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Impairment of steroidogenesis and follicle development after bisphenol A exposure during pregnancy and lactation in the ovaries of Mongolian gerbils aged females

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ABSTRACT

The ovaries regulate fertility and hormonal control in females, and aging is a crucial factor in this process, when ovarian function is drastically impacted. Exogenous endocrine disruptors may accelerate this process, acting as the main agents in decreased female fertility and hormonal imbalance, since they impact different features related to reproduction. In the present study, we demonstrate the implications of exposure of adult mothers to the endocrine disruptor bisphenol A (BPA) during pregnancy and lactation on their ovarian function during the transition to later in life (aging). The follicle population of BPA exposed ovaries showed impairment in the development of follicles to the mature stages, with growing follicles being halted in the early stages. Atretic and early-atretic follicles were also enhanced. Expression of estrogen and androgen receptors in the follicle population demonstrated impairment in signaling function: ERβ was highly expressed in follicles from BPA exposed females, which also showed a higher incidence of early atresia of developed follicles. ERβ1 wild-type isoform was also enhanced in BPA-exposed ovaries, compared to its variant isoforms. In addition, steroidogenesis was targeted by BPA exposure: aromatase and 17-β-HSD were reduced, whereas 5-α reductase was enhanced. This modulation was reflected in serum levels of estradiol and testosterone, which decreased in BPA-exposed females. Imbalances in steroidogenesis impair the development of follicles and play an important role in follicular atresia. Our study demonstrated that BPA exposure in two windows of susceptibility – gestation and lactation – had implications during aging, enhancing perimenopausal and infertile features.

1. Introduction

During adulthood, in healthy conditions, the ovarian reserve constitutes several stages of follicles in the process of development (Ford et al., 2020; Pan and Li, 2019), while the aging process naturally diminishes the follicle population in ovaries (Shifren and Schiff, 2000). These follicles are recruited throughout the woman's reproductive life through hormonal control, to assure reproductive function (Ford et al., 2020; Ouni et al., 2019a). In this regard, local estrogens and progesterone act in balance to progress each oocyte by the development of follicle features, under the regulation of the hypothalamic-pituitary axis (Brodowska et al., 2007; Emmen et al., 2005). In addition the main synthesis of female steroid hormones occurs in the ovarian follicles, as well as in the adrenal gland (Azziz, 2018). The enhancement of serum levels of these hormones is an outcome of follicle growth during the menstrual cycle and determines ovulation and the development of secondary characteristics, such as mammary and uterine development (Cunha et al., 2004; Dinny Graham and Clarke, 1997). Therefore,

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imbalances in follicular development and ovarian steroidogenesis directly affect female fertility and homeostasis (De Vos et al., 2010; Ouni et al., 2019b; Zhang and Liu, 2015).

Ovarian function may be impaired due to exposure to endocrine disruptors (Patel et al., 2015, 2017). These exogenous agents act directly in the ovarian tissue, diminishing ovarian reserve and, thus, depleting steroidogenic potential (Cho et al., 2020; Costa et al., 2014). Polycystic ovarian syndrome and ovarian cancer have been described as outcomes related to exposure to these compounds (Palioura and Diamanti-Kandarakis, 2015). Bisphenol A (BPA), an exogenous endocrine disruptor, disrupts estrogenic pathways associated with both the main steroid receptors, estrogen receptor alpha (ERα) and beta (ERβ), inducing different cellular effects (Dumitrascu et al., 2020; Nadal et al., 2018). In contrast, recent studies demonstrated the potential of BPA to activate different receptors in cells (Shafei et al., 2018), triggering important impacts in different levels of biological systems (Ruiz et al., 2021c).

Major effects of perinatal and neonatal exposure to BPA in ovaries have been widely described. Ovarian reserve is drastically decreased after BPA exposure (Cao et al., 2018), leading to infertile characteristics in adult females (Ehrlich et al., 2012). Atresia and apoptosis are frequently associated with BPA exposure in neonates and adults, decreasing the healthy follicular population in the ovaries (Bilgi et al., 2019; Cao et al., 2018). In addition, BPA exposure during the perinatal period impairs follicular activation by disrupting the transcription of *STAR* and *CYP* genes (Bloom et al., 2016; Mansur et al., 2016). These features implicate disrupted ovarian steroidogenesis (Bloom et al., 2016), since pre-formation of several steroidogenic enzymes that convert cholesterol by-products are damaged (Nakamura et al., 2010; Peretz et al., 2011). However, although these characteristics are well recognized in exposure during the perinatal window of susceptibility, the outcomes in the mother's microenvironment remain undiscovered and the effects in the aged gonad are not well documented.

Our study focused on females exposed to BPA during two main windows of ovarian tissue remodeling, pregnancy, and lactation, and examined ovarian status in aged individuals. During pregnancy, ovaries are responsible to secrete endocrine hormones to develop and mature secondary structures, remodeling itself to house a large corpus luteum, which produces progesterone, estrogens, and androgens (Ruiz et al., 2021c; Sitruk-Ware and El-Etr, 2013). In lactation, the process was coordinated by hypothalamus-pituitary axis, whereas ovaries were under remodeling and their hormonal secretion were decreased (McNeilly, 1997; Yu et al., 2010). Previous studies demonstrated that mothers exposed to BPA during both periods developed mammary carcinoma features through modulation of hormone receptors (Ruiz et al., 2021a; 2021b), as well as affects glucose metabolism in long-term (Alonso-Magdalena et al., 2015) contributing to the development of diabetes (Blesson and Yallampalli, 2015). Considering that aging is a factor in the development of ovarian dysfunction (Donnez and Dolmans, 2017; Liu and Li, 2010) and, consequently, infertility and systemic symptoms of lower hormonal production, understanding the impacts of endocrine disruptors during these phases is of particular relevance (Kling et al., 2019; Meldrum, 1993). Since perimenopause causes an imbalance in hormonal release and regulation of endocrine-dependent organs, as well as the viability of follicles for reproduction (Batrinos, 2013; Butler and Santoro, 2011), we aimed to describe the histopathological repercussions of BPA exposure in pregnancy and lactation in aged ovaries using a rodent model.

2. Methods

2.1. Animals and experimental design

To perform the experiment, fifteen female Mongolian gerbils (*Meriones unguiculatus*), at 3 months of age, were mated with fertile males. The first litter was discarded for induction of the second pregnancy. Eight days after the induction of the second pregnancy the females were separated from the males and divided into 3 experimental groups (n = 5): Control, gavage with water; Vehicle, gavage with corn oil; and BPA, gavage with 50 μg/kg of bisphenol A. The dose of BPA applied is higher than the dose considered safe by health authorities and has been described as demonstrating pro-tumorigenic potential (Kwon, 2022; Lorber et al., 2015; Ruiz et al., 2021a; Vom Saal and Vandenberg, 2021). The 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 reaching 18 months of age, characterizing the aging period. Euthanasia was performed only when the exclusive presence of cornified cells in the vaginal smear was observed for one week, aiming to standardize the hormonal status among the animals in the experiment. During euthanasia, the blood was collected for serum hormone analysis, and the ovaries were collected, weighed, and fixed in 4% paraformaldehyde for 24 h. Also, some ovaries (n = 5) were frozen (−150 °C) for molecular analysis in Western Blotting.

All procedures were performed according to the National Council of Control and Animal Experimentation (CONCEA, Brazil) and the study was approved by the local Ethics Committee on the Use of Animals (CEUA, IBILCE, UNESP), under number 217/2019.

2.2. ELISA assays

First, the serum concentration of testosterone and estradiol was investigated to identify changes in ovarian hormone production. For this, blood samples were centrifuged, and the serum was frozen at −80 °C. The hormone levels were quantified in triplicate by capture/sandwich ELISA using specific commercial kits, according to the manufacturer's instructions (Testosterone ELISA, RE52151, IBL International GmbH, Hamburg, Germany and 17beta-Estradiol ELISA, RE52041, IBL International GmbH, Hamburg, Germany). To conduct the ELISA tests, were pipetted 25 μl of sample with the 100 μl (17beta-Estradiol) or 200 μl (Testosterone) of enzyme conjugate into each well of the Microtiter Plate and incubated for 60 min (Testosterone) or 90 min (17beta-Estradiol) at room temperature. The plate was washed 3 times with the Wash Buffer, then added TMB Substrate Solution and incubated for 15 min (Testosterone) or 30 min (17beta-Estradiol) at room temperature. To finish it was added TMB Stop Solution and the optical density was measured at 450 nm. The analyses were performed in a SpectraMaxPlus 384 microplate reader (Molecular Devices, San Jose, CA, USA).

2.3. Histological procedures

To identify histological changes and quantify follicular populations the ovaries were fixed in paraformaldehyde 4%, for 24 h. Then, the ovaries were dehydrated in a gradual ethanol series, diaphanized in xylol, and embedded in paraffin Histosec (Merck) in a Leica Semi Enclosed System (TP1020, Leica Biosystems); the blocks were sectioned at 4 μm thickness and placed on silanized slides and destined to histological analysis.

2.3.1. Analysis of the follicular population and compartments volume-density

To understand the changes that BPA promotes in ovarian reserve, the follicle population was quantified. Eight HE- and PAS-stained sections from each animal were analyzed at intervals of fifteen sections at × 400 magnification using the slide scanner system (Olympus VS120–S5). The primordial and growing follicles were only considered in the count with the presence of the oocyte nuclei in the section analyzed. Mature follicles were counted when the nucleus or zona pellucida was present. Follicles were considered atretic by the presence of reactivity to PAS; early-atretic follicles were characterized by the presence of >50% of granulosa layer presenting positivity for active caspase-3

immunohistochemistry (Boone and Tsang, 1998; Feranil et al., 2005; Hurst et al., 2006).

Also, volume-densities of each histological compartment were assessed. Stereological analysis of relative frequency (%) was performed in each section (n = 15 sections per animal) by multipoint test system (400 points). The mean percentage of stroma (collagen, blood vessels), interstitial glands, and follicle population (primordial follicle to corpus luteum) were multiplied by total ovary weight, according to Hartanti et al. (2019), to determinate volume-density of each compartment.

2.3.2. Immunohistochemistry

To identify and quantify hormone receptors, steroidogenic enzymes, cell proliferation, and the early-atretic follicles was performed immunohistochemistry analysis. The slides were deparaffinized and hydrated. They were then subjected to peroxidase blockage in H_2O_2 5% diluted in methanol for 20 min, before subsequent antigen retrieval. The antigen retrieval was performed using citrate buffer or EDTA Tris at 98 °C for 40 min. The inhibition of nonspecific proteins was performed in 5% BSA or skimmed milk for 40 min. The primary antibodies were incubated overnight with specific dilutions: phospho-histone H3 (PHH3, rabbit polyclonal, 1:75, Ser10, 9701, Cell Signaling), active/cleaved caspase-3 (rabbit polyclonal, 1:50, NB100-56113, Novus Biological), androgen receptor (anti-AR, mouse monoclonal, sc-7305, 1:50, Santa Cruz Biotechnology), estrogen receptor alpha (anti-ERα, mouse monoclonal, sc-8005, 1:50, Santa Cruz Biotechnology), estrogen receptor beta (anti-ERβ rabbit polyclonal, PA1-310B, 1:50, Invitrogen, Thermo Fisher), aromatase (CYP19, mouse monoclonal, sc-74176, 1:50, Santa Cruz Biotechnology), 5-α-reductase (5α-Reductase 2 (H-100), rabbit polyclonal, sc-20659, 1:50, Santa Cruz Biotechnology), and 17BHSD (17β-HSD (M-174), rabbit polyclonal, sc-32872, 1:50, Santa Cruz Biotechnology). The steps described were interspersed with washes in PBS. The slides were then incubated with a post-primary antibody and Polymer kit, 1 h each at room temperature (Novolink™ polymer detection system 1, Leica Biosystems Newcastle Ltd.), and 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.

For quantification of the immunohistochemistry analysis, positive cells were counted from all follicles and interstitial glands present in one ovarian section from each animal (n = 5 per group). These analyses were performed in QuPath® software (Version 0.1.2, an open-source pathology software platform).

2.4. Western blotting

Although the immunohistochemistry analysis showed alterations in the ERβ, Western blotting was performed to identify which of the ERβ isoforms was altered. Frozen ovaries were used, and proteins were obtained with extraction buffer containing protease inhibitor (Protease Inhibitor Cocktail, P8340, Sigma Aldrich, St. Louis, MO, USA); subjected to electrophoresis in SDS page gel, and transferred to nitrocellulose membrane (Amersham Protram, 10,600,003, GE Healthcare, Darmstadt, Germany). The membranes were incubated with skimmed milk for 1 h at room temperature; incubated with ERβ antibody (anti-ERβ rabbit polyclonal, PA1-310B, 1:750, Invitrogen, Thermo Fisher) or GAPDH (mouse monoclonal MB374 Millipore, 1:10000) diluted in skimmed milk 1% in TBSt, overnight at 4 °C, and incubated with secondary antibodies (mouse Anti-mouse IgG, HRP-linked Antibody, #7076, 1:2000, Cell Signaling or Anti-rabbit IgG, HRP-linked Antibody, #7074, 1:2000, Cell Signaling) for 2 h at room temperature, according to the protocol described by Leonel and collaborators (2020). The steps described were interspersed with washes in TBSt. Then, the membranes were incubated with Luminol-Enhacer solution (Cod. XLS142L, Cyanagen, Italy) and peroxide solution (Cod. XLS142P, Cyanagen, Italy). Finally, the membranes were revealed in an imaging system machine (ChemiDoc MP, BioRad, Hercules, CA, USA). The density of the bands was quantified using ImageJ software (version 1.52a, Wayne Rasband, NIH, USA). Molecular weights of each band of isoforms were determined by Image Lab ® software (Bio-Rad Laboratories, Inc.).

2.5. Statistical analysis

Statistical analyses were performed in GraphPad Prism 5.00 software (GraphPad Software, San Diego, CA, USA). The analysis of data normality was performed by the Kolmogorov-Smirnov test. Data of IHC (ERα, ERβ, 17βHSD, PHH3), ELISA, and follicle population (parametric data) were analyzed by one-way ANOVA followed by Tukey's test. For IHC (AR, aromatase, and 5-α reductase) and Western blotting analysis (non-parametric data), the Kruskal-Wallis followed by Dunn's tests were applied. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Biometric data and reproductive parameters

Body weight, ovary, and ovary-uterus weights were evaluated and are demonstrated in Table 1. None of these parameters showed a significant difference between the experimental groups. However, the volume-density of each ovarian compartment detailed that in control group the enhanced volume-density were regarding follicular population, whereas in BPA group stromal compartment was increased.

In addition, reproductive parameters were compared by the number of pups per litter comparing the first pregnancy, when females were not exposed, and the second pregnancy, when the females were distributed into the experimental groups. Data shown in Fig. 1 (A) and Table 1 demonstrate a similar number of pups in the first pregnancy among groups (control: 6.40 ± 1.5; vehicle: 6.60 ± 0.8; BPA: 6.85 ± 1.5; number of pups per female), and a decreased number in the second pregnancy of females exposed to BPA (3.7 ± 1.11), compared to control (6.00 ± 0.7) and vehicle (5.6 ± 0.5).

3.2. Hormone levels

We performed ELISA assay to investigate serum hormone alterations among groups. The analyses of the ELISA assays showed a decrease in the serum levels of testosterone and estrogen. Regarding testosterone, the BPA group presented a significant difference from the control and vehicle groups (Fig. 2A). Regarding serum estradiol concentration, the BPA group showed a significant decrease compared to the vehicle and control groups (Fig. 2B).

3.3. Follicle population and proliferation

After established that hormonal alteration occurred, we evaluated the number of follicles per group (Fig. 3) due the hormonal influence.

Table 1
Biometric data of experimental groups. Data were shown in mean ± SD.

Biometric data	Control	Vehicle	BPA
Body weight (g)[a]	78.50 ± 4.99	87.60 ± 5.63	83.83 ± 2.79
Ovary (g)[a]	0.043 ± 0.012	0.034 ± 0.004	0.028 ± 0.004
Interstitial glands (g)[a,b]	0,0107 ± 0,001[a]	0,009 ± 0,0008[b]	0,0089 ± 0,0013[b]
*Stroma (g)***[b]	0,0133 ± 0,003[ab]	0,0103 ± 0,001[b]	0,0121 ± 0,009a
Follicles (g)[a,b]	0,0156 ± 0,004a	0,0102 ± 0,005b	0,0056 ± 0,007c
Ovary-Uterus (g)[a]	0.29 ± 0.051	0.29 ± 0.03	0.25 ± 0.01

[a,b] indicate statistical differences among groups.
[a] Parametric data. **Non-parametric data.
[b] Volume-density (relative frequence % x ovary weight).

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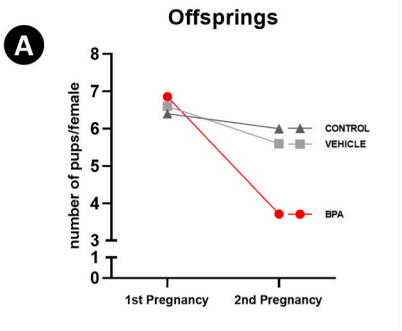

Fig. 1. Biometric and reproductive data. **(A)** Mean number of pups born per litter in first and second pregnancies of each experimental group (mean ± SD).

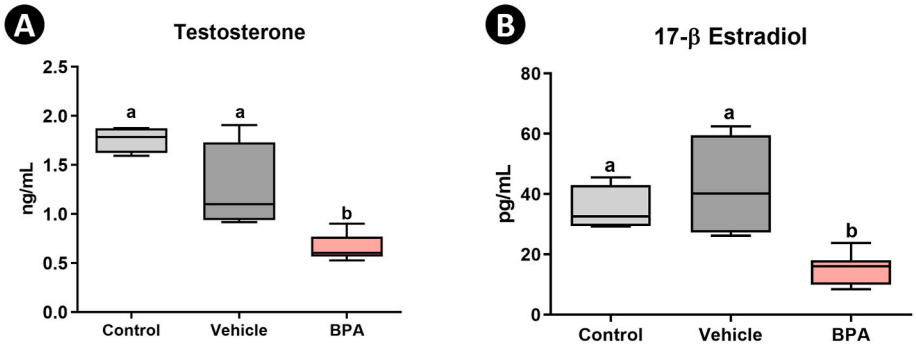

Fig. 2. Serum hormone levels. **(A)** Testosterone (ng/mL) and **(B)** 17-β-estradiol (pg/mL) of experimental groups. Different letters indicate statistical differences among groups ($p < 0.05$) (ANOVA test followed by Tukey's test).

Primordial and primary follicle types were enhanced in BPA exposed females, compared to control and vehicle groups (Fig. 3B). Secondary, pre-antral, and antral follicles, as well as corpus luteum numbers did not differ statistically among groups (Fig. 3C). Atretic follicles (Fig. 3D) were increased in the BPA group compared to other experimental groups. Furthermore, early atretic follicles, as demonstrated by active/cleaved caspase-3 (>50% of positivity for granulosa and theca cells, or oocyte positivity) and mature follicles in atresia (Fig. 3D') were enhanced in the BPA group. For early atretic follicles, this increase was almost 5 fold compared to the control and vehicle groups (Fig. 3D). In addition, analysis of proliferation demonstrated a decrease in PHH3 positive granulosa cells in growing follicles in BPA exposed follicles (Fig. 3E), which was not observed in granulosa and theca cells of mature follicles of the same group in comparison with other experimental groups.

3.4. Receptor expression

Quantification was performed of nuclear expression of androgen (AR) and estrogen (ERα and ERβ) receptors in different compartments (granulosa and theca) of growing and mature follicles (Figs. 4 and 5). AR was expressed in both compartments of both developmental stages. In growing follicles, AR was decreased in granulosa and theca cells of the BPA group, compared to control and vehicle groups (Fig. 4A), except for granulosa cells of the control group that did not differ statistically. ERα was decreased in follicles of the BPA group (Fig. 4B), with granulosa cells presenting the lowest values. Theca cells did not differ statistically among groups for this receptor. In addition, BPA exposure increased ERβ expression in both compartments of growing follicles compared to other groups (Fig. 4C–D).

In mature follicles (Fig. 5A–D), the BPA group presented the lowest values for AR-positive granulosa cells (Fig. 5A), whereas the expression did not differ among experimental groups for theca cells. Mature follicles showed high expression of ERα in granulosa and theca cells of control and vehicle groups (Fig. 5B), whereas in the BPA group a decrease was observed to both compartments. ERβ was also highly expressed in granulosa and theca cells of mature follicles exposed to BPA (Fig. 5C).

Due to the high expression of ERβ in ovaries of BPA-exposed females, as shown by immunohistochemistry, we decided to evaluate the different isoforms expressed by Western Blotting assay (Fig. 6). No significant differences among relative percentages were observed in isoforms 36 kDa (ERβ/36) and 44 kDa (ERβ/44) (Fig. 6A–B). However, BPA-exposed ovaries presented enhancement of the ERβ isoform 59 kDa (ERβ/59) (Fig. 6 C) compared to control and vehicle groups.

3.5. Steroidogenesis enzyme expression

The role of main steroidogenic enzymes in follicles and interstitial glands were reached to determinate the modulation and correlation with receptors expression and serum hormone levels (Fig. 7). Regarding expression localization, aromatase (Fig. 7A–B) and 5-α-reductase (Fig. 7C–D) were expressed in both compartments and 17-β-HSD (Fig. 7E–F) was only expressed in follicles. For aromatase, in the follicles its expression was decreased in the BPA group compared to control and vehicle groups (Fig. 7A). However, interstitial glands presented a drastic decrease in aromatase expression in BPA exposed females (Fig. 7A–B) compared to the enhanced values observed in the vehicle group. 5-α-reductase was also highly expressed in follicles of BPA exposed ovaries (Fig. 7C–D), compared to the other experimental groups. Interstitial glands did not present differences in 5-α-reductase expression among the groups. For 17-β-HSD expression, a decrease was observed in the BPA group compared to control and vehicle groups (Fig. 7E–F).

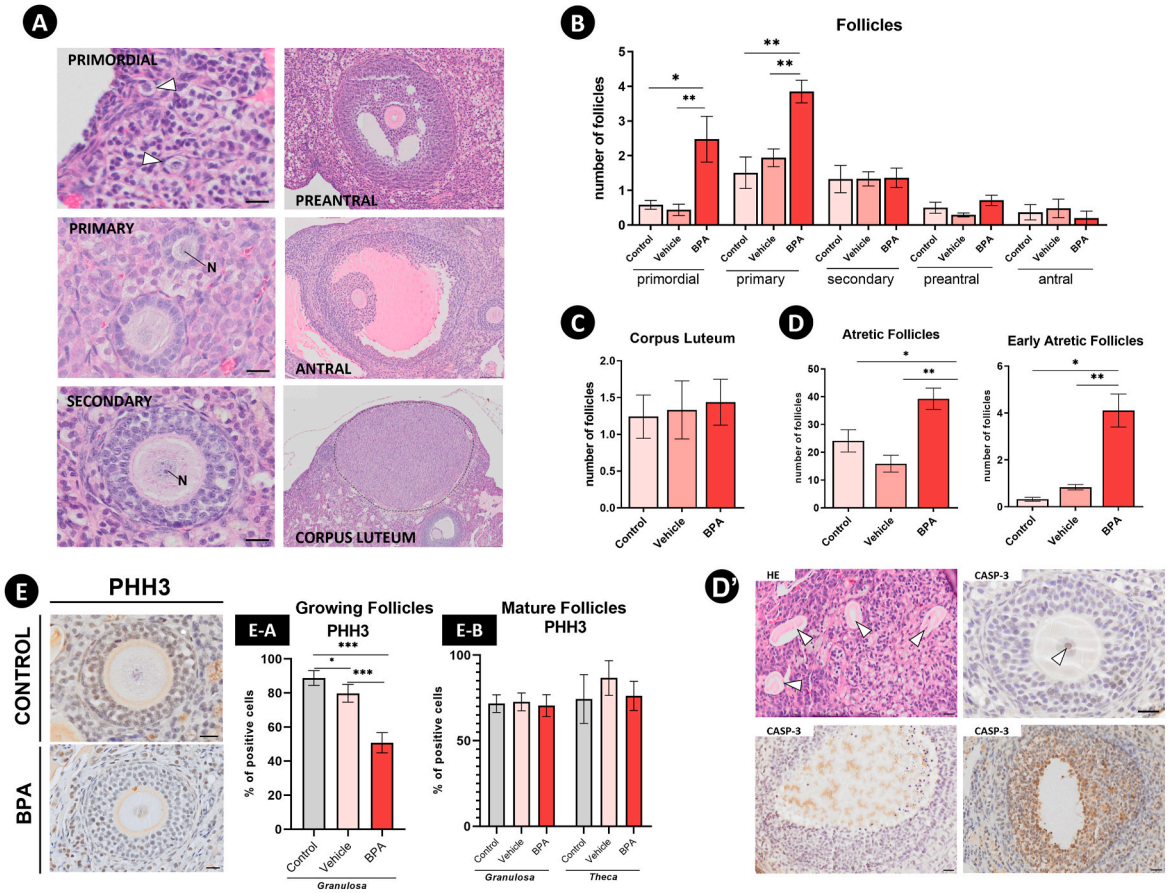

Fig. 3. Follicle population among aged females of experimental groups. **(A)** Representative pictures of follicle stages considered for quantification: primordial (arrowheads), primary, secondary, preantral, antral; and corpus luteum (dot-lined circle). **(B–D)** Number of follicles in different experimental groups. (C) Corpus luteum quantification. (D) Atretic and early-atretic follicle quantification. (D′) Representative pictures of atretic follicles identified by HE staining, and early atretic follicles that presented positive staining for active/cleaved caspase 3 in oocyte (arrowhead) or > 50% of granulosa cells. **(E)** PHH3. Quantification of proliferative index by PHH3 positive cell staining. (E–A) indicates PHH3 positive-granulosa cells in primordial/primary follicles, and (E–B) indicates PHH3 positive-granulosa cells in mature follicles and theca cells. Asterisks indicate significant differences among groups (*$p < 0.05$; **$p < 0.01$). Abbreviations: N – nucleus. Scale bars: 20 μm.

4. Discussion

The present study demonstrates the repercussions of exposure to BPA in pregnancy and lactation with consequences in the ovaries during aging. Here, we focus on the follicle population and steroidogenesis related to ovarian cells. Both processes seem to be impaired by chemical exposure to this endocrine-disruptor in two important windows of susceptibility (Ruiz et al., 2021c; Terry et al., 2019). Both, pregnancy and lactation, are sequential processes that concern in major remodeling of ovaries (Ruiz et al., 2021c; Sitruk-Ware and El-Etr, 2013) and hormonal regulation by hypothalamus-pituitary-ovaries axis (McNeilly, 1997; Yu et al., 2010). Follicle populations were drastically reduced in aged gerbils, and atresia was frequent. However, early follicle stages (primordial and primary) were present in high numbers, as well as atretic and early-atretic follicles in ovaries exposed to BPA. Even though published data state that BPA acts by diminishing the ovarian reserve (Cao et al., 2018), our data demonstrated that BPA altered follicular progress and recruitment, while diminishing follicle responsiveness to hormones.

Follicles are recruited for development, through sensitization to hormone levels (Matsuda et al., 2012; Pan and Li, 2019). Enhanced estrogen is the main signal for granulosa/follicular cell proliferation (Emmen et al., 2005; Tang et al., 2019), inducing follicle growth. Our results demonstrated decreased proliferation of these cells in BPA exposed females, associated with a drastic depletion in ERα expression in these cells. The loss of responsiveness to ERα in granulosa cells was related to late life-stages and enhanced apoptosis induction (Han et al., 2020), leading to the atretic process (Shifren and Schiff, 2000). In addition, ERs maintained progression of the pool of growing follicles (Yu et al., 2010). Decreases in this pool leads to impaired function, related to steroidogenesis (Bao et al., 2000; Payne et al., 1992), and an early atretic process (Gao et al., 2021).

The progression of follicular growth until pre-antral stages depends on androgen signaling (Cheng et al., 2002; Vendola et al., 1999). By this process, granulosa and thecal compartments are developed through a proliferative signal linked to AR (Asiabi et al., 2019). AR expression and signaling are disrupted by BPA, through low mRNA expression (Tucker et al., 2018) or impairment of associated molecular pathways (Rodríguez et al., 2010; Ruiz et al., 2021c). The progression from primordial to primary follicle stage was boosted by BPA, but AR expression was downregulated. Furthermore, enhancement of growing follicle atresia could be associated with the diminished expression of this receptor, since the absence of AR-androgen binding signal affects granulosa steroidogenesis and follicular maturation (Tetsuka and Hillier, 1997; Vendola et al., 1999). The imbalance of AR and ERα in BPA exposed ovaries (1) impaired development of the follicle pool, due their decreasing expression; and (2) was related to a steroidogenic deficient process, due to steroidogenic enzymes expression were modulation at aging after BPA exposure. This was reflected in serum levels of estrogen and testosterone, in which non-developed follicles presented reduced hormonal production capability in ovaries (Peretz et al., 2011).

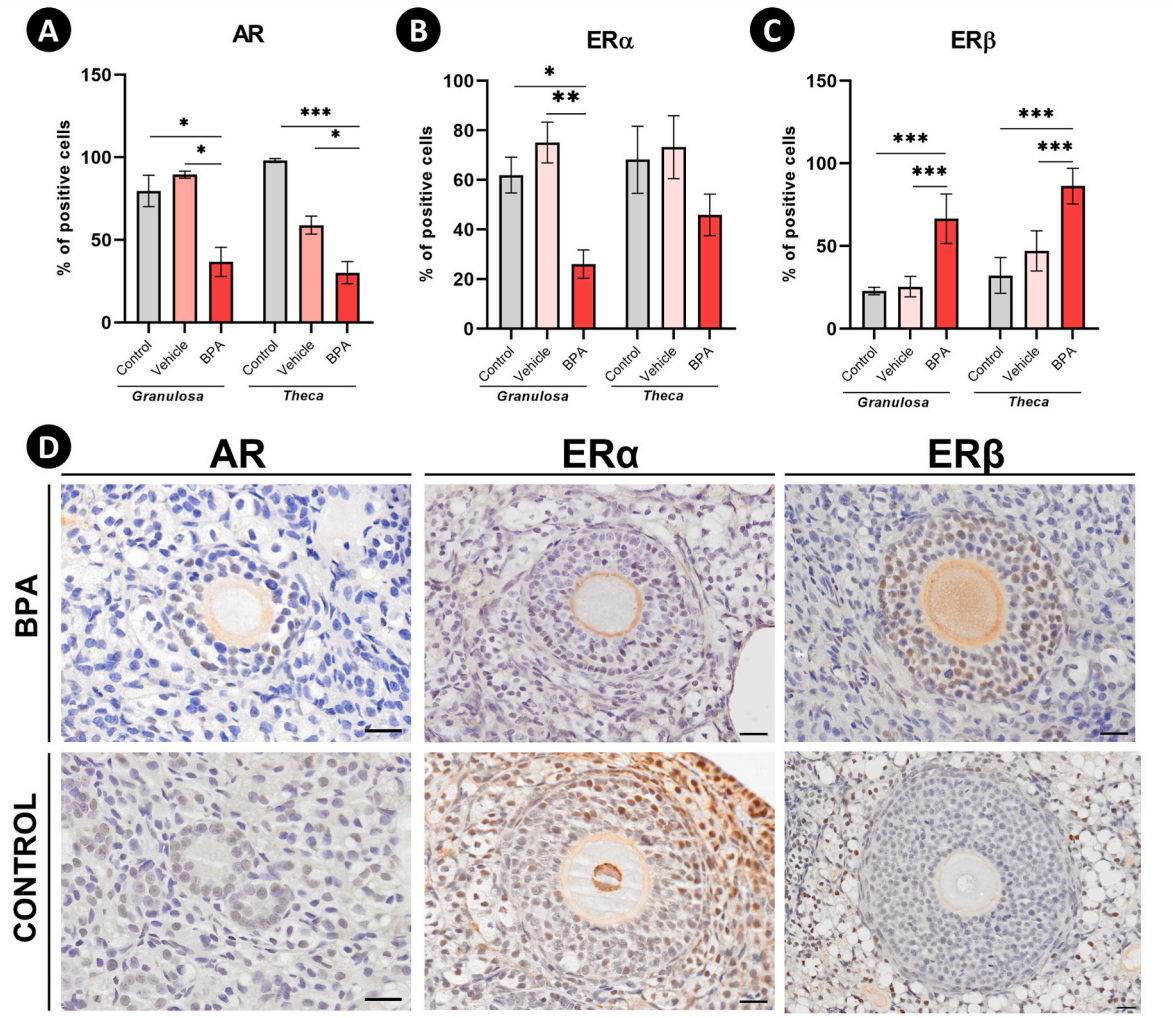

Fig. 4. Receptor expression in growing follicles (primordial, primary, and secondary). Quantification of expression in granulosa and theca compartments were performed for **(A)** AR; **(B)** ERα; and **(C)** ERβ. Asterisks correlate significant differences among groups (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). **(D)** Representative images of immunohistochemistry for AR, ERα, and ERβ expression in growing follicles of BPA and control experimental groups. Scale Bars: 20 μm.

Conversion of hormone compounds and their by-products in ovaries are characterized by several steroidogenic enzymes that are also associated with follicle activation. The enzyme 17-beta-HSD converts primary androgens, such as androstenedione, to testosterone (Miller and Auchus, 2011), whereas aromatase is responsible for converting testosterone to estradiol, mainly in granulosa cells (Tetsuka and Hillier, 1997). Although 5-alpha-reductase performs the conversion and production of testosterone to a potent androgen, dihydrotestosterone (DHT) (Gao et al., 2014), another pathway for the androgens, is enhanced in granulosa cells in polycystic ovarian syndrome (Jakimiuk et al., 2002). Herein, we found an imbalance in steroidogenic enzyme expression in follicles of BPA exposed females; while aromatase was decreased, 5-alpha-reductase was increased. These changes could implicate in main androgen conversion and higher local availability (Kaipia and Hsueh, 1997; Konieczna et al., 2018; Payne et al., 1992). Low aromatase reduces estradiol levels for follicle maturation and ovulation (Fan et al., 2019; Homburg, 2003), downregulating the systemic activity of estrogens. BPA alters aromatase expression mainly by its crosstalk with AR expression in cells (Bloom et al., 2016; Engin and Engin, 2021). Furthermore, the reduction in aromatase corroborates with decreasing estrogen serum levels in BPA exposed females. The drastic decrease in estrogen levels could accelerate the onset of menopausal symptoms, as well as enhancing the aging-related process (Chlebowski et al., 2020; Flores and Taylor, 2015; Shifren and Schiff, 2000; Tapiero et al., 2002). Previous studies highlighted the onset of carcinogenesis in mammary glands of aged females exposed to BPA in pregnancy and lactation windows (Ruiz et al., 2021a; 2021b). Thus, the present results corroborate with studies that correlate BPA exposure in females and the development of pathological features, such as mammary carcinogenesis, polycystic ovary syndrome, and ovarian insufficiency (Khan et al., 2021; Rutkowska and Diamanti-Kandarakis, 2016; Vabre et al., 2017), outcomes related to ovarian steroidogenesis imbalances.

BPA was also related to the decrease in 5-alpha reductase in the male prostate (Castro et al., 2013, 2018), which was contrary to our study. To the best of our knowledge, few studies in females exposed to BPA have described the dynamic of 5-alpha reductase (Castro et al., 2015; Veiga-Lopez et al., 2013). Despite this, several studies correlate exposure to BPA with polycystic ovarian syndrome (Rutkowska and Diamanti-Kandarakis, 2016; Wang et al., 2020). Upregulation of this enzyme raised interesting investigations in diverse scenarios of BPA exposure in females, such as the perinatal window of susceptibility (Pivonello et al., 2020). Furthermore, this modulation and comparison with male studies corroborates the multilevel concept of BPA action in biological systems (Ruiz et al., 2021c), and demonstrates the differences in males and females.

The function of the ovarian interstitial gland is poorly recognized, and few studies have described its morphology (de Souza et al., 2022) or observed its alterations (Jimenez, 2009; Miyabayashi et al., 2015).

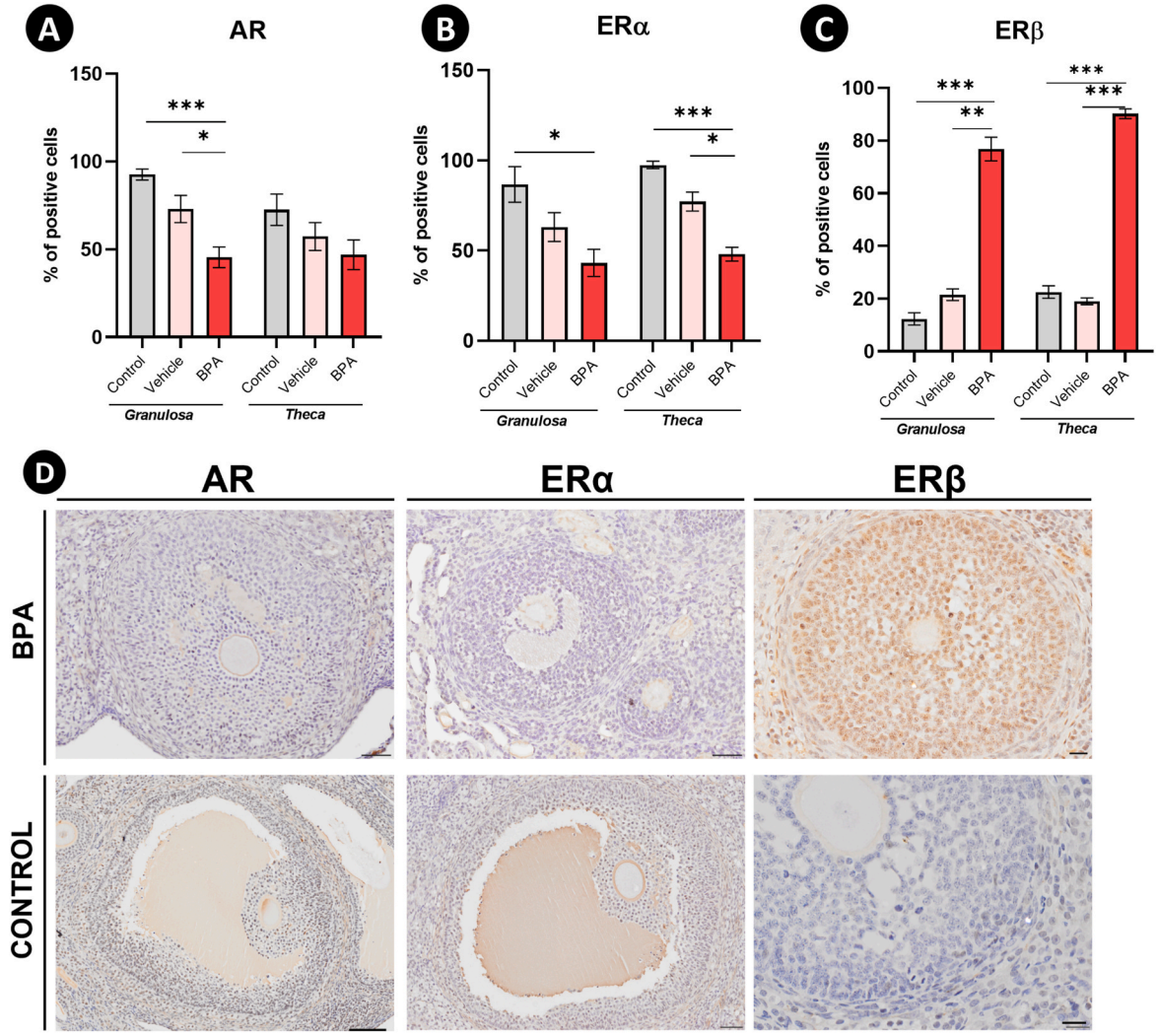

Fig. 5. Receptor expression in mature follicles (preantral and antral). Expression in granulosa and theca compartments were performed for **(A)** AR; **(B)** ERα; and **(C)** ERβ. Asterisks correlate significant differences among groups (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). **(D)** Representative images of immunohistochemistry for AR, ERα, and ERβ expression in growing follicles of BPA and control experimental groups. Scale Bars: 20 μm.

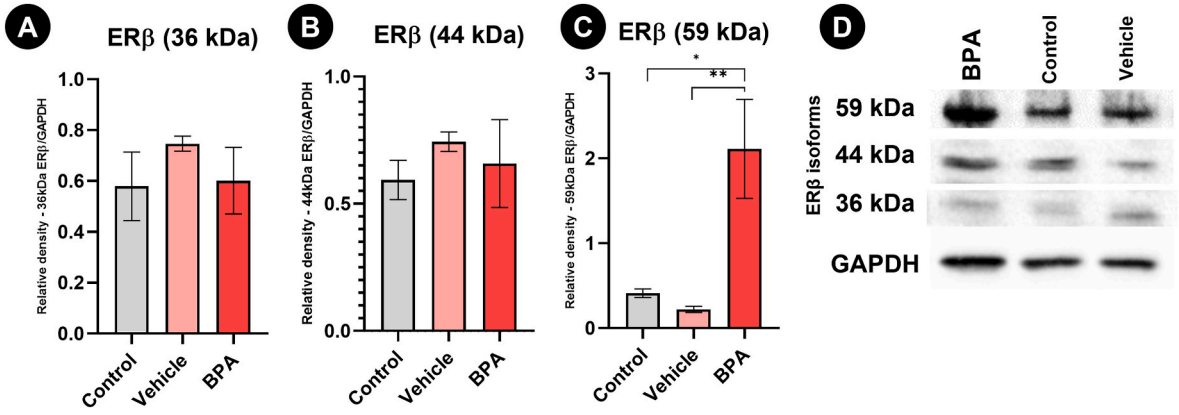

Fig. 6. Steroidogenic enzyme expression in ovaries of different experimental groups. **(A–B)** Aromatase. Note in (B) the expression in control and BPA groups in the follicle layers (granulosa and theca) and in the interstitial glands (surrounding the follicles). **(C–D)** 5-α reductase. Note in (D) the expression in control and BPA groups in the follicle layers (granulosa and theca) and in the interstitial glands (asterisks). **(E–F)** 17-β HSD. Note in (F) the expression in control and BPA groups in the follicle layers (granulosa and theca). Scale Bars: 20 μm.

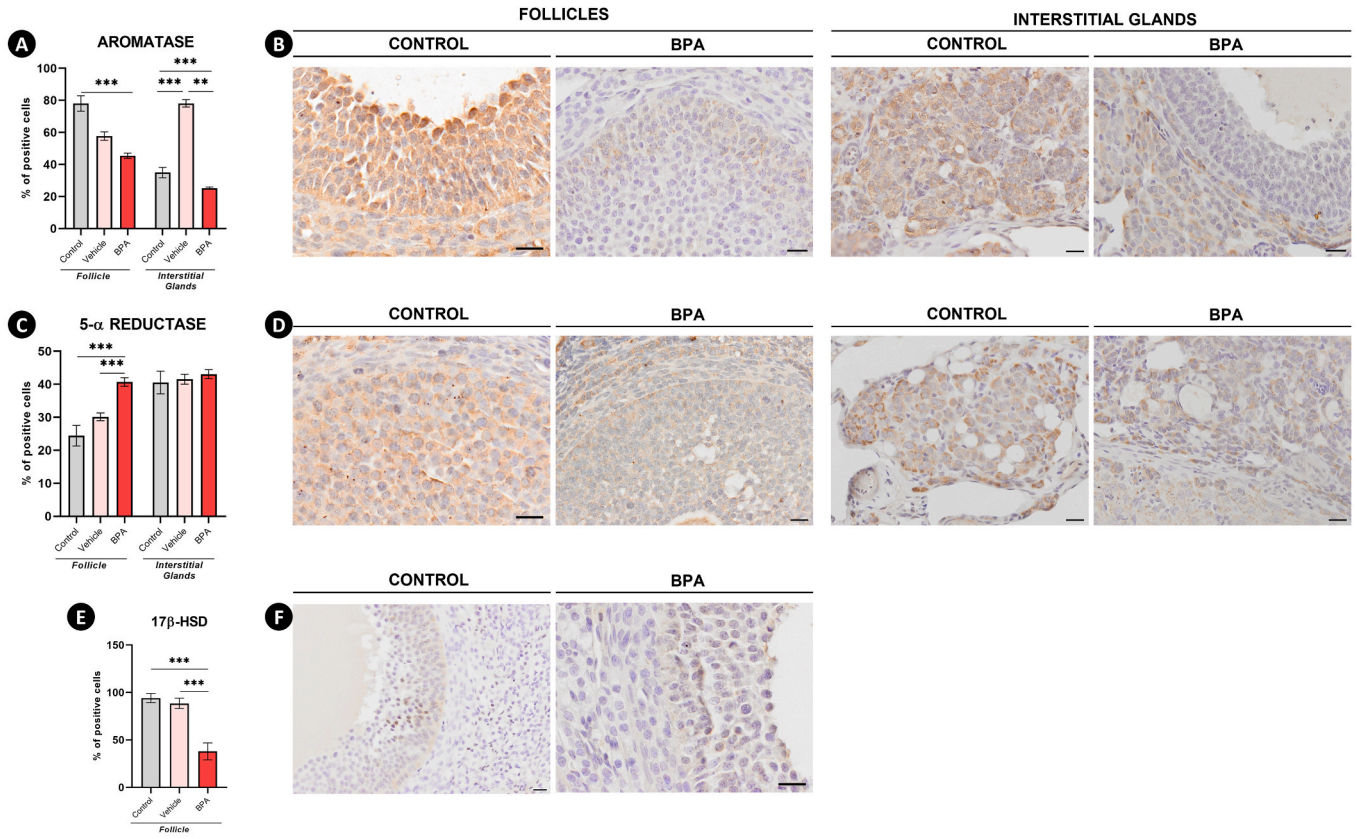

Fig. 7. Western Blotting assay. **(A–C)** Relative density of ERβ isoforms: 36 kDa, 44 kDa, 59 kDa. Endogenous control was performed by GAPDH expression for each group. Values are expressed as mean ± SEM, $p < 0.05$ (ANOVA followed by Tukey's test). **(D)** Representative blots for ERβ isoforms in 36 kDa, 44 kDa, 59 kDa.

However, our study describes some features regarding older females. The origin of these glands is directly related to the thecal and luteal cell source (Abd-Elkareem and Abou-Elhamd, 2019). Aging is an important factor to enhance this tissue in ovarian stroma (Shifren and Schiff, 2000). Interestingly, interstitial glands present a relevant role in female steroidogenesis (Miyabayashi et al., 2015). In Mongolian gerbils their responsiveness to estrogenic compounds was described (de Souza et al., 2022). According to our findings, BPA modulates the expression of aromatase in these glands in aged females. Since aging is a crucial issue to interstitial gland development and the disruption of BPA occurs by estrogenic pathways (Basak et al., 2020; Seachrist et al., 2016), modulation of this compartment could impact ovarian steroidogenesis. In our study, interstitial glands express aromatase and 5-α reductase, which are related to the final steps of steroidogenesis (Miller and Auchus, 2011). Thus, steroidogenic synthesis, by conversion of estradiol and androgens scope to these glands, impacts the final serum concentration of both hormones. Exposure to BPA in pregnancy/lactation windows impairs their normal endocrine homeostasis at aging due to a decrease in the synthetic ability of ovarian hormones, mainly by downregulation of aromatase expression (directly related to estradiol biosynthesis).

In addition to steroidogenic disruption, the ovarian follicle reserve was deregulated by BPA. Atresia is a response to desensitization of follicular cells due to endocrine signaling (Kaipia and Hsueh, 1997). In primordial and primary follicles, this signal is attended by granulosa cells (Cheng et al., 2002), whereas in more developed follicles the atretic process depends on crosstalk between theca and granulosa signaling (Matsuda et al., 2012). We found enhanced early atresia rates in developed follicles of BPA exposed ovaries, besides an increased number of complete atretic bodies in the same ovaries. A decrease in available follicles has been related to BPA after neonatal exposure, due to toxic exposure of early stage follicles (Rodríguez et al., 2010). Furthermore, our study demonstrated that BPA diminished the follicle population after exposure in adulthood, which could lead to infertile features, as previously demonstrated (Lin et al., 2021; Pivonello et al., 2020). This concept was also reinforced by our data on the decrease in the number of pups before and after BPA exposure, which could indicate a direct influence of this endocrine disruptor on reproductive ability of females.

In addition, BPA exposure reduced the potential for activation and growth progression of follicles, compared to other groups. A possible explanation is related to ERβ overexpression, which performs a pro-apoptotic role in granulosa cells (Langdon et al., 2020; Pierre et al., 2021). We demonstrated that ERβ isoform was highly expressed in BPA-exposed ovaries in the 59 kDa band, which is related to ERβ1, a wild type of ERβ (Ciucci et al., 2018; Leung et al., 2006). The other isoforms identified, 36 and 44 kDa, were recognized as variant isoforms of ERβ1, with deleted exon 5 (Lu et al., 1998). ERβ1-WT is known to perform pro-apoptotic signaling under disturbed conditions, precancerous lesions, and ovarian cancer (Al-Bader et al., 2011; Ciucci et al., 2018; Scobie et al., 2002). Therefore, the 3-fold enhancement of ERβ in granulosa and theca cells of BPA exposed females could express a possible pathway for this endocrine disruptor, not only to diminish fertility in females, but also to lead to carcinogenic development.

In summary, we demonstrated that exposure to BPA in two important windows of susceptibility, pregnancy and lactation, promoted the disruption of ovaries during aging. Despite the higher number of primordial and primary follicles found in the BPA group, their potential to be activated and develop, undertaking a steroidogenic role, is probably impaired. BPA provoked a disruption in the expression of ERα, ERβ, and AR, leading to a desensitizing status of growing follicles, associated with the atretic process of mature follicles. Furthermore, steroidogenesis was impaired in BPA exposed ovaries, modulating key steroidogenic converting enzyme expression in the process of estrogen and androgen synthesis. These features were reflected in the serum levels of these hormones and the progression of follicles to developed stages, impairing

fertility and hormonal activity in these females during aging.

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CRediT authorship contribution statement

Thalles F.R. Ruiz: Conceptualization, Methodology, Investigation, Writing – original draft, Funding acquisition. **Vitor Grigio:** Methodology, Investigation. **Luara J. Ferrato:** Methodology. **Lorena G. de Souza:** Methodology. **Simone J. Colleta:** Methodology. **Gustavo M. Amaro:** Methodology. **Rejane M. Góes:** Resources. **Patrícia S.L. Vilamaior:** Methodology, Resources. **Ellen C.R. Leonel:** Conceptualization, Methodology, Writing – original draft. **Sebastião R. Taboga:** Conceptualization, Investigation, Resources, Writing – original draft, Supervision, Project administration, Funding acquisition.

Data availability

Data will be made available on request.

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