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Inflammatory repercussions in female steroid responsive glands after perinatal exposure to bisphenol A and 17-β estradiol

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Abstract

The mammary gland (MG) and female prostate are plastic reproductive organs which are highly responsive to hormones. Thus, endocrine disruptors, such as bisphenol A (BPA) and exogenous estrogens, negatively affect glandular homeostasis. In addition to previously described alterations, changes in inflammatory markers expression also trigger the development of a microenvironment that contributes to tumor progression. The current work aimed to evaluate the inflammatory responses of the MG and prostate gland to BPA (50 µg/kg) and 17-β estradiol (35 µg/kg) exposure during the perinatal window of susceptibility. The results showed that at 6 months of age there was an increase in the number of phospho-STAT3 (P-STAT3) positive cells in the female prostate from animals perinatally exposed to 50 µg/kg BPA daily. In addition, the number of macrophages increased in these animals in comparison with nonexposed animals, as shown by the F4/80 marker. Despite an increase in the incidence of lobuloalveolar and intraductal hyperplasia, the MG did not show any difference in the expression of the four inflammatory markers evaluated: tumor necrosis factor-α, COX-2, P-STAT3, and F4/80. Analysis of both glands from the same animal led to the conclusion that exposure to endocrine disruptors during the perinatal window of susceptibility leads to different inflammatory responses in different reproductive organs. As the prostate is more susceptible to these inflammatory mechanisms, it is reasonable to affirm that possible neoplastic alterations in this organ are related to changes in the inflammatory pattern of the stroma, a characteristic that is not evident in the MG.

KEYWORDS
endocrine disruptor, female prostate, inflammation, mammary gland, morphology

Abbreviations: BPA, bisphenol A; E2, 17-β estradiol; IHC, immunohistochemistry; MG, mammary gland; P-STAT3, phospho-STAT3.

1 | INTRODUCTION

The endocrine system works by means of specialized regulatory mechanisms that control several vital functions. These mechanisms, however, may be affected if exogenous substances interfere in the system homeostasis. Thus, there is growing interest in chemicals released into the environment, since these can directly disrupt hormonal activity in organisms. Xenoestrogens, such as bisphenol A (BPA), are examples of these threats, as they are released by industrial synthetic compounds and cause damage to both male and female reproductive systems by means of hormonal signaling disruption (Sharpe & Skakkebaek, 1993). Hormone responsive glands, such as the mammary gland (MG) (Perrot-Applanat et al., 2018) and prostate (Prins et al., 2017), are particularly responsive to the effects of xenoestrogens. During BPA binding to hormone receptors, an inappropriate transcriptional conformation may take place, triggering alterations at gene expression levels (Acconcia et al., 2015). As a result, the proliferative and antiproliferative effects of ERα and ERβ are disturbed, leading to hyperplasia and preneoplastic lesions (Durando et al., 2006) even when the exposure occurs only during the perinatal period.

In rodents, MG development begins during the embryonic period. The epithelial parenchyma derives from ectoderm, and its stroma from mesoderm (Imagawa et al., 1990). During prenatal development, the gland grows internally as a rudimentary ductal structure, presenting allometric growth until puberty (Cardiff et al., 2018). The female prostate, also known as Skene's paraurethral gland, is present in several species—mice, dogs, bats—including humans (Biancardi et al., 2017b). It is an active organ with a secretion that has specialized functions in reproduction, such as improvement in motility and survival of spermatozoa in the female reproductive tract, as well as being a source of vaginal lubrication (Biancardi et al., 2017b). Prostate development starts in the urogenital sinus: the endodermic epithelium gives rise to prostatic buds of the mesodermal urogenital sinus mesenchyme (Staack et al., 2003), building the early prostate epithelium and stroma. In rodents, these events occur between embryonic Days 16.5 and 18.5 (Prins & Putz, 2008). Branching morphogenesis continues in the postnatal period until 2 weeks of age (Sugimura et al., 1986).

Therefore, the prenatal development of both glands is directly dependent on hormones and regulated by signaling pathways between the epithelium and stroma. It has been evidenced that estrogen receptors are present in the MG from the early developmental stages, confirming the susceptibility of this gland to variations in estrogen levels during development (Lemmen et al., 1999; Wadia et al., 2013). Consequently, exposure to xenoestrogens during this period causes lifelong negative impacts (Colborn et al., 1993). Previous results have also shown the vulnerability of the prostate after perinatal exposure to BPA because of estrogenic disruption (Rodríguez et al., 2016). This is due to the presence of a dynamic environment between the epithelium and stroma, where a basal membrane rupture occurs (Cowin & Wysolmerski, 2010), promoting contact between precursor epithelial cells and free BPA in the bloodstream.

Genetic, epigenetic, and morphological alterations are previously described long term effects in these glands (Doherty et al., 2010; Sanches et al., 2017). Furthermore, disturbances in specific signaling pathways of the epithelium/stroma relationship were described, which are crucial factors necessary for neoplastic development (Soto et al., 2013). Inflammatory conditions may also be a trigger for neoplastic development, since they promote oncogenic transformation of tissues (Bahiraee et al., 2019; Yu et al., 2009); in addition, genetic and epigenetic changes in malignant cells build an inflammatory microenvironment that supports tumor progression (Mantovani et al., 2008). As a result, it is possible that chronic inflammatory disturbances caused by endocrine disruptor exposure during the perinatal window of susceptibility may be related to long-term pathological disturbances (Ben-Baruch, 2003).

Since the development of neoplastic lesions is usually related to the presence of inflammatory cells and mediators, evaluation of inflammatory aspects is an interesting tool to unveil the mechanisms involved in preneoplastic lesions and tumor progression occasioned by endocrine disruptors on the MG and female prostate. In this respect, Mongolian gerbils constitute an interesting alternative for these studies. Females have a well-developed prostate, probably due to high testosterone levels in pregnant individuals in comparison to mice and rats (Santos et al., 2006). In this rodent model, prostatic buds originated from the urogenital sinus epithelium are distinguished on the 23rd intrauterine developmental day (Inomata et al., 1992). It becomes a laterally organized mesenchyme, called ventrolateral mesenchyme, described during the postnatal development (Sanches et al., 2016). Differently from the female rat prostate development, the periductal smooth muscle is continuous and the epithelium derives from 1 to 2 two prostatic buds during the intrauterine life, which go through branching and acini development during the second postnatal week (Sanches et al., 2016). They also demonstrate a spontaneous ability to easily develop neoplastic lesions, especially in the reproductive system (Campos et al., 2008; Kubiak et al., 2015; Quintar et al., 2017; Salyards et al., 2013). Thus, as an experimental model, gerbils mimic the natural environment of an individual susceptible to cancer (Custodio et al., 2010). With this, the real impact of BPA may be determined according to the presence or absence of inflammatory markers on exposed or intact control animals, allowing better understanding of the molecular mechanisms and morphological preneoplastic characteristics involved in tumor development in predisposed individuals.

Although the female prostate has been little explored, it is presumed that this gland shows similarities to the MG regarding inflammatory infiltrates and markers and the relation to the tumor microenvironment. Thus, the aim of this work was to evaluate, by means of morphological and immunohistochemical (IHC) analysis, the long term repercussions of perinatal exposure to endocrine disruptors BPA and 17-β estradiol on the MG and female prostate of Mongolian gerbils and the relation with inflammatory markers. Another objective was to establish comparison patterns between

inflammatory responses in both glands in each individual, since in this species females show a well-developed and functional prostate.

2 | MATERIALS AND METHODS

2.1 | Experimental design

The analyses were performed in the MG and prostate tissue obtained from female Mongolian gerbils (*Meriones unguiculatus*). All animal procedures were approved by the local ethics committee (CEUA—IBILCE/UNESP 113/2015). Pregnant 4-month old females were allocated to three groups ($n = 5$): intact control (not exposed to any treatment, IC), 35 µg/kg (Pinto et al., 2008) 17 β-estradiol (E2), and 50 µg/kg (Vandenberg et al., 2012) Bisphenol A (BPA). The compounds were diluted in 0.1 ml corn oil. On the 8th gestational day, day of embryo implantation in the uterine wall (Fischer & Floyd, 1972), gavage application began, three times/week for E2 and daily for BPA groups; the treatments provided to mothers continued until the final day of lactation, being the total gestational and lactational treatment length 39 days for both groups. After weaning, litters were separated from the mothers and one female was randomly chosen from each offspring group ($n = 5$) and used for analysis (Figure 1).

Perinatally exposed females were kept at a room temperature of 23–26°C, 50% relative humidity, and light/dark cycle of 12/12 h. Fresh water and commercial chow (Presence®) were provided ad libitum during the experiment. At 180 days of age the females were anesthetized with xylazine (3 mg/kg) and ketamine (10 mg/kg) and euthanized by decapitation. The euthanasia procedures were performed during the morning with the animals in the estrous phase of the cycle, indicated by vaginal smears. The left abdominal MG and prostatic complex (urethral segment, corresponding vaginal segment and prostatic ducts, and alveoli) were removed. These fragments were dissected using a Leica stereoscopic microscope (Leica).

2.2 | Morphological analysis

MG and prostate tissue were collected for morphological and IHC analysis. The glands were carefully removed and immediately fixed in paraformaldehyde 4% for 24 h. Next, samples were transferred to ethanol 70% and histologically processed under a semi enclosed system (TP1020; Leica Biosystems). The paraffin blocks were sliced (4 µm thick) and the sections placed on glass slides. An interval of 40 µm thick was taken between each of the three sections analyzed. Cytochemical staining was performed with hematoxylin and eosin for general morphological evaluation of the mammary tissue and localization of the secretory units. For detection and quantification of hyperplastic alterations, α-actin immunostaining was performed, to detect the basal limits of the epithelium. The slides were rehydrated and processed. Antigen retrieval was performed at 98°C in citrate buffer (pH 6.0), endogenous peroxidases blocked in 3% H2O2 at room temperature, and nonspecific proteins were blocked using 5% skimmed milk. Sections from three different depths were incubated overnight at 4°C with anti-α-actin antibody (mouse monoclonal, 1A4, 1:100, sc-32251; Santa Cruz Biotechnology). A polymer kit (Novo-castraNovolink RE7230-CE; Leica Biosystems) was applied for 1 h at room temperature, according to the manufacturer's instructions. Positive staining was detected using 3-30′-diaminobenzidine tetrahydrochloride (DAB) chromogen (NovolinkMaxDAB, RE7270-CE; Leica Biosystems) and counterstaining was performed with Mayer's hematoxylin.

In the MG, the incidence of lobuloalveolar hyperplasia was quantified by the number of animals showing hyperplastic tissue over the total animals in each group (3 sections/animal, 15 sections/group). Intraductal hyperplasia was estimated by the percentage of mammary epithelial structures with more than three layers of epithelial cells over the total number of epithelial structures in the tissue (Durando et al., 2006); this analysis was exclusively performed in animals without lobuloalveolar hyperplasia.

For morphological analysis of the prostate glands the presence of proliferative lesions (Guerra et al., 2019) was quantified. The individual incidence of acini showing lesions (prostatic intraepithelial neoplasia, epithelial proliferative hyperplasia) in the tissue's epithelial structures over the total number of acini was taken into consideration for quantifying the groups' prevalence of alterations. In total, 15 tissue sections were analyzed/group (3 sections/animal).

2.3 | Immunohistochemistry

For quantification of inflammatory proteins, the immunohistochemistry protocol previously described in Section 2.2 was performed. After blockage of nonspecific proteins, the Sections (3 sections/animal, 15 sections/group) were incubated overnight at 4°C with the following primary antibodies: anti-tumor necrosis factor-α (TNF-α) (rabbit monoclonal, immunoglobulin G [IgG], D2D4, #11948; Cell Signaling), anti-COX-2 (rabbit monoclonal, IgG, D5H5, #12282; Cell Signaling), anti-phospho-STAT3 (rabbit monoclonal, IgG, D3A7, #9145; Cell Signaling), and anti-F4/80 (rabbit monoclonal, IgG,

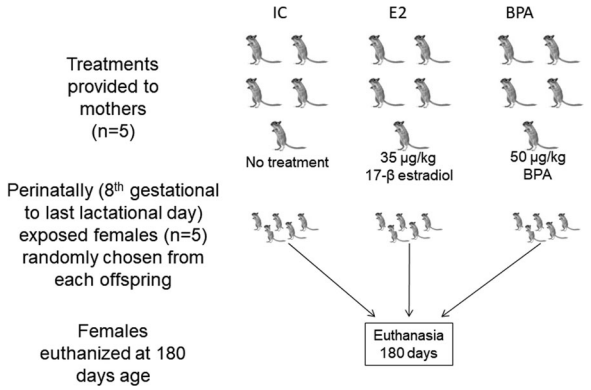

FIGURE 1 Experimental design

D2S9R, #70076; Cell Signaling). Then, the previously described method was adopted for detection.

Intracellular and tissue expression patterns were described for each protein analyzed. Quantification of cells expressing these markers was performed and the results were shown as number of positive cells per tissue area (cells/mm^2).

2.4 | Image analysis

For morphological analysis, slides of the MG and prostate were digitized (×400 magnification) using a BX61VS camera (Olympus Corporation) coupled to an Olympus VS120 Virtual Microscope Slide Scanning System (VS120-S5), from the same company. After tissue scanning, the total area of tissue containing the MG and prostate secretory epithelium was analyzed. The incidence of positive cells per area (cells/mm^2) was calculated in the Image Pro Plus software (Version 6.1 for Windows—Media Cybernetics) and the results are expressed as means ± *SE*.

2.5 | Statistical analysis

Statistical analysis was conducted using GraphPad Prism 5.0 software (GraphPad Software, Inc.). For each IHC assay, the mean ± *SEM* index obtained from the total tissue area in a given animal was taken into account. Quantitative analysis was based on parametric (analysis of variance followed by Tukey's test) or nonparametric (Kruskal—Wallis followed by Dunn's test) evaluation of differences between the groups. A p value of less than .05 was considered significant.

3 | RESULTS

3.1 | Morphological aspects

General morphology of the MG is shown in Figure 2. In the MG, as expected for adult virgin Mongolian gerbils, the tissue was characterized by the predominance of adipose tissue with foci of epithelial structures among adipocytes. The α-actin immunoreaction led to identification of the basilar limits of the epithelial structures, allowing ideal evaluation of its morphology and quantification of cell layers. Lobuloalveolar hyperplasia was absent among animals from the IC group (Figure 2b,c) and the incidence in E2 (Figure 2d) and BPA groups was 48.8% and 44.4%, respectively. The frequency of intraductal hyperplasia (Figure 2e,f) among morphologically normal glands was 4.6 ± 1.7, 21.5 ± 4.9, and 23.8 ± 5.7 for IC, E2, and BPA groups, respectively (Figure 2a).

Normal morphology of the prostate gland is shown in Figure 3b. Lesions consisted in epithelial proliferative hyperplasia (Figure 3c–e). The rupture of myoepithelial layer was also observed in the E2 group (Figure 3f). The total incidence of proliferative lesions was

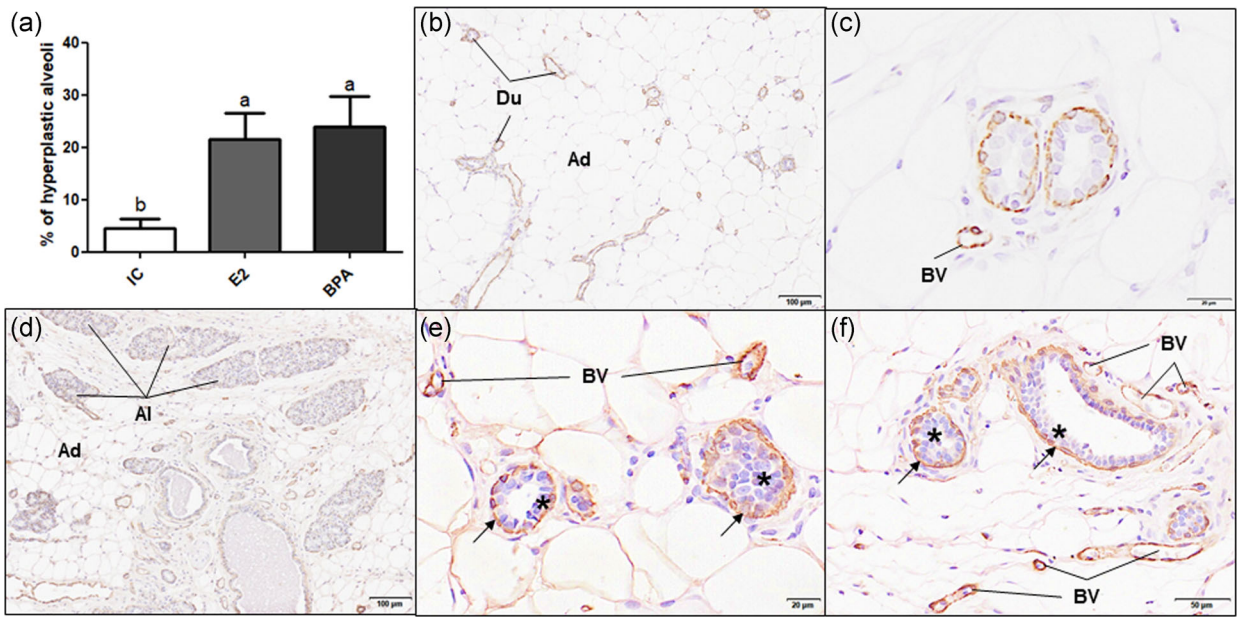

FIGURE 2 Morphological alterations in the mammary gland subjected to immunoreaction with α-actin. Hyperplastic alveoli incidence (a). The morphologically normal mammary tissue was characterized by the presence of few epithelial structure foci among adipose tissue (b: IC group) with continuous layer of myoepithelial cells (c: IC group). Lobuloalveolar hyperplasia was present in E2 and BPA groups and was characterized by an increase in epithelial structure foci among adipose tissue and the presence of secretion in the lumen (d: E2 group). Intraductal hyperplasia was characterized by the presence of more than 3 layers of epithelial cells in the epithelial structures (d, e: E2 group). Arrows: smooth muscle; asterisks: epithelium, Ad: adipose tissue, Al: alveoli, BV: blood vessel, Du: ducts. Bars: 100 µm in b and d; 20 µm in (c) and (e) and 50 µm in (f). ANOVA followed by Tukey's Multiple Comparison test (a) ($p < .05$). ANOVA, analysis of variance; BPA, bisphenol A; IC, intact control

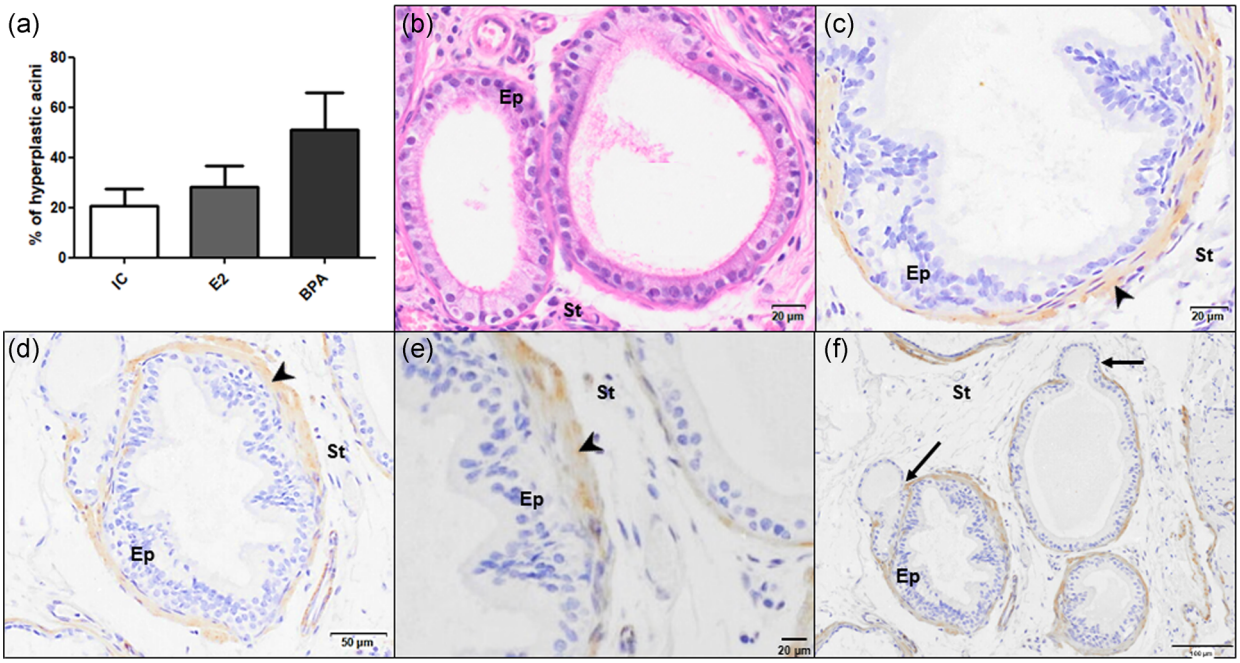

FIGURE 3 Morphology of the prostate gland. Hyperplastic acini incidence (a). Morphologically normal acini, stained by HE, are characterized by the presence of a simple cuboid/columnar epithelium (b: IC group). The immunoreaction for α-actin allows the identification of premalignant morphological alterations, such as epithelial proliferative hyperplasia (c: E2 group; d,e: BPA group). Rupture of the myoepithelial layer was present in acini with prostatic intraepithelial hyperplasia (microacinar budding) from the BPA group (f: arrows). Arrowhead: myoepithelial cell layer, Ep: epithelium, St: stroma. Bars: 20 µm in (b), (c), and (e), 50 µm in (d) and 100 µm in (f). ANOVA followed by Tukey's Multiple Comparison test (a). ANOVA, analysis of variance; BPA, bisphenol A; HE, hematoxylin and eosin; IC, intact control

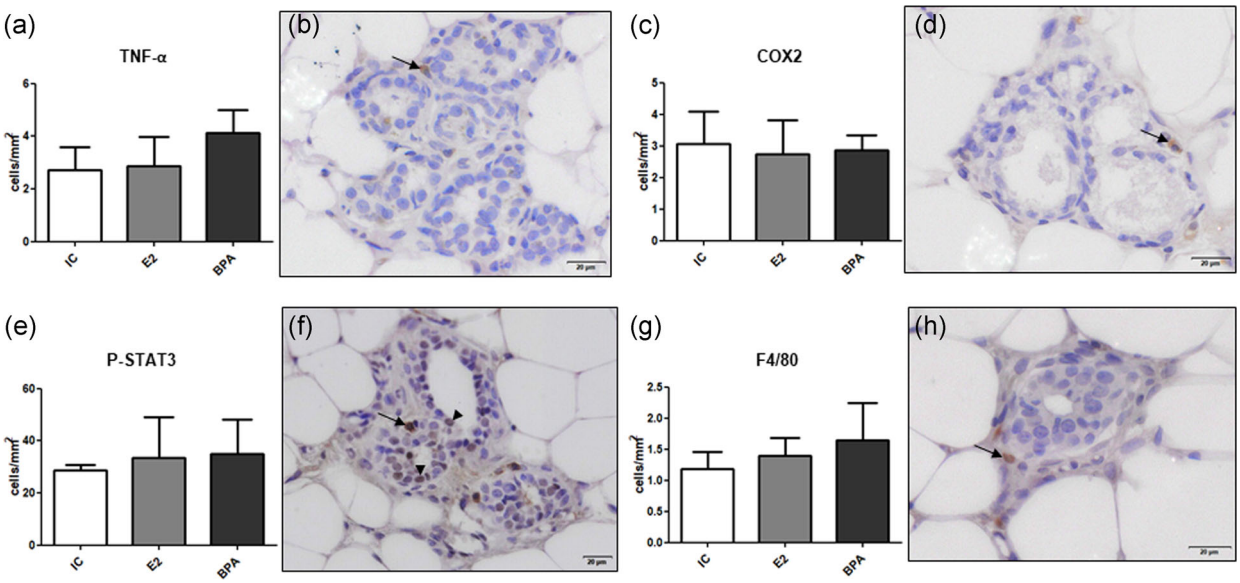

FIGURE 4 Incidence of inflammatory markers in the mammary gland. TNF-α (a,b: E2 group), COX-2 (c, d: IC group), phospho-STAT3 (e,f: E2 group), and F4/80 (g,h: BPA group) immunoreaction were quantified. All markers showed cytoplasmic staining in the stromal compartment (arrows) and phospho-STAT3 also showed nuclear staining in epithelial cells (arrowheads). ANOVA followed by Tukey's Multiple Comparison test (a,e,g) or Kruskal–Wallis test (c) ($p < .05$). Bars: 20 µm. ANOVA, analysis of variance; BPA, bisphenol A; IC, intact control; TNF, tumor necrosis factor

20.5 ± 15.1, 28.3 ± 18.1, and 50.9 ± 33.4 in IC, E2, and BPA groups, respectively (Figure 3a).

3.2 | MG expression of inflammatory markers

The quantification of inflammatory markers and illustrative pictures of their expression pattern in the MG are shown in Figure 4. All markers analyzed showed low to moderate cytoplasmic immunostaining, with a smooth scattered pattern throughout the cell. Positive cells were localized exclusively in the nonepithelial compartment of the MG, specifically among fibroblasts of the connective tissue surrounding epithelial structures among adipose tissue (Figure 4b,d,f,h). An exception in the staining pattern was observed during the P-STAT3 analysis, where nuclear staining was also present among epithelial cells (Figure 4f).

Although there was no difference between the groups in terms of positive cells/mm^2, the BPA group showed a slight increase in TNF-α, P-STAT3, and F4/80 (4.1 ± 0.8; 35.0 ± 13.0; and 1.6 ± 0.6, respectively) in comparison to the IC (2.7 ± 0.8; 28.5 ± 2.2; and 1.1 ± 0.2, respectively) and E2 groups (2.8 ± 1.1; 33.5 ± 15.6; and 1.3 ± 0.2, respectively) (Figure 4a,e,g). In contrast, the incidence of COX-2 was very similar between BPA (2.8 ± 0.4) and E2 (2.7 ± 1.0) groups, with a slight increase in the IC group (3.0 ± 1.0; Figure 4c).

3.3 | Prostate expression of inflammatory markers

The results of inflammatory marker expressions obtained after prostate gland analysis are shown in Figure 5. Similarly, as observed in the MG, the positive cells showed a cytoplasmic pattern of staining (Figure 5b,d,f,h), with the exception of P-STAT3 which was also present in the nucleus of epithelial cells (Figure 5f). Cytoplasmic stained cells were localized in the surrounding stroma, basally to the epithelium.

Despite the large differences between the IC (5.4 ± 0.5) and E2 (41.4 ± 21.0) and BPA (44.1 ± 14.4) groups, the analysis did not show statistical differences between them for TNF-α (Figure 5a). COX-2 also presented no changes in frequency of positive cells among the groups. This marker, moreover, showed the highest frequency, with an incidence of 33.5 ± 10.0 in the IC, 56.5 ± 12.7 in the E2, and 91.5 ± 29.8 in the BPA group (Figure 5c). In the prostate there was some smooth muscle staining of COX-2. Differently to the MG, there were differences in the number of cells/mm^2 among groups in the prostate regarding P-STAT3 and F4/80. P-STAT3 (Figure 5e) showed a higher incidence of positive cells in the BPA group (33.1 ± 6.7) in comparison to the IC (5.8 ± 1.2); both groups were similar to the E2 (17.6 ± 1.9). The frequency of macrophages, as shown by F4/80 (Figure 5g) staining also increased in the BPA group (59.4 ± 21.9) in comparison to the IC group (4.9 ± 1.3), both being similar to the E2 group (25.6 ± 9.5).

4 | DISCUSSION

Individual animal analysis showed that the pattern of morphological alterations and inflammatory responses varies between different steroid responsive secretory glands after perinatal exposure to endocrine disruptors. In terms of alterations related to proliferative lesions (hyperplasia), both the MG and prostate showed an increased

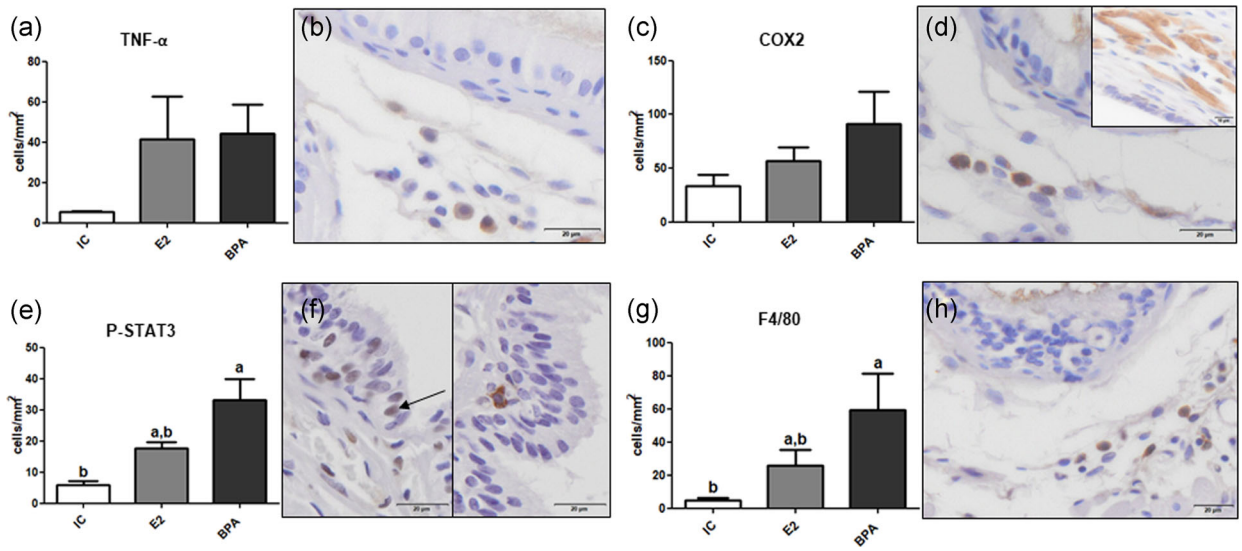

FIGURE 5 Incidence of inflammatory markers in the female prostate gland. TNF-α (a,b: E2 group), COX-2 (c,d: BPA group), phospho-STAT3 (e, f: E2 group), and F4/80 (g, h: BPA group) immunoreaction were quantified. Stromal inflammatory cells showed a cytoplasmic staining pattern for the markers; phospho-STAT3 also showed nuclear staining in epithelial cells (arrow). Different letters above bars indicate statistical difference. ANOVA followed by Tukey's Multiple Comparison test ($p < .01$). Bars: 20 μm. ANOVA, analysis of variance; BPA, bisphenol A; TNF, tumor necrosis factor

incidence. In the MG, lobuloalveolar hyperplasia was not observed in the animals from the IC group; this lesion was present in almost half the animals from the E2 and BPA groups. In the prostate, the increase in lesions in the BPA group was almost twofold in comparison to IC and E2 groups. Regarding inflammatory markers, surprisingly there were no differences in the protein expression in the MG between the groups. However, the prostate showed an increase in the incidence of macrophages and P-STAT3 positive cells in animals exposed to BPA in comparison to those from the IC group, suggesting that the pattern of inflammatory response is different in both glands and the increase in inflammatory cells and mediators is related to the incidence of preneoplastic lesions only in the prostate.

The concept that maternal exposure to adverse conditions impacts offspring health in adulthood has been related to the developmental origin of adult disease, also known as the "Barker hypothesis" (Barker, 2007). The proliferative inductive effects of BPA have previously been described in the MG (Lee et al., 2012; Leonel et al., 2020a; Qin et al., 2012). In vitro studies have also shown an increase in the mammary cell proliferation index in breast cancer (Sengupta et al., 2013) cell lines after exposure to BPA. MG exposure during development windows of susceptibility are a specific concern among research groups. The periods of differentiation and initial proliferation of mammary cells are critical windows of susceptibility to the long-term risk of malignancy development (Russo & Russo, 1996).

The use of α-actin as a marker enables adequate evaluation of glandular epithelial proliferative alterations by means of identification of the myoepithelial layer and quantification of epithelial layers (Duivenvoorden et al., 2018). In both, MG and prostate, with α-actin staining we can separate with clarity which cells compose the luminal and the stromal compartments; also, it allows to check for the continuity of the basal structure, which is directly related to the maintenance of the acini/alveolar structures and malignancy of lesions—myoepithelial cell layer acts as a barrier against cell invasion (Deckwirth et al., 2021). The presence of microacinar budding, shown in Figure 3f is an example of these lesions and was previously described as high-grade prostatic intraepithelial neoplasia (Tolkach & Kristiansen, 2018). In MG, both lobuloalveolar and intraductal hyperplasia were observed in this experiment and are sentinel signs of malignant lesion development (Keely, 2011). These alterations were observed in rats (Soto et al., 2008), mice (Vandenberg et al., 2008), and rhesus monkeys (Tharp et al., 2012) after perinatal exposure to low doses (0.5–50 μg/kg/day) of BPA. Our results corroborate those in the literature and the mechanisms involved in the induction of long-term proliferation are related to possible transcriptional effects of BPA (Wadia et al., 2013). Interestingly, according to Wadia et al. (2013), estrogen receptors are mainly expressed in stromal cells during in utero MG development, being increased in the epithelium after birth. This idea supports the hypothesis of a great influence of BPA on the stromal compartment that will later impact the epithelium homeostasis, since the BPA is known to exert disruption by means of estrogen receptor binding (Leonel et al., 2020a).

In males, the prostate has an important secretory function in the reproductive tract; particularly in humans, there is great concern related to the incidence of benign (Langan, 2019) and malign hyperplasia (Chang et al., 2014) in adults. In women, however, concern regarding hyperplastic alterations in this gland is more recent (Muto et al., 2017). The initial development of this organ takes place during the prenatal period and in rodents it has been shown to be susceptible to environmental factors. For example, female prostate glands experimentally exposed to disruptors during early development have also shown signs of developmental disruption (da Silva Gomes et al., 2020; Gomes et al., 2019). BPA also influences the homeostasis of this gland (Silva et al., 2019), as it also interacts with androgen receptors to prompt epithelial cells proliferation (Lee et al., 2003). In addition, not only androgens' but also estrogen receptors are expressed in the female prostate during its development, and in adults (Perez et al., 2011), allowing the BPA pathway mechanism to occur.

Chronic inflammation has been linked to both mammary (Qian et al., 2011) and prostatic (De Nunzio et al., 2016; Kramer & Marberger, 2006) hyperplasia and malignancy. The deep stromal/epithelial relationship during prostate and MG development and functioning in adulthood (Holloway et al., 2012) justifies this influence of stromal cells on epithelium. Thus, one may say that hyperplasia in these glands may be defined as an immune-mediated inflammatory disease (De Nunzio et al., 2016).

TNF-α is a member of the TNF/TNFR cytokine superfamily. It is involved in maintenance of the immune system and, consequently, in inflammation and defense. Knockout animals show defects in lymph node germinal center formation (Kuprash et al., 2005). We did not observe extracellular TNF-α staining in the MG or the prostate during our analysis; this factor was therefore present in the cytoplasm of immune cells in the stroma. This means that there was controlled production of TNF-α (Grivennikov et al., 2005), which may have avoided any excessive inflammatory response in both tissues. This absence of differences may be related to low activity exerted by TNF-α in a long-term response to perinatal BPA or E2. TNF-α has an active role in inflammation-associated cancers (Olivas & Price, 2020); here, however, despite the presence of proliferative lesions, there was no impact on TNF-α expression in either organ. In general, this factor may be directly involved in the initial development of malignancy, by contributing with an appropriate environment in the stroma (Balkwill, 2006). Thus, although the literature links TNF-α to a pro-carcinogenic environment, our results did not show any difference in the expression of this marker in the two hormonal responsive glands, showing that the early stage of initial hyperplasia here described is not in conjunction with changes in TNF-α expression.

Despite the lack of statistical differences between groups, we observed a slight reduction in COX-2 expression in the BPA exposed MG and, contrarily, an increase in the BPA exposed female prostate. This demonstrates that secretory organs from the same individual may present different patterns of inflammatory response when exposed to the same environment. The MG expression of COX-2 has been associated with a poor breast cancer prognosis in humans (Xu et al., 2018). The mechanism of

action by which this cytokine acts in inducing proliferative mammary disorders is in association with the prostaglandin cascade, generating "breast cancer inflammogenesis" (Harris et al., 2014). However, at least at 6 months of age, perinatal exposure to BPA did not impact the level of expression of this factor in the MG. Similar results were observed in the prostate where, despite a slight increase in the BPA group, there was no statistical difference in comparison to the IC group. These results are different from those described in adult rats, where exposure to BPA upregulated COX-2 (Wu et al., 2020); the authors attribute this increase to the higher levels of serum estrogen. Previous experimentation with Mongolian gerbils showed a decrease in serum levels of estradiol in E2 and BPA perinatally exposed animals (Leonel et al., 2020a, 2020b), which may explain the absence of differences in COX-2 expression among groups in the present work.

STAT proteins belong to a family of cytoplasmic transcription factors which have relevance in hyperplastic lesion development leading to cancer (Ashrafizadeh et al., 2019). STAT3 involvement in inflammatory responses is related to the expression of genes responsible for cancer progression (Aggarwal et al., 2006). For establishment of its signaling pathway, interleukin-6 binding to its receptor is necessary to create an extracellular signal by means of its transmembrane receptors (Subramaniam et al., 2013). This pathway also includes activity of Janus kinases, which activate STAT3 (Kim et al., 2012). In the nucleus, phosphorylated STAT3 binds to specific regions to control gene expression, which may vary among different tissues (Siddiquee & Turkson, 2008).

In the MG, no differences in the expression of P-STAT3 were observed among groups; the prostate, however, showed an increased number of stromal cells expressing this factor. BPA exposure increased STAT3 expression in cells from ovaries (Ptak & Gregoraszczuk, 2012), breast (Zhang et al., 2012), and prostate (Bishop et al., 2014). STAT3 has been linked to maintenance of an inflammatory environment and to malignant transformation in the prostate (Thompson et al., 2015). Perinatal exposure to BPA led to an increase in the inflammatory pattern of the prostate as mediated by P-STAT3. Both nuclear and cytoplasmic staining was observed. Nuclear staining was observed exclusively in the epithelium and is related to nuclear translocation, necessary for DNA binding after p48 cytoplasmic association (Ihle, 1995), indicating STAT3 activation by means of cytokine signaling from the surrounding stroma. Staining presented in the cytoplasm of stromal cells might be related to mediation of signaling from surface receptors not related to cell proliferation, but to immune cell activation and cytokine production (Takeda et al., 1999). Therefore, STAT3 is a molecular marker for prostate cancer (Huang et al., 2005) that did not respond to the perinatal proinflammatory/procarcinogenic compound BPA in the MG.

Specific staining with F4/80 was established for identification of mouse macrophages in general and monocytes (Austyn & Gordon, 1981). However, we observed specific staining in gerbil immune cells that corresponded to macrophages. No differences were presented among the groups regarding the number of F4/80 positive stromal cells; however, the presence of macrophages was higher in the MG from BPA perinatally exposed animals.

Macrophages are cells from the hematopoietic system that act in tissue remodeling, immune response, and homeostasis, being important sources of growth factors and cytokines (Varol et al., 2015). However, the immunogenic role of macrophages may be converted to a risky pathological threat (Wynn et al., 2013). In the male prostate, specifically, a proneoplastic function related to an increase in the epithelium proliferative pattern (Dang & Liou, 2018; Wu et al., 2019) has been attributed to macrophages. Furthermore, perinatal exposure to endocrine disruptors (Castro et al., 2020) has been associated with long-term changes in the incidence of macrophages, suggesting a modification in the stromal microenvironment and epithelium/stroma relationship. The influence of BPA on macrophages has been related to their self-renewal process (Ampem et al., 2019), by a mechanism that impacts the homeostasis of adipose tissue, as well as oxidative stress.

By glancing quickly at the graphs regarding all inflammatory markers, one may suggest that the prostate showed a stronger and more consistent response against perinatal disruptor exposure, with increases in all prostate glands from the treated groups. This suggests that the prostate is more susceptible than the MG to estrogenic compound exposure during perinatal development. It is, however, reasonable to recognize that the age at death may be crucial in the evaluation of perinatal exposure to disruptors; other experiments performed in Mongolian gerbils that analyzed older animals after early exposure to environmental disruptors (Biancardi et al., 2017a; Castro et al., 2020) showed significant results. Furthermore, this species is known for its ability to develop spontaneous hyperplastic lesions (Gisele et al., 2008; Quintar et al., 2017).

5 | CONCLUSIONS

These analyses led us to conclude that the female prostate shows a different pattern of inflammatory response to perinatal exposure to the endocrine disruptor BPA, with an increase in the expression of P-STAT3 and the incidence of macrophages. Although an increase was observed in the incidence of hyperplasia in both steroid responsive glands in the BPA and E2 exposed groups, only the prostate showed differences in the incidence of stromal inflammatory cells. This does not mean that malignancy is not imminent in the MG, but it may represent a less responsive tissue in which BPA prevents an ideal immune response.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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