen Cristina Rivas Leonel: Conceptualization, Investigation, Methodology, Writing manuscript, data curation and supervision.

Sebastião Roberto Taboga: Conceptualization, Investigation, Methodology, Writing manuscript, data curation and supervision. Acquisition of the financial support for the project leading to this publication (FAPESP and CNPq). Management and coordination responsibility for the research activity planning and execution.

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Declaration of competing interest

The authors declare no conflict of interest.

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