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RESEARCH ARTICLE

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Prenatal and pubertal exposure to 17α-ethinylestradiol cause morphological changes in the prostate of old gerbils

Fernanda G. Fleury[1] | Luísa R. F. Guimarães[1] | Elisa B. Rezende[1] | Tracy M. M. Martins[1] | Cássia R. S. Caires[2] | Fernanda C. A. dos Santos[3] | Sebastião R. Taboga[4,5] | Ana P. da S. Perez[6]

[1]Institute of Health Sciences, Medicine Course, Federal University of Jataí, UFJ, Jataí, Brazil

[2]Graduate Program in Health Sciences, Faculty of Medicine of São José do Rio Preto-FAMERP, São Paulo, Brazil

[3]Department of Histology, Embryology and Cell Biology, Federal University of Goiás, Samambaia II, Goiânia, Brazil

[4]Laboratory of Microscopy and Microanalysis, Department of Biology, São Paulo State University-UNESP, São José do Rio Preto, Brazil

[5]Department of Structural and Functional Biology, State University of Campinas-UNICAMP, Campinas, Brazil

[6]Medicine Course and Graduate Program of Animal Bioscience, Institute of Health Sciences, Federal University of Jataí, UFJ, Jataí, Brazil

Correspondence

Ana P. da S. Perez, Institute of Health Sciences, Medicine Course, Federal University of Jataí-UFJ, Jataí, Goiás. BR 364, Km 194, number 3800, Class of Teachers II, number 26, Jatobá campus-University City, Jataí, Goiás, 75801-615, Brazil.
Email: paulabio_perez@ufj.edu.br

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Abstract

This study evaluated such as exposure to ethinylestradiol during the prenatal (18th–22nd day) and pubertal (42nd–49th day) periods acts on the male ventral prostate and female prostate of 12-month old gerbils. We performed the analysis to serum hormone levels for estradiol and testosterone. The prostates were submitted to morphometric and immunohistochemical analyses. Exposure to ethinylestradiol during these developmental periods decreased the testosterone serum levels in males and increased the estradiol serum levels in females. Morphologically, prostate intraepithelial neoplasia and disorders in the arrangement of the fibrous components were observed in the prostate glands of both sexes of gerbil exposed to ethinylestradiol during development periods. In the male prostate, the ethinylestradiol promoted decreased in the frequency of positive epithelial cell for androgen receptor (AR) and increased the frequency of positive stromal cell for estrogen receptor α. However, in the female prostate, this synthetic estrogen caused AR upregulation and increased cell proliferation. This study shows that the exposure to ethinylestradiol during development phases alters the morphology and the hormonal signaling in the male and female prostates of old gerbils, confirming the action of ethinylestradiol as endocrine disruptor.

KEYWORDS

development, female, male, oral contraceptive, prostate

Abbreviations: AGD, anogenital distance; AR, androgen receptor; BSA, bovine serum albumin; DHT, dihydrotestosterone; EE, ethinylestradiol; EE PRE/PUB, female and male exposed to ethinylestradiol during the prenatal and pubertal period; EGF, epidermal growth factor; ERβ, estrogen receptor β; ERα, estrogen receptor α; FGF, fibroblast growth factor; HE, hematoxylin and eosin; IGF, insulin type growth factor; KGF, keratinocyte growth factor; p63, basal cell antigen; PBS, phosphate buffer saline; PCNA, proliferating cell nuclear antigen; PIN, prostatic intraepithelial neoplasia.

1 | INTRODUCTION

Endocrine disruptors are substances that, through several kinds of mechanisms, can cause modifications of hormonal physiology, affecting homeostasis (Schug et al., 2011). Ethinylestradiol (EE) is an exogenous estrogen that is found on most of oral contraceptives that are found in the market, and it is labeled as an endocrine disruptor.

Women who use contraceptives pills inappropriately can become pregnant and inadvertently continue to use the medication. This prenatal exposition is capable of causing several side effects in the reproductive system and is related with an increase of cancer incidence during adulthood. The pills contraceptives contain 20–50 μg of EE, corresponding to dose 0.2–0.7 μg/kg/day (Heneghan et al., 2019). In our study, the dose (15 μg/kg/day) this synthetic estrogen were 20–80 higher than exposure of women taking pills contraceptives. Yet studies have shown that changes can be caused by this endocrine disruptor mechanism while using inferior doses. In our study, we used a higher concentration with the goal to increase our chances of detecting the reproductive effects caused by this exposition (Mandrup et al., 2013).

Moreover, the synthetic estrogen, EE, is more persistent in the environment than natural estrogens. When women excrete the EE and release in the environment, it pollutant levels have been correlated with cancer susceptibility, as prostate cancer in male and breast cancer in women (Adeel et al., 2017).

Studies in Mongolian gerbils (*Meriones unguiculatus*) have shown that exposure to EE is also capable of affecting both male and female prostate development, predisposing the organ to morphological changes in later phases (Falleiros-Júnior et al., 2016; Perez et al., 2011, 2012, 2016). These changes can be accentuated when this exposure occurs during puberty, a period characterized by the morphofunctionality of the prostatic acinar (Sanches et al., 2016). During puberty the prostate is subject to high levels of sex hormones and might trigger potentially malignant lesions during aging (Perez et al., 2017).

As previously mentioned, the studies cited compared the effects of EE on the male and female prostates (Perez et al., 2011, 2012). It is known that the prostate gland is not exclusive to the male genital system; it is also known as Skene's gland and is found in several of mammalian species, including humans and rodents (Biancardi et al., 2017; dos Santos et al., 2003; Moalem & Reidenberg, 2009; Zaviacic & Ablin, 2000). The female prostate of the gerbil is histologically very similar to that of humans, being composed of ducts and acini coated by a columnar or cubic epithelium, as well as a fibromuscular stroma composed of fibroblasts, smooth muscle cells, nerves, and blood vessels (Biancardi et al., 2017; dos Santos et al., 2003; Santos & Taboga, 2006). The similar morphology between male and female prostates are possible indicators that the female prostate can also be submitted to pathological process such as adenocarcinoma, and prostatitis, although these have a lower prevalence (Sanches et al., 2019).

The gerbil is an ideal model animal for this kind of study not only for ther morphological similarity to the human prostate, but also it can be submitted to the same types of pathologies. Being, their prostate more suitable for our study. Furthermore, research that utilized a female human prostate are difficult to conduct and are usually *postmortem* samples. Unlike them, gerbil prostate is easier to work with in a laboratory environment (Sanches et al., 2019).

In rodents, prostatic development is initiated in the prenatal period, and the branching and glandular maturation, respectively, occur during the neonatal and pubertal periods (Prins & Putz, 2008; Sanches et al., 2016). In male gerbils, this development starts between the 20th and 21st day of the gestational period (Sanches et al., 2014) whereas in humans, the mains events of glandular development occur during the prenatal phase (Cunha et al., 2018).

Knowing that in the prenatal and pubertal periods occur crucial events for the prostatic development, these phases are considered critical windows for the exposure to EE. Previous studies from our research group have shown that exposure to EE during different phases of development, such as the prenatal period or puberty, promoted histopathologic alterations in the prostate of male and female gerbils. However, we do not know how EE acts on the morphological pattern of these glands when the exposure occurs at both development phases. This exposure will promote effects similar or different on the prostate tissue of senile males and females. To understand these issues, this study aims analyze the effects of exposure to this synthetic estrogen during the prenatal and pubertal periods on the morphological, morphometric, and immunohistochemical characteristics of the prostate of 12-month old male and female gerbils.

2 | MATERIALS AND METHODS

2.1 | Experimental design

Ten nulliparous adult female gerbils (90-days) were used in this study. Each female ($n = 10$) was maintained with an adult male ($n = 10$) to form different families. These animals were kept in the bioterium of São Paulo State University (UNESP, São José do Rio Preto, Brazil) and received rodent food ad libitum and filtered water in glass bottles. The animal handling and experiments were in accordance with the ethical principles of animal research and were approved by the Committee for Animal Research of this institution (Protocol No.: 020/09).

The families were separated into two experimental groups, being five female and five male for each group (Figure 1). In the EE PRE/PUB group, pregnant females received 15 μg/kg/day of EE (17α-ethinylestradiol; Sigma-Aldrich) by gavage diluted in 100 μl of mineral oil Nujol® (CAS 8020-83-5; Sigma-Aldrich) (Thayer et al., 2001). The exposure to EE occurred from the 18th to 22nd day of gestation, during the prostatic morphogenesis period (Sanches et al., 2014). After this exposure, five male and five female pups housed separately, from birth through weaning (30 days) and received the same dosage of EE from the 42nd to 49th day, which corresponds to puberty in gerbils (Pinto-Fochi et al., 2016). The control group no received treatment, being intact group. Animals in the control group were not exposed to synthetic estrogen. The males and females of both experimental groups were euthanized at 12 months of age. All animals were euthanized by CO_2 inhalation (chamber) followed by decapitation.

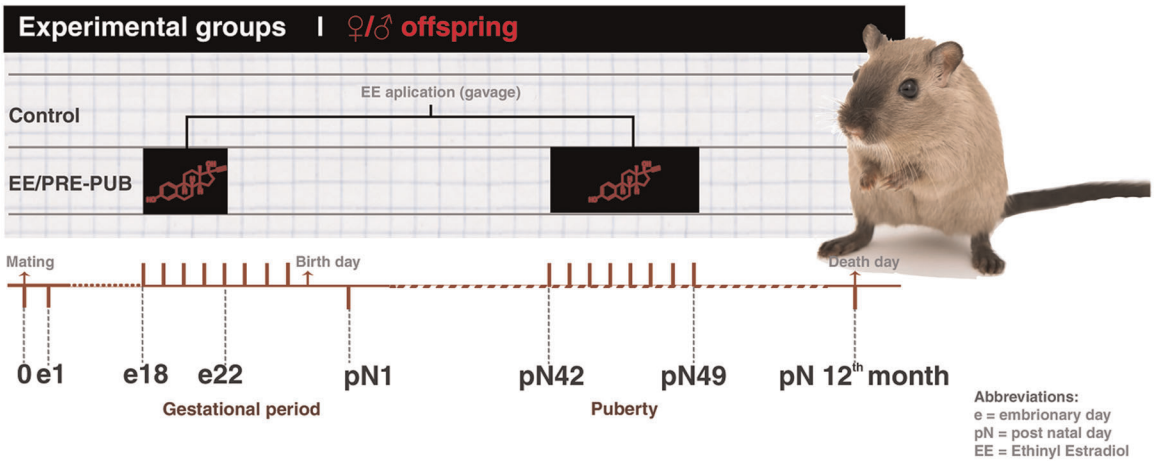

FIGURE 1 Schematic representation of the experimental design. Males and females received 15 µg/kg/day of 17α-ethinylestradiol (EE) during the embryonic period (e) and these gerbils were also exposed to EE between the 42nd and 49th days of life, which corresponds to during puberty (the EE PRE/PUB group). Gerbils in the control group were not exposed to EE. The gerbils (n = 5) were killed at 12 months (360 days) of age, in senescence. EE, ethinylestradiol

2.2 | Serum hormone levels

After the animals died the blood was collected from the cervical region of the head. The blood was centrifuged to 3000 rpm for 20 min and the serum obtained was stored at −80°C before sample analysis. We measured the hormonal levels (testosterone and 17β-estradiol) in duplicate, utilizing Elisa kits (Cayman Chemical Company). The detection limits for testosterone and 17β-estradiol were, respectively, 6 and 19 pg/ml. The assays were read using a SpectraMax Plus (384–405 nm) reader (Molecular Devices).

2.3 | Morphometry

After euthanized of the animals, the body weight of 12-month old male and female gerbils and the anogenital distance (AGD) utilizing a King Tools Digital Caliper (0–300 mm) were measured (Schwartz et al., 2019). Then, the male ventral prostate was separated of others prostatic lobes (Rochel et al., 2007) and weighted. For determine the relative weight realized the ratio between the weight ventral prostate by male weight body. In females, these procedures were performed on the prostate (Skene's gland) with the urethra attached. Before euthanasia, the females were cycled and sacrificed in the proestrous phase (Nishino & Totsukawa, 1996) to minimize variations in prostate morphology due to the phase of the estrous cycle (Fochi et al., 2008).

Tissue samples from the male ventral and female prostates were fixed in methacarn (1:3:6, acetic acid:chloroform:methanol) and 4% paraformaldehyde, submitted to histological steps, as dehydration, clarification, and embbebed in Paraplast (Merck). Compared to male prostatic lobes, only the ventral lobe was evaluated because it is very similar to the female prostate (Flamini et al., 2002). The glands were cut into 5 µm sections and stained with hematoxylin and eosin (HE) for morphological and morphometric analyses and Gömöri's reticulin technique was used for stereology analysis (Behmer et al., 1976). The Gömöri's reticulin technique was utilized for identified the types I and III (reticular) collagens fibers in prostatic tissue (Falleiros-Júnior et al., 2016; Perez et al., 2011, 2012).

In the morphometric analyses, the height of the epithelial layer and thicknesses of the muscle layer of the glands were measured. For this we utilized 10 microscopic fields of the prostatic gland with 40 measurements (µm) per animal/experimental group, obtaining 200 data points of each of the experimental groups (n = 5 animals for each sexes and experimental group).

During the stereology analysis, we compared the relative proportion (%) of each compartment prostatic (epithelium, lumen, reticular and collagen fibers, stroma, and blood vessels). For this, we analyzed 30 fields of each prostate/animal/experimental group, following the application of the M130 multipoint test system (Weibel, 1963).

The histological images were captured using a Zeiss Jenaval microscope, and the morphometric and stereological analyses were performed using Image-ProPlus software, version 4.5 for Windows (Media Cybernetics, Inc.).

To quantify the number of prostatic acini profiles per sectional area, we analyzed 30 random fields from each group (n = 5) using an optical microscope (DM750; Leica Microsystems) (Fochi et al., 2008).

2.4 | Immunohistochemistry

The sections were subjected to immunohistochemical technique for the detection of proliferating cell nuclear antigen (PCNA; mouse monoclonal IgG_{2a}, PC-10: sc-56; Santa Cruz Biotechnology), p63-basal cell (mouse monoclonal IgG2a, 4A4: sc-8431; Santa Cruz Biotechnology), smooth muscle α-actin (mouse monoclonal IgG2a 1C4: sc-32251; Santa Cruz Biotechnology), androgen receptor (AR; rabbit polyclonal IgG, N-20: sc-816; Santa Cruz Biotechnology), estrogen receptor α (ERα; rabbit polyclonal IgG, H-184: sc-7207; Santa Cruz Biotechnology), and estrogen receptor β (ERβ; rabbit polyclonal IgG, H-150: sc-8974; Santa Cruz

Biotechnology). Antigen retrieval was conducted in citrate buffer (pH 6.0) at 98°C for 20 min in steam pot, followed by washes with phosphate buffer saline (PBS). The samples were blocked with H_2O_2 (12%) in methanol for 15 min. All the antibodies were diluted in 1% bovine serum albumin (Sigma-Aldrich) in PBS (1:100 for PCNA, α-actin, p63 and AR; 1:50 for ERα and ERβ) and incubated at 4°C overnight. The sections were incubated in polymer (Novocastra, RE7260-K; Leica Biosystems) at 37°C for 45 min, then stained with DAB (diaminobenzidine; Sigma-Aldrich) and counterstained in Harris hematoxylin.

The images were captured using a photomicroscope (×400) and 2000 cells in five prostatic fields from each experimental group ($n = 3$) were analyzed to determine the frequency of positive cells (Perez et al., 2016). This analysis was performed using Image-ProPlus 6.0 software.

2.5 | Statistical analysis

Statistical analyses of the biometric, serological, morphometric, and immunohistochemical data were performed using *GraphPad Prism 5.0 software* (GraphPad 281 Software, Inc.). Firstly, the normality of the data was verified using a Kolmogorov-Smirnov test. The data that presented a nonparametric distribution were submitted to a Mann-Whitney test and those with parametric distribution to a test t. The significance level employed was 5% ($p \leq .05$), and the results were expressed as mean and ±*SD*.

3 | RESULTS

3.1 | Exposure to EE during the prenatal and pubertal periods did not alter the biometric parameters of male and female senile gerbils

The body weight and AGD data of the male and female senile gerbils presented no significant alterations between the experimental groups. In addition, the weights of the male ventral prostate and female prostate and the prostatic relative weights of both sexes did not differ between these groups (Table 1).

3.2 | EE exposure during development altered hormonal levels in senile male and female gerbils

Table 1 shows the hormonal levels of the experimental group for both sexes. Thus, we noted a decrease in the testosterone level in males and an increase in the estradiol levels in females in the EE PRE/PUB group compared to the respective control groups.

3.3 | Exposure to EE promoted the development of lesions and morphometric changes in the male ventral prostate

In the ventral prostate of the EE PRE/PUB group, we noted the presence of prostatic intraepithelial neoplasia (PIN) with atypical and hyperchromatic nuclei (Figure 2e–g). The thickness smooth muscle layer of the prostate EE PRE/PUB group reduced when compared to control group (Figure 2a–c and Table 2). The number of acini per glandular area was not significantly different between the experimental groups (Table 2). However, we observed a statistically significant decrease in the epithelial height and relative volume luminal compartment of the prostate of the EE PRE/PUB group compared to the control group (Table 2).

By using the Gömori's reticulin technique, we observed a disordered arrangement of types I and III (reticular fiber) collagens fibers in the prostatic stroma of the EE PRE/PUB group (Figure 2h). A significant reduction in the relative volume frequency of the luminal compartment and of type III collagen and a significant increase in the frequency of type I collagen and in the prostatic stromal compartment was observed in the ventral prostate of the EE PRE/PUB group compared to the control group (Table 2).

TABLE 1 Biometric and serological data of male and female 12-month old gerbils in the experimental group

Experimental group male			Experimental group female		
Parameters	**Control**	**EE/PRE-PUB**	**Parameters**	**Control**	**EE/PRE-PUB**
Body weight (g)	86.66 ± 6.11	71.33 ± 1.15	Body weight (g)	68.60 ± 7.12	61.20 ± 8.32
Ventral prostate (g)	0.02 ± 0.002	0.016 ± 0.008	Prostate + urethra (g)	0.04 ± 0.01	0.03 ± 0.003
Relative weight of ventral prostate ($\times 10^{-3}$)	0.25 ± 0.07	0.27 ± 0.09	Relative weight of prostate + urethra ($\times 10^{-3}$)	0.43 ± 0.22	0.47 ± 0.05
AGD (mm)	12.48 ± 1.49	12.47 ± 0.69	AGD (mm)	3.93 ± 0.49	4.30 ± 0.70
Testosterone (ng/ml)	2.16 ± 0.65	1.21 ± 0.53*	Testosterone (ng/ml)	0.81 ± 0.35	1.46 ± 0.56
Estradiol (pg/ml)	12.15 ± 7.20	16.00 ± 5.00	Estradiol (pg/ml)	8.30 ± 2.50	24.00 ± 9.14*

Note: The data are expressed as mean ± *SD* ($n = 5$).

*Significant difference between the groups ($p \leq .05$).

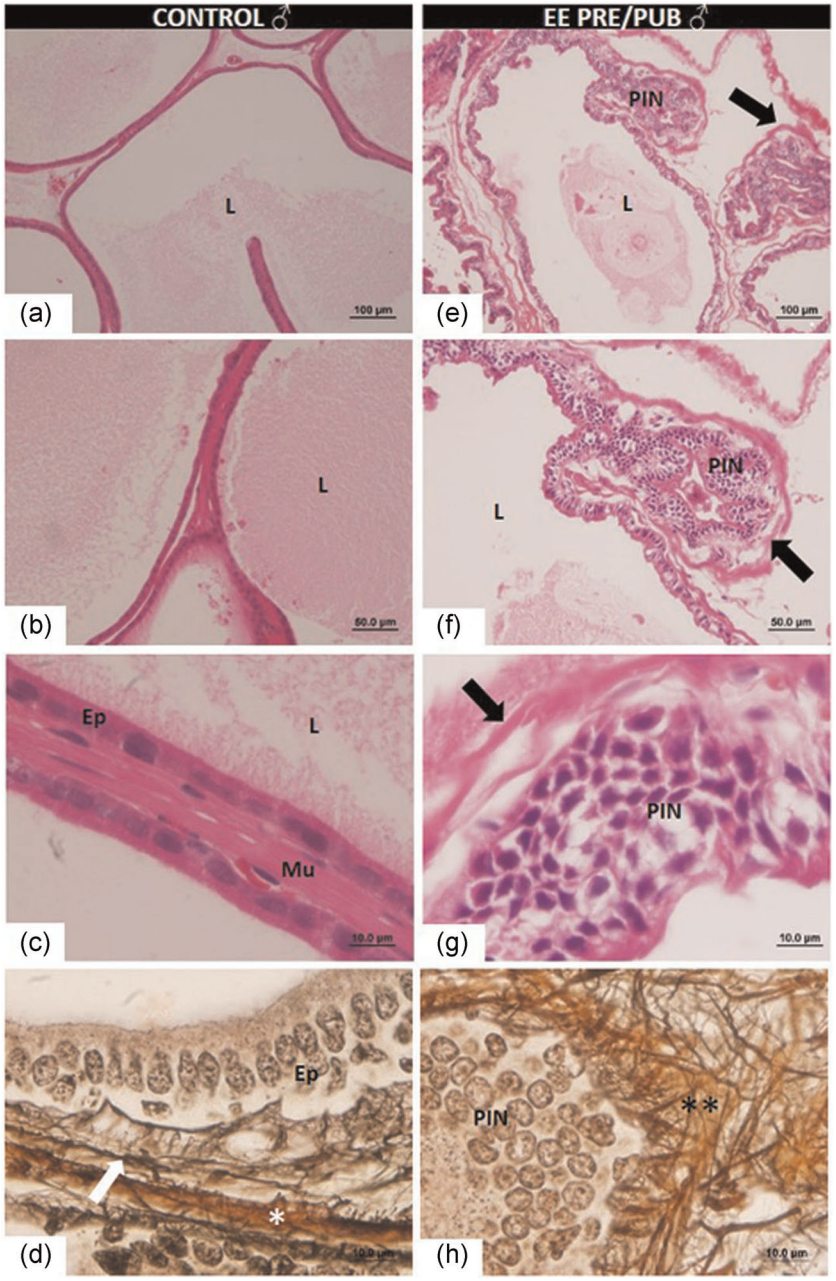

FIGURE 2 Histological sections of the ventral male prostate from 12-month old gerbils stained with HE (a–c, e–g) and Gömori's reticulin (d, h). *Control group* (a–c): prostatic epithelium (Ep) with the presence of a luminal region (L), smooth muscle layer (Mu), and stroma with blood vessels (vs). *EE PRE/PUB group* (e–g): The presence of PIN and irregularities in the muscle layer (arrows), *Control group* (d): prostatic epithelium (Ep), type III collagen (reticular) fibers (white arrow), and type I collagen fibers (white asterisk). *EE PRE/PUB group* (h): disarrangement of the type III collagen (reticular) fibers (asterisk). HE, hematoxylin and eosin; PIN, prostatic intraepithelial neoplasia

3.4 | An increase in the epithelial height and morphological changes were observed in the female prostate of gerbils exposed to EE

In the female prostate of the EE PRE/PUB group, we observed alterations in the fibromuscular stroma and in the glandular epithelium compared to the control group (Figure 3). In the stroma, we verified hyperplasia of muscle cells, whereas in the epithelium we observed regions of PIN (Figure 3e,f). Moreover, in the epithelium of this group, we noted vacuolated and pale areas characterized by an increased number of secretory vesicles (Figure 3g). The statistical analysis showed that there was no significant difference in the number of acini between the experimental groups, but that the epithelial height was higher in the prostate of the EE PRE/PUB group than the control group (Table 2).

In addition, the female gland of the EE PRE/PUB group exhibited a disarrangement in the organization of stromal components, such as the types I and III (reticular fiber) collagens fibers, compared to the control group (Figure 3d,h). We no observed significant difference in the frequency of the stromal compartments (Table 2).

3.5 | Changes in the immunoreaction of androgen and α estrogen receptors in the male ventral prostate of senile gerbils exposed to EE

The immunoreactivity of AR and ERα were noted in the prostatic epithelial and stromal compartments in senile gerbils from the experimental groups (Figure 4a,b and 4e,f). Whereas the frequency

TABLE 2 Morphometric and stereologic data of male and female 12-month old gerbils in the experimental group

Experimental group male			Experimental group female		
Parameters	**Control**	**EE/PRE-PUB**	**Parameters**	**Control**	**EE/PRE-PUB**
Acini/sectional area	45.79 ± 15.11	58.00 ± 39.00	Acini/sectional area	11.65 ± 3.50	14.23 ± 4.62*
Thickness of muscle layer (μm)	8.74 ± 3.00	5.34 ± 1.43*	Thickness of muscle layer (μm)	6.22 ± 3.13	7.14 ± 1.51
Epithelial height (μm)	16.27 ± 3.80	9.34 ± 2.55*	Epithelial height (μm)	14.81 ± 7.95	20.87 ± 15.05*
Stereology of epithelium (%)	26.92 ± 8.35	37.21 ± 11.23	Stereology of epithelium (%)	20.85 ± 12.38	35.81 ± 14.42*
Stereology of lumen (%)	38.65 ± 19.99	9.61 ± 3.54*	Stereology of lumen (%)	50.33 ± 20.53	25.43 ± 16.61*
Stereology of collagen fiber (%)	0.48 ± 1.36	6.74 ± 3.43*	Stereology of collagen fiber (%)	10.04 ± 6.05	15.02 ± 6.60
Stereology of reticular fiber (%)	20.10 ± 10.45	11.30 ± 3.65*	Stereology of reticular fiber (%)	5.81 ± 2.42	11.28 ± 4.92
Stereology of stroma (%)	11.53 ± 3.56	41.12 ± 20.56*	Stereology of stroma (%)	12.73 ± 4.71	17.01 ± 8.28
Stereology of blood vessel (%)	2.31 ± 0.82	5.73 ± 2.62*	Stereology of blood vessel (%)	7.77 ± 4.71	3.53 ± 2.46

Note: The data are expressed as mean ± *SD* ($n = 5$).

*Significant difference between the groups ($p \leq .05$).

immunopositive to AR was lower in the epithelial region, the frequency immunopositive to ERα was higher in the stromal region of the ventral prostate of the EE PRE/PUB group than the control group (Figure 4m,o).

We observed a positive ERβ immunoreaction in the nuclei of the epithelial cells in both groups, but in the cytoplasmic region of the prostatic epithelium this immunoreaction was evidence in the gland of the EE PRE/PUB group (Figure 4i,j). The frequency of this immunorreactivity was not significant (Figure 4q).

3.6 | Upregulation of the AR in the female prostate of senile gerbils exposed to EE during development

In the female prostate, we also verified immunoreaction of AR and ERα both in the epithelial and stromal regions (Figure 4c,d and 4g,h). The frequency immunopositive to AR was significantly higher in the stromal compartment of the female gland of the EE PRE/PUB group than the control group (Figure 4n).

Generally, the ERβ immunoreactivity is observed in the epithelial cells, however, in cytoplasm of the epithelial cells of the gland of the EE PRE/PUB group, this immunoreaction was evidence (Figure 4k,l,q).

3.7 | EE promoted no changes in the frequency of proliferative or basal cells, but decreased the immunoreactivity for α-actin in the muscular layer in foci of prostatic lesions in male senile gerbils

We observed the presence of proliferative cells in the prostatic epithelium and stroma of males in the experimental groups (Figure 5a,b), and by using immunohistochemistry for p63, it was possible to observe the basal cells in the glands of these animals (Figure 5e,f). However, these immunoreactions presented no changes after exposure to EE (Figure 5m,n).

In regions where we observed PIN and foci of inflammation, the immunoreactivity for α-actin was absent or lower in the muscular layer of the ventral prostate of the EE PRE/PUB males than in that of the control group males (Figure 5i,j).

3.8 | Exposure to EE caused an increase in epithelial cell proliferation and the immunoreactivity for α-actin in the muscle layer of the female prostate

Proliferative cells were observed in the prostatic epithelium and stromal regions using immunohistochemical staining for PCNA (Figure 5c,d), and we observed an increase in the frequency immunopositive of proliferative cells in the epithelium of the female prostate of senile gerbils exposed to EE compared to control group (Figure 5m). The frequency immunopositive of basal cells was not altered in the female prostate, which was verified by immunohistochemical staining for p63 (Figure 5g,h,n).

In the surrounding muscular layer to the regions with PIN, we noted an increase in the immunoreactivity for α-actin in the female prostate of the EE PRE/PUB group compared to the control group (Figure 5k,l).

4 | DISCUSSION

Ethinylestradiol affects the functions of the endocrine system organs and causes endocrine disruption (Saaristo et al., 2019; Schug et al., 2011). In our study, we observed that the exposure to EE during development periods is associated to decrease in the testosterone levels in male and increase in the estradiol levels in female of the old gerbils. We observed the development of lesions, morphometrical and stereological alterations in both the prostatic glands, further was noted that exposure to EE promoted down and upregulation of the AR frequency, respectively in male and female. The immunoreactivity for ERα and α-actin were lower in the ventral male prostate of EE/PRE-PUB group, whereas in the female prostate of this groups was observed an increase in epithelial cell

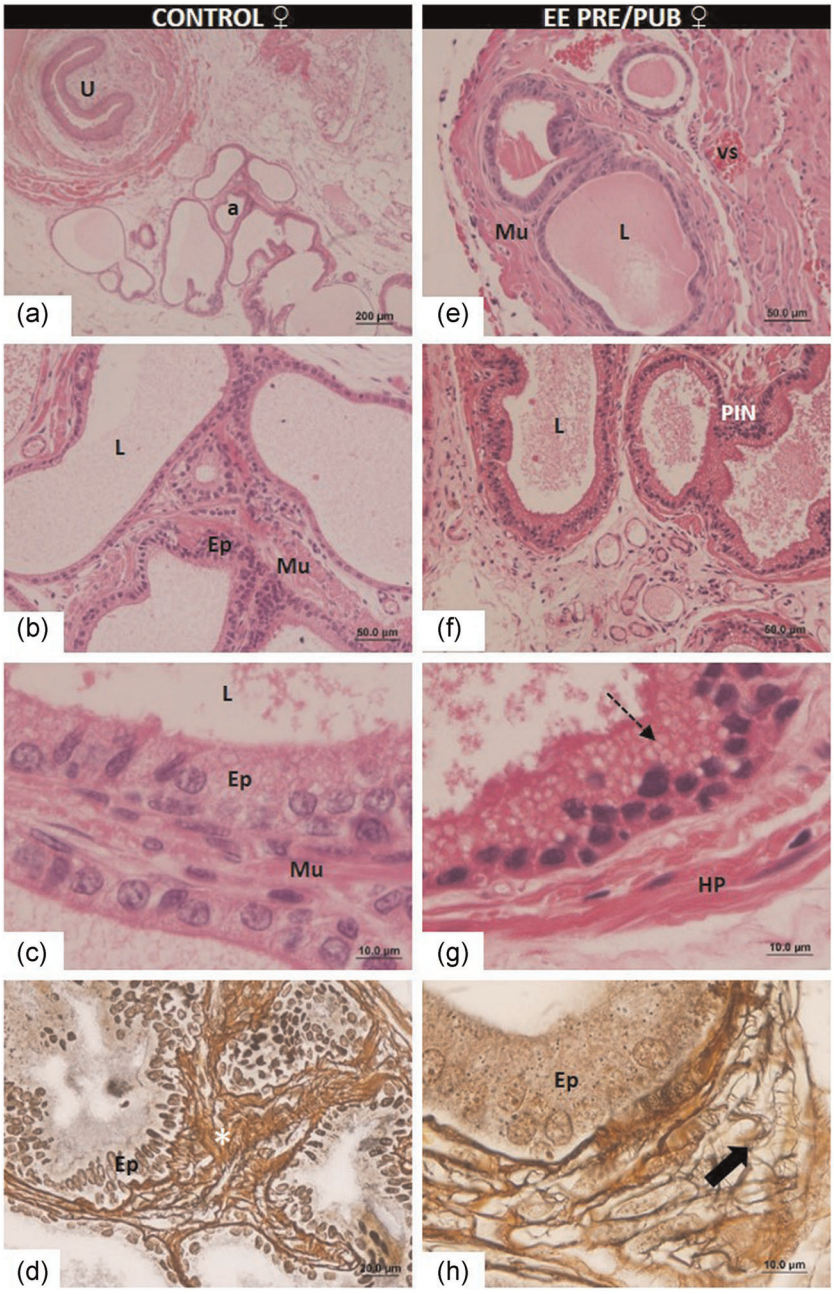

FIGURE 3 Histological sections of the female prostate from 12-month old gerbils stained with HE (a–c, e–g) and Gömori's reticulin (d, h). *Control group* (a–c): Female prostate with paraurethral (u) localization and the presence of the acini (a). Prostatic epithelium (Ep) with the presence of a luminal region (L), smooth muscle layer (Mu), and stroma with blood vessels (vs.). *EE PRE/PUB group* (e–g): The presence of PIN, an apparent increase in the number of secretory vesicles (dashed arrow) is indicated—observed in detail also, muscular HP. *Control group* (d): Prostatic epithelium (Ep), type III collagen (reticular) fibers (white asterisk). *EE PRE/PUB group* (h): Disarrangement of the type III collagen (reticular) fibers (arrow). HE, hematoxylin and eosin; HP, hyperplasia; PIN, prostatic intraepithelial neoplasia

proliferation and immunoreactivity for α-actin. The exposure to EE during developmental phases caused different morphophysiological changes in the ventral male prostate and female prostate during aging.

Among these morphological changes, we observed an increase in the epithelial height, an apparent increase in secretory vesicles and the presence of smooth muscle cell hyperplasia in the female prostate of EE PRE/PUB group. In adult females, the estrous phase showed maximum epithelial height and the typically secretory phenotype, been the phase characterized by intense prostatic development (Fochi et al., 2008). The female prostate normally develops in an environment with high endogenous estrogen concentrations (Biancardi et al., 2017) and possibly the exposure to EE, a synthetic estrogen, might have increased of the prostatic development during aging.

Testosterone levels increased in senile female gerbils exposed to EE only during the prenatal period (Perez et al., 2016). Androgen hormones induce the morphogenesis, maturation, and functionally of the female prostate (Biancardi et al., 2017). We verified an increase in the frequency AR in the prostatic stroma of EE PRE/PUB females. Testosterone is converted to dihydrotestosterone (DHT) by 5α-reductase, and DHT has a higher affinity for AR, inducing the transcription of growth factors such as epidermal growth factor, keratinocyte growth factor, fibroblast growth factor, and insulin type growth factor (Taboga et al., 2009). Since DHT is, a potent stimulator of prostatic stromal cells (Pejčić et al., 2017) these changes verified in the female prostate might be related to the development of smooth muscle cell hyperplasia in this gland. In addition, AR has a mediating action in the epithelial-stroma interactions, which might explain the

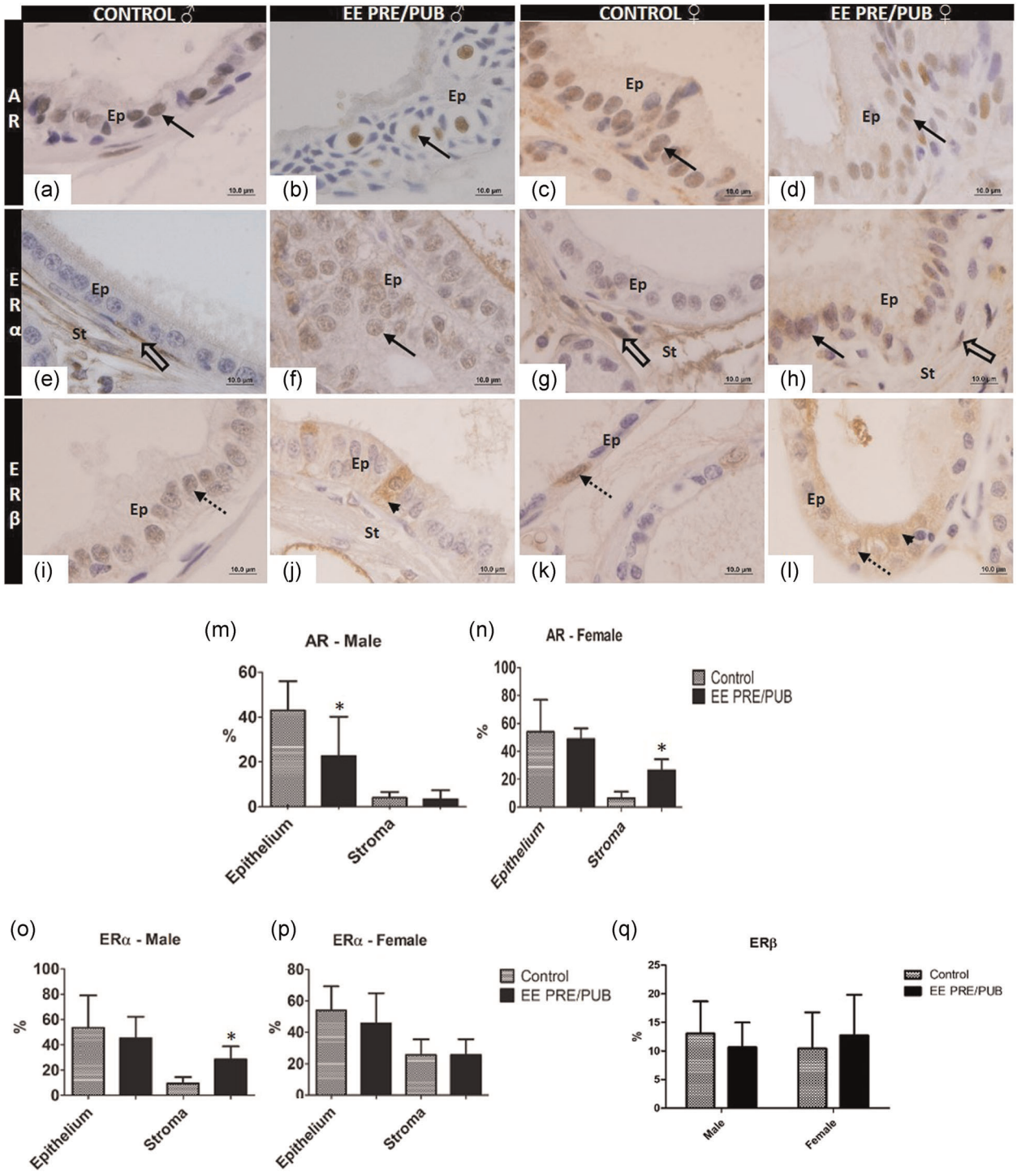

FIGURE 4 Histological sections of the ventral prostate and female prostate from senile gerbils submitted to immunohistochemical staining for AR (a–d), ERα (e–h), and ERβ (i–l). (a–d) Epithelium (Ep), stromal cells (St), and epithelial cells staining positive for AR (arrows). (e–h) Immunostaining for ERα in the epithelial (arrows) and stromal cells (large arrows). (i–l) Immunostaining for ERβ in the epithelial cells (dashed arrows) and cytoplasmic region (arrowhead). Frequency of positive cells for AR, ERα, and ERβ (m–q) in the male and female prostate of senile gerbils in the experimental group. The data are expressed as mean ± *SD* ($n = 5$). *Significant difference between the groups ($p \leq .05$). AR, androgen receptor; ERα/β, estrogen receptor α/β

alterations in glandular secretory activity and the development of prostatic lesions.

The increase in ERα immunoreactivity in the prostatic stroma of senile females in the group exposed to EE might reflect an inhibitory mechanism of proliferation promoted by AR pathways. Changes in the frequency of these receptors have been related to the estrogen imprint, in which the exposure to endocrine disrupters during the developmental period promotes, through epigenetics, a cellular

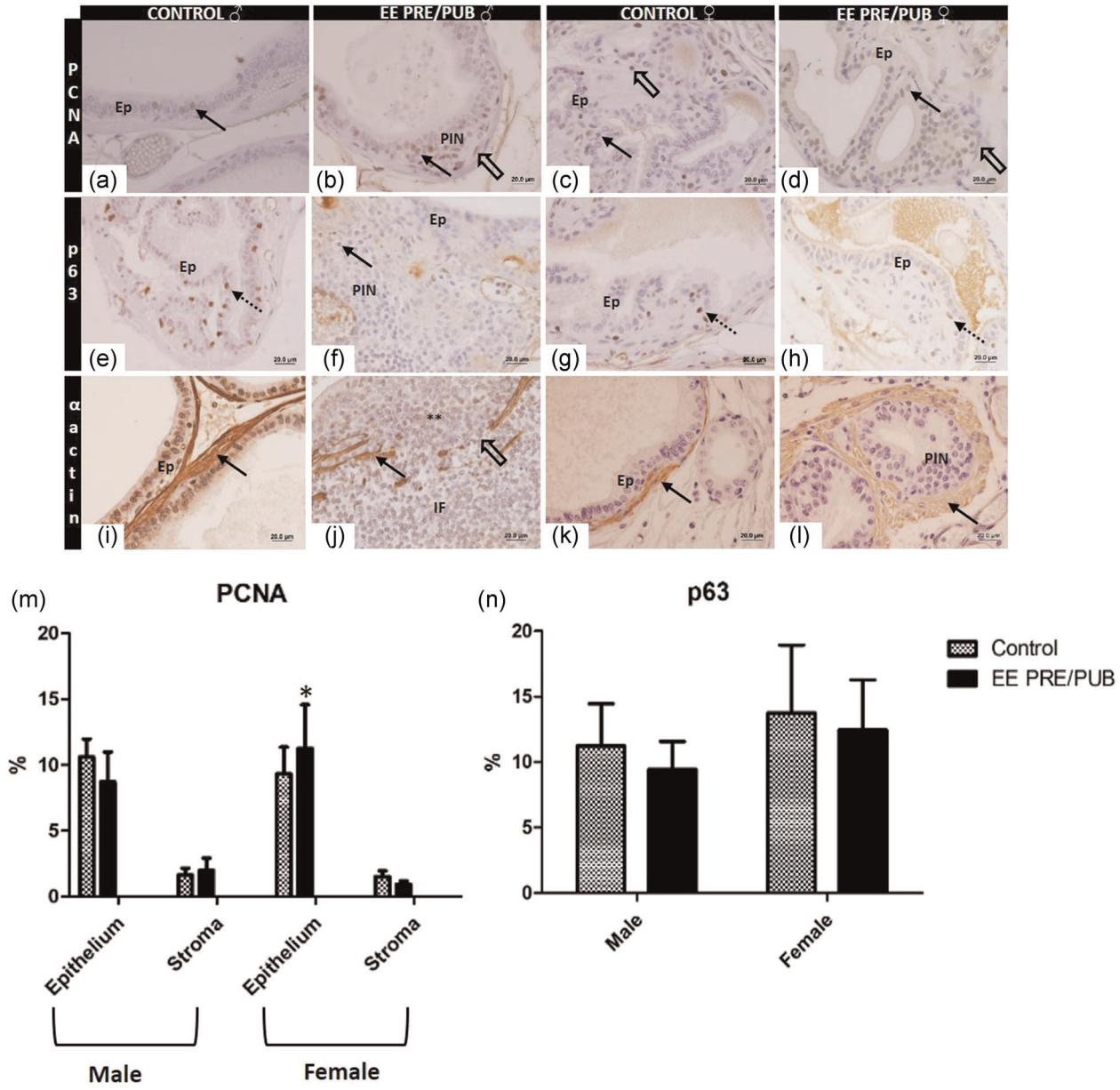

FIGURE 5 Histological sections of the ventral prostate and female prostate from senile gerbils submitted to immunohistochemical staining for PCNA (A-D), p63 (e–h), and α-actin (i–l). (a–d) Immunostaining for PCNA in the epithelial (Ep) (arrows), stromal cells (large arrows) and the presence of PIN. (e–h) Epithelial cell positive for p63 (dashed arrows) and the presence of PIN. (i–l) Immunostaining for α-actin in the muscular layer (arrows), the absence of immunostaining (large arrow) in the region with adenocarcinoma (asterisks), and the presence of IF. The frequency of positive cells for PCNA and p63 (m, n) in the male and female prostate of senile gerbils in the experimental group. The data are expressed as mean ± *SD* (n = 5). *Significant difference between the groups ($p \leq .05$). IF, inflammation; PCNA, proliferating cell nuclear antigen; PIN, prostatic intraepithelial neoplasia

reprogramming, altering the expression of specific genes, which can lead to prostatic morphological changes in gerbils, thus resulting in the emergence of potentially malignant lesions during later phases (Perez et al., 2011, 2012, 2016).

Male rodents, when exposed to high estrogenic doses during developmental phases, present permanent disturbances in the prostate gland, such as changes in development and differentiation and a reduced androgenic response in adulthood (Hess & Cooke, 2018; Risbridger et al., 2005). Another effect might be the increase in ERα expression, which subsequently causes prostatic morphogenesis to be directed by estrogens (Hess & Cooke, 2018). According Putz et al. (2001) neonatal low-dose exposure to exogenous estrogen (0.015–15 μg/kg/day) increased reproductive organ weights, as prostatic gland. In our study, the dosage was considered as low (15 μg/kg/day). However, the concomitant exposure in the prenatal and pubertal periods promoted similar effects to high estrogen dosages, such as decreased immunoreactivity of the AR in the epithelium and an increase in

the immunoreactivity of ERα in the prostatic stroma of senile males in the EE PRE/PUB group compared to controls.

Modification of the predominant ERα site might also be due to estrogen imprinting. ERα is related to abnormal cell proliferation pathways, the induction of inflammation, and the onset of potentially malignant lesions (Rochel-Maia et al., 2012). Increased immunosuppression of this associated with EE exposure during development might be related to the increase in the incidence of metaplasia and PIN in the prostate of adult (Perez et al., 2011) male gerbils and during aging (Perez et al., 2016), which is consistent with our observations during the morphological analysis of the male prostate in this study.

The p63 protein belongs to the same family of transcription factors as the p53 protein, which is associated with the aging process through its function in response to DNA repair (Nicolai et al., 2015). Therefore, these proteins are expressed in higher quantities in regions with neoplastic foci, and are important mechanisms of suppression in the case of malignant alterations (Pelúzio et al., 2006). The immunoreactivity of p63 was observed in the basal cells of the prostatic acinar epithelia of the females in the experimental groups. However, the frequency of this immunostaining was not altered by exposure to EE, as the presence of malignant lesions in the prostates of the analyzed groups was not observed.

The presence of epithelial proliferative cells and nuclear atypia was also observed in the male prostate of the EE PRE/PUB group gerbils, characterizing the presence of PIN (Shappell et al., 2004). The immunohistochemical technique for p63 has been considered an important tool for the histopathological diagnosis of the prostate gland (Fonseca-Alves et al., 2018). Although exposure to EE did not significantly alter the frequency of p63 in the prostatic epithelium of the male gerbils, it was possible to observe a few cells that were positive for this marker in foci with PIN in senile males exposed to EE during the prenatal period (Perez et al., 2017). This low immunoreactivity is often observed in high-grade PIN.

The exposure to low doses of EE during the prenatal (Perez et al., 2016), pubertal (Perez et al., 2017), and neonatal (Falleiros-Júnior et al., 2016) periods increases the frequency of PCNA immunoreactivity and is associated with proliferation of prostate epithelial cells in male gerbils during later phases. In our study, exposure to EE did not affect PCNA immunoreactivity in the stroma and prostate epithelium. Although the dosage of EE used in this study was low (Putz et al., 2001), the period of exposure was long, since it was carried out concomitantly at two stages of development: prenatal and puberty. In addition to the dosage used for EE, the exposure time and duration are important factors that influence the effects of this compound on the glandular tissue.

In a previous study, an increase in PCNA immunoreactivity was observed in the prostate of a group of senile female gerbils exposed to EE during the prenatal period (Perez et al., 2016), a result also observed in the present study. However, we quantified this immunoreactivity in the epithelial and stromal compartments of the prostates in the experimental groups, since the increase in the thickness of the epithelium of the EE PRE/PUB group might be related to a high proliferation index of these cells.

The female prostate of gerbils is predisposed to the development of different prostatic lesions in senile life (Custodio et al., 2010). In the present study, there was an increase in the relative volume of the epithelial compartment and a decrease in the luminal compartment in the prostate of the EE PRE/PUB group compared to the control group. This increase in the epithelial compartment might be a consequence of the increase in proliferation of the epithelial cells, as evidenced by the high PCNA immunoreactivity in these cells. PCNA is a crucial protein for DNA replication and repair, chromatin structure maintenance and cell-cycle progression (Glover et al., 2017). Chemical components, as synthetic estrogen, alter the levels of PCNA, interfering with cell cycle control pathways, resulting in the increase of the cell proliferation and the formation of neoplasia (Strzalka & Ziemienowicz, 2011). These changes contribute to the increase in prostatic lesions during senile life and are a consequence of exposure to the EE during phases of the development.

The reorganization of types I and III (reticular) collagens fibers, as well as the synthesis of new protein components and the degradation of the basement membrane, are events related to prostate neoplastic progression and factors that promote genetic reprogramming during the epithelial-stroma interaction (Gonçalves et al., 2014). Although the relative volume of types I and III collagens fibers did not differ significantly between the two experimental groups, in our study, it was possible to observe a disorganization of these fibrillary structures in the female prostate of senile gerbils in the EE PRE/PUB group. This disorganization is also associated with the appearance of neoplastic lesions in the glands of these animals during aging.

Previous studies have shown that exposure to EE during the prenatal period is more detrimental to the morphology of the ventral prostate of adult and senile male gerbils than to the morphology of the females prostate (Perez et al., 2011, 2012, 2016). When exposure occurred during puberty, there were divergences in the alterations in these glands; the effects on the ventral prostate of senile males were inhibitory, whereas in the female prostate, the effects were considered anabolic (Perez et al., 2017). Taking into account that during the pubertal period, the levels of endogenous estrogen increase in females, exposure to EE in this phase intensifies the effects of the hormone on the prostate gland. However, when exposure occurs concomitantly at different periods of development, an anabolic effect is not observed, as demonstrated in our study. In this case, the changes in both sexes were very similar, confirming that exposure to EE clearly modifies the prostate glands, however, the type and degree of the changes depends on the periods of exposure and might accentuate prostatic lesions during aging.

The results of this study shed light on the action of EE as an endocrine disrupter and highlight that the timing and duration of exposure to this compound are significant. Thus, we have gained a better understanding of the pathogenesis of prostatic lesions in both sexes, providing new avenues to be studied to reduce the incidence of this disease.

5 | CONCLUSION

The present study shows that exposure to EE during the prenatal and pubertal period promoted histopathological changes in the male and female prostate during aging. Many of these changes, caused by changes in the frequency of receptors that control prostate development in both phases, may predispose to potentially malignant lesions. The circumstances presented may be a means of understanding the action of EE as an endocrine disrupter, for this, the period and duration of exposure to the compound should be considered. Thus, we can better understand the pathogenesis of prostatic lesions in both sexes, providing new avenues to be studied to reduce the incidence of this disease.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

All of the authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript. Fernanda C. A.dos Santos, Luísa R. F. Guimarães, Elisa B. Rezende, and Tracy M. M. Martins performed the experiments, and Fernanda C. A.dos Santos and Ana P. da S. Perez wrote the manuscript. Cássia R. S. Caires, Fernanda C. A. dos Santos, and Sebastião R. Taboga equally contributed to the supervision of this study.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

ORCID

Tracy M. M. Martins https://orcid.org/0000-0003-0250-2234
Fernanda C. A. dos Santos http://orcid.org/0000-0002-4476-6046
Sebastião R. Taboga http://orcid.org/0000-0002-0970-4288
Ana P. da S. Perez http://orcid.org/0000-0002-2195-2452

REFERENCES

Adeel, M., Song, X., Wang, Y., Francis, D., & Yang, Y. (2017). Environmental impact of estrogens on human, animal and plant life: A critical review. *Environment International*, *99*, 107–119.

Behmer, A. O., Tolosa, E. M. C., & Neto, A. G. F. (1976). *Manual de práticas para histologia normal e patológica*. Edart-Edusp.

Biancardi, M. F., Santos, F. C. A. D., de Carvalho, H. F., Sanches, B. D. A., & Taboga, S. R. (2017). Female prostate: Historical, developmental, and morphological perspectives. *Cell Biology International*, *41*, 1174–1183.

Cunha, G. R., Vezina, C. M., Isaacson, D., Ricke, W. A., Timms, B. G., Cao, M., Franco, O., & Baskin, L. S. (2018). Development of the human prostate. *Differentiation*, *103*, 24–45.

Custodio, A. M. G., Santos, F. C. A., Campos, S. G. P., Vilamaior, P. S. L., Oliveira, S. M., Góes, R. M., & Taboga, S. R. (2010). Disorders related with ageing in the gerbil female prostate (Skene's paraurethral glands). *International Journal of Experimental Pathology*, *91*, 132–143.

dos Santos, F. C. A., Carvalho, H. F., Góes, R. M., & Taboga, S. R. (2003). Structure, histochemistry, and ultrastructure of the epithelium and stroma in the gerbil (*Meriones unguiculatus*) female prostate. *Tissue & Cell*, *35*, 447–457.

Falleiros-Júnior, L. R., Perez, A. P. S., Taboga, S. R., Santos, F. C. A. D., & Vilamaior, P. S. L. (2016). Neonatal exposure to ethinylestradiol increases ventral prostate growth and promotes epithelial hyperplasia and inflammation in adult male gerbils. *International Journal of Experimental Pathology*, *97*, 380–388.

Flamini, M. A., Barbeito, C. G., Gimeno, E. J., & Portiansky, E. L. (2002). Morphological characterization of the female prostate (Skene's gland or paraurethral gland) of *Lagostomus maximus* maximus. *Anatomischer Anzeiger*, *184*, 341–345.

Fochi, R. A., Perez, A. P. S., Bianchi, C. V., Rochel, S. S., Góes, R. M., Vilamaior, P. S. L., & Santos, F. C. A. (2008). Hormonal oscillations during the estrous cycle influence the morphophysiology of the gerbil (*Meriones unguiculatus*) female prostate (skene paraurethral glands). *Biology of Reproduction*, *79*, 1084–1091.

Fonseca-Alves, C. E., Kobayashi, P. E., Calderón, L. G. R., Felisbino, S. L., de Carvalho Rinaldi, J., Drigo, S. A., & Laufer-Amorim, R. (2018). Immunohistochemical panel to characterize canine prostate carcinomas according to aberrant p63 expression. *PLOS One*, *13*, e0199173.

Glover, M., Soni, S., Ren, Q., Maclennan, G. T., Fu, P., & Gupta, S. (2017). Influence of chronic inflammation on Bcl-2 and PCNA expression in prostate needle biopsy specimens. *Oncology Letters*, *14*, 3927–3934.

Gonçalves, B. F., de Campos, S. G. P., Costa, C. F. P., Scarano, W. R., Góes, R. M., & Taboga, S. R. (2014). Key participants of the tumor microenvironment of the prostate: An approach of the structural dynamic of cellular elements and extracellular matrix components during epithelial-stromal transition. *Acta Histochemica*, *117*, 4–13.

Heneghan, C., Aronson, J. K., Spencer, E., Holman, B., Mahtani, K. R., Perera, R., & Onakpoya, I. (2019). Oral hormone pregnancy tests and the risks of congenital malformations: A systematic review and meta-analysis. *F1000Research*, *7*, 17–25.

Hess, R. A., & Cooke, P. S. (2018). Estrogen in the male: A historical perspective. *Biology of Reproduction*, *99*, 27–44.

Mandrup, K. R., Jacobsen, P. R., Isling, L. K., Axelstad, M., Dreisig, K., Hadrup, N., & Boberg, J. (2013). Effects of perinatal ethinyl estradiol exposure in male and female Wistar rats. *Reproductive Toxicology*, *42*, 180–191.

Moalem, S., & Reidenberg, J. S. (2009). Does female ejaculation serve an antimicrobial purpose? *Medical Hypotheses*, *73*, 1069–1071.

Nicolai, S., Rossi, A., Daniele, N. D., Melino, G., Annicchiarico-Petruzzelli, M., & Raschellà, G. (2015). DNA repair and aging: The impact of the p53 family. *Aging*, *7*, 1050–1065.

Nishino, N., & Totsukawa, K. (1996). Study on the estrous cycle in the Mongolian gerbil (*Meriones unguiculatus*). *Experimental Animals*, *45*, 283–288.

Pejčić, T., Tosti, T., Tešić, Z., Milković, B., Dragičević, D., Kozomara, M., Čekerevac, M., & Džamić, Z. (2017). Testosterone and dihydrotestosterone levels in the transition zone correlate with prostate volume. *The Prostate*, *77*, 1082–1092.

Pelúzio, M. C. G., Volp, A. C. P., Queiroz, I. C., Brito, C. J., & Miranda, T. C. (2006). The suppressor proteins in malignant neoplasia—Knowing their role. *Revista Brasileira Nutrição Clínica*, *21*, 233–238.

Perez, A. P. S., Biancardi, M. F., Caires, C. R. S., Falleiros, L. R., Góes, R. M., Santos, F. C. A., & Taboga, S. R. (2017). Pubertal exposure to ethinylestradiol promotes different effects on the morphology of the prostate of the male and female gerbil during aging. *Environmental Toxicology*, *32*, 477–489.

Perez, A. P. S., Biancardi, M. F., Caires, C. R. S., Falleiros-Junior, L. R., Góes, R. M., Vilamaior, P. S. L., & Taboga, S. R. (2016). Prenatal exposure to ethinylestradiol alters the morphologic patterns and increases the predisposition for prostatic lesions in male and female gerbils during ageing. *International Journal of Experimental Pathology*, *97*, 5–17.

Perez, A. P. S., Biancardi, M. F., Góes, R. M., dos Santos, F. A., & Taboga, S. R. (2011). Exposure to ethinylestradiol during prenatal development and postnatal supplementation with testosterone causes morphophysiological alterations in the prostate of male and female adult gerbils. *International Journal of Experimental Pathology*, *92*, 121–130.

Perez, A. P. S., Biancardi, M. F., Vilamaior, P. S., Góes, R. M., Santos, F. C. A., & Taboga, S. R. (2012). Microscopic comparative study of the exposure effects of testosterone cypionate and ethinylestradiol during prenatal life on the prostatic tissue of adult gerbils. *Microscopy Research and Technology*, *75*(8), 1084–1092.

Pinto-Fochi, M. E., Negrin, A. C., Scarano, W. R., Taboga, S. R., & Góes, R. M. (2016). Sexual maturation of the Mongolian gerbil (*Meriones unguiculatus*): A histological, hormonal and spermatic evaluation. *Reproduction, Fertility, and Development*, *28*, 815–823.

Prins, G. S., & Putz, O. (2008). Molecular signaling pathways that regulate prostate gland development. *Differentiation*, *76*, 641–659.

Putz, O., Schwartz, C. B., Kim, S., LeBlanc, G. A., Cooper, R. L., & Prins, G. S. (2001). Neonatal lowand high dose exposure to estradiol benzoate in the male rat: I. Effects on the prostate gland. *Biology of Reproduction*, *65*, 1496–1505.

Risbridger, G. P., Almahbobi, G. A., & Taylor, R. A. (2005). Early prostate development and its association with late-life prostate disease. *Cell and Tissue Research*, *322*, 173–181.

Rochel, S. S., Bruni-Cardoso, A., Taboga, S. R., Vilamaior, P. S. L., & Góes, R. M. (2007). Lobe identity in the Mongolian gerbil prostatic complex: A new rodent model for prostate study. *The Anatomical Record*, *290*, 1233–1247.

Rochel-Maia, S. S., Santos, F. C. A., Alonso-Magdalena, P., Góes, R. M., Vilamaior, P. S. L., Warner, M., & Taboga, S. R. (2012). Estrogen receptors alpha and beta in male and female gerbil prostates. *Biology of Reproduction*, *88*, 7.

Saaristo, M., Johnstone, C. P., Xu, K., Allinson, M., & Wong, B. B. M. (2019). The endocrine disruptor, 17 ethinyl estradiol, alters male mate choice in a freshwater fish. *Aquatic Toxicology*, *208*, 118–125.

Sanches, B. D., Biancardi, M. F., Santos, F. C., Góes, R. M., Vilamaior, P. S., & Taboga, S. R. (2014). Budding process during the organogenesis of the ventral prostatic lobe in Mongolian gerbil. *Microscopy Research and Technique*, *77*, 458–66.

Sanches, B. D. A., Carvalho, H. F., Maldarine, J. S., Biancardi, M. F., Santos, F. C. A., Vilamaior, P. S. L., & Taboga, S. R. (2019). Differences between male and female prostates in terms of physiology, sensitivity to chemicals and pathogenesis: A review in a rodent model. *Cell Biology International*, *44*, 27–35. https://doi.org/10.1002/cbin.11214

Sanches, B. D. A., Maldarine, J. S., Zani, B. C., Biancardi, M. F., Santos, F. C. A., Góes, R. M., & Taboga, S. R. (2016). The expression of the androgen receptor and estrogen receptor 1 is related to sex dimorphism in the Gerbil prostate development. *The Anatomical Record*, *299*, 1130–1139.

Santos, F. C. A., & Taboga, S. R. (2006). Female prostate: A review about the biological repercussions of this gland in humans and rodents. *Animal. Reproduction*, *3*, 3–18.

Schug, T. T., Janesick, A., Blumberg, B., & Heindel, J. J. (2011). Endocrine disrupting chemicals and disease susceptibility. *The Journal of Steroid Biochemistry and Molecular Biology*, *127*, 204–215.

Schwartz, C. L., Christiansen, S., Vinggaard, A. M., Axelstad, M., Hass, U., & Svingen, T. (2019). Anogenital distance as a toxicological or clinical marker for fetal androgen action and risk for reproductive disorders. *Archives of Toxicology*, *93*, 253–272.

Shappell, S. B., Thomas, G. V., Roberts, R. L., Herbert, R., Ittmann, M. M., Rubin, M. A., Humphrey, P. A., Sundberg, J. P., Rozengurt, N., Barrios, R., Ward, J. M., & Cardiff, R. D. (2004). Prostate pathology of genetically engineered mice: Definitions and classification. the consensus report from the bar harbor meeting of the mouse models of Human Cancer Consortium Prostate Pathology Committee. *Cancer Research*, *64*, 2270–2305.

Strzalka, W., & Ziemienowicz, A. (2011). Proliferating cell nuclear antigen (PCNA): A key factor in DNA replication and cell cycle regulation. *Annals of Botany*, *107*, 1127–1140.

Taboga, S. R., Vilamaior, P. S. L., & Góes, R. M. (2009). Modulação androgênica e estrogênica na próstata: uma abordagem em modelos experimentais de roedores com enfoque na biologia estrutural. *Arquivos Brasileiros de Endocrinologia & Metabologia*, *53*(8), 946–955. http://www.scielo.br/scielo.php?script=sciarttextpid=S0004-27302009000800007lng=ennrm=isso

Thayer, K. A., Ruhlen, R. L., Howdeshell, K. L., Buchanan, D. L., Cooke, P. S., Preziosi, D., & vom Saal, F. S. (2001). Altered prostate growth and daily sperm production in male mice exposed prenatally to subclinical doses of 17alpha-ethinyl oestradiol. *Human Reproduction*, *16*, 988–996.

Weibel, E. R. (1963). Principles and methods for the morphometric study of the lung and other organs. *Laboratory Investigation*, *12*, 131–155.

Zaviacic, M., & Ablin, R. J. (2000). The female prostate and prostate-specific antigen. Immunohistochemical localization, implications of this prostate marker in women and reasons for using the term "prostate" in the human female. *Histology and Histopathology*, *15*, 131–142.

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