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Perinatal exposure to bisphenol A impairs the Mongolian gerbil ovarian follicle dynamics and its extracellular milieu

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ABSTRACT

Bisphenol A (BPA), a polycarbonate monomer commonly found in plastics, is an endocrine disruptor compound (EDC) that mimics the activity of steroid hormones. This study aimed to examine BPA's effects on Mongolian gerbils' ovary. Pregnant females (n = 5) were assigned to four groups and exposed by gavage to BPA at two doses (↓BPA, 50 μg/kg; ↑BPA, 5000 μg/kg), an oil vehicle (OC) or remained untreated (IC) from the eighth gestational day until weaning. One female from each offspring was euthanized at six months of age, and had their ovaries processed histologically. Cytochemistry was performed: HE for quantification and classification of follicles; Gomori's Trichrome for collagen fibers; Resorcin-Fuchsin, for elastic fibers; and reticulin, for reticular fibers. Immunohistochemistry was used to analyze estrogen receptor alpha (ERα) and beta (ERβ), and the proliferative index. Results showed no significant differences in primary or secondary follicle counts among the IC, OC, ↓BPA, and ↑BPA groups. Antral follicles showed a higher incidence in the ↑BPA (6,4 ± 1,0) compared to the IC (2,7 ± 0,4) and OC (2,8 ± 0,4) groups. Collagen fiber density was significantly higher in the ↑BPA group (38,0 ± 7,5) compared to the OC group (20,7 ± 6,2). Similarly, elastic fibers were increased in the ↑BPA group (7,4 ± 0,9) relative to ↓BPA (1,8 ± 0,4), OC (2,3 ± 0,5) and IC (2,6 ± 0,9), as were reticular fibers (↑BPA: 31,5 ± 1,8; ↓BPA: 17,8 ± 2,6; OC: 14,1 ± 2,3; IC: 12,9 ± 1,2). Additionaly, BPA-exposed groups exhibited fewer interstitial glands, suggesting a reduction in steroidogenic potential. The tissue response to estrogens was elevated among animals from the ↑BPA group within activated follicles, regarding receptors alpha and beta. Higher proliferation indexes were present within activated follicles and interstitial steroidogenic glands from the ↑BPA group as well. Therefore, dysregulation of estrogenic response and establishment of fibrosis observed in our results indicate deleterious effects on the ovary's morphophysiology, potentially leading to a pathological environment. This attests that perinatal exposure to BPA affects the offspring even in a long-term manner.

Introduction

Environmental contaminants have been researched worldwide due to their ability to reach a variety of species and environments through various contamination pathways, like water (Gonsioroski et al., 2020), soil (Liu et al., 2019), and food (Andújar et al., 2019). In this regard, an important contaminant is bisphenol A (BPA), a polycarbonate monomer in the bisphenol (bis hydroxy aryl alkanes) group, frequently used in the manufacturing of plastics and resins found in the inner and outer coating of food cans, PVC pipes, and even baby bottles, as discussed by Beserra and collaborators (Beserra et al., 2012). BPA is an example of endocrine disruptor compound (EDC), a substance that mimics the activity of endogenous steroid hormones (Ziv-Gal and Flaws, 2016). Therefore, it is able to bind estrogen receptors (ERα and ERβ) and consequently lead to alterations in cellular and tissue functions (Wetherill et al., 2007). Since the ovarian tissue presents significant steroidogenic activity, research has been conducted on this subject.

Exposure to EDCs during critical developmental stages, such as

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embryonic organogenesis, can cause negative impacts during infancy (Yolton et al., 2011); adulthood (Leonel et al., 2020) and senescence (Rattan et al., 2017). Additionally, Razzoli and collaborators (2005), have stated that exposure to BPA can significantly disrupt the reproductive behavior of females even at low concentrations. More specifically, previously published data suggest that exposure to EDCs during early embryonic development is more critical to mammary gland development in terms of adult neoplastic susceptibility (Leonel et al., 2020; Wormsbaecher et al., 2020). This is due to a transcriptional reprogramming of fibroblasts, as a result of primordial mesenchyme connections to these compounds (Palmer et al., 2006). This feature is extended to other estrogen susceptible organs, such as the ovaries. In these organs, folliculogenesis is impacted by in utero exposure to EDCs, such as phthalates (Zhang et al., 2023), which impair germ-cell cyst and primordial follicle assembly through autophagy mechanisms.

Such fibroblasts reprogramming affects the remodeling of ovarian tissue throughout the female mammalian reproductive life, characterizing a physiological age-associated stromal fibrosis (Briley et al., 2016). This is due to an alteration in the homeostasis between extracellular matrix (ECM) fibers synthesis and degradation (Wynn and Ramalingam, 2012). Interestingly, evidence of increased population of multinucleated macrophage giant cells and inflammation was found in 22-month-old mice ovarian tissue in comparison to younger animals (Briley et al., 2016). Nevertheless, these are extracellular features that favor the establishment of pathologies such as polycystic ovarian syndrome (Liu et al., 2007) and cancer metastasis (Yu et al., 2024). There is evidence that BPA exposure modulates changes in the ovarian ECM, as shown by variations in the expression of metalloproteinases within human granulosa-lutein cells in vitro (Dominguez et al., 2008). An experiment performed in BG-1 cells showed that BPA and nonylphenol, another EDC, favor ovarian cancer metastasis through regulation of epithelial-mesenchymal transition (Kim et al., 2015). Thus, the morphology of ovarian ECM in adult animals is an important source of information about the tissue mechanisms that organs elaborate in response to contaminants.

The reproductive function in the mammalian ovary is also impaired by EDCs exposure through different mechanisms (Peretz et al., 2011). Effects in the hypothalamus, pituitary, gonads and the reproductive tract have been described; the ovary is of specific interest because it is a dynamic organ that is constantly under morphological changes in response to hormone-regulated mechanisms throughout the reproductive cycle. Disruption in the steroidogenesis is frequently related to the fact that EDCs are able to bind estrogen receptors, acting either as xenoestrogens or anti-estrogens (Wetherill et al., 2007; Susiarjo et al., 2007). Exposure EDCs affects not only intracellular receptors but also the membrane G protein-coupled estrogen receptor 1 (GPER), which plays a key role in apoptosis (Huang et al., 2021) and proliferation (Hoffmann et al., 2017) mechanisms in ovarian cells. Unlike intracellular receptors, GPER mediates rapid non-genomic responses upon activation (Kang et al., 2010).

Folliculogeneis is also impaired by BPA (Souter et al., 2013). Mouse antral follicles showed increased signs of atresia (Peretz et al., 2012). In the mouse adult ovary, BPA impairs granulosa cells growth and viability through generation of reactive oxygen species, affecting normal follicle development (Wang et al., 2024). In addition, luteal regression through caspase-3 induced apoptosis was described in adult rats' ovaries (Lee et al., 2013).

EDCs have gained considerable attention within the research community mainly due to their contribution to the development of multiple disorders and chronic diseases related to endocrine and reproductive function, which incidence has grown in a fast manner (Barouki et al., 2012). Understanding how these compounds affect health in different species is thus dependent on the use of animals to reach the goal of unraveling the "predictive toxicology" (Patisaul et al., 2018), aiming to clarify the impacts in a multi-systemic manner. Since the biological complexity of the body is currently not reproducible in an in vitro manner, using living models has been the only choice. Therefore, some species have been applied as modeling systems to what happens in humans and to understand mechanisms of action, dose responses, windows of susceptibility, etc. One can mention zebrafish (*Danio rerio*) (Reif et al., 2016) and rodents such as mouse (*Mus musculus*) and rat (*Rattus norvegicus*) as well as their transgenic models (Boverhof et al., 2011). The Mongolian gerbil (*Meriones unguiculatus*) is a rodent native to semi-arid deserts with a geographical distribution spanning Africa, India, Mongolia, Southeast and Central Asia, Northeast China, and some regions of Western Europe (Field and Sibold, 1999). The Mongolian gerbil is a justified experimental animal model due to its frequent spontaneous development of neoplasms, which mirrors natural environments with a higher carcinogenesis (Custodio et al., 2010), which allows us to extrapolate the conclusions to other mammals, including humans.

The experiments performed here aimed to clarify the morphological impacts that perinatal exposure to different doses of BPA may cause in the adult ovaries. Our focus was on the analysis of follicle populations, the incidence and distribution of ECM fibers among the ovarian stroma and surrounding follicles, as well as the rates of estrogen signaling through the incidence of estrogen receptors and proliferation within follicular cells and stromal interstitial glands.

Material and methods

Animals and experimental design

The animal experimental procedures were conducted according to the National Council of Animal Experiment Control (CONCEA, Brazil, https://www.mctic.gov.br). The Ethics Committee on the Use of Animals (CEUA) from IBILCE/UNESP (protocol number 113/2015) and from UFG (028/2021) approved all the procedures described to be performed in the animals.

Female Mongolian gerbils (*Meriones unguiculatus*), 3 months age, were maintained in a controlled environment with room temperature of 23–26 °C, and light/dark cycle of 12/12 h. BPA free isolators (Alesco, Monte Mor, SP, Brazil) were used for keeping the animals in a rack with ventilation, fresh water and a commercial chow (Presence, Paulínia, SP, Brazil) provided *ad libitum*.

The animals were housed with fertile males. Pregnancy day 0 was established when sperm cells were present in the vaginal smears, that were performed every day. The pregnant females were assigned for four experimental groups and their female offsprings were the focus of this experiment.

Each experimental group (n = 5) consisted of pregnant/lactating females and their exposure to BPA started at the 8th gestational day. The intact control group (IC) did not receive any treatment. In the exposed groups, treatments were performed from the 8th gestational day until the end of lactation (46 days of treatment), with gavage being performed daily, in the morning. These groups were classified as: oil control (OC, 0.1 ml of corn oil; Mazola, Mairinque, SP, Brazil); low dose of BPA (↓BPA, 50 μg/kg BPA, Sigma Aldrich, St. Louis, USA, as stated by U.S. Food and Drug Administration (FDA) as a safe dose) diluted in 0.1 ml corn oil; and high dose of BPA (↑BPA, 5000 μg/kg BPA) diluted in 0.1 ml corn oil (Vandenberg et al., 2012).

After weaning, one female from each offspring was randomly chosen and used for analysis (n = 5); they were maintained under the previously described conditions until reaching 180 days of age. They were then anesthetized with xylazine (3 mg/kg) and ketamine (10 mg/kg) and euthanized by decapitation. The euthanasia procedures were performed only in animals in the estrous phase of the cycle, as indicated by vaginal smears performed daily after the females reached the target age.

Histological processing and cytochemical staining

Ovaries were collected and histologically processed for morphological analysis and immunohistochemistry. They were fixed in paraformaldehyde 4 % for 24 h and then transferred to alcohol 70 %.

Processing was performed under a semi-enclosed system (TP1020, Leica Biosystems, Buffalo Grove IL, USA) and the paraffin blocks obtained were subjected to microtomy (5 μm thick). The tissue slices were placed on slides and subjected to different cytochemical staining or to IHC procedures.

Hematoxylin and eosin staining was performed for general evaluation of the tissue and quantification/classification of the follicular population. Gomori's trichrome staining was performed in the slides for identification and quantification of the thick collagen fibers in the stroma, while resorcin-fuchsin was applied for elastic fibers quantification and reticulin for reticular fibers quantification. The quantifications were performed in three different depths of tissue, with a separation of at least 50 μm between each slice. One ovary from each animal was evaluated (n = 5).

Immunohistochemistry

Ovarian slices were subjected to antigen retrieval in citrate buffer (pH 6.0) at 98 °C. Then, a detection kit system (Novolink Polymer Detection System RE7150-K, Leica Biosystems, Newcastle, UK) was applied for identification of the proteins of interest. Briefly, endogen peroxidases and unspecific proteins were blocked. The sections were incubated overnight at 4 °C with primary antibodies against estrogen receptor alpha (anti-ERα, rabbit polyclonal, 1:50, IgG, MC-20, sc-542, Santa Cruz Biotechnology, Dallas, TX, USA), estrogen receptor beta (anti-ERβ, rabbit polyclonal,1:50, IgG, PA1-310B, Invitrogen, USA), and phospho-histone H3 (anti-P-H-H3, 1:100, rabbit polyclonal, H3, 1:75, Ser10, 9701, Cell Signaling, Danvers, USA). Positive staining was detected by 3–30′-diamino-benzidine tetrahydrochloride (DAB) chromogen (NovolinkMaxDAB, RE7270-CE, Leica Biosystems, Buffalo Grove IL, USA) and counter-stained with Harry's hematoxylin.

Quantification of the follicular population

Spherical follicles, attached to surrounding stroma cells, with a uniform distribution of granulosa cells and a spherical oocyte showing no retractions or vacuoles were considered normal. Normal follicles were counted and classified as primordial (oocytes surrounded by a single layer of flattened granulosa cells), primary (follicles surrounded by one layer of cuboidal granulosa cells), secondary (follicles surrounded by two or more layers of cuboidal granulosa cells) or antral (follicles containing antrum). Follicles were considered degenerated when there was the presence of disorganized granulosa cells layers or pyknotic oocyte nucleus. Only follicles presenting a visible oocyte nucleus were considered.

Quantification and distribution of fibers in the ovarian tissue

Microscopic fields were acquired in a digitalization system (Zeiss AxioScope A1 light microscope, Zeiss, Göttingen, Germany) at 400x magnification. All the ovarian tissue available (comprising a variable number of fields) was evaluated for quantifying ECM fibers through stereological analysis. The quantification was carried out to determine the frequency of collagen, reticular and elastic fibers according to the M130 multipoint test system (Weibel, 1963), adapted to the ovarian tissue. Points coinciding with areas of blue (Gomori's trichrome), blue/purple (Weigert's Resorcin-Fuchsin) or black (Gomori's reticulin) were counted and calculation of the frequency as a percentage over the total counted points was made. This analysis aimed to estimate the deposition of ECM fibers in the ovarian stroma among experimental groups.

Quantification of proliferation and hormonal receptors expression

The quantification of immunostaining was performed with QuPath® software by measurement of positive cells of MG. The nuclear staining was quantified by automatic counting of positive nuclei over the total number of nuclei present in the structure of interest (follicles or stromal steroidogenic glands). Within each experimental group, the total number of cells belonging to each category of follicles (primordial or activated) or to the glands was considered for statistical analysis.

Statistical analysis

The number of follicles in different developmental stages, number of follicular cells positive for estrogen receptors or the proliferation marker, or the total volume of tissue occupied by collagen, reticular or elastic fibers (%) were shown as means ± standard error (SE). Data were subjected to analysis using GraphPad Prism 5.00 for Windows (GraphPad Software, San Diego, CA, USA, https://www.graphpad.com). The normality was checked by the Kolmogorov-Smirnov test. Parametric data were analyzed by one-way ANOVA followed by Tukey's test. For non-parametric data, the Kruskal-Wallis test followed by Dunn's test was adopted. Differences were considered significant when $p < 0.05$.

Results

Follicular population

No differences were present between the groups when comparing the amounts of primordial (73.8 ± 5.2; 68.5 ± 3.4; 68.0 ± 3.2; 59.2 ± 8.3), primary (5.7 ± 1.2; 5.7 ± 1.5; 7.9 ± 2.2; 6.7 ± 2.3) or secondary follicles (15.0 ± 4.4; 15.7 ± 1.2; 16.7 ± 1.7; 21.5 ± 6.6), for IC, OC, ↓BPA, and ↑BPA, respectively (Fig. 1). Antral follicles showed a higher incidence in the ↑BPA (6,4 ± 1,0) compared to the IC (2.7 ± 0.4) and OC (2.8 ± 0.4) groups, while the ↓BPA groups was not different from the others (4.4 ± 1.1). Degenerated follicles were quantified as well but no differences were present between IC (2.4 ± 0.9), OC (3.8 ± 1.9), ↓BPA (3.1 ± 1.3) and ↑BPA (6.0 ± 1.6) groups.

Thick collagen, elastic, and reticular fibers

Cytochemical analysis performed with Gomori's trichrome revealed the presence of collagen fibers stained in blue (Fig. 2) among the ovarian stroma. Such structures were present as diffuse fragments of short and thick pieces of fibers, differently from what is usually present in other mammals' ovaries. The elastic fibers were identified in dark blue/purple by Weigert's Resorcin-Fuchsin stain within the ovarian stroma, while the reticular fibers were observed as very thin structures delineating follicles and groups of stromal cells by Gomori's reticulin technique (Fig. 2). A common aspect observed in ↑BPA group was a higher incidence of elastic and reticular fibers specifically related to less incidence of stromal steroidogenic glands.

The incidence of collagen fibers increased in the ↑BPA group (38.0 ± 3.7) compared to the OC group (20.7 ± 2.7); IC (21.7 ± 2.5) and ↓BPA groups (23.5 ± 3.1) were similar to the other groups. Elastic fibers increased in the ↑BPA (7.4 ± 0.9) compared to all the other groups (↓BPA: 1.8 ± 0.4; OC: 2.3 ± 0.5; IC: 2.6 ± 0.9). Similarly, reticular fibers were more frequent within the ovarian stroma in the ↑BPA group (31.5 ± 1.8) in comparison to all the other groups (↓BPA: 17.8 ± 2.6; OC: 14.1 ± 2.3; IC: 12.9 ± 1.2).

Expression of estrogen receptors

Immunohistochemistry was performed to quantify the amounts of estrogen receptors ERα and ERβ. The immunostaining pattern of ERα was characterized by nuclear localization within follicular granulosa cells (Fig. 3A-C). However, within stroma glands, the staining pattern was characterized by both nuclear and cytoplasmic localization in the same cell (Fig. 3A-C). ERβ, however, was characterized by an exclusive nuclear expression in granulosa cells, glandular stroma, and ovarian surface epithelium (Fig. 3D-F).

The percentages of granulosa cells positive for ERα in primordial

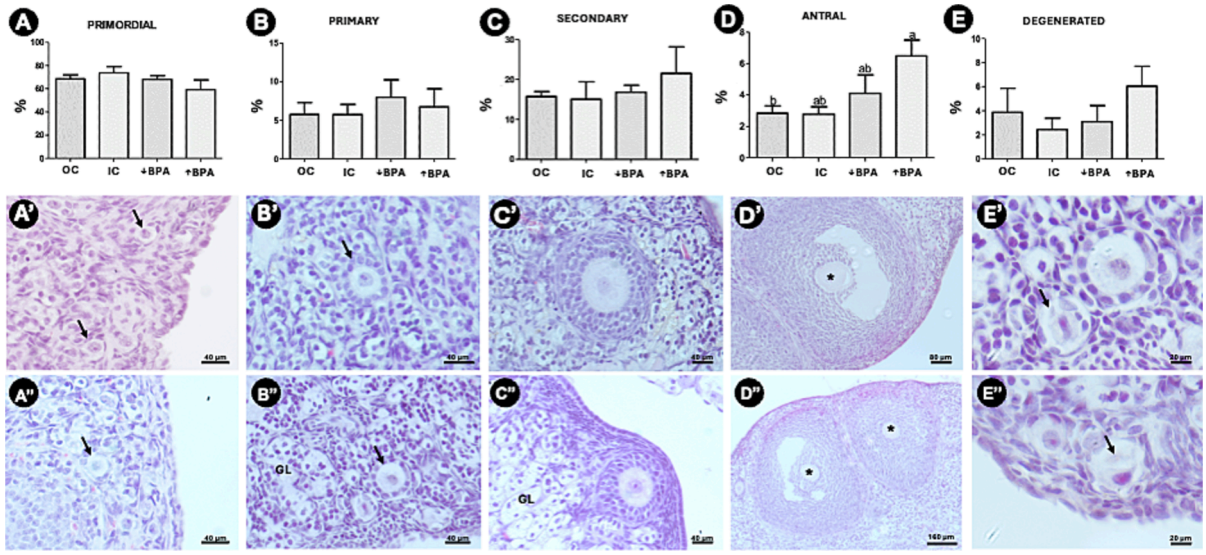


Fig. 1. Follicle population in the experimental groups. (A) Percentages of primordial follicles over the total number of follicles evaluated in ovarian tissue from animals from the experimental groups; (A') and (A'') illustrate primordial follicles from the OC and ↑BPA groups. (B) Percentages of primary follicles over the total number of follicles evaluated in ovarian tissue from animals from the experimental groups; (B') and (B'') illustrate primordial follicles from the ↓BPA and ↑BPA groups. (C) Percentages of secondary follicles over the total number of follicles evaluated in ovarian tissue from animals from the experimental groups; (C') and (C'') illustrate primordial follicles from the ↓BPA and ↑BPA groups. (D) Percentages of antral follicles over the total number of follicles evaluated in ovarian tissue from animals from the experimental groups; (D') and (D'') illustrate primordial follicles from the IC and ↑BPA groups. (E) Percentages of DEGENERATED follicles over the total number of follicles evaluated in ovarian tissue from animals from the experimental groups; (E') and (E'') illustrate primordial follicles from the OC and ↑BPA groups. Arrows: primordial (A', A''), primary (B', B''), and degenerated (E', E'') follicles. Asterisks: oocytes from antral follicles. GL: interstitial glands. In D, different letters indicate differences among groups. ANOVA followed by Tukey's test (D: $p < 0,05$).

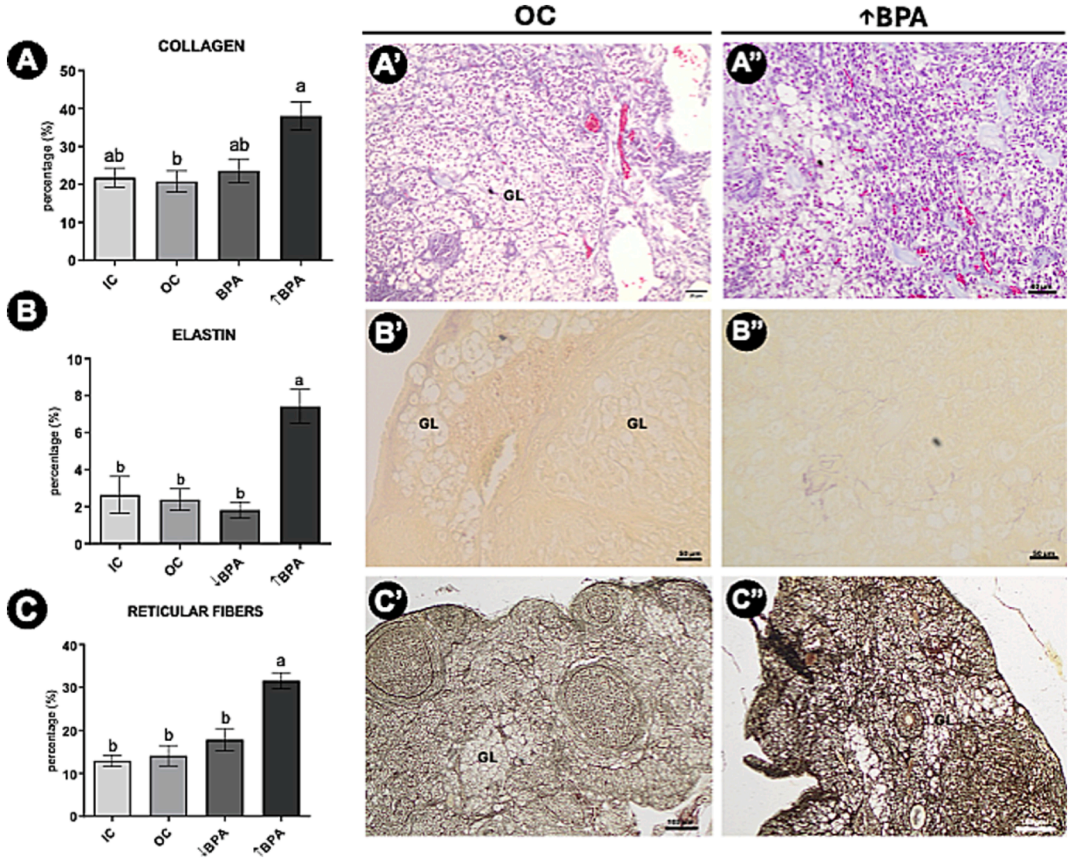


Fig. 2. Distribution of extracellular fibers. (A) Percentages of tissue volume occupied by collagen fibers. (A', B', C') and (A'', B', C'') illustrate tissue from the OC and ↑BPA groups, respectively. GL: interstitial glands. Different letters indicate differences among groups. ANOVA followed by Tukey's test (A: $p = 0.0061$, B: $p = 0.0003$, C: 0.0001).

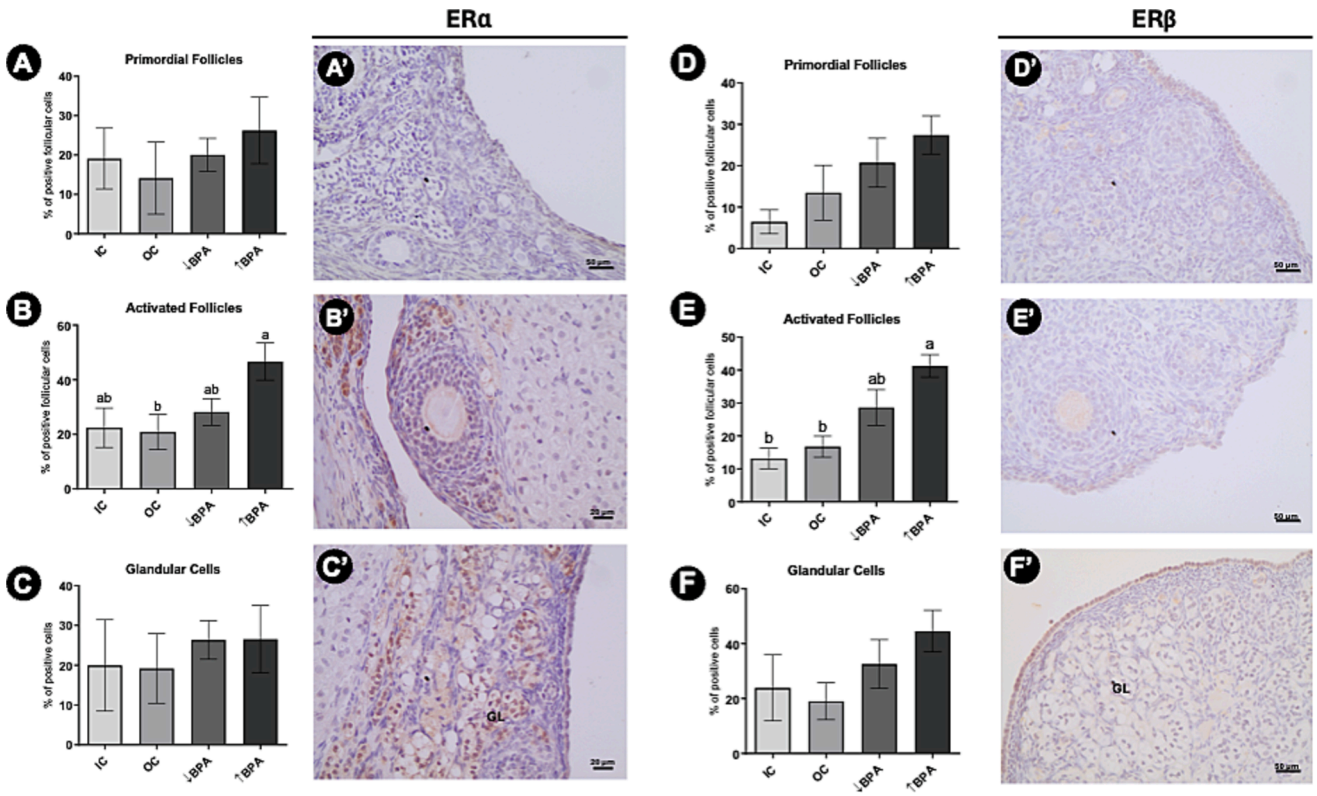


Fig. 3. Incidence of estrogen receptors. (A,B,C) Percentages of cells showing positive immunostaining for ERα over the total number of cells. (D,E,F) Percentages of cells showing positive immunostaining for ERβ over the total number of cells. (A', C', F') illustrate tissue from the ↑BPA group. (B', D',E') illustrate tissue from the OC group. GL: interstitial glands. Different letters indicate differences among groups. ANOVA followed by Tukey's test (B: $p = 0.02$, E: $p < 0.0001$).

follicles (19.1 ± 7.7, 14.13 ± 9.1, 20.0 ± 4.2, 26.25 ± 8.4), activated follicles (22.4 ± 7.2, 20.8 ± 6.4, 28.2 ± 4.8, 46.6 ± 6.8), and in steroidogenic stromal glands (19.9 ± 11.4, 19.1 ± 8.7, 26.3 ± 4.8, 26.5 ± 18.8), were evaluated for IC, OC, ↓BPA and ↑BPA, respectively. A significant difference between OC and BPA groups was observed for activated follicles only, while no significant differences were found relative to primordial follicles and stromal glands among the groups.

The quantifications of ERβ positive cells showed similar results as those seen for ERα. Primordial follicles (6.4 ± 2.8, 13.4 ± 6.6, 20.7 ± 5.9, 27.4 ± 4.6) and stromal glands (23.8 ± 12.0, 19.0 ± 6.7, 32.5 ± 8.8, 44.5 ± 7.5) did not show any difference between IC, OC, ↓BPA and ↑BPA experimental groups, respectively. However, for activated follicles, the group ↑BPA group (41.2 ± 3.4) showed a higher percentage of ERβ positive cells than the IC (13.1 ± 3.1) and OC (16.7 ± 3.1) groups; the ↓BPA group was not significantly different from the other groups (28.6 ± 5.4).

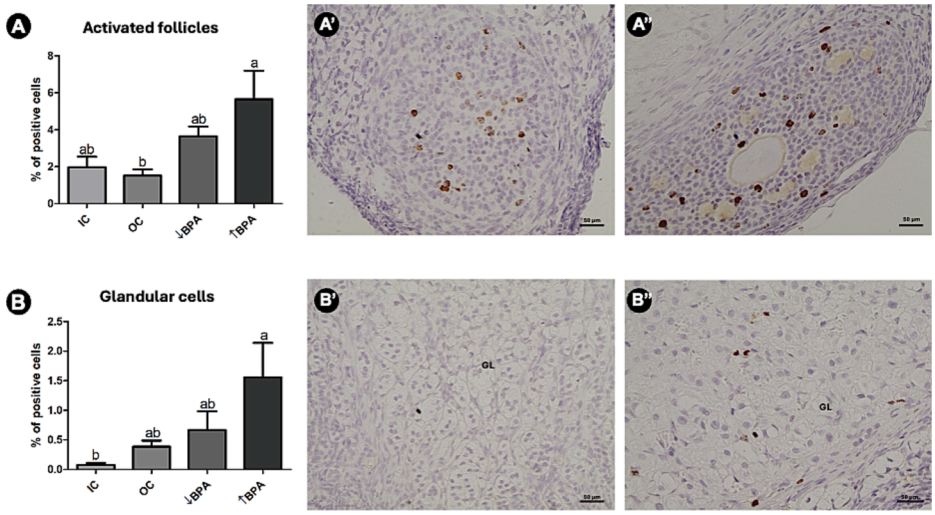


Fig. 4. Proliferating cells. (A,B) Percentages of proliferating cells over the total number of cells. (A') and (B') illustrate tissues from the IC group. (A'') and (B'') illustrate tissues from the ↑BPA group. GL: interstitial glands. Different letters indicate differences among groups. ANOVA followed by Tukey's test (A: $p = 0.02$, B: $p < 0.0001$).

Proliferation rates

The proliferative index was analyzed within follicular cells belonging to activated (secondary and antral follicles) growing follicles. The evaluation of primordial and primary follicles did not show positive staining for PHH3. Results showed an increase in the proliferative index in the ↑BPA group (5.65 ± 1.53) in comparison to the OC group (1.51 ± 0.34), with the IC (1.9 ± 0.56) and the ↓BPA (3.65 ± 0.34) groups showing results similar to the other groups (Fig. 4A).

Within stromal steroidogenic glands, however, analysis showed that the ↑BPA group (1.55 ± 0.58) had more proliferating cells than the IC group (0.07 ± 0.03). OC (0.38 ± 0.03) and ↓BPA (0.66 ± 0.31) groups were not different from the other groups (Fig. 4B).

Discussion

The perinatal exposure to a high dose of BPA altered ovarian morphological aspects that reflected in the follicular dynamics and, consequently, in the steroidogenic activity. Our results showed that, despite preantral follicles counting did not differ among the experimental groups, the number of antral structures was increased in the ovaries of animals exposed to daily 50 and 5000 μg of BPA in utero and during breastfeeding. ECM was also changed due to this EDC exposure, showing a higher incidence of collagen (I and III) and elastic fibers; it means that BPA influences the ovarian rigidity and flexibility and, consequently, the ability of follicles to grow, move, ovulate and of follicular cells to luteinize. Finally, our analysis showed that an increase of nuclear ERα receptors in cells from activated follicles was reflected in a rise of the proliferative index in follicular cells from the exposed group; simultaneously, the ↑BPA group showed higher levels of proliferating cells within ovarian steroidogenic interstitial glands, indicating the only aspect susceptible to BPA exposure in these structures. The increase in proliferation rates within glandular stromal structures observed in the present study may be related to the higher amount of ERα described for the ↑BPA group.

Despite little discussion on interstitial ovarian glands, they are important sites of murine active synthesis and secretion of steroid hormones in this organ, especially progesterone (Gray et al., 1995). In these cells, a classic morphological feature is the presence of intracellular lipid droplets, which provide cholesterol for progesterone synthesis on demand (Shen et al., 2016). In the present experiment, a clear visual increase of tissue area occupied by these glands was present in ↓BPA and ↑BPA groups. Also, we observed the expression of ERα and ERβ in the cytoplasm and the nucleus of interstitial gland cells, similarly as described in bovine ovarian tissue (Kenngott et al., 2016). In the feline ovary, due to the presence of steroidogenic enzymes such as P450c17 (CYP17) and 3β-hydroxysteroid dehydrogenase (3β-HSD), it was stated that interstitial gland cells arise from theca interna cells belonging to atretic follicles (Perez et al., 1999). In Mongolian gerbils, exposure to the estrogen ethinylestradiol during the pubertal and pre-pubertal windows of susceptibility led to a larger area of these glands in the ovarian tissue analyzed (Souza et al., 2022). Its function might thus be related to avoidance of pathologies related to aging, since the hypothesis that interstitial glands development may be a response to increased gonadotropic stimulation resulted from estrogenic drops in feline species (Perez et al., 1999).

BPA is ubiquitously present in human environments. While plastic and resin themselves are considered low-risk compounds, BPA monomers are released and can be absorbed through oral, dermal, or even pulmonary routes (Substance Report, 2022). The estimation of human daily intake of BPA is around 2×10^{-4} μg kg^{-1}, based on detection of metabolites in wastewater (Estévez-Danta et al., 2024) from an European cohort. This exceeds the safe limits established by European Food Safety Authority (EFSA) (E. and P.A. (CEP) EFSA Panel on Food Contact Materials, C. Lambré, J.M. Barat Baviera, C. Bolognesi, A. Chesson, P.S. Cocconcelli, R. Crebelli, D.M. Gott, K. Grob, E. Lampi, 2023), although the United States Environmental Protection Agency (US-EPA) (L. VEAL, United States environmental protection agency, US Environmental Protection Agency (EPA), 2005) has set an oral reference dose of 50 μg $kg^{-1}day^{-1}$.

BPA is strongly associated to polycystic ovary syndrome (PCOS) (Shi et al., 2024) and female reproductive insufficiency (Vabre et al., 2017). The mechanisms of such dysfunction in the human ovary are not yet well known but there is evidence that links follicular degeneration to granulosa cells apoptosis (Guedikian et al., 2018) due to oxidative stress (Sugino, 2005) and non-genomic signaling (Viñas and Watson, 2013). In the Mongolian gerbil, the present study showed that antral follicles were more frequent in the ↑BPA group, with no differences being presented in relation to the other follicular stages of development. This finding can be related to an increased activation pattern within follicles in animals exposed to high doses of BPA, not reflected in the secondary follicle population. In mammals, female germ cells and flattened granulosa cells housed as primordial follicles remain quiescent all along the reproductive life. However, follicular awakening relies on some mechanisms that may be affected by environmental aspects. According to a study that described the transcriptome of mouse follicle activation – primordial to primary transition –, this phenomenon is based on the activity of the Wnt signaling pathway and the expression of the ZFX and FRZB transcription factors in granulosa cells (Ford et al., 2022). The Wnt signaling pathway has demonstrated to be disrupted by BPA exposure in human ovarian cancer cells, through the increase of β-catenin protein levels, which contributes to the dormant follicle activation (Guo et al., 2020). However, despite previous publications describe that BPA decreases the primordial follicle pool in lamb newborns (Rivera et al., 2011). This was not evidenced in our experiment, reinforcing the hypothesis of increased levels of follicle activation caused by BPA.

Disruption in the maintenance of the dormant follicular population may lead to primary ovarian insufficiency, which is related to steroid secretion deficiency, elevated levels of gonadotropins and, consequently, impaired fertility (Nelson, 2009). In humans, this confers high risks of developing conditions such as osteoporosis, cardiovascular disease and endocrine disorders (Faubion et al., 2015). In this regard, the increase in the availability of interstitial glandular tissue may be assumed as an attempt to compensate fluctuations in steroidogenesis.

The ovarian ECM has specific elements that compose the stroma and surround the follicles. It participates actively in the ovary's dynamics during the prenatal development – germ cells migration –, follicular development, ovulation and luteinization (Rodgers et al., 1999). In this context, the ECM influences cells' behavior, providing a rigid or elastic mechanical support to them, depending on the context of the cycle. This includes simultaneous degradation – through metalloproteinases – and synthesis of the matrix (Rodgers et al., 1999). In a pathological context, human ovarian tumors show absence or reduction of collagen type IV during the early stages of tumorigenicity; however, its restoration during advanced stages of tumor development may boost metastasis (Ricciardelli and Rodgers, 2006; Capo-Chichi et al., 2002). Collagen types I and III, analyzed through Gomori's trichrome and reticulin, are widely present throughout the ovarian stroma. In the human ovarian impaired environment, there is an increase in collagen type III, through an intense synthesis of the procollagen peptide (Zhu et al., 1993). Our results provide evidence of an affected ECM, as seen by increases in collagen type I and III, confirming that the negative impacts of BPA are maintained even after several months of exposure. As a result, this dictates mechanical features of the ovary, in which high incidence of collagen type I and III characterizes a fibrotic environment that increases tissue rigidity in different mammalian species (Briley et al., 2016). A study performed on the human ovary extracellular proteomics revealed that collagen deposition is increased during the reproductive age and confirmed that steroids play a role in it (Ouni et al., 2020). Briefly, estrogens are able to increase the presence of thick fibers and decrease thin fibers. However, our results demonstrated that exposure to high BPA dose increased thin fibers, which were increased in the ↑BPA

group, that mimics an estrogenic environment. This work also discusses the presence of elastic fibers, which are expected to be upregulated under estrogenic environment (Ouni et al., 2020), corroborating our results. In mice lung the relationship between elastic fibers and BPA may be due to its influence in fibroblast homeostasis as a result of oxidative stress (Liang et al., 2024). According to earlier studies, oxidative stress induced by BPA occurs in a dose-dependent manner (Liang et al., 2024). Given that elastin synthesis is typically repressed in adulthood (Parks et al., 1993), the observed increase in elastin levels may be a response to environmental factors during early development. Changes in the degradation of these fibers through the expression of elastases is one hypothesis. Finally, elastogenesis is sensitive to increased concentrations of steroids. Glucocorticoids, for example, stimulate elastogenesis in the aorta (Foster and Curtiss, 1990), lungs (Noguchi, 1990) and cardiac fibroblasts (Bunda et al., 2009).

In a condition similar to the present methodology, the expression of estrogen receptors in the gerbil mammary epithelium was not different in the BPA exposed group (Leonel et al., 2020), contrary to our findings. Under exposure during pregnancy and lactation, there was an increase in the expression of ERβ within follicular cells; a reduction in serum levels of estrogen was also described (Ruiz et al., 2023). Estrogen receptors are ligand-dependent transcription factors that control expression of specific genes and synthesis of specific proteins. Increased expression of ERα and ERβ significantly impacts gene transcription related to follicular development and ECM remodeling.

Changes in the amounts of hormonal receptors impact gene expression, mainly those regulating signal transduction pathways, cell cycle progression and apoptosis. In the ovaries, ERα has a tumor-promoting role while ERβ has a tumor-suppressing role (Suzuki et al., 2008; Schüler-Toprak et al., 2023); in certain situations, ERβ acts as an ERα antagonist (Chen et al., 2008). Estrogen receptors, particularly ERβ, are involved in the transcriptional activation of genes crucial for folliculogenesis, such as those encoding aromatase (CYP19A1), which is essential for estrogen synthesis (Chauvin et al., 2022). In addition, estrogen signaling promotes granulosa cell proliferation and differentiation, which are vital for follicle growth and maturation (Chou and Chen, 2018). In general, reproductive function is impaired in ERβ knockout mice (Couse et al., 2005); still, attenuated response to FSH was observed in these mice, suggesting that increased expression in the ovaries may be related to an increase in estradiol secretion (Couse et al., 2005). If a decrease in the levels of ERβ is described in cystic follicles in PCOS ovaries (Zurvarra et al., 2009); contrarily, in ovarian cancer, the antiproliferative action of it is inhibited by pro-proliferative signaling pathways (Chu et al., 2004; Bao et al., 2000). Still, in human ovarian cancer, ERβ expression is associated with increased overall patient survival time (Halon et al., 2011). Previous experiments have linked endocrine disruptors exposure to the risk of developing breast cancer through mechanisms involving ERα signaling (Sang et al., 2021). Thus, despite our results describe an increase in the nuclear presence of ERα due to exposure to high levels of BPA, a concomitant rise in ERβ was observed.

Estrogen receptors also influence the expression of genes involved in ECM remodeling, including those encoding matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) (Nalvarte et al., 2016). A study showed that *Col11a1* was overexpressed in ERβ-null follicles, while *Col4* did not change in comparison to wild type cells (Zalewski et al., 2012). In ERαKO mice skin fibroblasts ECM fibers synthesis is increased, whereas it is decreased in the skin of ERβKO mice (Markiewicz et al., 2013). The authors attribute these characteristics to lower expressions of MMPs. However, while these mice showed decreased serum levels of estradiol, Mongolian gerbils perinatally exposed to BPA had a decrease on it (Leonel et al., 2020). Our results suggest that changes in MMPs may have influenced the deposition of collagen fibers in the ovarian tissue, since no evidence of estrogenic modulation on fiber synthesis was onberved.

This led us to hypothesize that the mechanisms involved in BPA long-term effects within follicular cells might be more related to metabolic disorders, such as PCOS. This would even agree with the increase of antral follicles in the ovarian tissue from ↑BPA group. The influence of BPA in the developments of PCOS has been widely discussed (for a review see (Xu et al., 2021). In this condition, the expression of estrogen receptors within developing follicles is altered (Xu et al., 2021; Jakimiuk et al., 2002).

The ability of BPA to produce opposite effects than those previously described may be related to differences in experimental approaches, such as dose and animal species applied. A previously published work using Mongolian gerbils to assess the impacts of BPA has described a decrease in the serum levels of estradiol in exposed groups (Leonel et al., 2020), differently to what has been found in other species. Thus, taking a specific and not usually applied species as experimental model, requires a more careful and conscious analysis of the results. This aspect must particularly be taken in consideration because Mongolian gerbils are physiologically different from humans in terms of reproductive cycles (Ruiz et al., 2023), levels of steroid hormones (Santos et al., 2006), and, of course, ovarian morphology.

Our results provide an insight into the long-term effects of a single EDC: BPA. However, individuals are routinely exposed to different contaminants that may interact synergistically or even antagonistically. Investigating realistic mixtures of toxicants could reveal different impacts (Defarge et al., 2018). This work evidences the clear impacts of BPA in the ovarian morphology, in terms of stromal fibers distribution and estrogenic signaling that impact cell proliferative status. Practically, our finds suggest that under the specific conditions applied in our experimental design, the main xenoestrogenic impacts led to alterations more commonly associated to metabolic disorders, such as PCOS, confirming the potential actions of BPA in the establishment of this condition in human patients. Expanding the analysis to additional stages of reproductive function by evaluating the reproductive ability of these animals would enhance the results and provide deeper insights into the mechanisms of BPA action during early development. Furthermore, molecular evaluations to quantify the expression of genes involved in ECM fiber aspects could further complement the findings.

Ethical Statement

The animal experimental procedures were conducted according to the National Council of Animal Experiment Control (CONCEA, Brazil, https://www.mctic.gov.br). The Ethics Committee on the Use of Animals (CEUA) from IBILCE/UNESP (protocol number 113/2015) and from UFG (028/2021) approved all the procedures described to be performed in the animals.

CRediT authorship contribution statement

Isabella Barbosa Melvin: Writing – original draft, Investigation, Formal analysis, Data curation. **Ana Carolina Camurça da Silva:** Investigation, Formal analysis, Data curation. **Thalles Fernando Rocha Ruiz:** Writing – original draft, Resources, Investigation. **Sebastião Roberto Taboga:** Writing – review & editing, Resources, Investigation. **Manoel Francisco Biancardi:** Resources, Methodology, Funding acquisition. **Fernanda Cristina Alcântara Santos:** Resources, Methodology, Funding acquisition. **Ellen Cristina Rivas Leonel:** Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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