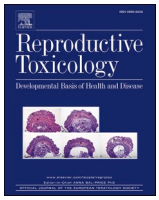
Reproductive Toxicology 137 (2025) 109013

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox



Late histopathological impacts on the female prostate of gerbils exposed to BPA during pregnancy and lactation

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ARTICLE INFO

Handling Editor: Anna K Price

Keywords:
Bisphenol
Dysregulation
Aging
Proliferation
Remodeling

ABSTRACT

The female prostate is regulated by steroid hormones, mainly androgens and estrogens. Exposure to exogenous chemical compounds leads to effects via endocrine pathways that alter prostate morphophysiology. Bisphenol A (BPA) is a widespread endocrine disruptor, which influences estrogenic pathways, facilitating pre-neoplastic and neoplastic alterations in hormone-sensitive organs. The aim of this study was to evaluate the influence of BPA on the prostate of aged female gerbils exposed to the compound during their gestational and lactational periods. The females were exposed to 50 μg/kg/daily during gestation and lactation, and the analysis was performed in the aged period (18-mo age). Our results showed that BPA increased epithelial alterations, observed by hyperplastic foci and a reduction in atrophies, commonly observed in aged female prostate. These data were supported by phopho-histone H3 immunostaining, that indicated proliferation of the epithelial and stromal compartments, leading to glandular hypertrophy. In addition, a pro-proliferative imbalance was observed through the differential expression of key proteins associated with cell death and proliferation, as well as in the expression of stromal components. Thus, the increase in cell proliferation implies the onset of epithelial alterations, as evidenced by the increased number of hyperplastic foci. The alterations reported after exposure to BPA demonstrated the increased likelihood of proliferative epithelial alterations, with tissue remodeling associated mainly with prostatic stroma.

1. Introduction

The female prostate is an exocrine gland, composed of an epithelium with columnar secretory and basal cells, surrounded by a fibromuscular stroma, and regulated by the action of steroid hormones [1,2]. When the female prostate was first described, it was called Skene's paraurethral gland [3]. The female prostate has already been described as a functional gland in several mammal species, which can be considered homologous to the ventral lobe of the male prostate and the human female prostate by functional and embryological homologies [3]. As a result, morphologic alterations during development of the female prostate are susceptible to hormonal signaling, as described for male prostate [4]. Steroid hormones, androgens and estrogens, control cell differentiation and proliferation, as well as the morphology of prostate cells [5], remodeling the compartments during reproductive cycles and lifetime.

With aging alters the functioning of the entire endocrine system, including the prostate, which generally involves a decrease in its function, leading to a decrease in tissue homeostasis and favoring the development of prostatic lesions [6]. Among several factors that cause this decline, hormonal alterations directly affect prostate homeostasis

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https://doi.org/10.1016/j.reprotox.2025.109013
Received 20 May 2025; Received in revised form 14 July 2025; Accepted 24 July 2025
Available online 25 July 2025

[7]. Androgenic hormones regulate cell fate, and changes in their concentration lead to alterations in the prostate tissue and cells, promoting pathological disorders [5,8]. On the other hand, estrogens act during puberty and cell differentiation during adulthood [9,10]. However, an 'aging' scenario, combined with other factors [11], such as hormone deprivation, can lead to the development of non-neoplastic morphological alterations in the prostate, such as atrophy or benign prostatic hyperplasia [12].

In addition, progression of pre- and malignant features on the female prostate have been related to exposure to chemicals and exogenous hormones. The endocrine disruptors are exogenous chemical compounds that alter the functioning of endocrine pathways, which can lead to morphophysiological alterations in the prostate [13]. These exogenous agents act by mimicking endogenous hormones, competing for the receptor binding site with these hormones, and inhibiting the action of these hormones or increasing their cellular response [14]. Bisphenol A (BPA) is one of the best-known endocrine disruptors, which acts mainly via estrogenic pathways, promoting pre-neoplastic and neoplastic alterations in hormone-responsive organs [13]. This substance is found in common materials, leading to exposure through food and drink [15,16]. Animals exposed to BPA early in life have shown increased morphological and functional alterations in the prostate. An increase in the development of prostatic lesions, such as hyperplasia and inflammatory foci, has also been observed when they reach with aging [17,18].

In the male prostate, it has been described that animals exposed to BPA in the perinatal period, show an increase in cell proliferation when reaching adulthood, in addition to a decrease in the amount of sperm, and consequently fertility, as well as other morphological and functional alterations [19,20]. The female prostate seems to be more sensitive to endocrine disruptors, as it undergoes changes through the estrogen pathways [21]. Exposure to BPA during development can also lead to an increase in cell proliferation in adulthood, which is seen in epithelial and stromal compartments [18]. This exposure can also promote an increase in the number of basal cells, which are associated with the incidence of invasive lesions [22]. Given that exposure to BPA induces alterations in the morphophysiology and developmental processes of the prostate, as previously delineated in aged males [23], it is suggested that similar exposure during other windows of susceptibilities for females may induce morphological changes in the female prostate. In studies performed with adult individuals, it was observed that exposure to BPA led to an increased propensity to prostatic alterations, such as hyperplasia, and inflammatory foci [17,18], which are associated with these morphological alterations, as well as causing changes in the expression of receptors related to hormonal regulation [24]. The literature contains some data related to exposure to BPA in females and its consequences [16,24–26], however, data on the effect of direct exposure to this compound during windows of susceptibility, such as pregnancy and lactation, are scarce, especially when related to aging. The windows of susceptibility are stages of development where the action of endocrine disruptors can have a greater impact, since hormonal alterations can represent an increased risk of developing alterations throughout life. Therefore, the aim of the current study was to analyze, through morphological and immunohistochemical analyses, the effects of exposure to BPA during the gestational and lactational windows of susceptibility on the female prostate of treated mother gerbils when they reach with aging.

2. Material and methods

2.1. Experimental design

Twenty-five female gerbils (Meriones unguiculatus) were kept until they reached reproductive age (around 3 months), when they were placed with males who were also of reproductive age. The animals were housed in polysulfone boxes, maintained at 25ºC and 40 –70 % relative humidity, on a 12 h light–dark cycle, with food and water ad libitum. The initial day of gestation was defined from the birth of the first generation, which was discarded. During the period of the second pregnancy (24–26 days), which begins 8 days after the birth of the first generation, and during lactation (21 days), treatment was performed by gavage. The mothers were divided randomly into three experimental groups with eight animals each: Control (submitted to 0.1 mL/day water gavage – n = 8), Vehicle-Oil (submitted to 0.1 mL/day corn oil gavage – n = 8), and Bisphenol A (BPA – submitted to 50 μg/kg/day diluted in 0.1 mL corn oil – n = 9). This dose has previously been described as the daily dose of BPA considered safe for humans [27–29], however, carcinogenic potential has already been observed for mammary gland [27]. The concentration of BPA used in this experiment was chosen in order to analyze the impact of a safe dose of this compound, administered during windows of endocrine sensitization, when these animals become aged. For the aged evaluation, the female prostates of the mothers exposed to the treatment were analyzed at 18 months (Fig. 1).

At the end of the experiment, at 18 months of age, the animals were anesthetized with isoflurane (3 % in O_2) and euthanized by decapitation. The collected prostates were fixed in 4 % paraformaldehyde (pH 7.2–0.1 M) for 24 h. After fixation, the female prostate was dehydrated in ethanol series, cleared in xylene, and embedded in paraffin utilizing a Leica (TP 1020) automatic processor and Leica (1150 H) for inclusion central. The blocks were sectioned using a Leica (RM 2255) automatic rotating microtome, 5 μm wide, for slide manufacture.

2.2. Stereological analysis

Stereological analysis of the histological sections stained with H&E was carried out to determine the relative frequency (%) of tissue compartments (epithelium, lumen, muscular stroma, and non-muscular stroma). For this, 10 fields were photographed in one section per animal, at 400x magnification, totaling 50 fields per group (n = 5), analyzed with a System Analyzer with Image-Pro Plus 6.0 software (VC Media Cybernetics, Inc., Bethesda, MD). For quantification, the relative frequency (%) was estimated using the M130 multipoint test system31.

2.3. Morphological analysis

Analysis of the multiplicity of epithelial prostatic alterations was also carried out, with the examination of 5 sections per animal (15 sections per experimental group). For histopathological analysis, Hematoxylin & Eosin (H&E) staining was used, as well as immunolabeling for α-SMA to identify budding. The analyses were performed using Olympus software, by digitizing the sections. The alterations were classified as described by Shappell et al., 2004 and Custodio et al., 2010 [30,31]. Prostatic alterations were classified as atrophy, budding development, planar and papillary atypia, prostatic hyperplasia, and *prostatic intraepithelial neoplasia* (PIN), the latter being classified as high (HGPIN) or low (LGPIN) grade.

Atrophy was characterized by the loss of cellular substances, which leads to a reduction in tissue, with a decrease in the amount of cytoplasm in the epithelium's secretory cells. This can occur in whole acinus epithelium or in isolated regions with only part of the epithelium altered. Epithelial hyperplasia was characterized by an increase in the number of cells, which leads to epithelial stratification and can affect both secretory cells and basal cells. This hyperplasia may, or not, be associated with cellular atypia. Planar and papillary atypia are based on alterations in the morphology of epithelial cells. The development of budding was characterized by the emergence of new acini (buds) from a pre-existing acinus, where there is a loss of muscle stroma in the region surrounding the acini. PINs, on the other hand, were characterized by exacerbated epithelial proliferation, which leads to stratification and growth of the epithelium towards the lumen. In PINs, the cell nucleolus becomes evident and may be associated with inflammatory cells. In addition, PINs are characterized by cellular and nuclear pleomorphism and loss of polarity. This type of alteration has been classified into two

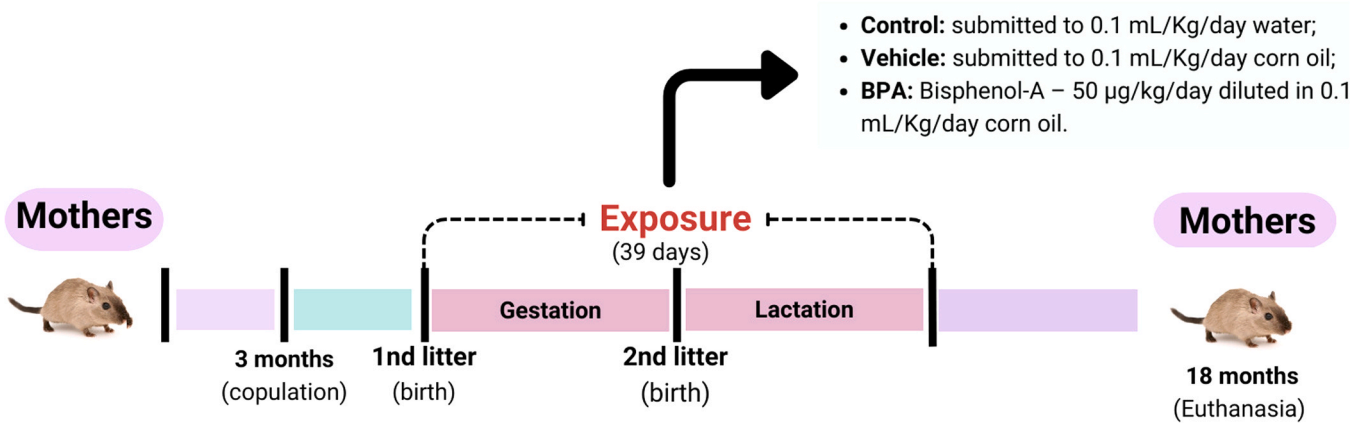

Fig. 1. Experimental Design.

types, according to grades: LGPIN and HGPIN. LGPINs have epithelial cells with enlarged nuclei and small, inconspicuous nucleoli, and this prominence of the nucleoli helps to distinguish them from HGPINs. In addition, HGPINs have a more complex architectural structure directed towards the lumen. HGPINs were characterized by the presence of large nuclei, relatively uniform in size, with prominent nucleoli. The multiplicity of these alterations was quantified, and the results are expressed as minimum-maximum (mean) for each experimental group.

Picrosirius red and Gomori Reticulin stains were used to assess the composition of the extracellular matrix (ECM), highlighting collagen fibers and reticular fibers, respectively. The multi-point technique was used to quantify the results. The relative frequency (%) was estimated using the M130 multi-point test system. Collagen fibers were quantified with and without the use of polarized light (picrosirius-P).

2.4. Immunohistochemical assays

For immunostaining, the following protocol was used: deparaffinization and hydration of the sections with baths in xylene (3 ×20 min), decreased ethanol baths (100 % - 3 ×5 min; 90 %, 80 %, 70 %, and 50 % - 1 ×5 min for each), and then rehydration (1 ×1 min); antigenic recovery in 10 mM citrate buffer at 95°C or 98°C for 30–60 min; the endogenous peroxidase was blocked with 10 % H_2O_2 in methanol (2 ×20 min); inhibition of non-specific proteins interactions were blocked by incubating the sections with 5 % skimmed milk in wash buffer for 30–60 min; the slides were then incubated with the primary antibody diluted in 1 % BSA overnight at 4ºC. The following antibodies were used for immunohistochemical analysis: phospho-histone H3 for cell proliferation (PHH3; rabbit, dilution 1:100, sc-56739, Santa Cruz Biotechnology), smooth muscle α-SMA (mouse monoclonal, dilution 1:75, sc-32251, Santa Cruz Biotechnology), p63 protein for basal cells (mouse monoclonal, dilution 1:75, sc-25268, Santa Cruz Biotechnology), activated caspase-3 for cell death (rabbit, dilution 1:75, NBP1–90125, Novus Biologicals), laminin (mouse monoclonal, dilution 1:50, NB600–883, Novus Biologicals), collagen IV (mouse monoclonal, dilution 1:50, sc-59814, Santa Cruz Biotechnology), tissue inhibitor of metalloproteinase 1 (TIMP-1, rabbit, dilution 1:50, D10E6, Cell Signaling Technology), and metalloproteases for tissue remodeling - MMP2 (mouse monoclonal, dilution 1:50, sc-13595, Santa Cruz Biotechnology), MMP3 (rabbit, dilution 1:50, D7F5B, Cell Signaling Technology), MMP9 (mouse monoclonal, dilution 1:50, sc-21733, Santa Cruz Biotechnology), and MMP13 (mouse monoclonal, dilution 1:100, sc-515284, Cell Signaling Technology). In negative controls, the incubation step in the primary antibody is replaced by incubation in 1 % BSA. After the primary antibody incubation, the secondary antibody was added to the samples and incubated for 45–60 min for polymer binding (polymer detection system Novolink™ (Leica Biosystems Newcastle 118 Ltd.; Newcastle, United Kingdom). Each step was interposed by washing with a buffer, PBS (pH 7.4–7.6) or TBS (pH 7.6–8). The protein-antibody link was stained with DAB (3'-3' diaminobenzidine; Novolink™ DAB, Leica Biosystems; Buffalo 121 Grove, USA), and counterstained with hematoxylin, with the exception of the immunostaining for collagen IV and laminin, which were not counterstained. The slides were then dehydrated and mounted with Canada Balsam.

To quantify the cells immunoreactive for PHH3, p63, TIMP-1, and MMPs (-2, −3, −9 and −13), positive and negative cells were counted in the epithelial and stromal compartments. The quantification was performed by selecting 10 random fields per animal (400X magnification) and calculated based on the number of positive cells divided by the total number of cells, presented as a percentage (%) of positive cells, using QuPath software. The other markers (activated caspase- 3, α-SMA, collagen IV, and laminin) were quantified using the Image-Pro ® Plus (v. 6.0) multi-field counting technique.

2.5. Immunofluorescence (IF)

To perform the immunofluorescence technique, the following antibodies were used: smooth muscle α-SMA (mouse monoclonal, dilution 1:75, Santa Cruz Biotechnology), phospho-histone H3 (PHH3; rabbit, dilution 1:100, Santa Cruz Biotechnology), and p63 protein (mouse monoclonal, dilution 1:75, Santa Cruz Biotechnology). This technique was employed to analyze the co-localization of PHH3 with p63, and PHH3 with α-SMA, using a similar protocol to IHC: deparaffinization and hydration of the sections with baths in xylene, alcohol, and water; antigenic recovery in 10 mM citrate buffer at 98°C for 60 min; inhibition of non- specific protein interactions were blocked by incubating the sections with 5 % skimmed milk in PBS or TBS for 60 min; the slides were then incubated with a mix of both antibodies (1:50 each) in BSA left overnight; on the following day a mix of BSA 1 % and Texas Red (TR; 1:50; Santa Cruz Biotechnology, USA; host: mouse) was added to the slides followed by the buffer wash and a mix of BSA 1 % and Fluorescein Isothiocyanate (FITC; 1:50, Santa Cruz Biotechnology, USA; host: rabbit); The slides were then washed and assembled with 6-diamidino-2-phenylindole (DAPI; Fluoroshield™ DAPI, histology mounting medium; Merck, Darmstadt, Germany), to highlight the nuclei. Peroxidase blocking was not performed. The slides were analyzed using a Zeiss AX10 fluorescence microscope (Zeiss, Oberkochen, Germany) and AxioVision Software (Zeiss). For quantification, counts were made using the multiple point technique with Image-Pro ® Plus (v. 6.0).

2.6. Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5.0 software (GraphPad Software, Inc., CA, USA), and the results were subjected

to the Shapiro-Wilk normality test, to choose the appropriate statistical test. Data with normal values were submitted to the parametric ANOVA test, followed by the Tukey test (post hoc) and, if not, the non-parametric Kruskal-Wallis test was used, followed by the Dunn test (post hoc). Data are expressed as mean ± standard deviation and $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Stereological and histopathological analysis

Analysis of the morphology of the acini (Table 1 and Fig. 2) demonstrated an increase in papillary atypia in the BPA group compared to the vehicle group (Fig. 2 – A, B). Similar results were observed for HGPIN (Fig. 2 – E) and hyperplasia (Figure F, G), where there was an increase in the BPA compared to the other groups. For the budding development, BPA increased the incidence in the female prostate compared to the control and vehicle groups (Fig. 2 - H). Atrophy was only increased in the control group, which was the main feature observed compared to the other groups (Fig. 2 - I).

The relative frequency of the histological compartments was analyzed (Fig. 2 – J-M). Multi-point analysis of the compartments showed a significant increase in epithelium in the vehicle group compared to control and BPA groups (Fig. 2 - J). This analysis also demonstrated a significant increase in the muscular stroma in the BPA group (Fig. 2 - K), as well as the non-muscular stroma, when compared to the other groups (Fig. 2 - L).

3.2. Immunohistochemical analysis

Immunostaining for PHH3 showed an increase in cell proliferation in the BPA group, compared to the control group (Fig. 3 – A, B). For the stromal compartment, the expression of PHH3 was significantly increased in BPA-exposed females compared to the control group (Fig. 3 – A, C). In the analysis for activated caspase 3, the BPA group presented an increase in the epithelium and stroma compared to the control group (Fig. 3 – D-F). The p63 protein, a basal cell marker, showed an increase in the number of basal cells in the BPA group, compared to the values observed in the control and vehicle groups (Fig. 3 – G, H). The smooth muscle cells present in the prostatic muscular stroma are marked by α-SMA, which means that its expression may indicate muscular stroma. Using this analysis, an increase in the muscular layer of prostate tissue was observed in the BPA group, compared to the other groups (Fig. 3 – I, J).

To assess the occurrence of proliferation of each main cell in the different compartments (epithelial and muscular stroma), a double immunofluorescence staining was performed for PHH3 and p63 (basal cells) or α-SMA (smooth muscle cells) (Fig. 4 – A-F). An increase in the proliferation of basal cells was observed in the BPA group compared to the other groups (Fig. 4 – D-F). Regarding proliferation of muscular stroma (Fig. 4 – A-C), the BPA-exposed female prostate presented an increase compared to the other groups, which corroborates the occurrence of cell proliferation in the muscle stroma.

Table 1
Histopathological Analysis. Multiplicity of prostatic alterations, evaluated with minimum-maximum (average) value. Letters indicate significant difference. *P < 0,05.

Prostatic Epithelial Alterations	CONTROL	VEHICLE	BPA
Papillary Atypia	0–7 (3.15)[a,b]	1–4 (2.33)[b]	1–7 (4.51)[a]
Planar Atypia	0–4 (1.4)	0–4 (2.79)	0–5 (2.88)
Hyperplasia	0–10 (2.91)[b]	1–5 (2.4)[b]	2–9 (4.77)[a]
LGPIN	0–2 (1.25)	0–2 (1.27)	0–3 (1.72)
HGPIN	0 (0)[b]	0 (0)[b]	0–2 (0.66)[a]
Budding	1–19 (5.76)[b]	1–7 (2.8)[c]	1–14 (8.1)[a]
Atrophy	2–17 (11.8)[a]	2–7 (3.46)[c]	2–16 (7)[b]
Microacinus	0–7 (2,4)	0–7 (2,1)	0–5 (2)
De Brie	0–7 (0,7)	0–2 (0,5)	0–5 (1)

3.3. Extracellular matrix (ECM)

The composition of the extracellular matrix (ECM) was analyzed considering laminin and collagen IV (Fig. 5) and collagen content type I and type III were evaluated by histochemical procedures (Fig. 5). Laminin immunostaining was increased in the BPA-treated group compared to the other groups (Fig. 5 – A, B). In addition, collagen type IV presented an increase in relative frequency in the female prostate exposed to BPA (Fig. 5 – C, D) in comparison to the other groups. For collagen type I, analysis of picrosirius red stain showed an increase in the BPA group – with (Fig. 5 – E, F) and without polarized light (Fig. 5 – G, H), as well as an increase in collagen type III analyzed by Gomori Reticulin staining in the same group (Fig. 5 – I, J).

The remodeling of ECM was analyzed by the expression of MMPs (-2, −3, −9, and −13) and TIMP by immunohistochemistry (Fig. 6). In relation to MMP2, there was an increase in the number of positive cells in the epithelium in the BPA group compared to the other groups (Fig. 6 – A-C). Similarly, increases in MMPs −3, −9, and −13 were found in epithelium and stroma in the treated groups (Fig. 6 – D-L). The opposite was observed for TIMP-1, in which there was a decrease in the BPA group compared to the control group (Fig. 6 – M, N).

4. Discussion

The present study demonstrated that BPA exposure during windows of pregnancy and lactation affects homeostasis of tissue compartments in the female prostate. Histopathological analysis indicated an increase in prostatic alterations, associated with the expression of the proliferative marker PHH3. Hyperplasia, HGPIN, and atypia were the main proliferative alterations found in BPA-exposed females. In contrast, an increase in the atrophic foci was observed in the control group. As described in previous studies, during aging the occurrence of atrophies in the epithelium is a common feature [10,32], due to unbalance of the proliferation-death ratio, to ensure a lower risk of developing hyperplasia and other proliferative alterations [31]. As already mentioned, atrophy is a common process with aging; however, the association with high rates of apoptosis can indicate that the prostate epithelium is not proliferating properly.

The expression of PHH3 and caspase 3 increased in BPA exposed female prostate. However, the expression of PHH3 is remarkable in the samples, indicating proliferative activity of epithelial cells and muscle stromal hypertrophy. The association between increases in the expression of PHH3 and the cell markers alpha-SMA and p63 indicates proliferative activity of both compartments. P63 is a basal cell marker and plays an important role in the development and proliferative activity of epithelium [33], essential for maintenance and integrity [34]. Exposure to BPA is related to modulation of the basal cell population, and these cells are commonly related to the appearance of proliferative *in situ* lesions [21]. Thus, with PHH3, an increase in p63 expression indicates a tendency towards stratification of the prostate epithelium [34], being a poor prognosis.

The increase in glandular epithelium could also be stimulated for the stromal compartment. Due to the epithelial-stromal interaction [35], the control of development and functioning require paracrine signaling. The increase in muscular stroma indicated by the association of increased ∝-SMA and PHH3 markers, could promote changes in prostatic epithelium, as described in previous studies [35]. Thus, BPA modulated the glandular hypertrophic process by enhancing both compartments. In contrast, although the activity of caspase 3, an apoptotic marker, also increased, it represented < 25 % of cells in the stromal compartment, compared to proliferative activity demonstrated by PHH3-positive cells (>50 % of cells). It has already been described that BPA acts mainly through alterations in estrogenic pathways, which leads to tissue

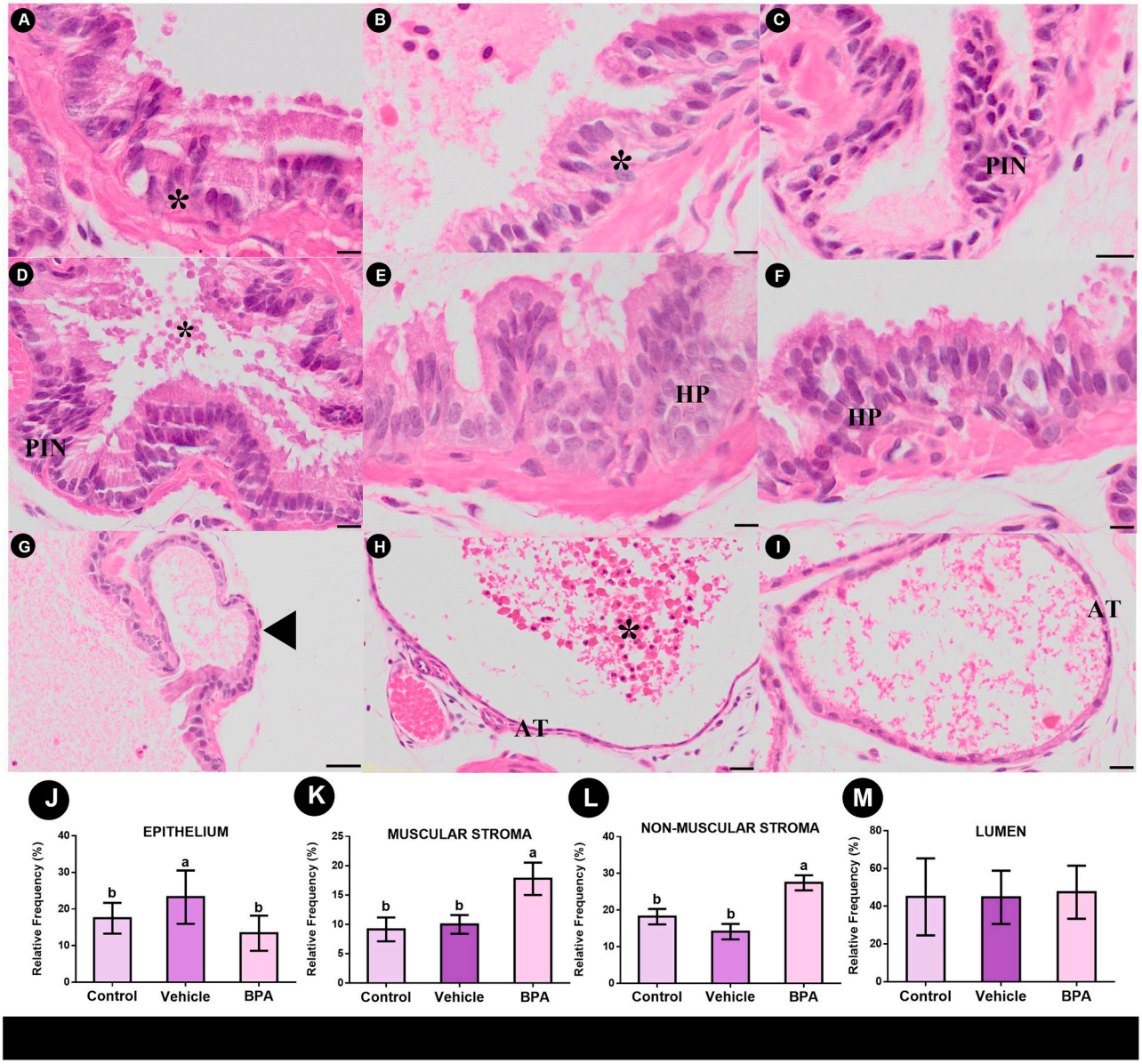

Fig. 2. Prostatic Epithelial Alterations and Stereological Analysis. **A-B.** Papillary atypia in the control group, indicated by the asterisk. **C-D.** LGPIN in BPA group; inflammatory cells are indicated by the asterisk. **E-F.** Hyperplasia in the BPA group. G. Budding development in vehicle group, indicated by the black arrowhead. **H-I.** Atrophic acini in BPA and control groups, inflammatory cells are indicated by the asterisk. **J.** Incidence of the epithelial compartment in the prostate of the different groups; **K.** Incidence of the muscular stromal compartment in the prostate of the different groups; **L.** Incidence of the non-muscular stromal compartment in the prostate of the different groups. **M.** Incidence of the lumen compartment in the prostate of the different groups. **HP:** Hyperplasia; **AT:** Atrophy. **Scale bars: A-F.** 20 μm, **G-H.** 50 μm. *P < 0,05.

proliferation in aging, especially in the female prostate [20].

Considering the cellular elements of stroma, the analyses related to ECM also indicated alterations after BPA exposure. The ECM mainly includes proteoglycans and fibrous proteins such as collagen and elastin, as well as laminin [36]. Collagen is the most abundant protein in the ECM, especially in the basement membrane, providing mechanical resistance and regulating cell adhesion [37], and it is also found in smooth muscles and around blood vessels. In prostate carcinoma [38], collagen synthesis increases, with type IV collagen degrading in advanced stages. Laminin, a glycoprotein in the ECM, is crucial for cell differentiation and maintaining cell adhesion in the epithelium [39]. Increased laminin expression could indicate increased cell motility, with a tendency for epithelial cells to invade the prostatic stroma [39]. Thus, the increase in all the markers related to ECM fibers (collagen fibers, especially types I and IV, reticular fibers, and laminin) reinforces the idea that BPA acted by altering the ECM, and these fibers have already been described as being associated with the development of adenocarcinomas [40].

Matrix metalloproteinases (MMPs) are a family of endopeptidases dependent on zinc (Zn^{2+}) or calcium (Ca^{2+}). They participate in the process of degrading extracellular matrix proteins and are responsible for initiating the process of tissue remodeling. The components degraded by MMPs include type I and IV collagens, elastin, and laminin [41,42]. As these are fundamental components for tissue remodeling, altering the homeostasis of MMP activity can lead to conditions that are harmful to prostate morphophysiology [43]. These components have already been described as important extracellular matrix components for the invasive capacity of cells, tumor growth, and the development of prostate cancer [44].

Among the MMPs, MMP2 is one of the most commonly associated

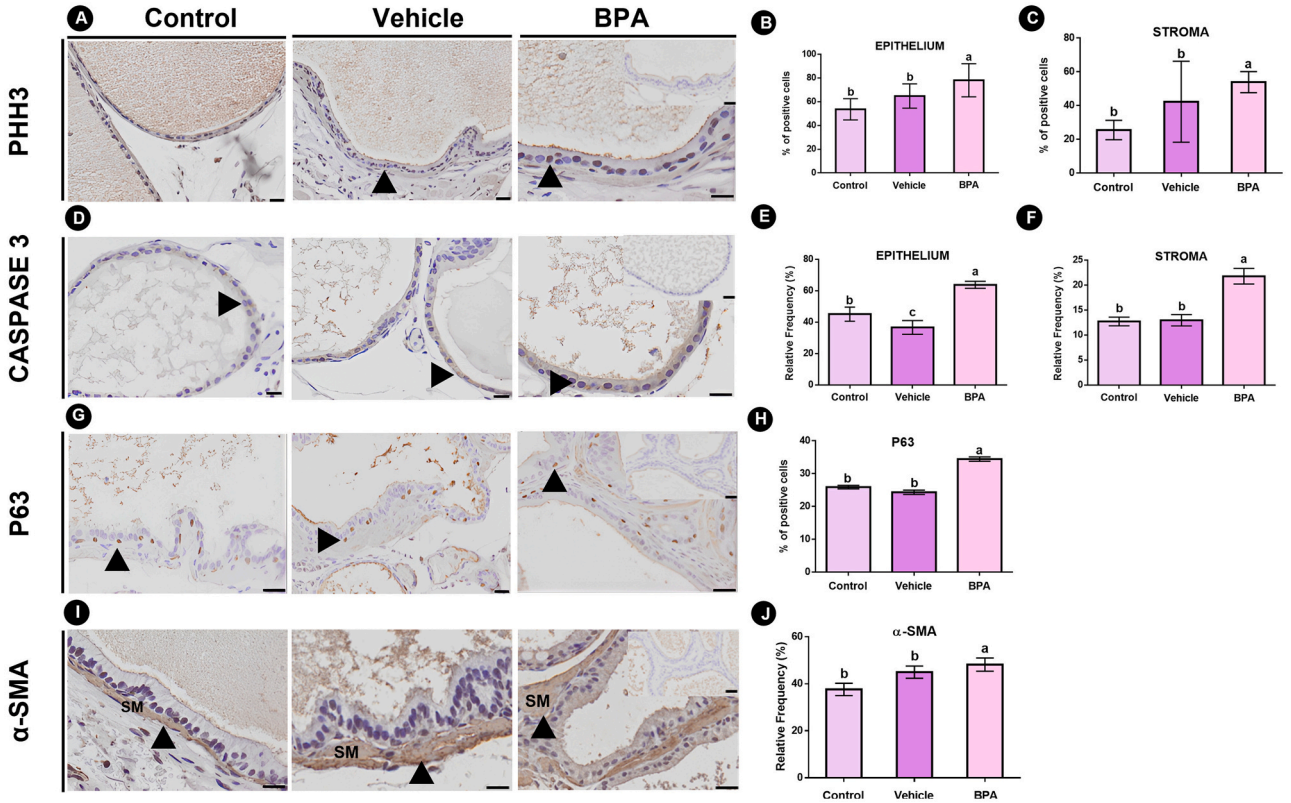

Fig. 3. Proliferation and Cell Death. **A-C.** PHH3 immunostaining, where we see positive cells (black arrows) and graphics comparing the groups, with letters indicating statistically significant differences; **D-F.** activated caspase 3 immunostaining, showing positive cells (black arrowheads) and graphics comparing the groups, with letters indicating statistically significant differences; **G-H.** p63 immunostaining, showing positive cells (black arrowheads) and graphics comparing the groups, with letters indicating statistically significant differences; **I-J.** α-SMA immunostaining, showing positive cells (black arrowheads) and graphics comparing the groups, with letters indicating statistically significant differences; **Scale bars: A.** 50 μm, 20 μm, 50 μm; **D.** 20 μm; **G.** 50 μm; **I.** 50 μm. **Inset:** negative control. *P < 0,05.

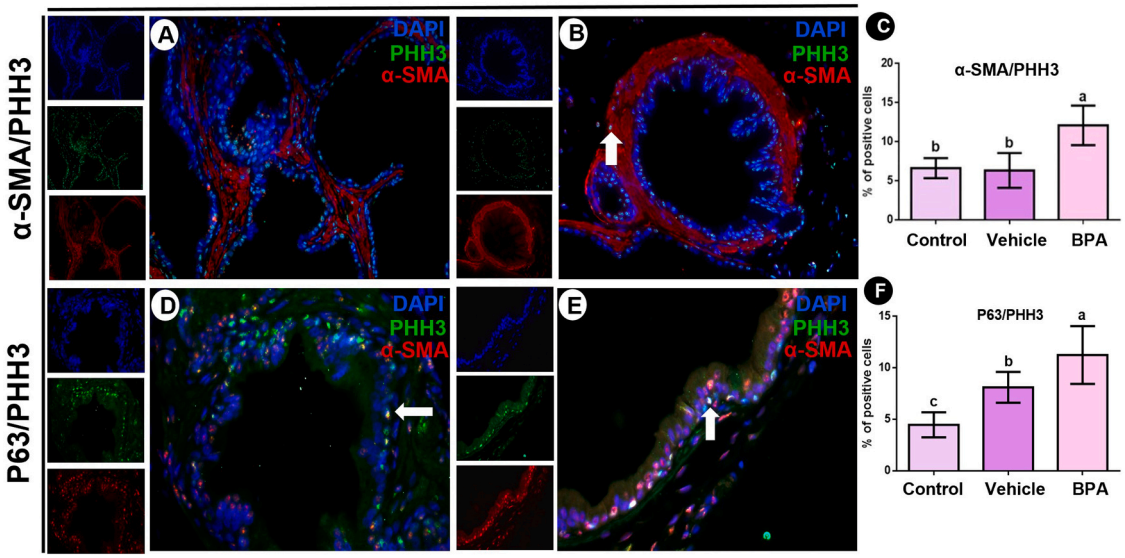

Fig. 4. Immunofluorescence results. **A.** Double-labeled immunofluorescence for α-SMA and PHH3 in the BPA group, with separate images and the overlay; **B.** Double-labeled immunofluorescence for α-SMA and PHH3 in the BPA group, with separate images and the overlay, showing the double labeling (white arrows); **C.** Graph for α-SMA and PHH3, comparing the groups, with letters indicating statistically significant differences; **D.** Double-labeled immunofluorescence for p63 and PHH3 in the BPA group, with separate images and the overlay, showing the double labeling (white arrows); **E.** Double-labeled immunofluorescence for p63 and PHH3 in the control group, with separate images and the overlay, showing the double labeling (white arrows); **F.** Graph for p63 and PHH3, comparing the groups, with letters indicating statistically significant differences. **Scale bars: A.** 100 μm; **B.** 20 μm; **D.** 20 μm; **E.** 20 μm. *P < 0,05.

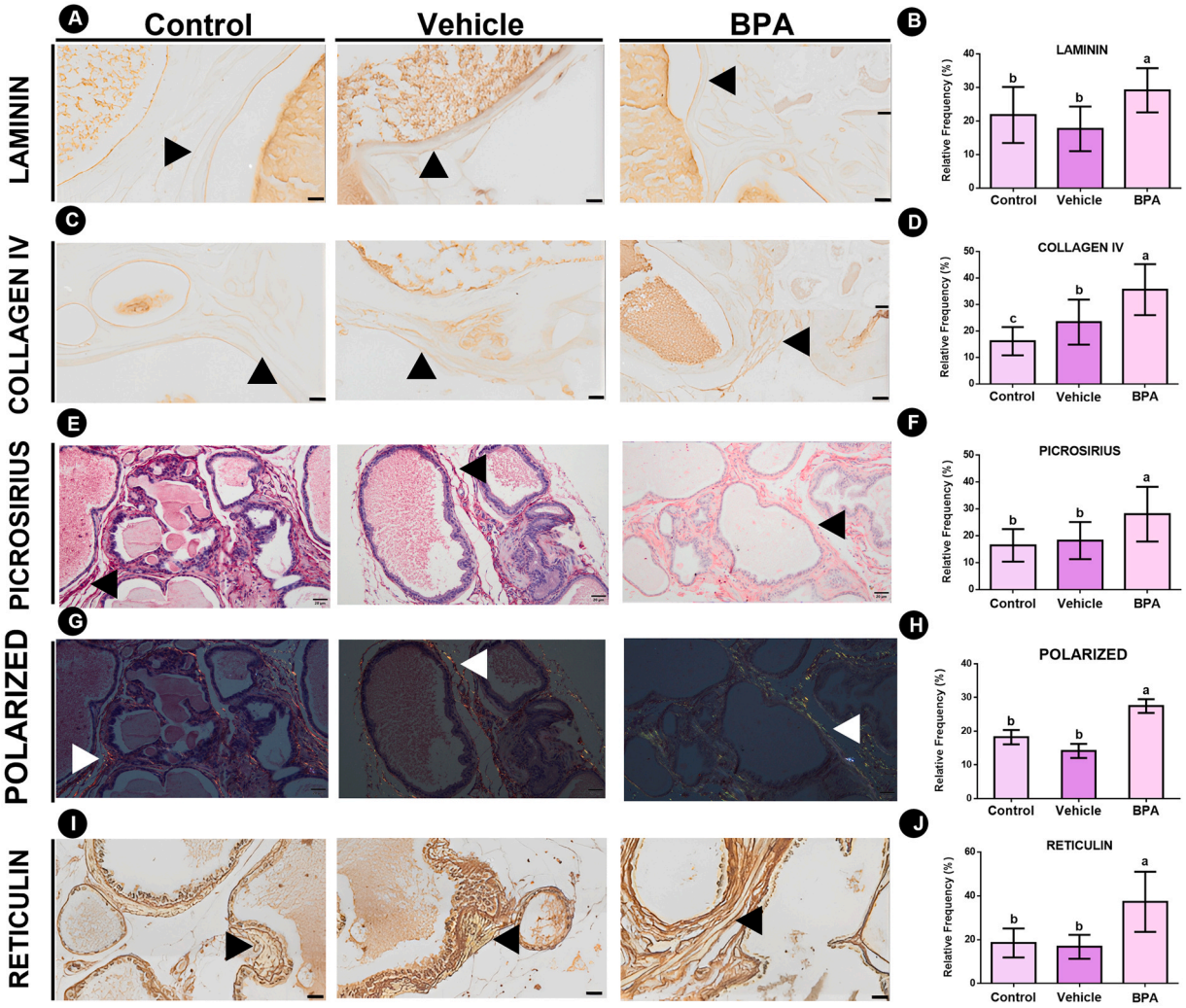

Fig. 5. Immunostaining for ECM components. **A-B.** laminin immunostaining, showing the marked fibers (black arrowheads) and graphics comparing the groups, with letters indicating statistically significant differences; **C-D.** collagen IV immunostaining, showing the marked fibers (black arrowheads) and graphics comparing the groups, with letters indicating statistically significant differences; **E-F.** Picrosirius staining showing the marked collagen fibers (black arrowheads) and graphics comparing the groups, with letters indicating statistically significant differences; **G-H.** Picrosirius staining in polarized light microscopy, showing the marked collagen fibers (white arrowheads) and graphics comparing the groups, with letters indicating statistically significant differences; **I-J.** Gomori Reticulin staining, showing the marked reticular fibers (black arrowheads) and graphics comparing the groups, with letters indicating statistically significant differences; **Scale bars: A.** 50 μm; **C.** 50 μm; **E.** 50 μm; **G.** 50 μm. Letters indicate statistically significant differences. **Inset:** Picrosirius red in polarized light and negative control. **Picrosirius-P:** Picrosirius staining in polarized light. *P < 0,05.

with the development of prostate cancer, mainly due to its action on cell growth and the production of cell junction proteins, as well as favoring cell invasion [45,46]. MMP2, in general, is responsible to cleave collagens type I, IV, and V, as well as elastin [47]. In addition, according to Nagase and collaborators (1998) [48], MMP2 can degrade the basal membrane. The increase in MMP2, as observed in the groups exposed to BPA, has already been described as being associated with cell growth and increased cell migration rates. In addition, MMP3 (degrades collagen types I and IV, fibronectin, and laminin) can generate different responses depending on the context: in the breast, it has a pro-tumorigenic action, for example [49], and the same effect has been described for the prostate, with MMP3 contributing to growth [50,51] and promoting invasion and migration [52], while in the development of skin cancer, it has the opposite response [53]. MMP9, on the other hand, is involved in cancer migration, invasion, and metastasis, as already described in prostate cancer [54]. MMP9 basically degrades the same elements as MMP2, except for type I collagen [47]. MMP9 is also fundamental in the female reproductive cycle since it is associated with the remodeling of endometrial tissue [55]. Unlike MMP2, which is mainly inhibited by the action of TIMP2, MMP9 is more affected by the action of TIMP1 [56]. Both are associated with increased aggressiveness and cancer survival [57]. The same is seen for MMP13, which is used as an indicator of metastasis in prostate cancer, since its expression increases in individuals with more advanced stages of this disease [58]. MMP13 is mainly responsible for cleaving collagen type II [59], and its activity is inhibited by TIMPs −1, −2, and −3 [60]. Its high expression has already been described as associated with the development and aggressiveness of tumors, as in the case of breast tumors [61,62].

TIMP1, as the name implies, has the function of inhibiting the action of metalloproteinases, which cause tissue remodeling from the degradation of the ECM, and is therefore associated with tumor invasiveness[65]. As previously mentioned, the composition of the ECM has a major influence on the development of tumors, such as prostate tumors [63]. TIMP1 has already been described as a regulator of the process of metastasis development in the prostate, not directly leading to its development, but due to its role in regulating MMPs [64,65]. The decrease in the presence of TIMP1 in the prostate, suggests a trend towards positive regulation of the action of MMPs, which is corroborated

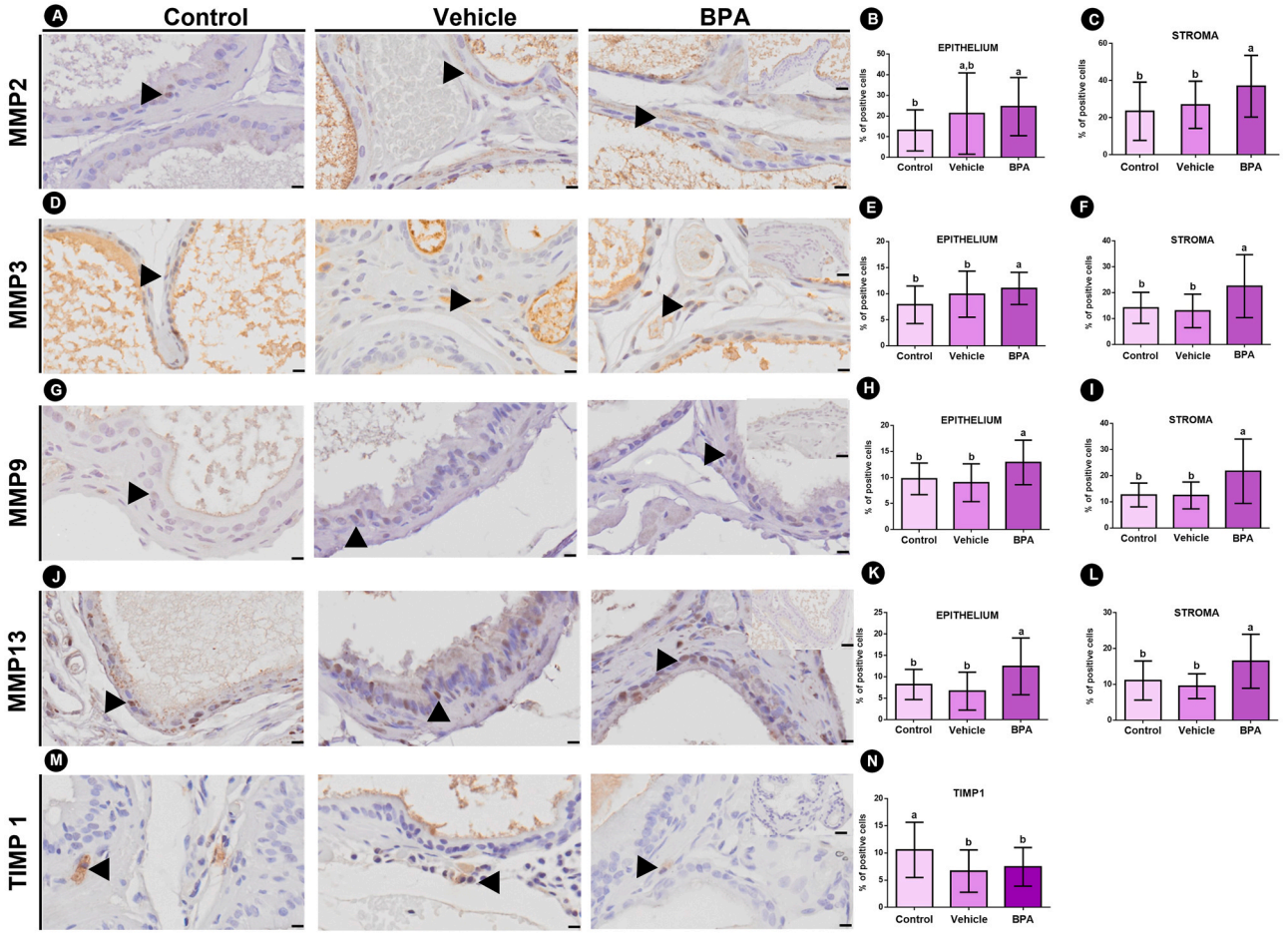

Fig. 6. Immunostaining for tissue remodeling markers. **A-C.** MMP2 immunostaining, showing positive cells (black arrowheads) and graphics comparing the groups, with letters indicating statistically significant differences; **D-F.** MMP3 immunostaining, showing positive cells (black arrowheads) and graphics comparing the groups, with letters indicating statistically significant differences; **G-I.** MMP9 immunostaining, showing positive cells (black arrowheads) and graphics comparing the groups, with letters indicating statistically significant differences; **J-L.** MMP13 immunostaining, showing positive cells (black arrowheads) and graphics comparing the groups, with letters indicating statistically significant differences; **M-N.** TIMP1 immunostaining, showing positive cells (black arrowheads) and graphics comparing the groups, with letters indicating statistically significant differences; **Scale bars: A.** 50 μm; **D.** 50 μm; **G.** 50 μm; **I.** 50 μm; **M.** 50 μm. Letters indicate statistically significant differences. **Insert:** negative control. *P < 0,05.

by the increase in the marking of MMPs −2, −3, −9, and −13 in the prostate of the treated animals. The increased presence of MMPs, in addition to the effects already mentioned, could lead to increased tumor migration [59].

5. Conclusion

In short, we concluded that exposure to BPA modulated the proliferation of the epithelial and stromal compartments in the female prostate, leading to glandular hypertrophy. This proliferation is highlighted by the increase in the multiplicity of prostatic lesions, as well as involving remodeling of the prostatic tissue when the individual reaches aging. The same remodeling process was observed in the stroma, where we found tissue remodeling associated with an increase in stromal fibers and extracellular matrix constituents. Thus, it was shown that exposure to BPA favors the development of proliferative prostatic alterations, with pro-proliferative modulation, involving remodeling of prostatic tissue. For future studies, it would be relevant to investigate the mechanisms of action of BPA, investigating the specific signaling pathways modulated by its action.

CRediT authorship contribution statement

Lorena Gabriela de Souza: Writing – original draft, Methodology, Investigation, Formal analysis. **Simone Jacovaci Colleta:** Methodology, Formal analysis. **Ellen Cristina Rivas Leonel:** Visualization, Methodology, Formal analysis. **Taboga Sebastião:** Writing – original draft, Visualization, Validation, Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Thalles Fernando Rocha Ruiz:** Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Brito-Filho Gervásio Evangelista:** Methodology, Investigation, Formal analysis. **Luara Jesus Ferrato:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis.

Ethics approval and consent to participate

The current study received Ethic Committee in Animal Utilization (CEUA) homologation, under protocol nº 217/2019.

Competing interests

The authors declare none.

Declaration of Competing Interest

TThe 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.

Acknowledgements

This work was financed by FAPESP (São Paulo Research Foundation) - Grant number: 2022/14750–0; PROPe-Unesp (São Paulo State University) - Notice number: 270538–39; CNPq (National Council for Scientific and Technological Development) - Contract number: 302938/2020–6 for SRT and 403028/2023–0. The procedures were approved by the Ethics Committee for the Use of Animals (CEUA- IBILCE/UNESP, process no 217/2019).

Data Availability

Data will be made available on request.

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