

RESEARCH

Mammary carcinoma in aged gerbil mothers after endocrine disruption in pregnancy and lactation

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Abstract

Compounds that trigger breast cancer onset and establishment are of great interest in biological research. Endocrine disruptors are relevant because they initiate carcinogenesis by changing endocrine pathways. Bisphenol A (BPA), as a ubiquitous xenoestrogen, is largely associated with dysfunctions in the female reproductive system and associated organs. This study proposes an investigation of the mammary gland (MG) in aged Mongolian gerbil (*Meriones unguiculatus*) mothers after their exposure to BPA in two windows of morphophysiological plasticity: pregnancy and lactation. A low dose (50 µg/kg) and a high dose (5000 µg/kg) of BPA were considered, and results showed few differences between them. As expected, we observed contrasts among control and BPA-exposed MG. The control groups presented a regressive phase with high apoptotic activity and elastic stroma. However, BPA damaged mammary tissue and provoked multifocal carcinoma development supported by an apparent epithelial-mesenchymal transition (EMT) and reactive stroma establishment. BPA remodeled stromal fibers deposition and enhanced the recruitment of tumor-associated cells, contributing to a tumoral microenvironment. Overexpression of TGF-β1 was induced by BPA in the epithelial compartment of exposed MG, and increased expression of metalloproteinases (MMP-2, MMP-3, MMP-9) was present in carcinoma cells. In conclusion, exposure of mothers to BPA during the gestational/lactational window of susceptibility leads to carcinogenic impacts with aging.

Key Words

- mammary gland
- bisphenol A
- TGF-beta
- epithelial-mesenchymal transition
- extracellular matrix

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Introduction

Carcinogenesis in the breast is a complex pathological process of the female reproductive system. Due to the occurrence of different types of breast cancer, diverse onset and dynamics are attributed to its progression and the regulation of tumor growth and metastatic dissemination (Alvarado-Cabrero *et al.* 2020). A central feature in

breast cancer is the deregulation of cell homeostasis and microenvironment, associated with tumor development (Zhang *et al.* 2017).

Considering the risk factors, age and parity are relevant aspects for the onset and advancement of breast cancer. In addition, due to the fact that the mammary gland (MG)

is one of the most plastic organs, oncogenesis in this tissue is closely related to stages of susceptibility that this gland undergoes during a woman's life (Terry *et al.* 2019). Pregnancy and lactation are two windows of susceptibility where MG is subjected to major morphological compartment rearrangements to perform its functionality (Faupel-Badger *et al.* 2013).

Several triggering factors have been attributed to carcinogenesis. Some of them are environmental chemicals that disrupt mammary tissue, leading to the development of endocrine-related cancers (Vandenberg *et al.* 2019), in women (Giulivo *et al.* 2016) and rodent models (reviewed by Seachrist *et al.* 2016). Endocrine-disrupting chemicals (EDCs) act by different pathways in the reproductive system, causing different consequences, mainly through establishing a tumoral microenvironment (Soto *et al.* 2013). Exposure to EDCs has also been implicated in increasing the incidence of breast cancer (Terry *et al.* 2019). Among EDCs, xenoestrogens, especially bisphenol A (BPA), promote disruptive effects by estrogenic routes, interacting with other receptors, such as androgen (AR) and mitogen receptors linked to MAPK/ERK, for promoting a disruptive activity (reviewed in Shafei *et al.* 2018a). Studies have demonstrated the development of carcinoma and tumor progression in the prostate (Huang *et al.* 2018), endometrium (Leung *et al.* 2020), and ovaries (Seachrist *et al.* 2016) after perinatal experimental BPA exposure.

In the MG, studies analyzing different perinatally exposed experimental models describe the impacts in offspring when mothers are exposed to BPA during pregnancy and lactation (Mileva *et al.* 2014, Mandrup *et al.* 2016, Leonel *et al.* 2020). These exposure periods are susceptible to the development of pathologies in MG, mainly neoplasia (Terry *et al.* 2019), being BPA a triggering agent for the incidence of breast cancer (Soto *et al.* 2013, vom Saal & Vandenberg 2021). In women, BPA has been associated with an increase in breast stromal density (Sprague *et al.* 2013), in addition to provoking modifications in molecular and epigenetic pathways that can trigger or promote breast cancer installation (Goodson *et al.* 2011, Soto *et al.* 2013, Dhimolea *et al.* 2014, Shafei *et al.* 2018b, Atlas & Dimitrova 2019). In fact, BPA has non-monotonic effects in the MG (Montévil *et al.* 2020), which may contribute to carcinogenic effects at different doses.

Thus, in the present study, we analyze MG from mothers directly exposed to BPA endocrine disruption during their gestation and lactation. We investigated long-term effects in the aging phase of a rodent experimental model, the Mongolian gerbil (*Meriones unguiculatus*), which shows spontaneous development of neoplasms

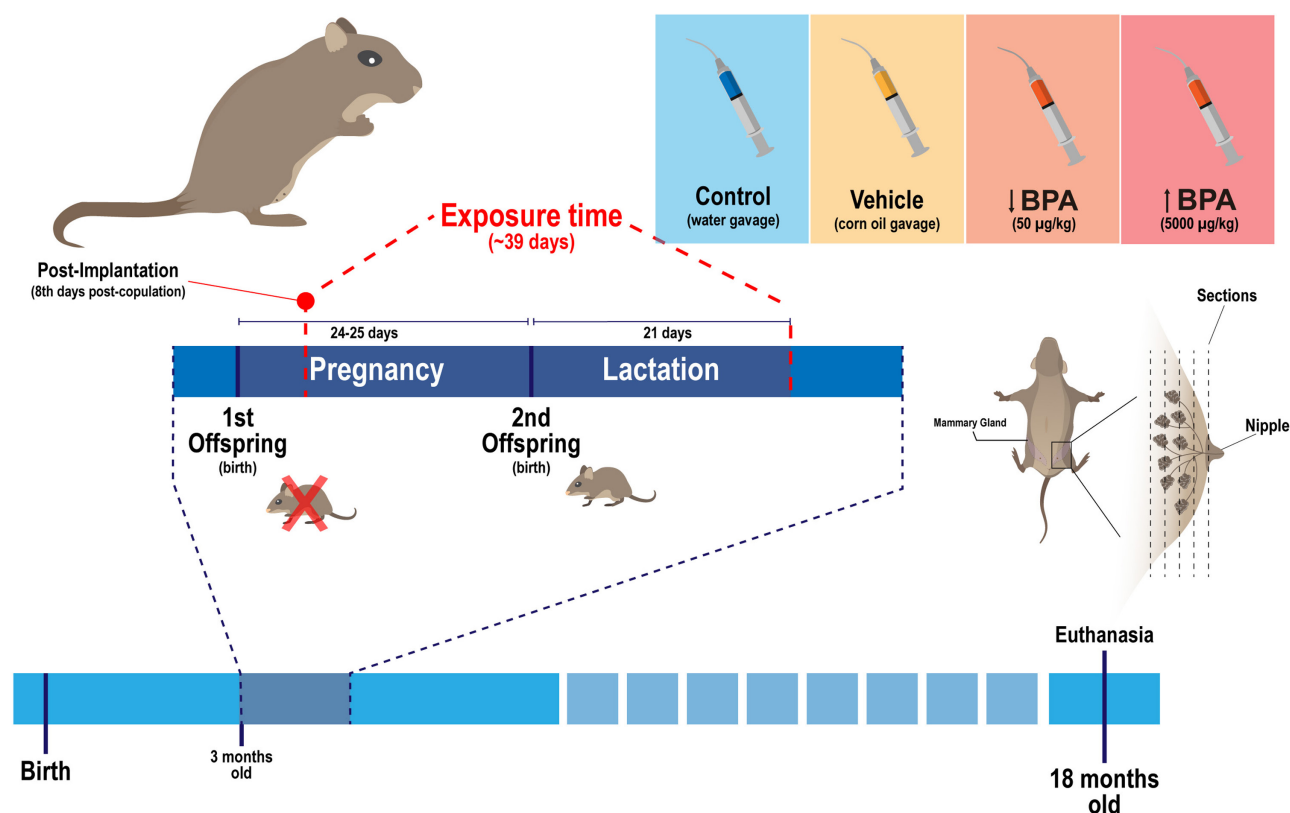
(Norris & Adams 1972, Custodio *et al.* 2010), thus mimicking a natural environment with a greater propensity for tumoral and inflammatory development. Also, aging in the gerbil is a critical point to the development of hormone-related disorders (Vincent *et al.* 1980, Campos *et al.* 2008), previously studied in the prostate from males and females (Skene's paraurethral glands) (Custodio *et al.* 2008, Biancardi *et al.* 2017).

The relevance of this study lies in the fact that aging is related to the establishment of a tumor favorable microenvironment (Li *et al.* 2020). Therefore, we hypothesize that gerbil mothers' exposure to BPA in gestational-lactational windows can be a risk factor for tumor development, increased with aging. In addition, we present relevant aspects of the MG neoplastic developmental process caused by an EDC and morphological aspects associated with the tumoral microenvironment.

Materials and methods

Experimental design

Twenty female Mongolian gerbils (*M. unguiculatus*) were used. The animals were kept in polysulfone isolators with wood shaving bedding. They were fed LABMIX® commercial chow (Agromix, SP, Brazil), containing only traces of soy and corn and fresh filtered water *ad libitum*. The 3-month-old females were housed with fertile males of the same age. The gerbil females were divided into four experimental groups ($n = 5$ for each group): control – subjected to daily gavage with water; vehicle – subjected to daily gavage with corn oil; treated groups: ↓BPA (50 µg/kg) – subjected to daily gavage with a dosage considered safe (not capable of provoking disruption), according to US EPA (Soriano *et al.* 2019) and EFSA (EFSA 2015); and ↑BPA (5000 µg/kg) – subjected to daily gavage of 100× the safety dose/high exposure, both diluted in 0.1 mL corn oil. The experimental design is shown in Fig. 1. Gerbil mothers were monitored and exposed to the treatments from the 8th day after the first parturition, when the second pregnancy started (first offspring was discarded), until the end of lactation. The treatment comprised 39 days of exposure in total (length of second pregnancy: 24–25 days, length of lactation: 21 days). The second offspring were separated (used in other studies), and all mothers were kept until 18 months of age (elderly females). One animal was maintained per cage to avoid social stress. Sacrification was performed during the morning, after confirmation of the exclusive presence of cornified cells in the vaginal smear. The animals were

**Figure 1**

Experimental design. Pregnant female gerbils were divided into four experimental groups ($n = 5$): control (water gavage); vehicle (corn oil gavage); $\downarrow$ BPA (50 $\mu\text{g/kg}$); $\uparrow$ BPA (5000 $\mu\text{g/kg}$). They were exposed from the 8th day after the birth of the 1st offspring (period corresponding to the pregnancy of the second offspring) until the end of lactation (total of 39 days). At 18 months of age, gerbil mothers were euthanized, and MG was transversally sectioned for histopathological analysis. A full color version of this figure is available at <https://doi.org/10.1530/ERC-21-0198>.

anesthetized in 3.0% isoflurane vaporized with oxygen and sacrificed by decapitation.

Histological procedures

The abdominal MG was collected. The entire gland with the skin and nipple was placed flattened in histological cassette and fixed with 4% paraformaldehyde for 24 h. Then, the skin was removed and nipples were maintained. Samples were washed in 70% ethanol and processed by routine histology in a Leica Semi Enclosed System (TP1020, Leica Biosystems). Taking the nipple as a reference, histological sections (4.5 μm thickness – serial) were made transversally to it (Fig. 1) and placed on silanized slides. All samples were collected within the MG sentinel lymph node region, visually observed during sacrifice. Hematoxylin and Eosin-Floxin (HE) staining was performed for histopathological analysis and description of tissue alterations using ordinary light microscopy and fluorescent light microscopy (Carvalho & Taboga 1996). Techniques for stromal connective fiber identification

were performed: Weigert resorcin-fuchsin for elastin and picrosirius for collagen fibers using polarized light microscopy (Junqueira *et al.* 1979).

Immunohistochemistry

The slides were subjected to immunohistochemistry (IHC). The procedure followed the steps described subsequently and was adapted according to the specificity of each primary antibody. The sections were first deparaffinized and rehydrated in a gradient of xylol, alcohol, and water followed by antigenic retrieval, in 10 mM citrate buffer or EDTA Tris, at 92°C or 98°C. Next, endogenous peroxidases were blocked with 10% H_2O_2 diluted in methanol. The inhibition of nonspecific proteins was performed in 5% BSA or skimmed milk. Subsequently, the following primary antibodies were incubated overnight with specific dilution: MMP-2 (mouse monoclonal, 8B4, 1:50, sc-13595, Santa Cruz Biotechnology), MMP-3 (rabbit polyclonal, PA5-27936, 1:100, Invitrogen, ThermoFisher), MMP-9 (mouse monoclonal, 2C3, 1:100, sc-21733, Santa

Cruz Biotechnology), α -smooth muscle actin (α -SMA) (mouse monoclonal, 1A4, 1:100, sc-32251, Santa Cruz Biotechnology), FAP (mouse monoclonal, F11-24, 1:100, sc-65398, Santa Cruz Biotechnology), TGF- β 1 (rabbit polyclonal, 3c11, 1:100, sc-146, Santa Cruz Biotechnology), active/cleaved caspase-3 (rabbit polyclonal, 1:100, NB100-56113, Novus Biological), phospho-histone H3 (PH-H3, rabbit polyclonal, 1:75, Ser10, 9701, Cell Signaling), p63 (mouse monoclonal, D-9, 1:100, sc-25268, Santa Cruz Biotechnology), pan-cytokeratin (mouse monoclonal, AE13, 1:100, sc-57012, Santa Cruz Biotechnology), and vimentin (mouse monoclonal, V9, 1:100, sc-6260, Santa Cruz Biotechnology). The steps described were interspersed with washes in PBS or TBS. The slides were then incubated with a post-primary antibody and Polymer kit (Novolink™ polymer detection system 1, Leica Biosystems Newcastle Ltd.) according to the manufacturer's descriptions. Detection of positive staining was performed with 3-30 diaminobenzidine tetrahydrochloride (DAB) (Novolink™ DAB, RE7270-CE, Leica Biosystems) and counterstaining with hematoxylin.

Histopathological analysis

The areas of different MG compartments (epithelium, stroma, and adipose tissue) were assessed by analysis of HE sections (three sections per animal from different depths) and evaluated by the adapted M130 multipoint test system (Leonel *et al.* 2020). The incidence and multiplicity of microscopic lesions in the MG of aged female gerbils were also evaluated. For this, α -SMA staining was applied for myoepithelial cells identification – aiming to evaluate the maintenance of basal membrane and structure of the epithelial unit – and mammary epithelial structures were divided into three classifications: normal morphology (without hyperplasia or rupture of the myoepithelial layer); hyperplasia (more than two layers of luminal epithelial cells, without disruption of the myoepithelial layer); and carcinoma/neoplasia (total or partial loss of myoepithelial layer and disruption of the epithelial compartment). The quantification was performed taking into account the incidence of normal alveoli, hyperplasia, and carcinoma regions in relation to all the epithelial structures in the same histological section. Data were expressed as a percentage of epithelial lesions found in the same section of MG.

The area of collagen and elastic fibers was quantified by the automated system from ImageJ software (version 1.52a, Wayne Rasband, 130 NIH). For these quantifications, 15 random fields (200 $\times$ magnification) were selected in three different sections, corresponding to different depths

of the MG sample, from the slides stained with picrosirius (for collagen analysis, under polarized microscopy) and resorcin-fuchsin (for elastic fibers analysis, under light microscopy).

The quantification of IHC positive staining for identification of cytoplasmic and nuclear markers was performed by applying QuPath software (version 0.1.2, an open-source pathology software platform). The total area of histological sections was considered for the quantification of the positive staining, and data were expressed in cells/mm².

Statistical analysis

Analyses of stereological parameters (incidence of compartments) and areas of collagen and elastic fibers were performed in three sections from different depths of the mammary gland sample. The mean of the three analyzed sections was calculated, and this mean was used as the individual value for each animal in statistical analyses. For IHC analyses, one random section per animal was analyzed, and values of cells/mm², presented as means $\pm$ S.E.M., were considered for the statistical analyses.

All data were checked for normality by the Kolmogorov-Smirnov test. Parametric data (epithelial area and lesions incidence, elastin area; IHC positive staining: PHH3, TGF- β 1, FAP, MMP-2, MMP-3, MMP-9) were analyzed by one-way ANOVA followed by Tukey's test. For non-parametric data (collagen area, stromal and adipose tissue areas; IHC positive stains: p63, active caspase-3), we applied the Kruskal-Wallis test followed by Dunn's test. Statistical differences among groups were considered significant when $P < 0.05$. For all statistical analyses, GraphPad Prism 5.00 for Windows (GraphPad Software, www.graphpad.com) was applied.

Ethical standards

The animals were kept at the Animal Breeding Center of the Institute of Biosciences, Humanities and Exact Sciences (IBILCE) of the São Paulo State University (UNESP) throughout the experiment, under controlled temperature ($24 \pm 2^\circ\text{C}$) and luminosity (12 h light:12 h darkness cycle). The procedures followed the standards according to the National Council of Animal Experiment Control (CONCEA, Brazil) and were authorized by the Ethics Committee on the Use of Animals (CEUA) from IBILCE/Unesp (Protocol number: 217/2019).

Results

Histopathological repercussions of BPA exposure in aged female MG

All tissue compartments showed differences when comparing control and vehicle groups to both BPA groups (Fig. 2A, B, C and D). The areas of epithelial and stromal compartments in both BPA groups were increased (Fig. 2C, D, E and F) in comparison to control and vehicle groups. At 18 months of age, MG from control and vehicle groups presented an increase in adipose tissue (Fig. 2G) compared to the BPA-exposed gerbils MG. The stromal area increased in both BPA groups, being statistically different from control and vehicle groups (Fig. 2F). MG epithelium occupation in BPA groups was increased almost to four-($\uparrow$ BPA) and three-fold ($\downarrow$ BPA) compared to control (Fig. 2E). These groups also showed disorganization of

the epithelial compartment with loss of differentiated luminal cells and alveolar shape, with the development of multifocal carcinoma regions. These regions showed heterogeneous nuclear atypia (Fig. 2H), with no apparent cytokeratin expression (Fig. 2I) and enhanced vimentin expression (Fig. 2J).

In comparison to control and vehicle groups (Fig. 3A and B), MG from BPA-exposed female gerbils showed an increase in hyperplastic structures and the presence of neoplastic features (Fig. 3C and D, respectively). Hyperplastic structures were identified by the presence of two (Fig. 3C) or more layers (Fig. 3D) of epithelial cells. Control and vehicle groups presented hyperplastic foci incidence (Fig. 3E) two-fold lower than BPA groups. Neoplastic structures showed discontinuity of the myoepithelial cell layer, as shown by α -SMA immunostaining; thus, a carcinoma/neoplastic phenotype was characterized by the absence

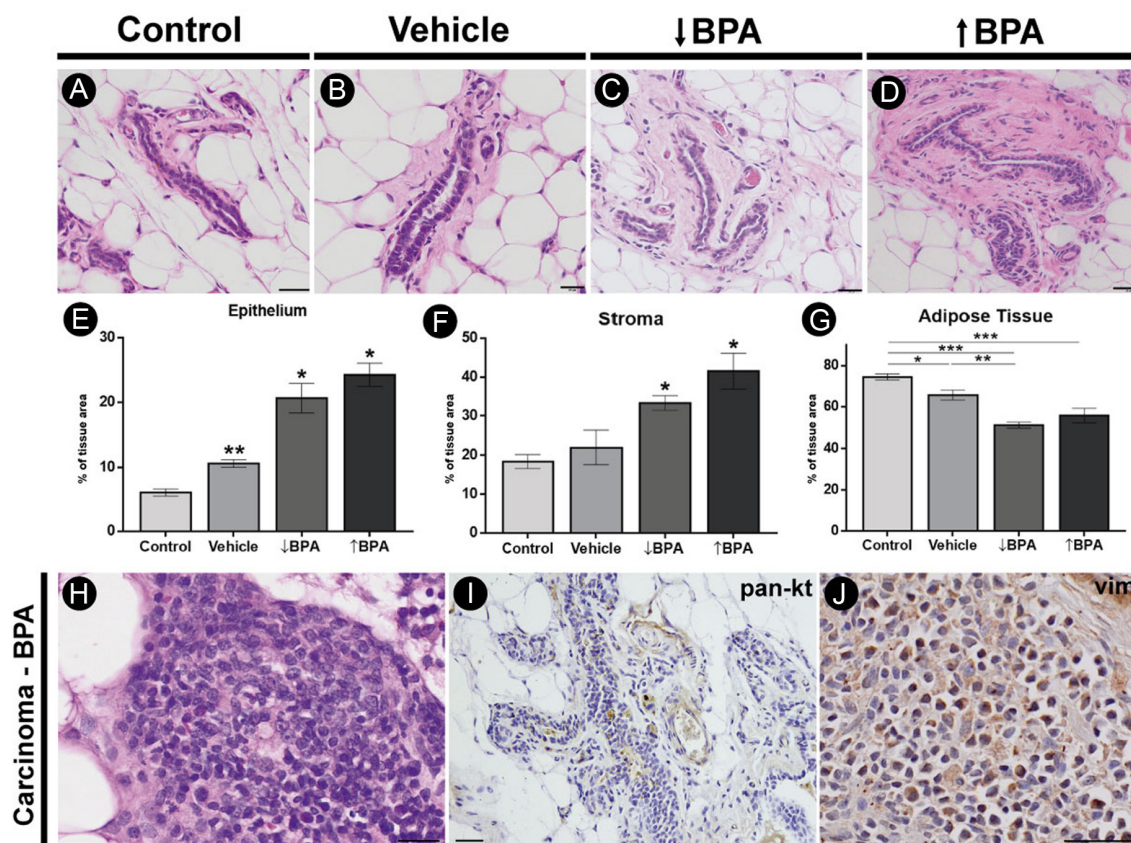


Figure 2

Tissue compartments and carcinoma features. (A, B, C, D) Morphology of normal mammary tissue in control (A), vehicle (B), $\downarrow$ BPA (C), and $\uparrow$ BPA (D) groups. (E, F, G) Incidence (percentage) of epithelial (E), stromal (F), and adipose tissue (G) compartments in different groups ($n = 5$). Asterisks indicate statistical differences among groups, considering significance when $P < 0.05$ (statistical analysis: parametric data: one-Way ANOVA followed by Tukey's test (epithelial percentage); non-parametric data: Kruskal-Wallis test followed by Dunn's test (stromal and adipose percentage)). (H, I, J) Carcinoma feature in MG of BPA-exposed aged females. Nuclear atypia and nuclear irregularity were observed (H) with the absence of pan-cytokeratin (I) and the presence of vimentin (J) expression. Staining: (A, B, C, D, H) HE. Scale bars: (A) 50 μ m; (B, C, D, H, J) 20 μ m; (I) 30 μ m. A full color version of this figure is available at <https://doi.org/10.1530/ERC-21-0198>.

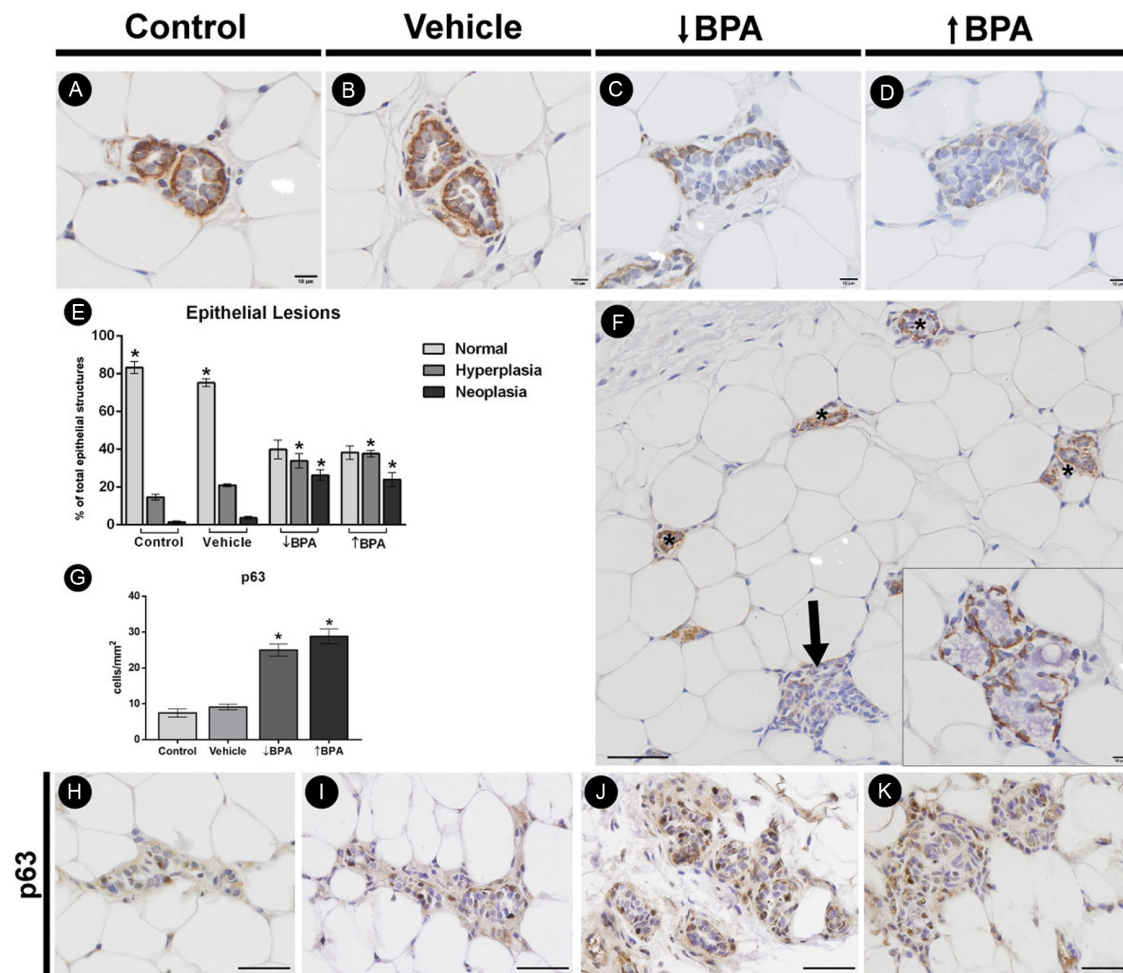


Figure 3

Mammary epithelial lesions, myoepithelial layer, and p63 expression. (A, B, C, D) Myoepithelial cell layer (α -SMA) in mammary tissues indicates continuity of myoepithelial layer in control (A) and vehicle (B), whereas, in $\downarrow$ BPA and $\uparrow$ BPA, there is a discontinuity (C) or absence (D) of this layer. (E) Epithelial lesions (% of total epithelial structures): normal, hyperplasia, and neoplasia were quantified. $\downarrow$ BPA and $\uparrow$ BPA groups presented a high incidence of both lesions, with multifocal carcinoma development (F, arrow) among normal alveoli (F, asterisks). Note alveoli with secretory activity (F-inset). (G) Incidence of p63 positive cells. (H, I, J, K) Expression of p63 in MG. Control (H) and vehicle (I) presented only nuclear staining, indicating basal epithelial cells. In $\downarrow$ BPA and $\uparrow$ BPA, in addition to nuclear staining, basal-like stemness was assigned by cytoplasmic staining in carcinomas. Asterisks indicate statistical differences among groups, considering $P < 0.05$ of significance ($n = 5$, parametric data: one-Way ANOVA followed by Tukey's test (epithelial lesions percentage); non-parametric data: Kruskal-Wallis test followed by Dunn's test (p63). Scale bars: (A, B, C, D, F-inset) 10 μ m; (F) 50 μ m; (H, I, J, K) 30 μ m. A full color version of this figure is available at <https://doi.org/10.1530/ERC-21-0198>.

of this layer around normal alveoli (Fig. 3F). In addition, $\downarrow$ BPA and $\uparrow$ BPA groups presented alveoli with secretory activity (Fig. 3F-inset), which was not observed in the other groups. These secretory structures were dispersed in MG parenchyma and identified in all $\downarrow$ BPA and $\uparrow$ BPA samples.

The vehicle group (Fig. 3I) demonstrated a slight increase in p63 basal cell expression but did not differ significantly from the control, which showed only nuclear staining (Fig. 3H). Contrarily, in the BPA groups, the expression increased almost three-fold compared to the control. Furthermore, in these groups, the expression

of p63 was nuclear and cytoplasmic in the regions of early carcinoma (Fig. 3J and K). $\uparrow$ BPA showed the highest expression rates but did not differ significantly from $\downarrow$ BPA.

The proliferative and apoptotic activity was evaluated by PH-H3 (Fig. 4A, B, C and D) and active caspase-3 (Fig. 4E, F, G and H) nuclear staining, respectively. The control group presented rates of PH-H3 positive cells almost ten-fold lower compared to BPA groups (Fig. 4I). Carcinoma in BPA groups and hyperplasia in both BPA and vehicle groups induced a high proliferative rate in comparison to the control group. $\downarrow$ BPA and $\uparrow$ BPA groups did not differ

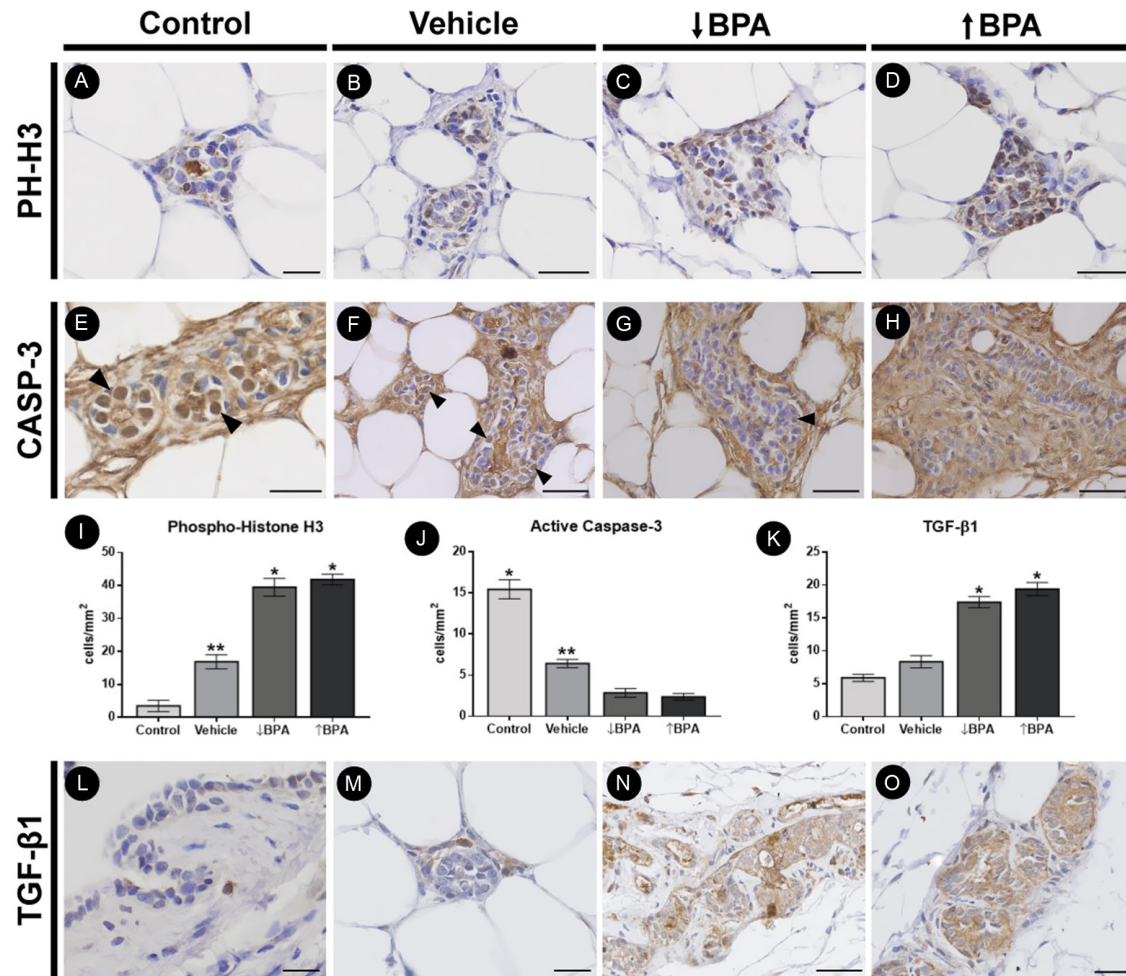


Figure 4

Proliferative and apoptotic activity and TGF- β 1 expression. (A, B, C, D) Phospho-histone H3 positive cells indicating proliferative activity and (E, F, G, H) active caspase-3 indicating apoptosis signaling (both nuclear staining – arrowheads). (I) and (J) show the proliferative/apoptotic opposite dynamics of mammary tissue among groups. Proliferation was increased in $\downarrow$ BPA and $\uparrow$ BPA groups, whereas in control, apoptosis was enhanced in epithelial cells. (K) TGF- β 1 expression in MG of aged females. Overexpression was observed in BPA groups. Control and vehicle groups presented positive cells only in the stroma (L and M, respectively), whilst BPA presented overexpression in the epithelial compartment (N and O). Asterisks indicate statistical differences among groups, considering $P < 0.05$ of significance ($n = 5$, parametric data: one-Way ANOVA followed by Tukey's test (PHH3, TGF- β 1); non-parametric data: Kruskal–Wallis test followed by Dunn's test (active caspase-3). Scale bars: (A, E, L, M) 20 μ m; (B–D, F–H, N, O) 30 μ m. A full color version of this figure is available at <https://doi.org/10.1530/ERC-21-0198>.

from each other and showed the highest values of PH-H3-positive cells in epithelial and stromal compartments (Fig. 4C and D). Nuclear expression of active caspase-3 was only observed in the epithelial compartment, from all groups. At 18 months of age, the control group presented a high number of apoptotic cells (caspase-3 positive cells) (Fig. 4J), while a drastic decrease in their expression was observed in $\downarrow$ BPA and $\uparrow$ BPA groups compared to control and vehicle. In multifocal carcinoma structures, present only in BPA-exposed gerbils MG, scarce expression of caspase-3 was observed in $\downarrow$ BPA (Fig. 4G) and was absent in $\uparrow$ BPA (Fig. 4H).

Expression of TGF- β 1 in MG of aging BPA-exposed females

TGF- β 1 was overexpressed in BPA groups (Fig. 4K). Gerbil MG of control and vehicle groups presented low rates of TGF- β 1-positive cells, which were restricted to the stromal compartment (Fig. 4L and M). However, BPA groups presented a drastic increase (three-fold) in TGF- β 1-positive cells with epithelial and stromal staining (Fig. 4N and O). Epithelial staining of TGF- β 1 was observed in normal and hyperplastic ducts, whereas carcinoma cells did not stain for this marker.

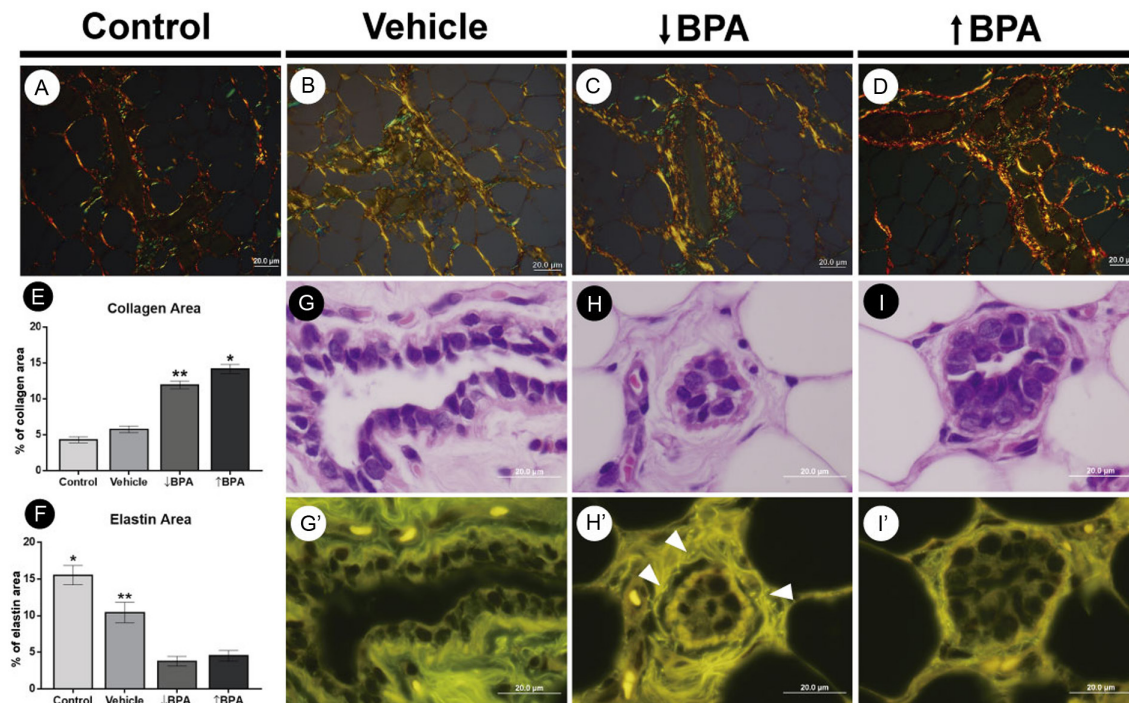


Figure 5

Stromal fibers in disrupted MG. (A, B, C, D') Collagen fibers were enhanced in BPA groups in the surrounding stroma. Furthermore, ↑BPA presented the highest values in % of collagen area and differed from all groups (E). (F) Elastin showed a continuous feature and its area % increased in control and vehicle (G and G') groups. (H and H', I and I') In addition to a decreased area, BPA groups presented rupture of elastin layer in MG stroma (arrowheads in H'). Staining: (A, B, C, D) Picosirius red under polarized microscopy; (G, H, I) H&E-Floxin under light and (G', H', I') fluorescent microscopy. Asterisks indicate statistical differences among groups, considering $P < 0.05$ of significance ($n = 5$, parametric data: one-Way ANOVA followed by Tukey's test (elastin area); non-parametric data: Kruskal-Wallis test followed by Dunn's test (collagen area). Scale bars: 20 μm . A full color version of this figure is available at <https://doi.org/10.1530/ERC-21-0198>.

Stromal fiber density of BPA-exposed MG in aged females

Collagen area occupation presented low values in control and vehicle groups (Fig. 5A and B), with more than two- and three-fold increase in ↓BPA and ↑BPA (Fig. 5C and D), respectively, compared to control group. Epithelial structures and carcinoma areas were surrounded by a collagen-rich stroma. The percentage of collagen and elastin fibers was different among groups (Fig. 5E and F). For elastin fibers, a high percentage was observed in control and vehicle, comparing to both BPA-exposed groups (Fig. 5G, G', H, H', I and I'). Furthermore, elastic fibers surrounding the stroma of intact ductal structures in BPA-exposed gerbil MG were ruptured (Fig. 5H').

Metalloproteases expression and FAP-positive cells in the stroma of normal and altered BPA-exposed MG

The BPA-exposed groups presented a higher incidence of cells expressing all MMPs compared to control and

vehicle groups (Fig. 6A, B and C). MMP-2 positive cells were observed in the stromal compartment (Fig. 6D and E). Cells in carcinoma regions presented poor staining (Fig. 6E). MMP-3 positivity was also observed in fibroblasts among carcinoma cells (Fig. 6G), stromal cells, and the non-disrupted myoepithelial layer (Fig. 6H and I). MMP-3 cytoplasmic expression was predominant but several cells also presented nuclear positivity (Fig. 6H). MMP-9 was observed in stromal cells and poorly diffused in ECM (Fig. 6J, K and L). Cytoplasmic staining of MMP-9 was observed in the disrupted epithelial compartment (Fig. 6K).

FAP expression (Fig. 6M, N, O, P and Q) in stromal cells was different between BPA-exposed groups and both control and vehicle groups (Fig. 6N and O). FAP-positive fibroblasts (i.e. cancer-associated fibroblasts, CAFs) increased around two- and three-fold in MG of ↓BPA and ↑BPA groups in comparison to control (Fig. 6M). Furthermore, FAP-positive cells in BPA-exposed gerbil MGs were predominant in surrounding stroma and carcinoma (Fig. 6Q).

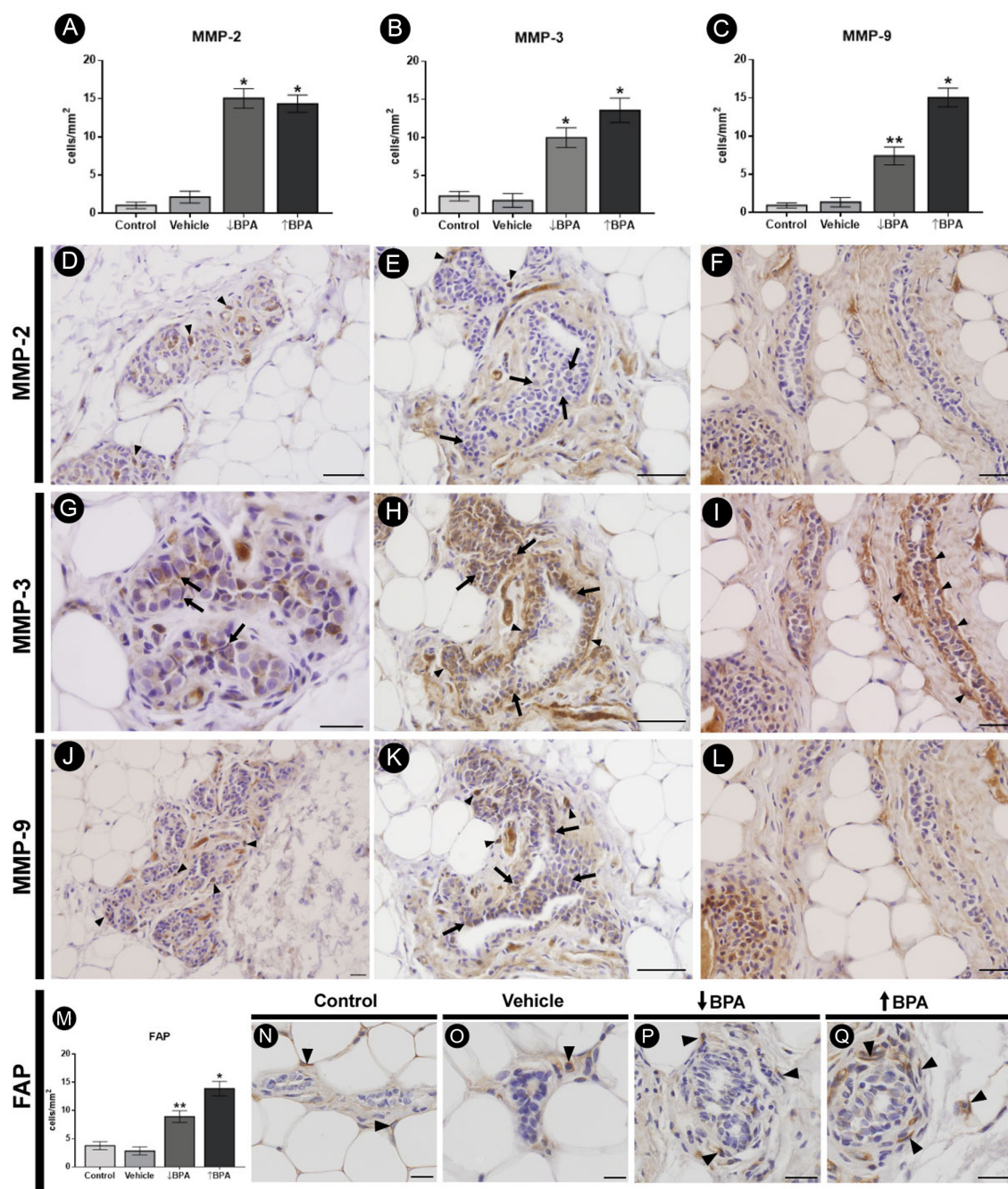


Figure 6

Metalloproteinases expression and FAP-positive cells in BPA-exposed MG. (A), (B), and (C) indicate the incidence of positive cells per mm² for MMP-2, MMP-3, and MMP-9, respectively. (D, E, F) MMP-2. Cytoplasmic staining in the stromal (arrowheads) and carcinoma cells (arrows) were observed. (G, H, I) MMP-3. (G) Fibroblasts (arrows) among disrupted epithelium expressing MMP-3 were found. (H-I) In normal alveoli and ducts, myoepithelial cells (arrowheads) express MMP-3. Arrows in both images demonstrate nuclear staining for MMP-3 in carcinomas. (J, K, L) MMP-9. Stromal (arrowheads) and carcinoma cells (arrows) present cytoplasmic staining for MMP-9. (M, N, O, P, Q) Incidence of FAP-positive cells. FAP-positive fibroblasts were observed only in the peripheric stroma of control and vehicle groups (N and O, arrowheads), whereas in both BPA groups, they were found among disrupted carcinoma (P and Q, arrowheads). Asterisks indicate statistical differences among groups, considering $P < 0.05$ of significance ($n = 5$, parametric data: one-Way ANOVA followed by Tukey's test). Scale bars: (D, E, F, G, H, I, J, K, L) 50 μ m; (N and O) 20 μ m; (P and Q) 30 μ m. A full color version of this figure is available at <https://doi.org/10.1530/ERC-21-0198>.

Discussion

Tumorigenic development was observed in MG of aged gerbils exposed to BPA

The female gerbils were exposed to BPA during two drastic remodeling periods for MG, pregnancy and lactation. We demonstrate the susceptibility of MG tissue to endocrine-chemical disruption and its potential to induce carcinogenesis during these exposure periods. Furthermore, aging is an aggravating factor to the onset of tumorigenic process (Fane & Weeraratna 2020). In the present study, we observed the development of multifocal neoplastic structures in MG of aged gerbils exposed to BPA during pregnancy and lactation. These exposure windows deserve to be focused on discussion related to the development of neoplastic triggering after pregnancy (Schedin 2006, Hsiao *et al.* 2010, Allouch *et al.* 2020). In addition, pregnancy-lactational window must be recognized as a vulnerability period, which impacts a third window related to aging. In fact, this study shows evidence that exposure to BPA (without the use of other carcinogenic inducers (Cheung *et al.* 2003)) is capable of promoting mammary cancer in a susceptible animal model. These negative impacts were demonstrated in MG of mothers directly exposed to BPA, a susceptible group not evaluated in the majority of published works, in which the main concern is the transgenerational effect (Yoshida *et al.* 2004, Wadia *et al.* 2007, Kunz *et al.* 2011, Lozada & Keri 2011, Gomez *et al.* 2017) resulted from disruption during the perinatal window of susceptibility.

The tumor development process was observed in the tissue, from stages for cancer establishment (nuclear atypia, phenotypic changes in epithelial cells, and hyperplasia) to alveolar remodeling and tumorigenic interaction of the epithelial and stromal compartments (Mallon *et al.* 2000). Hyperplasia was present in all groups but enhanced in females exposed to BPA. Hyperplastic structures were characterized by discontinuity of the myoepithelial cell layer, which acts as a barrier between the secretory luminal epithelial cells and the stroma (Li *et al.* 2020). The myoepithelial cell layer also signals the polarization of luminal epithelial cells (Gudjonsson *et al.* 2002). Thus, its loss leads to a disorder in the epithelial compartment where cells undergo proliferative disarray. Another marker considered for evaluating tumor progression to malignant and invasive phenotype was the cytoplasmic expression of p63 (Hsiao *et al.* 2010), which increased in BPA-exposed MG.

High neoplasia rates in BPA-exposed aged female gerbils demonstrate the carcinogenic potential of this xenoestrogen (Seachrist *et al.* 2016). *In vitro* studies showed the ability of BPA to induce neoplastic development in breast epithelial cells (Fernandez & Russo 2010). Shafei and colleagues reviewed BPA molecular mechanisms of action and related mammary disorders to epigenetic changes and anti-apoptotic activity (Shafei *et al.* 2018a). In the present study, the proliferation rates in the BPA groups were high; contrarily, the number of cells in the apoptotic process was reduced, establishing the tumorigenic process. MG from female Mongolian gerbil individuals at 18 months of age, when not exposed to the xenoestrogen BPA, presented similar characteristics of the perimenopause involution phase (Oh *et al.* 2016). This is also evidence that BPA alters cell machinery (Lee *et al.* 2013) to promote survival and transformation of cancer cells at a late age.

Also, an increase in proliferative activity was observed in MG of female gerbils from the vehicle group, mostly associated to hyperplasia. Indeed, this finding can be related to the activity of phytoestrogens found in the vegetable oil (Lorand *et al.* 2010), which could promote hyperplastic features in gerbils MG, as described (Leonel *et al.* 2020), and a morphological feature often associated to aging (Attia 1996, Gomez *et al.* 2017).

BPA induces epithelial-mesenchymal transition in MG of aged gerbils

The epithelial-mesenchymal transition (EMT) is one of the initial processes in tumor progression and invasion of adjacent tissues (Lee *et al.* 2017). When exposed to BPA, breast cancer cells modulate key signal-proteins of endocrine response to promote EMT signaling, such as loss of cytokeratin and vimentin expression (Patel *et al.* 2015), similarly to that observed in our study. TGF- β 1 is a cytokine with major responsibility for leading breast cancer cells to the EMT process, also contributing to the next invasiveness steps (Zhang *et al.* 2020). Its high expression in BPA groups, especially in $\uparrow$ BPA, corroborates the EMT mechanism observed in MG of aging gerbils. Dong and colleagues associated TGF- β 1 expression during mammary carcinoma development as a remarkable modulating signal for MMP-9 stromal overexpression in MG (Dong *et al.* 2019). The TGF- β 1 increase in response to stromal modifications indicates the relevance of epithelial-stromal interactions. Thus, the interaction between compartments supports carcinoma development induced by BPA.

Even though stromal features influence the EMT process, epithelial protein expression also contributes to tumorigenic development. High epithelial expression of TGF- β 1 in mammary cells of BPA-exposed gerbils indicates that this marker participates in susceptibility to BPA disruption (Betancourt *et al.* 2014). As TGF- β 1 amplifies the EMT process (Goulet & Pouliot 2021) and cancer modulation in ECM (Vandenberg *et al.* 2012), its expression in normal epithelial cells and tissue of BPA groups indicates its role as a mediator in the tumor induction by this xenoestrogen in gerbil MG. Clearly, the tumorigenic process has several molecular influences, but TGF- β 1 expression in disrupted tissue suggests its key role in BPA action.

Compared to non-exposed tissue of control and vehicle groups, in BPA groups, a proliferative pathway was induced (Dairkee *et al.* 2013) by means of a cell surviving mechanism (Muraoka-Cook *et al.* 2006) in an environment supposed to show apoptotic and regressive patterns. This cell survival leads to the perpetuation of a tumoral architecture that allows a clonal and disordered growth (Zhang *et al.* 2017) through the proliferative process, confirmed by an increase in PH-H3 positive cells in aged gerbil MG. BPA acts through estrogenic pathways to disrupt the cell cycle, such as in the prostate (Huang *et al.* 2018).

In addition, even though this EDC promotes mammary hyperplasia (Dumitrascu *et al.* 2020), in the present study, we denote its tumor development potential in aging through the EMT mechanism, which could develop into invasive and aggressive cancer.

The invasive profile can be monitored through p63 expression in carcinoma cells. Cytoplasmic and nuclear staining for this protein enables diagnosis of invasiveness and is present in early breast cancer (Sundqvist *et al.* 2020) and pregnancy-associated breast cancer (Hsiao *et al.* 2010). Here, after BPA exposure during pregnancy and lactation, gerbil MG tissue from aged females presented enhanced p63 expression mainly in the cytoplasm, indicating a poor prognosis. This increase is one of the mechanisms activated by the BPA disruption activity in mammary tissue, known as basal programming (Cheung *et al.* 2013), which contributes to the structural maintenance of carcinomas and leads to a collective stromal invasion. Furthermore, its expression in tissues prone to tumorigenesis can amplify the functions of TGF- β 1 as an EMT promoter, leading to invasiveness (Sundqvist *et al.* 2020). The expression of p63 demonstrates regulation of the EMT process in tumor tissue, exhibiting a permissive character to the collective invasion of cells in transformation associated

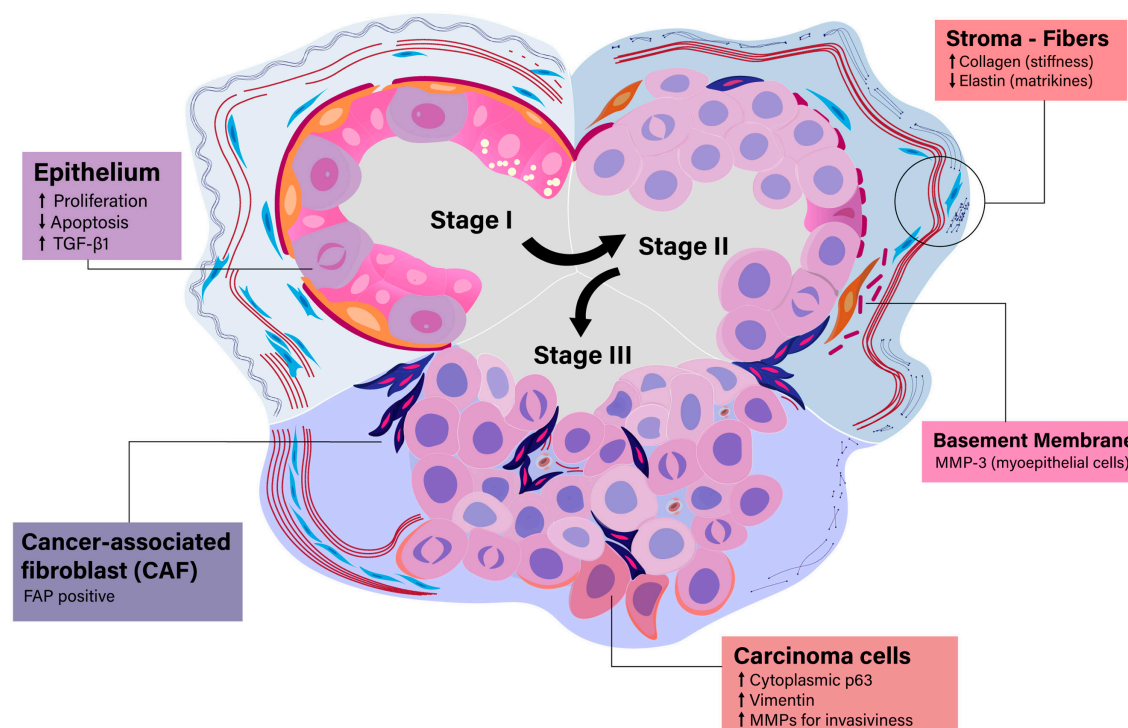


Figure 7

Graphical illustration of BPA-induced disruption in three stages of tumorigenesis in gerbils mammary gland. A full color version of this figure is available at <https://doi.org/10.1530/ERC-21-0198>.

with the remodeling of ECM in pro-tumor conditions (Gatti *et al.* 2019).

Stromal remodeling promotes a tumor microenvironment in MG exposed to BPA

A favorable tumor microenvironment is provided by disrupted stroma in several breast cancer types (Bussard *et al.* 2016). Collagen and elastin constitute the major fibrillar components of ECM, and its degradation and synthesis support tumor progression (Brassart-Pasco *et al.* 2020). In our study, this process occurred in the stroma of MG from BPA-exposed females with a strong correlation with MMP expression. Indeed, loss of fibrillar elastin in BPA leads to a decline in mechanical properties and fibrosis in the stromal compartment, that also affects the epithelial compartment (Brassart-Pasco *et al.* 2020). In contrast, the collagen area increased drastically in BPA groups, and this ECM remodeling is a remarkable step, previously described for breast cancer promotion (Gehmert *et al.* 2020).

Collagen fibers were frequent around carcinoma and normal mammary structures in MG from aged females. TGF- β 1 is known to increase the collagen deposition in ductal areas (Silberstein *et al.* 1990) creating a fibrotic aspect of ECM, stimulating proliferative disorders in the epithelial compartment and thus supporting tumor increase. Collagen and elastin dynamic in the MG of females exposed to BPA exhibit a tissue microenvironment that has undergone high remodeling. BPA promotes ECM stiffness in the MG of females exposed *in utero* through cellular machinery reprogramming for the synthesis of extracellular elements (Wormsbaeche *et al.* 2020). Thus, we propose that this EDC promotes a tumor-associated collagen signature that rearranges the collagen matrix of the stroma (Lyons *et al.* 2011), supporting the development of carcinoma, in addition to being able to influence local invasiveness (Provenzano *et al.* 2006).

Tumor growth invasiveness is promoted by the upregulation of ECM proteases. The reactive stroma concerns not only changes in the ECM fiber components but also those in cellular assembly (Mao *et al.* 2013), either by differentiation or by cell recruitment. Increases in the expression of MMP-2, MMP-3, and MMP-9 were observed in the MG of aged females exposed to BPA. Although each one presents a specific action in the mammary tissue, in the present study, these MMPs contributed to tumor growth and the installation of multifocal carcinoma in gerbil MG. MMP-2 and MMP-9, which showed an expressive increase in the BPA groups, were exclusively expressed in ECM and are related to stromal cells that promote its

remodeling (Di Cara *et al.* 2018). Both are proteases that aid the local invasion of tumor cells and tumor growth (Dong *et al.* 2019). However, interestingly, we detected MMP-3 expression in different cell types, which assisted in the understanding of remodeled tissue mechanisms. Cells potentially in the EMT process showed cytoplasmic MMP-3 staining, similarly to myoepithelial cells in the basal epithelium, when discontinuous or not.

In our study, the expression profile of these MMPs suggests two tumorigenic process mechanisms induced by BPA: (i) EMT reprogramming is supported by MMP-3 expression in the epithelium, and MMP-2 and MMP-9 expression in the stroma to develop a tumor feature; (ii) invasiveness and expansion of carcinoma occurred due to myoepithelial cell expression of MMP-3 and proteolytic activity in the basement membrane. The first mechanism is related to the main role of MMPs described during breast cancer establishment (Radisky & Radisky 2015). These proteases decrease cell adhesion (Marcus *et al.* 2019) and regulate signals of stemness (Radisky *et al.* 2017), leading to the consequent EMT process. Specifically, MMP-9, which is also overexpressed in other cancer types after BPA disruption (Kim *et al.* 2015), promoted the tumor signaling pathway of TGF- β 1. MMP-9 intensifies TGF- β 1 signaling during induction of the EMT process in breast tissue (Dong *et al.* 2019). This 'cell adhesion' effect can also be induced by MMP-2 (Di Cara *et al.* 2018). In terms of basement membrane, the second mechanism correlates to the degradation activity of MMPs. MMP-3 expression in myoepithelial cells of BPA-exposed MG promotes rupture of the last barrier between carcinoma and stroma (Deng *et al.* 2020). The surrounding stromal cells are recruited by BPA-induced carcinoma signaling pathways to express MMP-2 and MMP-9 for basement membrane discontinuity (Blavier *et al.* 2006). With basement membrane loss, disrupted epithelial cells are stimulated to a disordered proliferation and expansion toward stroma (Slepicka *et al.* 2019). BPA changes basement membrane deposition in MG, which impacts breast alveoli morphogenesis by epithelial cells (Marchese & Silva 2012). Thus, our results suggest that BPA disruption increases MMP-3 expression for tumor progression and collective invasion of the stroma, whereas MMP-2 and MMP-9 expression in stromal cells are increased to support invasion in MG from exposed aged females, mediating the epithelium-stroma interaction.

The epithelial-stroma interaction for tumor progression requires CAFs (Alexander & Cukierman 2020). Despite their multiple origins, CAFs are cellular elements that apparently arise from the establishment of reactive stroma in the breast (Elwakeel *et al.* 2019).

According to Wormsbaecheer and colleagues, the mammary fibroblast phenotype is altered when an *in utero* BPA exposure occurs, leading to subsequent ECM remodeling (Wormsbaecheer *et al.* 2020). It is important to note that the recruitment of these cells increases dramatically in gerbil MG exposed to BPA, as observed by the staining of FAP-positive cells (Tao *et al.* 2017), suggesting that the endocrine disruption acts in some pathway for the establishment of this cell type. One of the pathways of fibroblast activation in cancer is through induction by TGF- β 1 and FGF (Tao *et al.* 2017), both highly expressed in BPA-disrupted MG, which can be stated as a BPA endocrine disruption pathway for recruiting CAFs.

In summary, the gestational and lactational windows in MG represent an exposure period in which BPA imposes a delayed carcinogenic risk during aging. The analysis allowed us to identify cancer progression in gerbil MG associated with several elements that support tumoral invasiveness (Fig. 7). TGF- β 1 was highly expressed in the normal epithelium of disrupted tissue and seems to be a remarkable feature in BPA disruption, not addressed in aged females so far. These BPA-induced mechanisms contribute to EMT reprogramming and stromal signaling for tumor microenvironment establishment. Furthermore, the epithelial-stromal interaction, by molecular pathways and remodeling MMPs, emphasizes the alterations provoked by the studied EDC and the repercussions which favor carcinogenesis in disrupted mammary tissue.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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